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THE YEAST HANDBOOK

Carlos A. Rosa
Gábor Péter (Eds.)

Biodiversity and Ecophysiology of Yeasts

Springer

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The Yeast Handbook

Carlos Rosa, Gábor Péter (Eds.)

Biodiversity and Ecophysiology of Yeasts

With 40 Figures and 43 Tables

Springer

Dr. Gábor Péter
Budapest University of Economic
Sciences and Public Administration
Faculty of Food Sciences
National Collection of Agricultural and
Industrial Microorganisms
Somloi út 14-16
H-1118 Budapest
Hungary

Professor Carlos Rosa
Departmento de Microbiologia
ICB, CP 486
Universidade Federal de Minas Gerais
Belo Horizonte, MG
31270-901
Brazil

Cover pictures by courtesy of:
Vincent Robert, Centraalbureau voor Schimmelcultures (CBS), The Netherlands
Carlos Rosa, Universidade Federal de Minas Gerais, Brazil

ISBN-10 3-540-26100-1 Springer Berlin Heidelberg New York
ISBN-13 978-3-540-26100-1 Springer Berlin Heidelberg New York

Library of Congress Control Number: 2005930628

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Printed in Germany

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Editor: Dr. Sabine Schreck, Heidelberg
Desk Editor: Anette Lindqvist, Heidelberg
Production: SPI Publisher Services
Typesetting: SPI Publisher Services
Cover Design: Design & Production, Heidelberg

Printed on acid-free paper 39/3152-HM 5 4 3 2 1 0

Preface

Biological diversity or biodiversity, according to the United Nations Convention on Biological Diversity, "means the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part" and includes genetic diversity, species diversity, and ecological diversity. Authors trying to estimate the number of extant yeast species usually come to the conclusion that, similarly to other groups of microorganisms, a very small proportion of the yeasts, possibly only 1% of the species, have been described.

Studies on "yeast biodiversity" are more focused on taxonomic inventories, with emphasis on the description of novel species. Approximately 30% of known yeast species were described from a single strain, and information about the ecology and the genetic and physiological variability of these yeasts is missing or incomplete.

In the last few decades an increasing number of new yeast habitats have been explored. As a result, a large body of ecological information has been accumulated and the number of known yeast species has increased rapidly. The volume "Biodiversity and ecophysiology of yeasts" provides a comprehensive and up-to-date overview of several areas in the field of yeast biodiversity and ecology. The chapters are written by respected experts in various fields. The first chapters approach yeast biodiversity from different points of view, including phylogenetics and genomics. Some aspects of sugar and nitrogen metabolism are also discussed. Separate chapters are devoted to stress responses of yeasts, to environmental factors influencing them, to antagonistic interactions among them, to methods used for investigating yeast biodiversity, and to the role of culture collections in handling the ever-increasing number of yeast strains and relevant data.

The chapters dealing with yeast communities from different habitats include reviews on yeasts from invertebrates, the phylloplane, soil, freshwater and marine ecosystems, cactophilic communities, as well as Antarctic and tropical forest ecosystems. In some chapters the effect of human activity on yeast communities is also considered. The black yeasts are treated in a separate chapter, and finally the role of yeast biodiversity in biotechnology is reviewed.

We gratefully acknowledge the contributors to this book. We hope that it will provide a useful overview of the biodiversity and ecophysiology of yeasts, and that it will stimulate increasing efforts in yeast biodiversity research.

Carlos Rosa
Gábor Péter
May 2005

Acknowledgements

C.A. Rosa acknowledges the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for support of research on yeast biodiversity. Special thanks are extended to J.R. Nicoli, R.P. Martins, F.A. Silveira, M.A. Lachance, B.M. Borelli, A.O. Medeiros, L.C.R.S. Teixeira, I.C.A. Lacerda, B.S. Missagia, I.G. Gabler and F.C.O. Gomes for assistance during the review process of this book.

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Chapter 1

Yeast Biodiversity: How Many and How Much?

Marc-André Lachance

Department of Biology, University of Western Ontario, London, ON, Canada N6A 5B7 (e-mail: lachance@uwo.ca)

1.1 Introduction

Biodiversity is now a common word. Harper and Hawksworth (1995) tabulated the frequency of use of the term in *Biosis* and reported its first occurrence in 1988 followed by an increase to approximately 900 by 1994. A similar search of the PubMed database yielded a cumulative total of 1,361 hits by the end of 2003. By comparison, the number of articles using the word "yeast" is approaching 100,000. If the present trend continues, by the year 2016 searches for either word will produce in excess of 36,000 hits for that year only. The task at hand is to make similar predictions about yeast biodiversity.

Biodiversity means different things to different individuals. Gaston (1996) reviewed several definitions and concluded that the concept is an abstract expression of all aspects of the variety of life. Recent publications dealing with yeast *diversity*, had they appeared only 15 years earlier, might have used instead such terms as *taxonomy*, *ecology*, or *survey* (Nout et al. 1997; Buzzini and Martini 2000; Fell et al. 2000; Poliakova et al. 2001; Gadanho et al. 2003; Granchi et al. 2003; Lachance et al. 2003a; Ganga and Martinez 2004; Renker et al. 2004) or even *enzymology* (Lamb et al. 1999). The Convention on Biological Diversity (Anonymous 1992) defines biological diversity as "the variability among living organisms from all sources including, inter alia, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems." As with most things in our society, biodiversity became a tangible reality when it could be assigned a significant economic value. And as with most things in science, the recognition of biodiversity as a worthy research topic is predicated on measurability and the generation of testable hypotheses. The current urgency of the scientific study of biodiversity stems from the realization that only a small fraction (approximately 8%) of the total diversity of life is known (Stork 1999) and that species extinction is occurring at a measurable and increasing rate (Purvis and Hector 2000).

1.2 Measurement and Significance of Biodiversity

1.2.1 Levels of Diversity

The inclusion of any level of biological variation in the definition of biodiversity could lead to a trivialization of the concept, as variation is the very essence of biology. A more restrictive circumscription should limit the term to ecological and evolutionary variation. Theoretical ecologists, following the model of Whittaker (1960), often subdivide species diversity into three hierarchical components, namely within-sample (α), between-samples (β), and global (γ) diversity. These components may be considered additive ($\gamma = \alpha + \beta$, Crist et al. 2003). The main units of measurement are richness (simple species count) and heterogeneity (relative abundance of each species in a community). The two measures can be examined simultaneously in relative abundance plots, which contrast the number of species in a sample as a function of the number of individuals representing each species. Considerable interest in the underlying causes of such distributions was stimulated by the pivotal publication of MacArthur and Wilson's (1967) treatise on island biogeography. A recent model (Hubbell 2001) attributes a large portion of the species composition of a community to chance. Implicit to this view (but perhaps not sufficiently explicit) is that membership of a species in a community depends initially on its fundamental niche, in other words, the sum of its intrinsic properties. For example, the community of floral nectar rarely contains basidiomycetous yeasts. This is not due to chance, but to the fact that such a habitat favours fermentative, osmotolerant, copiotrophic species, which are found most often in the Saccharomycetales. In the neutral model, a community is seen as an assemblage of ecologically equivalent species, where the abundance of each species within a local community is not so dependent on the fundamental niche. Instead, species composition is affected by speciation in the metacommunity, the rate of influx of species, the size of the local community, and the local rate of extinction. This is almost entirely analogous to Kimura's (1983) neutral model of evolution. Natural selection remains the preliminary screen that causes rapid elimination of deleterious mutations and rapid fixation of adaptive alleles, just as the environment determines whether or not a species can enter a community. The majority of species in the community have already "passed the test" of selection, and are equally adapted. As is the case for selectively neutral mutations, the relative abundance of a species will be due not so much to some intrinsic property, but to chance.

Application of such theories to yeast communities is not yet completely practical. Most models of community ecology were developed for communities where members can be identified and enumerated rapidly, e.g., forest trees and insect biota. The recent development of identification methods based on DNA sequencing (Kurtzman and Robnett 1998; Fell et al. 2000) has not yet resulted in practical means of identifying yeasts instantly, in the field, but such technologies are no doubt forthcoming. An attempt to explore the factors that underlie community structure was made recently (Lachance et al. 2003a). The yeast biota of morning glory flowers and associated nitidulid beetles was characterized in a "forest island" (*kīpuka*) on the slope of the Mauna Loa volcano in Hawaii. The yeast community is highly

specialized, consisting almost entirely of members of two clades with affinities to *Metschnikowia* and *Wickerhamiella*: the former clade is vectored primarily by the beetles, and the latter by drosophilid flies. Although the resulting community is a mixture, each clade can be studied separately with selective media. The *Metschnikowia* clade members in the community consists of six species and whose frequencies follow the expected log series distribution, from abundant to rare. Two of the species (*Metschnikowia hawaiiensis* and *Candida kipukae*) are probably Hawaiian endemics. The others have also been found in Central America and are thought to have reached Hawaii in recent history. The six species are similar physiologically, suggesting that they might be mutually neutral with respect to niche. However, their distribution within the *kīpuka* is not random and follows closely the distribution of the host beetles. The latter consist of two major species, one Hawaiian endemic and one that was introduced in the early twentieth century. Maximum growth temperature and insect choice may be important factors in the local distribution, such that a completely neutral model would have to be ruled out. The study is in progress, and increased sampling is hoped to provide a test of the neutral hypothesis.

1.2.2 Diversity Within Species

Even if one agrees that species abundance is central to the characterization of biodiversity, genetic diversity is an essential feature of the species itself. Even orthodox proponents of the phylogenetic/autapomorphic species concept would have to agree that a "species" that is completely devoid of variation can hardly be regarded as a species (Wheeler and Meier 2000). Variation among members of a species has long plagued pragmatic systematists in their search for stable diagnostic (autapomorphic) characters (Lodder and Kreger-van Rij 1952). As DNA sequence analysis took the study of yeast diversity by storm, the recurring dream of an invariant species trait was temporarily rekindled (Kurtzman and Robnett 1998). However, the sequencing approach has in some instances brought to light considerable variability among individuals that share a common gene pool and thus are members of the same biological species. One response might be to denounce the biological species concept as antiquated and inoperable (Wheeler and Meier 2000). Another would be to accept that the genes that are most amenable to phylogenetic construction are not necessarily involved in conferring a common evolutionary destiny to members of a species, and that species cannot be defined on the basis of invariance in gene sequences.

In a study of the distribution of yeasts in seawater, Gadanho et al. (2003) subjected 234 isolates to microsatellite-primed PCR fingerprinting and demonstrated that in most cases multiple isolates of various basidiomycetous yeast species contain a substantial amount of genomic variation. Ascomycetous species recovered in that habitat exhibited less variation.

Intraspecific variability has been examined in the two species in the genus *Clavispora*, both of which occur in nature as heterothallic, haploid mating types. This offers the advantage that species boundaries can be assessed by mixing of compatible strains and observation of ascospores. *Clavispora opuntiae* has so far

been isolated exclusively from necrotic tissue or tunnels of moth larvae found in cacti. Hundreds of specimens have been recovered globally and preserved for study. Although the growth responses of most isolates are generally constant, polymorphisms have been detected at the level of the ribosomal DNA (rDNA) gene cluster (Lachance et al. 2000b). In some 500 isolates examined by restriction mapping, over 40 variants were recognized. These correlated to a large degree with geography, host plant species, and insect vectors. Most of the variation was shown by sequencing to be located in the intergenic spacer region, although a small amount of polymorphism was also detected in the large subunit rRNA gene. Strains representing the extremes of that variation had been shown previously to exhibit a lower degree of interfertility (Lachance et al. 1994) and perhaps represent the beginning of a speciation event.

C. lusitaniae is similar morphologically and physiologically to *C. opuntiae*, but exhibits much less habitat specificity, having been recovered in cactus fruit, agave rots, industrial wastes, clinical specimens, and several other sources. Mating compatibility and large subunit rDNA sequences were determined in 37 strains (Lachance et al. 2003c). The sequences could be assigned to ten types belonging to two families that differed by as much as 32 substitutions in the D2 domain. The variation was not correlated with mating intensity or abundance of mature asci.

Although these studies do not allow generalizations about the evolutionary or ecological significance of genetic diversity within yeast species, they would seem to support the view that variability is an intrinsic property of species.

1.2.3 Species Diversity

From the first to the current edition of the *The yeasts, a taxonomic study*, the number of species described has grown from 164 in 1952, to 349 in 1970, to 500 in 1984, and to 700 in 1998 (Lodder 1970; Kurtzman and Fell 1998). Extrapolation of these numbers leads to the prediction that an eventual 2016 edition would contain approximately 1,000 species. However, this number may very well be exceeded in the forthcoming fifth edition, planned for 2005. The increase is due to several factors, including methodology and species concepts. In the first edition (Lodder and Kreger-van Rij 1952), species were circumscribed on the basis of morphology and a small number of growth tests. The doubling in the number of species found in the second edition was due in part to the use of a much larger battery of nutritional properties. Early application of molecular approaches had a considerable impact on the third edition, but was not entirely accountable for species proliferation, as the shift to a genomic basis for species delineation also caused the merger of physiological or morphological variants into larger and more diverse species. The publication of the fourth edition coincided with early application of DNA sequencing in yeast identification and phylogenetic reconstruction, although the full impact of this approach came later. Again, the result is a mixture of species fusions and subdivisions.

The definition of species is fundamental in the generation of meaningful estimations of biodiversity, which accounts in part for the heartiness of the debate on that subject (Wheeler and Meier 2000). The species problem as it applies to bacterial and

fungal diversity has been discussed by O'Donnell et al. (1995), who pointed out the lack of a common standard. Although species concept controversies are not alien to yeast systematics, many practitioners agree that species should, whenever possible, represent cohesive evolutionary units. Individual researchers may disagree on how best to document the boundaries of such units, but the result is nonetheless a relatively stable consensus. As the issue is far too complex to be examined here in detail, it will be expedient to assume, rightly or wrongly, that taxa which are recognized at any given time constitute genuine and meaningful species.

Sequence analysis resulted in an enormous increase in the ease and speed of identification, making intense biodiversity surveys almost manageable. Many species descriptions currently being published come from material collected in the past and stored in collections in the hope that new technologies would eventually facilitate meaningful species assignments. The sequencing approach has fulfilled this need. Unfortunately, the clarifications brought forward by sequencing have done little to improve our understanding of the natural history or ecology of the species being described. Unless the ecological context of species is also documented, Linnean binomials will remain no more than mere labels of little relevance to biodiversity. By their very nature as unicellular heterotrophs, yeasts are inexorably dependent on other fungi, bacteria, animals, and plants for their existence, and ideally species descriptions should include data on these interactions. The old precept, "everything is everywhere", although no longer tenable, sadly continues to influence yeast taxonomy. An inordinate amount of energy is devoted to transforming sequence data into "correct" trees, at the expense of the yeasts themselves and their biology. Another important consequence of the dependence of yeasts on other life forms is the urgency of documenting their natural history before their very habitats disappear. Unless conservation efforts are intensified, it will become easier to determine the rate of extinction of yeasts than to estimate the number of extant species. The fact that the construction of a comprehensive inventory of life on Earth is seen as a priority by an increasing number of researchers, governments, and granting agencies (Mulongoy et al. 1999) should be viewed with optimism. Equally encouraging is the emergence of more frequent studies aimed at characterising whole yeast communities in relation to their insect vectors. A case in point is a recent description of 16 closely related species originating from fungivorous beetles and their habitats (Suh et al. 2004). Members of the Coleoptera associated with tree decay have long been known to harbour numerous yeast species, as evidenced by the work of pioneers such as L.J. Wickerham, J.P. van der Walt, and H.J. Phaff. These yeasts are suspected to engage in intimate symbiotic relationships with insects, although the nature of the interaction remains elusive in most cases. Recent studies of yeasts found in tropical bees led to the discovery of the genus *Starmerella* (Rosa and Lachance 1998), the nucleus being a growing clade whose membership has increased from 12 described species in 1998 to 29 putative species at the last published count (Rosa et al. 2003). Studies of nitidulid beetles associated with ephemeral flowers have resulted in the near doubling of described species of *Metschnikowia* (Lachance et al. 2003b) and a significant expansion of the formerly monotypic genera *Kodamaea* (Lachance et al. 1999) and *Wickerhamiella* (Lachance et al. 2000a).

1.3 Predicting the Number of Yeast Species

Hawksworth (1991) attempted to predict the number of fungal species on the basis of an estimated 69,000 described species and the ratio of fungi to other life forms. By reference to vascular plants, he proposed a conservative estimate of 1.5 million fungal species, and a comparison with insect species extended the range to three million. The proportion of fungi described (in 1991) was thus thought to be approximately 5% of the total fungal biota. A hasty transposition of Hawksworth's reasoning to the yeasts generates a forecast of approximately 12,000 species.

Hughes et al. (2001) pointed out that microbes may be too diverse to enumerate exhaustively and argued for a statistical approach. One tool used in reaching this objective is the accumulation curve, where species abundance is plotted as a function of sampling intensity. Species sampling follows a rarefaction pattern in which the rate of increase in the detection of species obeys the law of diminishing returns. Well-sampled habitats produce curves that can be fitted to saturation models such as the Michaelis–Menten equation or the negative-exponential function, characterized by growth towards an asymptotic maximum. Poorly sampled habitats produce nearly linear curves. Gadanho et al. (2003) applied this approached to yeasts in seawater and estimated that the 31 species recovered represented approximately 60% of the existing species in their study site. In order to extrapolate beyond a single site, Lachance (2000a) used random internal sampling of collection records to generate accumulation curves. The data were fitted to trend line functions available in Microsoft Excel and the curves were extrapolated to large sample sizes. For insects associated with ephemeral flowers, data from eight localities worldwide (26 yeast species in total) led to the prediction that sampling from 50 localities would raise the number to 42 species. Simulations based on yeasts from tree fluxes were validated by predicting, from eight samples, the total number of yeast species (45) present in 47 actual samples. Extrapolation to 1,000 samples predicted that the number of species would rise to approximately 500. In Fig. 1.1, the same data were analysed using the Michaelis–Menten model. Linear regressions of the double-reciprocal plots of species richness as a function of sampling intensity predict asymptotic maxima of 40 species for floricolous insects and 182 species for tree fluxes, which is not entirely inconsistent with the previous predictions. Using these numbers and an estimate of 1,000 currently described yeast species, a simple rule of 3 predicts that the number of yeast species on Earth is in the order of 1,500 on the basis of the insect model, and 15,000 on the basis of the flux model. The lower value comes from a highly specific yeast community, whereas the upper boundary is characteristic of a more generalistic community. Other well-sampled, highly specific communities follow a conservative pattern similar to that of flower insects. Calderone (2002) recognised 13 *Candida* species as human pathogens compared with eight in 1988. The highly specialized nature of yeasts associated with humans, combined with the extremely high sampling intensity makes the current number of species a good approximation of the saturation point. In the case of the moderately specific community of yeasts associated with necrotic cacti, 3,701 samples yielded fewer than 80 species (Starmer et al. 1990).

The broad range of predictions from 1,500 to 15,000 yeast species in total reflects the fact that the average degree of specificity for all yeast communities is not known.

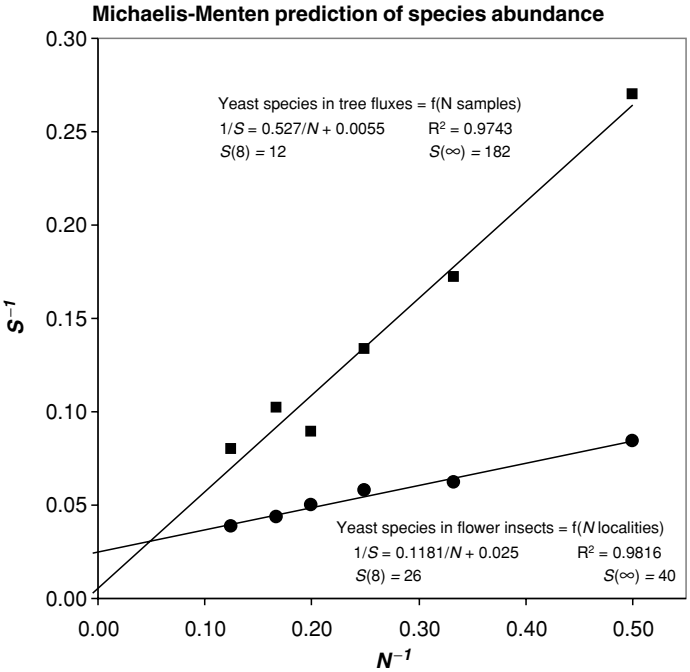

Fig. 1.1. Prediction of yeast species abundance in tree sap fluxes and in insects associated with morning glories based on pseudoreplicated collections from N=8 localities. As the data follow Michaelis–Menten kinetics, double reciprocal plots were used to predict $S(\infty)$, the number of species that would be found in similar habitats in an infinite number of localities. The corresponding linear equations and their regression coefficients are given. The equation S (total) = $1000(S(\infty)/S(8))$ was used to estimate of the total number of extant yeast species assuming that the global depletion curve is similar, which of course may not be the case

Furthermore, the numbers and kinds of habitats remaining to be studied are unknown. As floricolous insect and tree flux communities are greatly affected by the activity of members of Coleoptera, whose number of described species is in the order of 350,000, one might predict similar numbers for yeasts; however, the proportion of beetles that harbour yeasts remains to be established. Although tree-boring species and floricolous nitidulids frequently contain yeasts, the very speciose family Chrysomelidae seldom does. It is therefore not reasonable to assume that the number of yeasts is commensurate to that of beetles as a whole.

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Yeast Systematics and Phylogeny – Implications of Molecular Identification Methods for Studies in Ecology

Cletus P. Kurtzman[1] and Jack W. Fell[2]

[1] *Microbial Genomics and Bioprocessing Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, 1815 N. University St., Peoria, IL 61604, USA*
(e-mail: kurtzman@ncaur.usda.gov)
[2] *Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, University of Miami, Key Biscayne, FL 33149, USA*
(e-mail: jfell@rsmas.miami.edu)

2.1 Introduction

A major factor that determines the validity of studies in yeast ecology is the correct identification of species in the ecosystem. Before the present era of yeast taxonomy, which uses gene sequences and other molecular criteria, identifications were of necessity based on phenotypic tests. Although phenotype can sometimes be used to correctly identify species, molecular comparisons have shown that many earlier identifications based on phenotype have been incorrect. While this does not mean that earlier work in yeast ecology is invalid, it does say that conclusions drawn from this work may need to be reexamined following more accurate identification of species. In particular, the often-asked question "Is everything everywhere?" cannot be adequately addressed until taxa are correctly identified. In this chapter, we will discuss molecular methods now used for identification of yeasts, what we perceive of their genetic resolution, their impact on systematics, and finally a description of some of the rapid molecular methods that are applicable to the large species populations often examined in ecological studies.

2.2 Molecular Identification of Species

The transition from phenotypic identification of yeasts to molecular identification began with determination of the mole percent guanine (G) plus cytosine (C) ratios of nuclear DNA. These analyses demonstrated that ascomycetous yeasts range from approximately 28 to 50 mol% G+ C, whereas basidiomycetous yeasts range from approximately 50 to 70 mol% G+ C. Depending on the analytical methods

used, strains differing by 1–2 mol% are recognized as separate species (Price et al. 1978; Kurtzman and Phaff 1987). The need for quantitative assessment of genetic similarity between strains and species was satisfied, in part, by the technique of nuclear DNA reassociation or hybridization. DNA from the species pair of interest is sheared, mixed, made single-stranded, and the degree of relatedness determined from the extent of reassociation. Many different methods are used to measure this process, which can be done spectrophotometrically or through use of radioisotopes or other markers (Kurtzman 1993a).

A major question has been how to interpret DNA reassociation data. Measurements of DNA complementarity are commonly expressed as percent relatedness. This usage can be misleading because DNA strands must show at least 75–80% base sequence similarity before duplexing can occur and a reading is registered on the scale of percent relatedness (Bonner et al. 1973; Britten et al. 1974). Experimental conditions can greatly influence the extent of duplex formation, but under optimum conditions, different methods of assessing DNA relatedness do give essentially the same result (Kurtzman 1993a). Percent DNA relatedness provides an approximation of overall genome similarity between two organisms, but the technique does not detect single gene differences or exact multiples of ploidy, although aneuploidy can sometimes be detected (Vaughan-Martini and Kurtzman 1985).

On the basis of shared phenotype, strains that showed 80% or greater nuclear DNA relatedness were believed to represent members of the same yeast species (Martini and Phaff 1973; Price et al. 1978). This issue was also examined on the basis of the biological species concept (Dobzhansky 1976), asking what is the fertility between strains showing varying degrees of DNA relatedness (Kurtzman 1984a, b, 1987; Kurtzman et al. 1980a, b). In one of these studies, the heterothallic species *Pichia amylophila* and *P. mississippiensis*, which showed 25% DNA relatedness, gave abundant interspecific mating, but ascus formation was limited and no ascospores were formed. Similar results were found for crosses between *P. americana* and *P. bimundalis* (21% DNA relatedness) and between *P. alni* and *P. canadensis* (*Hansenula wingei*), the latter pair showing just 6% DNA relatedness. The varieties of *Issatchenkia scutulata*, which exhibit 25% DNA relatedness, behaved somewhat differently. Crosses between *I. scutulata* var. *scutulata* and *I. scutulata* var. *exigua* gave an extent of mating and ascospore formation comparable to that of intravarietal crosses. Ascospore viability from these intervarietal crosses was about 5%, but sib-matings of the progeny had 17% ascospore viability. However, backcrosses to the parentals gave poor ascosporulation and very low viability, which suggests that these two varieties represent separate species. *Williopsis saturnus* is a homothallic species with five varieties that range in DNA relatedness from 37 to 79% (Kurtzman 1987). Intervarietal fertility is reduced and varies depending on the strains crossed. Consequently, the preceding studies show that mating among heterothallic as well as homothallic taxa can occur over a wide range of DNA relatedness values, but that highly fertile crosses, which demonstrate conspecificity, seem to require 70–80% or greater DNA relatedness between strains. Because species barriers are complex and involve a number of factors, the numerical range of 70–100% DNA relatedness as indicative of conspecificity should be viewed as a prediction.

Nuclear DNA reassociation studies have had a marked impact on recognizing yeast species, but the method is time consuming and the extent of genetic resolution goes no further than that of closely related species. Gene sequencing offers a rapid method for recognizing species and resolution is not limited to closely related taxa. Peterson and Kurtzman (1991) determined that domain 2 of large subunit (26S) ribosomal RNA (rRNA) was sufficiently variable to resolve individual species. Kurtzman and Robnett (1998) expanded the preceding work by sequencing both domains 1 and 2 (approximately 600 nucleotides) of 26S ribosomal DNA (rDNA) for all known ascomycetous yeasts, thus providing a universally available database for rapid identification of known species, the detection of new species, and initial phylogenetic placement of the species. Fell et al. (2000) published the D1/D2 sequences of known basidiomycetous yeasts, thus completing the database for all known yeasts. Resolution provided by the D1/D2 domain was estimated from comparisons of taxa determined to be closely related from genetic crosses and from DNA reassociations. In general, strains of a species show no more than zero to three nucleotide differences (0–0.5%), and strains showing six or more noncontiguous substitutions (1%) are separate species. Strains with intermediate nucleotide substitutions are also likely to be separate species. One impact of the D1/D2 database has been to permit detection of a large number of new species, which has resulted in a near doubling of known species since publication of the most recent edition (fourth) of *The yeasts, a taxonomic study* (Kurtzman and Fell 1998). Another use is that the nontaxonomist can now quickly and accurately identify most known species, as well as recognize new species, by sequencing approximately 600 nucleotides and doing a BLAST search in GenBank.

The internal transcribed spacer regions ITS1 and ITS2, which are separated by the 5.8S gene of rDNA, are also highly substituted and often used for species identification, but for many species, ITS sequences give no greater resolution than that obtained from 26S domains D1/D2 (James et al. 1996; Kurtzman and Robnett 2003). However, Fell and Blatt (1999) were able to resolve cryptic species in the *Xanthophyllomyces dendrorhous* species complex that had been unresolved from D1/D2 sequence analysis, and Scorzetti et al. (2002) reported ITS sequences to provide somewhat greater resolution among many basidiomycetous species than was found for D1/D2, although, a few species were less well resolved by ITS than by D1/D2. Consequently, it appears useful to sequence both D1/D2 and ITS when comparing closely related species. The intergenic spacer (IGS) region of rDNA tends to be highly substituted and sequences of this region have been used with good success to separate closely related lineages of *Cryptococcus* (Fan et al. 1995; Diaz et al. 2000), *Xanthophyllomyces* (Fell and Blatt 1999), *Mrakia* (Diaz and Fell 2000) and *Saccharomyces* (Kurtzman et al., unpublished). Because of the occurrence of repetitive sequences and homopolymeric regions, the IGS region tends to be difficult to sequence for some species. Small subunit (18S) rDNA, which has been extremely important in broad-based phylogenetic analyses, is generally too conserved to allow separation of individual species (James et al. 1996; Kurtzman and Robnett 2003).

The focus of our discussion on species identification from gene sequences has been on rDNA. A major advantage of rDNA is that it is present in all living organisms, has a common evolutionary origin, occurs as multiple copies and is easy

to sequence because primer pairs for conserved regions can generally be used for all organisms. However, gene sequences other than those of the rDNA repeat have been used for separation of species from many kinds of fungi (Geiser et al. 1998; O'Donnell et al. 2000), including the yeasts. Belloch et al. (2000) demonstrated the utility of cytochrome oxidase II for resolution of *Kluyveromyces* species, Daniel et al. (2001) successfully used actin-1 for species of *Candida*, and Kurtzman and Robnett (2003) showed the usefulness of elongation factor 1-α and RNA polymerase II for resolution of *Saccharomyces* species. At present, the main impediment to widespread use of gene sequences other than rDNA is developing sequencing primers that are effective for essentially all species, and construction of databases that include sequences from all known species. Daniel et al. (2001) and Daniel and Meyer (2003) have made considerable progress in development of an actin sequence database for species identification, although no primer set has been effective for all species, thus requiring additional primers to obtain these sequences. The need for multiple primers seems to be a problem common to sequencing of protein encoding genes because of frequent nucleotide substitutions. Resolution of taxa from actin is somewhat greater than from D1/D2, but not surprisingly, clear separation of closely related species is not always certain.

Separation of species using single gene sequences is not always reliable. Different lineages may vary in their rates of nucleotide substitution for the diagnostic gene being used, thus confusing interpretation of genetic separation, and hybrids are common and appear to be part of the speciation process. For example, Vaughan-Martini and Kurtzman (1985) proposed from DNA reassociation studies that *Saccharomyces pastorianus* is a natural hybrid of *S. cerevisiae* and *S. bayanus*. Peterson and Kurtzman (1991) confirmed the proposal by showing that the D2 domain rRNA sequence of *S. pastorianus* is identical to that of *S. bayanus,* but divergent from *S. cerevisiae*. The three varieties of *Candida shehatae* may also represent hybrids, or are examples of a lineage with a slow rate of nucleotide substitution in the rDNA. From DNA reassociation, the varieties show approximately 50% relatedness, but they have essentially identical domain 2 large subunit sequences (Kurtzman 1990). Groth et al. (1999) discovered a natural chimeric isolate of *Saccharomyces* with genetic material from three species, and Nilsson-Tillgren et al. (1981) presented evidence that several natural and industrial yeast strains are hybrids. Kurtzman et al. (2005) reported that *Kazachstania heterogenica* appears to be a natural hybrid that shares an RNA polymerase II gene with *K. pintolopesii*. In an additional study, Lachance et al. (2003) found interfertile strains of *Clavispora lusitaniae* that are highly polymorphic in the D1/D2 domain. Detection of unexpected divergence in a gene sequence should be possible from its lack of congruence with other gene sequences. Single gene sequences are extremely useful for rapid species identification, but from the foregoing examples, caution in interpretation of species identity is required.

Other molecular-based methods commonly used for species identification include species-specific primer pairs and probes, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP), and karyotyping. Species-specific primers are effective when used for PCR-based identifications involving a small number of species or

when a particular species is the subject of the search (Fell 1993; Mannarelli and Kurtzman 1998). Otherwise, there is the likelihood that PCR mixtures containing large numbers of species-specific primer pairs will lead to uncertain banding patterns. Microsatellite-primed RAPDs (Gadanho et al. 2003) and AFLP fingerprints (de Barros Lopes et al. 1999) have been effectively used in some laboratories. One concern in using these latter two techniques is reproducibility between laboratories because small differences in PCR conditions may impact the species-specific patterns that serve as a reference. Karyotyping with pulsed-field electrophoresis and RAPD on mitochondrial DNA can serve in the initial characterization and identification of yeast species. However, the interpretation of the chromosome band patterns and mitochondrial restriction fragments for taxonomic purposes is complicated by the high degree of polymorphism, such as chromosomal rearrangements, within some yeast taxa (Spírek et al. 2003).

2.3 Molecular Phylogeny and Systematics of the Yeasts – an Overview

In the previous section, we discussed various molecular methods for species identification. In addition, many phylogenetic relationships among the yeasts and other fungi have been resolved from analysis of gene sequence divergence. These studies presume that horizontal gene transfer among different lineages has been limited, which can be tested by comparing the congruence of phylogenies derived from different genes. Most of the analyses have used rDNA sequences, but there are generally no major differences in tree topologies whether the analyses are from rDNA sequences or from those of other genes (Geiser et al. 1998; Liu et al. 1999; O'Donnell et al. 2000; Kurtzman and Robnett 2003). Although phylogenetic trees derived from analyses of various genes are generally congruent, support for basal lineages from single gene analyses is often weak (Kurtzman and Robnett 2003; Rokas et al. 2003). Because of this weak support, branching order is uncertain, leading to ambiguity of what constitutes a genus, a family or an order. Hawksworth et al. (1995) addressed this issue in part by stating "there are no universally applicable criteria by which genera are distinguished, but in general the emphasis is now on there being several discontinuities in fundamental characters ...". Many systematists now regard these fundamental characters as gene sequences. However, a number of factors impact our recognition of genera and higher levels of classification. Phylogenetic trees determined from single genes are seldom robust, leading to uncertainty whether neighboring species groups are a separate genus or members of a more broadly based genus. Multigene analyses generally strengthen support for basal lineages. Kurtzman and Robnett (2003) examined relationships among the approximately 80 species of the "*Saccharomyces* complex" from multiple genes. Combined analysis of 18S, 26S, 5.8S/conserved ITS and mitochondrial small subunit rDNAs with elongation factor 1-α and cytochrome oxidase II gave high bootstrap support for moderately deep lineages, which were interpreted as genus-level, but not for more basal lineages.

Rokas et al. (2003) screened the published genome sequences from seven *Saccharomyces* species and that of *Candida albicans* and selected 106 widely distributed

orthologous genes for phylogenetic analysis. The resulting analyses showed that a dataset comprising a concatenation of a minimum of nearly any 20 genes gave well-supported trees that were comparable to those of a dataset comprising 106 genes. This work clearly illustrates that a much larger number of genes is required for reconstructing phylogenies than is currently being analyzed in most laboratories. Whether 20 gene sequences will strongly resolve species clades larger than *Saccharomyces* needs to be determined. However, partial genome sequence analysis appears sufficient to resolve phylogenetic relationships within different groups of yeasts. Another factor that impacts resolution, as well as circumscription of genera, is the issue of missing taxa. It seems likely that fewer than 1% of extant species are known, which can be inferred from the high frequency of long single-species branches in phylogenetic trees. Consequently, the majority of the yeasts are yet to be discovered and characterized, and their addition to future phylogenetic analyses is likely to influence our perception of genera, even those that are presently circumscribed from multigene analyses.

2.4 Ascomycetous Yeasts

The distinction between yeasts and dimorphic filamentous fungi has often been uncertain. Some authorities have viewed the yeasts as primitive fungi, whereas others perceived them to be reduced forms of more evolved taxa (Cain 1972; Redhead and Malloch 1977). Phylogenetic analyses of rDNA sequences demonstrated the ascomycetous yeasts, as well as yeast-like genera such as *Ascoidea* and *Cephaloascus*, to comprise a clade that is a sister group to the "filamentous" ascomycetes (euascomycetes). *Schizosaccharomyces*, *Taphrina*, *Protomyces*, *Saitoella, Pneumocystis,* and *Neolecta*, a mushroom-like fungus, form a divergent clade basal to the yeast-euascomycete branch (Hausner et al. 1992; Hendriks et al. 1992; Kurtzman 1993b; Nishida and Sugiyama 1993; Wilmotte et al. 1993; Kurtzman and Robnett 1994, 1995, 1998; Landvik 1996; Sjamsuridzal et al. 1997; Sugiyama 1998; Kurtzman and Sugiyama 2001). Nishida and Sugiyama (1994) have termed the basal ascomycete clade the "archiascomycetes." Some members of the yeast clade, such as certain species of *Ascoidea* and *Eremothecium,* show no typical budding, whereas budding is common among the so-called black yeasts in the genera *Aureobasidium* and *Phialophora*, as well as in certain other dimorphic euascomycete genera. Similarly, vegetative reproduction by fission is shared by *Dipodascus* and *Galactomyces*, members of the yeast clade, as well as by the distantly related genus *Schizosaccharomyces*. Consequently, yeasts cannot be recognized solely on the basis of the presence or the absence of budding, but with a few exceptions, ascomycetous yeasts can be separated phenotypically from euascomycetes by the presence of budding or fission and the formation of sexual states unenclosed in a fruiting body.

During the past 10 years, the widespread use of molecular taxonomic methods has resulted in the discovery and description of a large number of new taxa, bringing the total of ascomycetous species to nearly 1,000. Many of these new species are readily detected by sequencing a single species-resolving gene, such as domains D1/D2 of large subunit rDNA, keeping in mind the exceptions discussed in the previous section. If we accept that fewer than 1% of extant species are known and that current sequencing technologies allow rapid detection of new species, the limiting

factor for presenting new species is the time required for formal description, which includes information on vegetative and sexual states, fermentation and assimilation reactions, and ecology, where known.

From single gene analyses, such as the D1/D2 phylogenetic trees presented by Kurtzman and Robnett (1998), it is apparent that many of the ascomycetous yeast genera are not well circumscribed, but actual boundaries are often not clear. Multigene sequence analyses have been applied to just a few genera, such as those of the "*Saccharomyces* complex", which includes *Saccharomyces*, *Kluyveromyces*, *Tetrapisispora*, *Torulaspora*, and *Zygosaccharomyces*, as well as the neighboring genera *Eremothecium*, *Hanseniaspora*, and *Saccharomycodes* (Kurtzman and Robnett 2003). In this multigene study, approximately 80 species were compared from the combined signal of seven genes. The analysis gave 14 phylogenetically defined clades, most of which had strong bootstrap support. From this study, the major genera *Saccharomyces*, *Kluyveromyces*, and *Zygosaccharomyces* were shown to be polyphyletic, leading to reclassification of certain of the species in the new genera *Naumovia*, *Nakaseomyces*, *Vanderwaltozyma*, *Zygotorulaspora*, and *Lachancea*, and expansion of the earlier described genus *Kazachstania* (Kurtzman 2003) (Fig. 2.1). Lineages basal to the branches supporting the 14 clades generally had low bootstrap support, leaving uncertain the genetic relationships among the genera. The genus *Eremothecium* appears separate from the family *Saccharomycetaceae* and was maintained in the *Eremotheciaceae*. Similarly, the sister genera *Hanseniaspora* and *Saccharomycodes*, which reproduce by bipolar budding rather than multilateral budding typical of the *Saccharomycetaceae*, were retained in the family *Saccharomycodaceae*. As demonstrated from this analysis, as well as that of Rokas et al. (2003), a relatively large number of gene sequences will be required to understand phylogenetic relationships among the yeasts. Currently accepted ascomycetous yeast genera are listed in Table 2.1 with their proposed assignments to orders and families. Because of weak basal resolution in phylogenetic trees, many of the genera cannot be reliably assigned to families. Furthermore, on the basis of the large amount of phylogenetic divergence conveyed by present datasets, it seems likely that many new families will need to be described.

From D1/D2 sequence analysis, the greater than 100 species assigned to the genus *Pichia* are seen to be distributed across the *Saccharomycetales* (Kurtzman and Robnett 1998). Major species groups in *Pichia* are centered on *P. membranifaciens*, *P. anomala*, and *P. angusta* (*Hansenula polymorpha*), the latter species representing the majority of methanol-assimilating taxa. Some of the species will be maintained in *Pichia* and some will need to be placed in new genera as stronger datasets become available. A few of the outlying species have already been assigned to new genera. *P. pastoris*, the outlying member of the methanol-assimilating yeasts, was transferred to *Komagataella* (Yamada et al. 1995a), and support for this genus as a distinct clade recently increased with the discovery of two additional species of *Komagataella* (Dlauchy et al. 2003; Kurtzman 2005). *P. burtonii*, now transferred to *Hyphopichia*, is phylogenetically distant from the three main clades of *Pichia*, as are the D-xylose-fermenting species *P. stipitis* and *P. segobiensis*. An additional change was the assignment of *P. ohmeri* to the genus *Kodamaea* (Yamada et al. 1995b). Support for this genus has increased with the discovery of additional species closely related to

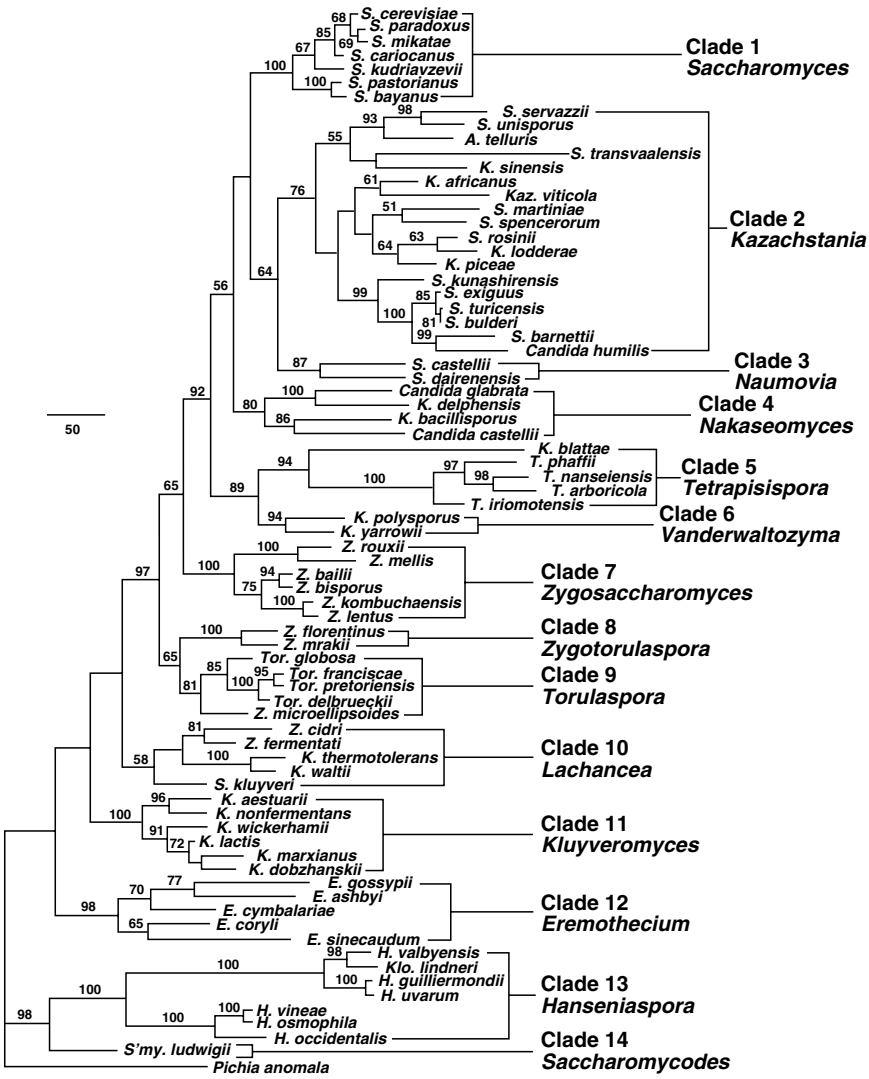

Fig. 2.1. Maximum parsimony tree resolving species of the "*Saccharomyces* complex" into 14 clades, which are interpreted as phylogenetically circumscribed genera. The analysis resulted in the description of five new genera. Earlier generic assignments are given for each species. This phylogenetic tree was derived from analysis of a dataset comprised of nucleotide sequences from 18S, 5.8S/alignable ITS, and 26S (three regions) *rDNAs*, elongation factor 1-α, mitochondrial small subunit rDNA and COXII. Branch lengths are based on nucleotide substitutions as indicated by the *bar*, and bootstrap values under 50% are not given. *Pichia anomala* is the outgroup species in the analysis. (Modified from Kurtzman 2003; Kurtzman and Robnett 2003)

Table 2.1 Classes, orders and families of yeasts and yeast-like genera of the *Ascomycota*

Neolectomycetes
- *Neolectales* Landvik, O.E. Eriksson, Gargas & P. Gustafsson
  - *Neolectaceae* Redhead
    - *Neolecta* Spegazzini (T)

Pneumocystidomycetes
- *Pneumocystidales* O.E. Eriksson
  - *Pneumocystidaceae* O.E. Eriksson
    - *Pneumocystis* P. Delanöe & Delanöe (A)

Schizosaccharomycetes
- *Schizosaccharomycetales* Prillinger, Dörfler, Laaser, Eckerlein & Lehle ex Kurtzman
  - *Schizosaccharomycetaceae* Beijerinck ex Klöcker
    - *Schizosaccharomyces* Lindner (T)

Taphrinomycetes
- *Taphrinales* Gäumann & C.W. Dodge
  - *Protomycetaceae* Gray
    - *Burenia* M.S. Reddy & C.L. Kramer (T)
    - *Protomyces* Unger (T)
    - *Protomycopsis* Magnus (T)
    - *Saitoella* S. Goto, Sugiyama, Hamamoto & Komagata (A)
    - *Taphridium* Lagerheim & Juel ex Juel (T)
    - *Volkartia* Maire (T)
  - *Taphrinaceae* Gäumann & C.W. Dodge
    - *Lalaria* R.T. Moore (A)
    - *Taphrina* Fries (T)

Saccharomycetes
- *Saccharomycetales* Kudryavtsev
  - *Ascoideaceae* J. Schröter
    - *Ascoidea* Brefeld & Lindau (T)
  - *Cephaloascaceae* L.R. Batra
    - *Cephaloascus* Hanawa (T)
  - *Dipodascaceae* Engler & E. Gilg
    - *Dipodascus* Lagerheim (T)
    - *Galactomyces* Redhead & Malloch (T)
    - *Geotrichum* Link:Fries (A)
  - *Endomycetaceae* J. Schröter
    - *Endomyces* Reess (T)
    - *Helicogonium* W.L. White (T)
    - *Myriogonium* Cain (T)
    - *Phialoascus* Redhead & Malloch (T)
  - *Eremotheciaceae* Kurtzman
    - *Coccidiascus* Chatton emend. Lushbaugh, Rowton & McGhee (T)
    - *Eremothecium* Borzi emend. Kurtzman (T)
  - *Lipomycetaceae* E.K. Novak & Zsolt
    - *Babjevia* van der Walt & M.Th. Smith (T)
    - *Dipodascopsis* Batra & Millner (T)
    - *Lipomyces* Lodder & Kreger van Rij (T)
    - *Myxozyma* van der Walt, Weijman & von Arx (A)
    - *Zygozyma* van der Walt & von Arx (T)
  - *Metschnikowiaceae* T. Kamienski
    - *Clavispora* Rodrigues de Miranda (T)
    - *Metschnikowia* T. Kamienski (T)

Continues

Table 2.1 Classes, orders and families of yeasts and yeast-like genera of the *Ascomycota*—*cont'd*

Pichiaceae Zender
- *Brettanomyces* Kufferath & van Laer (A)
- *Dekkera* van der Walt (T)
- *Pichia* Hansen (*pro parte*) (T)
- *Saturnispora* Liu & Kurtzman (T)

Saccharomycetaceae G. Winter
- *Kazachstania* Zubkova (T)
- *Kluyveromyces* Kurtzman, Lachance, Nguyen & Prillinger (T)
- *Lachancea* Kurtzman (T)
- *Nakaseomyces* Kurtzman (T)
- *Naumovia* Kurtzman (T)
- *Saccharomyces* Meyen ex Reess (T)
- *Tetrapisispora* Ueda-Nishimura & Mikata (T)
- *Torulaspora* Lindner (T)
- *Vanderwaltozyma* Kurtzman (T)
- *Zygosaccharomyces* Barker (T)
- *Zygotorulaspora* Kurtzman (T)

Saccharomycodaceae Kudryavtsev
- *Hanseniaspora* Zikes (T)
- *Kloeckera* Janke (A)
- *Saccharomycodes* Hansen (T)

Saccharomycopsidaceae von Arx & van der Walt
- *Saccharomycopsis* Schiönning (T)

Saccharomycetales incertae sedis
- *Aciculoconidium* King & Jong (A)
- *Ambrosiozyma* van der Walt (T)
- *Arxula* van der Walt, M.Th. Smith & Y. Yamada (A)
- *Ascobotryozyma* J. Kerrigan, M.Th. Smith & J.D. Rogers (T)
- *Blastobotrys* von Klopotek (A)
- *Botryozyma* Shann & M.Th. Smith (A)
- *Candida* Berkhout (A)
- *Citeromyces* Santa María (T)
- *Cyniclomyces* van der Walt & Scott (T)
- *Debaryomyces* Lodder & Kreger-van Rij (T)
- *Hyphopichia* von Arx & van der Walt (T)
- *Kodamaea* Y. Yamada, T. Suzuki, Matsuda & Mikata emend. Rosa, Lachance, Starmer, Barker, Bowles & Schlag-Edler (T)
- *Komagataella* Y. Yamada, Matsuda, Maeda & Mikata (T)
- *Kuraishia* Y. Yamada, Maeda & Mikata (T)
- *Lodderomyces* van der Walt (T)
- *Macrorhabdus* Tomaszewski, Logan, Snowden, Kurtzman & Phalen (A)
- *Nadsonia* Sydow (T)
- *Nakazawaea* Y. Yamada, Maeda & Mikata (T)
- *Ogataea* Y. Yamada, Maeda & Mikata (T)
- *Pachysolen* Boidin & Adzet (T)
- *Phaffomyces* Y. Yamada, Higashi, S. Ando & Mikata (T)
- *Schizoblastosporion* Ciferri (A)
- *Sporopachydermia* Rodrigues de Miranda (T)
- *Starmerella* Rosa & Lachance (T)
- *Starmera* Y. Yamada, Higashi, S. Ando & Mikata (T)
- *Stephanoascus* M. Th. Smith, van der Walt & Johannsen (T)
- *Sympodiomyces* Fell & Statzell (A)

Table 2.1 Classes, orders and families of yeasts and yeast-like genera of the *Ascomycota*—*cont'd*

Trichomonascus Jackson (T)
Trigonopsis Schachner (A)
Wickerhamia Soneda (T)
Wickerhamiella van der Walt (T)
Yamadazyma Billon-Grand emend. M. Suzuki, Prasad & Kurtzman (T)
Yarrowia van der Walt & von Arx (T)
Zygoascus M.Th. Smith (T)

[1](A) = Anamorphic genus, (T) = Telemorphic genus.
[2]Anamorphic and teleomorphic genera are placed together in the same family when relationships are known. For many anamorphic and teleomorphic genera, phylogenetic relationships are unclear and the genera are placed in Saccharomycetales *incertae sedis* until family relationships become known.

K. ohmeri (Rosa et al. 1999). On the basis of single gene analyses, species of the *Lipomycetaceae* and such genera such as *Yarrowia*, *Citeromyces*, and *Saccharomycopsis* appear to be natural groups. *Metschnikowia*, which is characterized by elongated, needlelike ascospores, is represented by a large number of phylogenetically divergent species, but molecular data are insufficient to determine if the genus is monophyletic. Consequently, multigene sequence analysis will be required to resolve relationships between the preceding genera as well as for determining relationships within the genera.

2.5 Basidiomycetous Yeasts

The division *Basidiomycota* is a group of approximately 30,000 described species, with a distinct sexual cycle that includes the production of spores on a clublike structure (basidium). The majority of the species, which are easily recognized as mushrooms, bracket fungi, rusts, and smuts, produce filamentous hyphae and do not have a yeast phase. The recognition of a phylogenetic connection between yeasts and basidiomycetes was slow to evolve. An initial observation of the presence of ballistoconidia led Kluyver and van Niel (1924, 1927) to suggest that *Sporobolomyces* was related to the basidiomycetes. An often overlooked basidiomycete connection was provided by Nyland's (1949) description of the teliosporic genus *Sporidiobolus*. Subsequently, Banno's (1967) description of a teliosporic life cycle in *Rhodosporidium toruloides* gave a solid recognition to the presence of basidiomycetes among the yeasts. That discovery was followed by descriptions of several teleomorphic genera, including *Filobasidium* (Olive 1968), *Leucosporidium* (Fell et al. 1969), *Filobasidiella* (Kwon-Chung 1975), *Cystofilobasidium* (Oberwinkler et al. 1983) and *Bulleromyces* (Boekhout et al. 1991). The phylogenetic relationship between the genera and to the anamorphic species remained open to conjecture until sequence analyses became readily available.

Many researchers explored basidiomycete phylogeny, and a particularly significant report (Swann and Taylor 1995) of 18S rDNA analysis found that basidiomycetous

yeasts occur in three classes: *Uredinomycetes*, *Hymenomycetes*, and *Ustilagenomycetes*. The *Hymenomycetes* are generally associated with the jelly fungi (*Tremellales*). Yeasts are found within four major clades of the *Hymenomycetes*: *Tremellales*, *Trichosporonales*, *Filobasidiales*, and *Cystofilobasidiales*. The *Uredinomycetes*, which are often linked with the rust fungi, include four major clades of yeasts and related genera: *Agaracostilbales*, *Microbotryales*, *Sporidiobolales*, and the *Naohidea* clade. The majority of the *Ustilaginales* are plant and fungal parasites, with the smuts as well-known examples. Sampaio (2004) reported three major groups in the Ustilaginales: Entorrhizomycetidae, Exobasidiomycetidae, and Ustilaginomycetidae. Yeasts are found in the latter two subclasses.

A list of the genera assigned to the three classes (Table 2.2) was modified from the information provided by Scorzetti et al. (2002) and Sampaio (2004). An observation of note is the presence of anamorphic genera *Cryptococcus*, *Rhodotorula*, and *Sporobolomyces* in more than one phylogenetic group. Historically, anamorphic genera were described on phenotypic characteristics. For example, the genus *Rhodotorula* was originally delineated by the characteristic red color of the colony, although species with white and cream colonies were subsequently included in the genus (Weijman et al. 1988). A cursory identification of a red yeast as *Rhodotorula* has a high probability of being correct, however, the color is not phylogenetically specific. These phenotypic names are temporarily being maintained, with conversion to teleomorphic nomenclature as sexual cycles and species relationships are determined. For example, Sampaio et al. (2004) found the complete sexual cycle of

Table 2.2 Classes and orders of yeasts and yeast-like genera of the *Basidiomycota*

Hymenomycetes
Cystofilobasidiales Boekhout & Fell
Cystofilobasidium Oberwinkler & Bandoni (T)
Cryptococcus Vuillemin (A)
Guehomyces Fell & Scorzetti (A)
Itersonilia Derx (A)
Mrakia Y. Yamada & Komagata (T)
Phaffia Miller, Yoneyama & Soneda (A)
Tausonia Bab'eva (A)
Udeniomyces Nakase & Takematsu (A)
Xanthophyllomyces Golubev (T)
Filobasidiales Julich
Cryptococcus Vuillemin (A)
Filobasidium Olive (T)
Trichsporonales Boekhout & Fell
Cryptococcus Vuillemin (A)
Trichosporon Behrend (A)
Tremellales Rea emend. Bandoni
Auriculibuller Sampaio (T)
Bullera Derx (A)
Bulleribasidium Sampaio, Weiss & Bauer (T)
Bulleromyces Boekhout & Fonseca (T)
Cryptococcus Vuillemin (A)

Table 2.2 Classes and orders of yeasts and yeast-like genera of the *Basidiomycota*—*cont'd*

Cuniculitrema Sampaio & Kirschner (T)
Dioszegia Zsolt emend.Takashima, Deak & Nakase (A)
Fellomyces Y. Yamada & Banno (A)
Filobasidiella Kwon-Chung (T)
Holtermannia Saccardo & Traverso (T)
Kockovaella Nakase, Banno & Y. Yamada (A)
Sirobasidium Lagerheim & Patouillard (T)
Sterigmatosporidium Kraepelin & Schulze (T)
Tremella Persoon (T)
Trimorphomyces Bandoni & Oberwinkler (T)
Tsuchiyaea Y. Yamada, Kawasaki, M. Itoh, Banno & Nakase (A)

Uredinomycetes

Agaricostilbales Oberwinkler & Bauer
Agaricostilbum Wright emend. Wright, Bandoni & Oberwinkler (T)
Bensingtonia Ingold emend. Nakase & Boekhout (A)
Chionosphaera Cox (T)
Kondoa Y. Yamada, Nakagawa & Banno emend. Fonseca et al. (T)
Kurtzmanomyces Y. Yamada, M. Itoh, Kawasaki, Banno & Nakase emend. Sampaio (A)
Sporobolomyces Kluyver & van Niel (A)
Sterigmatomyces Fell emend. Y. Yamada & Banno (A)
Microbotryales
Bensingtonia Ingold emend. Nakase & Boekhout (A)
Curvibasidium Sampaio & Golubev (T)
Leucosporidiella Sampaio (A)
Leucosporidium Fell, Statzell, Hunter & Phaff (T)
Mastigobasidium Golubev (T)
Reniforma Pore & Sorenson (A)
Rhodotorula Harrison (A)
Rhodosporidium Banno (T)
Sporobolomyces Kluyver & van Niel (A)
Naohidea clade
Bannoa Hamamoto (T)
Erythrobasidium Hamamoto, Sugiyama & Komagata (T)
Naohidea Oberwinkler (T)
Rhodotorula Harrison (A)
Sakaguchia Y. Yamada, Maeda & Mikata (T)
Sporobolomyces Kluyver & van Niel (A)
Sporidiobolales Sampaio,Weiss & Bauer
Rhodotorula Harrison (A)
Rhodosporidium Banno (T)
Sporidiobolus Nyland (T)

Ustilaginomycetes

Rhodotorula Harrison (A)
Sympodiomycopsis Sugiyama, Tokuoka & Komagata (A)
Malassezia Baillon (A)
Pseudozyma Bandoni emend. Boekhout (A)
Tilletiopsis Derx ex Derx (A)

[1](A) = Anamorphic genus, (T) = Telemorphic genus.
[2]Some genera, such as the anamorphic genus *Cryptococcus*, are presently polyphyletic as defined, and members of the genus are found in more than one teleomorphic order.

Rhodotorula fujisanensis, for which they described the genus and species *Curvibasidium cygneicollum*. Wholesale description of new anamorphic genera on the basis of clade relationships should be avoided. These nomenclatural changes would result in temporary taxonomic fixes that would be confusing and potentially phylogenetically incorrect.

The development of extensive basidiomycetous rDNA (ITS and D1/D2) databases (Fell et al. 2000; Scorzetti et al. 2002) provided a springboard for sizeable expansion in the rate of the descriptions of new species and genera. The number of genera increased from 33 (Kurtzman and Fell 1998) to approximately 55 (Sampaio 2004). The increase in number of species can be exemplified by the genus *Trichosporon*: 19 (Guého et al. 1998) to 36 (Fell and Scorzetti 2004). Importantly, the resulting phylogenetic trees indicate the extent of genetic diversity and the extent of relationships between species, including the anamorphic and teleomorphic species.

The definition of a basidiomycetous species, based on sequence analysis, needs considerable attention. As previously discussed, zero to three nucleotide differences among ascomycetous yeasts in the D1/D2 region generally signifies strains within a single species. This general concept is not always applicable among basidiomycetes. Several significant examples exist, which demonstrate that other genetic regions must be examined to distinguish taxa. *Mrakia gelida* and *M. frigida* are identical in the D1/D2 and significantly different in the ITS and IGS regions (Diaz and Fell 2000). Similarly, the pairs *Filobasidiella neoformans*: *F. bacillispora* and *Phaffia rhodozyma*:*Xanthophyllomyces* type strains differ by one base pair in the D1/D2 domains and significantly in the ITS and IGS regions (Fell and Blatt 1999; Scorzetti et al. 2002).

2.6 Rapid Identification of Yeasts from Ecological Studies

Prior to the existence of molecular phylogeny, ecological research on basidiomycetous yeasts was hampered by reliance on phenotypic characteristics. As a consequence, there was a generalized concept that many of the species have worldwide distributions in diverse environments. This concept was particularly true for species such as *Cryptococcus albidus* and *Rhodotorula glutinis*. Fonseca et al. (2000) dispelled this concept by demonstrating that *C. albidus* is a complex of 12 species. This concept is further dispelled as established species and their phenotypic synonyms are being examined, e.g., *Rhodosporidium* (Sampaio et al. 2001), and as new species are being described that are phenotypically indistinguishable from related and unrelated species, e.g., *Trichosporon* (Middelhoven et al. 2004).

A major ecological problem is that estimates indicate that only 1% of the yeast species in nature have been described. Yeast ecology, therefore, is at a stage of discovery. The ability to undertake biocomplexity studies, viz., environmental/population interactions, is difficult, if the individual players (species) are unknown. A case in point is an ongoing study of yeast populations in the Florida Everglades (Fell and Statzell-Tallman, unpublished). This study involves quarterly (seasonal) sampling in a subtropical Everglades watershed that ranges from freshwater marshes to seawater mangrove habitats. The number of cells ranges from 100 to 2,700 per liter of water. These variations in density correlate with sample location and season of the year.

Preliminary data demonstrate that the biodiversity in the water is comprises 23 genera and 120 species of basidiomycetes and ascomycetes. The undescribed species in these collections represented 54% of the taxa. More than 800 strains were sequenced during this study. In order to examine a large number of strains, ecological studies are most efficiently undertaken with the assistance of a high throughput sequencer. These instruments can sequence approximately 1,000 strains/day. Throughputs of this magnitude are beyond the capabilities of the average single researcher to manipulate and analyze on a daily basis; however, large numbers of strains can be sequenced on a weekly and monthly basis.

Studies of complex unknown communities, such as illustrated with the Florida Everglades program, are most efficiently studied by direct sequence analysis of the species. In contrast, monitoring of ecological niches with known community structure can employ a variety of techniques, including temperature and denaturing gradient gel electrophoreses (TGGE and DGGE), single-strand conformation polymorphism (SSCP), terminal RFLP (T-RFLP), amplified rDNA restriction analysis (ARDRA) and amplified ribosomal intergenic spacer analysis (ARISA). The application of these techniques to fungal ecology was reviewed by Anderson and Cairney (2004). By necessity, all of these techniques must be confirmed by sequence analysis. Therefore, the bias and research concentration in our laboratories has been directed to sequence-based identification methods.

PCR-based species-specific primers represent the least expensive and easiest method for identification of small numbers (one to ten) of taxa (Chapman et al. 2003). Multiplex PCR of larger numbers of primers in a single reaction is difficult owing to various factors, such as formation of primer dimers and differences in temperature requirements for primer hybridizations. Our laboratories have been exploring a high-throughput probe hybridization method for detection of multiple species in multiple samples. The method (Diaz and Fell 2004; Page and Kurtzman, 2005) is an adaptation of the Luminex xMAP technology (Luminex Corporation), which consists of a combination of 100 different sets of fluorescent beads covalently bound to species-specific capture probes. Upon hybridization, the beads bearing the target amplicons are classified in a flow cytometer by their spectral addresses with a 635-nm laser. The hybridized biotinylated amplicon is quantitated by fluorescence detection with a 532-nm laser. The multiplex assay is specific and fast: species that differ by one nucleotide can be discriminated and the assay can be performed, after amplification, in less than 50 min in a 96-well format with as many as 100 different species-specific probes per well. The advantage of this method for ecological research is that multiple species can be identified from multiple samples, such as water and soil. In practice, DNA is extracted followed by the Luminex xMAP procedures. This technique is applicable to a variety of ecological monitoring strategies. For example, we have been successfully employing this method for the analysis of sewage-associated bacteria in waters adjacent to marine recreational beaches (Fell et al., unpublished).

In summary, rapid detection and accurate identification of yeasts is now possible through use of a variety of molecular methods. Application of these methods will bring a greater degree of clarity to studies in yeast ecology, which previously was not possible when yeasts were identified from the phenotype.

Acknowledgements

J.W.F. acknowledges support from the National Science Foundation (USA), Biodiversity Surveys and Inventories Program (DEB 0206521). The mention of firm names or trade products does not imply that they are endorsed or recommended by the US Department of Agriculture over other firms or similar products not mentioned.

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Chapter 3

Yeast Biodiversity and Culture Collections

VINCENT ROBERT, JOOST STALPERS, TEUN BOEKHOUT AND SHU-HUI TAN

Centraalbureau voor Schimmelcultures (CBS), P.O. Box 85167, 3508 AD, Utrecht, The Netherlands
(email: robert@cbs.knaw.nl)

3.1 Introduction

It is in the nature of humans to collect, to classify, to organize and to summarize their findings. From the early days of taxonomic surveys and collecting fieldtrips, scientists and amateurs have been gathering samples and evidence related to their discoveries. In those days, the only way to keep track of previous findings was to describe the morphology, the ecology and some basic physiological properties. Species of yeasts were then often described on the basis of one or several specimens but the latter were not always mentioned and/or properly described, analyzed and preserved. When it became mandatory to designate type specimens, for every new species to be described and to keep them available for future reexaminations, the need for proper storage of the material was raised. The first and most obvious way of doing this was by the drying of living specimens and the creation of herbaria. Dried, nonliving material is of great importance in mycology but of limited use for yeasts, since the physiological, biochemical, genetic and ecological properties cannot be properly studied or reexamined. In order to perform more advanced investigations and analyses on previously collected specimens, they had to be kept alive and maintained in a condition as close as possible to their "original" state. This is where the culture collections (CCs) came into the picture, which still play a key role in the preservation of the yeast biodiversity (Agerer et al. 2000; Hawksworth 2004). Proper preservation methods have been mainly developed by scientific staff working for CCs (Mikata and Banno 1989; Tan et al. 1991, 1994, 1995, 1998; Tan 1997; Tan and van Ingen 2004). CCs often host a pool of scientists who study the strains of the collection and produce a wealth of new and valuable data on these strains. CCs are not only large biological storage and distribution facilities, they are also information and references centers that provide advice, courses, training and valuable information through their catalogues and more recently the Internet. Consequently, other researchers working in academic institutions, clinics or industry benefit greatly from the services of CCs.

3.2 Strains and Species

In the past, most members of the scientific community considered the species as the basis of the exchange of information. Nowadays, researchers are increasingly using information at the strain level. The definition of a yeast species is a summary made by systematicians and is based on the observation of taxonomically informative characteristics of one or several strains, thought to belong to the same biological, phenetic and/or phylogenetic entity. These entities can be large, like *Saccharomyces cerevisiae*, or small when a species is known from a single strain only. The size or the volume of species clusters does not only depend on the number and the heterogeneity of the strains used for its circumscription, but also on the variability of the set of characteristics that is considered. Known taxonomic information associated with the species is not always relevant or sufficient for workers in other disciplines. For example, people working in industry, in ecology, in biotechnology or in clinical settings may not be satisfied with a species name. They may require data related to the resistance to heat in sterilization procedures that can be strain- and not species-dependent, or they may want to know the transformation potential of strains in complex ecological cycles. Clinicians are usually interested in the pathogenicity and the resistance of the strains against antibiotics. As many properties can be variable within a given species, it is of the utmost importance to record and keep data related to the strains and to be able to dynamically create homogeneous groups of strains that share the same phenotypic profiles. Species are often subjective entities and their circumscriptions, definitions and volumes are variable and may evolve with time, the methods used to study them and the understanding of the taxonomists. When properly preserved, strains may keep the same properties and can therefore be used and reused in many different types of studies, such as physiology, genetics or biotechnology. All previously recorded data associated with a given strain can be complemented with new ones. This allows continuous updating and improvement of our understanding of the mechanisms underlying the basal processes connected with metabolism, pathogenicity and many other strain-related characteristics and properties. It also permits some cross-character correlations to be performed that would not be possible or desirable otherwise when working with species only.

Strain preservation, characterization, identification, analysis and distribution are the core business of CCs. Therefore, and even more than herbaria and museums, CCs remain the ideal place for taxonomic endeavors. In the past, systematic work was usually the main task of CCs, but this is not the case anymore in many cases, as applied research is becoming more and more important. In addition, many CCs tend to specialize (viz., mutant collections, specialized applications in fermentation industries, screening for new biologically active compounds). However, several major yeasts CCs have hosted most of the editors of major monographs on yeasts [e.g., Lodder 1934, 1970; Diddens and Lodder 1942; Wickerham 1951; Lodder and Kreger-van Rij 1952; Kreger-van Rij 1984; Yarrow (in Barnett et al. 1983, 1990a, 2000a); Kurtzman and Fell 1998]. This made sense since those researchers had access to all type strains and had the possibility to review them by

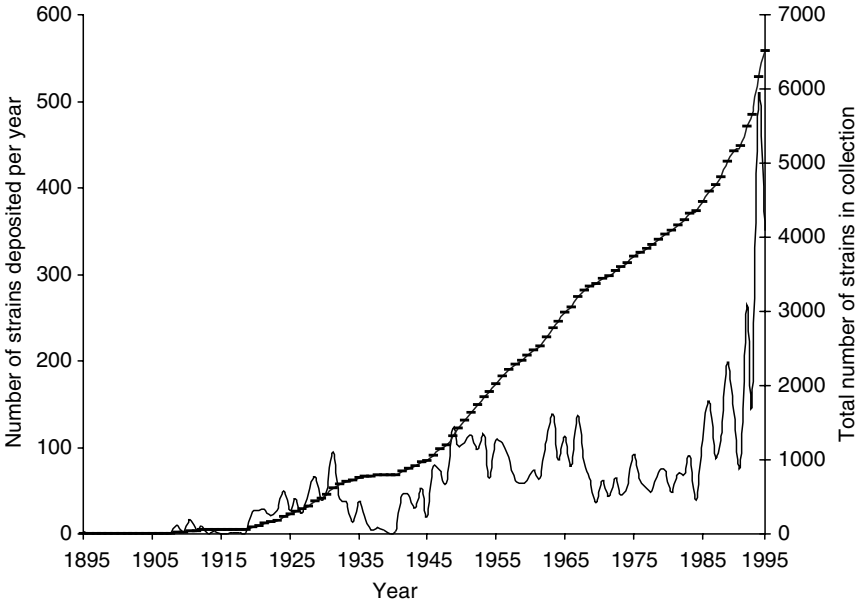

Fig. 3.1 Number of strains deposited in the yeast collection of the Centraalbureau voor Schimmelcultures (CBS) between 1895 and 2004. The *smooth line* represents the annual deposit, while the *line with bars* represents the cumulative curve

applying the new methods that became available. Since the early days of yeast taxonomy, the methods employed for the characterization of yeasts have constantly evolved. In the early days, only microscopically and macroscopically visible morphological features were recorded. Later, some basic physiological tests were introduced and a number of them are still used in today's standard testing panels. More recently, biochemical data were added (viz., cell wall carbohydrates, coenzyme Q, GC contents), followed by sequence data of the ribosomal genes. Certainly, one can expect more new features to be included in the descriptions panels, such as transcriptome, proteome and metabolome data. Modern CCs are not only able to keep strains in the original condition, but they also archive all previously recorded data at the unit level (strains); therefore, they allow researchers to build on existing knowledge and to concentrate their efforts on new developments. Since species are usually or ideally based on several units (strains, specimens), the addition of new data cannot be linked to them in a straightforward manner, as previously collected data should be reviewed again. With the growing number of species described and accepted, namely182 in 1952 (Lodder and Kreger-van Rij), 341 in 1970 (Lodder), 518 in 1984 (Kreger-van Rij), 597 in 1990 (Barnett et al. 1990a), 678 in 2000

(Barnett et al. 2000a) and more than 900 in 2004 (Robert et al. 2004), it has become almost impossible to reanalyze all these species and their related strains, thus stressing the value of maintaining strain-related data.

Recently, a trend emerged to isolate more strains from the environment and to explore new niches (see the annual and cumulative deposit at the Centraalbureau voor Schimmelcultures,CBS, CC in Fig. 3.1).

There are three good reasons for this. The first one is that new methods such as DNA sequencing are now relatively cheap, easy, reliable and, thanks to the pioneering work of some yeast taxonomists, complete databases are available for identification. The second reason is that the increased rate of destruction of our environment makes it important to investigate "unconventional" niches. The third one relates to the work of biotech companies that are isolating and screening large numbers of strains for biologically interesting compounds. Figure 3.2 shows the geographic origin of the currently available strains in the CBS CC. Although the analysis is obviously biased since only 66% of the exact geographic coordinates of the strains are available, it can easily be seen that most activity has been performed in some "hot spots" such as western Europe, Japan, the USA, South Africa and some coastal regions.

Countries in dark shades have been poorly investigated until now, usually because they lack the facilities needed, research labs and CCs. The presence of active researchers working in previously nonsampled areas can be very "rewarding", and result in the discovery of many species new to science. The example of J. van der Walt in South Africa is very striking. Before he started collecting, very few strains, if any, were isolated from South Africa. During his long and fruitful career, he and his colleagues collected, described and published many new findings that were all deposited in CCs and are still available now. H.J. Phaff and M.-A. Lachance, among many others, also did pioneering work by collecting previously ignored or less sampled areas or substrates like cacti or insects. From our experience of fieldtrips to South America, Africa and Asia, we estimate that roughly 50% of the material collected, which mainly originated from flowers, represented new species. Strikingly, a number of isolates originating from different continents belonged to the exact same new species (not yet published but available from the CBS CC). Suh et al. (2004) and Suh and Blackwell (2005) found that almost one third of their 650 isolates from beetles represented new species. The same kinds of findings were obtained by other researchers working on beetles who found the same species distributed all over the American continent (Lachance et al. 1998; Rosa et al. 1999). This suggests that even with a statistically nonrepresentative number of isolates, we can still discover new species that have a pandemic distribution (Robert et al. 1998; Hawksworth 2004; Suh et al. 2004; Suh and Blackwell 2005). This means that our understanding of yeast biodiversity is still extremely poor, which is in agreement with a number of publications in which the importance of fungal biodiversity, particularly in tropical and threatened or fragile environments, was discussed (Hawksworth 1991, 2001, 2004; Bills and Polishook 1994; Hawksworth and Rossman 1997; Hyde and Hawksworth 1997; Rossman 1997; Lachance and Starmer 1998; Prance et al. 2000).

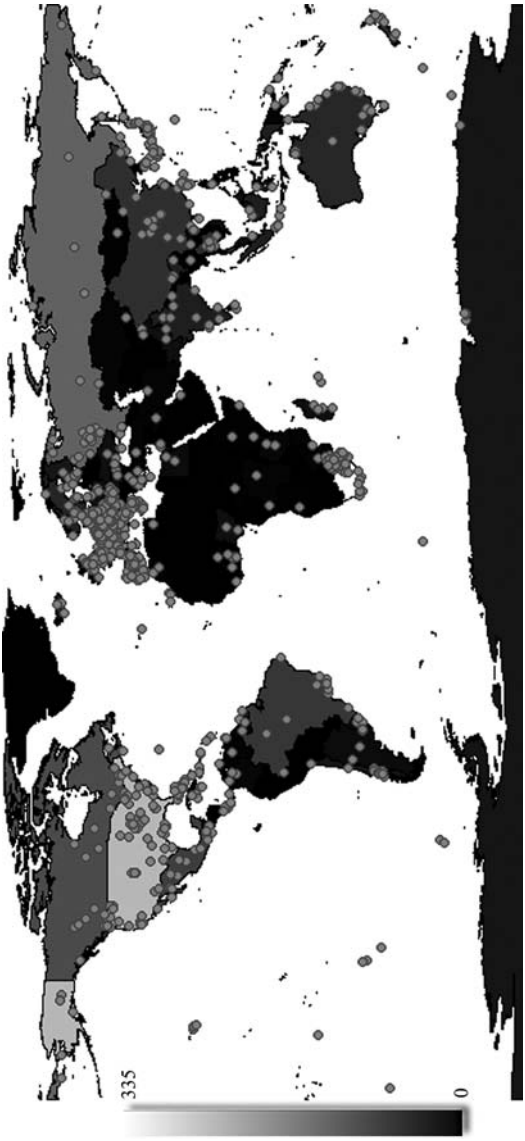

Fig. 3.2. Geographical distribution of the isolation localities of yeast strains deposited at the CBS collection. *Dots* represent localities where the strains were isolated. Countries from where many strains have been isolated are in *white* (South Africa, Japan and many countries in western Europe) although they are not always visible owing to the large number of strain-related dots. Countries in *black* have been sampled less

3.3 Culture Collections, Diversity and Expertise

CCs are hosting a wide range of activities and scientific disciplines. Statistics maintained by the World Data Centre for Microorganisms (WDCM, at http://wdcm.nig.ac.jp) show this diversity of objectives and focus points amongst the various CCs. Four hundred and eighty-nine CCs from over 65 countries are registered in the WDCM system. Most of them are governmental bodies (33%), semigovernmental bodies (7%) or linked to universities (27%). Only a small proportion of the CCs are supported by private institutions or industry (5%). There was no information available for the other 28%. A total of 2,700 researchers, technicians and administrative staff are employed by the CCs and about one third of them produce a catalog. Slightly fewer than half of the CCs provide storage, distribution, identification, training and consulting services. As can be seen from Tables 3.1 and 3.2, most of the collections are located in Europe, Asia or America. Very few are situated in Africa, although this continent is likely to hold a large portion of the world's biodiversity of yeasts. The geographic distribution of the CCs can be directly correlated to the origin of the yeast strains present in the CBS CCs (Fig. 3.2). This implies means that huge effort should be invested to set up CCs in Africa and other less developed parts of the world and to properly train the staff to enable them to collect the many yeast strains that are available in their countries. However, the setup and maintenance of new CCs is costly, and it is unlikely that many African governments will be able or tempted to support such infrastructures, especially in times where struggles against poverty, hunger and disease are legitimate priorities. Presently, existing well-established CCs can help in the training of scientists from less developed countries and can provide them with the facilities they need to store and study their strains. It is also the duty of CCs to initiate and support forays in those unexplored areas, which is in agreement with the Rio Convention on Biodiversity (CBD).

In Table 3.2, some of the largest CCs that accept, maintain and/or work with yeasts are listed with their URLs. CCs that are not members of the World

Table 3.1 Geographic origins and type of services provided by the culture collections (*CCs*). (Data from World Data Centre for Microorganisms)

Region	Patent deposit	Storage service	Distribution service	Identification service	Training offered	Consultancy service	No. of CCs/ no. of cultures
Africa	1	4	4	6	23	8	10/8,540,
Asia	13	54	52	59	168	121	161/248,845
Europe	47	86	93	92	243	232	161/487,405
America	7	42	56	53	164	132	114/319,179
Pacific	1	14	17	22	28	36	43/88,206
Total	69	200	222	232	626	529	489/1,152,175

Table 3.2 Some of the major CCs working with yeasts strains ordered by descending number of publicly available strains.

Acronym	Name	Country	No. of yeasts	Internet address
NRRL	National Center For Agricultural Utilization Research	USA	14,500	http://nrrl.ncaur.usda.gov
CBS	Centraalbureau voor Schimmelcultures	Netherlands	6,600	http://www.cbs.knaw.nl
UCD	University of California Davis, Herman J. Phaff Collection	USA	6,000	http://www.phaffcollection.org
UWO-PS	Department of Biology, University of Western Ontario	Canada	>5,000	
DBVPG	Dipartimento di Biologia Vegetale e Biotecnologia Agroambientale	Italy	4,500	http://www.agr.unipg.it/dbvpg/home.html
CCY	Culture Collection of Yeasts	Slovakia	3,800	http://www.chem.sk/activities/yeast/ccy/
BCCM/IHEM	Scientific Institute of Public Health – Louis Pasteur	Belgium	3,500	http://www.belspo.be/bccm
AMFC	Australian Medical Fungal Collection, Westmead Hospital in Sydney	Australia	3,500	http://www.mmrl.med.usyd.edu.au
NBRC (former IFO)	NITE Biological Resource Center	Japan	>3,150	http://www.nbrc.nite.go.jp
NCYC	National Collection of Yeast Cultures	UK	3,100	http://www.ncyc.co.uk
CECT	Coleccion Espanola de Cultivos Tipo	Spain	2,500	http://www.uv.es/cect
JCM	Japan Collection of Microorganisms, Bioresource Center	Japan	2,400	http://www.jcm.riken.jp
VKM	All-Russian Collection of Microorganisms	Russia	2,300	http://www.vkm.ru
BCCM/MUCL	Mycotheque de l'Universite catholique de Louvain	Belgium	2,200	http://www.belspo.be/bccm
ATCC	American Type Culture Collection	USA	>2,000	http://www.lgcpromochem.com
LCC	Labatt Culture Collection, Technology Development	Canada	2,000	http://www.labatt.com
ZIM	Culture Collection of Industrial Microorganisms	Slovenia	1,740	http://www.bf.uni-lj.si/zt/bioteh/chair/index.html

Continues

Table 3.2 Some of the major CCs working with yeasts strains ordered by descending number of publicly available strains.—*cont'd*

Acronym	Name	Country	No. of yeasts	Internet address
BCRC	Bioresource Collection and Research Center	Taiwan	1,564	http://www.ccrc.firdi.org.tw
PYCC	Portuguese Yeast Culture Collection	Portugal	>1,500	
NCAIM	National Collection of Agricultural and Industrial Microorganisms	Hungary	>1,200	http://ncaim.uni-corvinus.hu
VTT	VTT Biotechnology, Culture Collection	Finland	1,120	http://www.vtt.fi/bel/services/culture_collection.htm
CLIB	Collection de Levures d'Interet Biotechnologique Collection of Yeasts of Biotechnological Interest	France	1,000	http://www.inra.fr/clib/
TISTR	TISTR Culture Collection Bangkok	Thailand	650	http://www.tistr.or.th
AWRI	Institute of Molecular and Cellular Biosciences, The University of Tokyo	Japan	600	http://www.awri.com.au
CCOC	Coleção de Culturas Oswaldo Cruz	Brazil	>500	http://sicol.cria.org.br/crb?INCQS
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH	Germany	500	http://www.dsmz.de
IAM	Institute of Molecular and Cellular Biosciences, The University of Tokyo	Japan	450	http://www.iam.u-tokyo.ac.jp/misyst/ColleBOX/en/about.html
KCTC	Korean Collection for Type Cultures	South Korea	225	http://www.brc.re.kr
KACC	Korean Agricultural Culture Collection	South Korea	62	http://kacc.rda.go.kr
CBTC-CCAS	The Cell Bank of Type Culture Collection of Chinese Academy of Sciences	China	?	http://www1.im.ac.cn/typecc/en.html

Federation of Culture Collections (WFCC) have been omitted, as they usually do not have the required structure for distribution, and indeed they rarely distribute strains on a regular basis to third parties. There are also large collections outside the public domain (e.g., pharmaceutical or biotech companies) that are not included in the statistics displayed in Tables 3.1 and 3.2.

Out of the 1,152,175 microbial strains that are maintained in public CCs worldwide, about 400,000 are fungi, including some 80,000 yeasts. The largest yeast CCs are biodiversity collections that maintain living-type specimens and related strains. The North American collections, with the NRRL, the UCD, the UWO and the ATCC, maintain almost 30,000 strains of yeasts. The ATCC is a generalist collection with a strong focus on *Saccharomyces* mutants. The others are general biodiversity collections but are also developing projects with industry. In South America some large institutions, like the Fiocruz in Brazil, the Corpoica in Colombia and several others in Mexico, Cuba, Chile and Argentina, have CCs. Europe hosts various yeast CCs. The CBS is the largest among them and focuses, like BCCM/MUCL, IGC, DSMZ and VKM, mainly on type strains and general yeast biodiversity, but they also maintain industrially important strains, notably under the Budapest Treaty. The NCYC, DBVPG, CECT, NCAIM, ZIM and CLIB, as well as some others, are more specialized CCs and work in close connections with food and agriculture related industries, e.g., the wine and beer industries. In Asia, several major CCs have a long history in zymology and many taxonomists have been active in both the NRBC (former IFO) and the JCM in Japan. In addition to industry-associated research, they remain active in the field of biodiversity studies in general and develop projects linking several Asian institutions through bilateral and/or multilateral projects (e.g., through the MIRCEN Network). Institutions like Biotech and TISTR in Thailand host growing collections of yeasts that are used to screen the biodiversity for potential industrial and pharmaceutical applications. Researchers associated with the Chinese Academy of Science in Beijing are involved in studies on China's vast yeast biodiversity, which resulted in the discovery of many new species. As said before and with the notable and important exception of South Africa, Africa lacks important yeast-associated CCs.

3.4 Preservation and Stability

Proper preservation of yeast cultures is essential if one wants to maintain them for future investigations. As the cultures need to keep the same morphological and physiological properties, preservation studies and media optimization have received considerable attention at CCs. The early-used methods consisted of regular subculturing of the strains, thus allowing for important stability and mutational problems to occur. The introduction of lyophilization and cryopreservation techniques permitted major enhancements of the stability of the strains. A better understanding of the freeze-drying (lyophilization) techniques, especially provoked by the development of cryomicroscopes, has resulted in the possibility to dry recalcitrant organisms and a prolonged shelf life of freeze-dried specimens as well.

Freeze-drying is a rather complicated series of processes, aimed at the drying of organisms to such an extent that the metabolic activity is strongly reduced.

The result should allow for the storage of these organisms at a practical temperature and guarantee a successful revival after a prolonged period. The process starts with a suspension of living cells in a lyoprotectant. This suspension is frozen and subsequently dried by providing an open connection to a colder condenser under a relatively low vacuum. The drying occurs in two steps: primary drying, in which the frozen free water (crystalline) is removed, and secondary drying at higher temperatures. During primary drying the cells dehydrate by exposure to an eutectic solution with a high osmotic value, which freezes later to become a glass. The aim of secondary drying is to reduce the residual moisture content of the primary dried material in order to obtain a stable glass at preservation temperature. Residual moisture content is expressed as a percentage of the absolute dry weight. For fungi the tolerance of residual moisture content is between 1 and 2.5%. More severe drying will result in irreversible damage to the cells owing to denaturation of proteins and lipids, while higher moisture contents will reduce the shelf life of the material. Most yeast strains can now be lyophilized reliably.

Although for distribution freeze-drying has advantages over freezing, the method of choice for the preservation of yeasts is freezing at ultralow temperatures. It is the stablest and most reliable technique and it also allows for a better preservation compared with periodical subculturing. Yeast researchers can concentrate on their research without worrying about the stability of the strains that are provided by CCs.

The usage of the latter methods is of the utmost importance, not only for the quality of the stored microbiological materials, but also because they allowed for a drastic reduction of the time spent in subculturing.

3.5 Distribution

One of the major functions of CCs is the distribution of strains. Nowadays, the quality of the material, such as correct identity and physiological condition, is only a part of the task of the CCs. More and more governmental and postal regulations have important implications for CCs. For example, quarantine organisms, which can cause harm to humans, animals or local crops, or organisms that potentially can be used by terrorists are not easily distributable anymore and are subject to restrictions. There are now a number of obligations that are imposed by international treaties such as the Convention on Biodiversity, the Budapest Treaty (patents) or by transport regulations (IATA, road transport). Because of all these elements, qualified personnel are needed and special protocols have to be followed, which has considerable implications and costs.

The Convention on Biological Diversity (CBD), now ratified by over 185 countries, was established to support the conservation and utilization of biodiversity, ensuring fair and equitable sharing of benefits arising from the latter and was implemented in 1993. It has three major objectives, namely, conservation of biodiversity, sustainable development of genetic resources and fair and equitable sharing of resultant benefits. The CBD assigns sovereign rights to the country of origin and requires that prior informed consent (PIC) be received from the country in which access to organisms is requested. Mutually agreed terms (MAT) on the conditions

under which access is granted and on which benefits will be shared, should they occur from the use of the organisms, must be put in place. Benefit sharing may include monetary elements, but may also include information, technology transfer and training. The supply of organisms must also be under agreed terms under material transfer agreements (MTA) between supplier and recipient to ensure benefit sharing with, at least, the country of origin.

CCs must put in place mechanisms to comply with the provisions of the CBD, but they cannot completely police the distribution of strains. What they can do is to restrict further distribution, to allow traceability, to raise the attention of the client to the obligations related to the CBD and to provide the means to do so. When authorized they can also negotiate between the client and the country of origin.

Key developments for the endorsement of a voluntary code of conduct for access and benefit sharing have been made thanks to the Microorganisms Sustainable Use and Access Regulation International Code of Conduct (MOSAIC) project and the Bonn Guidelines.

3.6 Underpinning Science and Industry

Preserving strains under good and stable conditions at CCs contributes largely to scientific research in general by providing valuable quality material for the technologists to develop new or better products. However, CCs are not just biological stores, as the research staff of CCs also contribute to the value of the collections by bringing new methodological data (Barnett et al. 1985, 1987, 1990b, 1994, 1996, 2000b; Mikata and Banno 1989; Tan et al. 1991, 1994, 1995, 1998; Deak 1992, 1995; Robert et al. 1997, 2004; Tan 1997; Esteve-Zarzoso et al. 1999; Boekhout et al. 2002; Robert and Szoke 2003; Tan and van Ingen 2004), expertise and new isolates (Suh et al. 2004; Suh and Blackwell 2005). These interests have sparked the development of research programs at leading collections, which include investigations into phylogeny, taxonomy and nomenclature, strain preservation techniques, functional genomics, screening and bioinformatics.

In order to make CCs ever more useful and to share the important information that is kept at the strain and species levels, CCs have developed software and Internet websites. As an example, the CBS codeveloped the BioloMICS software (Robert and Szoke 2003) for the management of strains and species data, the statistical analysis of those data and for identification and classification of the isolates. Detailed information about more than 900 yeast species and 6,600 yeast strains is available online (http://www.cbs.knaw.nl/yeast.htm). Hundreds of characters are annotated for each record, which include morphological, physiological and molecular data. This information is freely accessible for each record, as is textual, bibliographic, geographic and taxonomic information. The database includes thousands of macroscopic and microscopic images and a bibliography of almost 10,000 references. The taxonomic database features 23,500 scientific names (including anamorph and teleomorph names, as well as synonyms). It is also possible for users to align their own sequences with a database containing up to 450,000 fungal sequences. The BioloMICS software also allows for online polyphasic similarity-based identifications, which can be performed using any combination of geographic, morphological, physiological or

molecular data. The CBS in The Netherlands, CABI in the UK and Landcare Research in New Zealand have launched the Mycobank website (Crous et al. 2004; www.mycobank.org) that allows the online registration of new fungal names as well as associated data. For the yeasts, this website is coupled to the yeast BioloMICS database and new species are made available online directly. We hope that this will be a highly valuable tool for all yeast researchers, especially those working on biodiversity and ecology.

3.7 Conclusions

The results obtained from biodiversity and functional studies of yeasts should be investigated in a more global and comprehensive approach, allowing a better understanding of the roles of these organisms in nature and their relationships to other living beings. Attaining this seemingly idealistic goal necessitates the use of collaborative teams of scientists as well as the training of additional taxonomists. Ecologists, taxonomists, botanists, zoologists and chemists must prioritize the niches that must be explored in the light of endangered habitats and ecosystems. These scientists should work with statisticians to define sampling plans, with field technicians to assist in the collection, isolation and the characterization of the isolates, with biochemists to search for interesting properties, and with computer scientists to construct useful databases. They must also convince politicians of the importance of long-term, comprehensive studies in order to finance the development and continued existence of large, multidisciplinary scientific teams and CCs. International collaboration will be crucial, not only for financing these scientifically difficult studies, but also because scientific expertise and technology and financial and ecological resources are scattered over different countries throughout the world. CCs are at the heart of this problem and should act and be seen as the core basic instrument allowing us to reach the previously mentioned targets.

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Chapter 4

Genomics and Biodiversity in Yeasts

M. Bolotin-Fukuhara

Laboratoire de Genetique Moleculaire, Institut de Génétique et Microbiologie, CNRS UMR 8621, Batiment 400, Université Paris-Sud, 91405 Orsay Cedex, France (e-mail: monique.bolotin@igmors.u-psud.fr, Tel.: +33-1-69156201, Fax: +33-1-69157296)

4.1 Introduction

The first complete nucleotide sequence of a yeast – *Saccharomyces cerevisiae* – genome was published in 1997 (The Yeast Genome Consortium 1997). It was also the first case of complete genome sequencing of a eukaryotic genome. The analysis of this yeast genome has revealed a set of conceptually new and unexpected features whose meanings were not fully understood for quite a while (number of genes, gene duplications, segmental duplications, etc.). By a concerted effort of the yeast research community the sequencing program was then developed to a systematic functional analysis, achieving a functional description of a large part of the genetic information.

The full eukaryotic genome sequences which followed this first attempt (*Caenorhabditis elegans*, by the *C. elegans* Sequencing Consortium 1998; *Drosophila melanogaster*, by Adams et al. 2000; *Arabidopsis thaliana*, by the *Arabidopsis* Genome Initiative 2000; *Homo sapiens*, by Lander et al. 2001 and Venter et al. 2001; *Anopheles gambiae* by Holt et al. 2002; *Mus musculus* by Waterston et al. 2002) belonged to organisms dispersed in the evolutionary tree of life. It is only recently that a set of ascomycetes complete genome sequences appeared in the literature and their availability is a real bonus to undertake comparative genomics studies. Yeasts share a common way of life as unicellular eukaryotic organisms (developmental steps are minimum, cell-to-cell communications is restricted to specific pathways such as sexual conjugation, killer system, etc.), but are largely dispersed along the evolutionary tree. The five yeasts analyzed by Dujon et al. (2004) appear molecularly more diverse than the whole phylum of chordates. During this long period of evolution and speciation, they also found their specific ecological niches and lifestyle. Several yeast genome sequences are now available (see Fig. 4.1 for their phylogenetic relationship). This information can be used to identify the molecular events that drove their evolution, and also to analyze the process of environmental adaptation. For the past few years, biologists have studied evolutionary developmental mechanisms also called "Evo-Devo" (reviewed by Hall 2003). A focus of this complex problem has been the mechanisms by which the transcriptional regulatory systems

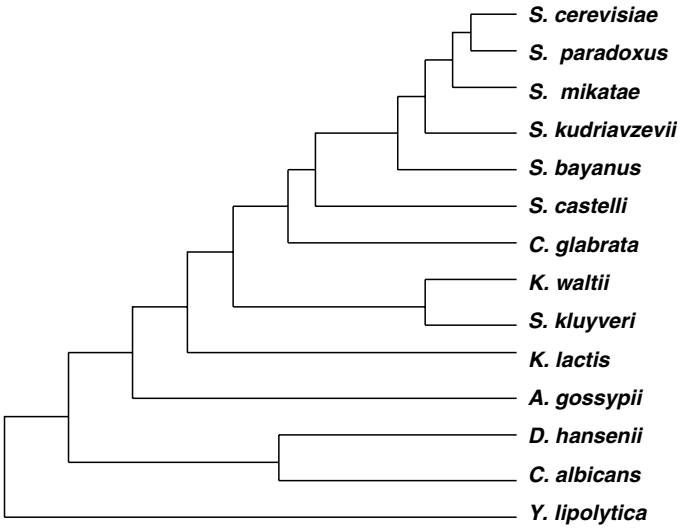

Fig. 4.1. Schematic phylogenetic relationships of the genome-sequenced yeasts. (Taken from Wolfe 2004)

evolved (published works are largely based on the studies of homeotic genes), and the contributions of these regulatory changes in the evolution of phenotype (Wray 2003). Such studies can now be undertaken with yeasts, based this time on complete genome sequence, in order to understand what could be called "Evo-Adapt" mechanisms. There are several recent reviews which deal with yeast genome evolution (Herrero et al. 2003; Piskur and Langkjaer 2004; Wolfe 2004). This short review is not exhaustive but intends to present our personal, perhaps biased, view of the present developments and perspectives of this new exciting field.

4.2 The Which and Why of Complete Yeast Genome Sequencing

The *S. cerevisiae* genome sequencing project was proposed 2 decades ago by Goffeau to the European Community and received strong support from the yeast research community. *S. cerevisiae* was not only an essential industrial yeast, but also "the" model yeast for basic research at that time. Sequencing of other yeast genomes started later, but the choice of species and the work calendar were not coordinated, and were rather dictated by circumstances and specific interests of different research groups. The genome sequencing of *Schizosaccharomyces pombe*, another model yeast for some aspects of cell biology, such as "cell cycle", and of the medically important *Candida albicans* (Tzung et al. 2001) were the first to be undertaken. The complete analysis of the *S. pombe* genome came out in 2002 (Wood et al. 2002). The determination of the complete nucleotide sequence of several *sensu stricto* and *sensu lato Saccharomyces* was undertaken with particular interest focused on intergenic regions. Since these regions diverge much faster than coding sequences, the

detection of conserved features in such regions required a comparative study of closely related yeasts (Cliften et al. 2003; Kellis et al. 2003; see Sect. 5.1 for more details). The *Ashbya gossypii* (*Eremothecium gossypii*) project also started a few years ago and the results were published recently (Dietrich et al. 2004). The choice of this yeast was based on several features of particular interest, including the very small size of its genome, the high degree of open reading frame (ORF) conservation with respect to *S. cerevisiae* and its filamentous form. More recently the genome sequence of *Kluyveromyces waltii* (= *Lachancea waltii*) was reported (Kellis et al. 2004). Sequence analysis of *K. waltii* and *A. gossypii* confirmed the view that the complete genome duplication occurred before the *S. cerevisiae* speciation, a hypothesis proposed earlier by Wolfe and Shields (1997). In the meantime, the French consortium "Génolevures" explored the hemiascomyceteous phylum (that includes most of the commonly known yeast species) by very partial shotgun sequencing of 13 different species well dispersed along the evolutionary tree of hemiascomycetes (The Génolevures Consortium 2000). This project was later completed by total genome sequencing of four selected species chosen on the basis of their adaptation to different environmental conditions as well as their supposed positions in the evolutionary tree (*C. glabrata*, *K. lactis*, *Yarrowia lipolytica*, and *Debaryomyces hansenii*) (Génolevures II, Dujon et al. 2004). Finally, the genome of *Hansenula polymorpha* (= *Pichia angusta* = *Ogataea polymorpha*) was sequenced and analyzed because of the well-known industrial interest in this yeast (Ramezani-Rad et al. 2003). In the near future, many more yeast sequences are expected to be released, increasing our repertoire and the possibilities of comparative genomic analysis.

An important fallout of all these projects was the improvement of annotation of the *S. cerevisiae* genome based on genome sequence comparison. This allowed the number of coding sequences to be reduced from the initial 6,200 to about 5,700–5,800 (Blandin et al. 2000; Brachat et al. 2003; Cliften et al. 2003; Kellis et al. 2003).

Speaking of a complete nucleotide sequence, the published data are not of the same level of completeness. In most cases, sequences are based on shotgun sequencing (usually between 5 and 8 times genome coverage), which leaves unfinished pieces and makes assembly difficult, resulting in a collection of large contigs. For the moment only a few genomes have been fully assembled as was done for *S. cerevisiae*: such is the case for *A. gossypii, S. pombe*, and the Génolevures II program (*C. glabrata, K. lactis, Y. lipolytica*). According to the type of analysis to be done, this (in)completeness has some importance as will be discussed in Sect. 4.1.

A comparison of genomes revealed first that all ascomycetes genomes were in the range 9–20 Mb, a small variation compared with the size of most eukaryotic genomes. Compared with higher eucaryotes, these small genomes also share common features. The number of ORFs varies around 6,000 (*A. gossypii*, the smallest one, contains 4,718 ORFs, while *Y. lipolytica* is supposed to encode 6,703 ORFs). Goffeau's description for *S. cerevisiae*, i.e., "life with 6,000 genes" (Goffeau et al. 1996) seems to apply to other yeasts. In addition, intergenic regions are generally short, and there are few introns, or repetitive elements, even if the genome of *Y. lipolytica* tends to be slightly more expanded than the others. Transposons are scarce and only a few cases of horizontal transfer have been detected.

Despite these common characteristics of their genome, each yeast species shows a distinct physiology. Their niches are diverse: trees and fruits (*S. cerevisiae*), dairy products (*K. lactis*), decaying organic compounds (*Y. lipolytica*), seawater (*K. aestuari*), high-salt food (*D. hansenii*) or highly acidic media (*Zygosaccharomyces bailii*). Some can be pathogens (*C. glabrata, C. albicans*). Even within the sole *Saccharomyces* group a quantitative difference of phenotype is evident, such as the low level of glucose repression typically observed in *S. kluyveri* (= *Lachancea kluyveri*) (Moller et al. 2002). Sugar assimilation patterns also illustrate the diversity, offering a convenient key for yeast classification. A general description of sugar assimilation patterns of yeasts can be found in Kurtzman and Fell (1998).

To summarize, the hemiascomycetous yeasts share many basic features of the genome, and contain roughly the same number of ORFs, whereas their physiology varies greatly. We may ask how yeast generates this diversity from the largely similar genomic components. The question can now be approached by both bioinformatics and experimental tests. We will consider here three possible mechanisms, not mutually exclusive, that may be at the basis of this adaptation process. The observed phenotypic variations could involve (1) large chromosomal rearrangements (such as translocations for example), (2) gene duplication/gene loss, possibly associated to functional differentiation, and (3) modified expression of the same gene subsets under the control of different regulatory mechanisms (variation of regulatory networks). Many earlier studies focused on the second mechanism, but more recent works have analyzed the two other possibilities. The respective weights of these different mechanisms in the functional evolution of yeasts are for the moment difficult to estimate. The aim of this review is to sum up the recent approaches dealing with these mechanisms.

4.3 Gross Chromosomal Rearrangements

In 2000, Fischer et al. analyzed the genomes of six closely related *Saccharomyces* species belonging to the *sensu stricto* complex for the possible presence of gross chromosomal rearrangements and identified several translocations (mostly reciprocal). These rearrangements happened at specific points ("hot spots") and were associated to ectopic recombination between Ty elements or duplicated pairs of genes. Highly polymorphic chromosomes appear to be a common characteristic of industrial *Saccharomyces* strains (Codon et al. 1998; for a review, see Mortimer 2000) where Ty-mediated reciprocal translocations have been identified (Codon et al. 1997; Rachidi et al. 1999; Infante et al. 2003). In some cases, these rearrangements have been shown to increase adaptative evolution. During long-term cultivation experiments in glucose-limited medium, sequence alterations occurred which have been correlated with adaptive changes of physiology (Dunham et al. 2002). In another observation on a wine yeast strain, a reciprocal translocation led to a promoter change in the *SSU1* gene, coding for a sulfite transporter involved in the resistance to sulfite, a preservative used in winemaking (Perez-Ortin et al. 2002).

Such gross rearrangements have been postulated as a potential mechanism of speciation, since these strains can mate, but interspecific pairings produce a sterile hybrid. A closer examination of the chromosome structure of different *sensu stricto*

yeast strains (Fischer et al. 2000) revealed that gross rearrangements were not present in all the species since the chromosomal maps of *S. paradoxus* and *S. kudriavzevii* were collinear with the *S. cerevisiae* map. Such a result showed that gross rearrangements such as translocations did not drive alone the speciation process. Taking a direct experimental approach, Delneri et al. (2003) engineered a *S. cerevisiae* strain by modifying its chromosomes to make them collinear with two *S. mikatae* strains. When interspecific hybrids were produced from these strains, their progeny were mostly viable, but they were extensively aneuploid, while the crosses between the noncollinear version of the otherwise isogenic strains indicated that reciprocal translocations caused a marked reduction in hybrid fertility. This confirms that translocations can contribute to the reproductive isolation between species, in combination with other mechanisms such as mismatch repair (Hunter et al. 1996).

4.4 Gene Duplications Leading to Adaptation and Biodiversity

Gene duplications have long been thought to be a major driving force in biological evolution. This mechanism provides an extra copy which can later be modified to produce a "novel function" (Ohno 1970) without losing – initially at least – the original function and therefore without risk. The importance of this mechanism in evolution has already been evidenced in many cases (Papp et al. 2003; Gu et al. 2004; Zhang and Kishino 2004, references therein).

4.4.1 Gene Duplication in Functional Evolution

Wolfe and Shields (1997) have proposed that a global genome duplication occurred prior to the speciation of *Saccharomyces* (*sensu stricto*). This seems to be confirmed by the analysis of the complete sequence of *K. waltii* (Kellis et al. 2004). Further analyses of the various genomes now at our disposal revealed a large variety of events which may superpose their effects prior or posterior to the global duplication event. Rearrangements and gene loss followed the complete genome duplication (Wong et al. 2002). Spontaneous local duplications have also been shown experimentally to happen quite frequently (Koszul et al. 2004) and could play an important role in reshaping the genome. Paralogous genes thus generated can follow separate fates. The two copies can evolve to different functions as mentioned before, or keep the same function. Alternatively one of the copies can be lost by deletion or inactivated by mutation leaving in place a pseudogene (also called a "relic" when highly degenerate). Some relic genes have been identified recently (Lafontaine et al. 2004).

There are no general rules that allow us to predict the functional fate of duplicated genes or the proportion of duplicates which are functionally differentiated. In addition, the notion of functional differentiation is loosely defined since it may mean the same function with a different expression (as for example the pair of anaerobic/aerobic genes of *S. cerevisiae* which play the same function but are expressed in two different physiological conditions), or a duplicate gene can actually change to exert a function different from that of the original gene. This question was

approached experimentally by examining the function of pairs (or more) of gene families as described, for example, in Llorente et al. (1999). In this case the two most closely related pairs of three genes were shown to have the same function with no differences in expression, while the third one, which acquired another domain (gene fusion), seemed to encode a protein with a different (and yet unknown) function. Delneri et al. (1999) analyzed the family of aryl alcohol dehydrogenase and showed that in terms of response to oxidative stress, the apparent genetic redundancy is more apparent than real since only one gene seems to be the key player; nevertheless, the real function of these genes has still to be established. In another report on the *THI5* gene family (involved in thiamine biosynthesis), only very subtle differences in the regulation were revealed (Wightman and Meacock 2003). In the last two cases, the genes are located within the subtelomeric regions, which may act as a "nest" for the formation of newly duplicated sequences, as proposed by Piskur and Langkjaer (2004) in their review. Such (evolutionary) very young copies did not have enough time to differentiate. With a more theoretical approach, Langkjaer et al. (2003) tried to assess the initial assumption that "rarely paralogues are preserved because they differentiate and become functionally specialized" but they could only deduce by phylogenetic studies whether the gene duplicated before or after the divergence of two yeast lineages. Using a different approach, Zhang and Kishino (2004) tried to predict the fate of a duplicate with respect to the recombination rate of the genomic region around this gene. An alternative way of functionally classifying proteins is to rely on their capacity of protein–protein interactions independently of sequence data. Baudot et al. (2004) developed a bioinformatic method called PRODISTIN which was applied to 899 duplicated genes. Their main conclusion is that the fate of duplicate genes is distributed quite unequally between three categories: the majority (63%) of protein pairs are involved in the same biological process, 7.5% may be involved in different aspects of the same biological process. Only a limited fraction (22%) acquire new biological functions. If this is confirmed by other methods, one may consider that the role of duplication in functional evolution, based on acquisition of new functions, has probably been quantitatively overestimated.

Despite these attempts, most of our means to understand functional evolution of a biological pathway are restricted to a systematic comparative analysis in different species of the presence/absence/copy number of the genes involved in that pathway. This approach has been successfully applied to several biological processes, such as mating type genes, silencing and subtelomeric gene families (Fabre et al. 2004), the MAT locus and HO endonuclease (Butler et al. 2004) or DNA replication, repair and recombination (Richard et al. 2005), allowing stimulating hypotheses to be formulated for the acquisition of new functions. This comparison also led to the observation that the simultaneous loss of genes of the GAL pathway in some species was a way to their adaptation to specific ecological "niches" (Hittinger et al. 2004). In our laboratory we are examining carbon and oxygen metabolism on the same basis (Bolotin-Fukuhara et al. 2005; Bolotin-Fukuhara and Bao, unpublished data) and some of the data which complete the analysis on anaerobiosis by Gojkovic et al. (2004) are presented later.

Before any conclusions are drawn however, one should be aware that this type of analysis (presence/absence of genes/copy number) relies entirely upon the quality of

the sequence in terms of assembly and polishing. When the genome coverage is low, the absence of the homologue may only mean that either the corresponding sequence is absent, or that the partial sequence did not allow detection of the homologue. The same holds true for the detection of duplicates. This problem was encountered in the Génolevures I program, for which functional analysis was not very informative (Gaillardin et al. 2000). Only completely finished sequences can lead to precise and definitive conclusions. Therefore, the recent works described in the preceding discussion relied to a great part on the completely sequenced and assembled genomes from the Génolevures II project. This point has been discussed in detail in Bolotin-Fukuhara et al. (2005).

4.4.2 Central Metabolism

The sugar sources which can be assimilated vary greatly according to yeast species (Barnett 1976), but glucose remains a universal carbon source of yeasts. Glucose is directed to pyruvate through glycolysis and most yeasts metabolize pyruvate partly though fermentation and partly through respiration (such is the case of *K. lactis* or *P. stipitis)* or use it exclusively for respiration as does *Y. lipolytica* and other typically respiratory species. *S. cerevisiae* and closely related species are, in contrast, predominantly fermentative yeast in which respiratory pathways are strongly repressed by glucose (Crabtree effect positive). Very complete carbon source assimilation spectra are listed in Kurtzman and Fell (1998). A more recent review addressing carbohydrate and energy-metabolism in nonconventional yeasts (Flores et al. 2000) concluded that "basic knowledge is missing on many components of these pathways and that studies on regulation of critical steps are scarce." The existence of genomic data offers, therefore, an opportunity to reinvestigate these important pathways.

In a previous review (Bolotin-Fukuhara et al. 2005), we compared the presence/absence of genes involved in glycolysis, the tricarboxylic acid cycle and related pathways. We took advantage of the fact that the three yeasts which were analyzed, *S. cerevisiae*, *K. lactis* and *Y. lipolytica* represent three different levels of balance between fermentation and respiration. A decade ago the glycolytic pathway was studied in *K. lactis* by screening for Rag mutants (RAG means resistance to antimycin on glucose and allows mutants of the glycolytic pathways to be selected). Fourteen complementation groups have been identified in this screen (Wesolowski-Louvel et al. 1992) and later molecular analysis revealed a low level of gene redundancy as compared with that in *S. cerevisiae*. In the latter most of the glycolysis genes are duplicated or even multiplicated (such as multiple hexose transporters). The systematic analysis made on the three genomes confirmed this trend (Bolotin-Fukuhara et al. 2005). Extending this type of analysis to the yeasts whose genome is sequenced reinforces this idea since the genes of the upper part of the glycolysis pathway (Table 4.1) are generally not redundant in these yeasts, as opposed to *S. cerevisiae*. This may be explained – among other hypotheses – if we assume that storage reactions have to be more active in fermentative yeasts like *S. cerevisiae* since the energetic yield of glycolysis is smaller than in respiratory metabolism. It is interesting to note that this low redundancy of the genes involved is not restricted to the species placed in the phylogenetic tree before the general duplication of the genome.

Table 4.1 Distribution of some genes involved in carbon metabolism in yeast. Yeast species are placed in the order of the phylogenetic tree presented in Fig. 4.1. The yeast species abbreviations are *Sc* for *Saccharomyces cerevisiae*, *Sa* for *S. castellii*, *Cg* for *Candida glabrata*, *Kw* for *Kluyveromyces waltii*, *Sy* for *S. kluyveri*, *Kl* for *K. lactis*, *Ag* for *Ashbya gossypii*, *Dh* for *Debaryomyces hansenii*, *Ca* for *C. albicans* and *Yl* for *Yarrowia lipolytica*. Genes encoding enzymes involved in carbohydrate storage and the pyruvate decarboxylase genes. A *question mark* indicates that the data are unclear (partial sequence only, ambiguity in allele attribution, weak similarity which does not discriminate between the pairs, etc., see also the discussion in Sect. 2)

	Part A: carbohydrate storage									
Enzyme activity	**Sc**	**Sa**	**Cg**	**Kw**	**Sy**	**Kl**	**Ag**	**Dh**	**Ca**	**Yl**
Phospho-glucomutase	2: *PGM1* *PGM2*	2	2	1	1	1	1	1	1	1
Glycogen synthase	2: *GSY1* *GSY2*	2	2	1	1	1	1	1	1	1
Glycogenin glucotransferase	2: *GLG1* *GLG2*	1?	1	1	1	1	1	2	1?	1
alpha, alpha-trehalose-phosphate synthase (regulatory activity)	2: *TPS3* *TSL1*	1	2	1	1	1	1	1	1	1
alpha, alpha-trehalase	2: *NTH1* *NTH2*	1	2	1	1	1	1	1	1	1
	Part B: pyruvate decarboxylase									
	3: *PDC1* *PDC5* *PDC6*	3?	2	1	3	1	2	3	2	1

Therefore, the situation may reflect particular physiological requirements of each species. A species such as *S. castellii* does not maintain the complete set of duplicate genes perhaps because its metabolism is less fermentation-oriented than that of *S. cerevisiae* and its *sensu stricto* relatives. This absence of redundancy may also be linked to the reduced glucose repression in *S. kluyveri*, a *sensu lato Saccharomyces* like *S. castellii*.

The general redundancy of glycolysis genes in *S. cerevisiae* may suggest that it is a way of increasing the metabolic flux of this pathway. However, no direct evidence has been provided yet for actually increased levels of most enzymes. A few attempts at genetic engineering have been made on key metabolic points which are often critical in industrial processes. Such is the case of pyruvate decarboxylase (PDC) genes. PDC is a key enzyme that directs pyruvate to fermentation rather than respiratory pathways. Indeed, construction of *S. cerevisiae* strains with reduced specific Pdcp activity has been attempted (Flikweert et al. 1999; Remize et al. 2000). PDC is encoded by three genes in *S. cerevisiae* (a major form, *PDC1*, and a minor form, *PDC5*, Hohmann and Cederberg 1990; the third enzyme, *PDC6*, is inducible by sulfur limitation, Fauchon et al. 2002), while in *K. lactis*

there is only one single gene (Bianchi et al. 1996). In this case, the reduced copy number of *PDC* genes is probably not associated with increased respiratory activity. In other species (Table 4.1), the number of PDC genes is variable and is not correlated with the intensity of the respiratory metabolism of each species. Experimental data also point to this conclusion. Moller et al. (2004) identified three PDC genes in the yeast *S. kluyveri* and measured the enzyme activity under various conditions. They could show that *S. kluyveri* and *S. cerevisiae*, grown under comparable conditions, had different flux distributions at the pyruvate branch point, and hypothesized that factors other than the Pdcp activities are responsible for this difference.

4.4.3 The Case of Aerobiosis/Anaerobiosis

Yeasts cannot grow on strict anaerobiosis, with the exception of some *Saccharomyces* yeasts. Among the yeasts for which the genome has been completely sequenced, there are two types of physiology: *S. cerevisiae* and the *Saccharomyces sensu stricto* species (*S. bayanus*, *S. kudriavzevii*, *S. Paradoxus* and *S. mikatae*) and *sensu lato* species (*S. kluyveri* and *S. castellii*) are able to grow anaerobically, while *K. lactis*, *D. hansenii*, *C. glabrata*, *A. gossypii*, *C. albicans* and *Y. lipolytica* are not. As an example of functional evolution, the study of these genomes may shed light on the question of how some yeasts have acquired (or lost) the capacity to grow anaerobically. For *S. cerevisiae*, a systematic study has been done to compare the genes expressed in anaerobiosis versus aerobiosis by a genome-wide transcriptional analysis (Ter-Linde et al. 1999). Only a small number of genes responded to these different physiological conditions. The study revealed *ROX1* (the repressor of hypoxic and specific anaerobiosis genes) as a main site of response, mediating the other targets. This was further confirmed by transcriptome studies of Rox1p targets (Ter Linde and Steensma 2002). Some pathways are usually dependent upon oxygen and anaerobic yeasts developed a way to circumvent oxygen limitation. Such is the case of the fourth step of the pyrimidine biosynthesis pathway, the dihydroorotate dehydrogenase (DHODase) encoded by the *URA1* gene in *S. cerevisiae*. In relation with the DHODase, Gojkovic et al. (2004) recently proposed an explanation why some yeasts are obligate aerobe. In most species this enzyme activity is dependent upon oxygen, because they possess only a mitochondrial form of the enzyme. In *S. cerevisiae* (and in other *Saccharomyces* yeasts) there is a cytoplasmic form of the enzyme. This cytoplasmic enzyme is phylogenetically related to a bacterial DHODase and Gojkovic et al. postulated that yeast has acquired the corresponding gene by horizontal transfer. An elegant confirmation of the model was presented on the basis of an analysis of the *S. kluyveri* genome, which possesses both mitochondrial and cytoplasmic forms of DHODase. Each of the corresponding genes was separately disrupted and the effects were analyzed (Gojkovic et al. 2004) to substantiate the proposal.

Another oxygen-dependent pathway is the sterol biosynthesis pathway. When it is functional, yeast does not take up a significant amount of exogenous sterols (a phenomenon called aerobic sterol exclusion, Lewis et al. 1985). In anaerobiosis however, *S. cerevisiae* has to rely on external sterol supply for growth. Only if ergosterol is added to the medium *S. cerevisiae* grows in the absence of oxygen. Wilcox et al.

(2002) have shown that a few genes are essential for sterol influx: the two paralogous sterol transporters Aus1p and PdrIIp, as well as the cell-wall protein called Dan1p and the transcription factor Upc2p. Another hypoxic transcription regulator, Sut1p, is probably also involved in the process (Alimardani et al. 2004). In a previous comparison (Bolotin-Fukuhara et al. 2005), we came to the conclusion that the sterol uptake pathway was probably absent in the two aerobic yeasts *K. lactis* and *Y. lypolitica*. In order to generalize the role of sterol transport in anaerobic growth of yeasts, this line of study has been extended to other yeasts. Table 4.2 indicates the presence/absence of the corresponding genes in the complete panel of yeasts whose complete genome has been sequenced. It seems reasonable to conclude that all *sensu stricto Saccharomyces* yeasts have the complete set of genes for sterol uptake as well as the cytoplasmic form of *URA1*, which account for their ability to grow anaerobically, although this conclusion has restrictions inherent to the analysis of incomplete genomes as discussed before (see Sect. 4.1). Interestingly, other yeasts that are less closely related to *S. cerevisiae* (*S. castellii, S. kluyveri* and *C. glabrata*) have only one copy of the sterol transporters (Aus1p/PdrIIp), suggesting that the duplication of sterol transporter gene is probably a late event in evolution. The presence of the special cell-wall protein Dan1p is considered to be essential for anaerobic growth (Alimardani et al. 2004). Indeed, the corresponding gene has been detected only in *sensu stricto Saccharomyces* and in *S. castellii* (this yeast can grow anaerobically according to Langkjaer et al. 2003) and, surprisingly, in *K. waltii* (but only as a fragment; the capacity of anaerobic growth of this species is not known). *C. glabrata* seems to possess most of the genes necessary for sterol import despite its inability to grow anaerobically. The absence of the *DAN1* gene was a possible explanation, but a closer examination revealed that this species did not contain the cytoplasmic *URA1* gene form which, according to the Gojkovic hypothesis (Gojkovic et al. 2004), should be present. A general scheme of introduction of new anaerobic traits during the evolution of yeasts is represented Fig. 4.2. This complexity only confirms that many events superimposed their effect on lineage evolution and took place during the general evolution of yeasts as was discussed previously. While information gained from the sequence data and their bioinformatic analysis provides ideas about the process of acquisition of the capacity of anaerobic growth, experimental confirmation is required to confirm/invalidate the different hypotheses proposed.

4.5 Changes in Regulatory Circuits for Adaptation to New Environments and Physiology

As described before, modification of expression of a subset of genes by changing their (coordinated) regulation is one of the possibilities of adaptation of a species to a different environment. On *S. cerevisiae*, much effort has been made in the past year to unravel the regulatory circuitries through either theoretical or experimental approaches with the aim to predict the behavior of *S. cerevisiae* in different conditions. Even though global pictures of the regulatory circuitry of *S. cerevisiae* are emerging (Lee et al. 2002), we have still a long way to go to reach this goal. Extending these approaches to the other yeasts further requires identification of the *cis* and *trans* elements involved in transcriptional control, followed by a comparative

Table 4.2 Presence/absence of genes involved in key pathways required for anaerobiosis in yeasts. Same as in Table 4.1. *Sp* is *S. paradoxus*, *Sm* is *S. mikatae*, *Sk* is *S. kudriavzevii* and *Sb* is *S. bayanus*. The presence/absence of putative orthologues of *AUS1/PDRII* was concluded after examining the whole family of transporters. The *numbers* in *parentheses* indicate the number of copies which were found. An *asterisk* indicates that the information was obtained from fragments of genes and not complete sequences. A *question mark* means that the conclusion is ambiguous (usually because of the presence of only a fragment of the sequence)

Genes (as in Sc)	Sc	Sp	Sm	Sk	Sb	Sa	Cg	Kw	Sy	Kl	Ag	Dh	Ca	Yl
UPC2	Yes	Yes	Yes	Yes*	Yes*	Yes	Yes?	No	Yes?	No	No	No	No	No
DAN1	Yes	Yes	Yes	Yes	Yes	Yes?[a]	No	Yes	No	No	No	No	No	No
AUS1/PDRII	Yes (2)	Yes (2)	Yes (2)	Yes (2)	Yes (2)	Yes (1)	Yes (1)	No	Yes (1)	No	No	No	No	No
SUT1	Yes	Yes	Yes	Yes	Yes	No	Yes?	No	No	No	No	No	No	No
URA1 (cytoplasmic)	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	No	No	No	No
URA1 (mitochondrial)	No	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

[a]Two putative proteins were identified which match with Dan1p but not with other Dan proteins, nor between themselves.

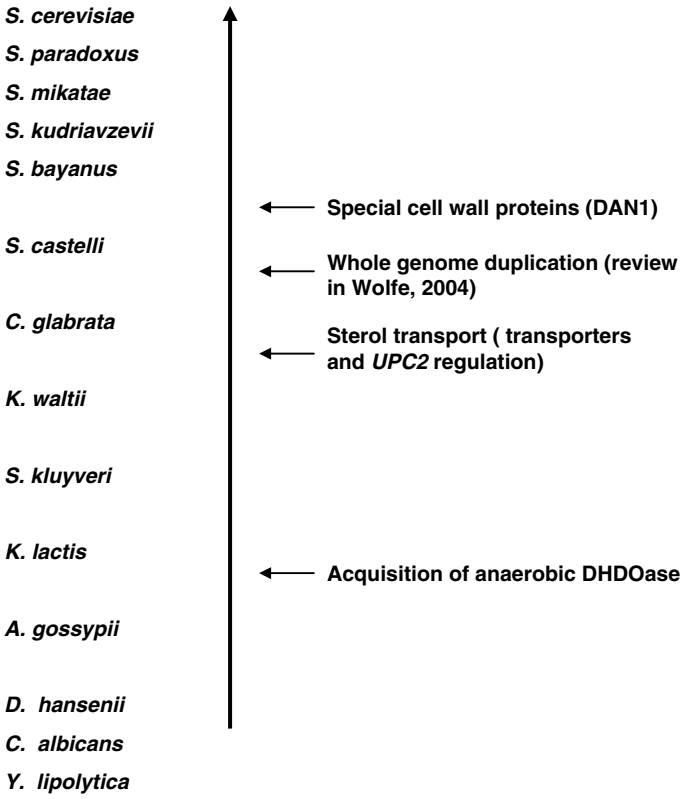

Fig. 4.2. Introduction of new anaerobic traits during the evolution of yeasts. The acquisition of traits related to anaerobiosis such as a new cytoplasmic form of the dihydroorotate dehydrogenase, or different functions related to sterol uptake, are indicated along the evolutionary tree of yeasts as represented Fig. 4.1

analysis of their regulatory networks. The availability of several complete genomic sequences of yeasts now offers the necessary tools to reach this ultimate goal.

4.5.1 Identification of *cis*-Regulatory Elements by a Comparative Genome Approach

It is not an easy task to systematically identify the *cis*-regulatory elements in a genome. Several methods have been developed for this purpose. One may wonder if comparison of genomic sequences can help to find out functionally conserved intergenic sequences, in the way we are searching for orthologous genes. The difficulty comes from the facts that (1) intergenic regions diverge rapidly as compared with coding sequences and (2) the regulatory sequences are generally short and often tolerant to some sequence variation. Comparison between species for which coding sequences are already quite different will not be helpful because the signal-to-noise

ratio of these short sequences is not significant enough. In contrast, exploiting sequences of closely related yeasts may be less informative for functional domain analysis of proteins but should allow the detection of conserved signals ("phylogenetic footprints") in intergenic regions. Previous work (Moses et al. 2003) has indeed shown that binding sites for transactivating elements evolve slower than their surrounding sequences. Therefore, we expect that an improved analysis of intergenic sequences may detect regulatory elements.

This was the rationale followed by two groups who chose to analyze *Saccharomyces sensu stricto* species *S. paradoxus*, *S. mikatae* and *S. bayanus* in one case (Kellis et al. 2003) and several yeasts of the *sensu stricto* and *sensu lato Saccharomyces* groups (*S. mikatae*, *S. kudriavzevii*, *S. bayanus*, *S. castellii* and *S. kluyveri*) in the other (Cliften et al. 2003). The *sensu lato* strains were included here in order to help pinpoint some sequences when the background intergenic sequences are not divergent enough.

The two groups developed different algorithms to identify the binding sites of transcription factors and proposed 72 and 79 *cis*-binding sites respectively. However, while Kellis et al. (2003) emphasized that "such an approach has the power to identify key functional elements without previous biological knowledge," many biological results (functional significance of neighboring genes, chromatin immunoprecipitation, ChIP-chip assays, coregulation, etc.) were in fact used in this work to select the best candidates. The regulatory sequences thus identified are thought to be of biological significance, because the result of the search included the already known, functionally characterized elements. Further experimental data may still be necessary to substantiate this approach.

4.5.2 The Repertoire of Transactivators

Transcriptional regulators have been well described in *S. cerevisiae* and for many of them their binding sites and (at least some of) their cellular functions have been identified. Homologues of these regulators can be searched for in the genomes of other yeast species. This systematic work has been achieved on the basis of the Génolevures I data (Bussereau et al. 2004). From these data it was inferred that if the DNA-binding motifs are well conserved, the rest of the sequences diverge very rapidly. This means that functional homologues could probably be identified rather easily in the species close to *S. cerevisiae* as was the case for the *cis* sequences reported in Sect. 5.1. For more distant species the task may be more difficult, but such systematic analysis, combined with phylogenetic studies, is now in progress (Bolotin-Fukuhara et al., unpublished data). The number and distribution of transactivators between the different DNA-binding classes is also of interest, and in particular for the group which is only found in ascomycetes, such as the zinc-binuclear cluster group (Todd and Andrianopoulos 1997). A few questions may be of particular interest: is the number of transactivators belonging to each class more or less constant along the phylogenetic tree? Are these proteins evolving by domains (disappearance or addition of new motifs) to produce new proteins not directly recognizable anymore but able to function in higher eucaryotes? Along this evolution what functions tend to be conserved more than others? Are they specific of ascomycetes?

The transcriptional repressor Rox1p is a good example of the problems raised in transcriptional regulator analysis. In *S. cerevisiae,* this repressor has an essential regulatory role as a function of oxygen (see Sect. 4.3). Several genes expressed in anaerobiosis are repressed by Rox1p in aerobiosis. Searching for Rox1p homologues in the *Saccharomyces* species allows the identification of a putative protein which is highly conserved in the *sensu stricto* group (Fig. 4.3a). However, this conservation becomes less obvious in *S. castellii* and *S. kluyveri*, with the exception of the readily recognizable HMG box motif (Fig. 4.3b). This illustrates the difficulty to identify transactivator homologues along the evolutionary tree (even in related species). Yet, all the yeasts mentioned previously can grow anaerobically and we expect the presence of a functional homologue of Rox1p. In other yeast species which cannot grow in anaerobiosis, the presence of a Rox1p-like protein may be questioned. Sequence analysis reveals in all cases the presence of a protein which contains a HMG motif but has no other sequence similarities (Bolotin-Fukuhara et al. 2005). One may wonder what is the biological meaning of such a finding. Obviously, experiments have to be done to decide if these proteins are functional equivalents of Rox1p. A similar question has been asked about the Hap4p protein, which is the regulatory and transactivator part of a transcriptional regulation complex (the HAP complex). For a long time this protein has been known only in *S. cerevisiae*. This seemed to make sense because its role in the cell is to allow the transcriptional reprogramming when the cell goes from a fermentative to a respiratory metabolism (DeRisi et al. 1997; Buschlen et al. 2003; Lascaris et al. 2003). However, we were able to isolate a *HAP4* functional homologue from the respiratory yeast *K. lactis* (Bourgarel et al. 1999) and more recently identified such proteins from all ascomycetes whose genome is sequenced using two short conserved motifs detected by comparing the *K. lactis* and *S. cerevisiae* sequences. A *HAP4* homologue from *H. polymorpha*, a respiratory yeast species more distant from *S. cerevisiae* than *K. lactis* in the phylogenetic tree, was shown to be fully functional despite the absence of one of the conserved motifs (Sybirna et al. 2005). The questions raised for *ROX1* can consequently also be raised for *HAP4* and probably many other transactivators.

4.5.3 Comparison of Regulatory Networks in Different Yeasts

As discussed before, it is possible to identify conserved *cis*-binding motifs specific for transcriptional regulators which themselves seem to be conserved in most cases. The next step is therefore to know to what extent the regulatory network controlled by a given transactivator is conserved in related species. Two possibilities can be considered: (1) either the same set of genes are coregulated or (2) only the global function of the network (such as energy control and production of specific metabolites) is conserved but is achieved through the regulation of a modified set of genes. To discriminate between these two possibilities, experiments are needed and are awaiting the necessary experimental genome-wide tools. Some information comes from the studies of *K. lactis*. From this yeast, several transactivators homologous to *S. cerevisiae MIG1* (Cassart et al. 1995), *HAP4* (Bourgarel et al. 1999) and *CAT8* (Georis et al. 2000) have been cloned and analyzed. While *CAT8* controls the key enzyme of gluconeogenesis in *S. cerevisiae*, the *K. lactis* homologue did not show this function.

```
S. cerevisiae
S. paradoxus      MNPKSSTPKIPRPKNAFILFRQHYHRILIDEWTAQGVEIPHNSNISKIIGTKWKGLQPED
S. mikatae        MNPKSSTPKIPRPKNAFILFRQHYHRILIDEWTAQGVEIPHNSNISKIIGTKWKGLQPEE
S. kudriavzevii   -------------------------------------------------------------
S. bayanus        -------------------------MWWNSDEWTTQGVEIPHNSNISKIIGTKWKGLQPEE
                  ***********************     **** *************************

S. cerevisiae     KAHWENLAEKEKLEHERKYPEYKYKPVRKSKKKQLLLKEIEQQQQQQQKEQQQ-------
S. paradoxus      KAHWESLAKKEKLEHERKYPEYKYKPIRKSKKKQLLLKEIEQQQQQQQQQQQQ-------
S. mikatae        KAHWEDLAKKEKLEHERKYPEYKYKPIRKSKKKQLLLKEVEEQQQQQ---TQQ-------
S. kudriavzevii   --------------------------------------GDLILALWWN-----------------
S. bayanus        KSHWESLAKKEKLEHERKYPEYKYKPIRKSKKRQLLLKEMEQQQQAQQQQAQQPLQQQQP
                  * *** **  ***************** *****  ***     *** *    **

S. cerevisiae     ----------QKQSQPQLQQPFNNNIVLMKRAHSLSPSSSVSSSNSYQFQLNNDLKRLPIP
S. paradoxus      ---------PQPQPQSQLQQPFNNNIILMKRAHSLSPSSSVSSSNSYQFQLNNDFKRLPIP
S. mikatae        ----------AQTQPQPQLQQPFNNNIVLMKRAHSLSPSSSVSSSNSYQFQLNNDFRRLPIP
S. kudriavzevii   ------------------------------------SSSISSSSSYQYQLNNDFRRLPNP
S. bayanus        LQQQQPLQQQQQQQPQLQPLNSNNIILMKKAHSLSPSSSVSSSNSYQYQLNNDFRRLPNP
                                                 *** *** ********* *** *** ***** **** *

S. cerevisiae      SVNTS--NYMVSRSLSGLPLTHDKTARDLPQLSSQLNSIPYYSAPHDPSTRHHYLNVAQA
S. paradoxus       SVNSP--NYMVSRSLSGLPLSHEKTARDLPQLSSQLNSIPYYSAQHDRSTRHHYLNVAQA
S. mikatae         SVSSP--NYMVSRPLNGLPLAHDKAVRDLPQLSSQFHSIPHYSAPHDSATRHHYLNIAQA
S. kudriavzevii    SVNSP—NYMASRPLNGLPLAHEKRAGDLPQLSSQLHAIPYFPATHDSTARHH-FLNVAQ-
S. bayanus         SINSPAGNYLVSRPSNGLPLTHDRTARDLPQISSQLHPMPYYSSTYDSTTRNHFLNIAQA
                   *     *  **   **** *     *** ***    *      *    *  ** **

S. cerevisiae      QPRANSTPQLPFISSIINNSS-QTPVT-TTTTSTTTATSSPGKFSSSPNSSVLENNRLNS
S. paradoxus       QPRANSTPQLPLISSIINNNS-QTPVT-TTTTTTTTATSSPSKFSSSPNSSILENNRLNS
S. mikatae         QPRANSTPQLPLISSIINSSN-QTSVTNSTATTTTTATSSPSKLSSSPNSSIFENNRLNS
S. kudriavzevii   -PRANSTPQLPLISSIIENSNNQTPVT-NTTTASTTAASSPNKVSSSPNSSISENNRLNG
S. bayanus         QPRANSTPQLPLISSIINNNNNNNNQT-PATTTTTTETSSPGKFTSSPNSSVSENNRLNS
                  ***************** *                    **  ***    ******  ******

S. cerevisiae      INNSNQYLPPPLLPSLQDFQLDQYQQLKQMGPTYIVKPLSHTRNNLLSTTTPT-HHHIPH
S. paradoxus       INISNQYLPPPLLPSLQDFQLDQYQQLKQMGPTYIVKPLSHTRNNLLSTTTPT-HHHIPH
S. mikatae         INNSNQYLPPPLLPSLQDFQLDQYQQLKQMGPTYIVKPLSHTRNNLLSTTTPT-HHHIPH
S. kudriavzevii   INSLNQYLPPPLLPSLQDFQLDQYQQLKQMGPTYIVKPLSHARNNLLSTTTPT-HHHIPH
S. bayanus         INNSNQYLPPPLLPSLQDFQLDQYHQLKQLGPTYIVKPLSHTRGNLLSTAAPTHHHHIPH

S. cerevisiae      IPNQNIPLHQIINSSNTEVTAKTSLVSPK
S. paradoxus       IPNQNIPLHQIMNSSNTEITAKTSLVSPK
S. mikatae         IPNQNIPLHQIINS-NNEVTAKTSLVSPK
S. kudriavzevii   ISNQNISLHQIINANNNEVTAKTSLVSPK
S. bayanus         IPNQNIPLHQLMNASNNEVTAKTGLISPK
                  * **** ***  *    * **** * ***
```

(Continued)

```
S. cerevisiae      ------------------------------------------------------------
S. kluyveri        ------------------------------------------------------------
S. castellii       MTRSLSVADITNTFDSPTMSNENNHSIFTTATSLPPITDRLNLPNMVMVSSSIDDNNNGA

S. cerevisiae      MNPKSSTPKIPRPKNAFILFRQHYHRILIDEWTAQGVEIPHNSNISKIIGTKWKGLQPED
S. kluyveri        -----MEEKIPRPRNAFILFRQHHHKLLIDQLREEGKPIPHNSEISKLLGTRWRSLSTDE
S. castellii       IHNRGKLEKIPRPKNAFILFRQHYHKELIDEWTKAGTDIPHNSEISKILGVKWKNISENE
                          ***** ********* *  ***     *  ***** ******  *

S. cerevisiae      KAHWENLAEKEKLEHERKYPEYKYKPVRKSKKKQLLLKEIEQQQQQQQKEQQQQKQSQPQ
S. kluyveri        KSYWDSKAQLEKEEHEKKYPDYKYRPVRRKRR---------------------ANSVPG
S. castellii       KTHWSDLAKEEKLEHLKKYPQYKYKPTRRKPRK------------------VPKKLKHA
                   *  *  **  ** ** **** ***** *

S. cerevisiae      LQQPFNNNIVLMKRAHSLSPSSSVSSSNSYQFQLNNDLKRLPIPSVNTSNYMVSRSLSGL
S. kluyveri        IPTTFQQHPVMSN---------------SVPMPIHPTVYRAETEYIHEPHFPPTSAESIY
S. castellii       TPWGFQKLSNFQQQ------------QHQQQEMIQPNYVRTDMNGFYQHQQHQQPQQHIN
                       *                                  *

S. cerevisiae      PLTHDKTARDLPQLSSQLNSIPYYSAPHDPSTRHHYLNVAQAQPRANSTPQLPFISSIIN
S. kluyveri        SIHNHSRNNIYPPL---LPAFQFWRAPQQ--------------------QLQQMRGSTS
S. castellii       QFPSYEASGFQNPN----MTLINKQVPSVP-------------------RILQPNALPR
                                             *

S. cerevisiae      NSSQTPVTTTTTSTTTATSSPGKF-SSSPNSSVLENNRLNSINNSNQYLPPPLLPSLQDF
S. kluyveri        SGDGRNYTGSSSGSFSSTSTNSSG-SYSTAGSLLSNNLPHFPYQDKQLQQPHQHPHQQQQ
S. castellii       DASYHPSPPLHSQNIMYQQPNGPIPSYSMVVHNSSRNQQPYMQQGSQSNIVIKSNYDNNT
                                             * *        *         *

S. cerevisiae      QLDQYQQLKQMG--PTYIVKPLSHTRNNLLSTTTPTHHHIPHIPNQNIPLHQIINSSNTE
S. kluyveri        QQQSFTHPYRHMTQPQQQQGQQLQYQQHLLQQQGQQHQHLQLQQQQPSQHQEQLHFASMQ
S. castellii       NVGNNHILRLPYPRTSSLPDRMTQPPVLLPSLLSPSSSNRELARQLPIPPLSAINLN---
                                                 *

S. cerevisiae      VTAKTSLVSPK----
S. kluyveri        VRSPPAAMYSFEREK
S. castellii       -RAGSEEYFKK----
```

Fig. 4.3. (Cond't) Clustal W alignments of Rox1p putative proteins from different yeast species. **a** Rox1p homologues alignment in the *Saccharomyces sensu stricto* species. An *asterisk* indicates identities. The 5′ part of the Rox1p homologues of *S. kudriavzevii* and *S. bayanus* could not be identified from the sequences available. The box underscores the HMG motif contained in the Rox1p sequence. **b** Alignment of the *S. cerevisiae* protein with homologues from *S. castellii* and *S. kluyveri*. An *asterisk* indicates identities. The 5′ part of the Rox1p homologues of *S. kudriavzevii* and *S. bayanus* could not be identified from the sequences available. The box underscores the HMG motif contained in the Rox1p sequence

Further analyses have revealed some functional conservations in the pathway (in particular the relation *SNF1*/*CAT8*) as well as differences in specific gene regulation (Lodi et al. 2001; Charbon et al. 2004). Clearly the range of function of *CAT8* is different in *K. lactis*. The same holds true for the other transactivators. The invertase gene is tightly regulated by *MIG1* in *S. cerevisiae*, but is not regulated in *K. lactis* (Georis et al. 1999). Disruption of the components of the *K. lactis* HAP complex (Nguyen et al. 1995; Bourgarel et al. 1999) does not lead to any growth defect on respiratory carbon sources, and the *CYC1* gene regulated by the HAP complex in *S. cerevisiae* is not regulated in *K. lactis* (Ramil et al. 1998). The possibility to study global gene expression with DNA arrays in the different yeasts should help us to unravel this complexity and better understand the evolution of regulatory circuits.

4.6 Conclusions

Yeast species live in different ecological niches, but their genomes reveal many conserved characteristics. This short review has tried to analyze and evaluate how the species exploit this basic genomic information to adapt to their new environment. Function evolution can be acquired by different mechanisms, based on gross chromosomal rearrangements, gene gain or loss or differences in gene expression. Much of the information we now have is predictive and is based mostly on bioinformatics analysis. While these data are very helpful for analyzing the mass of information now available, this new research field badly needs experimental data to confirm/invalidate those predictions. Fortunately, the "omics" tools necessary to obtain such data are or will soon be available for these so-called nonconventional yeasts (complete sequence, transcriptomics, proteomics, metabolomics, etc.). Some of these are even amenable to experimentation by classical and molecular genetics. These possibilities open the way to many comparative functional studies and will certainly change the respective importance of the different yeasts, building up new model yeasts for specific studies.

Acknowledgements

The author is grateful to H. Fukuhara, the Génolevures Consortium and colleagues from IFR115 "Genomes" for helpful and stimulating discussions during the course of this work.

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Chapter 5

Methods for Investigating Yeast Biodiversity

K. Boundy-Mills

Phaff Yeast Culture Collection, Department of Food Science and Technology, University of California Davis, Davis, CA 95616, USA
(e-mail: klbmills@ucdavis.edu, Tel.: +1-530-7545575, Fax: +1-530-7524759)

Abbreviations

ASBC	American Society of Brewing Chemists
CFU	Colony forming units
CLEN	Cadaverine lysine ethylamine nitrate
CLPP	Community level physiological profiling
DG18	Dichloran 18% glycerol agar
DGGE	Denaturing gradient gel electrophoresis
DIC	Differential interference contrast
FAME	Fatty acid methyl ester
FISH	Fluorescence in situ hybridization
G+C	Guanine plus cytosine
GPS	Global Positioning System
IGS	Intergenic spacer
ITS	Internal transcribed spacer
MPN	Most probable number
mRNA	Messenger RNA
NSA	Niger seed agar
OD_{600}	Optical density at 600 nm
OIV	Office International de la Vigne et du Vin
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
Q-PCR	Quantitative PCR
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal RNA
SGA	Sabouraud's glucose agar
SSCP	Single-strand conformation polymorphism
TGGE	Temperature gradient gel electrophoresis
TGYA	Tryptone–glucose–yeast extract agar
VNC	Viable nonculturable
X-α-gal	5-Bromo-4-chloro-3-indolyl-α-galactoside
YEPD	Yeast extract peptone dextrose
YM	Yeast extract–malt extract medium

5.1 Introduction

Much can be learned from a comparison of the methods used for microbial analysis of various materials by different specialists. Methods that are reproducible, specific, and accurate for one substrate can be appropriately modified for a new substrate. For this reason, in this chapter, some aspects of standardized industrial and clinical methods will be compared with those used for sampling natural ecosystems. Microbial methods developed for enumeration and detection of bacteria that can be or are being applied to yeast are also discussed. This chapter is not intended to be a complete description of all yeast sampling, detection, and enumeration methods that have been used. Rather it is a cross-section of methods used in a variety of applications, chosen to illustrate the many factors to consider when designing your own experimental protocol. Topics in this chapter include sampling, plating, microscopy, and culture-independent methods. The sampling, detection, and enumeration methods most appropriate for a specific application depend on what question is being asked. Different methods would be used for general surveys of the most common species present in a habitat than for detection of a low-abundance pathogen, for example. Details of detection and enumeration methods developed for sampling of specific habitats such as the phylloplane, marine and fresh water, and cacti can be found in other chapters of this book.

In recent decades, increasing numbers of researchers have discovered the rich field of yeast ecology. Yeasts interact in fascinating ways with other organisms and with their environment, whether in a wine fermentation, a mycosis, or decaying plants. Much of our knowledge of yeast ecology has been gleaned through culture-dependent enumeration and detection methods; however, inherent restrictions placed by culture-dependent methodologies have limited our progress in understanding the ecology of yeast habitats. While they have limitations of their own, recently developed culture-independent methods are providing new insights into yeast ecology.

A broad diversity of microbial species including yeast play crucial roles in biogeothermal cycles and cycling of organic compounds, as well as human endeavors, including the food, biotechnology, and pharmaceutical industries. The global production of fermented beverages, involving primarily the yeast species *Saccharomyces cerevisiae*, amounts to billions of dollars annually. Non-*Saccharomyces* yeast species (nonconventional yeast) are being used in an increasing array of applications, including chemicals, enzymes, feed additives, fuels, flavors, and neutraceutical and pharmaceutical compounds (reviewed in Abbas 2003). Furthermore, the growing population of immunocompromised individuals has resulted in an alarming increase in the occurrence of mycoses. Pathogens and opportunistic pathogens are wreaking havoc on the medical care system. The influence of yeasts on human society is not a recent development. *S. cerevisiae* has been called the "oldest cultivated plant" (Braidwood 1953; Rose 1960) owing to its role in beverage fermentations. Moreover, it has been proposed that humans relinquished their nomadic lifestyle in favor of an agricultural one primarily to cultivate grain for fermented beverages (Braidwood 1953). Although this may be pressing the point, few would deny that yeast-fermented grain beverages are far more enticing than nonfermented ones.

Despite the huge influence of yeasts on natural ecosystems and human society, it is estimated that less than 5% of yeast species have been described (Staley et al. 1997). It is not known if this small percentage is representative of overall yeast diversity. The number of yeast species described to date (over 800) (Barnett et al. 2000; Kurtzman and Fell 2000 and subsequent publications) has been limited by several factors, including the limited number of yeast ecologists, systematists, and taxonomists. Another contributing factor is the constraints placed by the culture-dependent methods used to detect and characterize yeasts. The similar rich media and aerobic incubation conditions used for yeast surveys may not allow growth of many yeast species. For example, the large budding yeast species *Cyniclomyces guttulatus* was observed in the intestinal tract of rabbits in 1845 (Remak 1845), but was not successfully cultured for over 100 years. Repeated attempts to culture this organism on commonly used media such as malt extract were not successful, until the correct growth conditions were determined, which include humidity, temperature, pH, and nutritional requirements (Shifrine and Phaff 1958). Similarly, the yeast species *Coccidioascus legeri* was observed long ago within the cells of the intestinal epithelium of *Drosophila funebris* (Chatton 1913), and can be observed in Geimsa-stained gut smears of live *Drosophila* (Ebbert et al. 2003), but has eluded cultivation and characterization.

A revolution is currently under way in microbial ecology, owing to several culture-independent molecular, biochemical, and microscopy methods developed for the study of microbial ecology in various fields ranging from natural ecology to pathology to food spoilage to fermentation science. For example, unprecedented biological diversity is being revealed in many ecosystems through molecular methods such as the polymerase chain reaction (PCR) followed by hybridization or sequencing. Huge numbers of undescribed species are being detected solely on the basis of ribosomal DNA sequences. Unfortunately, while a ribosomal sequence may allow presumptive phylogenetic placement of a species, proper characterization of these uncultivable (or more properly, "not yet cultivated"), low-incidence, fastidious, or otherwise recalcitrant species must await further technological developments, including cultivation methods.

5.2 Sampling Methods

The sampling method used in any given application depends on what question is being asked. Similar questions are asked in a number of natural and anthropogenic contexts, including:

- What are the dominant yeast species in a habitat, and what is their relative and absolute abundance at various times?
- What are the nondominant species or strains?
- What vectors deliver these yeasts to this habitat?
- Is a specific species present, and in what abundance?

As fungi, yeasts are saprophytic, and thrive in habitats containing a simple carbon source. A host of yeast habitats have been surveyed by yeast ecologists, as described in other chapters of this book: the phylloplane, cactus, marine and fresh

water, insects, soil, forests, and extreme environments. These surveys have revealed that many yeast species are specialists, meaning they are found almost exclusively in specific habitats. New species, and new habitats of known species, have recently been discovered from sampling of habitats as diverse as leaves (Wang and Bai 2004), bees (Rosa et al. 2003; Brysch-Herzberg 2004), beetles (Lachance and Bowles 2002), cork (Villa-Carvajal et al. 2004), pickles (Tominaga 2004), and soft drinks (Stratford et al. 2002). Future analysis of unexplored habitats, such as insects and other invertebrates, will undoubtedly uncover additional species. Even frequently studied habitats such as soil contain undescribed species (Renker et al. 2004).

Certain food and beverage industries have developed standardized methods for microbial sampling, detection, and enumeration for quality control purposes, such as to confirm sanitation of equipment, or to follow the course of fermentation. These methods are more critical in some industries than in others. For example, the temporal and regional variation of product in the wine industry is not only accepted but celebrated, and ascribed such terms as vintage or *terroir*. Consumers enjoy the result of variation in fermentation conditions. On the other hand, the brewing industry, particularly any internationally marketed brand, depends on consistency of their product across the globe and over the years, and sanitation of the facility is more crucial for product quality and safety. The American Society of Brewing Chemists (ASBC) has, since 1945, produced a regularly updated handbook (ASBC 2003) with detailed protocols for the detection and enumeration of yeasts and bacteria from equipment, ingredients, and at various stages of the production process. Specific methods for microbial analyses of process water and compressed air supplies are even described. Internationally standardized methods for microbial analysis of wine have also been published, such as those by the Office International de la Vigne et du Vin (OIV) (OIV 2004). Owing to high risks of spoilage, methods for microbial analysis of dairy products (IDF 1987; APHA 2001) are particularly stringent. In the 1930s and 1940s, the International Association of Milk and Food Sanitarians and the American Public Health Association tested various media formulations to find one that could be used for aerobic plate counts of the broadest variety of fresh foods. "Standard methods agar" (now called plate count agar) was designated as the official medium for aerobic plate counts (Walter 1967). Detailed sampling, dilution, and plating methods for meats, produce, dairy products, water, food ingredients, and processed foods have been compiled by the International Commission on Microbial Specifications for Foods (ICMSF 1978, 1986). Despite publication of these recommended protocols, a review of 100 studies of plate counts of fresh meat and poultry products since 1985 found that 15 different plating media were used, as well as many different incubation temperatures and times (Jay 2002).

Studies of biodiversity in natural ecosystems must consider many of the same issues as industrial analyses, namely, the heterogeneity of samples, and temporal and spatial variability of populations within the substrate. Sampling, detection, and enumeration techniques must be carefully designed to take these factors into consideration. For example, when describing a new species, it has been recommended that the description be based on the isolation and characterization of a number of strains gathered at different times from different locations in order to obtain a clear view of the habitat preference and the biogeography of the species (Phaff et al. 1978). In

addition, the biogeography of a species has larger ramifications. The effect of geographic isolation of populations on the speciation of larger organisms has long been known. The effect of spatial separation of two or more populations on microbial speciation has recently been recognized (Papke and Ward 2004).

5.2.1 Surface Sterilization and Aseptic Sample Collection

A variety of methods have been used for gathering a sample for microbial analysis. In cases where the sample is inherently of very small volume and homogenous, such as the nectar in a flower, the liquid can be withdrawn with a sterile capillary pipet, and plated directly on isolation medium (Herzberg et al. 2002). Substances such as tree sap flux (Lachance et al. 2001) or insect frass (Phaff and do Carmo-Sousa 1962) have been directly placed or streaked onto the surface of plates. When determining the microbial flora present on plant surfaces, the plant material such as a leaf or flower petal can be pressed to the surface of a plate (Brysch-Herzberg 2004). Yeasts on the tarsi and other outer surfaces of insects have been isolated using a "walk plate," in which an insect such as a fruit fly (Lachance 1995) or bee (Rosa et al. 2003) is allowed to walk on the surface of an agar plate for 15-60 min, then removed. The material deposited by the insect may be spread over the surface of the plate with a sterile loop (Lachance et al. 1999). Yeasts that produce forcefully ejected ballistospores can be isolated from substances such as leaves using the ballistospore-fall method, described by Derx (1930). A leaf or other specimen is suspended above an agar plate, and the ballistospores are deposited onto the surface of the plate.

Other sampling methods must be used when sampling a larger specimen. With the exception of a laboratory fermenter, with its aerator aerating and impeller impelling, few microbial microcosms of significant size exhibit a uniform distribution of oxygen, nutrients, pH, moisture, or cell density. Furthermore, yeast are immobile, and thus the distribution of microorganisms is most likely not homogenous. For example, yeasts are most abundant on the surface of a grape in areas where juice might escape, such as around the stem (Belin 1972).

Thus it is important to select a sampling method that addresses the question at hand. If one is interested in determining what microbes may be deposited by or consumed by certain insects, the surface of a rot should be sampled. Identification of yeasts consumed by larvae feeding within the rot would require sampling of deeper tissues. Collection of several samples over a period of time would reveal the temporal succession of microflora. A survey of all yeasts present in the rot would require homogenization of a cross-section of rot, including sections both near the surface and within the rot.

This concept is reflected in recommended methods for obtaining a representative sample of brewing yeast. Yeast inocula used in brewing, whether a slurry or a compressed cake, "can easily become stratified" (ASBC 2003). Combination of a series of samples gathered aseptically from various sections of the yeast cake or yeast slurry is therefore recommended. Sampling techniques used in the brewery include letting several liters of liquid flow through the tap before collecting a sample (ASBC 2003). Similarly, in a clinical setting, patients are told to collect mid-stream urine. Examples of recommended methods for sampling various food products include

drilling to the center of a container of frozen eggs using a sterile bit, slicing a sector of Gouda cheese with a sterile blade, or aseptically collecting one center and one peripheral sample from canned meats (ICMSF 1986).

Sampling must also consider the differentiation between surface contaminants and autochthonous inhabitants. Often samples are surface-sterilized prior to removal of a sample for microbial analysis. Brewers may disinfect the zwickel cock with 70% ethanol, followed by flaming with a propane torch (C. Wallin, personal communication). Quality control technicians in a dairy packaging facility, on the other hand, may use an iodine solution to sanitize the sampling spigot. Certain clinical specimens are collected after swabbing the area with iodine, ethanol, or 2-propanol solutions to remove surface bacterial and other contaminants (ALA 1985). Similar methods can be used for surface sterilization of natural hosts of yeasts, but selection of a method requires careful consideration of the biology of the specimen being sampled. For example, sampling the intestinal microflora of small insects such as *Drosophila* must be performed very soon after collecting the specimen, because the ingested yeast are digested quickly (Shehata and Mrak 1951). Soaking certain insects in 70% ethanol is best preceded by immobilization by freezing for a few minutes to suppress the insect's regurgitation and ingestion reflex. Alternatively, the insect can be anesthetized with carbon dioxide prior to pressing the surface of the insect to an agar plate (Brysch-Herzberg 2004). Discussions with specialists with knowledge of the biology of the host organisms are highly valuable. Botanists can provide advice on the season and time of day when certain flowers will be in bloom and open, as well as where they may be found. Entomologists can be asked what season, weather conditions, and times of day are optimal for finding certain insect species, where they may be feeding, what luring and trapping methods are best used for that insect species, what body cavities may most likely harbor microbes, and how they can be dissected.

Different researchers may use different methods for surface sterilization of the same substance. For example, Rogers et al. (2004) compared several methods of surface sterilization of glacial ice cores prior to plating or PCR analysis, including exposure to bleach, ethanol, UV radiation, acid and base, and hydrogen peroxide. Treatment with bleach was found to be most effective in killing surface contaminants with the least loss of viability of interior microorganisms.

Aseptic sampling can involve a variety of implements, such as a sterile spatula, an inoculation loop, a needle, a swab, a pipet, or the collection of a liquid sample. The sample volume depends on the concentration of cells and the detection method to be used. Yeast in liquids with low concentrations of particulate matter, such as beverages or marine water, can be visualized and enumerated directly by microscopy, if present in sufficient abundance.

One of the most extensively studied yeast habitats is the wine fermentation. The resident yeast community has been examined from the grape in the vineyard through the stages of fermentation to the final product (Amerine and Kunkee 1968; Kunkee and Amerine 1977; Martini et al. 1980; Kunkee and Bisson 1993; Fleet et al. 2002; Ganga and Martinez 2004). As has been reviewed extensively, it is clear that the abundance and identity of yeast species shifts as the biochemical composition of the medium changes. Apiculate yeasts are common on the surface of the

grape, and in the early stages of fermentation. As the sugar level drops, and the ethanol level rises, ethanol-sensitive species subside, and *S. cerevisiae*, the champion of ethanol tolerance, predominantes. Successional stages of microbial flora have been observed in a number of other habitats, including, for example, a single slime flux over the course of a year (Phaff et al. 1964), a commercial malt whisky fermentation (van Beek and Priest 2002), degrading wood (Gonzalez et al. 1989), amapa fruit (Morais et al. 1995), and cocoa fermentation (Schwan and Wheals 2004). The biochemical and physiological environment of a microorganism is altered by the metabolic activity of the microbes themselves, which can affect several factors, including macronutients and micronutrients, pH, temperature, metabolites, and killer toxins. The community development in many cases "can be thought of as an orderly sequence of chemical changes each catalyzed by species waiting their turn in an imaginary eco-queue" (Giraffa 2004). Careful observation of the physiological state of a substrate is necessary to determine the optimal sampling strategy for enumeration and detection of yeasts. If the temporal course is of interest, samples should be collected before or shortly after the introduction of a yeast to a substrate, and at several points during and after the development of the yeast population.

Carefully recorded sampling details will not only allow future reisolation of specific species, but will also reveal details of the preferred habitat of a yeast species, successional stages, and geographic distribution. It has been shown that the yeast community present on grapes varies with factors such as climate, including temperature, rainfall, and geographic location (Parish and Carroll 1985; Longo et al. 1991); application of antifungal compounds (Monteil et al. 1986); grape variety and vineyard age (Martini et al. 1980; Rosini 1982; Pretorius et al. 1999); and soil type (Farris et al. 1990). Data that should be recorded when sampling any substance for yeast include geographic location (Global Positioning System, GPS, coordinates if possible), habitat sampled, state of the hosts and vectors present (whether flowers are in bloom, whether cactus flesh is rotting, presence of any insects), composition of any diluent used before plating or microscopy, surface sterilization method, medium formulation of plates used, and the length of any time delay between sampling and plating. Metadata that may be noted include references for host species identification, such as a field guide, and the reference datum used for GPS coordinates.

5.2.2 Sample Homogenization

The importance of collecting a representative sample, or a statistically significant number of samples, cannot be overstated. Yeasts and other microbes are not often evenly distributed in a substance. Scanning microscopy studies of leaves (Beech and Davenport 1970) and grapes (Belin 1972) have shown that yeasts adhere tightly to surfaces in the form of microcolonies. Bacteria are known to cluster in the rhizosphere around the roots of plants (Curl and Truelove 1986); yeasts may behave similarly. In certain foods, yeasts are also highly localized at high densities in reticulate structures (Fleet 1999). Geostatistical methods have been developed to describe spatial distribution of soil microorganisms, and power analyses can be used to determine the optimal sample size (Klironomos et al. 1999).

Where appropriate, a representative sample can be homogenized before analysis. This will give an estimate of the overall abundance of various species throughout the sample. Various methods have been used to homogenize specimens before enumeration by plating or other methods. The sample can be ground if necessary, then combined with a known volume of one of the diluents described later. The sample can be shaken manually or in a wrist-action shaker, pummeled in a peristaltic agitator such as a Stomacher, stirred on a magnetic stirrer, swirled in an orbital shaker, or pureed in a blender. Low-tech methods are also used: sterile gloves were used to hand-squeeze aseptically collected grapes in a study of *S. cerevisiae* isolated from indigenously fermenting musts (Cappello et al. 2004). Homogenization methods must be carefully selected. Fleet (1999) has summarized in detail the implications of erroneous enumeration of cell density owing to improper maceration and dilution steps when analyzing microbes in foods.

Fortes et al. (2001) have detected the pathogen *Cryptococcus neoformans* in the hollows of trees in Brazil. In cases such as this in which homogenization of a large sample, such as a tree, is not practical, a number of samples must be collected from different parts of the same rot and analyzed. Spatial heterogeneity results from inherent variability in the structure and composition of organic matter, differences in the external environment, and also results from microbial activity.

5.2.3 Sample Concentration

Many methods of enumeration of yeasts require a particular cell concentration range. Dilute liquid specimens can be concentrated by centrifuging at 3,000*g* or higher for several minutes, removing the supernatant, and resuspending the pellet in the remaining liquid (OIV 2004).

Yeast are known to be present in both freshwater (van Uden and Ahearn 1963; Spencer et al. 1964) and marine environments (Fisher and Brebeck 1894; van Uden and Fell 1968), and are known to be the dominant fungi in oceans (Sieburth 1979). However, direct plating is difficult owing to low cell counts, ranging from ten to 1,000 colony forming units (CFU) per liter (van Uden and Fell 1968). For enumeration of yeasts in liquids with low cell counts, whether marine water or beverages after bottling, membrane filtration is used to concentrate the cells to a detectable level. For most plating methods including membrane filtration, a target of 30–300 CFU per plate (Fugelsang 1997) or 20–200 CFU for wine (OIV 2004) or wastewater (Greenberg et al. 1992) or 25–250 CFU for various prepared foods (Zipkes et al. 1981) has been recommended as a statistically significant and countable range. A measured volume of liquid is passed through a sterile filter. Cellulose acetate or cellulose nitrate membranes with 0.45-μm porosity are recommended for beer samples (ASBC 2003). Antifoam can be placed in the receiving flask when filtering beer samples (ASBC 2003); this may be helpful for filtration of other liquids with the potential of foam formation. In the brewery, saline solution consisting of 0.85% NaCl is used to wash the membrane before transfer to an agar plate (ASBC 2003). Membrane filtration has also been used in the analysis of marine yeasts (van Uden and Fell 1968; Gadanho et al. 2003). After filtration, the membrane is placed on the surface of an appropriate agar medium, and colony formation on the surface of the membrane is monitored.

5.2.4 Sample Dilution

Desiccated substances, such as tree exudates, have been rehydrated with sterile water (Phaff and Starmer 1987). Samples with high concentrations of yeasts, such as decaying fruits, must be diluted before plating in order to obtain a countable and significant number of colonies upon plating. Serial dilution involves preparation of a series of tenfold dilutions in sterile diluent. Aliquots of each dilution are plated on appropriate media, and the number of colonies is used to calculate the concentration of yeasts in the original sample, expressed as CFU per milliliter.

Various diluents have been utilized for enumeration of yeasts in different contexts. Distilled water is not recommended in most cases owing to osmotic shock effects. Saline solution (0.85% NaCl) is used by brewers (ASBC 2003); 0.85% NaCl or Ringer's solution have been recommended for wine (OIV 2004); 0.1% peptone solution is used in analysis of foods (Mian et al. 1997); milk or Butterfield's phosphate-buffered water are used as diluents in analysis of diary products (Maturin and Peeler 1998). A low concentration of surfactant such as Tween 80 (0.01–0.05%) can be included in the diluent to aid in separation of cell clumps and filamentous structures (Deak 2003). Diluents with lower water activity must be used with osmotolerant yeast, such as *Zygosaccharomyces rouxii*, which may be encountered in very high sugar environments. Glucose concentrations of 20–30%, or glycerol concentrations of 18–26%, have given good recovery of this species from fruit juice concentrates and syrups (Hernandez and Beauchat 1995; Hocking et al. 1992).

Yeast in diluent must be plated promptly, particularly in saline solutions, which have been shown to have an adverse effect on viability, even for salt-tolerant yeast such as *Debaryomyces hansenii* (Andrews et al. 1997). Using the traditional plating method, 50–100 μL of a series of tenfold dilutions is placed on the surface of agar plates and spread with a flame-sterilized bent-glass rod while spinning the plate. Alternatively, a pour plate can be prepared by adding 15–20 mL of molten agar to up to 1 mL of liquid sample, and swirling to distribute the sample in the medium. These procedures involve a considerable expenditure in disposable plastics and media. The "track-dilution technique" is a modification of this method that decreases the time and expense involved (Jett et al. 1997). One 10-μL aliquot of each of six tenfold dilutions is spotted along one edge of a square agar plate. The plate is tipped so that the dilutions migrate in parallel tracks down the plate. After incubation, the dilutions with a countable numbers of colonies are selected and enumerated. Use of this method with wine-associated yeast cultures appears effective (D. Mills, personal communication).

Spiral plate counting is used in the food industry for enumeration of bacteria, and could also be used for enumeration of yeasts. Four logs of dilution of a sample are delivered on a single plate using a spiral plater. The dispensing stylus deposits decreasing amounts of a sample onto the surface of a rotating agar plate as it moves from the center of the plate outward. A sector with a reasonable number of colonies is selected for counting. The number of colonies in that sector is divided by the volume of sample dispensed on that sector to obtain the cell density, expressed as CFU per milliliter.

5.3 Direct Observation Methods

Antonie van Leeuwenhoek first viewed "animalcules," including brewing yeast, in the 1680s using his meticulously ground glass lenses. The microscope continues to be an extremely valuable tool in the microbiologist's laboratory. Direct observation of yeasts in a variety of substrates is used for detection and enumeration.

5.3.1 Microscopy

Light microscopy is useful for detection and enumeration of yeasts when the concentration of yeasts in the sample exceeds 5×10^5 cells/mL. Cell concentrations can be adjusted by concentration or dilution as described before. A detailed summary of yeast specimen preparation and photomicrography methods is presented in Barnett et al. (2000). The four optical systems commonly available and used to visualize yeast are bright field, dark field, phase contrast and differential interference contrast (DIC). Barnett et al. (2000) prefer the use of DIC for visualization of nonfilamentous vegetative cells, bright field for ascospores, and phase contrast for filaments.

In a clinical setting, a presumptive identification of certain fungal genera can be made on the basis of microscopic examination of clinical specimens (summarized in Koneman et al. 1997). For example, *Candida* spp. are seen as pseudohyphae, and sometimes as budding yeast forms. *Cryptococcus* spp. are seen as spherical and irregular-sized yeast forms, some with a thick polysaccharide capsule, and buds attached by a narrow constriction. In the winery, apiculate yeast species and *Brettanomyces*/*Dekkera* species also have characteristic cell morphologies. Some processing of specimens may be required to reveal the presence of yeasts. Potassium hydroxide is added to clinical specimens to dissolve epithelial and bacterial cells, allowing visualization of alkali-resistant yeast spores and hyphae by microscopy (Reilly 1991).

Electron microscopy has also been used to detect yeast cells in substrates such as ancient wine (McGovern 2003).

5.3.2 Direct Enumeration

Direct microscopy is used for the enumeration of many types of liquid suspensions of cells including yeast in various substrates. Brewers (ASBC 2003) and enologists (Fugelsang 1997; OIV 2004) commonly use a hemocytometer with Neubauer ruling, which has two etched 1-mm^2 grids of 400 squares each in a 0.1-mm-deep well, giving a volume of 0.1 μL. A sample of appropriate cell concentration is prepared. After homogenization and degassing, a sample is placed in the counting chamber and visualized under bright field illumination. The ASBC manual describes detailed protocols for reproducible counting, including whether to count cells touching the boundary lines, and when to count buds as additional cells. The number of cells per grid is multiplied by 10^4 and any dilution factor to obtain the number of cells per milliliter in the original sample.

The cell density of pure cultures in liquid medium can be estimated spectrophotometrically by measuring the optical density at 600 nm (OD_{600}). Cultures should be

diluted so that the OD_{600} is less than 1. The OD_{600} should be calibrated against another method of determining cell density, such as direct counting in a hemocytometer or plating for viable colonies. For the species *S. cerevisiae*, for example, an OD_{600} of 1 corresponds to roughly 3×10^7 cells/mL (Treco and Winston 2001).

5.3.3 Viability Staining

Brewers, enologists, histologists, and others use a variety of stains such as methylene blue, Ponceau-S, and Walford's stain to distinguish between viable and nonviable yeast cells. Of the various staining procedures available, methylene blue is preferred in both the wine and the brewing industries (Smart et al. 1999; ASBC 2003; OIV 2004). Methylene blue stains dead cells blue, while viable cells remain unstained. Various formulations are used, with and without buffers, but the preferred method for brewing yeast is the Fink–Kühles phosphate-buffered methylene blue method (Fink and Kühles 1933).

5.3.4 Fluorescent Labeling

DNA or RNA hybridization to probes specific for a species or group of species is an important tool for microbial ecology. Originally, radioactive labels were required, but more recently probes have been tagged at the 5′ end with markers such as fluroescein or rhodamine. After dot blotting, the cells are lysed to release nucleic acids, and the probes are hybridized. Changes in the relative amount of hybridization to ribosomal RNA (rRNA) reflect changes in the abundance and/or the rRNA content of the population being probed (Theron and Cloete 2000). Hybridization of monoclonal antibodies or nucleotide probes can also be performed in situ on environmental samples, which provides information on the spatial distribution of the species of interest. This method, called fluorescent in situ hybridization (FISH), has been used to study the spatial distribution of bacteria in minimally disturbed habitats such as in seafood (Connil et al. 1998), wine (Sohier and Laonvaud-Funel 1998), and cheese (Kolloffel et al. 1999), and yeast in tissue sections (Lischewski et al. 1996, 1997), *Aureobasidium pullulans* on leaf surfaces (Li et al. 1997), and *Brettanomyces* in wine (Stender et al. 2001). Use of FISH to analyze yeast on the phylloplane is described elsewhere in this book (see Chap. 13).

5.4 Culture-Dependent Methods

In the past, surveys of the yeast species resident in a habitat, host, or vector such as plants, insects, and soil were only possible through plating a specimen, incubating to allow for colony formation, noting the number and type of distinguishable colony morphologies, and identifying one or more isolate of each colony morphology (Phaff et al. 1978; Phaff and Starmer 1987). These types of plating methods involving selective plating and direct viable counts are relatively inexpensive, and provide information on the active, heterotrophic organisms in a population. These methods have given us innumerable insights into the life of yeasts, such as the range of species present, the substrates that support growth of certain yeast species, and the vectors

that distribute yeast to new habitats. We have learned that many yeast species are associated with a specific habitat and geographic range, which allows the isolation of a particular species at will from its known habitat. Culture-dependent methods have also resulted in the isolation of thousands of pure cultures that can be further characterized and utilized by future generations. In cases where the spectrum of resident species is well known, such as beverage fermentations, culture-dependent detection and enumeration techniques are preferred because they are reproducible, inexpensive, and accurately reflect the prevalence of the species of interest.

However, culture-dependent methods have limitations. Oftentimes different yeast species have indistinguishable colony morphologies, or one species may exhibit two or more colony morphologies, leading to inaccuracies in determining relative species abundances. Furthermore, there are very likely many species that do not grow under the growth conditions used, including temperature, pH, and a host of macronutrients and micronutrients. Cells growing in microcolonies or biofilms must be dislodged from their support medium in order to be enumerated accurately. Plate growth favors species that grow quickly. Taken together, these limitations influence the apparent diversity of the microbial community being analyzed. It may be impossible to estimate how many species are *not* detected using these methods.

It is now widely accepted that plate culturing techniques reveal only a portion of the true yeast diversity of a natural or man-made ecosystem: those individuals that are viable, and culturable. Organisms that are stressed, are in the so-called viable nonculturable (VNC) state, or that cannot grow under the conditions used are not detected. This in part explains why only a small fraction of the yeast species believed to exist have been described. A sublethally injured cell, including cells in the VNC state, may not be detected, but can survive until conditions improve. This has been observed in the case of lactic acid bacteria during wine storage and ageing (Millet and Lonvaud-Funel 2000), and has also been demonstrated for non-*Saccharomyces* yeast in wine fermentation (Cocolin et al. 2000a). Yeast cells that have been sublethally injured by heat or osmotic shock may grow poorly on plates, but can be revived by various resuscitation techniques (Fleet 1992; Deak and Beauchat 1996), indicating that they still influence the ecosystem.

5.4.1 Liquid and Solid Media

Being fungi, yeasts are saprophytic. Yeasts cannot perform photosynthesis or nitrogen fixation, and thus require carbon and nitrogen sources for growth. Yeasts also require a range of vitamins, minerals, and other growth factors, the range of which depends on the yeast species. Several rich media formulations have all the necessary factors required for the growth of many known yeast species. Media used for isolation and enumeration are generally complex and nutritionally rich. Common ingredients include a carbon source (e.g., glucose, fructose, sucrose), a nitrogen source, such as a digested protein (e.g., peptone, tryptone, casitone), and a complex supplement (e.g., yeast extract, malt extract). Selective and differential media are used to detect specific species or groups of yeasts. Some of the more commonly used rich media are described later. A sample of the selective and differential media that can be used in detection of specific species is also presented.

Malt extract medium, an early yeast medium formulation, was developed for the benefit of the brewing industry. This medium was made from diastatic malt, and was used either in a solid (malt extract agar) or in a liquid form. Wickerham modified this recipe by including yeast extract and peptone, resulting in yeast extract–malt extract (YM) medium still commonly used for maintaining and storing yeast cultures (Wickerham 1951). YM and many other standardized isolation and selection media formulations are commercially available in premixed form.

Several formulations of rich media are used by researchers in various fields. Clinical yeast isolations are often performed using Sabouraud's glucose agar (SGA) (Odds 1991). Brewers often use universal beer agar (ASBC 2003). In addition to malt extract and YM media, commercially available rich media useful for the cultivation of yeasts include yeast extract, peptone, and dextrose (YEPD), potato dextrose agar (PDA), and tryptone–glucose–yeast extract agar (TGYA). The development of these and other media has been described (King et al. 1986; Jarvis and Williams 1987; Fleet 1990; Deak 1991). Variants on TGYA have been developed, with differences in glucose concentration, or supplemented with chloramphenicol to retard growth of bacteria, or acidified to pH 3.5. One variant, yeast extract glucose chloramphenicol agar, lacking tryptone, has been recommended by the International Organization for Standardization as an international standard medium (ISO 1987) for use in food analysis. Yeasts from high-sugar environments such as nectar (Herzberg et al. 2002) have been grown on a medium containing 40% sugar (Hautman 1924).

The presence of bacteria and spreading molds in certain foods, decaying fruits, and soil complicates plating-based enumeration of yeasts. Supplements added to media include antibiotics such as oxytetracycline or chloramphenicol to decrease the growth of bacteria. Propionic acid or calcium propionate decrease mold growth significantly, but also limit growth of some aerobic yeast (Buhagiar and Barnett 1971). Rose Bengal (Martin 1950), ox gall (Miller and Webb 1954), eugenol, dichloran (Bell and Crawford 1967), or oligomycin can be added to inhibit rapidly spreading molds. Deak et al. (1998) have recommend that media containing Rose Bengal should be kept in the dark, to prevent degradation of Rose Bengal to a mold-inhibiting derivative. Studies of the recovery of yeasts from food products, including cheese and other foods, have indicated that media containing these additives perform more favorably than previous media acidified with organic or inorganic acids (Beauchat 1993). Researchers in five countries recently evaluated 11 selective media for the enumeration of yeasts from blue-veined cheese (Viljoen et al. 2004), which contains high levels of *Penicillium* species, bacteria, and often yeasts as natural contaminants, causing spoilage or affecting the final flavor. The tested media contained various additives such as biphenyl, molybdate, oxytetracycline, gentamycin, chloramphenicol, and eugenol to control bacteria or molds. Biphenyl was superior to Rose Bengal in controlling mold growth. The results indicated that dichloran 18% glycerol agar (DG18) and malt extract with biphenyl were superior in mold inhibition and yeast recovery from blue cheese, while Rose Bengal chloramphenicol agar, dichloran Rose Bengal chloramphenicol (King et al. 1979), and malt extract agar supplemented with sodium chloride and oxytetracycline were also acceptable for sampling this substrate. The use of plates containing Rose Bengal and/or chloramphenicol in recent

ecological studies (Lachance et al. 2001; Rodrigues et al. 2001; Rosa et al. 2003; Villa-Carvajal et al. 2004) marks a shift from the long-standing use of acidified media for control of bacteria.

When the spectrum of yeast species commonly found in a particular substrate has been extensively studied, it is possible to design a selective minimal medium in which only one or a few of the resident species are able to grow, based on the carbon and nitrogen assimilation patterns or other growth characteristics. Positive selection can be used to enumerate only yeast that can grow on a particular carbon or nitrogen source. For example, the cactus-specific yeast *Candida sonorensis* has been enumerated on yeast nitrogen base agar with 0.5% methanol as the sole source of carbon (Miller et al. 1976). Negative selection can be used to eliminate the growth of yeast species that can only grow in the presence of a particular nutrient, such as the sulfur-containing amino acids required by *Pichia amethionina* (Starmer et al. 1978). Media and growth conditions have been developed for selection of psychrotrophic, acid-resistant, or xerotolerant yeasts (Fleet 1992; Deak and Beauchat 1996). Several selective media are used in the brewery to detect wild *Saccharomyces* and non-*Saccharomyces* species. Detection of contaminating wild yeast is particularly crucial in brewing because pitching yeast is often reused for several fermentations. Several selective media have been designed for the detection of wild yeast contaminants in the presence of brewing yeast (ASBC 2003). Lysine medium contains L-lysine as the sole nitrogen source (Heard and Fleet 1986; Walters and Thiselton 1953). Wild non-*Saccharomyces* species grow on this medium, while brewing and wine strains of *S. cerevisiae* do not. Lin's wild yeast medium contains fuchsin sulfite and crystal violet, and restricts growth of brewery yeasts and permits growth of wild yeasts. Medium containing dextrin as the sole carbon source can be used to detect superattenuating yeast (Ingledew and Casey 1982). CLEN medium contains cadaverine, lysine, ethylamine, and nitrate as nitrogen sources, and allows some wild yeast to grow, while brewing yeasts are unable to grow. Additional differential media include Schwarz differential medium (Brenner et al. 1970), and MYGP plus copper sulfate medium (Taylor and Marsh 1984) (MYGP is another name for YM medium, so named because it contains malt extract, yeast extract, glucose and bacto peptone.) Certain contaminating yeast can also be detected by incubation at 37°C. A comparison of several of these methods for detection of nonbrewing yeasts in pitching yeast was performed by van der Aa Kuhle and Jespersen (1998). In an analysis of 101 pitching yeast samples from 45 breweries, contaminating yeast were most often detected on MYGP plus copper sulfate medium. Because different contaminants grow on different media, analysis on multiple media has been recommended (van der Aa Kuhle and Jespersen 1998).

Molybdate agar containing 0.125% propionate can be used to distinguish several yeast species found on tropical fruit (Rale and Vakil 1984). Kish et al. (1983) developed ethanol sulfite agar, containing 12% v/v ethanol and 150 mg/L sulfite to detect *S. cerevisiae* when outnumbered by apiculate yeasts. Acid-resistant yeasts can be enumerated on *Z. bailii* agar (Erickson 1993), or TGYA. Xerotolerant yeast can be enumerated on DG18 (Deak and Beauchat 1996). *Zygosaccahromyces* differential medium was developed to detect the spoilage yeast *Z. bailii* in wine (Schuller et al. 2000).

Agar medium can be supplemented with indicator compounds to distinguish between yeast species, or strains within a species. For example, acid-producing yeast species can be identified on plates containing a pH indicator such as bromocresol green or bromophenol blue. 5-Bromo-4-chloro-3-indolyl-α-galactoside (X-α-gal) is used to differentiate between melibiase-secreting lager yeast and nonsecreting ale yeast. Ale yeast colonies grown on medium containing X-α-gal remain white, while lager yeast colonies turn blue-green. The pathogen *Cr. neoformans* is identified in clinical specimens by formation of a black pigment on medium containing 3,4-dihydroxyphenylalanine (Chaskes and Tyndall 1975). *Dekkera*/*Brettanomyces* differential medium was developed to detect low levels of *Brettanomyces* and *Dekkera* species, which can cause phenolic taint in wine (Rodrigues et al. 2001). This medium is partially selective for *Brettanomyces* and *Dekkera* owing to the presence of ethanol as the sole carbon source and inclusion of cycloheximide, and is differential for the detection of these genera based on colony morphology, the time required for colony growth, the color change of a pH indicator, and the distinctive odor released by metabolism of *p*-coumaric acid to phenolic compounds. Another example of a differential medium is Niger seed agar (NSA), which was developed for testing environmental samples for the presence of *Cr. neoformans*, which forms brown colonies on this medium (Staib 1962). NSA has been used to detect *Cr. neoformans* in various trees and bird droppings (Pfeiffer and Ellis 1992; Sorrell et al. 1996; Lazera et al. 1998, 2000; Fortes et al. 2001). This medium can also be used to screen clinical specimens (Sukroongreung et al. 2001).

In the analysis of clinical specimens, SGA is frequently used as a primary isolation medium. However, differentiation of *Candida* species on the basis of colony morphology is not possible on this medium. Different yeast species plated on CHROMAgar Candida (CHROMAgar Co., Paris, France), which contains a proprietary chromogenic substrate, produce distinctive colony colors. Because this medium contains chloramphenicol to suppress bacterial growth, it can be used as both a primary isolation plate and a differential screening plate. Of 22 clinically relevant species screened, only *C. albicans* formed green colonies on this medium, allowing a presumptive identification of this species from clinical samples (Odds and Bernaerts 1994). This medium has been particularly useful to identify mixed infections, allowing appropriate antifungal treatment (Yera et al. 2004). The range of colony colors obtained with different yeast species may make this medium useful as a primary isolation plate for ecological surveys. Molds, however, are able to spread on this medium.

Variations on the standard Petri plate include Petrifilm (3M, St. Paul, MN, USA) and SimPlate (BioControl Systems, Bellevue, WA, USA), which are reported to produce comparable enumeration results to standard plate counts in the analysis of various foods (Beauchat et al. 1990, 1991, 1998). The thin, flexible Petrifilm yeast and mold count plate may prove convenient in remote locations for isolation of yeasts from certain natural habitats. A thin, clear film is peeled back to expose a water-soluble gel matrix. After addition of a liquid specimen, the film is replaced and the plate is incubated. After growth, colonies can be removed for purification and analysis. This film compares favorably to conventional plate counts in the enumeration of yeasts from various foods (Beauchat et al. 1990). Limitations include the inability to

streak for separation of colonies, and differences in colony morphology from those seen on standard Petri plates owing to the contact of the cover membrane with the growth medium.

5.4.2 Growth Conditions

In general, yeasts are grown in the laboratory under conditions similar to those of their natural habitat. Nutrients and other growth conditions can be manipulated to select for growth of particular species or groups of species. Enrichment cultures are used to isolate and characterize any number of niche-specific microbes, ranging from pesticide-degrading bacteria to thermophilic bacteria to halotolerant yeast. Simply stated, a specimen is placed in media with restrictive growth conditions, and passaged for several generations until the organisms best able to grow predominate. The restrictive growth conditions can be the presence of a certain compound as the sole carbon and/or nitrogen source, the presence of a growth-inhibiting compound, low water activity, or high or low temperature. The fermentation of wine can be considered one of the earliest enrichment culture techniques used by man. While *S. cerevisiae* is present in very low numbers on grapes (Rosini et al. 1982; Martini et al. 1996), the proportion of this ethanol-tolerant species relative to that of other yeast species rises dramatically as the ethanol concentration increases. Indigenous fermentation has been used as an enrichment technique to isolate wild strains of *S. cerevisiae* from aseptically gathered grapes (Cappello et al. 2004; Versavaud et al. 1995). An enrichment technique was also used to isolate novel limonene-degrading yeast species (Ngyuen Thanh et al. 2004).

The growth of most yeast species is not inhibited by a pH value as low as 3.0, although the optimal pH range for growth of most species is 4.5–6.5 (Phaff et al. 1978). Low pH media have been used in yeast surveys for decades to inhibit growth of bacteria (Miller et al. 1962).

Yeast can generally be grown at temperatures close to that of their natural habitat. This is particularly important for yeasts isolated from low temperatures, such as the *Cr. vishniacii* isolated from soil in Antarctica (Vishniac and Hempfling 1979), the type strain of which grows well at 10°C but poorly at 20°C. Many species isolated in temperate zones grow well at 20–25°C, and poorly at 30°C. A few psychrophobic yeasts, isolated from warm-blooded animals, require incubation temperatures above 30°C (Travassos and Cury 1971).

Aerobic plating conditions are used predominantly for quality control in the food industry (APHA 2001) and also in most surveys of yeast biodiversity. However, the presence of oxygen severely inhibits growth of many microbial species. The use of anaerobic in addition to aerobic conditions may improve yeast surveys of potentially anaerobic or microaerobic environments such as deep water, vertebrate and invertebrate guts, and the interior of plant rots. Under field conditions where anaerobic incubators are impractical, convenient anaerobic devices such as a candle jar or the Gas Pak chamber or bag (BD Biosciences) can be used for generation of anaerobic, microaerophilic, or CO_2-enriched conditions. Alternatively, a novel paraffin wax overlay method has been used to exclude oxygen from pour plates, allowing the isolation and enumeration of purple non-sulfur bacteria from flooded paddy soil (Archana et al. 2004).

Many yeast species form colonies on agar plates within 2–3 days. However, when exposed to a variety of stresses (including lyophilization and cryopreservation), colony formation by even fast-growers may be delayed. Slow-growing yeast species such as *Z. bailii* or *Dekkera bruxellensis* may require up to 14 days to form colonies (Millet and Lonvaud-Funel 2000; Rodrigues et al. 2001). An appropriate incubation time must be selected to allow growth of yeasts of interest.

Manipulation of incubation conditions may reveal the presence of rare or slow-growing yeasts. A decrease in incubation temperature from 25 to 17°C, an increase in humidity, and periodic replacement of the plates used in a ballistospore-fall plate (Nakase and Takashima 1993) resulted in the isolation of four novel slow-growing *Sporobolomyces* yeast species from plant leaves in Japan (Wang and Bai 2004).

Some yeast species require medium amendments for satisfactory growth on agar media. For example, *Schizosaccharomyces octosporus* will grow poorly on minimal medium unless it is supplemented with 15 mg adenine/L (Northam and Norris 1951). The yeast *Debaryomyces mycophilus*, associated with wood lice, is only able to grow on media supplemented with chelated iron (Thanh et al. 2002). While most halotolerant yeast species can grow well on media lacking sodium chloride, strains of the species *Metschnikowia bicuspidata* will not grow on media with salt concentrations less than 2% (Lachance et al. 1976). In contrast, sugar-tolerant yeast species such as *Z. bisporus* or *Z. rouxii*, found in high-sugar environments such as honey, do not grow well when placed directly on standard media containing much lower sugar concentrations. It has been recommended to use media with 30–40% sugar for primary isolation of yeasts from these environments, followed by transfer to lower-sugar media (Phaff and Starmer 1987). The yeast-like organism *Eremascus albus*, however, does not have the ability to adapt to lower-sugar media (Phaff and Starmer 1987).

5.4.3 Liquid Culture Methods

The most probable number (MPN) method was developed for the enumeration of microorganisms. This method is particularly useful for enumeration of yeasts in a specimen containing a high particulate content which would preclude membrane filtration or microscopy, such as must or solid food. The specimen is homogenized, and a series of tenfold dilutions are prepared. When inoculated in culture medium, the more concentrated cell suspensions will result in growth, while the more dilute suspensions will not. Using a table based on McCrady's probability calculations, the positive and negative growth results from the dilutions are used to extrapolate the concentration of cells in the original sample (Greenberg et al. 1992). This method is used in the analysis of food (Harrigan 1998), wine (Fugelsang 1997; OIV 2004) and wastewater (Greenberg et al. 1992). When used in conjunction with selective media, the MPN technique can be used to enumerate minority spoilage or fermenting yeast (Loureiro and Malfeito-Ferreira 2003).

Just as a pure microbial culture displays a distinct pattern of carbon source utilization, a mixed population of microbes also exhibits a distinct pattern (Garland and Mills 1991). Changes in microbial populations are reflected in changes in carbon utilization patterns, which are measured using a technique called community level physiological profiling (CLPP). Biolog (Hayward, CA, USA) distributes

fungal-specific microtiter plates, SFN2 and SFP2, with three replicates of 31 environmentally relevant carbon sources in liquid media (Classen et al. 2003). After inoculation with environmental samples, the ability of the resident microbial community to utilize the various substrates is monitored. Multivariate analysis is used to determine functional diversity of environmental populations. This method has been used to assess metabolic diversity in environmental substrates including plant rhizospheres (Ellis et al. 1995; Garland 1996; Grayston and Campbell 1996; Grayston et al. 1998). The API system (bioMerieux, France), including strips for identification of yeast species, could be used to measure functional diversity of yeasts and other microbial communities (Torsvik et al. 1990). The advantages of this method are that it is fast, reproducible, relatively inexpensive, and can distinguish differences in microbial communities. Because CLPP is a culture-dependent method, however, it has the limitations common to these methods: the profile only represents the culturable portion of the community, and it favors fast-growing organisms (Kirk et al. 2004). Moreover, the in vivo metabolic diversity may not reflect in situ diversity.

5.5 Indirect Detection Methods

Although brewing yeast were some of the first microorganisms visualized by van Leeuwenhoek, many of the recent advances in microbiology first emerged in studies of prokaryotes and were then applied to eukaryotes. Yeast researchers have applied these new methods, from DNA–DNA hybridization to ribosomal sequencing, resulting in new knowledge of yeast biodiversity. Some recent advances in detection and enumeration of mixed microbial populations have also been based on methods used to characterize pure cultures.

5.5.1 Biochemical-Based Methods

Signature fatty acids are indicative of specific taxonomic groups, and form a relatively constant proportion of cell biomass when standardized culture conditions are used. Just as species can be differentiated by fatty acid methyl ester (FAME) analysis, a change in the fatty acid profile of a community can be detected. Zelles (1999) has reviewed the use of FAME analysis to characterize microbial communities. FAME analysis does not require culturing, and the detection of signature fatty acids indicates the presence of certain taxonomic groups. However, this method has limitations, including the fact that the cellular fatty acid composition can vary under different growth conditions, such as temperature and nutrition.

5.5.2 Molecular Methods

Molecular, DNA-based methods of detection and enumeration have gained popularity in recent years owing to the ease of use, specificity, and, unlike biochemical methods such as FAME analysis, independence of the metabolic state of the cell. PCR-based methods are particularly powerful owing to the sensitivity of these methods, as will be discussed later. Many methods are based on PCR amplification of DNA from a mixed population, followed by separation by a variety of techniques.

5.5.2.1 Community Profiling Methods

Two DNA-based methods developed for the analysis of pure cultures have recently been modified for profiling the genetic diversity of communities of microorganisms: guanine plus cytosine (G+C) content and nucleic acid reassociation. While they do not provide detailed information on the identity of individual species present, they can be used as a measure of the genetic diversity of a microbial community, and can detect shifts in microbial community profiles.

The G+C content of DNA is characteristic of a yeast species, with ascomycetous yeast species having G+C contents of 27–50%, and basidiomycetous species having contents of 48–70%. The overall G+C content of mixed populations, as well as other methods, has been used to study the differences in microbial diversity between forest cover and pasture in Hawaiian soil (Nusslein and Tiedje 1999). This method has the advantage in that it is not subject to PCR amplification biases, but it does require large amounts of DNA.

The rate of DNA–DNA reassociation of two yeast strains is an extremely valuable measure of genetic similarity of closely related species and strains. DNA reassociation rates have also been used to assess the genetic complexity of DNA from an environmental sample, which reflects the biodiversity of the resident microbial community (Torsvik et al. 1990). The rate of reassociation of DNA extracted from an environmental sample depends on the complexity of the DNA sequences present: as the complexity increases, the rate of DNA reassociation decreases (Theron and Cloete 2000). The time needed for half the DNA to reassociate ($C_o t1/2$) is considered a diversity index, which can be compared with that of other environmental samples. Moreover, the rate of reassociation of DNA isolated from two different environmental samples has been used to measure the similarity of their microbial communities (Griffiths et al. 1999).

5.5.2.2 Detection of Species or Groups of Species

Hybridization-based molecular methods can be used to detect specific species or groups of species in environmental or other samples. While these methods can provide detailed information on the species present in a sample, they suffer from lack of sensitivity: nondominant species may not be detected. PCR amplification of target sequences prior to hybridization can often be used to eliminate this problem. DNA can be amplified directly to detect all species present, whether active or dormant, and complementary DNA from reverse transcription of messenger RNA (mRNA) can be amplified to detect active microbial communities.

Flow cytometry has been used to detect, enumerate, and characterize liquid suspensions ranging from bacteria to mammalian cells (reviewed in Davey 2002), and has been used extensively for analysis and sorting of cells, including yeast. For example, Jespersen et al. (1993) used an enrichment technique followed by flow cytometry to detect as few as one wild yeast cell in a background of 106 brewer's yeast cells. The Luminex 100 is a flow-cytometer system utilizing a set of color-coded fluorescent latex beads, each of which can be tagged with a different nucleotide probe. Using species-specific capture probes based on ribosomal sequences, this method

has been used for detection and enumeration of yeasts of the genus *Trichosporon* in a mixed population (Diaz and Fell 2004). The Luminex system is extremely rapid, sensitive, and can distinguish between sequences differing by a single nucleotide. The range of species being detected must be determined in advance.

PCR methods have been developed for the detection and identification of a single species or group of species. Species-specific primers have been developed for the identification of pure cultures, as discussed elsewhere in this book. These primers have been useful in detecting specific species in mixed population and environmental samples. For instance, *S. cerevisiae* was detected in an ancient wine jar from the tomb of King Scoprion I in Egypt, dated to 3,000 B.C. (Cavalieri et al. 2003) using primers that amplify the *S. cerevisiae* ITS1, 5.8S, and ITS2 region (Guillamon et al. 1998). A mixed microbial community can be profiled by amplifying a target sequence, then separating the amplicons by a variety of methods.

Variations on PCR-based detection methods introduce even more sensitivity. A nested PCR method developed for detection of *Dekkera*/*Brettanomyces* strains was sensitive to as few as ten cells in sherry (Ibeas et al. 1996). In multiplex PCR, primers to several target genes are combined in one PCR amplification. This has been used, for example, to detect and discriminate between pathogenic *Escherichia coli* strains on the basis of the presence of six strain-specific toxins and virulence factors (Watterworth et al. 2004).

Quantitative PCR (Q-PCR) can be used to detect the presence of specific organisms in environmental samples. An extremely sensitive and accurate Q-PCR method has recently been developed for the detection of six pathogenic *Candida* species in drinking water (Brinkman et al. 2003). Species-specific PCR primers were designed to amplify a region of the D1/D2 domain of the large (26S) ribosomal subunit. This filter-based method could detect as few as 1–3 cells per filter. Another Q-PCR assay using species-specific primers to the D1/D2 domain of the 26S rRNA gene was developed to detect and enumerate the spoilage yeast *D. bruxellensis* from wine (Phister and Mills 2003).

Ribosomal intergenic spacer (IGS) analysis and automated ribosomal IGS analysis have been used to profile bacterial communities. In these methods, the IGS between the 16S and 23S ribosomal subunits is amplified by PCR, denatured, and analyzed on a polyacrylamide gel under denaturing conditions. The IGS region of different species differs in length and sequence. These differences are detected directly by silver staining of the gel, or by automated detection of a fluorescently labeled primer (Fisher and Triplett 1999). These methods have been used to compare microbial diversity in soil (Borneman and Triplett 1997; Ranjard et al. 2000) and the rhizosphere of plants (Borneman and Triplett 1997).

Molecular detection methods have the distinct advantages of being culture-independent, and, if DNA sequencing is involved, can also provide information on the phylogenetic placement of species detected. These molecular methods have revealed the presence of previously undetected and uncharacterized species. However, DNA-based methods have limitations and potential biases at each step, including cell lysis, DNA extraction, and purification. The efficiency at which cells or mycelia are lysed can vary within and between microbial groups (Prosser 2002): spores and mycelia lyse differently, and mycelia of different ages lyse differently. DNA and RNA extrac-

tion methods that result in DNA shearing, such as bead beating, can also lead to biases (Wintzingerode et al. 1997). Certain contaminants in DNA preparations such as humic acids from soil can interfere with PCR amplification. Subsequent purification steps can lead to loss of DNA or RNA. Furthermore, detection of cells that have been stressed or injured can be difficult.

Wintzingerode et al. (1997) have summarized factors that can result in differential PCR amplification of templates within a mixed population, including different affinities of primers to various templates, different copy number of templates, and varying levels of hybridization efficiency and primer specificity. Furthermore, sequences with lower G+C content may separate more easily, and therefore be preferentially amplified (Wintzingerode et al. 1997). This could conceivably lead to biases in amplification in mixed populations of ascomycetous and basidiomycetous yeasts, which differ in G+C content.

DNA microarrays bring a new level of specificity to DNA–DNA hybridization studies. Because a single array can contain thousands of DNA sequences, a large number of target sequences can be detected in an environmental sample simultaneously. These target sequences can be either species-specific probes to detect and quantify particular species, or they can be function-specific probes, such as metabolic genes, to detect functional diversity. Microarrays have been used to detect and quantify several bacterial species in a sample with high specificity (Cho and Tiedje 2001). Direct profiling of microbial communities in sediment samples was performed by hybridization of extracted rRNA to microarrays containing oligonucleotides specific for major microbial groups (El Fantroussi et al. 2003).

Target regions to be amplified are often rRNA genes or internal transcribed spacer (ITS) regions, because these are present in all organisms, they are not subject to horizontal transfer, and sequence databases are publicly available. Although only a small proportion of yeast species have been described, certain DNA regions such as the D1/D2 region of the large (26S) ribosomal subunit as well as the ITS1 region have been sequenced in the majority of known yeast species. This information is extremely useful for identification of yeast species, as discussed elsewhere in this book, as well as for the detection of specific species within a mixed population. Moreover, community-level fingerprints representative of microbial diversity can be obtained using PCR amplification of target DNA such as D1/D2 or ITS regions, followed by separation of amplicons by two similar methods, denaturing gradient gel electrophoresis (DGGE) (Muyzer et al. 1993) or temperature gradient gel electrophoresis (TGGE). The difference between these two methods is the nature of the gradient: denaturing chemicals (urea and formamide) in the former, and temperature in the latter. In both techniques, the forward primer contains a 35–40 base pair GC clamp, which ensures that part of the amplified DNA fragment remains double-stranded as it passes through the denaturant. Amplicons are separated on a polyacrylamide gel. As they pass through an increasing concentration of denaturants, domains melt in sequence-specific manner, with AT-rich domains denaturing before GC-rich ones. The bands can be excised, reamplified, and sequenced to identify the species. The number and intensity of the bands is indicative of the genetic diversity of the sample. Theoretically, a single base-pair difference can be detected. DGGE and TGGE are reliable, reproducible, rapid, and multiple samples can be analyzed

simultaneously. DGGE and TGGE have been used extensively to detect bacterial and/or fungal diversity in a range of habitats, such as the guts of pigs (Simpson et al. 2000) and chickens (van der Wielen et al. 2002), soil (Gomes et al. 2003), and rhizosphere (Duineveld et al. 1998, 2001; Smalla et al. 2001). Cocolin et al. (2000a) used this method to analyze the yeast species present at successional stages in wine fermentations. Nonculturable yeast were detected late in the fermentation of a *Botrytis*-affected wine (Mills et al. 2002). Prakitchaiwattana et al. (2004) compared DGGE with traditional plating methods to study the yeast ecology of wine grapes. DGGE was less sensitive than agar plating methods, detecting only populations of more than 104 cells/mL, but a greater diversity of species was detected. DGGE has also been used to detect yeast in the fermentation of *Coffea arabica* (Masoud et al. 2004) and sourdough (Meroth et al. 2003a).

Theoretically, the PCR primers selected should amplify all species of interest in the microbial community. Universal or group-specific ribosomal DNA regions are often used. For instance, primers to the bacterial nitrite reductase gene (*nirK*) were used to detect denitrifying bacteria in soils. The sequences of most of the of 56 PCR products analyzed were similar, but not identical, to those of known species, indicating the presence of uncultivated denitrifying species (Henry et al. 2004). In some cases, reverse-transcriptase PCR (RT-PCR) of short-lived mRNA is used to preferentially detect metabolically active cells (Sheridan et al. 1998). For example, an RT-PCR method was recently published for the detection of *Cr. neoformans* in clinical specimens, based on the amplification of the capsular *CAP10* gene mRNA (Amjad et al. 2004). Very little target DNA is required.

Single-strand conformation polymorphism (SSCP) is also based on the electrophoretic separation of DNA molecules on the basis of small differences in DNA sequence. Polyacrimide gel electrophoresis is used to separate single-stranded PCR-amplified DNA on the basis of differences in their secondary structure (Lee et al. 1996) caused by differences in sequence. In addition to several bacterial studies, this method has been used to study the arbuscular mycorrhizal fungi species present in roots (Simon et al. 1993; Kjoller and Rosendahl 2000). SSCP has also been used to detect polymorphisms in clinical isolates of *C. albicans* (Graser et al. 1996). Limitations of this method are similar to those of DGGE/TGGE. Unlike DGGE/TGGE, however, SSCP does not require a GC clamp or the use of gradient gels.

Restriction fragment length polymorphism (RFLP) patterns, in addition to amplified rDNA restriction analysis, are very useful to identify pure microbial cultures, including yeasts (Esteve-Zarzoso et al. 1999). RFLP patterns have also been used to detect changes in bacterial community structure (Massol-Deya et al. 1995), but cannot be used to quantify diversity or detect specific phylogenetic groups (Liu et al. 1997). The use of a 6-base rather than a 4-base recognition restriction enzyme would result in a less complex pattern when mixed microbial communities are analyzed.

The RFLP method has been modified to address some of these limitations. In terminal RFLP, one of the PCR primers is labeled with a fluorescent dye, which allows the detection of only the terminal restriction fragment. This results in a simplified banding pattern, with one labeled fragment representing each sequence

present (Tiedje et al. 1999). A similar method, terminal fragment length polymorphism analysis, has been developed for the differentiation of *Malassezia* yeast species (Gemmer et al. 2002). This nested PCR approach utilizes a second PCR reaction rather than restriction digestion, and could be useful for the detection and discrimination of other fungal species.

5.6 Concluding Remarks

Both culture-based and culture-independent detection and enumeration methods have great utility in the detection and enumeration of yeasts, but they also have limitations. A polyphasic approach is recommended to draw on the strengths of both these methods. As a case in point, in a study of the microbes responsible for the traditional fermentation of cassava dough, some *Lactobacillus* species detected by DGGE were not recovered on plating, while some species recovered from enrichments were not detected by DGGE (Miambi et al. 2003). A combination of DGGE/TGGE and plating methods was more informative than either method alone for the identification of dominant populations within the community in studies of sausage (Cocolin et al. 2000b), cheese (Ercolini et al. 2002; Ogier et al. 2002; Randazzo et al. 2002), and doughs (Meroth et al. 2003b). Prakitchaiwattana et al. (2004) compared DGGE with cultural isolation of yeasts from wine grapes, and found that DGGE was less sensitive to low-concentration organisms, but detected a greater diversity than plating. The use of a combination of culture-dependent and molecular methods is recommended in detecting the spectrum of yeast species that may be present in a substrate.

When examining a substrate that may contain nonculturable organisms, microscopy methods such as FISH in addition to plating have been successful in the study of the microflora of cheese (Kolloffel et al. 1999) and wine (Millet and Lonvaud-Funel 2000). However, FISH has a low level of sensitivity, making it difficult to enumerate nondominant populations. Furthermore, cells with low rRNA levels, or damaged or stressed cells, may be difficult to detect by FISH. DNA-based methods that do not require cultivation of microorganisms, such as shotgun metagenome sequencing, are revealing aspects of the metagenome of mixed microbial populations such as soil (Rondon et al. 2000) and marine water (Venter et al. 2004). While information on individual species is difficult to obtain by shotgun sequencing, particularly for organisms such as yeast that have multiple chromosomes, a billion base pairs of nonredundant sequence revealed a plethora of information on the gene content, diversity, and relative abundance of organisms present in sea water (Venter et al. 2004), including the presence of 148 previously unknown bacterial phylotypes and 782 new rhodopsin-like photoreceptors. While these studies do not result in isolation of type cultures, amazing insights into the lives of yeasts can be obtained by these methods.

While PCR-based methods are powerful and useful methods, limitations of these methods include PCR biases (Ercolini et al. 2002), labor, and the difficulty in detecting organisms representing less than 1–2% of the population (MacNaughton et al. 1999). Also, one band may represent more than one species, and one species may give multiple bands on the gel. Intragenomic sequence heterogeneity in the

ribosomal region has been demonstrated with many bacterial species (Coenye and Vandamme 2003) and also in the yeast species *Clavispora lusitaniae* (Lachance et al. 2003). This sequence heterogeneity within a strain could result in multiple bands.

Some methods introduced for the analysis of bacterial communities hold great promise for use in detection and enumeration of pure and mixed communities of yeasts. These include microarrays containing species-specific probes to a number of species, and the many variants of PCR analysis. However, the use of culture-dependent methods is far from obsolete. Culture-based methods using new selective and differential media and variations in growth conditions will continue to be valuable tools of the yeast ecologist.

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Sugar Metabolism in Yeasts: an Overview of Aerobic and Anaerobic Glucose Catabolism

Fernando Rodrigues, Paula Ludovico and Cecília Leão

Life and Health Sciences Research Institute (ICVS), Health Sciences School, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
Fernando Rodrigues (e-mail: frodrigues@ecsaude.uminho.pt)
Paula Ludovico (e-mail: pludovico@ecsaude.uminho.pt)
Cecília Leão (e-mail: cleao@ecsaude.uminho.pt, Tel.: +351-253-604818, Fax: +351-253-604809)
F.R. and P.L. contributed equally to this chapter.

6.1 Introduction

Yeasts are ubiquitous unicellular fungi widespread in natural environments colonizing from terrestrial, to aerial to aquatic environments, where the successful colonization is intimately related to their physiological adaptability to a highly variable environment. The metabolic pathways of the central carbon metabolism are basically identical between different yeast species, suggesting that these microorganisms might constitute a metabolic homogenous group. Nonetheless, the mechanisms for nutrient uptake, the number of different isoenzymes and most importantly the regulation of fermentation and respiration differ substantially (Flores et al. 2000) and make yeasts a highly heterogeneous and complex metabolic group.

In yeasts, like other heterotrophic organisms, the energy and carbon metabolism are intimately interconnected, i.e., anabolism is coupled with catabolism. ATP is provided by the oxidation of organic molecules that also act as carbon sources for biosynthesis, and ultimately it is used as energetic currency for all kinds of cellular work.

In the natural environment yeast species have a broad set of carbon sources (e.g., polyols, alcohols, organic acids and amino acids) that can support their growth but preferentially they metabolize sugars. The information related to the metabolism of different carbon sources is huge, the most widely studied being sugars such as hexoses (glucose, fructose, galactose or mannose) and disaccharides (maltose or sucrose) as well as compounds with two carbons (ethanol or acetate). The metabolic networks employed for the metabolism of hexoses and disaccharides share the same pathways (most metabolic building blocks are derived from intermediaries of glycolysis, the tricarboxylic acid cycle (TCA), and the pentose phosphate pathway) and differ only in the initial basic steps of metabolism. However, significant changes could be observed when the metabolism of sugars is compared with that of the

two-carbon compounds. In this case, the TCA, the pentose phosphate pathway together with gluconeogenesis and the glyoxylate cycle are essential for the provision of anabolic precursors.

Yeast environmental diversity mostly leads to a vast metabolic complexity driven by carbon and the energy available in environmental habitats. It is the scope of this chapter to contribute a comprehensible analysis of yeast metabolism specifically associated with glucose catabolism in *Saccharomyces cerevisiae*, under both aerobic and anaerobic environments. A brief introduction to glycolysis together with the most relevant effects triggered by oxygen and glucose are presented in order to center the reader in the problems discussed later. Most of our attention is given to the metabolic flux on the pyruvate branch point, with reference to alcoholic fermentation and respiration. As a last issue we address the most pertinent features of anaerobic metabolism, culminating with the hitherto unexplained metabolic requirements for fully anaerobic growth.

6.2 A Brief Comment on Pasteur, Crabtree and Custer Effects

Yeasts may be physiologically classified with respect to the type of energy-generating process involved in sugar metabolism, namely non-, facultative- or obligate-fermentative (van Dijken and Scheffers 1986). The nonfermentative yeasts have exclusively a respiratory metabolism and are not capable of alcoholic fermentation from glucose (e.g., *Rhodotorula glutinis*), while the obligate-fermentative yeasts – "natural respiratory mutants" – are only capable of metabolizing glucose through alcoholic fermentation (e.g., *Candida slooffii* = *Kazachstania slooffiae*). Most of the yeasts identified are facultative-fermentative ones, and depending on the growth conditions, the type and concentration of sugars and/or oxygen availability, may display either a fully respiratory or a fermentative metabolism or even both in a mixed respiratory-fermentative metabolism (e.g., *S. cerevisiae* or *Pichia jadinii* – the latter is herein always referred to as *C. utilis*).

The sugar composition of the media and oxygen availability are the two main environmental conditions that have a strong impact on yeast metabolic physiology. There are three frequently observed effects associated with the type of energy-generating processes involved in sugar metabolism and/or oxygen availability: Pasteur, Crabtree and Custer. The known Kluyver effect is beyond the scope of the metabolic overview of this chapter (see the review in Fukuhara 2003 for more details).

6.2.1 Pasteur Effect

In modern terms the Pasteur effect refers to an activation of anaerobic glycolysis in order to meet cellular ATP demands owing to the lower efficiency of ATP production by fermentation compared with respiration. In 1861 Pasteur observed that *S. cerevisiae* consume much more glucose in the absence of oxygen than in its presence. However, there are several misinterpretations concerning the results presented by Pasteur that were reviewed in depth by Lagunas (1981, 1986, and references therein). This author gathered all the information referred to *S. cerevisiae* and showed that the initial descriptions of Pasteur were an artefact due to anaerobic growth impairment

by the lack of sterols and fatty acids. In fact, *S. cerevisiae* only shows a Pasteur effect under special experimental conditions, specifically at low growth rates (sugar-limiting continuous culturing) and at resting-cell conditions, where a high contribution of respiration to sugar catabolism occurs owing to the loss of fermentative capacity.

6.2.2 Crabtree Effect

The Crabtree effect is currently defined as the occurrence of alcoholic fermentation under aerobic conditions (for a review see Pronk et al. 1996, and references therein). After the initial descriptions of Crabtree, it was shown that *S. cerevisiae* catabolyzes glucose mainly by a fermentative process, and this effect was presented as the Crabtree effect (Swanson and Clifton 1948). From all the theories presented in the literature one should stress that the Crabtree effect has received special attention from several research teams and, even today, a lack of consensus in terms of its definition is observed. De Deken (1966) described the Crabtree effect as "...the phenotypic expression of a regulatory system involved in the synthesis of cytochromes..." Glucose repression became accepted as synonymous of the Crabtree effect until the 1980s with the emergence of the theory involving an "overflow/limited respiratory capacities" in the branching point of pyruvate metabolism (Kappeli 1986). The Crabtree effect was divided into short- and long-term effects, mainly based on continuous culturing studies where under steady-state conditions the growth rate can be experimentally manipulated (Petrik et al. 1983). The short-term effect is defined as the capability of triggering alcoholic fermentation upon the sudden condition of glucose excess, whereas the long-term effect is characterized as the respiration-fermentative metabolism observed with batch cultivations or with continuous culturing above critical dilution rates.

6.2.3 Custer Effect

The Custer effect is known as the inhibition of alcoholic fermentation by the absence of oxygen. A clear example of the Custer effect is found in yeasts belonging to the *Brettanomyces* and *Dekkera* genera that ferment glucose into ethanol and acetic acid under aerobic conditions (Wijsman et al. 1984; van Dijken and Scheffers 1986). However, upon a shift to an anaerobic condition, fermentation is strongly inhibited. This phenotype can be fully rescued by the reintroduction of oxygen into the culture media or by the addition of H^+ acceptors such as acetoin (which is reduced to 2,3-butanediol) or other aliphatic carbonyl compounds (Sheffers 1966; Wijsman et al. 1984). From all the previous considerations it is thought that the Custer effect is caused by reductive stress. It seems that yeasts displaying this effect are somehow incapable of closing the redox balance through the production of glycerol or other highly reduced compounds.

6.3 Glycolytic Metabolic Central Block: a Brief Summary

After glucose uptake, intracellular glucose is fated to be dissimilated and/or assimilated by metabolic processes (see reviews in Lagunas 1993; Boles and Hollenberg

1997; Forsberg and Ljungdahl 2001; Jeffries and Jin 2004). In this section we will briefly summarize the glycolytic pathway that constitutes the central block of hexose and disaccharide metabolism and that has been extensively revised during the last few years (for reviews see Gancedo and Serrano 1989; Richard 2003; Kruckeberg and Dickinson 2004).

Once inside the cell, glucose is phosphorylated by kinases to glucose 6-phosphate and then isomerized to fructose 6-phosphate, by phosphoglucose isomerase. The next enzyme is phosphofructokinase, which is subject to regulation by several metabolites, and further phosphorilates fructose 6-phosphate to fructose 1,6-bisphosphate. These steps are the first part of glycolysis that requires energy in the form of ATP.

The subsequently acting enzymes are aldolase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase. The last block of glycolysis leads to pyruvate formation associated with a net production of energy and reducing equivalents.

Essentially the glycolytic pathway is common to all yeast species, the carbon flux regulation being done at the level of the pentose phosphate pathway. Several flux analyses have shown that approximately 50% of glucose 6-phosphate is metabolized via glycolysis and 30% via the pentose phosphate pathway in Crabtree negative yeasts. However, about 90% of the carbon going through the pentose phosphate pathway reentered glycolysis at the level of fructose 6-phosphate or glyceraldehyde 3-phosphate. The distribution of carbon flux between glycolysis and the pentose phosphate pathway seems to play a more important role in glucose dissimilation in Crabtree negative yeasts than in Crabtree positive ones (Bruinenberg et al. 1983; González-Siso et al. 2000). Specifically, *S. cerevisiae*, a Crabtree positive yeast, was shown to have low catabolic fluxes through the pentose phosphate pathway (Blank and Sauer 2004). These observations indicate that the pentose phosphate pathway in Crabtree positive yeasts is predominantly used for NADPH production but not for biomass production or catabolic reactions, as will be discussed later for redox balances.

6.4 From Pyruvate to Acetyl–Cofactor A

At the pyruvate (the end product of glycolysis) branching point, pyruvate can follow three different metabolic fates depending on the yeast species and the environmental conditions (Pronk et al. 1996). On the other hand, the carbon flux may be distributed between the respiratory and fermentative pathways.

Pyruvate might be directly converted to acetyl–cofactor A (CoA) by the mitochondrial multienzyme complex pyruvate dehydrogenase (PDH) after its transport into the mitochondria by the mitochondrial pyruvate carrier. Alternatively, pyruvate can also be converted to acetyl–CoA in the cytosol via acetaldehyde and to acetate by the so-called PDH-bypass pathway (reviewed in Pronk et al. 1996). The PDH-bypass pathway requires the activity of three different enzymes: (1) pyruvate decarboxylase, which converts pyruvate to acetaldehyde; (2) acetaldehyde dehydrogenase (ALD), which converts acetaldehyde to acetate; and (3) acetyl-CoA synthetase (ACS), which converts acetate to cytosolic acetyl–CoA that can then be transported

unidirectionally into the mitochondria via the carnitine acetyltransferase system (Kispal et al. 1991; Pronk et al. 1996) (Fig. 6.1). It is still matter of debate if the flux distribution during pyruvate metabolism determines whether the pyruvate flows inward through the PDH complex or through the PDH-bypass pathway and thereby the split between respiration and fermentation (Pronk et al. 1996).

One of the mechanisms underlying the regulation of pyruvate flux through the different routes is the regulation of the enzymes involved and their kinetic properties (Pronk et al. 1996). Compared with cytosolic pyruvate decarboxylase, the mitochondrial PDH complex has a higher affinity for pyruvate and therefore most of the pyruvate will flow through the PDH complex at low glycolytic rates. However, at increasing glucose concentrations, the glycolytic rate will increase and more pyruvate is formed, saturating the PDH bypass and shifting the carbon flux through ethanol production and beginning the fermentation (Fig. 6.1).

In addition to the different enzyme affinities for pyruvate, the enzymatic activities may also play a role in the regulation of pyruvate flux. In *S. cerevisiae*, high glucose concentrations induce an increase of 3–4 times the pyruvate decarboxylase activity and a decrease of acetaldehyde dehydrogenase activity, favoring the alcoholic fermentation. On the other hand, at low glucose concentrations, pyruvate is mainly converted to acetyl–CoA by the PDH complex. From an energetic point of view PDH-bypass is less efficient owing to the consumption of an ATP molecule, which is converted into AMP by the acetyl-CoA synthetase (Steensma 1997).

As already discussed and particularly in the yeast *S. cerevisiae*, the external glucose level controls the switch between respiration and fermentation. Although

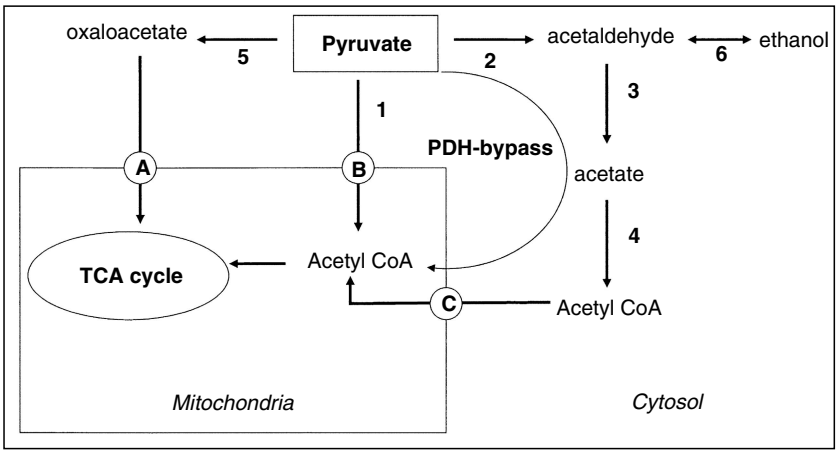

Fig. 6.1. Scheme of pyruvate branch point pathways. Pyruvate formed in glycolysis is converted to acetyl–cofactor A (*CoA*) and/or oxaloacetate, both intermediates of the trycarboxylic acid cycle. *1* pyruvate dehydrogenase complex, *2* pyruvate decarboxylase, *3* acetaldehyde dehydrogenase, *4* acetyl–CoA synthetase, *5* pyruvate carboxylase and *6* alcohol dehydrogenase. *A* mitochondrial oxaloacetate carrier; *B* mitochondrial pyruvate carrier and *C* carnitine acetyltransferase

significant ethanol production is generally absent in Crabtree negative yeasts during aerobic conditions (de Deken 1966; González-Siso et al. 1996), yeast species such as *Pichia anomala, C utilis* and *Kluyveromyces lactis*, during aerobic conditions and high glucose concentration, temporarily (during the exponential growth phase) produce ethanol. However, it has been shown that this transient ethanol production is mainly due to inadequate aeration (Kiers et al. 1998).

6.5 Tricarboxylic Acid Pathway

Acetyl–CoA generated either by the PDH complex or by PDH bypass is the link between glycolysis and the tricarboxylic acid (TCA) cycle. The main catalytic function of the TCA cycle is to provide reducing equivalents to the respiratory chain through the oxidative decarboxylation of acetyl–CoA. However, the TCA cycle also functions in furnishing biosynthetic metabolism and, with the exception of isocitrate, every TCA cycle intermediate is commonly used by other metabolic reactions. The eight enzymes from the TCA cycle are encoded by 15 nuclear genes in *S. cerevisiae* (McAlister-Henn and Small 1997; Przybyla-Zawislak et al. 1999). The first reaction of TCA is catalyzed by citrate synthase (encoded by *CIT1*, *CIT2* and *CIT3*) and it is the condensation of acetyl–CoA and oxaloacetate resulting in the formation of citrate (McAlister-Henn and Small 1997). Only Ct2p is a nonmitochondrial protein peroxisomally localized contributing to the efficiency by which the cells use two-carbon compounds in anaplerotic pathways especially in the glyoxylate cycle.

The second reaction of the TCA cycle is catalyzed by aconitase, leading to the conversion of citrate into isocitrate. Aconitase (encoded by *ACO1*) is located both in mitochondria and in cytosol.

The next step of the TCA cycle is the oxidative decarboxylation of isocitrate to α-ketoglutarate. There are three known isoenzymes of isocitrate dehydrogenase, a mitochondrial NAD^+-specific one and two $NADP^+$-dependent ones (one mitochondrial and the other cytosolic). A number of pieces of evidence point to the role of mitochondrial NAD^+-specific isocitrate dehydrogenase in the regulation of the rate of mitochondrial assembly besides its specific role in the TCA cycle (Kruckeberg and Dickinson 2004).

The formation of succinate is catalyzed by α-ketoglutarate dehydrogenase, which promotes the oxidative decarboxylation of α-ketoglutarate via succinil–CoA to succinate, which is then converted to fumarate by succinate dehydrogenase. The next step of the TCA cycle is the conversion of fumarate to malate by the enzyme fumarase, which exists as separate cytosolic and mitochondrial forms. There is not yet a clear explanation for the existence of these two forms; however, the localization and distribution of fumarase appears to be unique because there is only one translation product which is targeted to mitochondria (Sass et al. 2001).

Malate dehydrogenase catalyzes the last step of the TCA cycle and leads to the oxidation of malate to oxaloacetate. There are three isoenzymes of malate dehydrogenase: a cytosolic, a mitochondrial and a peroxissomal one; however, the mitochondrial one accounts for 90% of malate dehydrogenase activity when glucose is being metabolized (Steffan and McAlister-Henn 1992).

TCA cycle flux appears to be constricted at two steps on the basis of the limited availability of the substrates oxaloacetate and α-ketoglutarate. As represented in Fig. 6.1, in *S. cerevisiae*, the synthesis of oxaloacetate from cytosolic pyruvate catalyzed by pyruvate carboxylase constitutes the anaplerotic pathway for the replenishment of this TCA-cycle intermediate. The TCA cycle works in a two-minicycles model interconnecting these two substrates (oxaloacetate and α-ketoglutarate) and their transamination products (glutamate and aspartate) (Yudkoff et al. 1994; Rustin et al. 1997). This model is consistent with the unique regulation of the first three enzymes of the TCA cycle in yeasts (McCammon et al. 2003).

The genes encoding TCA cycle proteins might also be regulated by glucose levels. In *S. cerevisiae* the depletion of glucose increases 3–10 times the TCA messenger RNAs (DeRisi et al. 1997). Oxygen limitation could also induce a shift in the TCA pathway, which operates as a cycle during aerobic growth and as a two-branched pathway under oxygen limitation, sustaining only the synthesis of the biomass precursor's oxaloacetate and α-ketoglutarate as will be discussed hereafter. However, TCA operating as a two-branched pathway was also identified in *S. cerevisiae* during aerobic fermentation on glucose (Gombert et al. 2001).

6.6 Aerobic Metabolism: Oxidative Phosphorilation and Redox Balance

6.6.1 Oxidative Phosphorilation

The mitochondrial oxidative phosphorylation is a complex and highly controlled network through which ATP synthesis must be continuously adapted to changes in the cell energy demand to sustain growth and/or homeostasis. During respiratory metabolism, both cytosolic and mitochondrial NADH are reoxidized by the respiratory chain. However, *S. cerevisiae*, in contrast to many eukaryotic cells including other yeast species, lacks the multi-subunit complex-I-type NADH dehydrogenase (Nosek and Fukuhara 1994). Instead, *S. cerevisiae* contains a single-subunit NADH:ubiquinone oxidoreductase, which couples the oxidation of intramitochondrial NADH to the respiratory chain. This enzyme (encoded by *NDI1*), referred to as the "internal NADH dehydrogenase," catalyzes the transfer of two electrons from intramitochondrial NADH to ubiquinone (de Vries and Grivell 1988; Marres et al. 1991).

Nonetheless, yeast mitochondria, like those of plants (Moller et al. 1993), not only contain the internal mitochondrial NADH dehydrogenase, but also a mitochondrial external NADH dehydrogenase activity (von Jagow and Klingenberg 1970). *S. cerevisiae* has two genes encoding external NADH dehydrogenase isoenzymes, *NDE1* and *NDE2*, both of them typical aerobic expressed genes (Luttik et al. 1998; Small and McAlister-Henn 1998). Like the internal NADH dehydrogenase, the external isoenzymes do not pump protons (von Jagow and Klingenberg 1970). Therefore, *S. cerevisiae* has a low ATP stoichiometry of oxidative phosphorylation. Notwithstanding this low stoichiometry of oxidative phosphorylation, complete respiratory dissimilation of a glucose molecule yields approximately 16 ATP molecules (four ATP molecules from substrate-level phosphorylation – two from glycolysis and

two from GTP formed in the TCA cycle – and about 12 ATP molecules from oxidative phosphorylation). This is eightfold higher than the maximum ATP yield from glucose dissimilation via alcoholic fermentation. The much higher ATP yield from respiratory sugar dissimilation is reflected in the biomass yields of sugar-limited cultures: the typical biomass yield on glucose of respiring cell cultures is fivefold higher than that obtained in fermenting cell cultures (Verduyn 1991).

Another peculiar feature of the *S. cerevisiae* respiratory chain is that the complexes bc1 (III) and cytochrome c oxidase (IV) were shown to assemble into large supercomplexes (Schagger and Pfeiffer 2000). The respiratory chain of *S. cerevisiae* differs from that of other fungi and of plants not only in the presence of the NADH dehydrogenase not coupled to the proton pump but also owing to the absence of a cyanide-insensitive alternative oxidase which catalyzes the direct oxidation of ubiquinone by molecular oxygen without generating a proton motive force (Vanlerberghe and McIntosh 1997).

Resting cells of the yeast *S. cerevisiae* have shown that the overall rate of oxidative phosphorylation is short-term controlled (1) downstream from the ATP synthase by the cytosolic ATP turnover, (2) upstream from the respiratory chain by reducing equivalent availability and allosteric activation of dehydrogenases and (3) by the ionic permeability of the inner mitochondrial membrane (Beauvoit et al. 1993).

Dejean et al. (2000) have shown that mitochondria are the major energy dissipative system in a fully aerobic metabolism and that energy dissipation can be regulated by the decrease in mitochondrial enzyme content to maintain the oxidative phosphorylation regime.

6.6.2 Redox Balances

The pyridine-nucleotide cofactors NAD^+/NADH and $NADP^+$/NADPH play a central role in yeast metabolism. NADH is preferentially used in dissimilatory metabolism, whereas NADPH is generally required for assimilatory reactions (van Dijken and Scheffers 1986). In *S. cerevisiae*, *C. utilis*, and probably in the yeasts in general, NADH and NADPH cannot be interconverted owing to the absence of a transhydrogenase activity (Bruinenberg et al. 1983). The maintenance of a redox balance is a prerequisite for living cells in order to sustain the regular metabolic activity and enable growth. Hence, since biological membranes are impermeable to pyridine-nucleotides, to maintain the redox balances, the reduced coenzymes must be reoxidized in the compartment in which they are produced.

Cytosolic NADPH is produced by the oxidative part of the pentose phosphate pathway, which branches off from glycolysis at the level of glucose 6-phosphate. Also, $NADP^+$-dependent isocitrate dehydrogenase and $NADP^+$-dependent acetaldehyde dehydrogenase can contribute to NADPH production (Bruinenberg et al. 1983; Minard et al. 1998). *S. cerevisiae* cannot directly couple the oxidation of NADPH to the respiratory chain (Bruinenberg 1986) and therefore is unable to directly oxidize the surplus of cytosolic NADPH. Consequently, the pentose phosphate pathway, which produces the NADPH required for biosynthesis, cannot function as a dissimilatory route in *S. cerevisiae* (González-Siso et al. 1996). Also in *S. cerevisiae* fermentative sugar dissimilation, the role of NADPH is limited, as the major alcohol

dehydrogenases are strictly NAD^+-dependent (Ciriacy 1979). In contrast, *K. lactis* uses the pentose phosphate pathway for glucose dissimilation when glycolysis is blocked, suggesting that, in this yeast species, oxidation of the cytosolic NADPH generated in the pentose phosphate pathway can be efficiently coupled to the mitochondrial respiratory chain (González-Siso et al. 1996). On the other hand, mitochondrial NADPH dehydrogenases, which couple the oxidation of cytosolic NADPH to the mitochondrial respiratory chain, are common in plants, and this mitochondrial NADPH oxidizing activity has also been reported in *C. utilis* (Bruinenberg 1986; van Urk et al. 1989) but it is absent from *S. cerevisiae* (de Vries and Marres 1987; van Urk et al. 1989; Small and McAlister-Henn 1998).

S. cerevisiae has several mechanisms to reoxidize NADH allowing the metabolism to proceed (Bakker et al. 2001). The reduction of NAD^+ occurs both in the cytosol by glycolysis and in the mitochondria by the PDH complex and dehydrogenases of the TCA cycle. Both pools of NADH can be oxidized by the mitochondrial respiratory chain with oxygen as the terminal electron acceptor (de Vries and Marres 1987; Luttik et al. 1998; Overkamp et al. 2000; Bakker et al. 2001).

Aerobically, several systems for conveying excess cytosolic NADH to the mitochondrial electron transport chain exist in *S. cerevisiae* (Bakker et al. 2001). The two most important systems in this respect seem to be the external NADH dehydrogenase (Nde1p/Nde2p) (Luttik et al. 1998; Small and McAlister-Henn 1998) and the glycerol 3-phosphate shuttle (Larsson et al. 1998) although other shuttles could in particular situations play some role (Bakker et al. 2001) (Fig. 6.2). While the external NADH dehydrogenase is suggested as the main system employed for oxidation of cytosolic NADH, the glycerol 3-phosphate shuttle is proposed to be of some importance at low growth rates and perhaps its real significance is only expressed during starvation conditions. The relative importance of these two systems under different conditions is still to a large extent an unsolved matter. Since the external NADH dehydrogenase and the glycerol 3-phosphate shuttle fulfill the same physiological function some kind of regulatory interactions between the two systems would be expected. However, it was shown that the glycerol 3-phosphate shuttle system, which involves the flavin adenine dinucleotide (FAD) dependent Gut2p (Pahlman et al. 2001), is a more efficient system used under conditions where the availability of energy is limited and in fact this system has a higher ATP/O ratio compared with the external NADH dehydrogenase (Larsson et al. 1998). On the other hand, the external NADH dehydrogenase is superior in terms of producing ATP at a high rate and it is therefore the preferred alternative under most other conditions. Nonetheless, all known pathways of respiratory NADH oxidation in *S. cerevisiae* converge at the ubiquinone pool of the respiratory chain.

Kinetic interactions between Nde1p/Nde2p and Gut2p have been demonstrated since the deletion of either of the external dehydrogenases causes an increase in the efficiency of the remaining enzyme (Pahlman et al. 2001). Moreover, the same authors showed that the activation of NADH dehydrogenase inhibited Gut2p, although this inhibition was not a consequence of the direct action of NADH on Gut2p (Pahlman et al. 2001). Most interestingly is the recent work of Bunoust et al. (2005) where they described that electrons coming from Nde1p have the right of way over those coming from either Gut2p or Ndi1p. Therefore, they proposed that the

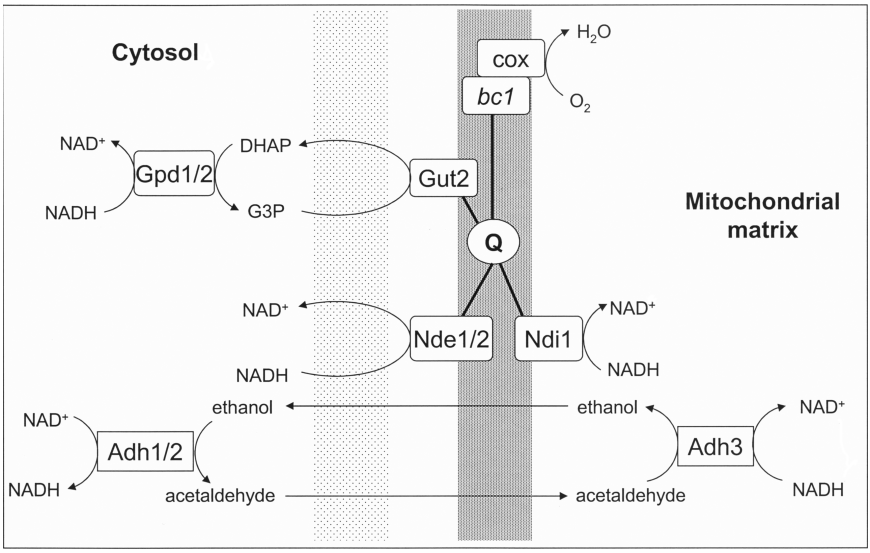

Fig. 6.2. Scheme of redox balance systems in *Saccharomyces cerevisiae*. *Adh* alcohol dehydrogenase; *bc1* bc1 complex; *cox* cytochrome c oxidase; *Gpd* soluble glycerol 3-phosphate dehydrogenase; *Gut2* membrane-bound glycerol 3-phosphate dehydrogenase; *Nde* external NADH dehydrogenase; *Ndi1* internal NADH dehydrogenase; *Q* ubiquinone (Adapted from Bakker et al. 2001)

metabolic organization of the respiratory chain is such that it allows a selection and a priority in electron supply, pointing to a new mechanism of regulation of the yeast oxidative metabolism.

The regulation between the two NADH reoxidation systems has also to contemplate the glucose catabolic repression especially when we are talking about *S. cerevisiae*. At high glucose concentrations respiration is only partly repressed (Blomberg et al. 1988; Larsson et al. 1998). These traits will render the glycerol 3-phosphate shuttle inactive since one of its components, Gut2p, is subject to glucose repression (Sprague and Cronan 1977). To a large extent, under these conditions, cytosolic redox balance is restored by ethanol and glycerol formation, though the activity of the external NADH dehydrogenase might explain the lower glycerol formation obtained during aerobic compared with anaerobic batch cultures of *S. cerevisiae* (Rigoulet et al. 2004). On the other hand, during aerobic growth at low glucose concentrations in chemostat cultures, both the external NADH dehydrogenase and the glycerol 3-phosphate shuttle seem operative simultaneously (Rigoulet et al. 2004) (Fig. 6.2). However, the NADH dehydrogenase activity appears more important under these conditions.

6.7 Anaerobic Metabolism

As referred to before, yeasts may be physiologically classified with respect to the type of energy-generating process involved in sugar metabolism, namely non-,

facultative- or obligate-fermentative. Within these classes only the last two have a fully operative pathway that can provide free-energy-transduction under anaerobic growth. Alcoholic fermentation is acknowledged as the catabolism of glucose to ethanol. According to the stoichiometry of alcoholic fermentation two molecules of ATP are produced per molecule of glucose converted into ethanol functioning as the main energy supply for maintenance and growth. Additionally, the alcoholic fermentation is a redox-neutral process, since the NADH produced during the oxidation of glyceraldehyde 3-phosphate by glyceraldehyde 3-phosphate dehydrogenase is subsequently reoxidized in the reduction of acetaldehyde to ethanol by alcohol dehydrogenase (van Dijken and Scheffers 1986) (Fig. 6.2). However, one cannot overlook that growth is associated with anabolic processes and yeasts have an overall biomass composition that is more oxidized than that of compounds like glucose, which implies that anabolic processes lead to a surplus of reducing equivalents. In contrast to aerobic conditions, the absence of oxygen abolishes the possible oxidation of reduced pyridine nucleotides in the respiratory chain coupled to oxidative phosphorilation. One common way to satisfy the redox balances during growth under anaerobic conditions is the split of glucose metabolism towards glycerol. Glycerol is produced by reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol 3-phosphate followed by a dephosphorylation of glycerol 3-phosphate to glycerol. The first step is catalyzed by NAD^+-dependent glycerol 3-phosphate dehydrogenase (encoded by the two isogenes *GPD1* and *GPD2*), whereas the second reaction is catalyzed by the activity of glycerol 3-phosphatase (encoded by *GPP1* and *GPP2*) (Eriksson et al. 1995; Larsson et al. 1993; Norbeck et al. 1996).

Additionally, the up-regulation of *GPD2* associated with an anaerobic growth impairment of *S. cerevisiae* gpd2 mutant clearly establishes the metabolic relevance of this isoenzyme (Ansell et al. 1997; Bjorkqvist et al. 1997; Nissen et al. 2000). In the same line of thought, whereas the Gpp2p seems not to play a relevant role in glycerol production under anaerobic conditions, mutants lacking *GPP1* display a growth defect under identical conditions. One should stress that the anaerobic growth defects presented by the latter mutant are not due to redox balance impairments but are mainly due to growth-inhibiting levels of intracellular glycerol 3-phosphate (Pahlman et al. 2001). Moreover, anaerobic conditions do not affect expression of *GPP2*, while *GPP1* is induced (ter Linde et al. 1999). Hence, the glycerol biosynthesis has defined physiological roles in the metabolic adaptation of *S. cerevisiae*. The correlation between glycerol production and growth under anaerobic conditions is clearly illustrated by the restoring of anaerobic growth defects of mutants with impaired glycerol production by the presence of the electron acceptors acetoin (3-hydroxy-2-butanone) and acetaldehyde. These two compounds alleviate the imposed cellular reductive stress since they enzymatically oxidize intracellular NADH to NAD^+. Furthermore, the increasing flux towards glycerol biosynthesis with the increasing of growth rate is well established. This metabolic shift is the result of an increasing drain from the catabolic pathways to support the higher level of biosynthetic molecules such as RNA and proteins (Nissen et al. 1997).

On the other hand, several yeast species, including *S. cerevisiae*, are well known by their capability of producing acetic acid under both aerobic and anaerobic/

oxygen-limiting conditions. In *S. cerevisiae*, the further metabolism of acetic acid through acetyl–CoA synthetase (encoded by *ACS1* and *ACS2* genes) is the only source of cytosolic acetyl–CoA, an imperative building block of fatty acid biosynthesis (van den Berg and Steensma 1995; Flikweert et al. 1996). When acetaldehyde is converted into acetic acid a surplus of two reducing equivalents is produced. The formation of such a product poses an additional problem in the redox balances, once again circumvented by the production of glycerol. However, if the yeasts cannot cope with the surplus of reducing equivalents the anaerobic activity will come to a standstill, as discussed hereafter. From an anabolic point of view, when acetaldehyde is converted into acetate, NADPH is generated. From the five currently known isogenes of acetaldehyde dehydrogenases, only *ALD4*, *ALD5* and *ALD6* gene products have a role in acetate production during glucose fermentation under both aerobic and anaerobic/oxygen limiting conditions (Remize et al. 2000; Saint-Prix et al. 2004). While the last one encodes a cytoplasmic isoform of acetaldehyde dehydrogenase, the first two encode mitochondrial proteins (Meaden et al. 1997; Tessier et al. 1998; Wang et al. 1998). Moreover, this reaction together with the ones catalyzed by malic enzyme (encoded by *MAE1*) and $NADP^+$-dependent isocitrate dehydrogenase (encoded by *IDP1*) are currently known to generate mitochondrial NADPH, necessary for biosynthetic purposes.

TCA pathway activity is maintained during fermentation for primary fuel biosynthetic reactions supplying cells with four and five carbon compounds, namely oxaloacetate and 2-oxoglutarate, the precursors of aspartate and glutamate. The TCA pathway is known to operate in a branched fashion under anaerobiosis or conditions of glucose repression (Gombert et al. 2001) where succinate dehydrogenase is nonfunctional (Camarasa et al. 2003). Hence, the flux from 2-oxoglutarate to oxaloacetate is zero. Therefore, the split of the carbon flux in the TCA cycle results in the formation of one oxidative branch leading to the formation of 2-oxoglutarate (Nunez de Castro et al. 1970) and one reductive branch that culminates with the formation of fumarate (Atzpodien et al. 1968). The key enzyme of the redutive branch is the fumarate reductase encoded by *FRDS* and *OSM1* genes (Arikawa et al. 1998). The relevant role of this reaction in the reoxidation of intracellular NADH is highlighted by the inability to grow under anaerobic conditions of fumarate reductase null mutant that is restored by the addition of the oxidized form of methylene blue or phenazine methosulfate, which nonenzymatically oxidizes cellular NADH to NAD^+ (Enomoto et al. 2002). It may perhaps be considered that the role of the enzyme pyruvate carboxylase is to catalyze the reaction of pyruvate to oxaloacetate at the expenses of one molecule of ATP and carbon dioxide (de Jong-Gubbels et al. 1998). This anaplerotic reaction functions to further replenish the TCA pathway for biosynthetic purposes or organic acid production as referred to previously.

The synthesis of precursors for biomass building blocks actively results in the net formation of NADH in the mitochondrial matrix (Visser et al. 1994). The reductive branch, thermodynamically favorable, and the *ADH1*/*ADH3* shuttle (Fig. 6.2) are two feasible routes for the oxidation of the surplus of NADH produced in mitochondria by both the oxidative TCA branch and the synthesis of amino acids. It should be noted that, stoichiometrically, the formation of succinate by the reductive pathway or by the oxidative pathway results, respectively, in two molecules of FAD

oxidized or in five molecules of reduced NADH per molecule of glucose consumed. The surplus of reducing equivalents may be reoxidized by the *ADH1*/*ADH3* shuttle involving the mitochondrial alcohol dehydrogenase, adh3p, that results in a net production of NADH in the cytosol and that can be circumvallated by glycerol production (Nissen et al. 1997; Bakker et al. 2000). In fact, Nissen et al. (1997) showed that the *ADH1*/*ADH3* shuttle is responsible for the regeneration of NAD^+, whereas the reductive branch is associated with succinate production. Nevertheless, the *ADH1*/*ADH3* shuttle does not strictly control the redox balance under anaerobic conditions, since a null *ADH3* mutant still shows anaerobic growth, albeit slower than that of the wild type (Bakker et al. 2000). The TCA pathway during fermentation also leads to the formation of organic acids, mainly citrate, malate and succinate (Heerde and Radler 1978), and depending on the different yield products there are differences in the way that cells have to cope with the redox balance. As yet, however, the nitrogen source strongly influences cellular metabolism and product formation in *S. cerevisiae*, especially owing to redox contour-balancing under anaerobic conditions (Albers et al. 1996). A high demand of NADPH is required for glutamate formation when cells are using ammonium as the only nitrogen source. In contrast, when glutamate is used as a nitrogen source the reduced synthesis of 2-oxoglutarate from glucose causes fewer reducing equivalents to be formed, which reduces the glycerol yield and hence increases the ethanol yield (Albers et al. 1996; Camarasa et al. 2003).

The regulatory pattern of malic enzyme (encoded by *MAE1*) suggests the specific physiological function in the provision of intramitochondrial NADPH or pyruvate in anaerobic metabolism; however, this enzyme does not have a strict function in redox metabolism, since null mutant in *MAE1* shows identical glycerol production under anaerobic conditions (Boles et al. 1998).

6.8 Yeast Growth Ability under Anaerobic Conditions: Is There a Common Prerequirement?

Of the 678 recognized species, around 60% are considered to be fermentative on the basis of taxonomic tests such as gas production in Durham tubes (Barnett et al. 2000). However, this number is even higher since, under certain conditions, some of those species considered as nonfermentative are also able to ferment glucose (van Dijken et al. 1986; Visser et al. 1990). While the ability to ferment glucose under oxygen limitation turns out to be a common feature of the different yeast species, apparently the capability of growth under anaerobic conditions is not widespread among these microorganisms (Visser et al. 1990). In fact, only very few yeast species are capable of fast growth under those conditions and *S. cerevisiae* stands out as the yeast generally acknowledged as a facultative anaerobe (Verduyn et al. 1990; Visser et al. 1990). It is commonly accepted that facultative anaerobes have the ability to grow both under aerobic and anaerobic conditions using, respectively, molecular oxygen or another compound as the final electron acceptor of the reducing equivalents that overflow from anabolic processes. Owing to the fact that anaerobic growth is associated with a low energy yield compared with that observed under complete oxidative processes, these microorganisms display two common characteristics:

(1) the rate of sugar-substrate consumption is higher under anaerobic processes than that observed under aerobic conditions and (2) oxygen is used as a preferential source of the final electron acceptor.

As clearly pointed out by Lagunas (1979, 1981, 1986), *S. cerevisiae* can roughly meet the previously described criteria for the reasons given hereafter. Even though *S. cerevisiae* is capable of rapid anaerobic growth it requires an external supply of sterols and fatty acids (Verduyn et al. 1990). The biosynthetic pathways involved in the production of these compounds, essential for membrane turnover, require molecular oxygen (Andreasen and Stier 1953, 1954). Besides the expected benefits that would be produced from a shift from an anaerobic (where energy is the limiting factor) to an aerobic environment (with the putative ability to produce 1.4–5.4 times more ATP per mole of sugar) they are almost irrelevant in *S. cerevisiae* (Lagunas 1979, 1986, and references therein; Rodrigues et al. 2001). It should be stressed that in this species only around 5-10% of glucose, maltose or fructose is not metabolized via alcoholic fermentation under aerobic batch growth. The ratios between the flux of those sugar consumptions under aerobic and anaerobic conditions are, respectively, 1.05, 0.90 and 1.08 (Lagunas 1979). Ultimately, a rate value close to 1 means that no Pasteur effect occurs for the metabolism of the sugars described before.

Nevertheless, the biosynthetic oxygen requirements of facultative-fermentative yeasts are extremely small. Therefore, for reliable and meaningful identification of yeast species with the ability to grow under strictly anaerobic conditions special precautions (e.g., use of oxygen-resistant tubing and ultrapure nitrogen gas for sparking) are needed during the setup process of growth to minimize oxygen entry to the extent that these small oxygen requirements become apparent (Visser et al. 1990; Rodrigues et al. 2001). Albeit, in some cases the requirements of molecular oxygen may be substantial, whereas, for instance, an insignificant leakage of oxygen between 0.3 and 6 μmol of O2 h^{-1}, corresponding to normal strict anaerobic conditions, is sufficient to allow the yeasts *Zygosaccharomyces bailii* and *C. utilis* to grow (Rodrigues et al. 2001). In spite of the linearity of the growth kinetics characteristics of oxygen limitation, *C. utilis* requires 1.5-fold more time than *Z. bailii* to finish cell proliferation, highlighting the differences in oxygen demand of both species for growth. This raises the general question, what are the further limitations that explain the anaerobic growth incapability of "non-*Saccharomyces*" yeasts?

During the last few years, different research groups have raised several hypotheses, most of them related to the inability to fulfill the biosynthetic requirements under anaerobic conditions. Ergosterol and unsaturated fatty acids were shown to be essential for *S. cerevisiae* and *S. kluyveri* (Moller et al. 2001). Strengthening the specific requirements of *S. cerevisiae* for ergosterol and unsaturated fatty acids, Wilcox et al. (2002) showed that the ATP-binding transporters Aus1p and Pdr11p, associated with sterol uptake, are essential for anaerobic growth. However, it seems that *Schizosaccharomyces japonicus* does not require ergosterol for growth (Bulder 1971). On the other hand, the discovery by Nagy et al. (1992) that *S. cerevisiae* has one dihydroorotate dehydrogenase (DHODase) (encoded by the gene *URA1*), which catalyzes the single redox reaction converting dihidroorotate into orotate, not dependent on the functionality of the respiratory chain, opened a new line of research into the ability of yeasts to grow under anaerobic conditions. In this line, it was possible

to turn the oxygen-dependent yeast *Pichia stipitis* into an anaerobic culturable yeast just by expressing the *URA1* gene from *S. cerevisiae* (Shi and Jeffries 1998). More recently, it was experimentally shown that the genome of *S. kluyveri* also encodes a DHODase, functionally equivalent to that of *S. cerevisiae* (Gojkovic et al. 2004). The nice study of Gojkovic et al. (2004) illustrated that while aerobic yeasts, such as *S. pombe* and *C. albicans* contain just a mitochondrial DHODase, with a quinine-type electron acceptor, the facultative anaerobes yeasts, such as *S. cerevisiae*, *S. bayanus* and *S. castellii* contain a cytosolic DHODase, with a fumarate-type electron acceptor, whereas *S. kluyveri* contains both. This study pointed to horizontal gene transfer from bacteria to the progenitor of these lineages, and raised the hypothesis that a remodeling of the biochemical pathways during yeast diversification took place, allowing the progressive reduction of oxygen requirements for growth. As yet, however, the capability of *Z. bailii* to grow under anaerobic conditions in yeast–peptone–glucose opposed to defined media is still unexplained since it is not due to uracil auxotrophy (Rodrigues et al. 2001). The involvement of additional biosynthetic requirements may perhaps be considered in other cases.

Another even more generalized limitation to anaerobic growth may be related to either/both a sufficiently high rate of ATP formation of a given substrate or/and an impaired mitochondrial ADP/ATP translocation, as well as constraints on redox metabolism (van Dijken and Scheffers 1986; Verduyn et al. 1990; Visser et al. 1994; Trezeguet et al. 1999). Under anaerobic conditions and in contrast to aerobic conditions, the TCA cycle has only an anabolic function and the cell growth is completely dependent on ethanol formation for the provision of energy. In theory it is conceivable that, for certain yeast species, the glycolytic flux is not sufficient to drive the free energy for cellular processes. Verduyn et al. (1992) showed that the *S. cerevisiae* fermentation rate limits the supply of ATP for maintenance purposes above that required for anabolic reactions, to a value of approximately 17 mmol ATP Eq g^{-1} h^{-1}. However, when the maintenance requirement increases to a threshold value (e.g., triggered by the uncoupling effect of weak carboxylic acids or by the decrease of the pH of the culture medium below 2.8), glycolytic flux can no longer provide further ATP, leading to cytosolic acidification and subsequently cell death (Verduyn et al. 1990). It is to be stressed that the previous considerations are not related to the well-known Custers effect on yeast which is characterized by fermentation inhibition upon the transition to an anaerobic environment. In *Torulaspora delbrueckii*, it seems that the increase of glycolytic flux under anaerobic conditions is not sufficient to cope with growth demands (Hanl et al. 2004). On the other hand, an impairment of mitochondrial ADP/ATP translocation may lead to growth arrest in the absence of oxygen. In *S. cerevisiae* it was shown that the blockage of ADP/ATP translocators by bongkrekic acid induced a reduction of approximately 50% in the growth rate under anaerobic conditions (Visser et al. 1994). This dependence was further established by the inability of *S. cerevisiae* null mutant in *AAC* genes (*AAC1*, *AAC2*, *AAC3*; coding ADP/ATP translocators) to grow under those conditions. One should emphasize that the same authors showed that this triple mutant was still able to grow on fermentable sources in the presence of oxygen and that *AAC3* was induced under anaerobic conditions (Kolarov et al. 1990; Lawson et al. 1990; Drgon et al. 1991). Furthermore, in *S. cerevisiae* batch cultivations, the

surplus of reducing equivalents formed in anabolic reactions has to be balanced by the formation of glycerol estimated in approximately 5% of glucose breakdown. In this species, the null mutant in glycerol 3-phosphate dehydrogenase is incapable of growth under anaerobic conditions, and the simple deletion of the *GPD1* gene leads to a decrease of 5 times the growth rate (Nissen et al. 2000). More surprisingly, the same authors showed that the expression of a cytoplasmic transhydrogenase from *Azotobacter vinelandii* could not rescue the phenotype, showing that the NAD^+ pool that limited growth was lower than the threshold value favoring the transhydrogenase reaction. Therefore, an impaired redox metabolism may, in some cases, be behind the inability to grow anaerobically.

From everything discussed here, the hitherto unexplained inability of most yeast species to grow under anaerobic conditions is still an unsolved puzzle. Scientific efforts must, therefore, focus on an integrated approach keeping the essential strait balance between the two complementary approaches – the yeast physiology and the yeast molecular biology – and thus driving the expected increase in knowledge of yeast performance.

Acknowledgment

The authors thank Agostinho Almeida for critical English revision of the text.

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Chapter 7

Diversity of Nitrogen Metabolism Among Yeast Species: Regulatory and Evolutionary Aspects

Francine Messenguy[1], Bruno André[2] and Evelyne Dubois[1]

[1]*Institut de recherches Microbiologiques J-M Wiame, Avenue Emile Gryzon 1, 1070 Brussels, Belgium*
(e-mail: fanarg@ulb.ac.be, Tel.: +32-2526-7277, Fax: +32-256-7273)
[2]*Laboratoire de Physiologie Moléculaire de la Cellule, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, 6041 Gosselies, Belgium*

7.1 Introduction

Yeasts are capable of utilizing a wide diversity of nitrogen compounds as nitrogen sources, and the ability or inability to utilize one or several of these compounds can be used in classifying yeasts. It is worth noting that nitrogen compounds can also be used as a carbon source or be incorporated into proteins, but these metabolisms will not be considered in the present chapter.

More than 50 different nitrogen sources are known to be utilizable by yeast species, including L-amino acids, D-amino acids, pyrimidines, purines, polyamines, amines, nitrate and nitrite. However, the nitrogen utilization profiles markedly vary according to yeast species, many of which are much more versatile than *Saccharomyces cerevisiae*. The utilization of nitrogen compounds as a nitrogen source has been well studied in *S. cerevisiae*, and less extensively in other yeasts. In 1986, Large (1986) presented an extensive survey of organic-nitrogen-compound utilization by yeast strains from various genera. This article focused on the diversity of nitrogen compound degradation, especially in its chemical and metabolic aspects. Since 1986, mainly for *S. cerevisiae*, many new data have become available concerning the levels of transport and the control of nitrogen metabolisms allowing the establishment of metabolic and regulatory networks and an integration of nitrogen metabolism. In contrast, for other yeast species the study of nitrogen metabolism and its regulation did not raise the same interest, and only dispersed data with no integration are available in the literature, with the exception of nitrate/nitrite metabolism in *Hansenula*. However, since the genome sequence of 24 yeasts (as stated by the consortium Génolevures) was recently performed, the comparison between genes from *S. cerevisiae* and related hemiascomycetous yeasts or a more distant yeast species such as *Schizosaccharomyces pombe* could lead to the development of new approaches to study the diversity of nitrogen metabolism among yeasts. Such

comparisons could bring to light the reason for nonutilization of some nitrogen compounds, and could also provide new data on molecular evolution.

Degradation of nitrogen-containing compounds as the sole source of nitrogen for growth leads to the formation of three key nitrogen compounds in the cell, ammonia, glutamate and glutamine. These are interlinked by three enzyme systems: NADP-dependent glutamate dehydrogenase (GDH) (ammonia→glutamate, reaction 1), NAD-dependent GDH (glutamate→ammonia, reaction 2) and the glutamine synthetase/glutamate synthase system (reactions 3, 4) as illustrated in Fig. 7.1. The major pathway for glutamate synthesis is through combination of ammonia with α-ketoglutarate. Glutamine is synthesized by the combination of ammonia with glutamate by glutamine synthetase. The glutamine synthetase coupled to the glutamate synthase system is an alternative pathway to produce glutamate, which is common in bacteria (Tempest et al. 1970), but is present only in a small number of yeasts. Although glutamine synthetase is widespread in yeasts, glutamate synthase activity has only been detected in *S. cerevisiae* (Roon et al. 1974) and in certain *Schizosaccharomyces* species (Brown et al. 1973). Comparison among five yeast genomes that have been completely sequenced revealed the presence of ortholog genes to *GLT1* (coding for glutamate synthase in *S. cerevisiae*) in *Candida glabrata, Yarrowia lipolytica*, and *Kluyveromyces lactis*. It is probably also present in *Debaryomyces hansenii*, because there are two contiguous open reading frames whose products share strong similarity to *S. cerevisiae* glutamate synthase. The importance of this alternative pathway in *S. cerevisiae* is supported by the fact that a mutant lacking NADP–GDH grows on ammonia at approximately half the rate of the wild type. However a double *gdh1*, *glt1* mutant still presents residual growth with ammonia as the sole nitrogen source, indicating the existence of another alternative pathway. A computer search identified a new gene, *GDH3*, whose product shows similarity to *GDH1* product, and the triple mutant strain was strict glutamate auxotroph (Avendano et al. 1997). In contrast, in *S. pombe, C. glabrata, Y. lipolytica, K. lactis*, and *D. hansenii,* there is only one putative gene encoding the NADP-dependent GDH.

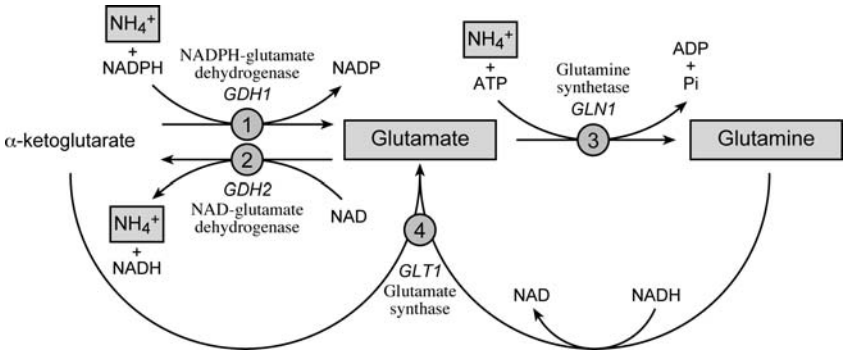


Fig. 7.1. Central pathways for nitrogen metabolism. The *Saccharomyces cerevisiae* gene for each of the enzymatic steps is designated in *italics*

7.2 Nitrogen Transporters

Transport of amino acids for breakdown to supply nitrogen for the synthesis of other molecules involves transporters, such as Gap1 and Agp1. The general amino acid permease (Gap1) (Grenson et al. 1970; Jauniaux and Grenson 1990) can recognize most if not all amino acids, including ones not present in proteins (e.g., citrulline, ornithine, γ-aminobutyric acid), D-isomers, β-alanine, and many toxic amino acid analogs. Contrary to a widely held belief, Gap1 displays a very high affinity for most of its substrates (apparent K_m in the micromolar range). This permease is most active under limiting nitrogen supply conditions, e.g., when the sole nitrogen source is ammonia at low concentration, urea, or proline. The main function of Gap1 under these conditions is probably to scavenge traces of amino acids to be used as a source of nitrogen. When amino acids (other than proline) are present at relatively high concentrations, Gap1 is inactive and broad-range-specificity amino acid permeases are induced. Among the latter is Agp1, an amino acid permease able to recognize most neutral amino acids. The affinity of Agp1 for these amino acids is lower (K_m about 100–200 μM) than the affinity of Gap1 (Iraqui et al. 1999b; reviewed in Boles and Andre 2004). Proline uptake is also mediated by Put4, also able to recognize γ-aminobutyric acid (GABA) (Vandenbol et al. 1989), alanine and glycine (Regenberg et al. 1999). Other amino acid permeases first believed to recognize only a single or a few amino acids (Bap2, Bap3, Gnp1, Tat1, Tat2, Dip5) actually recognize larger sets of amino acids, although their specificity ranges do seem narrow as compared with Agp1. Bap2 preferentially transports branch-chained amino acids, and glutamine and asparagine are efficiently transported into the cell by the products of *GNP1* and *AGP1* genes (Zhu et al. 1996; Schreve et al. 1998). Tat1 transports at least leucine and tyrosine, Tat2 mainly transports tryptophan, and Dip5 transports dicarboxylic amino acids (Schmidt et al. 1994; Regenberg et al. 1998). GABA is transported into the cell by the inducible Uga4 high affinity permease, and urea entry is mediated by the inducible permease Dur3. *DAL4* encodes the allantoin permease, a member of the uracil/allantoin permease family, and *DAL5* the allantoate and ureidosuccinate permease (Andre 1995). Besides these transporters, *S. cerevisiae* also synthesizes specific permeases mediating high-affinity uptake of arginine (Can1, Alp1), lysine (Lyp1), histidine (Hip1), and methionine (Mup1, Mup2) (reviewed in Boles and Andre 2004). Ammonia transport requires three permeases encoded by the *MEP1*, *MEP2*, and *MEP3* genes (Marini et al. 1997).

Genes encoding amino acid transporters have also been reported in yeasts other than *S. cerevisiae*. The gene *CaGAP1* of *C. albicans* can functionally complement a *S. cerevisiae gap1* mutant. Mutation of the *CaGAP1* gene has an effect on citrulline uptake in *C. albicans*. Transcription of *CaGAP1* is regulated by the quality of the nitrogen source, and is dependent on the Cph1-mediated Ras1 signaling pathway. Defective filamentation or abnormal colony morphology in homozygous and heterozygous *CaGAP1* disruptants is found under certain conditions (Biswas et al. 2003). The *C. albicans CaCAN1* gene, encoding a high-affinity permease for arginine, lysine, and histidine, can complement a *S. cerevisiae* strain lacking basic amino acid permeases. The CaCan1 protein sequence is strongly homologous to both permeases for basic amino acids, Can1 and Lyp1 of *S. cerevisiae* (Sychrova and

Souciet 1994). The expression of CaCan1 was influenced by the available nitrogen source, being almost negligible when cells were grown in the presence of ammonia (Matijekova and Sychrova 1997). In *S. pombe*, there is only a single high-affinity system for the transport of basic amino acids (Sychrova et al. 1992).

Tight regulation at the level of transporter synthesis or at the level of transporter activity and stability in response to nitrogen availability allows fine-tuning of nitrogen import. These aspects will be discussed in a further section.

7.3 Nitrogen Sources and Their Degradative Pathways

Yeasts are capable of utilizing the diversity of nitrogenous compounds that they find in their rich natural environment. Figure 7.2 summarizes the nitrogen compounds known to serve as a source of nitrogen for yeasts. An exhaustive list of these nitrogen compounds is presented in Large (1986). All these degradative pathways lead to ammonia or glutamate or both. Ornithine, GABA, tryptophan, tyrosine, phenylalanine, isoleucine, leucine, valine, alanine, methionine, and aspartate provide by transamination, glutamate or the α-ketoacid analog of the corresponding amino acid, whereas glutamine, asparagine, threonine, cytosine, adenine, cytidine, guanine, and urea lead to ammonia production by deamination. In *S. cerevisiae,* two asparaginases have been implicated in asparagine metabolism (Jones 1977), one of these, asparaginase I (*ASP1*) is cytosolic and acts only on intracellular L-asparagine.

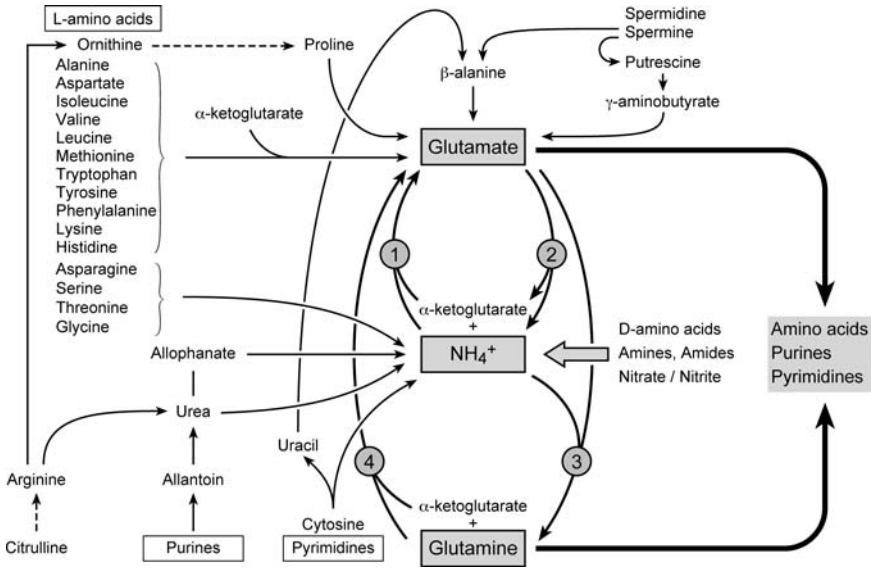


Fig. 7.2. Schematic representation of the main reactions involved in nitrogen utilization in yeasts grown on various nitrogenous compounds. See the text for specific pathways used by the different yeasts

Asparaginase II is produced extracellularly and can hydrolyze both D-asparagine and L-asparagine in the growth medium. Some strains of *S. cerevisiae* lack asparaginase II (*ASP3*) (Dunlop et al. 1976), but this enzyme occurs in a wide range of yeasts (Imada et al. 1973). Degradation of serine and threonine is catalyzed by the catabolic L-serine (L-threonine) dehydratase (*CHA1*), which converts serine in one step into pyruvate and ammonia (Ramos and Wiame 1982; Petersen et al. 1988). GABA is degraded in two steps to glutamate. Gene *UGA1* encodes 4-aminobutyrate aminotransferase, and gene *UGA2* encodes succinate semialdehyde dehydrogenase (Ramos et al. 1985). Arginine is cleaved by arginase (*CAR1*) to ornithine and urea. The ornithine so formed is converted via transamination (*CAR2*) to glutamate and glutamate semialdehyde. *S. pombe* genome contains two putative orthologs of gene *CAR1*. One of these genes (SPB26C9.02c) has been cloned by functional complementation of a *car1* mutant strain of *S. cerevisiae* (van Huffel et al. 1994). Further catabolism of ornithine proceeds via the amino acid proline, whose catabolism occurs in the mitochondria (Brandriss and Magasanik 1980). Proline is oxydized by proline oxydase (*PUT1*) to Δ-pyrroline 5-carboxylate, which is then oxydized to glutamate by the mitochondrial pyrroline 5-carboxylate dehydrogenase (*PUT2*). Citrulline is used as a nitrogen source by conversion to arginine by the two last arginine biosynthetic enzymes, argininosuccinate synthetase (*ARG1*) and argininosuccinase (*ARG4)*. Urea produced by the degradation of arginine or purines, or present in the medium, can be processed via two different routes depending on the yeasts. Schizosaccharomycetacae are the only urease-positive ascomycetous yeasts reported (Booth and Vishniac 1987), but basidiomycetous yeasts such as *Cryptococcus neoformans* and *Rhodotorula* spp., also present urease activity (Seeliger 1956; Sen and Komagata 1979). In the other yeasts (listed in Fig. 7.7), including *Y. lipolytica,* the reaction proceeds via the intermediate formation of allophanic acid and the process requires ATP. The two enzymes, urea carboxylase and allophanate hydrolase (*DUR1*,*2*) are tightly associated in *S. cerevisiae* in a multienzyme complex (Sumrada and Cooper 1982; Whitney and Cooper 1972). *DUR1,2* orthologs can be found in *D. hansenii, Y. lipolytica, C. glabrata* and *K. lactis*.

The catabolism of the branched-chain (leucine, isoleucine, valine) and aromatic amino acids (tryptophan, phenylalanine, tyrosine) in *S. cerevisiae* proceeds through the Erlich pathway involving three enzymatic steps (Dickinson 2000; Dickinson et al. 2003). A first transamination produces the α-ketoacid analogs of the amino acids, a decarboxylation step yields the corresponding aldehydes, and in a third step, the resulting aldehydes are reduced to alcohols, collectively referred to as fusel oils. This general scheme of amino acid degradation has long been proposed from studies of metabolic intermediates and end-product formation. But only in recent years, the enzymes involved in these degradative pathways and their genetic determinants have been studied. Degradation of the branched-chain amino acids starts with the reversible transfer of the α-amino group to α-ketoglutarate to form glutamate and the respective branched-chain α-keto acids (α-ketoisocaproic from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine). These reactions are mainly catalyzed by the branched-chain aminotransferase isozymes, mitochondrial Bat1 and cytosolic Bat2; *bat1 bat2* double mutants are indeed auxotrophic for isoleucine, valine, and leucine (Eden et al. 1996; Kispal et al.

1996; Prohl et al. 2000). Bat1 and especially Bat2 diversely contribute also to the formation of higher alcohols from branched-chain amino acids (Eden et al. 2001; Yoshimoto et al. 2002), but their respective contribution to branched-chain amino acid utilization as nitrogen sources remains to be investigated. There are significant differences in the way each α-ketoacid is subsequently decarboxylated. In the leucine degradation pathway, the major decarboxylase is encoded by *KID1/YDL080c* (Dickinson et al. 1997). In valine degradation, any one of the three isozymes of pyruvate decarboxylase encoded by *PDC1*, *PDC5*, and *PDC6* can decarboxylate α-ketoisovalerate (Dickinson et al. 1998), and in isoleucine catabolism, any member of the family of decarboxylases encoded by *PDC1*, *PDC5*, *PDC6*, *KID1*, and *ARO10/YDR380w* is apparently sufficient for the conversion of isoleucine to active amyl alcohol (Dickinson et al. 2000). In aromatic amino acid metabolism also, two isozymes can catalyze in vitro the reversible conversion of tryptophan, phenylalanine, and tyrosine to indol-3-pyruvate, phenylpyruvate, and hydroxyphenylpyruvate, respectively: the constitutive aromatic amino acid aminotranferase I, encoded by *ARO8,* and the inducible aromatic aminotranferase II, encoded by *ARO9* (Kradolfer et al. 1982; Iraqui et al. 1998; Urrestarazu et al. 1998). Both catalyze the last step of phenylalanine and tyrosine biosynthesis, as *aro8* or *aro9* single mutants are prototrophs, whereas *aro8 aro9* double mutants are phenylalanine and tyrosine auxotrophs (Urrestarazu et al. 1998). Aro9 catalyzes the first step of tryptophan degradation as *aro9* mutant strains grow poorly on tryptophan, whereas the enzyme is dispensable for growth on phenylalanine or tyrosine, indicating that Aro8 or other transaminases can also ensure their degradation (Iraqui et al. 1999a). In both phenylalanine and tryptophan catabolism the decarboxylation step can be carried out by any one of the gene products of *PDC1*, *PDC5*, *PDC6*, or *ARO10* (Dickinson et al. 2003). In fact, it has been shown for phenylalanine that the inducible Aro10 is the physiologically relevant 6-phenylpyruvate decarboxylase in wild-type cells; in *aro10* mutants, an alternative activity was observed requiring the combined presence of Kid1 and at least one of the three pyruvate decarboxylases, Pdc5, Pdc1, or Pdc6 (Vuralhan et al. 2003). According to Dickinson et al. (2003), any one of six alcohol dehydrogenases (encoded by *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, or *SFA1*) is sufficient for the final stage of aromatic and branched-chain amino acid catabolism that converts an aldehyde to a long-chain or complex alcohol. This preliminary work, however, does not determine what are the physiologically relevant alcohol dehydrogenases that contribute to fusel oil formation in wild-type cells.

Glycine can serve as the sole nitrogen source, but only very poorly (Sinclair and Dawes 1995) (Fig. 7.3). This degradation involves a mitochondrial glycine decarboxylase complex, which catalyzes the breakdown of glycine to CO_2 and ammonia, yielding the activated one-carbon unit 5,10-methylenetetrahydrofolate and NADH. In *S. cerevisiae* the GCV complex comprises four subunits: the T-protein (*GCV1*), the P-protein (*GCV2*), the H-protein (*GCV3*) and the L-protein (*LPD1*) (Ross et al. 1988; Sinclair et al. 1996; McNeil et al. 1997; Nagarajan and Storms 1997). Possibly the physiological role of the GCV complex is more attuned to one-carbon metabolism rather than for provision of nitrogen. Nonetheless it is possible to show a non-growth phenotype of the *gcv1*, *gcv2*, *gcv3*, or *lpd1* mutants on a medium with glycine as the sole nitrogen source.

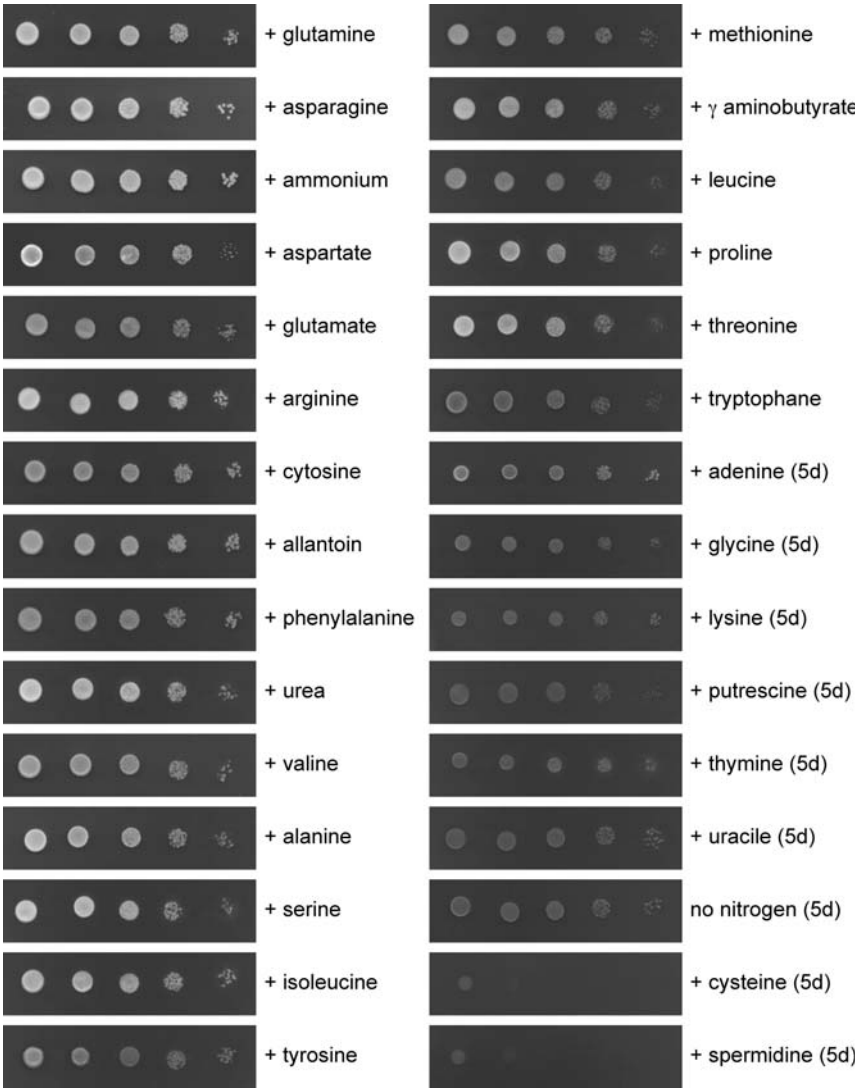


Fig. 7.3. Growth tests of a wild-type *S. cerevisiae* strain on various nitrogen sources. Tenfold serial dilutions of cells from the haploid strain ∑1278b were plated and incubated at 30°C for 3 days (except when otherwise indicated) on minimal medium with 2% glucose and the different nitrogen sources as indicated (1mg/ml). The growth tests are classified according to the growth rates on each nitrogen source. The growth reference is the same minimal medium without nitrogen

Many yeasts, but not *S. cerevisiae*, are able to utilize histidine or lysine as the sole nitrogen source. The histidine degradative pathway remains uncharacterized, whereas the lysine degradative pathway has been studied. Among the archiascomycete and hemiascomycete yeasts *S. pombe*, *Y. lipolytica*, *Debaryomyces*, and different *Candida*, *Pichia*, and *Kluyveromyces* are lysine utilizers. The lysine degradation pathway has been investigated in *Y. lipolytica* by the group of Gaillardin (1976). The first enzyme – lysine N6-acetyltransferase – is encoded by the *LYC1* gene, which has been cloned and sequenced (Beckerich et al. 1994). The second step involves a N6-acetyllysine aminotransferase providing glutamate by transamination of α-ketoglutarate. Lysine N6-acetyltransferase activity was found in *C. tropicalis* (Large 1986), which grows on lysine as the sole nitrogen source. For other yeasts no information about enzyme activity is available. Recently a comparison of genomic sequences revealed in *S. cerevisiae* the presence of a sequence YGR111w presenting a very weak similarity to *S. pombe* Lyc1. This sequence is also present in *C. glabrata* and *K. lactis*. The gene encoding N6-acetyllysine aminotransferase has not yet been characterized. It is thus still unclear why some yeasts grow on lysine and others do not. The ability to degrade purines to provide nitrogen is widespread. Adenine deamination by adenine deaminase (*AAH1*) leads to the formation of hypoxanthine, which is then oxidized to uric acid via xanthine dehydrogenase (gene uncharacterized in any yeast). Guanine deaminase (*GUD1*) converts guanine to xanthine. Uric acid is degraded via allantoin and allantoate to urea. These different steps are catalyzed by urate oxydase (gene uncharacterized in any yeast), allantoinase (*DAL1*), and allantoicase (*DAL2*). Gene *DAL3* encodes ureidoglycolate hydrolase, which converts ureidoglycolate to glyoxylate, ammonia, and CO2.

Many yeasts can use cytosine and uracil as good nitrogen sources, but thymine is less frequently used (LaRue and Spencer 1968). However *S. cerevisiae* grows well on cytosine but not on uracil. Cytosine deaminase activity (*FCY1*) provides ammonia and uracil. When uracil is used as a nitrogen source, it is degraded in three steps to β-alanine and ammonia (as shown in Fig. 7.2).

Since all nitrogen sources are not used with equal efficiency, the quality of different nitrogen sources has been evaluated by growth tests (serial dilutions) of a wild-type *S. cerevisiae* strain (Σ1278b background) on different nitrogen compounds compared with the residual growth without nitrogen in the medium (Fig. 7.3). This residual growth could result from the storage of nitrogen compounds in the cell, but the presence of nitrogen traces in the agar cannot be excluded. The best nitrogen sources are glutamine, asparagine, and ammonia (doubling time about 2 h), followed by aspartate, glutamate, arginine, cytosine, allantoin, and phenylalanine. A significant reduction in growth rate is observed on urea, valine, alanine, serine, isoleucine, tyrosine, methionine, and GABA. On leucine, proline, threonine, or tryptophan the doubling time is at least 3 h. Adenine and glycine are very poor nitrogen sources, and lysine, putrescine, thymine, and uracil cannot serve as a nitrogen source. Cysteine and spermidine are even inhibitors of residual growth. Growth on histidine was not tested.

Although the genera *Saccharomyces* and *Schizosaccharomyces* are unable to use nitrate or nitrite as nitrogen sources, more than 150 species of different genera can grow on these compounds (Fig. 7.4). Curiously some yeasts are able to use nitrite but not nitrate. Recently, the availability of classical genetics and molecular biology tools for the yeast *Hansenula polymorpha* (renamed *Pichia angusta* = *Ogataea poly-*

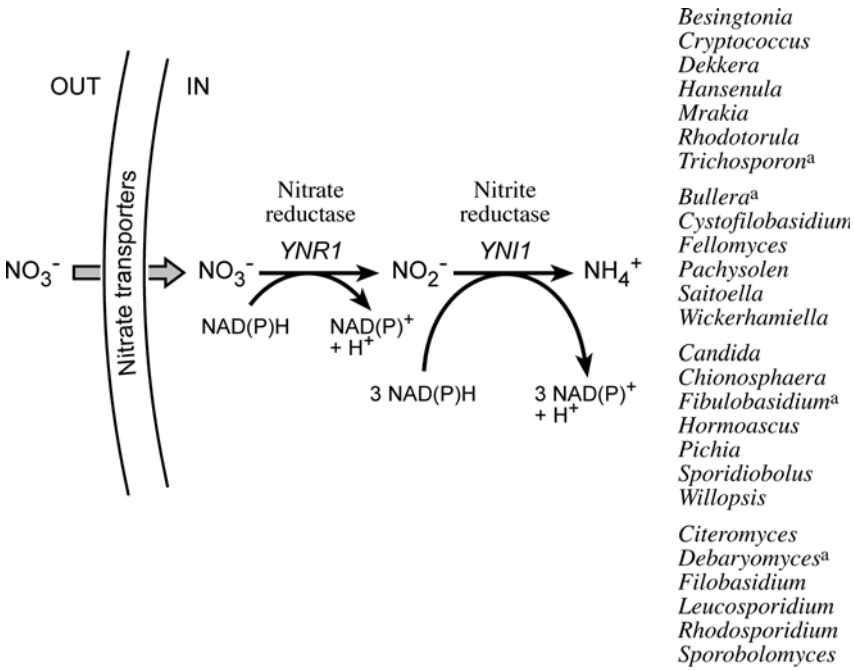


Fig. 7.4. Nitrate/nitrite utilization pathway and yeast utilizers. Some of the species of this genera utilize nitrite but not nitrate. The genes for each enzymatic step are in *italics*

morpha) has shed some light on this metabolic pathway in yeast (reviewed in Siverio 2002). Nitrate is transported in the cell by Ynt1, a high-affinity nitrate transporter, which is also able to transport nitrite. Ynt1 belongs to the major facilitator superfamily (MFS). In addition, nitrite uptake takes place by a nitrite-specific transporter (Machin et al. 2004). Nitrate assimilation in yeast follows the same pathway as that described for plants and filamentous fungi. Nitrate is converted to ammonia by two successive reductions catalyzed, respectively, by nitrate and nitrite reductase. *YNR1* gene is the only one that encodes nitrate reductase, and *YNI1* gene encodes nitrite reductase. These genes lie closely together in a cluster also containing two other genes encoding transcriptional regulatory factors. Regulation of this pathway will be addressed in the following section.

7.4 Regulation and Coordination of Nitrogen Catabolism

7.4.1 Control of Gene Expression in Response to the Quality of the Nitrogen Source

Selective nitrogen source utilization in *S. cerevisiae* is accomplished through a physiological process designated nitrogen catabolite repression (NCR) (Cooper 1982; Wiame et al. 1985). NCR consists in the specific inhibition of transcriptional

activation systems of genes encoding the permeases and catabolic enzymes needed to degrade poor nitrogen sources (e.g., allantoin, proline, GABA). When readily used nitrogen sources (e.g., Asn, Gln or, ammonia in some strains) are available, NCR-sensitive genes are expressed at low levels. Upon depletion of these repressive nitrogen sources, NCR is relieved and transcription of NCR-sensitive genes is increased. There are two different criteria that can be used to judge the quality of a particular nitrogen source. The growth rate that can be supported by a source of nitrogen would seem to be the simplest criterion for quality. As shown in Fig. 7.3 the differences in the growth rate are often small and it is therefore difficult to use the growth rate to make clear distinctions between the qualities of different nitrogen sources. A second criterion is based on the level to which systems for use of alternative nitrogen sources are derepressed during growth on a particular nitrogen source. The relation between the quality of the nitrogen source and the level of gene activation is illustrated in Fig. 7.5. On glutamine, the level of NCR-sensitive genes such as *MEP2* and *DAL5* is very low, compared with the level on proline, which is the nitrogen source leading to optimal derepression of these genes. Arginine and glutamate lead to a moderate increase of expression of NCR-sensitive genes, independently of the genetic background of the strain. Some observations (see later) provide evidence that intracellular concentration of glutamine would be the signal responsible for NCR establishment. However, although wild-type strains of S288c or Σ1278b background grow on ammonia as efficiently as on glutamine, there seems

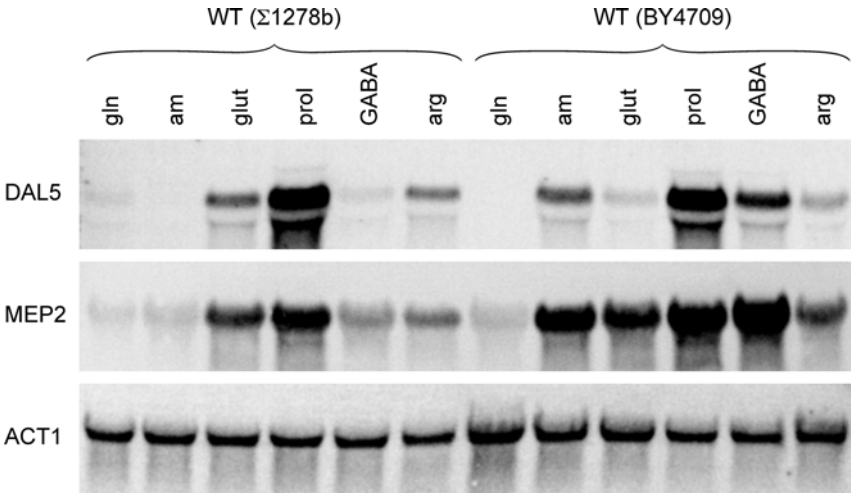


Fig. 7.5. Expression of nitrogen catabolite repression (*NCR*) sensitive genes of two wild-type *S. cerevisiae* strains on different nitrogen sources by Northern blot analysis. Total RNAs were extracted from strains ∑1278b and S288c and 30 μg was analysed by Northern blotting with *DAL5*, *MEP2*, and *ACT1* (encoding actine as a control) DNA probes. These strains were grown on the following nitrogen sources: glutamine (*gln*), ammonia (*am*), glutamate (*glut*), proline (*prol*), γ-aminobutyrate (*GABA*), and arginine (*arg*)

to be a difference in regulation between the two strains. In the Σ1278b background, ammonia exerts a strong repression on *MEP2* and *DAL5* genes, whereas in the S288c background, expression of these genes is partially derepressed on that medium (Fig. 7.5). In the Σ1278b genetic background, it was reported that ammonia could regulate nitrogen repression without being converted into glutamine (Dubois et al. 1977; ter Schure et al. 1998).

Modulation of NCR gene expression results from interplay between four GATA factors, each containing a GATA binding zinc-finger motif that has been conserved in organisms from yeast to metazoans. Gln3 and Nil1/Gat1 act as transcriptional activators, and for some NCR genes their action is counteracted by two negative GATA factors, Dal80/Uga43, and Nil2/Gzf3. The *NIL1, DAL80*, and *GZF3* genes are subject to nitrogen regulation, whereas *GLN3* is expressed constitutively regardless of the nitrogen source (Coffman et al. 1997; Soussi-Boudekou et al. 1997). There is thus a network of cross-regulating- and autoregulating GATA factors. A major role of this regulation might be to ensure a proper balance between the concentrations of the four GATA factors, this balance being tightly adapted according to nitrogen supply. It is noteworthy that NCR-sensitive genes are activated either by Gln3, or by Nil1, or by both factors, but the organization of the promoters does not provide a clue to predict by which factor(s) the gene will be regulated. Indeed all the genes responding to Gln3 and Nil1 activation contain a sequence named UASNTR, consisting of at least two GATAAG or GATTAG sequences. Some promoters require an auxiliary binding site, TTGT/GT, or a binding site for Abf1 or Rap1. The amplitude of response for each NCR-sensitive gene depends on the number and the position of these regulatory elements (reviewed in Magasanik and Kaiser 2002). The nitrogen-utilizing genes, encoding either transporters or degradative enzymes and responding to NCR regulation, are listed in Fig. 7.6 (shaded in gray). In addition to the four GATA family transcription factors, nitrogen catabolic gene expression is negatively regulated by Ure2. In a *ure2* mutant strain, repression no longer occurs (Drillien et al. 1973; Dubois and Grenson 1974; Grenson et al. 1974; Courchesne and Magasanik 1988).

It has been shown that the TOR kinases have an essential role in preventing the expression of nitrogen-regulated genes in cells using glutamine as a source of nitrogen (Beck and Hall 1999). Treatment of yeasts with rapamycin, an immunosuppressant drug, results in the activation of the expression of nitrogen-sensitive genes. Tor1/2 proteins inhibit expression of NCR genes by sequestering the GATA binding transcription factors Gln3 and Nil1 in the cytoplasm. In the presence of a good nitrogen source, Gln3 is phosphorylated in a TOR-dependent manner and is thereby tethered to the cytoplasmic Ure2 protein. Upon rapamycin treatment, Gln3 is dephosphorylated by the type-2A-related phosphatase Sit4, released from Ure2 inhibition, and translocated into the nucleus, where it activates target genes (Bertram et al. 2000). Recent data suggest that the TOR pathway senses glutamine (Crespo et al. 2002). This signaling mechanism had been extrapolated to the cell response to nitrogen starvation and to nitrogen availability. However, recently the Cooper group showed that on a poor nitrogen source Gln3 is localized in the nucleus despite its hyperphosphorylation (Cox et al. 2004). These data suggest that rapamycin treatment, a short-term response, and growth on poor nitrogen sources,

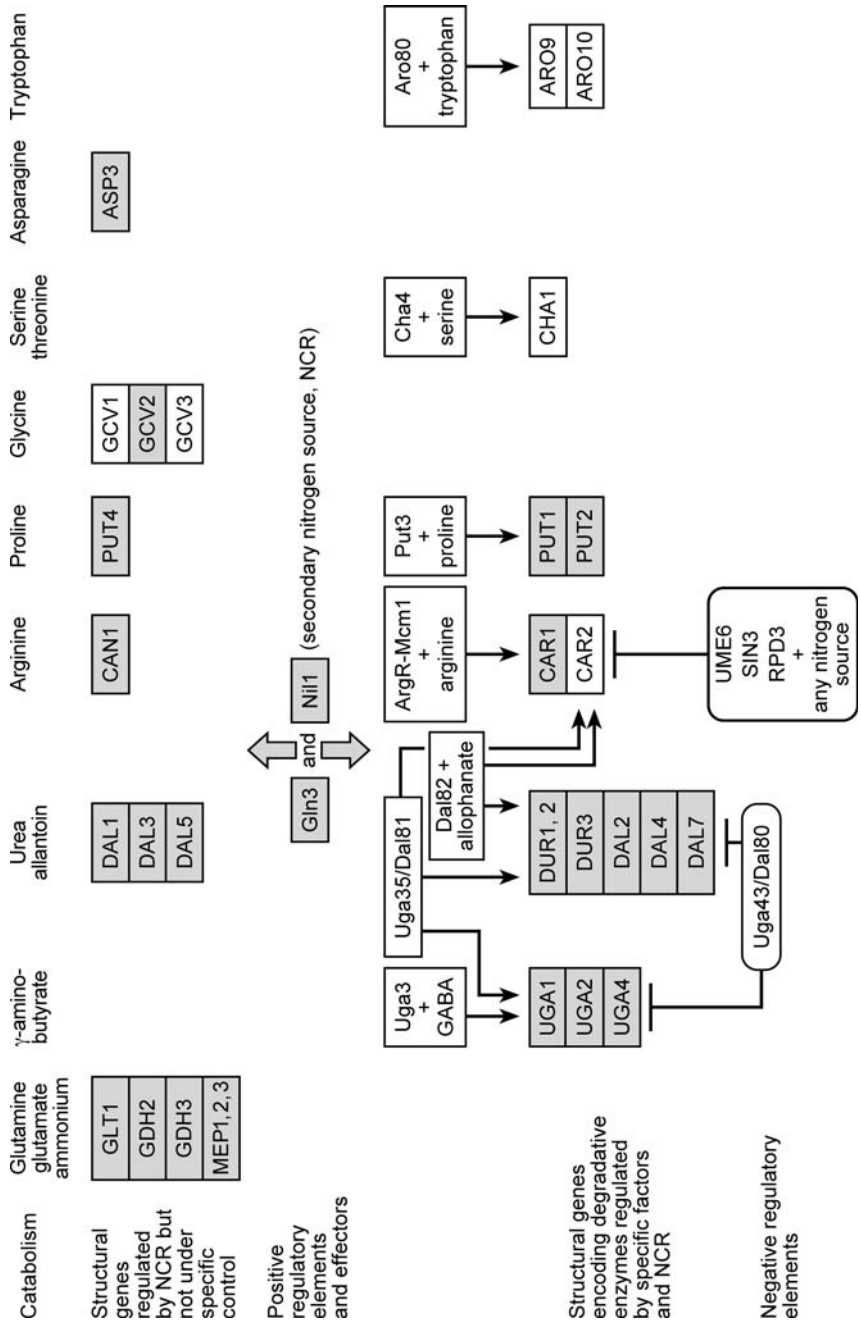


Fig. 7.6. Regulatory network controlling structural genes involved in the catabolism of various nitrogen sources. The products of the genes present in the figure are described in the text. The genes regulated by NCR are shaded in *gray*

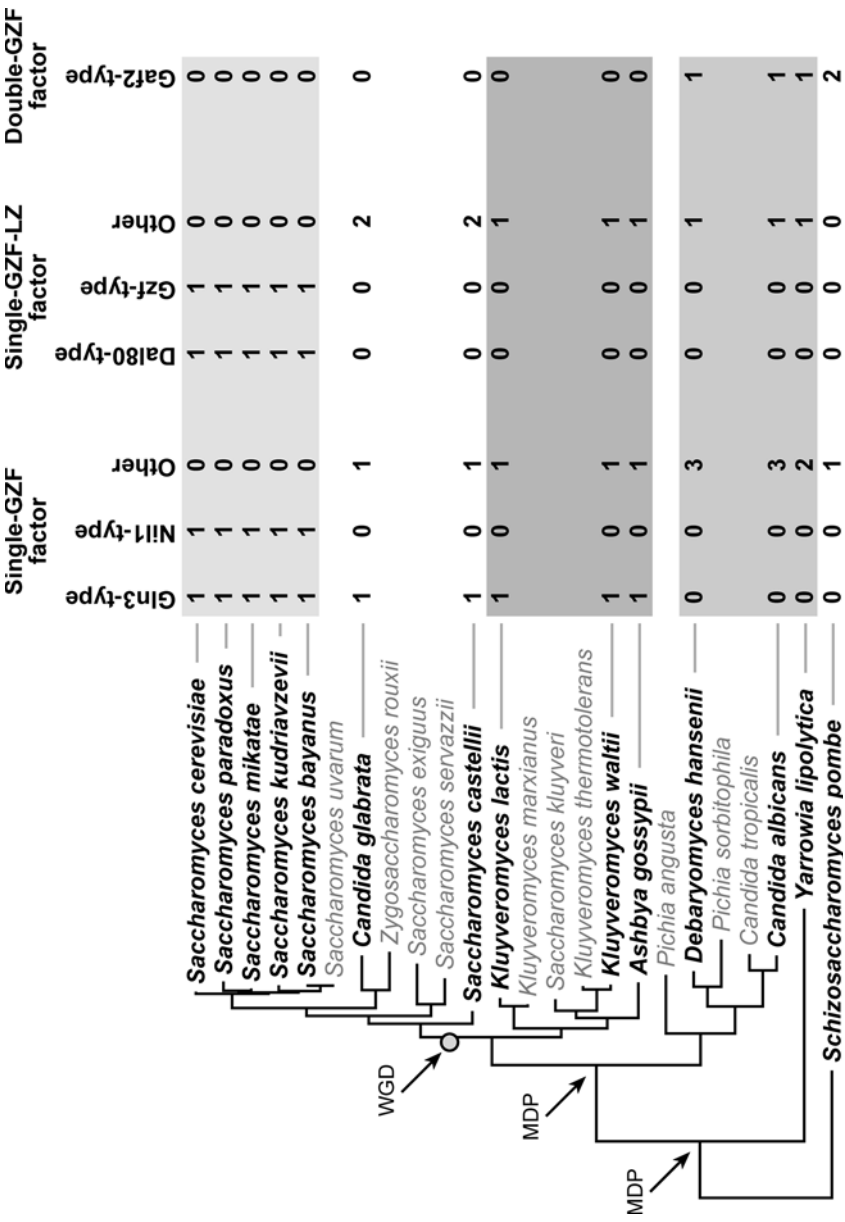
a long-term response, generate the same cellular response but likely do so by different mechanistic pathways (reviewed in Cooper 2002; Crespo and Hall 2002; Magasanik and Kaiser 2002; Rohde and Cardenas 2004).

Interestingly, Ure2 does not only inhibit Gln3 or Nil1, but could fulfill other cellular functions. Ure2 exhibits primary sequence and three-dimensional homologies to known glutathione S-transferases. Rai et al. (2003) showed that Ure2 is required for detoxification of glutathione S-transferase substrates and cellular oxydants. *ure2* mutants are hypersensitive to cadmium and nickel ions and hydrogen peroxide. *ure2* mutations possess the same phenotypes as mutations in known *S. cerevisiae* and *S. pombe* glutathione S-transferase genes, suggesting that Ure2 could serve as glutathione S-transferase in yeast. Whether this enzyme activity of Ure2 is involved in NCR regulation is still unknown. In addition the *ure2* mutation prevents the inactivation of glutamine synthetase when glutamine is added to wild-type cells grown with glutamate as a nitrogen source (Legrain et al. 1982; Coschigano and Magasanik 1991), suggesting a broader role for Ure2 in the control of nitrogen metabolism.

The presence of Ure2 orthologs in ascomycetous yeast species was searched for by comparative sequence analysis. A single Ure2 protein with typical structural features allowing clear distinction with classical glutathione S-transferase enzymes was found in all the yeast species analyzed and presented in Fig. 7.7, except in *S. pombe*. There is thus no clear coevolution between Ure2 and Gln3, i.e., yeast species like *D. hansenii* without any true Gln3 ortholog have a Ure2 protein. This is consistent with the ability of Ure2 proteins to control the subcellular location of GATA factors other than Gln3, e.g., Nil1 in *S. cerevisiae* (see later). Interestingly, *S. pombe* has one protein with a single GATA zinc finger (GZF) domain but no Ure2 ortholog.

GATA factors have been studied in other yeast species, e.g., *S. pombe* (Gaf2) and *C. albicans* (Gat1) as well as in *Ustilago maydis* (Urbs1). The Gaf2 and Urbs1 factors have two GZF domains, a property shared by several other proteins found in *D. hansenii*, *C. albicans*, and *Y. lipolytica* (Fig. 7.7). The *S. pombe* Gaf2 is transcribed constitutively, irrespective of the nitrogen source in the medium and up till now there is no evidence of a role of Gaf2 in the control of nitrogen-utilizing genes (Hoe et al. 1996). In contrast, Gat1 of *C. albicans* seems to be implicated in nitrogen regulation. The growth of mutants lacking Gat1 is reduced when isoleucine, tyrosine, or tryptophan are the sole sources of nitrogen, and *gat1* mutants are unable to activate expression of *GAP1, UGA4*, or *DAL5*, nitrogen-responsive genes in *C. albicans*. This regulatory defect does not prevent filamentation of *gat1* mutants in nitrogen repressing or nonrepressing conditions, demonstrating that NCR does not influence dimorphism. However, the mutants are highly attenuated in a murine model of disseminated candidiasis, suggesting an important role for nitrogen regulation in the virulence of *C. albicans* (Limjindaporn et al. 2003).

All yeast species analyzed shown in Fig. 7.7 contain three to five proteins of the GZF domain family. These proteins may be classified in three categories: (1) some factors (hereby named GZF) contain a single GZF domain, like the positive factors Gln3 and Nil1 of *S. cerevisiae*; (2) some (named GZF–GZF) contain a duplicated GZF domain, e.g., the *S. pombe* Gaf2 protein; (3) others (named GZF–LZ) contain a single GZF domain associated with a leucine zipperlike (LZ) domain likely


Single-GZF factor
Single-GZF-LZ factor
Double-GZF factor
Gln3-type
Nil1-type
Other
Dal80-type
Gzf-type
Other
Gaf2-type
Saccharomyces cerevisiae 1 1 0 1 1 0 0
Saccharomyces paradoxus 1 1 0 1 1 0 0
Saccharomyces mikatae 1 1 0 1 1 0 0
Saccharomyces kudriavzevii 1 1 0 1 1 0 0
Saccharomyces bayanus 1 1 0 1 1 0 0
Saccharomyces uvarum
Candida glabrata 1 0 1 0 0 2 0
Zygosaccharomyces rouxii
Saccharomyces exiguus
Saccharomyces servazzii
Saccharomyces castellii 1 0 1 0 0 2 0
Kluyveromyces lactis 1 0 1 0 0 1 0
Kluyveromyces marxianus
Saccharomyces kluyveri
Kluyveromyces thermotolerans
Kluyveromyces waltii 1 0 1 0 0 1 0
Ashbya gossypii 1 0 1 0 0 1 0
Pichia angusta
Debaryomyces hansenii 0 0 3 0 0 1 1
Pichia sorbitophila
Candida tropicalis
Candida albicans 0 0 3 0 0 1 1
Yarrowia lipolytica 0 0 2 0 0 1 1
Schizosaccharomyces pombe 0 0 1 0 0 0 2
WGD
MDP
MDP

involved in dimerization, e.g., the negative factors Dal80 and Gzf3 of *S. cerevisiae* (Fig. 7.7). Yeast species closely related to *S. cerevisiae* (*sensu stricto*) have a Gln3 and a Nil1 ortholog, with the Nil1 orthologs displaying much higher divergence than the Gln3 orthologs. More distant species (down to *A. gossypii* in Fig. 7.7) have a Gln3 ortholog plus a second GZF factor that may not be considered as a true Nil1 ortholog. Still more distant species have one to three GZF factors which do not correspond to true Gln3 or Nil1 orthologs (Fig. 7.7). These distant species also contain a single GZF–GZF protein. In contrast, the *S. pombe* proteome contains only one GZF and two GZF–GZF factors. This yeast species does not contain any member of the GZF–LZ family. All other yeast species indicated in Fig. 7.7 have at least one GZF–LZ factor. In species deriving from the one that has undergone whole genome duplication (WGD) (Wolfe and Shields 1997; Kellis et al. 2004), two genes encoding a GZF–LZ protein are present. In the *Saccharomyces sensu stricto* species, a clear divergence between Dal80-type and Gzf3-type GZF–LZ proteins is observable, with a much higher divergence among Gzf3 than Dal80 orthologs. These results suggest the following evolutionary scenario. Ancestral yeast species likely contained a single GZF factor gene. Duplication events probably led to the appearance of GZF paralogs and/or to one or several genes encoding GZF–GZF proteins. After the separation of lineages leading to *S. cerevisiae* and *S. pombe*, one of the GZF-encoding genes likely evolved into one coding for a GZF–LZ factor that has acquired a negative regulatory function. After separation of the lineages leading to *S. cerevisiae* and *C. albicans*, one of the GZF proteins evolved to the main regulator Gln3 likely involved in transcriptional activation of the other genes encoding GZF or GZF–LZ proteins (as is the case in *S. cerevisiae*). Finally, after WGD, the duplicated GZF–LZ factor genes were maintained, while the extra copies of each of the two GZF-encoding genes disappeared. The duplicated GZF–LZ factors finally evolved to generate the Dal80 and Gzf3 proteins known in *S. cerevisiae* and the *sensu stricto* species.

The *S. pombe* homologs of the TOR genes have been characterized. The *S. pombe TOR2* gene is essential for growth, whereas *TOR1* is required only under starvation, osmotic, and oxydative stresses. So far, no role for the Tor proteins in the control of nitrogen-gene expression has been reported (Weisman and Choder 2001). It was found from a comparison of yeast genomes that Tor1 and Tor2 proteins are present

Fig. 7.7. Phylogenetic distribution of GATA zinc-finger (*GZF*) domain proteins among diverse yeast species. The tree represents yeast species whose genome has been completely sequenced (indicated in *bold*) (Goffeau et al. 1996; Wood et al. 2002; Cliften et al. 2003; Kellis et al. 2003; Dietrich et al. 2004; Dujon et al. 2004; Kellis et al. 2004; Jones et al. 2004) plus several others whose genome sequencing is in progress by the Genolevure consortium (http://cbi.labri.fr/Genolevures/). The estimated position of the whole genome duplication (*WGD*) event is indicated, as well those of the main divergence points (*MDP*). The *columns* on the *right* represent the number of (1) proteins with a single GZF domain which are highly related to Gln3 or Nil1 and those which are not highly similar to Gln3 nor to Nil1 outside of their GZF domain, (2) proteins with a single GZF plus a leucine zipperlike (*LZ*) domain which are highly similar to Dal80 or Gzf3 and those which are not highly related to Dal80 nor to Gzf3 outside of their GZF and LZ domains, and (3) proteins with a double GZF domain similar to that of *S. pombe* Gaf2 protein

in *C. glabrata,* whereas in *K. lactis* and *D. hansenii* only the Tor2 ortholog was found, and in *Y. lipolytica* there is only one Tor protein with equivalent similarity to *S. cerevisiae* Tor1 and Tor2 proteins.

7.4.2 Control of Gene Expression by Specific Effectors: Arginine, Proline, Allantoin/Urea, γ-Aminobutyrate, Serine, Tryptophan, Amino acids, and Nitrate/Nitrite

In addition to general nitrogen control, some degradative pathways respond to specific inducers. Figure 7.6 summarizes the network of catabolic pathways regulated by NCR and by specific factors and effectors.

The metabolism of arginine presents the unique feature of possessing a regulatory mechanism coordinating its biosynthesis and its degradation. This coordination is achieved through the involvement of the same regulatory elements in the control of both pathways. In the presence of arginine, Arg80/ArgRI, Arg81/ArgRII, Mcm1, and Arg82/ArgRIII are required to repress the synthesis of five anabolic enzymes and to induce the synthesis of two catabolic enzymes. Arg81, a zinc cluster protein, is the sensor of arginine and interacts with the two MADS box proteins Arg80 and Mcm1 to form a complex (called ArgR–Mcm1) at the "arginine boxes" present in all the arginine coregulated genes. Arg82, an inositol polyphosphate kinase, acts as a chaperone for Arg80 and Mcm1 by stabilizing them in the nucleus. The inositol polyphosphate kinase activity of Arg82 is not required for arginine control (Messenguy et al. 1991; Amar et al. 2000; Dubois et al. 2000; El Bakkoury et al. 2000; Messenguy and Dubois 2000). Expression of *CAR1* and *CAR2* genes is not only regulated specifically by arginine and by the quality of the nitrogen source, but also in response to nitrogen availability by the histone deacetylase complex Ume6–Sin3–Rpd3. Ume6 is a DNA-binding protein belonging to the Zn2C6 family of transcription factors, interacting with DNA at a sequence named URS1 (Strich et al. 1994). Ume6 recruits the Rpd3–histone deacetylase complex by interacting with Sin3 (Kadosh and Struhl 1997). The role of the Ume6–Sin3–Rpd3 complex in the control of arginine catabolism is to block the expression of *CAR1* and *CAR2* promoters as long as exogenous nitrogen is available (Messenguy et al. 2000).

Vissers et al. (1982) investigated the repression of arginine biosynthesis (ornithine carbamoyltransferase, OTCase) and induction of the catabolism (arginase) in response to arginine, in the presence or absence of ammonia in the growth medium, in 28 yeast species. These species were obligate aerobes, such as *D. hansenii,* facultative anaerobes with a strong Pasteur effect, such as *K. lactis*, and facultative anaerobes with a weak Pasteur effect, such as *S. cerevisiae* and *S. pombe*. Most yeasts showed the classical repression of anabolic OTCase and induction of catabolic arginase, when arginine was added to the growth medium. In addition, they were subject to NCR, as judged by the increase in arginase synthesis when arginine was the only source of nitrogen compared with ammonia plus arginine as the nitrogen nutrient. No specific regulators involved in arginine regulation have been identified so far in these yeast species. Moreover, the promoter of the *S. pombe CAR1* gene does not contain the arginine boxes defined as the targets of the ArgR–Mcm1 proteins in the promoters of the arginine coregulated genes in *S. cerevisiae*. In addition,

the heterologous expression of *S. pombe CAR1* gene in *S. cerevisiae* is independent of the Arg81 gene product (van Huffel et al. 1994).

The expression of *PUT* genes involved in proline degradation is regulated by the Put3 activator protein, which responds to the presence of proline in the medium and increases the transcription of the *PUT1* and *PUT2* genes (Wang and Brandriss 1987). Put3 appears to respond to two signals, the presence of proline and the absence of preferred nitrogen sources, which are the conditions for maximal activation of *PUT1* and *PUT2*. Hyperphosphorylation of Put3 is correlated with growth on nonpreferred nitrogen sources (Huang and Brandriss 2000; Saxena et al. 2003). Put3 containing a six-cysteine, two-zinc domain (Zn2Cys6) constitutively binds to the upstream activation sequences in the promoters of both *PUT1* and *PUT2* genes in vitro and in vivo but activates transcription only in the presence of proline (Axelrod et al. 1991). The presence of the specific inducer causes a conformational change in the Put3 protein, allowing Put3 to shift from an inactive to an active state (des Etages et al. 1996, 2001).

Among the genes involved in allantoin and urea degradation, some, such as *DAL5*, are only NCR-sensitive, some, such as *DAL3*, are also down-regulated by Dal80, and others, such as *DAL7* and *DUR80*, are in addition regulated in response to the inducer, allophanate or its analog oxalurate (Yoo and Cooper 1991). Induction requires the dodecanucleotide sequence, UISall, and two transactive factors, Dal82/DurM and Dal81/DurL/Uga35. The precise function of Dal81 is still unknown, although this protein contains a zinc cluster motif, whereas Dal82, despite the absence of any known DNA-binding motif, binds UISall, present in the promoters of some *DAL* genes and also in the *CAR2* promoter (Dorrington and Cooper 1993; Park et al. 1999). Dal82 was recently shown to bind to UISall, and in vitro the 85 N-terminal amino acids of Dal82 are sufficient to bind UISall (Scott et al. 2000). In the absence of inducer, even on a poor nitrogen source, Dal80-mediated repression prevails and allantoin pathway genes are expressed at a low basal level (Rai et al. 1999). In the presence of an inducer, Gln3–Nil1-mediated transcription prevails and the expression of these genes occurs at a high level. This suggests that the role of the inducer would be to release repression by Dal80 at the GATAA sequences, allowing the binding of the two GATA activators, although this has never been demonstrated.

GABA induces transcription of the *UGA* genes required for its utilization as a nitrogen source. These genes contain in their promoters a conserved GC-rich sequence (UASgaba) essential to induction by GABA. To be effective, UASgaba requires two positive-acting proteins that both contain a zinc cluster motif, namely pathway-specific Uga3 and pleiotropic Dal81/Uga35 (Andre et al. 1995; Talibi et al. 1995). Alone the UASgaba element is sufficient to enable transcription of a reporter gene in the presence of GABA, even on a good nitrogen source; however, in the full-length promoters of *UGA* genes, the regulation is more complex, because another UASgata element, located just upstream from UASgaba, plays a determining role in producing a high level of induced expression. In the absence of an inducer, Dal80/Uga43 would prevent Gln3 or Nil1 from binding, whereas in the presence of the inducer Uga3 and Uga35/Dal81 factors acting through UASgaba promote some levels of transcriptional activation on their own, but could also facilitate binding of

Gln3 or Nil1 to the adjacent UASgata. This synergistic action leads to the optimal expression of *UGA* genes, only when GABA is the unique nitrogen source.

The transcription of *CHA1,* the gene encoding the catabolic L-serine (L-threonine) deaminase responsible for utilization of serine/threonine as nitrogen sources is activated by Cha4, a zinc cluster protein. Two serine–threonine response elements have been identified in the *CHA1* promoter, to which Cha4 is bound constitutively (Holmberg and Schjerling 1996). Regulation of *CHA1* basal expression involves assembly of a positioned nucleosome over the TATA element which requires the RSC complex (Moreira and Holmberg 1998, 1999) Upon activation, this nucleosome is removed, but so far no role for Cha4 in this chromatin remodeling has been reported.

Transcription of *ARO9* and *YDR380w/ARO10* is induced by aromatic amino acids by the transacting factor Aro80, a zinc cluster protein (Iraqui et al. 1999a). *aro80* cells are unable to grow on tryptophan medium, but their growth is only slowed down on a medium containing tyrosine or phenylalanine as a nitrogen source. A 36-base-pair element present in *ARO9* and *ARO10* 5′ regulatory regions is necessary and sufficient to mediate transcriptional activation of a reporter gene in response to aromatic amino acids. UASaro could thus be the DNA element to which Aro9 binds.

In response to external amino acids, *S. cerevisiae* induces the expression of amino acid permease genes, such as *AGP1*, *BAP2*, *BAP3*, *TAT1*, *TAT2*, and *GNP1*, and the dipeptide and tripeptide permease gene *PTR2* (reviewed in Boles and Andre 2004). The presence of external amino acids is transmitted to the transcription machinery through the Ssy1 amino acid sensor (de Boer et al. 1998; Didion et al. 1998; Jorgensen et al. 1998; Iraqui et al. 1999b; Klasson et al. 1999; Forsberg et al. 2001) Ssy1 is part of a multicomponent membrane-associated signaling complex (SPS), which includes at least two other proteins, Ptr3 and Ssy5 (Forsberg et al. 2001). Ssy1 contains 12 transmembrane spans (TM1–TM12), and its long N-terminal cytosolic tail likely plays a crucial role in signaling (Bernard and Andre 2001) together with Ptr3. The signal generated by Ssy1 and Ptr3 would be transmitted to Ssy5, which appears to interact with Ptr3. A next step in the signaling pathway is the endoproteolytic processing of transcription factors Stp1 and Stp2 (Andreasson and Ljungdahl 2002), requiring the SCFGrr1 ubiquitin–ligase complex. Recent data indicate that Ssy5 would be the endoprotease cleaving Stp1 (Abdel-Sater et al. 2004a) After processing, truncated Stp1 is translocated into the nucleus, and acts via an upstream sequence (UASaa) to activate gene expression. UASaa consists of at least two copies of the 5′-CGGC-3′ tetranucleotide separated by four to nine nucleotides (de Boer et al. 1998; Abdel-Sater et al. 2004b). Another transcription factor, Uga35/Dal80, is also important for induction of Ssy1-regulated genes.

Ssy1, Ptr3, and Ssy5 orthologs are present in all yeast species shown in Fig. 7.7, but *S. pombe* (Souciet et al. 2000; Cliften et al. 2003; Kellis et al. 2003). Hence, yeast species always have the three or none of the Ssy1–Ptr3–Ssy5 proteins, a result compatible with the three proteins being associated into a complex (SPS) (Forsberg et al. 2001). The Ssy5 orthologs share a well-conserved C-terminal protease domain and a rather divergent N-terminal domain possibly involved in the interaction with Ssy1 and Ptr3. The Ptr3 orthologs exhibits structural similarities with WD40 proteins and

they are also more closely related in their C-terminal regions. Comparison of the Ssy1 orthologs revealed that the large N-terminal tails and the extrapeptide regions between TM7 and TM8 have poorly similarity, while the extrapeptide regions between TM5 and TM6 are highly conserved. As shown in Fig. 7.8, the Ssy1 N-terminal end from *D. hansenii, C. albicans* and *Y. lipolytica,* present some degree of conservation despite their difference in length. The *C. albicans* Ssy1 end-terminal tail is composed of two regions, one present in *Y. lipolytica* Ssy1 (dark gray) and the other in *D. hansenii* Ssy1 (medium gray). Furthermore, all Ssy1 proteins contain an invariant triad (L/IFP) at their N-terminal end. Recently, the *SSY1* ortholog of *C. albicans* was functionally characterized (Brega et al. 2004). This protein (Cys1) is required for induction at the transcriptional level of several amino-acid permease genes as well as for filamentation in serum and amino acid based media. Interestingly, the range of amino acids to which Cys1 responds markedly differs from that of Ssy1.

The genes, *YNT1, YNR1*, and *YNI1*, encoding the enzymes for the nitrate-assimilation pathway are clustered in *H. polymorpha*. The genes encoding the transcriptional factors, *YNA1* and *YNA2*, two zinc cluster proteins, are located in the same cluster (Avila et al. 1998; Siverio 2002), but these genes are independently transcribed. Yna1 and Yna2 present similarity to the transcriptional factors nirA and nit4, involved in the nitrate induction in *Aspergillus nidulans* and *Neurospora crassa*, respectively. Deletion of *YNA1* or *YNA2* impairs growth on nitrate and nitrite, and leads to the absence of expression of *YNT1, YNR1*, and *YNI1*. A similar specific regulation occurs in *Hansenula* (*Pichia*) *anomala* (Garcia-Lugo et al. 2000). It has been shown that nitrate reductase has no role in the transcriptional induction of the nitrate-assimilatory genes in *H. polymorpha*, in contrast to data reported for filamentous fungi (Hawker et al. 1992; Siverio 2002). Expression of these genes is also subject to NCR, but only in *H. polymorpha*. Indeed there is a striking drop in nitrate-utilizing-enzyme activities when the cells are grown on glutamine plus nitrate compared with the levels on nitrate, and the fact that rapamycin abolishes NCR reveals the involvement of a TOR signaling pathway in the control of nitrate-assimilation genes (Navarro et al. 2003). It was reported that *NMR1* gene could be important in mediating the negative effect of the optimal nitrogen source on the

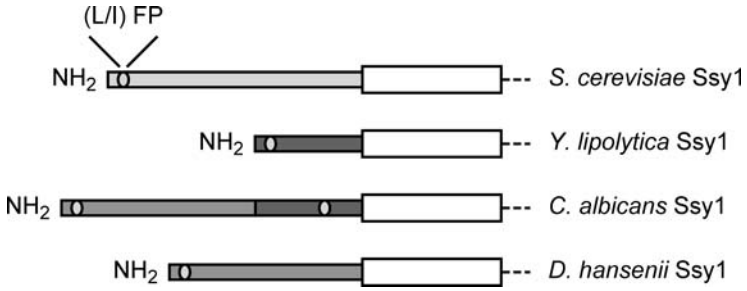


Fig. 7.8. Organization of Ssy1 transporters in different yeast species and comparison of their N-terminal tails

nitrate-assimilation pathway, since an *nmr1* mutant exhibits activation of these genes in the presence of nitrate plus ammonia (Serrani et al. 2001).

7.4.3 Control at the Level of Enzyme Activity and Enzyme Localization

S. cerevisiae has developed several regulatory mechanisms which exclude interference between biosynthesis and degradation of arginine. In addition to coordinated repression of biosynthesis and induction of catabolism by arginine and the ArgR–Mcm1 complex, this yeast has a peculiar mechanism to avoid the formation of a futile urea cycle when yeast is growing on arginine as the sole nitrogen source (Messenguy and Wiame 1969) Arginase, the first enzyme of the arginine degradative pathway, converts this amino acid into ornithine and urea. This enzyme is able to inhibit OTCase, the biosynthetic enzyme which converts ornithine and carbamoylphosphate to citrulline and phosphate. In the presence of ornithine and arginine, the respective substrates of the two enzymes, OTCase and arginase, both trimeric enzymes, form a one-to-one enzyme complex in which the activity of OTCase is inhibited, whereas arginase remains catalytically active. This regulation prevents the recycling by OTCase of ornithine produced by arginase, thus further excluding interference between anabolism and catabolism of arginine, since both enzymes are cytosolic in *S. cerevisiae*. This regulation was found to occur in vivo in eight out of 32 yeast species tested (Vissers et al. 1982). Obligate aerobes were devoid of this regulation (such as *D. hansenii*). Among fermenting species, *Schizosaccharomyces* and budding genera had different properties: all *Schizosaccharomyces* species were devoid of this regulation, whereas all species of budding yeasts tested showing a weak or absent Pasteur effect had this regulation (*Saccharomyces fermentati* = *Torulaspora delbrueckii*, *Saccharomyces kloekerianus* = *T. globosa*). Strains showing a strong Pasteur effect and taxonomically related to *Saccharomyces* (*Kluyveromyces*) had the regulation. It was also reported that *S. pombe* arginase is a hexamer instead of being a trimer, which can explain why *S. cerevisiae* OTCase is not inhibited by *S. pombe* arginase in vitro (El Alami et al. 2003). The absence of this epiarginasic regulation in some species can be linked to a mitochondrial localization of OTCase, whereas arginase is cytosolic, as for example in *D hansenii*, *H. anomala*, and *S. pombe* (Jauniaux et al. 1978). So yeasts can use different methods to control efficiently the flux of metabolites (in this case arginine and ornithine), and an efficient compartmentation of enzymes and metabolites can be sufficient to replace the sophisticated regulatory mechanisms operating in *S. cerevisiae*. Metabolites such as amino acids can be sequestered in the vacuole, so allowing them to function as a nitrogen reserve even under conditions when enzymes that are capable of catabolizing them are present at high levels in the cell. When growing on ammonia as a source of nitrogen, basic amino acids (arginine, lysine, histidine) are 90% vacuolar, threonine, serine, glycine, alanine, valine, tyrosine, and phenyalanine are 75% vacuolar, leucine and isoleucine are equally distributed between the vacuole and the cytoplasm, whereas glutamate and aspartate are mainly cytosolic (Messenguy et al. 1980). Since most of these amino acids can supply nitrogen, their release from the vacuole to the cytoplasm under conditions of starvation would then allow their nitrogen to be immediately available. However, when the cells are shifted

from a minimal medium containing ammonia to a minimal medium devoid of a nitrogen source, not all amino acids are released immediately from the vacuole (Messenguy et al. 2000). Interestingly, nitrogen deprivation leads to a rapid consumption of cytosolic glutamate without release from the vacuole. Glutamine is 80–90% vacuolar when the cells are grown on ammonia medium but is quickly released in the cytoplasm and immediately utilized as a nitrogen source upon nitrogen starvation. In contrast, the vacuolar arginine, whose pool concentration is much higher than that of glutamine, is released more slowly. After 40 min of starvation only 15% of the vacuolar arginine is consumed without accumulation in the cytosol. In contrast, when cells growing on arginine as the sole nitrogen source are shifted to a medium without nitrogen, the very high arginine vacuolar pool decreases rapidly because arginine is consumed very efficiently by the highly induced cytosolic arginase (Kitamoto et al. 1988). Thus, mobilization of vacuolar reserves may depend on environmental growth conditions.

7.4.4 Control at the Level of Protein Stability

In addition to control at a transcriptional level, many plasma membrane transporters are subject to tight control at the membrane trafficking level and modulation of intrinsic activity. Typical physiological conditions inducing these controls include changes of substrate concentration and availability of alternative nutrients. These changes of conditions often provoke the downregulation of specific transporters eventually accompanied by upregulation of others which are more appropriate to the new conditions. Downregulation involves the onset or acceleration of endocytosis of the transporters and subsequent targeting to the vacuole where it is degraded. The same physiological signal can also induce diversion of the neosynthesized transporter from the Golgi apparatus to the endosomal/vacuolar degradation pathway without passing through the plasma membrane (reviewed in Haguenauer-Tsapis and Andre 2004).

S. cerevisiae possesses about 20 different amino acid permeases, the substrate specificities of which overlap in many cases. These proteins are not simultaneously present in the cell, most are differentially regulated according to the nitrogen and/or amino acid content of the growth medium. In conditions of poor nitrogen supply the synthesized Gap1 permease accumulates at the plasma membrane in an active and stable form (Grenson 1983a; de Craene et al. 2001; Soetens et al. 2001). Upon the addition of ammonia, Gap1 is internalized by endocytosis and targeted to the vacuole for degradation (Nikko et al. 2003). Ubiquitination of Gap1 involving the ubiquitin ligase (Npi1/Rsp5) is essential to this ammonia-induced downregulation (Hein et al. 1995; Springael and Andre 1998). This downregulation may also be triggered by addition of amino acids (Stanbrough and Magasanik 1995). *NPR1* encoding a protein kinase is another gene involved in the posttranscriptional control of Gap1. In *npr1* mutants growing on a poor nitrogen source, the amount of *GAP1* transcript is unaltered but Gap1 is inactive (Grenson 1983a). Other ammonia-sensitive permeases, including the proline permease Put4 (Vandenbol et al. 1990), the ureidosuccinate, and allantoate permease Dal5 (Rai et al. 1988), and the inducible GABA permease Uga4 (Andre et al. 1993) are also dependent on Npr1 to be active.

The negative effect of an *npr1* mutation on Gap1 activity is suppressed by mutations inhibiting Gap1 downregulation such as *npi1*. This suggests a role of Npr1 in the control of Gap1 trafficking (Grenson 1983b). Recently it was shown that Npr1 phosphorylation is regulated by nitrogen through the TOR signaling pathway. Nitrogen starvation or growth on proline results in Npr1 dephosphorylation (Schmidt et al. 1998). During the downregulation of Gap1 following the addition of high concentration of amino acids, other broad-specificity amino acid permeases such as Agp1, Tat2, Tat1, and Bap2 are induced (Iraqui et al. 1999b). These permeases, therefore, take on the functions of Gap1. Unlike Gap1, these permeases are synthesized and active in cells grown on YPD medium. Shifting cells from YPD to nitrogen-starvation conditions results in the derepression of Gap1 and the rapid downregulation of Tat2 and Bap2. This downregulation process may also be induced by adding rapamycin to the medium. The requirements for ubiquitin, Rsp5, and lysines in the downregulation of Tat2 and Bap2 have been extensively studied (Beck et al. 1999; Omura et al. 2001; Umebayashi and Nakano 2003; reviewed in Boles and Andre 2004). The fate of newly synthesized transporters present in the secretory pathway may also depend on environmental conditions: the protein may be targeted to the cell surface, or directly diverted to the multivesicular bodies/vacuole for degradation without passing through the plasma membrane. For example, in cells grown on glutamate as the sole nitrogen source Gap1 is synthesized but is inactive because it is diverted from the secretory pathway to the vacuole without passing through the plasma membrane (Roberg et al. 1997a, b). Bul1 and Bul2 are redundant proteins required for the direct sorting of Gap1 to the vacuole and for its polyubiquitylation, suggesting that polyubiquitylation is required for direct sorting of the permease to the vacuole (Helliwell et al. 2001). The sorting of Tat2 is also regulated by cellular nitrogen status (Beck et al. 1999). Tryptophan availability in the medium plays an important role in controlling the fate of newly synthesized Tat2 (Umebayashi and Nakano 2003). In the presence of low concentrations of tryptophan Tat2 is targeted to the cell surface, whereas on media containing a high concentration of tryptophan Tat2 is sorted from the secretory pathway to the vacuole. Lipid rafts and ubiquitin play key roles in this regulated permease sorting.

7.5 Concluding Remarks

In the last decade biochemical and genetic studies allowed us to get deeper insight into nitrogen metabolic pathways, mainly in *S. cerevisiae*. Genes encoding enzymes catabolyzing aromatic and branched-chain amino acids have been characterized and new regulators involved in their control have been identified. In contrast, in other yeast species little new information has been provided about nitrogen degradative pathways, with the exception of nitrate/nitrite utilization and its regulation in *H. polymorpha*. It seems that most efforts were aimed at elucidating the complex and multiple regulations controlling nitrogen utilization in *S. cerevisiae*, and even for this yeast we still have a long way to go. Now that the genome sequences of many yeasts are known, it is rather easy to identify orthologs of genes encoding nitrogen-utilizing enzymes and their regulatory factors. However, biochemical and genetic studies are still necessary to test their functionality. This is especially true for the regulatory

factors, in which the best conserved sequences are the DNA-binding motifs, suggesting a putative regulatory function but not necessary involved in the control of the same pathway. Moreover, this comparative genomic analysis will not lead to an understanding of why yeasts use a particular nitrogenous compound or not, unless the genes and gene products involved in this degradation are characterized at least in one species, and this is far from being the case for many pathways. Hopefully, the novel approach to "evolutionary genomics" proposed by the Génolevures consortium will provide a rationale and better criteria to further unravel the full diversity of nitrogen utilization among yeast species.

Acknowledgements

We are very grateful to Stephan Vissers and Antonio Urrestarazu for reading the manuscript and for helpful suggestions. We would like to thank Jean-Pierre ten Have for the figures.

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Chapter 8

Environmental Factors Influencing Yeasts

Tibor Deak

Department of Microbiology and Biotechnology, Corvinus University Budapest, Faculty of Food Sciences, Somloi ut 14/16, Budapest 1118, Hungary
(e-mail: tibor.deak@uni-corvinus.hu)

8.1 Introduction

Yeasts are ubiquitous microorganisms that form part of the microbiota of most if not all natural ecosystems. A wide variety of yeast species occur in soil, fresh water and marine water, they are normal inhabitants on plants, are commonly associated with animals, and are also found frequently in man-made habitats such as foods. The conditions prevailing in these natural and artificial habitats determine the metabolic activity, growth and survival of yeasts. A variety of abiotic and biotic factors influence the life of yeasts, and exert stress conditions which the cells must withstand and adapt to or otherwise they die. Death of individual cells may not bring about the extinction of a whole population as there is a large variation in resistance to stress factors among cells. Also, these factors change with time and show spatial heterogeneity; their effect is manifested differently in microenvironments. So, when looking at the environmental relations of yeasts, we should deal with a very complex interrelation of factors that are only partially understood. Nevertheless, basic knowledge of these is important for understanding the ecology and biodiversity of yeasts as well as to control the environmental factors in order to enhance the exploitation of yeasts or to inhibit or stop their harmful and deleterious activity.

In this chapter our current knowledge of the environmental relations of yeasts is summarized. First, the most relevant physical, chemical and biotic factors and their interactions are described in a changing environment. Further on, responses of yeast cells to stress conditions are discussed at single cell and population level. In-depth treatment of the genetic background of variation, and the mechanism of adaptation to stress factors would be beyond the limits of this chapter. Further chapters in this book are devoted to their discussion. Reference is made to relevant literature and to the large amount of data covered and summarized in previous works and reviews (Phaff and Starmer 1987; Rose 1987; Watson 1987; Boddy and Wimpenny 1992; Fleet 1992; Deak and Beuchat 1996; Deak 2004).

8.2 Physical Factors

The most important physical factor influencing the life of yeasts is temperature. Other factors exerting less definite, and also less studied effects are light, radiation and pressure. In a wider sense, geography, locality and climate can be considered as ecological factors.

8.2.1 Temperature

The temperature limits and range for growth of yeasts vary with species. Most yeasts are mesophilic, and grow best at temperatures between 20 and 30°C. In a study covering nearly 600 strains of more than 100 species including genera of *Saccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Candida* and others (Vidal-Leira et al. 1979), the upper limit for growth of 98% of yeasts fell between 24 and 48°C, for a few it was below 24°C but for none was it above 50°C (Table 8.1). Yeasts such as *Leucosporidium scottii*, *Mrakia frigida* and a few others can be considered psychrophilic, having a minimum growth temperature as low as –1 to 4°C and a maximum at about 20°C. At 37°C only a limited number of species can grow, mostly those associated with warm-blooded animals, at least temporarily, such as *Candida albicans* and a number of other opportunistic pathogenic yeasts. Most strains of *Saccharomyces cerevisiae* occurring widely in industrial fermentation can grow at 37°C, whereas growth of *S. bayanus* in a similar environment is limited up to 30–35°C. As to the lower limit of growth temperature, it may extend a few degrees below 0°C for psychrophiles provided the water suspension remains fluid, such as the salty seawater in arctic regions. A number of yeasts can be considered psychrotrophic, possessing a range of growth temperatures between 0 and 25°C, whereas some mesophilic species may also grow at 5–10°C as the lowest limit. Even those yeasts isolated from the water and marine animals of the Arctic Ocean, as well as from frozen foods, grow faster at higher temperatures, and develop colonies in 1 week at 20°C but in 2 weeks at 5°C. Some yeasts are characterized with a rather wide

Table 8.1 Maximum growth temperatures of some yeast species

Species	T_{max} (°C)
Kluyveromyces marxianus	44–47
Candida glabrata	43–46
Candida albicans	42–46
Pichia guilliermondii	38–43
Pichia anomala	35–37
Yarrowia lipolytica	33–39
Metchnikowia pulcherrima	31–39
Candida zeylanoides	32–34
Candida vini	27–31
Leucosporidium scottii	22–24

Adapted from Vidal-Leira et al. (1979)

range of growth temperature, e.g., for *Pichia membranifaciens* it extends from 5 to 45°C, whereas others have much narrower limits, e.g., 28–42°C for *Candida sloofii*. Few yeasts only can grow above 40°C and none can be considered termophilic. *Kluyveromyces marxianus* is rather thermoduric; many strains of this species can grow up to 45–47°C; a few strains of exceptional thermotolerance were found to be able to grow and ferment at 52°C (Banat and Marchant 1995). Temperatures above 50°C are lethal for yeast cells and not even the sexual spores show much higher heat resistance.

The minimum, optimum and maximum limits of the growth temperatures are not absolute degrees as they are influenced by the physiological state of the cells as well as other environmental conditions. Inhibitory factors such as low water activity, low pH and the presence of antimicrobial substances, e.g., ethanol, will limit the range of the growth temperature. In turn, temperatures below or above the optimum would decrease the resistance of yeasts to other stress factors.

Temperature would not only determine whether or not a yeast strain is able to grow but it also influences the rate of growth (or its reciprocal, the generation time) once it started. Within the range of the growth temperature, changing of the growth rate can be described by the equation developed by Arrhenius according to which the growth rate increases linearly with temperature in the optimum range, approaching the minimum it decreases steadily, whereas moving from the maximum it decreases abruptly.

8.2.2 Light and Solar Radiation

Yeasts are not photosynthetic organisms; hence, illumination is not vital for their existence. Few observations refer to any effect of light on them, but they point to the possible killing effect of UV wavelengths of sunlight. This may explain the relative abundance of pigmented species (e.g., *Cryptococcus, Rhodotorula*) on surfaces of plant leaves. A systematic study on a phylloplane microbial community suggested that the position of a leaf within an apple tree canopy substantially affects the resident population (Andrews et al. 1980). Differences in the density of phylloplane microbiota could be attributed to a variation in the exposure to light and rainfall and also to the accessibility of leaves to airborne microbes (see Chap. 13).

8.2.3 Pressure

Under natural land conditions, atmospheric pressure does not affect the life of yeasts. When they occur in deep sea, the cells should withstand high hydrostatic pressure. Yeasts are frequently found in shallow marine environments but only recently have isolates been obtained from sediments and benthic microorganisms from deep sea of 2,000–6,500 m. Red yeasts (*Rhodotorula* and *Sporobolomyces*) were commonest among the isolates, some of them represented new species, such as *Kluyveromyces nonfermentans* (Nagahama et al. 1999, 2001). Unlike bacteria, however, no data refer to the physiology of yeasts occurring in the deep sea. Our knowledge of baroresistance of yeast cells comes from studies on the possible application of high hydrostatic pressure in food preservation (Smelt 1998). High

pressure exerts a strong effect on cell structures, and the viability of yeasts decreases with increasing pressures above 100 MPa; between 200 and 300 MPa cells are killed (Palhano et al. 2004). Unexpectedly, when cells were exposed to mild stress (hydrogen peroxide, ethanol or cold shock), it induced higher resistance to pressure. This hints to the function of a general mechanism of stress response in yeast cells, similar to that protecting them against other stress factors (see Sect. 8.5).

8.2.4 Geography, Climate

By and large, geography, locality and climate act as ecological determinants on the biodiversity and communities of yeasts. These large-scale phenomena manifest themselves through changes of temperature, rainfall, wind, solar radiation, drought, soil composition, vegetation, insect vectors and the like. Several studies have shown the impact of these factors on the ecology of yeasts at the microhabitat level (Andrews et al. 1980; Barker et al. 1987; Starmer et al. 1992; Chand-Goyal and Spotts 1996; Sláviková and Vadkertiová 1997).

8.3 Chemical Factors

Among these factors are included the availability of nutrients and water, the acidity and pH, the oxygen relations, as well as the effect of inhibitory and antimicrobial substances. Mutual interactions between these factors as well as the physical environment bring about very complex relations. Their manifestation is hardly known in natural habitats and has been mostly studied under laboratory conditions.

8.3.1 Nutrients

Yeasts require for maintenance and growth some sources of carbon, nitrogen, mineral salts and certain vitamins and growth factors. The different ability of yeasts to utilize various nutrients is one of the most important ecological factors to determine habitat specificity.

The most important carbon and energy sources are carbohydrates, mostly sugars such as hexoses and oligosaccharides. Glucose and a number of simple sugars can be utilized by many yeasts both fermentatively and oxidatively. A wide range of other carbon sources (e.g., alcohols, organic acids) can be metabolized only by aerobic respiration. A faulty generalization arising from the vigorously fermentative group of *Saccharomyces* species is that all yeasts are capable of fermenting. In reality, about half of the currently known yeast species lack the ability to ferment and carry out aerobic oxidation exclusively. Not even the fermentative species can permanently survive under anaerobic conditions because some constituents of membrane lipids can be synthesized only aerobically. Both the aerobic and the anaerobic utilization of various carbon sources has been traditionally used for the physiological characterization and identification of yeasts; the list of these compounds extends to over 40 (Yarrow 1998; Barnett et al. 2000).

Polysaccharides and other macromolecules such as proteins and lipids are not commonly used nutrients of yeasts. Few species produces a substantial amount of

hydrolytic enzymes extracellularly. The number of amylolytic yeasts is small and none are known to hydrolyze intact cellulose. It seems, however, that protease, peptidase or lipase activities are widespread in yeasts. In studies on tropical yeast communities, 40–60% of isolates was found to possess proteolytic enzymes and around 30% produced pectinases (Abranches et al. 1997; Trindade et al. 2002). This empowers the yeasts to expand their ecological niche and make better use of the available carbon sources.

All yeasts can utilize inorganic nitrogen sources such as ammonium salts, many of them also nitrates. Amino acids, urea and other organic nitrogen sources can be utilized by various species. Some yeasts can grow without any external vitamin sources, others may require biotin, thiamin, nicotinic acid or some other growth factors. Inorganic phosphate salts satisfy the need of all yeasts; sulfate, sulfite and thiosulfate can serve as a sulfur source. A number of other inorganic elements, e.g., potassium, magnesium, iron and zinc, are required in low concentration. These requirements are generally met in most natural habitats of yeasts. Under laboratory conditions care should taken to satisfy specific needs of certain yeasts, but most of them can be cultivated on relatively simple growth media. If an essential micronutrient is lacking some members of the natural community may be lost in laboratory culture.

8.3.2 Water

Nutrients are taken into yeast cells in the form of water solutions and water itself is an essential requirement for life. Water should be fluid and free (not chemically bound) in order to be absorbed. The availability of water is usually expressed in terms of water activity (a_w); the more exact physicochemical term water potential (ψ) is used less frequently in microbial ecology (Marechal et al. 1995).

Yeasts living in marine habitats and large bodies of fresh water must cope with the highly diluted nutrients, and are rarely exposed to the osmotic pressure exerted by concentrated solutes that more frequently occur in habitats of solid matrices, such as soil or foodstuffs. Most yeasts can develop well at water activities around 0.95–0.90 (Table 8.2). A particular group of yeasts, however, is notable of being able to grow at much lower a_w values. In the earlier literature, an a_w value of 0.61 was given as the minimum value for growth. This was not confirmed in recent reinvestigations that resulted in values around 0.70 (Jermini and Schmidt-Lorenz 1987). This group of yeasts is often called osmophilic and halophilic depending on whether the solutes are sugars or salts, respectively, bringing about low water activities, corresponding to 55–65% sugar or 15–25% salt concentrations. A more general and appropriate term is xerotolerant for most strains of the species in question can grow in dilute solutions and do not require increased solute concentrations (Silva-Graca et al. 2003). *Zygosaccharomyces rouxii*, *Z. mellis, Z. bisporus*, *Debaryomyces hansenii*, *Candida versatilis*, *C. lactiscondensi* and *C. halonitratophila* are the species most notable for being xerotolerant; several other yeasts, among them certain strains of *S. cerevisiae* as well as *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Pichia anomala* and others possess less tolerance to a_w lower than 0.80.

Xerotolerant yeasts are of special importance to the food industry because they are able to cause spoilage of foods preserved by added sugar or salt. It was found

Table 8.2 Minimum a_w for growth of yeasts in media adjusted by different solutes

Yeast species	Minimum a_w for growth controlled by			
	Glucose	Fructose	Sucrose	NaCl
Candida lactiscondensi	0.79	0.78	0.70	0.92
Candida versatilis	0.79	0.80	0.79	0.84
Debaryomyces hansenii	0.84	0.86	0.81	0.84
Hanseniaspora uvarum	0.90	0.93	0.90	0.95
Pichia membranifaciens	0.90	0.92	0.90	0.94
Rhodotorula mucilaginosa	0.90	0.92	0.90	0.90
Saccharomyces cerevisiae	0.89	0.91	0.91	0.92
Torulaspora delbrueckii	0.86	0.89	0.87	0.90
Zygosaccharomyces bisporus	0.85	0.85	0.79	0.95
Zygosaccharomyces rouxii	0.79	0.82	0.79	0.86

Data from Tokouka and Ishitani (1991)

that various processing factors such as temperature, pH and the composition of the food interact with water activity with regard to the inhibitory effect of growth (Tokouka 1993); it can be supposed that these factors interact whenever low water activity is encountered in the environment.

The physiological background of resistance to low a_w stress conditions has also been the subject of a wide range of studies. Production of compatible solutes (glycerol, mannitol), active pumping out of sodium ions or their exchange for K^+, induction and differential expression of stress-responsive genes have been supposed as protective mechanisms; however, different species show contrasting reactions (Ramos 1999; Hohman 2002). Extreme osmotic stress can exceed the osmoregulatory capacity of cells and cause loss of viability (Table 8.3). Beyond industrial concern, understanding the phenomenon of xerotolerance is of relevance for the biodiversity of yeasts in natural habitats such as plant nectars and saps or salt ponds, where ecological factors are exerted in high solute concentrations.

Table 8.3 Viability of *Saccharomyces cerevisiae* in relation to osmotic stress

Water activity (a_w)	Water potential (MPa)	Cell viability (% related to control)	
		Glycerol	Poly(ethylene glycol) 600
0.90	–14.5	92	59
0.80	–30.8	65	58
0.70	–49.2	55	40
0.60	–70.5	20	28
0.50	–95.7	10	0

Data from Marechal et al. (1995)

8.3.3 Oxygen Relations

Yeasts are basically aerobic organisms although they are most noted for the vigorous fermentation of sugars – a remarkable characteristics of *Saccharomyces* species and a number of other genera but this certainly does not hold good for all yeasts as pointed out in Sect. 8.3.1. Most basidiomycetous yeasts, *Cryptococcus*, *Rhodotorula* and others, are strictly aerobic and not able to ferment. Even the fermentative yeasts are facultatively anaerobic and under aerobic conditions they switch to respiration under the well-known metabolic regulation, the Pasteur effect. This regulation is more complex, however, because in addition to oxygen the concentration of glucose is also an effector in that at high glucose concentrations yeasts start alcoholic fermentation even under aerobic conditions (Crabtree effect) (Gancedo 1998) (see also Chap. 6).

In most natural habitats normal atmospheric conditions prevail, with high oxygen and low carbon dioxide concentrations. In an aquatic environment the dissolved oxygen in water may be a factor influencing metabolism and growth. The degree of saturation depends on the climate, in particular temperature, and other factors, e.g., the stirring of water.

Carbon dioxide is a metabolic product of various microorganisms, including alcoholic fermentation of yeasts. Under natural conditions, CO_2 accumulates rarely in inhibitory concentrations. Yeasts living in the intestinal tract of insects may be subjected to high CO_2 concentrations. More often, being easily soluble in water but depending on the pH, carbon dioxide forms bicarbonate ions, which inhibit growth of yeasts (Curran and Montville 1989; Dixon and Kell 1989).

8.3.4 Acidity and pH

In general, yeasts prefer a slightly acidic medium and have an optimum pH between 4.5 and 5.5 (Table 8.4). However, they tolerate a wide range of pH and grow readily at pH values between 3 and 10. Moreover, yeasts show a remarkable tolerance to pH, and several species can grow at strong acidic pH values as low as 1.5. The actual pH range of growth for a given species depends upon the kind of acid dissociating in the medium. Acetic acid is generally more inhibitory than lactic, propionic, citric and other organic as well as inorganic acids. As in the case of each environmental factor, the impact of pH on growth is influenced by other factors. For instance, at

Table 8.4 Effect of pH and a_w on the specific growth rates of *Zygosaccharomyces rouxii*

pH	Specific growth rate (μh^{-1}) at a_w values				
	0.957	0.923	0.904	0.880	0.843
2.5	0.21	0.15	0.12	0.08	0.05
3.5	0.33	0.23	0.18	0.14	0.08
4.5	0.34	0.24	0.19	0.14	0.09
5.5	0.30	0.21	0.17	0.12	0.07

a_w values adjusted with 300, 500, 600, 700 and 800 g l^{-1} final sugar concentrations obtained by mixing 30% glucose and 70% fructose; temperature 25°C. (Adapted from Membré et al. 1999)

low temperatures the minimum pH permitting growth is higher. In this respect, the interaction of yeasts with other organisms in microbial communities should be considered. Yeasts and lactic acid bacteria occur together in many natural habitats because they possess many common ecological requirements. The concentration of lactic acid produced by bacteria and the corresponding low pH could be inhibitory for fermentative yeasts; on the other hand, yeasts pursuing aerobic metabolism can utilize lactic acid oxidatively. A particular group of yeasts, genus *Dekkera* (anamorph *Brettanomyces*) is noted for a peculiar metabolic regulation that increases alcoholic fermentation under aerobic conditions and also results in the production of a high amount of acetic acid. In narrow habitats (e.g., in laboratory culture) the concentration of acetate may accumulate up to a level that kills the cells of the producing yeast.

8.3.5 Antimicrobial Compounds

In addition to acetate, lactate and others, some weak organic acids exert specific inhibitory effects towards yeasts, such as benzoic and sorbic acid. These are widely used preservatives in the food industry, but are rarely encountered in natural habitats. Plant and animal tissues, however, contain a variety of compounds that may inhibit yeast growth. Spices and herbs are particularly rich in phenolic and aromatic compounds, essential oils, volatile fatty acids, oleoresins and other constituents that have antifungal activity (Kim et al. 2004). Antibiotics are well known, and some of them are strong fungicides; these and other metabolites of microorganisms, among them yeast products, are discussed in the next paragraph.

The main product of alcoholic fermentation of yeasts, ethanol, exerts a toxic effect on various organisms (not to mention humans), among them yeasts and the producing strain itself. The ethanol tolerance of yeasts has been the subject of extensive studies especially concerning the production of wine (Fleet and Heard 1993). Natural residents on grapes such as *Hanseniaspora* (*Kloeckera*) and *Candida* species that start the spontaneous fermentation of grape juice are relatively sensitive to ethanol, and die out soon at concentrations around 5–8%. Most strains of the true wine yeast, *S. cerevisiae,* can tolerate 13–15% ethanol, some strains up to 18% or somewhat higher. As in the case with many inhibitors, tolerance to ethanol is affected by other environmental factors, in particular temperature and pH (Fleet 2003), and according to recent results some strains of *Candida stellata* and *Hanseniaspora* spp. may show resistance to ethanol comparable to that of *S. cerevisiae* (Table 8.5).

8.3.6 Interactions Between Environmental Factors

Under natural conditions the effect of no environmental factor manifests itself alone isolated from other factors. Many of these come into force together and simultaneously, mutually influencing the effect of the others; moreover, their interaction is dynamic and changes in time and space. The outcome of interaction is hardly predictable when several factors come into play. For practical applications, the food industry is very interested in the combination of physical and chemical treatments

Table 8.5 Minimum inhibitory concentration of ethanol on yeast growth

Species	Ethanol % (v/v)
Candida utilis	6.1–6.5
Kluyveromyces marxianus	8.6–9.4
Pichia anomala	10.0–10.9
Schizosaccharomyces pombe	11.8–12.5
Hanseniaspora valbyensis	11.9–13.2
Saccharomyces cerevisiae	11.3–13.7

Glucose-peptone broth, pH 5.6, 30°C. (Adapted from Antoce et al. 1997)

in order to better retain the safety, quality and stability of minimally processed foods (Tapia de Daza et al. 1996). Extensive experiments have been carried out and complex statistical methods as well as predictive mathematical models have been developed for their evaluation (Kalathenos et al. 1995; Table 8.6). The interaction between temperature, water activity, pH, salt, sugar and preservatives has been studied in various combinations with different types of food. Reference is made only to some examples relating to the growth inhibition of spoilage yeasts (Praphailong and Fleet 1997; Charoenchai et al. 1998; Betts et al. 2000; Battey et al. 2002). Although these studies have greatly increased our understanding of the interaction between environmental factors on the spoilage yeasts from the point of view of how to inhibit their activity, they bear little on what reactions and interactions really occur in natural habitats (Fleet 1998). Nevertheless, the concerted action of the very same

Table 8.6 Combinations of ethanol, fructose, pH and a_w on the doubling time of *Saccharomyces cerevisiae*

Ethanol (% v/v)	Fructose (% w/v)	pH	a_w	Doubling time (h)
0	2.5	3.5	0.991	1.69
4	2.5	5.5	0.985	2.19
8	2.5	5.5	0.973	2.34
12	2.0	3.2	0.962	8.49
0	4.0	2.5	0.996	2.49
0	16.0	2.5	0.984	2.68
0	32.0	2.5	0.969	2.93
0	50.0	2.5	0.952	6.73
0	50.0	5.5	0.952	5.72
0	50.0	8.0	0.952	5.71
3	8.0	4.0	0.983	1.88
3	8.0	7.0	0.983	2.64
3	40.0	4.0	0.953	4.29
3	40.0	7.0	0.953	6.60

Selected values from a multifactorial response surface experiment conducted at 25°C in Bacto yeast nitrogen base broth adjusted to various treatment combinations. (Adapted from Kalathenos et al. 1995)

and similar factors plays a prominent role in determining the biodiversity and life of yeast communities in natural habitats as well, but they have been studied and explored less intensively until now. For example, it has been shown that the composition of yeast communities associated with sugarcane is under the influence of temperature, pH and the concentration of sugars as they change with various parts of the plant and the time passed since plantation (de Azeredo et al. 1998). Specific yeast communities have also been described for a great variety of plant microhabitats (fruits, flowers, leaves, tree saps, decaying tissues, etc; Spencer et al. 1992; Rosa et al. 1995; Santos et al. 1996), and these studies revealed habitat specialization being influenced by a variety of the respective biotic and abiotic factors. From these studies it also emerged that a biotic factor, the insect vectors, is of particular significance in shaping yeast communities. This will be discussed in more detail in the next section.

8.4 Biotic Factors

In natural and artificial ecosystems yeasts are always subjected to interactions with other organisms. These effects can be mutual or unidirectional, neutral, synergistic or antagonistic, and involve interactions of yeasts with themselves, bacteria, filamentous fungi and higher organisms (Lachance and Starmer 1998).

8.4.1 Yeasts and Yeasts

Abundant literature deals with the products of yeasts lethal to other yeasts, called killer toxins. These polypeptides are genetically determined on plasmids or chromosomes, and about a dozen types of them have been described (Magliani et al. 1997; Marquina et al. 2002; Schmitt and Breinig 2002). Growth inhibitory or lethal action of killer toxins impacts mainly on yeasts; earlier claims to extend it to bacteria and eukaryotes other than yeasts have been verified with certain plant pathogenic and wood decaying fungi (Walker et al. 1995). A killer positive property is widespread among yeasts. Strains of the producing species are resistant, while other species can be sensitive or neutral to the toxin. In natural communities 9–27% of species were shown to produce toxin, in some cases, e.g., in fermenting grape juice, toxigenic strains reached 50–75%, whereas the ratio of sensitive yeasts varied between 10–40% (Starmer et al. 1992; Vagnoli et al. 1993). Indigenous species are less sensitive than members of different communities; within the same habitat 3–10% of killer sensitive species occur but 20–40% among yeasts from different localities and habitats (Abranches et al. 1997; Trindade et al. 2002). The ecological role of killer yeasts in natural communities can be attributed to the competition with sensitive species leading to their exclusion from sources of nutrients (Starmer et al. 1987) (see also Chap. 10).

Killer yeasts often play a role in the competitive interaction between yeast species associated with fruits. Initially, ripening fruits are always colonized by apiculate yeasts belonging to the genera *Hanseniaspora* and *Kloeckera*. These fermentative species have a narrow assimilative profile and are replaced, after a few days, by yeasts utilizing a broader range of substrates and/or fermenting more strongly (Abranches at al. 2001). Killer strains may also facilitate the dominance of wine

yeasts during the spontaneous fermentation of grape juice. In other fermentation this may not be the case (Lachance 1995), and the succession of yeast species in the course of fermentation is governed by the competition for nutrients and the tolerance to ethanol. The successional development in grape juice of *Hanseniaspora* (*Kloeckera*), *Metschnikowia*, *Pichia* and *Candida* species and finally the true wine yeast *S. cerevisiae* has been thoroughly demonstrated and generally attributed to the degree of ethanol tolerance of the respective yeasts (Fleet and Heard 1993). In commercial wine production, however, the spontaneous course of events is disturbed by human intervention. This takes place by the treatment of grape juice with sulfur dioxide and the inoculation of selected wine yeast starter in order to control fermentation (Fugelsang 1997).

Predation among yeasts has been considered as a unique and rare phenomenon, but recent findings show that it may be a widespread property of filamentous species of *Saccharomycopsis* and related yeasts (Lachance and Pang 1997). Yeast and moulds may serve for prey and they are attacked by haustoria-like outgrowths that penetrate and kill other cells. The ecological impact of predacious yeasts remains to be assessed. As a peculiar property, predation has also been demonstrated in several yeast species. These belong mostly to *Saccharomycopsis* and related hyphal genera (Lachance and Pang 1997). Predatory species were found to be sulfur auxotrophs, and it is believed that the ecological significance of predation lies in obtaining nutrients.

8.4.2 Yeasts and Molds

Yeasts may rely on nutrients produced by molds, e.g., take up simple sugars liberated by the polysaccharide-splitting enzymes of molds. Recently, strict dependence of a yeast, *Debaryomyces mycophilus*, was shown on the iron-containing siderophore, a metabolic product of some common soil fungi such as *Cladosporium cladosporoides*, *Aspergillus alliaceus*, and *Penicillium* spp. (Than et al. 2002).

Some yeast species, in particular *Pichia guilliermondii* and *P. anomala*, inhibit the growth of certain moulds attacking fruits during postharvest storage (Wisniewski and Wilson 1992; Suzzi et al. 1995). Conversely, a large number of mycelial fungi can attack yeasts. Parasitism is commoner among the basidiomycetes than in other fungal groups; nearly 50% of the basidiomycete fungi tested positive (Hutchinson and Barron 1996). Mycoparasitic fungi utilize yeasts as a nutrient source either by lysing yeast cells or by penetrating the cell wall, similar to the way they attack plants and nematodes.

8.4.3 Yeasts and Bacteria

Reference has been made (Sect. 8.3.4) to the association of yeasts with lactic acid bacteria in a number of spontaneous and controlled fermentations. Further examples for mutualistic and synergistic interactions are given here. In kefyr grains their interaction is synergistic; the vitamins provided by yeasts and the lactate produced by bacteria are mutually utilized (Leroi and Courcoux 1996). A similar association develops in sour dough between the maltose fermenting lactobacilli and glucose

fermenting yeasts (Gobetti et al. 1994). In the fermentation of sauerkrauts and pickles, both fermentative and oxidative yeasts live together with lactic acid bacteria; the yeasts often form films on the surface of salt brine where the aerobic decomposition of lactic acid may open a way to spoilage (Buckenhüskes 1997). In red wine, the malolactic fermentation by *Oenococcus* (*Leuconostoc*) *oenos* is facilitated by vitamins and amino acids produced by yeasts (Alexandre et al. 2004). In oriental fermentation of rice, soy, vegetables and even fishes, mixed communities of molds, yeasts, lactic acid and other bacteria and bacilli participate with manifold interactions among them (Nout 2003). In the ripening of sausages, cheeses and other dairy products, yeasts develop interactive associations with bacteria and molds alike (Viljoen 2001).

Lactic acid bacteria are known for the production of various bacteriocins, the direct effect of which on yeasts is not verified (Magnusson et al. 2003). Hydrogen peroxide, often liberated by catalase-negative lactic acid bacteria, may have a lethal effect on yeasts. In turn, disregarding the inhibitory effect of ethanol, no specific compounds are produced by yeasts that are antagonistic to bacteria.

Special and strong metabolic interactions, mutualistic relations and competition take place in biofilms developing on solid surfaces where mixed communities of cells get in close spatial contact. Biofilms are formed mostly by bacteria, with yeasts often contributing to them. Yeasts producing extracellular slime and capsules play an important role in the adhesion to sites. A complex structure of biofilms occurs on the mucosal membranes covering the gastrointestinal tract and other body cavities of macroscopic organisms and they have become the subject of intensive studies from the medical point of view (Costerson and Lewandowski 1997; Watnick and Kolter 2000; El-Azizi et al. 2004). On soil particles, living and inanimate underwater objects and other natural habitat biofilms are common life forms of microorganisms. The formation of biofilms facilitates the colonization of habitats and offers protection to stress reaction caused by the changing physical and chemical conditions of the environment.

8.4.4 Yeasts, Plants and Animals

Yeasts are generally saprotrophic organisms, but a few species are parasitic on plants or pathogenic to animals. Typical examples are, respectively, *Nematospora coryli* and *Metschnikowia bicuspidata*. For humans, *Cryptococcus neoformans* and *Candida albicans* are the most threatening pathogenic yeasts, although in recent years the number of opportunistic pathogen species has greatly increased (*C. glabrata*, *C. tropicalis*, *C. krusei* and others). Adaptive evolution of tolerance to antifungal agents may be responsible for the emergence of these yeasts as epidemiological agents (Ahearn 1998). Pathogenicity of yeasts to warm-blooded animals is beyond the scope of the present chapter. Other manifold relations between yeasts and invertebrates will not be discussed either; this topic is covered extensively by Phaff and Starmer (1987) and Ganter in this Volume (see Chap. 14). However, one aspect of this interrelation is of prominent significance from an ecological point of view, i.e., the role of insect vectors in transmitting and distributing yeasts in different habitats.

Insects serve not only for vectors but also feed on yeasts. In both ways they contribute actively in structuring yeast communities (Morais et al. 1995; Lachance et al.

2001). Association between yeasts, bees and flies, in particular *Drosophila* species, may be highly specialized and results in coadaptation of the partners (Starmer and Fogelman 1986; Rosa et al. 2003). Extensive studies carried out by Phaff's group on yeasts associated with cacti also revealed the intimate relations with drosophilas and other insects visiting the plant (Phaff and Starmer 1987; Starmer et al. 1991). Similar close relations have been detected between yeasts, flowers and insects that can be considered as natural ecosystems (Lachance et al. 2001, 2003).

8.5 Effect of Environmental Factors on Populations

Much of our knowledge of the effect of environmental factors on yeasts comes from observations obtained with populations. In natural conditions communities are formed from mixed populations of species, whereas the laboratory pure cultures represent in the best-case clones of a single species. Investigations provide average responses of all cells in a population but no information on individual cells or subpopulations. It has become possible only recently, with the development of the technique of fluorescent flow cytometry, to sort individual cells and get information on the heterogeneity of the population (Attfield et al. 2001). Long before, it was recognized that heterogeneity is a natural attribute of a cell population. For instance, studies on lethal stress factors often resulted in survival curves with a shoulder and/or a tail, revealing the different resistance among cells in a culture. S-shaped survival curves are obtained frequently after heat treatment or irradiation. Heterogeneity may be due to mutation and other genetic changes, and this forms the basis of natural selection, adaptation and evolution (Zeyl 2000). However, heterogeneity also occurs in isogenic populations and manifests itself in differential sensitivity to stress conditions which can be fundamental to the fitness and persistence of an organism in its habitat (Booth 2002). The reasons for phenotypic heterogeneity are manifold. Cell-to-cell variations come from differences in cell cycle stages, growth phases and growth rate, the age of cells and other properties that show a stochastic distribution (Sumner and Avery 2002). Yeast cells have a definite life span which lasts until the cessation of cell division. The age of a cell is marked by the number of bud scars (in the case of *S. cerevisiae* the average is about 20). Aging is genetically determined, and with aging changes take place in physiological processes, metabolic regulation and stress responses (Sinclair et al. 1998; Jazwinski 1999). The practical significance of heterogeneity among cells is the ability of a fraction of the population to survive exposure to stress factors that kill the majority of the cells. When the survivors outgrow into a new population, it will have the same degree of variation as the original culture.

In addition to phenotypic heterogeneity, yeast populations are capable of developing an adaptive response to stress factors. Starvation is one of the most common stresses encountered in natural habitats. Starving yeast cells enter into a stationary phase and maintain viability until they resume growth again (Werner-Wasburne et al. 1993). Sublethal stress imposed by heat, osmotic shock, toxic chemicals or other inhibitors confers yeasts with resistance to withstand subsequent higher doses of stress. In recent years several studies have focused on the elucidation of the mechanism of adaptation. Induction of adaptive responses include the synthesis of

heat-shock proteins, activation of plasma membrane H^+-ATPase and the accumulation of trehalose conferring cells to maintain intracellular pH homeostasis and protecting membrane integrity and functional proteins (Mager and Ferreira 1993; Ribeiro et al. 1999; Piper et al. 2001; Cabral et al. 2004). In-depth studies have revealed a common pattern of stress sensor system and signal transduction pathways which activate the genetic transcription of many genes in response to environmental stress (Bauer and Pretorius 2000; Rossignol et al. 2003; Garay-Arroyo et al. 2004; Zuzuarregui and del Olmo 2004). It is beyond the scope of this chapter to go into more detail, and we conclude that physiological adaptation of yeast cells is crucial to maintain viability and is essential for the cells to survive stressful environmental transitions (see also Chap. 9 and Chap. 15).

Despite the armory of protective responses to stress factors, yeast cells suffer sublethal injury as a result of exposure to adverse conditions. Plasma membrane, enzyme proteins, DNA and gene transcription may be damaged rendering cells to lose the ability to grow and retain viability. Injured cells may be able to repair damage and resume growth under appropriate conditions (Fleet and Mian 1998). The viable but nonculturable state of cells has been the subject of numerous studies. It may draw a false picture in the assessment of pathogens after clinical treatment or food preservation, and also of the real composition of microbial communities occurring in a natural habitat. If selective media are used without resuscitation, injured cells would not be able to form colonies; hence, plate counts less then real are obtained (Beuchat 1984).

8.6 Concluding Remarks

Major advances have been made in understanding the ecology of yeasts in various habitats and ecosystems. However, the majority of yeast ecological studies focused on the identification of species, often determining the most frequent isolates only. Moreover, much of our knowledge is derived from laboratory studies on isolated strains about how the various growth factors regulate growth and survival. Despite enormous progress, much has to be learned on the responses to environmental stresses and interactions of individual cells, their population and mixed communities of different species occurring in most natural habitats.

According to an excellent analysis by Fleet (1998), to understand the growth and activities of yeasts in their habitats further information and data are required on:

- The physiological properties of species that permit their growth and activity in the ecosystem
- Quantitative data on the limits for growth of environmental factors regarding populations and communities
- The specific interactions between different strains and species of yeasts and between other microorganisms occupying a common habitat
- The dynamics and changing of activity, growth and survival of species and populations in time and space in response to the changing environment

Rarely does a single species occur in any habitat, rather populations of different species, yeasts and other microorganisms are assembled in communities; therefore,

the activity and growth of any one strain or species is influenced by the presence of other microorganisms. Little is known about how cells sense and communicate with each other and the mechanism regulating population growth.

In colonizing the habitat yeasts grow in spatial heterogeneity and form microcolonies and biofilms, also with other microorganisms. In the complex structure of the biofilm spatially organized mixed populations develop where metabolic interactions and interspecies competition and cooperation manifest themselves. Almost nothing is known about yeast biofilms, apart from the special cases of dental plaques and human body cavities (El-Azizi et al. 2004).

Species communities in an ecosystem are not static in time. The sequential development of strains and species lead to a continuously changing association both qualitatively and quantitatively in response to the changing environment. Moreover, the cells are simultaneously exposed to a combination of stress factors, and their effect may be additive or synergistic. The changing microenvironment and interactions between biotic and abiotic factors will determine the actual niche of an organism in the habitat.

Recent advances in the techniques of molecular biology will allow the assessment of the composition of species in mixed populations and their localization in space and the change with time. Culture-independent methods (e.g., the direct epifluorescent filter technique, DEFT, flow cytometry, fluorescent in situ hybridization, FISH) as well as refined PCR-based methods (denaturing gradient gel electrophoresis, DGGE, thermal gradient gel electrophoresis, TGGE, amplified fragment length polymorphism, AFLP) have recently been applied to study bacterial biodiversity in natural ecosystems (Schloter et al. 2000; Giraffa and Neviani 2001; Giraffa 2004), but their use for investigating yeasts has just started (Cocolin et al. 2000; Brul et al. 2002; Zuzuarregui and del Olmo 2004).

In microbial populations a large genetic variation is always present, which is the prerequisite for biological evolution. New molecular analytical approaches will allow us to understand the underlying genetic mechanisms and the impact of the microenvironment on the diversification of genes and their expression (Gibson 2002; Rodriguez-Valera 2004). The increasing number of completely sequenced yeast species ranks these organisms in the frontiers of research on environmental genomics (Zeyl 2000, 2004; Kellis et al. 2003; Querol et al. 2003). The interplay between genetic microdiversity and the influences of the microenvironment guarantees the creation, existence and maintenance of rich biodiversity in populations and the development of new evolutionary lines.

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Yeast Responses to Stresses

An Tanghe[1], Bernard Prior[2] and Johan M. Thevelein[1,3]

[1]*Laboratorium voor Moleculaire Celbiologie, Institute of Botany and Microbiology, Department of Biology, Katholieke Universiteit Leuven, 3001 Leuven–Heverlee, Flanders, Belgium (e-mail: an.tanghe@bio.kuleuven.ac.be; e-mail: johan.thevelein@bio. kuleuven. ac.be, Tel.: +32-16-321507, Fax: +32-16-321979)*
[2]*Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa (e-mail: bap@sun.ac.za)*
[3]*Department of Molecular Microbiology, Vlaams Interuniversitair, Instituut voor Biotechnologie, Kasteelpark Arenberg 31, 3001 Leuven–Heverlee, Flanders, Belgium*

9.1 Introduction

In their natural habitat, yeasts are continuously exposed to a myriad of changes in environmental conditions. These changes may occur suddenly or may take place over an extended period of time and they can constitute a single specific change or a combination of changes. It is clear that extreme changes, e.g. in physical and chemical conditions, will always represent a stress to the cells and will require specific response mechanisms in order to protect and adapt the cells to the new condition. The same is true for starvation and any dramatic change in the nutrient supply. Such conditions are generally referred to as stress conditions and the adaptation mechanisms they evoke as stress responses (Hohmann and Mager 2003). On the other hand, in the case of moderate changes in the growth conditions the amount of stress experienced by the cells is not always clear. The difference between response to stress and adaptation to a new growth condition is not clear-cut and probably in reality also represents a gradual transition. Yeast cells must always be on guard to protect themselves in order to continue to survive, grow and multiply. For example, yeasts found on the surface of sugar-containing plant material can expect to be exposed to high sugar concentrations under dry conditions that might change rapidly with rainfall. Similarly on the same plant material, fermentation of the sugar can result in an elevated ethanol concentration and a reduced pH that also impose stress on the yeast. Once the nutrients are exhausted, a prolonged nutrient starvation period usually follows. In order to survive and multiply under dramatically variable conditions, microorganisms like yeasts have developed a complex set of sensing and signalling mechanisms that enable them to rapidly adapt their physiology to the new conditions.

9.2 Types of Stress

A large number of stress-inducing conditions have been described and may be either physical or chemical or biological in nature. They include changes in temperature, osmotic pressure, pH and concentration of water, ions and solutes, as well as exposure to extremes of radiation, pressure and toxic chemicals, to oxidative conditions and to nutrient starvation. Stress is also imposed on pathogenic yeasts by the host defence mechanisms. For example, the high levels of reactive oxygen species produced by neutrophil cells called an oxidative burst are an important host defence mechanism used to eliminate pathogenic yeasts (Moye-Rowley 2003).

Some stress effects are implicated in more than one type of stress. For example, NaCl elicits both an ionic and an osmotic stress. The yeast responds physiologically in a similar way to an ionic and a nonionic solute such as sugar by initially shrinking, excluding the extracellular osmotica followed by intracellular accumulation of compatible solutes to restore cell volume and turgor. However, with NaCl additional stress is placed on the yeast by the intracellular accumulation of Na^+ that the cell must export in order to prevent general cellular damage (Wadskog and Adler 2003).

In nature yeasts might be exposed to various types of stress simultaneously or sequentially. *Saccharomyces cerevisiae* typically grows under fermentative conditions where high sugar and ethanol concentrations occur and the yeast needs to tolerate stress imposed by osmotic pressure, water stress and ethanol toxicity. During a wine fermentation the yeast is initially exposed to a sugar concentration often greater than 250 g/l that results in an osmotic stress response. As the sugar is fermented to ethanol, the osmotic stress is reduced but simultaneously ethanol increases, imposing water and ethanol stress on the yeast. In order to cope with the differing stresses, the yeast expresses a sequence of different stress response genes (Zuzuarregui and del Olmo 2004). Respirative yeasts, on the other hand, are exposed to reactive oxygen species as by-products of their cellular metabolism that impose an oxidative stress which can occur in combination with other stresses such as nutrient starvation in the stationary growth phase.

Yeasts that commonly proliferate in extreme environments would be expected to adapt more easily to environmental stress when compared with less tolerant yeasts. These yeasts have been studied poorly or not at all at the molecular level and while there are response mechanisms that appear to be conserved in all yeasts investigated to date, it appears that some response mechanisms might be specific to a yeast species and related to the particular habitat where the yeast is found. Investigation of the genomic makeup of the highly osmotolerant yeast *Zygosaccharomyces rouxii* has revealed that many of the genes involved in salt stress response in the less osmotolerant *S. cerevisiae* are also present in this yeast (Iwaki et al. 1998, 1999). However, the regulation of these genes might be different: in *Z. rouxii* induction of glycerol 3-phosphate dehydrogenase does not seem to respond to osmotic stress as observed in *S. cerevisiae* (van Zyl et al. 1991; Albertyn et al. 1994b; Iwaki et al. 2001). *Z. rouxii* also possesses a NaCl-induced glycerol uptake system that has not yet been reported to be present in *S. cerevisiae* (van Zyl et al. 1990).

In nature many types of stress would be expected to be imposed over an extended period, whereas in the laboratory because of limitations of available time and facili-

ties, most stress experiments are conducted over a much shorter period. *S. cerevisiae* has been found to adapt differently to short-term and long-term stress. For example, when hyperosmotic conditions are imposed on *S. cerevisiae,* growth ceases, the yeast rapidly loses water and plasmolysis occurs. This is followed by a recovery phase where compatible solutes are produced and accumulated until osmotic homeostasis is achieved to enable cell proliferation to recommence. The time required to recover is dependent upon the degree of osmotic stress imposed. The greater the stress imposed, the longer the time that the yeast requires to recover (Albertyn et al. 1994a).

9.3 General and Specific Stress Responses

An overview of the best studied examples of stress responses in *S. cerevisiae* is shown in Fig. 9.1.

9.3.1 General Stress Response

It has been discovered that yeast cells respond to a variety of stress conditions with a similar response at the transcriptional level and that this response is mediated

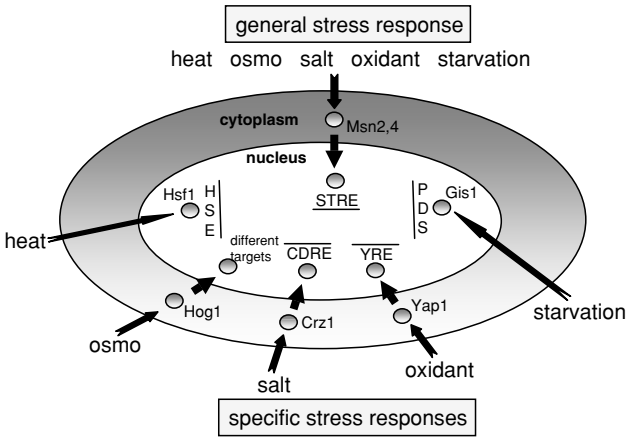

Fig. 9.1. Best-established stress response mechanisms in *Saccharomyces cerevisiae*. The general stress response is triggered by a variety of stressful conditions. It involves transfer of the Msn2 and Msn4 transcription factors into the nucleus, where they induce transcription by binding to the stress-response elements (*STRE*) in the promoters of target genes. In the specific stress responses heat-shock factor Hsf1 activates heat-shock-induced transcription through the heat-shock elements (*HSE*), whereas in the osmostress response the Hog1 protein migrates to the nucleus, where it interacts with several transcription factors to activate transcription of osmostress-induced genes. Salt stress specifically triggers entry of the Crz1 transcription factor into the nucleus, where it interacts with the calcineurin-dependent-response element (*CDRE*) in target gene promoters. Oxidative stress specifically causes accumulation of the Yap1 transcription factor in the nucleus, where it induces transcription of antioxidant genes through interaction with Yap1-response elements (*YRE*). Nutrient starvation activates the Gis1 transcription factor, which induces stationary-phase genes through interaction with the postdiauxic shift element (*PDS*)

by the widely distributed stress-response element (STRE) promoter (Marchler et al. 1993; Ruis and Schuller 1995). Recent work using genome-wide gene expression analysis has shown that about 9–14% of all genes in *S. cerevisiae* and *Schizosaccharomyces pombe* are involved in this general stress response (Gasch et al. 2000; Chen et al. 2003). Major targets of the general stress response in *S. cerevisiae* are the transcription factors Msn2 and Msn4, which bind to the STREs in the promoters of about 200 target genes, and are required for induction of a large number of stress-induced genes (Martinez-Pastor et al. 1996). Other genes depend on other transcription factors and many genes are also repressed as part of the general stress response. Genes induced are involved in carbohydrate metabolism, metabolite transport, fatty acid metabolism, maintenance of the cellular redox potential, detoxification of reactive oxygen species, autophagy, protein folding and degradation, cell wall modification, DNA-damage repair, secretion, vacuolar and mitochondrial functions, intracellular signalling, and a relatively large number of genes with unknown function. Genes repressed are mainly involved in protein synthesis (e.g. ribosomal protein genes) and other growth-related functions (Gasch et al. 2000). The transcriptional response to stress conditions is often transient with the largest change occurring rapidly after the stress (Rep et al. 1999). This allows rapid adaptation of the cells to the new environmental conditions. Initially it was thought that the general stress response might be triggered by a common effect of the different stress treatments by which it is activated, such as protein denaturation, change in intracellular pH, change in cyclic AMP level, etc. However, it appears that at least part of the integration of the response to different types of stress conditions might also occur at the level of the targets themselves, e.g. through different specific STREs occurring in the promoter of the same target gene. A typical consequence of the general stress response is the occurrence of cross-protection: submission of the cells to one stress condition renders them not only more resistant to the same stress condition but also to other stress conditions (Lewis et al. 1995).

Another pathway found in *S. cerevisiae* that is activated by different stress conditions, such as heat shock, low osmolarity, cell wall perturbing agents, but also internal signals generated in morphogenetic processes, is the cell integrity pathway. It appears to adapt the cell wall assembly to external stress conditions as well as to growth and developmental processes that require cell wall remodelling (Gustin et al. 1998). The Wsc1 and Mid2 plasma membrane proteins are considered to be sensors for cell wall strength. They control activity of protein kinase C, Pkc1, which in turn controls the expression of cell wall biosynthesis genes through an MAP kinase cascade (Levin et al. 1994; Heinisch et al. 1999).

In *S. pombe* the Sty1 MAPK pathway responds to a variety of stress conditions and also plays a role in cell cycle control and developmental switches (Degols et al. 1996). Although Sty1 is the homologue of Hog1 in *S. cerevisiae*, the latter appears to be more specifically involved in the response to osmostress (see further).

9.3.2 Response to Heat Stress

Apart from the general stress response (Wieser et al. 1991) heat stress is known to induce a specific response which is commonly known as the heat shock response and apparently occurs in all organisms (Lindquist 1986). In eukaryotic cells the heat

shock response is mediated by the heat shock transcription factor, which in yeast is encoded by the *HSF1* gene (Sorger 1991). Upon activation by heat shock, the Hsf1 transcription factor binds to heat-shock elements (HSE) in the promoters of target genes (Morimoto 1993). Many of the Hsf1 target genes are chaperones which often display a basal expression level that is also important for normal cellular functioning by stabilizing and refolding protein-folding intermediates or facilitating protein degradation (Morimoto et al. 1997). This explains why *HSF1* itself is also an essential gene under normal growth conditions. Interestingly, several heat shock genes contain both HSE and STRE in their promoter and these are used redundantly upon heat shock induction (Treger et al. 1998).

9.3.3 Response to Osmostress

The main pathway triggered by osmostress in *S. cerevisiae* is the HOG or 'high-osmolarity-glycerol' response pathway (Hohmann 2002). It causes rapid accumulation of the compatible solute glycerol and this is brought about by several distinct mechanisms. First, the *GPD1* gene encoding the main rate-limiting enzyme of glycerol biosynthesis, glycerol-3-phosphate dehydrogenase, is rapidly induced (Albertyn et al. 1994b). Second, the flux through glycolysis is stimulated at the level of phosphofructokinase which is apparently required for 'overflow' of glycolysis into elevated glycerol production (Dihazi et al. 2004). Third, the accumulation and release of glycerol from the yeast cells is favoured by the rapid closure and opening of the Fps1 glycerol channel in the plasma membrane in response to hyper- and hypoosmostress (Luyten et al. 1995). Osmostress probably affects the Fps1 channel directly. The stimulation of glycerol biosynthesis is triggered by a complex signalling pathway, starting with the putative osmosensors Sln1 and Sho1 in the plasma membrane which each stimulate a pathway leading to activation of the MAP kinase Pbs2, which in turn phosphorylates the MAP kinase Hog1. This leads to its accumulation in the nucleus and subsequent activation of several transcription factors, such as Sko1 and Hot1, but also the Msn2 and Msn4 factors (Hohmann 2002). The HOG signalling pathway also affects other targets besides glycerol production, such as cytoskeletal organization and plasma membrane composition.

Several components of the glycerol synthesis and HOG pathways have been identified in other yeast species. The genomes of both *S. pombe* and *Z. rouxii* contain a *GPD1* homologue but the expression of the gene in *Z. rouxii* in response to osmotic stress differs from that in *S. cerevisiae* and *S. pombe* (Ohmiya et al. 1995; Iwaki et al. 2001). A number of *FPS1* homologues have been found in other yeasts but surprisingly when the homologue was deleted in *S. pombe,* the yeast still released glycerol under hypoosmostress, suggesting that other mechanisms might regulate glycerol in this yeast (Kayingo et al. 2004). *HOG1* homologues are found in *Candida albicans* and *Z. rouxii* and their deletion also results in reduced glycerol production and osmosensitivity (San Jose et al. 1996; Alonso-Monge et al. 1999; Iwaki et al. 1999).

9.3.4 Response to Salt Stress

Addition of high salt concentrations not only evokes osmotic stress but also ionic stress. The plasma membrane transport systems such as the highly abundant

H^+-ATPases, sodium transporters and Na^+/H^+ antiporters of *S. cerevisiae* are prominent cell components involved in excluding NaCl from the cell, thereby maintaining a high intracellular K^+-to-Na^+ ratio and ion homeostasis (Serrano et al. 1986; Garciadeblas et al. 1993; Banuelos et al. 1998). Expression of Ena1, the major plasma membrane Na^+ efflux pump in yeast cells, is controlled at the transcriptional level by a complex network of pathways, including the HOG, PKA and Ca^{2+}/calcineurin pathways (Serrano and Rodriguez-Navarro 2001). Na^+ stress stimulates through the Ca^{2+}/calcineurin pathway entry of the Crz1 transcription factor into the nucleus, where it induces *ENA1* expression through the calcineurin-dependent-response element (CDRE) in the promoter (Mendizabal et al. 2001). The role of Crz1 in NaCl tolerance is supported by the failure of the *crz1Δ* mutant to induce *ENA1* expression and by their hypersensitivity to NaCl stress (Mendizabal et al. 1998). Accumulation of Na^+ in the vacuole is a second protection mechanism used to maintain a low cytosolic Na^+ concentration upon NaCl stress (Nass et al. 1997). On the other hand, comparison of transcriptional responses in *S. cerevisiae* with isoosmotic sorbitol and NaCl have shown similar expression patterns (Rep et al. 2000; Causton et al. 2001). Only ten additional genes were induced significantly more strongly by 0.7 M NaCl than an isoosmotic concentration of sorbitol (Rep et al. 2000).

9.3.5 Other Forms of Water Stress

Besides osmotic water stress also nonosmotic forms of water stress have been described (Hallsworth 1998). A net loss of water from the cell due to high levels of extracellular solutes unable to freely penetrate the cell membrane that reduce cell turgor has been described as an osmotic form of water stress (Hallsworth 1998). On the other hand, chaotropic compounds such as ethanol that diffuse through the plasma membrane impose a nonosmotic water stress. Ethanol reduces the intracellular water activity by decreasing the strength of hydrogen bonding and perturbing the structure and function of hydrated macromolecules, including nucleic acids, proteins and lipids. As is found with osmotic stress, compatible solutes are able to protect cell metabolism against nonosmotic water stress (Hallsworth 1998). Since ethanol is formed in high concentrations in natural fermentations of *S. cerevisiae* it is of paramount importance for the ecology of this yeast species. Apparently, the build-up of the high ethanol concentration combined with the high intrinsic ethanol tolerance of *S. cerevisiae* cells is the major factor that inhibits the growth of competing microorganisms and makes *S. cerevisiae* the dominating microorganism near the end of natural fermentations of sugar-rich media (Bauer and Pretorius 2000).

9.3.6 Response to Oxidative Stress

A cellular imbalance between the level of oxidants and the capacity of antioxidants and repair systems will lead to oxidative stress. Accumulation of reactive oxygen species, including different oxidation states of dioxygen (O_2) and singlet oxygen, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive

hydroxyl radical (OH^*) at toxic levels have been implicated in oxidative stress (Halliwell and Gutteridge 1999). These reactive oxygen species are produced during respiration when O_2 is incompletely reduced and during fatty acid metabolism in the peroxisome (Toledano et al. 2003). They attack molecules containing aromatic rings, such as purine or pyrimidine bases, lipids, metal-containing proteins or amino acids such as cysteine and methionine. The concentration required to elicit an oxidative stress varies. Peroxides such as lipid hydroperoxides and H_2O_2 in the respective toxic concentrations of 0.05 and 5 mM have been used to investigate the oxidative stress response in *S. cerevisiae*, whereas menadione is toxic in the 100 μM range to *S. cerevisiae* (Toledano et al. 2003). H_2O_2 has been used extensively to generate oxidative stress in yeasts. The molecular response mechanisms to these various oxidants have a number of common features in *S. cerevisiae*, whereas other components of the response mechanism differ between the oxidants, suggesting the presence of both common and oxidant-specific defence mechanisms (Moye-Rowley 2003). Oxidative stress causes massive induction of the genes encoding the defence systems, as well as additional genes encoding heat shock proteins, trehalose biosynthesis enzymes and enzymes of the pentose phosphate pathway which are important for the generation of reducing power (Gasch et al. 2000). The major transcription factor in yeast specifically activated by oxidants is Yap1. Oxidative stress causes Yap1 to accumulate in the nucleus and this is due to inhibition of its rapid nuclear export rather than to stimulation of its import (Kuge et al. 1997). Yap1 binds to its recognition element, YRE, present in the promoters of the antioxidant biosynthesis genes (Kuge and Jones 1994; Wu and Moye-Rowley 1994). Although Yap1 is thought to be a redox sensor itself, it is still not known whether peroxides directly oxidize Yap1. Moreover, different oxidants appear to affect Yap1 in different ways (Kuge et al. 2001). Yap1 acts in association with Skn7 (Lee et al. 1999). However, the action of Skn7 is not specific to the oxidative stress response. In addition to Yap1, Skn7 also associates with other transcription factors that regulate stress and metabolic responses (Toledano et al. 2003).

9.4 Stress-Protection Mechanisms

In yeast, stress-induced cellular injury is to a certain extent specific for one type of stress, but can also be common for different types of stress. Different stress conditions seem to cause injury through common mechanisms, which renders it plausible that the mechanisms of protection and repair are likewise partially overlapping (Davidson et al. 1996; Beck et al. 2000; Mager et al. 2000; Pahlman et al. 2001; Rep et al. 2001; Tanghe et al. 2003). The major yeast stress-protection mechanisms recognized so far, i.e. trehalose accumulation, synthesis of molecular chaperones, synthesis of antioxidant proteins, accumulation of compatible solutes, synthesis of hydrophylins and adaptation of plasma membrane composition, have indeed been shown to protect the yeast cell against various stress types. On the other hand, expression of aquaporins has been identified as a mechanism specifically protecting against freeze stress and not against other stress conditions (Tanghe et al. 2003).

9.4.1 Trehalose Accumulation

The nonreducing disaccharide trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is widespread in nature. For a long time it had been assigned a role only as a storage compound, but more recently its unique stress-protection properties have been revealed (reviewed in François and Parrou 2001; Elbein et al. 2003). In baker's yeast, for instance, improved stress tolerance is often correlated with higher trehalose levels as a function of the growth condition (Hottiger et al. 1987, 1994; de Virgilio et al. 1994; van Dijck et al. 1995). Further evidence for a stress-protective role of trehalose has been provided with yeast strains genetically engineered in trehalose metabolism, revealing a clear link between trehalose levels and tolerance to different stress types, including freeze, heat, dehydration, ethanol, osmotic and oxidative stress (Attfield et al. 1992; Eleutherio et al. 1993; Kim et al. 1996; Hounsa et al. 1998; Shima et al. 1999; Fillinger et al. 2001; Alvarez-Peral et al. 2002). This observation has also been extended to other yeast species, such as *S. pombe* (Ribeiro et al. 1997), *C. albicans* (Arguelles 1997), *Z. rouxii* (Kwon et al. 2003) and *Hansenula polymorpha* (Reinders et al. 1999).

How trehalose provides protection to cells is not entirely clear. Both in vitro and in vivo evidence has been obtained for a dual mechanism: preservation of the intracellular water structure and stabilization of membranes and proteins by replacing water (Sano et al. 1999). In line with the stress-protective effect of both endogenous (Hirasawa et al. 2001) as well as exogenous trehalose (Diniz-Mendez et al. 1999), it has been proposed that the protection exerted by trehalose requires its presence at both sides of the plasma membrane (de Araujo 1996).

9.4.2 Synthesis of Molecular Chaperones

Denaturation of proteins is a major injury factor following stress and, not surprisingly, the action of molecular chaperones is a major stress tolerance mechanism in yeast cells. Molecular chaperone proteins stabilize macromolecules to prevent them from aggregating. They recognize, selectively bind and reassemble proteins with an aberrant structure (Lindquist and Craig 1988; Buchner 1996). They might help to maintain a low degree of protein denaturation and reassemble damaged proteins during and after the imposition of the stress. Historically, molecular chaperones were discovered to be heat-shock-induced proteins but they are also involved in resistance to various other types of stress and also play an important role in many basic cellular functions where protein folding is involved.

There is increasing evidence that trehalose and molecular chaperones act synergistically as stress protectants (reviewed in François and Parrou 2001). During heat stress, trehalose has been shown to suppress the aggregation of denatured proteins in yeast, maintaining them in a partially folded state from which they can be activated by molecular chaperones (Elliott et al. 1996; Singer and Lindquist 1998a). However, the refolding activity of molecular chaperones itself is inhibited by trehalose. Hence, the rapid mobilization of trehalose upon removal of the stress condition is essential for the concerted action of trehalose and molecular chaperones in preventing cellular stress-induced injury (Singer and Lindquist 1998b).

Exposure of yeast cells to a mild dose of a particular type of stress results in the acquisition of resistance against a subsequent treatment with the same or another type of stress (Lewis et al. 1995). Accumulation of trehalose and synthesis of molecular chaperones are most likely to be the main players in these so-called acquired stress resistance and cross-protection phenomena (Soto et al. 1999).

The importance of trehalose and molecular chaperones as general stress protectants is also illustrated by the fact that the general stress resistance of yeast and other fungal cells strongly depends on the growth conditions. In yeast and many other fungi, slower growth is generally correlated with higher general stress resistance (Thevelein 1996) as well as high trehalose and molecular chaperone levels.

The concerted action of trehalose and molecular chaperones can nevertheless not account for all the stress resistance observed in yeast. There are studies reporting inconsistencies between stress tolerance and levels of trehalose and molecular chaperones, which apparently indicate the existence of other factors that are important or required for maintenance of viability under these conditions (van Dijck et al. 1995). Besides differences in trehalose and molecular chaperone levels, cells present in different growth phases or cultured under different conditions are likely to differ also in other properties which might contribute to stress tolerance. The importance of such other factors has only recently been revealed but their precise contribution is not clear yet (Versele et al. 2004). Evidence is forthcoming that the accumulation of antioxidant proteins, compatible solutes and hydrophylins, as well as the adaptation of plasma membrane composition together may at least account partly for the tolerance of yeast cells against different stress conditions.

9.4.3 Antioxidant Proteins and Other Molecules

The main antioxidant defences are a suite of metal-containing antioxidants: superoxide dismutases and catalases, and the thiol dependent antioxidants thioredoxin and glutathione. The breakdown of H_2O_2 to O_2 is catalysed by two catalase enzymes located in the cytosol and peroxisome in *S. cerevisiae*. Of the two superoxide dismutases found in yeast, the cytosolically located enzyme removes superoxide anions from the cytoplasm, whereas mitochondria are protected from superoxides generated during respiration by a mitochondrial-located enzyme (Jamieson 1998). Glutathione, α-tripeptide γ-L-glutamyl-L-cystinylglycine, is probably the most abundant redox-scavenging molecule in yeast. This molecule acts as a radical scavenger with the redox-active sulphydryl group reacting with oxidants to produce reduced glutathione (Jamieson 1998). Genes involved in glutathione synthesis have been identified in *S. cerevisiae* and mutants are hypersensitive to superoxide generators. Thioredoxin is a small sulphydryl-rich protein which acts as a reductant for thioredoxin peroxidase and for ribonucleotide reductase. The precise physiological function of thioredoxin is uncertain as deletion of the genes encoding thioredoxin is not lethal in *S. cerevisiae*. A number of other proteins, such as pentose phosphate pathway enzymes, metallothioneins and peroxidases, are also apparently involved in the protection of yeasts against oxidants (Juhnke et al. 1996; Jamieson 1998).

9.4.4 Compatible Solutes

Another damaging factor associated with many types of stress is osmotic disturbance. To be able to respond to changes in the osmotic pressure of their environment, yeast cells have so-called osmolytes, osmoprotectants or compatible solutes at their disposal (Kempf and Bremer 1998). In response to high osmolarity stress, they are able to accumulate those solutes through uptake and synthesis, whereas upon return to low-osmolarity conditions they can rapidly release them. Their accumulation is also correlated with higher tolerance to various other stresses, including heat stress, desiccation stress and freeze stress (Welsh 2000). The nature of these solutes in microorganisms is diverse, ranging from amino acid derived osmoprotectants such as proline and glycine betaine to sugar-related osmoprotectants such as trehalose, fructans and polyols. In *S. cerevisiae* and other yeasts, glycerol has been demonstrated to serve as the major compatible solute although other polyols such as arabitol and mannitol may also act as compatible solutes (Brown and Simpson 1972; Albertyn et al. 1994b; Tamás et al. 1999; Hohmann and Nielsen 2000). Notwithstanding its minor role as an osmolyte in yeast cells, proline at high levels has been reported to protect mature yeast ascospores against desiccation (Ho and Miller 1978) and intracellular accumulation of proline has been shown to improve freeze tolerance (Morita et al. 2003).

9.4.5 Hydrophylins

Proteins that meet the hydrophylin criteria – a high percentage of glycines and a high hydrophylicity – have mainly been found in plants but also in fungi (Garay-Arroyo et al. 2000). They are suggested to possess both water- and protein-binding regions, enabling them to protect enzymes from water loss, as has been demonstrated for some enzymes upon dehydration and freezing in vitro (Honjoh et al. 2000; Covarrubias et al. 2001). The so-called late embryonic abundant (LEA) proteins also belong to the family of hydrophylins. In baker's yeast, the LEA-like protein Hsp12 (Mtwisha et al. 1998) has been demonstrated to increase dessication-, ethanol- and barotolerance (Sales et al. 2000; Motshwene et al. 2004).

9.4.6 Plasma Membrane Composition

As the plasma membrane of unicellular organisms is in close contact with the surrounding medium, it is likely that its characteristics will influence the tolerance of the cells to all kinds of environmental challenges. A correlation has indeed been found between membrane composition and tolerance to heat, oxidation, ethanol and salt in several instances (Steels et al. 1994; Chi and Arneborg 1999; Swan and Watson 1999; Allakhverdiev et al. 2001; Sakamoto and Murata 2002). In addition, different types of stress have been shown to influence membrane composition (Low and Parks 1987; Singh et al. 1990; Sajbidor and Grego 1992). Fluidity and permeability are the most studied membrane characteristics. Freeze resistance, for instance, has been positively correlated with membrane fluidity (Calcott and Rose 1982) and membrane water permeability (Lewis et al. 1994; Tanghe et al. 2002). The precise

relationship between these membrane characteristics and stress tolerance is, however, not always clear (Swan and Watson 1997).

9.4.7 Aquaporins

Recently, a link was found between freeze tolerance and expression of the aquaporin encoding genes *AQY1* and *AQY2* (Tanghe et al. 2002). This observation supports a role for plasma membrane water transport activity in determination of freeze tolerance in yeast. It is proposed that rapid osmotically driven efflux of water during the freezing process might reduce intracellular ice crystal formation and resulting cell damage. This is in accordance with the observation that aquaporin-mediated improvement of freeze tolerance is limited to fast freezing conditions (Tanghe et al. 2004). Changes in aquaporin expression levels do not seem to alter other cell characteristics, including tolerance to other stress types (Tanghe et al. 2002).

9.5 Growth Conditions, Stress Tolerance and Activity of the Protein Kinase A Pathway

In *S. cerevisiae* there is a striking correlation between the growth conditions, the stress tolerance of the cells and the apparent activity of the protein kinase A (PKA) pathway. Although a relationship between stress tolerance and growth conditions has also been noted in other yeast species, very little is known about the underlying mechanisms in these species. When *S. cerevisiae* cells grow rapidly on fermentable sugars, like glucose and fructose, they display low stress tolerance. When they grow slowly on nonfermentable carbon sources or when they are in a stationary phase they display high stress tolerance (Schenberg-Frascino and Moustacchi 1972; Plesset et al. 1987). The connection between stress tolerance and the PKA pathway has been revealed by mutations either reducing or enhancing activity of the pathway. For instance, mutations in adenylate cyclase (Cyr1/Cdc35), in its activators Ras1,2 or Cdc25, or mutations in the catalytic subunits of PKA will reduce activity of the pathway and enhance stress resistance. On the other hand, mutations in the Ras inhibitors Ira1 and Ira2, or in the regulatory subunit Bcy1 of PKA, will enhance activity of the pathway and reduce stress resistance (Sass et al. 1986; Toda et al. 1987a, b; Cameron et al. 1988; Park et al. 1997). Extensive characterization of these mutants has revealed many other targets of the PKA pathway: trehalose and glycogen content, starvation survival, cell wall strength, sporulation and pseudohyphal growth capacity, etc. (Broach and Deschenes 1990; Chi and Arneborg 1999; Thevelein and de Winde 1999). The stress tolerance level and the status of the other targets of the PKA pathway indicate that in cells growing on rapidly fermented sugars, the activity of the pathway is apparently high, whereas in respiratively growing cells or in the stationary phase the activity of the pathway must be low. Downregulation of stress tolerance by the PKA pathway occurs at the level of the Msn2 and Msn4 transcription factors, which are excluded from the nucleus when PKA activity is high (Görner et al. 1998). A second system downregulated by the PKA pathway consists of the postdiauxic shift (PDS) element, which closely resembles the Msn2/Msn4-controlled STRE (Boorstein and Craig 1990). Downregulation

of the PDS element by PKA occurs through inhibition of the Rim15 protein kinase which is required for the activity of Gis1, the transcription factor that induces PDS-controlled genes (Reinders et al. 1998; Pedruzzi et al. 2000).

The presence or absence of a fermentable sugar in the growth medium is not the sole determinant for the level of stress tolerance in yeast cells. When yeast cells are starved of an essential nutrient, like nitrogen, phosphate or sulphate, in the presence of a fermentable sugar, they arrest growth and become highly stress tolerant. Hence, it is the combination of a fermentable sugar and active growth that in some way keeps stress tolerance low. The pathway involved has been called the 'fermentable-growth-medium-induced pathway' (Thevelein 1994). As a result, a rapid drop in stress resistance cannot only be induced by addition of a fermentable sugar like glucose to respiring cells (van Dijck et al. 1995) but also by addition of a nitrogen source or phosphate to cells starved in the presence of glucose for nitrogen (Donaton et al. 2003) or phosphate (Giots et al. 2003), respectively. Mutants and multicopy suppressor genes have been isolated that were more resistant to fermentation-induced loss of stress resistance ('fil') and they were identified as components of the cAMP–PKA pathway (Kraakman et al. 1999; Versele et al. 1999; van Dijck et al. 2000).

What is the reason for the correlation between stress tolerance and the growth conditions? It has been argued that the higher stress tolerance (which is due to the expression of stress-protection-related genes) on respirative carbon sources indicates that the cells are more 'stressed' on these carbon sources. If the amount of stress that cells experience can be deduced from the number of stress-protection mechanisms that are induced then this conclusion seems to be warranted. However, an alternative conclusion is that the cells induce stress-protection mechanisms because they expect to experience more stress; hence, as a preparation to survive stress. Resting stages, such as stationary-phase cells or ascospores in yeast, and seeds, cysts and other survival forms in other organisms are highly stress resistant clearly as a preparation for stressful conditions rather than as a reaction to the stress that is experienced. Hence, the higher stress resistance in yeast cells growing on nonfermentative carbon sources (in nature this will generally be ethanol) might be a preparation for the stress that the cells will experience due to the accumulation of the ethanol or as a preparation for the subsequent stationary phase which inevitably follows exhaustion of the ethanol.

Another point that needs attention is the general inverse relationship that exists in nature between metabolic activity and stress resistance. The more actively cells are metabolizing and multiplying the less stress resistant they are. Several explanations can be proposed for this observation. For yeasts cultivated in conditions that allow rapid growth, investment in maximal proliferation of the cells might be much more useful for survival in the long term than investment in higher stress resistance for the individual cells. Stress-protection mechanisms might also be incompatible with metabolic activity. Evidence in this respect has been reported for trehalose, which in high levels confers stress protection but reduces the activity of molecular chaperone proteins (Singer and Lindquist 1998a). Hence, folding activity of proteins, catalytic activity of enzymes and actually any protein activity might be hampered by stress-protection mechanisms. This would leave a yeast with a limited (or obligate?) choice between rapid proliferation and preparation for stress survival.

9.6 Stress and the Distribution of Yeasts in Nature

With respect to stress under natural conditions, it is important to emphasize that research on the molecular basis of stress responses and tolerance in the laboratory is generally conducted on uniform cell suspensions of yeast cells complemented with plate assays of growth. In nature yeasts usually grow in mixed populations and sometimes in special communities, such as biofilms. Cells in such mixed or specialized communities might respond quite differently to stress conditions. This is shown, for instance, by the much greater resistance to antifungals of *C. albicans* cells growing in biofilms (Baillie and Douglas 1998). The type of solid substrate on which the cells are growing and interactions with other microorganisms might also influence stress tolerance and stress response. Very little is known in this respect.

The ability of yeasts to tolerate stress and to grow in extreme environments is often species-specific (see Chaps. 15, 16). Certain yeast species like many other microorganisms have developed mechanisms that enable them to grow in extreme environments that exclude most other species. Well-known examples include the highly osmotolerant yeasts *Z. rouxii* and *Debaryomyces hansenii* (Brown 1978; Blomberg and Adler 1992). Pathogenic yeasts, such as *C. albicans*, can proliferate in the stressful environment of the host because part of its virulence factors constitute stress-protection mechanisms (Calderone and Fonzi 2001). Undoubtedly, the capacity to proliferate under specific stress conditions has a major impact on the distribution of the yeast in nature. Yeasts also have the ability to survive beyond the limits of growth in extreme conditions, such as low and high temperature, dehydration, extreme pH, excessive ethanol and limited nutrients. The survival mechanisms to these extreme conditions can be expected to be related in many instances to the adaptation mechanisms that yeasts use to grow under stressful conditions. The capacity of the yeast to survive under extreme conditions will also impact on its distribution in nature but this will be more difficult to reveal compared with that of yeast species able to multiply under extreme conditions.

9.7 Conclusions and Perspectives

We have shown in this review that the responses of yeasts to stress involve multiple components of cell metabolism. Most studies have focussed on the specific interaction of a certain aspect of metabolism and a stress condition. Recent investigations based on a global approach to the study of gene expression in some yeasts have shown that yeasts possess general stress responses as well specific responses to each stress type. These observations imply that the yeast cell is programmed to withstand the myriad of stresses that the yeast can expect during its lifetime. Some yeast species withstand the extremes of stress more successfully than others. However, in many instances we do not understand the basis for the greater stress resistance of one species compared with another. Furthermore, yeasts in nature seldom occur in pure culture but usually exist in complex communities consisting of numerous species together with other microorganisms. How these communities respond to stress has been poorly studied and the interactions between yeasts and their environment represent a future challenge for the yeast biologist to investigate. The tools to understand these

complex questions are now becoming available. With the recent explosion of yeast genomic sequences and improvements in computing power, the stage is now set not only to investigate specific changes to stress but rather to take an integrative approach that studies the interplay of genes, proteins, molecules and organelles with the environment, thereby obtaining a global picture of how the yeast operates under stress.

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Chapter 10

Antagonistic Interactions Among Yeasts

W.I. Golubev

Russia Collection of Microorganisms, Institute for Biochemistry and Physiology of Microorganisms, Pushchino, 142290, Russia
(e-mail: wig@ibpm.pushchino.ru)

10.1 Introduction

In every biocenosis, competition for nutrients and space is one of the major factors that determines which organisms succeed and become established. The reasons for such ecological success vary. In many cases, the microbes often alter the environment with their metabolic products, securing their dominance in the habitat as they create conditions unfavourable for the survival of other microorganisms. Such interaction is known as antagonism. Some of these antimicrobial compounds are nonspecific. For example, the antibacterial activity of yeasts is commonly caused by pH changes in the medium as a result of organic acid production or by producing high concentrations of ethanol. This chapter will focus on much more specific interactions stemming from antifungal agent secretion by yeasts.

By now two classes of such extracellular agents of yeasts are known: (glyco)proteins and glycolipids (Fig. 10.1). The proteinaceous compounds having fungicidal or fungistatic action are termed mycocins (zymocins, killer toxins) and they have been investigated for some 40 years, whereas the fungicidal activity of their extracellular glycolipids has only recently come to light.

10.2 Mycocinogeny

The synthesis of proteinaceous antimicrobial compounds (killer phenomenon) is not unique for yeasts; they are produced by various species ranging from bacteria to mammals (Nissen-Meyer and Nes 1997). As for microorganisms, secretion of proteins with a toxicity specific for related organisms, which is associated with specific immunity, is known in smut fungi (Koltin 1988), paramecia (Quackenbush 1988), slime molds (Mizutani et al. 1990) and bacteria (James et al. 1991). The bacterial protein antibiotics are termed bacteriocins and, to emphasize the general nature of such antagonistic interactions, it is preferable to call yeast toxins mycocins and killers mycocinogenic strains. Mycocins are antifungal proteins whose activity is directed against organisms which are taxonomically related to mycocin producers. The latter themselves are immune to their own mycocin but not to others mycocins.

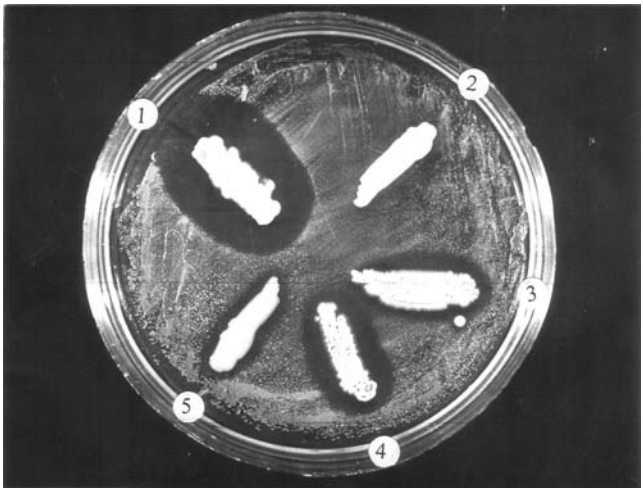

Fig. 10.1. Mycocin and cellobiolipid secretion by *Filobasidium capsuligenum* VKM Y-1439 (*5*) and *Cryptococcus humicola* 9-6, VKM Y-1613, 2238 (*1*, *3*, *4*) strains. They were streaked onto the surface of the medium that had been seeded with a lawn of a test culture that is insensitive to *Cystofilobasidium bisporidii* VKM Y-2700 (*2*) mycocin

In addition, there are some (neutral) strains which neither secrete mycocin nor are sensitive to it. Mycocins do not act against bacteria or protozoa, and no pharmacological activity has been detected in tests with animal organs (Ohta et al. 1984; Pfeiffer et al. 1988). A few reports of the antibiotic action of yeast strains against a wide variety of prokaryotic and eukaryotic organisms have come from an unwarranted interpretation of any observed growth inhibition as mycocinogeny (Polonelli and Morace 1986). No attempts were made to characterize the toxic substances cited in these reports and the growth inhibition was most likely to be due to metabolic products other than mycocins. With respect to antifungal activity, it is necessary to distinguish the mycocinogeny from other inhibition effects, for example, the arresting of growth from mating pheromones. The broad anti-yeast activity of *Metschnikowia pulcherrima* is associated with excretion of the iron-binding agent pulcherriminic acid (Vustin et al. 1990; Nguyen and Panon 1998).

10.2.1 Assay for Mycocinogenic Activity

Both the level and the expression of mycocinogenic activity depend on a number of variables. One of the most important conditions for its detection is the pH of the test medium. Killer activity is expressed under acidic conditions, usually between pH 3 and 6. As a rule, mycocins are most active at pH 4-5 (Woods and Bevan 1968; Young and Yagiu 1978; Middelbeek et al. 1979; Tolstorukov et al. 1989). Because of their proteinaceous nature, mycocins are inactivated at high temperatures, and in most cases 15–20°C is optimal for incubation when assaying for mycocinogenic activity. Adding glycerol (5–15%) to the medium produces broader inhibition zones around

Kluyveromyces and *Pichia* mycocinogenic strains and increases the sensitivity of the bioassay significantly (Lehmann et al. 1987b; Golubev and Blagodatskaya 1993). The antifungal action of mycocins produced by halotolerant yeasts (*Candida, Debaryomyces* and *Pichia* spp.) is evoked and enhanced in the presence of increasing (4–12%) NaCl concentrations (Kagiyama et al. 1988; Suzuki et al. 1989; Gunge et al. 1993; Llorente et al. 1997). Mycocins are stabler in a solid medium than in a liquid medium, and agitation can cause their inactivation (Woods and Bevan 1968; Wilson and Whittaker 1989). The concentration of sensitive cells influences the sensitivity of the bioassay; when the lawn of the target strain is too dense and the inoculum of the mycocinogenic strain is small, the inhibition zone can be narrow and rapidly overgrown or not developed at all. In addition, the composition of the medium and buffer solution may affect to the sensitivity of the assay (Panchal et al. 1985). In most cases, glucose–yeast extract–peptone agar with sodium citrate–phosphate buffer are used. The assay conditions are particularly important for detecting mycocinogenic strains with low activity and those organisms that are only slightly sensitive.

However, using optimal conditions does not guarantee successful screening, because the principal trait of mycocins is the specificity of their toxicity. Hence, the choice of appropriate sensitive strains is crucial for detecting mycocinogenic strains. Their incidence was found to be much higher when screening for mycocinogenic activity with target cultures of the same species (or one closely related taxonomically) as that being tested (Thornton 1986; Heard and Fleet 1987; Golubev and coworkers 1990, 1993).

After the discovery of mycocinogeny in *Saccharomyces cerevisiae* (Makower and Bevan 1963), it soon became evident that the production of mycocins is a general phenomenon for most, if not all, yeasts. At present, mycocinogenic strains have been found in over 100 species from more than 20 genera among both ascomycetous and basidiomycetous yeasts (Table 10.1). Several types of mycocinogenic strains were identified in some species (*Cryptococcus laurentii, Pichia membranifaciens*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*).

10.2.2 Characteristics of Mycocins

All mycocins are either proteins or glycoproteins that often consisted of two or three subunits. Most yeasts secrete mycocins with a molecular mass of about 10–30 kDa, although those of *Kluyveromyces lactis*, *P. acaciae*, *P. anomala* and *P. inositovora* are much higher, about 100 kDa or greater (Sawant et al. 1989; Stark et al. 1990; McCracken et al. 1994; Klassen and Meinhardt 2003). The best known and most fully studied K1 mycocin (20 kDa) of *Saccharomyces cerevisiae* is secreted as a molecule consisting of α and β disulfide-bonded unglycosylated polypeptides with a relatively high content of hydrophobic and charged amino acids (Zhu et al. 1987). Its precursor has been synthesized in cells as a large single-strand polypeptide that has a δ–α–γ–β domain organization. The δ domain is a leader sequence that mediates folding and secretion; the α and β domains are separated by an interstitial glycosylated γ region which is required for maturation (Lolle and Bussey 1986). The β subunit is a lectin-like domain and is essential for recognition and binding to whole

Table 10.1 Yeast species for which mycocinogenic activity has been reported

Yeast species	References
Bullera alba	Golubev et al. (1997a, b)
B. hannae	Golubev et al. (1996)
B. sinensis	Golubev et al. (1997a)
B. unica	Golubev and Nakase (1998)
Candida albicans	Rogers and Bevan (1978)
C. berthetii	Buzzini and Martini (2000b)
C. cacaoi	Aguiar and Lucas (2000)
C. diversa	Abranches et al. (2000)
C. freyschussii	Buzzini and Martini (2000a)
C. glabrata	Sriprakash and Batum (1984)
C. homilentoma	Carreiro et al. (2002)
C. maltosa	Polonelli et al. (1987)
C. naeodendra	Suzuki et al. (1989)
C. nodaensis	Aguiar and Lucas (2000)
C. oleophila	Buzzini and Martini (2000b)
C. parapsilosis	Zekhnov et al. (1989)
C. silvae	Buzzini and Martini (2000b)
C. sonorensis	Starmer et al. (1987)
C. species	Yokomori et al. (1988)
C. stellata	Abranches et al. (2000)
C. versatilis	Vaughan-Martini et al. (1988)
Cryptococcus aerius	Carreiro et al. (2002)
C. albidus	Starmer et al. (1987)
C. aquaticus	Pfeiffer et al. (2004)
C. flavus	Buzzini and Martini (2000b)
C. laurentii	Golubev and Kuznetsova (1989)
C. luteolus	Buzzini and Martini (2000b)
C. nemorosus	Golubev et al. (2003a)
C. perniciosus	Golubev et al. (2003a)
C. podzolicus	Golubev (1991a)
Curvibasidium pallidicorallinum	Golubev (1992a)
Cystofilobasidium bisporidii	Kulakovskaya et al. (1996)
Cyst. infirmominiatum	Golubev et al. (2003b)
Cyst. species	Gacser et al. (2001)
Debaryomyces carsonii	Polonelli et al. (1987)
D. castellii	Vustin et al. (1993)
D. etchelsii	Buzzini and Martini (2000b)
D. hansenii (*C. famata*)	Suzuki et al. (1989); Aguiar and Lucas (2000)
D. occidentalis	Chen et al. (2000)
D. polymorphus	Vaughan-Martini et al. (1988)
D. vanrijiae	Vustin et al. (1993)
Fellomyces penicillatus	Aguiar and Lucas (2000)
Filobasidium capsuligenum	Golubev and Kuznetsova (1991)
Hanseniaspora uvarum (*Kloeckera apiculata*)	Rosini and Cantini (1987); Schmitt et al. (1997)
H. vineae (*K. africana*)	Abranches et al. (2000)
H. valbyensis (*K. japonica*)	Starmer et al. (1987)
Issatchenkia orientalis (*C. krusei*)	Lehmann et al. (1987a)
I. scutulata	Buzzini and Martini (2000b)
I. terricola	Abranches et al. (2000)

Table 10.1 Yeast species for which mycocinogenic activity has been reported—*cont'd*

Yeast species	References
Kloeckera lindneri	Abranches et al. (2000)
Kluyveromyces aestuarii	Vaughan-Martini and Rosini (1989)
K. dobzhanskii	Vaughan-Martini and Rosini (1989)
K. lactis (*C. sphaerica*)	Vaughan-Martini et al. (1988); Stark et al. (1990)
K. loddereae	Vaughan-Martini and Rosini (1989)
K. marxianus (*C. pseudotropicalis*)	Lehmann et al. (1987b); Polonelli et al. (1987)
K. phaffii	Comitini et al. (2004)
K. polysporus	Kono and Himeno (1997)
K. thermotolerns (*C. dattila*)	Choi et al. (1990)
K. waltii	Kono and Himeno (1997)
K. wickerhamii	Vaughan-Martini and Rosini (1989)
K. wikenii	Rosini and Cantini (1987)
Pichia acaciae	Bolen et al. (1994)
P. americana	Buzzini and Martini (2000b)
P. amethionina	Starmer et al. (1987)
P. anomala	Sawant et al. (1989)
P. antillensis	Starmer et al. (1987)
P. bimundalis	Polonelli et al. (1987)
P. burtonii	Buzzini and Martini (2000b)
P. cactophila	Starmer et al. (1987)
P. canadensis	Lehmann et al. (1987a)
P. ciferrii	Nomoto et al. (1984)
P. fabianii	Polonelli et al. (1987)
P. farinosa	Price et al. (1999)
P. guilliermondii (*C. guilliermondii*)	Zekhnov et al. (1989)
P. haplophila	Aguiar and Lucas (2000)
P. holstii	Polonelli et al. (1987)
P. inositovora	Klassen and Meinhardt (2003)
P. jadinii	Vaughan-Martini et al. (1988)
P. kluyveri	Zorg et al. (1988)
P. membranifaciens (*C. valida*)	Golubev and Blagodatskaya (1993)
P. mexicana	Starmer et al. (1987)
P. minuta	Polonelli et al. (1987)
P. ohmeri	Zekhnov et al. (1989)
P. opuntiae	Starmer et al. (1987)
P. petersonii	Nomoto et al. (1984)
P. pini	Zekhnov et al. (1989)
P. punctispora	Golubev and Blagodatskaya (1994)
P. quercuum	Zekhnov et al. (1989)
P. spartinae	Polonelli et al. (1987)
P. stipitis	Laplace et al. (1992)
P. subpelliculosa	Young and Yagiu (1978)
P. thermotolerans	Ganter and Starmer (1992)
Pseudozyma antarctica	Buzzini and Martini (2000b)
Rhodotorula dairenensis	Golubev (1989)
R. glutinis	Aguiar and Lucas (2000)
R. graminis	Buzzini and Martini (2000b)
R. lactosa	Buzzini and Martini (2000b)
R. lignophila	Buzzini and Martini (2000b)
R. mucilaginosa	Golubev and Churkina (1997)

Continues

Table 10.1 Yeast species for which mycocinogenic activity has been reported—*cont'd*

Yeast species	References
R. pallida	Golubev (1991b)
Saccharomyces cerevisiae	Wickner (1996)
S. exiguus (*C. holmii*)	Nagornaya et al. (1989); Vital et al. (2002)
S. paradoxus	Naumov (1985)
S. unisporus	Nagornaya et al. (1989)
Schizosaccharomyces pombe	Bonilla-Salinas et al. (1995)
Sporidiobolus pararoseus	Janderova et al. (1995)
S. salmonicolor	Golubev and Tsiomenko (1985)
Tilletiopsis albescens	Golubev (1998b)
T. flava	Golubev and Churkina (2001)
Torulaspora delbrueckii	Bonilla-Salinas et al. (1995)
Trichosporon asteroides	Buzzini and Martini (2000b)
T. capitatum	Morace et al. (1983/1984)
T. jirovecii	Carreiro et al. (2002)
T. pullulans	Golubev et al. (2002)
Williopsis pratensis	Vustin et al. (1991)
W. saturnus	Kimura et al. (1995); Komiyama et al. (1995)
W. saturnus var. *mrakii*	Kimura et al. (1999)
W. saturnus var. *subsufficiens*	Shemyakina et al. (1991)
Zygosaccharomyces bailii	Weiler and Schmitt (2003)
Z. fermentati	Buzzini and Martini (2000b)
Z. florentinus	Aguiar and Lucas (2000)
Z. microellipsoides	Buzzini and Martini (2000b)
Zygowilliopsis californica	Vustin et al. (1988); Theisen et al. (2000)

cells, while the α subunit acts at the cytoplasmic membrane to produce a collapse of a transmembrane proton gradient (Sturley et al. 1986; Douglas et al. 1988; Zhu and Bussey 1991). Unlike most reported mycocins, some produced by *Pichia* and *Williopsis* species show a higher thermostability and function over a wide pH range (Ashida et al. 1983; Ohta et al. 1984; Vustin et al. 1989). This stability appears to be due to the large number of disulfide bonds in these cysteine-rich molecules.

10.2.3 Modes of Action

After exposure to most mycocins, growing sensitive cells exhibit a reduction in intracellular pH and leakage of potassium ions, ATP and other cellular intermediates. Amino acid transport and proton pumping into the culture medium are also inhibited (Skipper and Bussey 1977; Middelbeek and coworkers 1980a–c; Ashida et al. 1983). All of these effects are indicative of an increase in proton permeability in mycocin-sensitive cells (de la Pena et al. 1981). Apparently, mycocin either inhibits a component of the proton pump or becomes incorporated into the cytoplasmic membrane and creates ion-permeable channels (Kagan 1983; Martinac et al. 1990; Santos and Marquina 2004). The K1 mycocin was shown to induce aberrant activity of ion channels, which leads to dysregulated potassium homeostasis (Ahmed et al. 1999).

The overall effect of pore formation disrupts the cell electrochemical potential across the plasma membrane and results in cell death (Fig. 10.2). Many, but not all mycocins, have a membrane-damaging activity. The *Kluyveromyces lactis* toxin, for example, does not elicit leakage of potassium ions and ATP but causes an arrest in the G1 phase of the cell cycle (Butler et al. 1991); while the mycocin of *Williopsis saturnus* var. *mrakii* inhibits β-1,3-glucan synthesis (Yamamoto et al. 1986) and the KT28 mycocin of *Saccharomyces cerevisiae* inhibits DNA synthesis (Schmitt et al. 1989).

Regardless of the mode of action of mycocins, it occurs in two phases. The first stage of cell interaction is a rapid and energy-independent binding to the cell wall surface. The second stage, the time-lag and energy-dependent process, involves mycocin translocation to the cytoplasmic membrane and interaction with a membrane receptor (Al-Aidroos and Bussey 1978; Bussey et al. 1979). Binding is pH-dependent and may be responsible for the pH range of mycocin activity on yeast cells. Binding sites may be particular cell wall receptors, which have other functions, such as the uptake of nutrients. Until now, the primary functions of these receptors have not been elucidated for any yeasts.

Comparison of the mycocin killing spectra on whole cells and on spheroplasts showed that it is the cell wall which determines the specificity of sensitivity to mycocins. *Saccharomyces cerevisiae* mycocin K1 can kill cells of the same species and those of *Candida glabrata*, but has a wide spheroplast-killing action and can

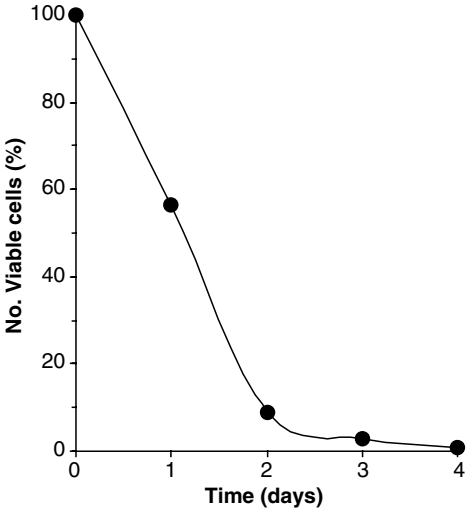

Fig. 10.2. Killing of *Mrakia nivalis* VKM Y-1443 cells incubated with *Cryptococcus aquaticus* VKM Y-2428 mycocin-containing culture filtrate (pH 4.5, 18°C). Survival is expressed as the percentage of cells able to produce colonies, where 100% is the number of colonies counted immediately before addition of the mycocin-containing filtrate

destroy the spheroplasts of *C. albicans*, *C. utilis*, *Kluyveromyces lactis* and *Debaryomyces occidentalis*. However, the whole cells of these species are insensitive to this mycocin (Zhu and Bussey 1989). Mutations that change the molecular structure of cell wall glucans and mannans alter their capacity to act as binding sites of mycocins and induce resistance to them (Schmitt and Radler 1988; Nakajima et al. 1989). As yeast and hyphal forms of the same organisms vary in the composition of their walls they can differ in sensitivity to mycocins (Golubev and Boekhout 1992). Any of the principal cell wall components can be involved in their binding. For mycocins produced by *Hanseniaspora uvarum*, *P. anomala*, *P. membranifaciens*, *S. cerevisiae* (K1 and K2 types) and *Williopsis saturnus,* the binding components have been identified as β-1,6-glucans; those for mycocins of *Debaryomyces occidentalis*, *S. cerevisiae* (K28 type) and *Zygosaccharomyces bailii* are mannans, whereas chitin may represent the binding site for mycocins of *Kluyveromyces lactis* and *P. acaciae* (Hutchins and Bussey 1983; Schmitt and Radler 1988; Sawant and Ahearn 1990; Radler et al. 1993; Takita and Castilho-Valavicius 1993; Kasahara et al. 1994; McCracken et al. 1994; Schmitt et al. 1997; Santos et al. 2000).

10.2.4 Genetic Basis for Mycocinogeny

Mycocin determinants can be either cytoplasmically or chromosomally inherited. In most cases mycocin production is presumed to be determined by nuclear genes but these have clearly been identified in a few species only (Sriprakash and Batum 1984; Goto et al. 1990; Kimura et al. 1993; Suzuki and Nikkuni 1994).

Much more attention is given to the extrachromosomal genetic elements, double-stranded RNA (dsRNA) viruses and linear double-stranded DNA (dsDNA) plasmids which are responsible for a mycocinogenic phenotype. The virally encoded mycocinogeny has been most intensively studied in *S. cerevisiae* (Wickner 1996), but has also been described for *Hanseniaspora uvarum* (Zorg et al. 1988) and *Zygosaccharomyces bailii* (Schmitt and Neuhausen 1994). In addition, the mycocin-coding dsRNA viruses have been identified in the basidiomycetous yeasts, *Cystofilobasidium bisporidii* (Karamysheva et al. 1991), *C. infirmominiatum* (Golubev et al. 2003b), *Sporidiobolus salmonicolor* (Kitaite and Čitavičus 1988) and *Trichosporon pullulans* (Golubev et al. 2002). Currently, these isometric dsRNA viruses with undivided genomes are classified in the genus *Totivirus* in the family *Totiviridae* (Ghabrial 1994). In contrast to most plant and animal RNA viruses, yeast viruses are noninfectious and are transmitted by vegetative cell division or through sexual fusion. The mycocins are encoded by different satellite dsRNA viruses (denoted M) which are dependent on another group of helper dsRNA viruses (denoted L). These encode capsid and RNA polymerase for replicating both virus types. Curing of viruses from mycocinogenic strains results in loss of activity and immunity to their own mycocins.

Mycocinogenic strains of *Kluyveromyces lactis* (Schaffrath and Breunig 2000), *P. acaciae* (Bolen et al. 1994) and *P. inositovora* (Ligon et al. 1989) contain two types of linear dsDNA plasmids differing in size. The loss of these plasmids also gives rise to cultures which are not only incapable of producing mycocins, but are also susceptible to them.

10.2.5 Taxonomic Implications of Sensitivity to Mycocins

By virtue of the fact that cell walls of yeast taxa vary in structure and chemical composition (Kreger-van Rij and Veenhuis 1971; Weijman and Golubev 1987; Fleet 1991), and as different wall components are involved in mycocin binding, it is reasonable to infer that mycocin activity against whole cells may be restricted to taxonomically related organisms, so that mycocin sensitivity patterns are of taxonomic importance (Golubev and Boekhout 1995). The differences between yeasts in this respect are associated with a whole range of taxonomic and phylogenetic markers, such as septal pore ultrastructure, monosaccharide composition of extracellular polysaccharides, and sequence similarity of small and large subunit ribosomal RNAs (Golubev 1998a). However, it must be emphasized that there are no definite taxonomic levels criteria for the action of mycocins, as their host ranges vary considerably. Although the mycocins are active against organisms phylogenetically and taxonomically related to the mycocinogenic strains, the degree of relatedness may vary from strains of the same species to species of related genera or even higher taxa. Apparently, the diversity of cell-wall receptors involved in binding of mycocins can be both unique and common for certain taxa and may provide the basis of differences in the ranges of mycocin action. In practice, this feature dictates that the use of each mycocin as a taxonomic tool must be preceded by a careful study of its killing pattern. The differences in host ranges may serve to resolve different levels of taxonomic organization. Broad-spectrum mycocins are apparently of most interest for overall phylogenetic evaluations, whereas narrow-spectrum ones may be used for clarification of the taxonomy of closely related organisms.

As a rule, ascomycetous yeasts are insensitive to mycocins produced by basidiomycetous yeasts and conversely. The exceptions are the mycocins of *Williopsis pratensis*, *Bullera alba* and *Curvibasidium pallidicorallinum*. That of *W. pratensis* is active not only against ascomycetous yeasts but also against some sporidiobolaceous species (Vustin et al. 1991), whereas that of *B. alba* is active against some ascomycetous yeasts (mainly the members of the Lipomycetaceae) in addition to basidiomycetous species (Golubev et al. 1997a). The mycocin produced by *C. pallidicorallinum* (previously identified as *Rhodotorula fujisanensis*) mainly acts against sporidiobolaceous yeasts and also shows a weak activity against some tremellaceous yeasts (Golubev 1992a). In most cases, all strains of the same species and closely related species of the same genus have identical responses to specific mycocins. However, some taxa are heterogeneous not only within a genus but also within a species. The major reason for such variability is the heterogeneity of many yeast taxa. Most teleomorphic taxa are homogenous, unlike the anamorphic taxa in which heterogeneity in sensitivity patterns is much more widespread. The additional reasons are the immunity of mycocinogenic strains. The responses to mycocins that are free from the effects of immunity are of taxonomic interest. There is the problem associated with distinction of immunity from resistance to mycocins. Resistance and immunity are similar phenotypically, and plate assays do not allow one to distinguish between these two types of insensitivity which differ both in their genetic mechanisms and in their taxonomic significance. Resistant yeasts lack the specific receptors necessary for the adsorption, and hence action, of mycocin.

Mycocinogenic strains, like sensitive ones, still possess these receptors, but the mycocins contain a component which gives immunity (specific for only one mycocin type) to mycocinogenic cultures. Immunity appears to be conferred at the cytoplasmic membrane level by a component which may act as a competitive inhibitor of mycocin by saturating membrane receptors (Boone et al. 1986; Hanes et al. 1986; Douglass et al. 1988). The so-called neutral strains contain genetic determinants for mycocin synthesis, either the mycocin is produced in an inactive form or it is not secreted. Consequently, such strains retain their immunity (Bussey et al. 1982; Wingfield et al. 1990). Thus, the insensitivity caused by immunity is a clone-related property, which coupled with possible cross-immunity between immunologically similar mycocins, may interfere with the resistance shown at the cell-wall level and conferred by the nuclear genotype. All these reasons taken together give a complicated picture of mycocin-sensitive relationships. Consequently many authors consider the sensitivity patterns to be strain-related and propose using them for fingerprinting in order to biotype the strains of a species (Morace et al. 1983/1984; Lehmann et al. 1987a; Vaughan-Martini et al. 1988; Vaughan-Martini and Rosini 1989).

Current molecular studies have shown that many classical features used to define taxa, such as the formation of pseudomycelium, the presence and morphology of spores, fermentation and assimilation of sugars and nitrate, have limited value. In this situation, sensitivity testing by the use of a set of mycocinogenic strains with known and different host ranges could be a very useful taxonomic tool. Although immunity to mycocins and the possible occurrence of resistant mutants limits the value of mycocin sensitivity patterns for identification, these difficulties can be overcome by using a panel of mycocinogenic strains with known and different host ranges. Mycocinotyping may then become an additional taxonomic tool with which to examine yeast classification.

10.3 Extracellular Glycolipids

While mycocinogeny was described at the beginning of 1960s, the antibiotic effect of extracellular glycolipids was only discovered 30 years later (Golubev 1992b), although these yeast compounds had long been known (Spencer et al. 1979). They were commonly considered as emulsifying agents associated with the growth of microorganisms on water-insoluble substrates. Thus far, the antibiotic activity of extracellular glycolipids has been recorded in only a few species: *Cryptococcus humicola* (Puchkov et al. 2002), *Pseudozyma flocculosa* (Cheng et al. 2003), *P. fusiformata* (Golubev et al. 2001) and *Sympodiomycopsis paphiopedili* (Golubev et al. 2004). The first species belongs to the tremellaceous yeasts, while the others are phylogenetically distributed among the Ustilaginomycetes. In this connection it should be mentioned that such compounds have long been known in smut fungi (Lang and Wagner 1987).

10.3.1 Characteristics of Glycolipids

Almost all known antifungal glycolipids contain cellobiose (4-*O*-*β*-D-glucopyranosyl-D-glucose) glycosidically linked to saturated fatty hydroxy acids (C16, C18).

So, *Ustilago maydis* produces a mixture of cellobiolipids in which cellobiose is esterified with 15,16-dihydroxyhexadecanoic or 2,15,16-trihydroxyhexadecanoic acid termed "ustilic acids". Similar compounds were also shown to be secreted by several yeasts. In *Cr. humicola* they are composed of a highly acetylated cellobiose linked to 2,16-dihydroxyhexadecanoic acid. The acyl chain forming aglycon can be replaced by 2,18-dihydroxyoctadecanoic, 2,16,18-trihydroxyoctadecanoic or 2,17,18-trihydroxyoctadecanoic acid (Puchkov et al. 2002). The structure of *P. flocculosa* glycolipid was 2-(2′,4′-diacetoxy-5′-carboxy-pentanoyl)octadecyl cellobioside (Cheng et al. 2003). The fatty acid moiety of *S. paphiopedili* cellobiolipid (Fig. 10.3) was 2,15,16-trihydroxyhexadecanoic acid (Kulakovskaya et al. 2004). The glycolipids secreted by *P. fusiformata* were only incompletely characterized but they are also cellobiose lipids (Golubev et al. 2001).

As with mycocins, antifungal activity of extracellular glycolipids occurs under acidic conditions, but in contrast to them, glycolipids have a much broader range of action. Their killing patterns are not taxonomically specific, but also include many of both ascomycetous and basidiomycetous yeasts, as well as mycelial fungi (Fig. 10.4, Table 10.2). Increased sensitivity of related organisms can only be noted (Golubev and Shabalin 1994; Golubev et al. 2001, 2004; Kulakovskaya et al. 2003).

10.3.2 Mode of Action

Cellobiolipids have a fungicidal action which involves a gross increase of the nonspecific permeability of the cytoplasmic membrane. Sensitive cells treated with cellobiolipids showed a leakage of intracellular compounds, including electrolytes

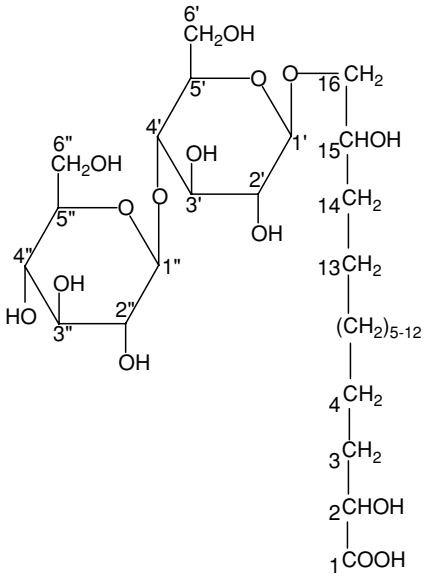

Fig. 10.3. Extracellular cellobiose lipid of *Sympodiomycopsis paphiopedili* VKM Y-2817

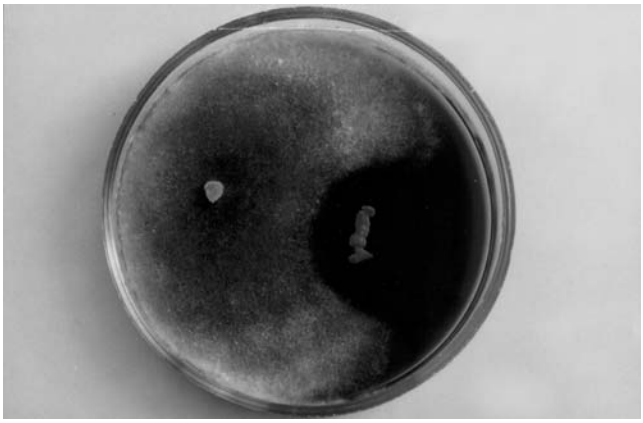

Fig. 10.4. Inhibitory activity of *Sympodiomycopsis paphiopedili* (streak) against *Sclerotinia sclerotiorum* (lawn). Glucose–peptone agar (pH 4.0, 20°C)

and ATP (Puchkov et al. 2001; Kulakovskaya et al. 2003). Probably, cellobiolipids can intercalate into the lipid matrix. This intercalation leads to permeability changes which disturb the membrane order and integrity, resulting in cytoplasmic disorder, cell disintegration and eventually in cell death.

Table 10.2 The genera of fungi (251 species tested) sensitive to the cellobiolipid of *Sympodiomycopsis paphiopedili*

Agaricostilbum	*Gymnosporangium*	*Schizosaccharomyces*
Arthroascus	*Holtermannia*	*Sclerotinia*
Arxula	*Issatchenkia*	*Septobasidium*
Bensingtonia	*Itersonilia*	*Sorosporium*
Bullera	*Kluyveromyces*	*Sphacelotheca*
Bulleromyces	*Kockovaella*	*Sporidiobolus*
Candida	*Kurtzmanomyces*	*Sporisorium*
Citeromyces	*Leucosporidium*	*Sporobolomyces*
Clavispora	*Lipomyces*	*Stephanoascus*
Cryptococcus	*Lodderomyces*	*Sterigmatomyces*
Cystofilobasidium	*Malassezia*	*Taphrina*
Debaryomyces	*Mastigobasidium*	*Tilletia*
Dekkera	*Mastigomyces*	*Tilletiaria*
Diaporthe	*Metschnikowia*	*Tilletiopsis*
Dioszegia	*Microbotryum*	*Torulaspora*
Dipodascus	*Mrakia*	*Trichosporon*
Endomyces	*Myxozyma*	*Trimorphomyces*
Endophyllum	*Nadsonia*	*Tsuchiyaea*
Erythrobasidium	*Pichia*	*Udeniomyces*
Exobasidium	*Protomyces*	*Ustilago*
Farysia	*Pseudozyma*	*Wickerhamia*
Fellomyces	*Puccinia*	*Williopsis*
Filobasidiella	*Rhodosporidium*	*Xanthophyllomyces*
Filobasidium	*Rhodotorula*	*Zygosaccharomyces*
Guilliermondella	*Saturnispora*	*Zygozyma*

Obviously, a difference in the cytoplasmic membrane lipid composition (specifically, sterol content, Avis and Bélanger 2002) among fungi is the major factor in determining their level of sensitivity to cellobiolipids. A difference in the cell envelope composition and surface charge may possibly also cause some variations in the sensitivity of fungi to these membrane-damaging compounds.

10.3.3 Genetic Basis

The synthesis of extracellular glycolipids may be controlled by chromosomal genes, since neither dsRNA viruses nor DNA plasmids were detected in any species secreting antifungal cellobiolipids (Golubev and Shabalin 1994). This is consistent with the observation that such a phenotype is cureless (Golubev and coworkes 2001, 2004). Moreover, multiple genes are probably involved in glycolipid production in *P. flocculosa* (Cheng et al. 2003).

10.4 The Ecological Role of Antagonistic Yeasts

Negative antagonistic interactions between microbes are more frequent than positive ones; and, indeed, production of antibiotic substances has long been established as a commonplace phenomenon among many groups of prokaryotic and eukaryotic microorganisms. Although yeasts are consistent and normal members of the microbial biota associated with plants, animals, soils and waters in all geographic areas, until recently they have been considered mainly as suppliers of growth-promoting substances (vitamins, sterols) for other members of biocenoses. The discovery of antagonistic activity in yeasts has important implications for a proper understanding of their role in natural communities. At the present time, only mycocinogeny can be considered from this viewpoint as secretion of fungicidal glycolipids by yeasts has only been revealed quite recently and the impact of this phenomenon on natural fungal communities remains to be studied.

Mycocin-producing strains of yeasts may be isolated from various sources, but they occur much more frequently in habitats where yeast populations reach relatively high densities, so that competition is more intense. Table 10.1 shows that most of the species listed are associated with plants and food; while mycocinogenic cultures have not been found among specific soil species (*Cryptococcus terreus*, *Lipomyces* spp., *Schizoblastosporion starkeyi-henricii*) that have been isolated exclusively from soils which usually have low yeast concentrations (Golubev, unpublished data). During the 4-year study of yeast communities from the phyllosphere and soil of the Prioksko-terrasny biosphere reserve (Russia), mycocinogenic strains were found to comprise one quarter of all isolates from steppe plants that had total yeast counts in the range 10^5–10^6 CFU/g, whereas no mycocinogenic yeasts were isolated from soils where the total numbers of yeasts were about 10^4 CFU/g (Golubev and Golubeva 2004).

Mycocinogeny has a marked impact on competition between related yeasts for their favoured ecological niche. This is because sensitivity to mycocins is specific, and only the yeast cells containing receptors for the mycocins are sensitive to them. The competitive advantage of mycocin production has been exemplified by

demonstrating the predominance of mycocin-producing *Trichosporon pullulans* isolates both in the spring exudates from birch and under laboratory-simulated conditions (Golubev et al. 2002). During the initial phase of the exudation, when the density of yeast populations is low (10^2 CFU/ml), the incidence of *T. pullulans* mycocinogenic strains was 3–4%; but towards the end of the exudation period the density increases to as much as 10^7 CFU/ml (Golubev et al. 1977). These represented 20–40% of all *T. pullulans* isolates. Mycocinogenic cultures are able to exclude sensitive ones from industrial fermentations (Vondrejs 1987).

Mycocinogenic yeasts isolated from particular habitats were found to have greater killing activity against yeasts of other habitats than against those in their own habitat. The activity (number killed per number tested) of *P. kluyveri* against yeasts from a fruit habitat was 12%, while the activity against yeasts from other habitats was 64% (Starmer et al. 1987). Further, only 9% of the yeasts originating from fruit were sensitive to *P. kluyveri* mycocinogenic strains, whereas 42% of the strains from habitats other than fruit were sensitive to the *P. kluyveri* strains tested (Starmer et al. 1992). Analogously, almost all soil isolates were sensitive to mycocins produced by yeasts originating from the phyllosphere but there were many resistant strains among isolates from plants, indicative of a selection within communities to the mycocins present (Golubev and Golubeva 2004). Clearly, the production of mycocins has an important function for mycocinogenic cells by defending an ecological niche against invading cells which have the same nutritional requirements and may occupy the same sites. In other words, mycocinogeny has a role in maintaining community composition by excluding "foreign" competitor yeasts from particular habitats.

The energy and cellular machinery used to produce mycocins cannot also be used for reproduction. Hence, mycocin synthesis may reduce the growth rate and competitiveness of mycocin producers (Pintar and Starmer 2003). Probably, the cost of mycocin production, together with spatial and temporal heterogeneity of habitats, makes possible the coexistence of mycocinogenic and sensitive populations, as is observed in natural communities.

10.5 Applications of Antagonistic Yeasts

Since its initial discovery, mycocinogeny has come to the attention of specialists in genetics, virology, biochemistry and molecular biology, who have used it as an excellent model to study host–virus interactions in eukaryotic cells (Wickner 1996), the mechanisms of regulation in eukaryotic protein processing (Riffer et al. 2002) and mycocin-based cloning vectors which are highly efficient for the effective secretion of heterologous proteins (Heintel et al. 2001). Antagonistic yeasts have also attracted attention not only from those doing applied research, but from those doing fundamental research as well.

10.5.1 Food and Fermentation Industries

Many commercial yeast strains used in the production of wine, beer and bread have been found to be sensitive to mycocins, and hence wild mycocinogenic yeasts can

cause protracted or block fermentations and negatively affect the quality of products. To protect industrial fermentations against contaminating yeasts, many attempts have been made to use suitably constructed strains with mycocinogenic activity as starter cultures (van Vuuren and Jacobs 1992; Javadekar et al. 1995; Schmitt and Schernikau 1997). In addition, mycocinogenic yeasts have been used for biotyping patented industrial strains (Buzzini and Martini 2000a; Buzzini et al. 2001).

10.5.2 Medicine

Considerable effort has also been devoted to biotyping medically important yeasts. Mycocin sensitivity patterns have been used as epidemiological markers for the intraspecific discrimination of pathogenic strains (Caprilli et al. 1985; Polonelli et al. 1985; Boekhout and Scorzetti 1997; Golubev et al. 2000). The use of mycocins as novel agents has also been proposed for treating fungal infections (Polonelli et al. 1986; Seguy et al. 1998). This use may be effective for treating superficial lesions, but mycocins cannot be used orally or intravenously, as they are protease-sensitive, antigenic, inactive at 37°C and active within a narrow pH range only. However, it was possible to obtain anti-idiotypic antibodies which apparently share the active site of the *P. anomala* mycocin and have anti-*Candida albicans* activity (Polonelli et al. 1997). Viral dsRNAs from yeasts were shown to have interferon-inducing activity (Nosik et al. 1984).

10.5.3 Agriculture

Mycocinogenic yeasts are commonly considered as promising natural biocontrol agents of plant-pathogenic fungi and in feed and food preservation from yeast spoilage (Petersson and Schnurer 1995; Walker et al. 1995; Kitamoto et al. 1999; Lowes et al. 2000). However, rare mycocins (for example, HMK mycocin of *Williopsis mrakii*) that have the broad spectra of activity, pH and temperature stability may be suitable for this purpose. Nearly all mycocins have narrow spectra of activity and are unstable. For this purpose, glycolipid-secreting yeasts are more promising as their thermostable cellobiolipids have much broader spectra of antifungal activity (Golubev et al. 1997b; Avis and Bélanger 2002).

Acknowledgement

J.A. Barnett is acknowledged for correcting the English text.

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Chapter 11

Yeasts in Soil

Alfred Botha

Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa
(e mail: abo@sun.ac.za, Tel.: +27-21-8085851, Fax: +27-21-8085846)

11.1 Introduction

Most reviews on the microbial ecology of soil barely mention the existence of soil yeasts, while the majority of ecological studies conducted on these yeasts were merely surveys utilizing classic microbiological techniques, indicating the presence of yeasts culturable on the isolation media used by the researcher. Thus, not all yeasts occurring in a particular soil sample may have been observed. Also, relatively little is known about the interactions of soil yeasts in situ, since the majority of studies in this regard were conducted in vitro or with soil microcosms under controlled conditions in the absence of a plethora of factors that may impact on soil yeast metabolism, when these organisms are growing in their natural habitat.

Nevertheless, ecological studies of culturable soil yeast populations provide some insight into the distribution of yeasts in soils and the role of soil yeast communities. In addition, in vitro studies on cardinal growth temperatures, nutrient assimilation and antibiotic resistance may provide us with a glimpse of the intrinsic abilities of particular yeasts (Lachance and Starmer 1998). Although not always a true indication of the ecological niche, the latter may provide insight into the fundamental niche of a yeast species. Similarly, binary interactions of yeasts with other microbes studied in vitro may help us to understand interactions of soil yeasts in their natural habitat (Fracchia et al. 2003). However, it must always be borne in mind that soil yeasts, in their natural habitat, may often intimately co-exist with a wide diversity of algae, moulds, prokaryotes, protists, macroscopic and microscopic fauna, as well as plant roots.

Growth and survival of a particular yeast species in soil may therefore not solely depend on the intrinsic abilities of the yeast, but is the cumulative result of a number of interactions within the soil microbial community. With the previous considerations as a background the purpose of this study was to review our current knowledge on soil yeast ecology and to provide a base for further investigations into this largely unexplored field of study.

11.2 Soil as a Habitat

A review on the ecology of soil yeasts would be incomplete without a brief discussion on their habitat, in which these fungi co-exist with other organisms. Soils consist of mineral and organic particles forming heterogeneous aggregates of various sizes, which contain a complex network of pores (Gray and Williams 1979; Young and Crawford 2004). The latter may be filled with atmospheric gasses, water vapour or aqueous solutions of various salts. Characteristic of soil as a habitat are the remarkable spatial and temporal heterogeneities regarding physico-chemical properties including available nutrients, pore size, temperature and water availability. This results in similar heterogeneities in microbial numbers and diversity, as well as biological processes brought about by soil microbes playing a pivotal role in ecosystem function.

Within the ecosystem, organic carbon acting as nutrient source for soil-borne microbial decomposers ultimately originates from plants (Wardle et al. 2004). The activity of these decomposers in turn indirectly regulates plant growth and community composition by determining the supply of available soil nutrients to plants. Plants may also provide organic carbon directly to organisms in the rhizosphere, such as root herbivores, pathogens and symbiotic mutualists like mycorrhizal fungi. These root-associated organisms and their consumers influence plants directly. In addition, they also influence the flow of energy and nutrients between plants and the decomposers. Considering these interactions of plants with soil microbes, the findings that vegetation type, or even individual plants, may influence the composition of soil microbial communities (Saetre and Bååth 2000; Grayston et al. 2004) come as no surprise.

It must be remembered, however, that despite the carbon inputs originating from plants and deposited in soil as dead organic matter, the amounts of available nutrients in soils are limited (Gray and Williams 1971; Poindexter 1981; Williams 1985). Water extracts from soils may contain less than 2 and 5 $\mu g\ ml^{-1}$ amino acids and carbohydrates respectively (Ko and Lockwood 1976). It is therefore understandable that soils are generally viewed as being in a state of oligotrophy (Williams 1985).

Soil yeasts are known to be able to grow under oligotrophic conditions (Kimura et al. 1998), but are also isolated from relatively nutrient rich habitats such as the rhizosphere (Kvasnikov et al. 1975) and organic debris like dung and decaying toadstools (Fell and Statzell-Tallman 1998). However, considering the previously mentioned heterogeneity of soil as a habitat, it can be expected that yeast numbers and species would be unevenly distributed in soil and would show temporal variation, depending on ever-changing environmental conditions.

11.3 Yeast Distribution in Soil

It is commonly known that yeasts occur in a wide range of soil types from a vast diversity of geographical areas ranging from the artic zones to the tropics (Carmo-Sousa 1969; Phaff and Starmer 1987; Spencer and Spencer 1997; Lachance and Starmer 1998). Many different ascomycetous and basidiomycetous yeasts were found in soil. In most cases, however, yeast numbers and species composition were found to be distributed quite unevenly and the numbers of these fungi are low com-

pared with that of prokaryotes and moulds. The number of yeasts that mostly occur in the top 10 cm of soil as a result of their ability to grow aerobically on a wide diversity of carbon compounds may range from less than 10 to 10^6 culturable cells per gram of soil. It was found that nutrient-rich moist soil may support a wider diversity of yeast species than nutrient-poor arid soils (Spencer and Spencer 1997). About 25–50% of the yeasts in nutrient-rich moist soils were found to be able to ferment carbohydrates. More yeasts are usually found in the soil beneath plants bearing fruits rich in carbohydrates, since the latter may act as a nutrient-rich yeast inoculum when the spoiled fruit is deposited in the soil (Phaff et al. 1966). Soil further away from a plant usually contains fewer yeast species associated with that particular plant. This phenomenon was already demonstrated in the 1950s for oak and pine trees and the associated genera *Saccharomyces* and *Schizosaccharomyces* (Carmo-Sousa 1969). More recently it was demonstrated that *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* can be isolated from oak-associated soils using enrichment culturing medium containing 7.6% (v/v) ethanol (Sniegowski et al. 2002).

Other yeast species, belonging to the genera *Cryptococcus*, *Debaryomyces*, *Lipomyces* and *Schizoblastosporion* were repeatedly isolated from various soils, indicating that the ecological niches of these fungi occur in soil (Phaff and Starmer 1987). The ability of these autochthonous soil yeasts to survive in this habitat was ascribed to a number of traits. For example, most of these yeasts possess a wide spectrum of metabolic activities enabling them to assimilate the hydrolytic products of plant materials, generated by moulds and prokaryotes. *Lipomyces* and *Williopsis saturnus* are able to produce resistant spores. Some like *Lipomyces*, *Cryptococcus* and *Rhodotorula* produce exopolymeric capsules and it was suggested that this would enable them to survive better in habitats that are poor in available nutrients. It was found that semi-arid soils, low in nutrients and moisture, were mostly populated by cryptococci and related basidiomycetous yeasts (Spencer and Spencer 1997). Interestingly, it was found that some soil cryptococci are able to produce extracellular polymeric substances (EPS) and form biofilms when cultivated in flow cells under oligotrophic conditions (Joubert et al. 2003). The formation of these biofilms is a known mechanism whereby microbes are able to sequester and concentrate nutrients while growing in low-nutrient environments (Decho 1990). Figure 11.1 illustrates the ability of a common soil inhabitant, *Cryptococcus laurentii*, to grow under oligotrophic conditions as a biofilm on soil particles such as sand grains.

11.4 The Role of Yeast Communities in Soil

11.4.1 Dissipation and Transformation of Energy Through the Ecosystem

Within a functional soil ecosystem, dissipation and transformation of energy continually occurs away from the primary producers, i.e. plants, into organisms farther along the food chain, thus supporting a wide diversity of heterotrophs, including microbes and macroscopic fauna (Coleman and Crossley 1996). The primary agents of decomposition, able to degrade compounds directly or indirectly derived from plants, were found to be bacteria and fungi. However, the yeasts are also part of the fungal domain and the vast majority of yeasts discovered so far are saprotrophs

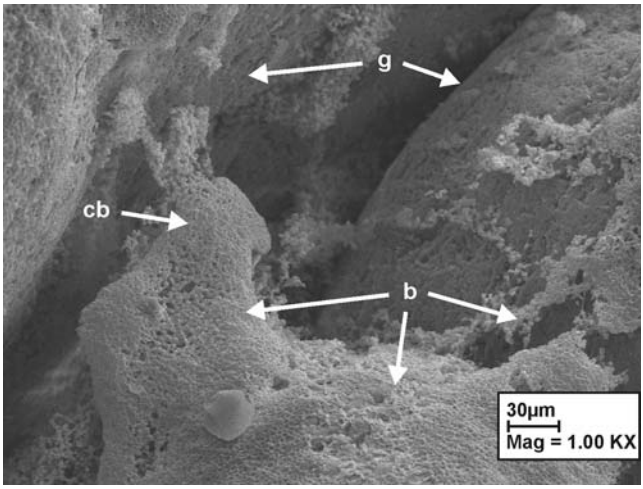

Fig. 11.1. Scanning electron micrograph illustrating biofilm formation (*b*) by *Cryptococcus laurentii* under oligotrophic conditions on sand grains (*g*), as well as a connective bridge (*cb*) formed between the sand grains as a result of excessive extracellular polymeric substances (*EPS*) production by the yeast. Sand grains with attached yeast cells were mounted onto stubs, sputter-coated with gold, and viewed unfixed and fully hydrated with a LEO 1430 VP scanning electron microscope operated at 7 kV. (Photograph; L. Joubert, Department of Microbiology, University of Stellenbosch)

contributing to mineralization processes in the environment by utilizing a wide range of organic carbon compounds. Some of these yeasts have the ability to ferment carbohydrates, but many are able to respire both carbohydrates and non-fermentable organic compounds (Kurtzman and Fell 1998a). The occurrence of autochthonous soil yeasts (Lachance and Starmer 1998) indicates that yeasts do play a role in the decomposition and dissipation of energy within the soil ecosystem as mentioned before for bacteria and fungi in general.

Most of the yeast species frequently encountered in soil (Table 11.1) are able to aerobically utilize L-arabinose, D-xylose and cellobiose. These carbohydrates are known to be products of the enzymatic hydrolysis of lignocellulosic plant materials by bacteria and moulds (Bisaria and Ghose 1981; Tomme et al. 1995). Some of the frequently encountered yeasts in soil were also found to assimilate intermediates of lignin degradation i.e. ferulic acid, 4-hydroxybenzoic acid and vanillic acid (Middelhoven 1993; Sampaio 1990). However, despite their ability to utilize the degradation products of woody material, it was suggested that soil yeasts do not play a major role in the decomposition of organic matter, because of their relatively low numbers compared with those of moulds and prokaryotes occurring in the same habitat (Phaff and Starmer 1987). Nevertheless, in some habitats, such as in the arctic zones, yeasts may be the dominant culturable soil microbes (Wynn-Williams 1982). This will enable them to make a significant contribution to decomposition of soil organic matter.

Table 11.1 Selected characteristics of the most abundant soil yeast species found during ecological surveys

Species according to Kurtzman and Fell (1998a)	Original identification during survey	Reference	Veg[a]	F[b]	Carbohydrate assimilation[c]			Aromatic compound assimilation[d]		
					xyl	ara	cel	pHA	VA	FA
Cryptococcus albidus	*Cryptococcus albidus/ diffluens/ terricolus*	Di Menna (1965); Bab'eva and Azieva (1980); Moawad et al. (1986); Polyakova et al. (2001)	F; G; T; V	–	+	+	+	+	+	+
Cryptococcus curvatus	*Candida curvata*	Di Menna (1965); Moawad et al. (1986)	F; G; V	–	+	v	+	+	–	–
Cryptococcus gastricus	*Cryptococcus gastricus*	Bab'eva and Azieva (1980)	T	–	+	+	+	+	–	–
Cryptococcus gilvescens	*Cryptococcus gilvescens*	Polyakova et al. (2001)	T	–	–	+	+	ND	ND	ND
Cryptococcus humicolus	*Candida humicola*	Di Menna (1965)	G; V	–	+	+	+	+	–	–
Cryptococcus laurentii	*Cryptococcus laurentii*	Bab'eva and Azieva (1980); Sláviková and Vadkertiová (2000)	F; T	–	+	+	+	+	–	–
Cryptococcus podzolicus	*Candida podzolica*	Bab'eva and Reshetova (1975)	V	–	+	+	+	–	–	–
Cryptococcus terreus	*Cryptococcus terreus*	Di Menna (1965)	G; V	–	+	+	+	+	+	v
Filobasidium uniguttulatum	*Cryptococcus uniguttulatus*	Bab'eva and Azieva (1980)	T	–	+	+	–	–	–	–
Cystofilobasidium capitatum	*Cystofilobasidium capitatum*	Sláviková and Vadkertiová (2000)	F	–	+	v	+	–	–	–
Leucosporidium scottii	*Leucosporidium scottii*	Sláviková and Vadkertiová (2000)	F	–	+	v	+	+	+	v

Continues

Table 11.1 Selected characteristics of the most abundant soil yeast species found during ecological surveys—*cont'd*

Species according to Kurtzman and Fell (1998a)	Original identification during survey	Reference	Veg[a]	F[b]	Carbohydrate assimilation[c]			Aromatic compound assimilation[d]		
Mrakia frigida	*Candida curiosa/gelida*	Bab'eva and Azieva (1980)	T	+	+	+	+	–	–	–
Rhodotorula aurantiaca	*Rhodotorula aurantiaca*	Sláviková and Vadkertiová (2000)	F	–	+	v	v	+	–	–
Rhodotorula glutinis	*Rhodotorula glutinis*	Moawad et al. (1986); Sláviková and Vadkertiová (2000)	F; V	–	v	v	v	+	+	v
Rhodotorula mucilaginosa	*Rhodotorula mucilaginosa*	Polyakova et al. (2001)	T	–	+	v	v	+	+	+
Schizoblastosporion starkeyi-henricii	*Schizoblastosporion starkeyi-henricii*	Di Menna (1965)	G	–	–	–	–	+	–	–
Sporobolomyces roseus	*Sporobolomyces roseus*	Polyakova et al. (2001)	T	–	v	v	v	+	+	+
Trichosporon cutaneum	*Trichosporon cutaneum*	Di Menna (1965); Sláviková and Vadkertiová (2000)	G; F; V	–	+	+	+	+	–	–

[a]General type of vegetation covering the soil that was sampled; *F* forest, *G* grass, *T* tundra, *V* various
[b]Ability of species to ferment carbohydrates according to Kurtzman and Fell (1998b); + able to ferment at least glucose, – unable to ferment carbohydrates
[c]Ability of species to aerobically assimilate carbohydrates according to Kurtzman and Fell (1998b); *xyl* D-xylose, *ara* L-arabinose, *cel* cellobiose, + positive, – negative, *v* variable
[d]Ability of species to aerobically assimilate aromatic compounds according to Middelhoven (1993) and Sampaio (1999); *pHA* *p*-hydroxybenzoic acid, *VA* vanillic acid, *FA* ferulic acid, + positive, – negative, *v* variable, *ND* not determined.

As part of the soil microbial biomass, yeasts may serve as a food source for bacteria and microbivorous eukaryotes. Invertebrate fauna such as micro-arthropods and nematodes, as well as protista are major soil predators (Bardgett and Griffiths 1997) and are known to graze upon soil yeast populations, thereby ensuring the continual dissipation and transformation of energy through the soil ecosystem. Both ascomycetous and basidiomycetous yeasts were digested in the gut of the soil millipede *Pachyiulus flavipes* (Byzov et al. 1998), while nematodes, belonging to the genera *Alaimus*, *Panagrellus* and *Rhabditis* were found to graze upon yeasts e.g. *S. cerevisiae* (Hechler 1970; Yeates 1971). Another soil nematode, *Caenorhabditis elegans*, was found to graze upon *C. laurentii* and *C. kuetzingii* (Nicholas 1984; Mylonakis et al. 2002). Under laboratory conditions, *C. elegans* was able to survive on both these yeast strains as the sole food source, maintaining brood sizes similar to when it is cultivated on *Escherichia coli*, the usual laboratory food source for this nematode.

Protista, of which the activity is limited to the water film within soil pores, may contribute up to 30% of the total net nitrogen mineralization that takes place in soil (Bardgett and Griffiths 1997). Although it is known that mycophagous protista do exist, very little is known about their interactions with yeasts. However, the fact that the majority of yeasts are found in the top cm of soil and that protists are more numerous in this part of the soil suggests that interactions between these two groups are inevitable within the natural soil environment. *Acanthamoeba* is a protist occurring in soil (Sawyer 1989) and it was found to feed on yeasts (Allen and Davidowicz 1990). Benting et al. (1979) found that when *Acanthamoeba polyphaga* was incubated with some strains of *Cryptococcus neoformans*, the amoeba was able to phagocytize and kill up to 99% of the yeast cells of within 9 days.

Yeasts were also found to be the nutrient source of bacteria, a number of Gram-positive bacteria isolated from soil were found to cause lysis of *S. cerevisiae* (Goto-Yamamoto et al.1993). It was also demonstrated that a group of Gram-negative bacteria commonly found in soil, the *Myxobacteria* (Reichenbach 1999), are able cause lysis of a wide diversity of yeasts including *Cryptococcus albidus*, *Filobasidium capsuligenum* (syn. *Candida japonica*), *Geotrichum candidum*, *Rhodosporidium toruloides, Rhodotorula glutinis* and *S. cerevisiae* (Yamanaka et al. 1993).

11.4.2 Dissolution of Rock and Release of Nutrients

It has also become apparent that some yeasts, because of their oligotrophic capabilities, may not only occur within soil, but are able to grow on rocks above ground (Sterflinger and Prillinger 2001; Burford et al. 2003). Such epilithic and endolithic fungi comprise a significant proportion of the microbial community in a wide range of rocks, including granite, gypsum, limestone, marble, sandstone and siliceous rock types such as silica, silicates and aluminosilicates. The yeasts and other fungi occurring in these rocks may contribute to weathering thereof, soil formation, as well as to the supply of soluble nutrients to the soil microbial community (Gadd and Sayer 2000; Burford et al. 2003). Dissolution of rock as a result of fungal metabolic activity may therefore result in phosphorous, sulphur and trace elements becoming bio-available. It was found that this dissolution is the result of the reactions of

metabolic end-products such as H^+ and organic acids, as well as siderophores. Solubilization of insoluble metal compounds in ores may therefore be the result of protonation of the anion of the metal compound, decreasing its availability to the cation. Sources of protons are the proton-translocating ATPase of the fungal plasma membrane and organic acids produced by these fungi. Soluble metal complexes may also be formed between organic acid anions and metal cations, depending on the relative concentrations of these ions, pH and the stability constants of the various complexes. Another mechanism whereby metals, specifically iron(III), are brought into solution and made bio-available is by reacting with low molecular mass (500–1,000 Da) iron-chelating compounds called siderophores. These ferric-specific ligands were found to be produced by a wide diversity of microbes (Neilands 1981), including basidiomycetous yeasts, such as *Leucosporidium scottii*, *Rhodotorula glutinis, R. mucilaginosa* and *Sporobolomyces roseus* (Atkin et al. 1970) which are commonly found in soil (Table 11.1).

Interestingly, both ascomycetous and basidiomycetous genera such as *Candida*, *Lipomyces, Rhodotorula* and *Trichoderma* were isolated from rock substrates (Sterflinger and Prillinger 2001; Burford et al. 2003), with euascomycetes taxa found in an even wider diversity of rock types. The latter taxa, which include meristematic fungi and the so-called black yeasts, may grow yeast-like in culture and represent the genera *Aureobasidium*, *Exophiala*, *Hormonema*, *Hortaea*, *Lecythophora*, *Phaeotheca*, *Rhinocladia* and *Sarcinomyces*. The melanized cell walls of these fungi, chlamydospore formation, as well as their clump- or yeast-like morphology with optimal surface-to-volume ratio, were found to make them well adapted for epilithic stress conditions, such as high levels of UV irradiation, large variations in temperature and available water, as well as low nutrient conditions (see Chap. 20).

11.4.3 Soil Aggregate Formation

Another way in which yeast populations may interact with their physico-chemical environment, and thus impact on biological processes in soil, is by producing EPS. Production of these extracellular compounds enables soil yeasts from the genera *Cryptococcus* and *Lipomyces* to play a role in the formation of soil aggregates, thereby impacting on soil structure (Bab'eva and Moawad 1973; Vishniac 1995). Figure 11.1 illustrates a connective bridge formed between two sand particles, as a result of cryptococcal EPS production, thereby contributing to aggregate formation. A high degree of aggregate stability and favourable soil structure was found to be associated with a decrease in erodibility, enhanced porosity and water-holding capacity, as well as improved soil fertility (Bronick and Lal 2004).

11.5. Ecological Interactions

11.5.1 Interactions with the Physico-Chemical Environment

Using sterile sandy loam soil as a growth medium it was demonstrated that the growth rate of the common soil yeast *C. albidus,* over the first 24 h after inoculation, was higher in the presence of 3–9% than in the presence of 1 or 2% added water

(Vishniac 1995). After monitoring yeast populations in different forest soils, seasonal changes in population sizes were partly ascribed to changes in soil moisture content (Sláviková and Vadkertiová 2000). Other factors are also known to impact on soil yeast populations. After sampling different soil types, it was found that a positive correlation (at 1% level) exists between soil yeast population size and both organic carbon (r=0.884) and organic nitrogen content (r=0.829) of the soil (Moawad et al. 1986). While no significant correlation was observed between the number of soil yeasts, soil $CaCO_3$ content, pH or soil texture. However, the study did not take into account the seasonal changes in the physico-chemical composition of the soils.

An investigation of the post-fire effects of vegetation covering different soils during a hot dry summer on the sizes of culturable yeast populations in the top 100 cm of soil revealed that yeast populations started to recover during the onset of the cooler, wetter weather of autumn (Cilliers et al. 2004). During this period the availability of macronutrients (P, K, Na, NO_3) was an important determinant of soil yeast population sizes. After further recovery during winter and spring, the most important determinants of soil yeast population sizes, however, were Mg, Na and soil texture.

Soil yeast populations, on the other hand, were also found to impact on the chemical composition of soils. Results indicated that nitrification of added ammonium, the hydrolysis of urea and the subsequent nitrification of released ammonium, all of which were stimulated after sugar beet emendation, may have largely been due to increased soil yeast numbers (Wainwright and Falih 1996). During these experiments representatives of *G. candidum* and *Williopsis californica* were found to increase in number in the emended soil.

Most of the previously mentioned studies on yeast interactions with their physico-chemical environment highlighted interactions of yeast populations in soil, rather than the interactions of individual species or strains in this habitat. The majority of studies conducted on the interactions of individual yeast strains with their physico-chemical environment were done in vitro mostly either to elucidate the physiology of the yeast cell while growing in monoculture (Rose and Harrison 1987) or to classify pure yeast cultures during taxonomic investigations (Kurtzman and Fell 1998a). These studies provided insight into the intrinsic abilities of particular species and were essential in the rapid development of yeast biotechnology. In addition, the studies highlighted the diversity of yeast species occurring in nature. During the course of these studies, however, in vitro investigations into cardinal growth temperatures, nutrient assimilation and antibiotic resistance may have provided only limited insight into the ecological niche of certain yeast species (Lachance and Starmer 1998). For example, while the ability of *Cryptococcus podzolicus* to assimilate carbohydrate products of the enzymatic hydrolyses of lignocellulosic plant materials (Table 11.1) may explain its presence in soil containing decaying plant material, the inability of this species to utilize monomeric aromatic acids associated with podzolic soils (Lunström et al. 2000) as sole carbon sources (Sampaio 1999) does not explain its presence in these soils (Bab'eva and Rheshetova 1975). Similar anomalies were uncovered regarding the ability of soil yeasts in their natural habitat to respond to perturbations with heavy metals. During enumeration of yeasts in virgin soil with a relatively low natural Cu content (approximately 2 mg kg^{-1} Cu), plate counts were dominated by hymenomycetous strains able to tolerate

Cu levels of up to 500 mg l^{-1} Cu in a liquid medium (Cornelissen et al. 2003). When 1,000 mg kg^{-1} Cu was added to the soil, a shift in the soil yeast community occurred, resulting in urediniomycetous strains, with similar high Cu tolerance levels, dominating the enumeration plates. The survival and growth of a particular yeast strain in soil therefore seems not to depend solely on the intrinsic abilities of the particular strain to maintain itself within the chemical environment. Other factors, such as interactions with members of the soil microbial community, may also be determinative factors in shaping the species composition of soil yeast populations.

11.5.2 Interactions with Soil Organisms

A list of the possible microbe–microbe interactions that terrestrial yeasts may participate in was provided by Lachance and Starmer (1998). These included amensalism, commensalism, competition, mutualism or symbiosis, neutralism, parasitism, predation and synergism. Taking the wide diversity of organisms into account that intimately coexist with soil yeasts in their natural habitat, it is conceivable that at a particular time a yeast in its ecological niche may participate in more than one of these microbe–microbe interactions. In addition, these yeasts are known to be grazed upon by soil fauna and protists. Therefore, to study a single interaction between two soil organisms in their natural habitat would seem to be a daunting task. Nevertheless, evidence for the intrinsic ability of some soil yeasts to participate in a number of these interactions was uncovered in different studies.

5.2.1 Amensalism

It was found that a yeast commonly found in soil, *C. laurentii*, is able to inhibit growth of filamentous fungal post-harvest pathogens on damaged fruit (Roberts 1990; Chand-Goyal and Spotts 1997). Environmental conditions, such as temperature (Roberts 1990) and available nutrients in agar plates (own unpublished results), were found to impact on the antagonistic effect of *C. laurentii* on filamentous fungi. No in situ experiments on yeast antagonism, however, have been conducted in bulk soil in the absence of plant roots. Also, the exact mode of action of this antagonistic effect of yeasts is still unclear, although it has been suggested that amensalism (chemical interference) such as killer activity or the production of enzymes, e.g. glucanases, may play a role (Roberts 1990; Fredlund et al. 2002; Masih and Paul 2002). In addition, the antagonistic effect has been ascribed to nutrient competition.

5.2.2 Competition

Considering the oligotrophic state of most soils (Williams 1985) the environmental factors most likely to be in short supply, and hence the objects of competition, are carbon and nitrogen sources (Lockwood 1992). Competition for ferric iron was also found to occur quite commonly in soil. It is known that such competition for nutrients impacts on the species composition of soil microbial communities. For example, it was demonstrated that yeasts such as *Candida tropicalis* emerge as dominant *n*-alkane-utilizing microbes when soil microcosms containing inocula from petro-

leum contaminated soil were treated with a mixture of n-alkanes (Schmitz et al. 2000). Competition experiments in soil microcosms with *n*-alkane-utilizing microbes from different culture collections confirmed that yeasts overgrow bacteria in sandy soil. This was partly ascribed to the acidification of the soil as a result of yeast metabolic activity, because when bentonite, a clay mineral with high ion-exchange capacity, was added to the soil, yeasts and bacteria belonging to the genus Pseudomonas coexisted in similar numbers. It was also found that *n*-alkane-utilizing yeast strains from culture collections, representing different yeast species, showed different levels of competitiveness in these *n*-alkane-treated microcosms. Strains of *Arxula adeninivorans*, *Candida maltosa* and *Yarrowia lipolytica* overgrew strains of *Candida shehatae*, *C. tropicalis* and *Pichia stipitis*. However, *C. maltosa* and *Y. lipolytica* were able to coexist in equal number within these soil microcosms.

5.2.3 Predation

While studying binary interactions of predatory yeasts with their prey on agar plates, it was found that some *Saccharomycopsis* strains are able to prey on a range of ascomycetous and basidiomycetous yeasts by forming infection pegs to penetrate and kill these organisms (Kreger-van Rij and Veenhuis 1973; Lachance and Pang 1997; Lachance et al. 2000). It can therefore be assumed that such interactions will also occur in soil under the appropriate environmental conditions, especially since two of the *Saccharomycopsis* species associated with predation, i.e. *S. fermentans* and *S. javanensis*, have been isolated from soil (Kurtzman and Smith 1998).

5.2.4 Synergism and Antagonism Regarding Nematodes

Recently a synergistic relationship was discovered involving ethanol production by *S. cerevisiae* resulting in enhanced growth of *Acinetobacter*, a bacterium which may occur in the same ecological niche as the yeast (Smith et al. 2004). The ethanol was found to also enhance pathogenicity of the bacterium towards the soil-borne predator *C. elegans*. It was suggested that by producing ethanol the yeast may therefore be able to indirectly reduce the numbers of its predator.

When preyed upon some yeast species may also exert a lethal effect directly on their predator. For example, when *C. neoformans*, a yeast periodically isolated from soil, was used as a food source for the soil nematode *C. elegans,* it caused distention of the nematode intestine when ingested (Mylonakis et al. 2002). The lethal effect was ascribed to the yeast capsule, as well as to a series of genes associated with mammalian virulence. However, killing of the nematode by an acapsular strain of *C. neoformans* was also observed. It was speculated that the nematode was killed either by toxins produced by the yeast, or by toxic components released during degradation of the cell wall within the nematode.

11.6 Yeasts Associated with the Rhizosphere

The rhizosphere is the narrow zone of soil extending for a few millimetres from the plant root surface out into the surrounding soil (Huang and Germida 2002). It may

also be considered as an interface through which energy is channelled away from the plant to the soil biota of the belowground ecosystem, because up to 18% of the carbon assimilated during photosynthesis is released from the roots. This may result in larger microbial populations in the rhizosphere than in the bulk soil away from the roots. As a result of root metabolic activity the chemical characteristics of the rhizosphere, such as pH, redox potential, as well as concentration and composition of organic compounds, may differ from those of the bulk soil. It was also proposed that, depending on soil conditions, the rhizosphere may select for specific microbial populations. This phenomenon was demonstrated when 18S ribosomal DNA (rDNA) fragments, that were amplified from soil and maize rhizosphere DNA, were cloned and sequenced (Gomes et al. 2003). On the basis of the sequence similarity of these rDNA fragments with known 18S rDNA sequences in a database, it was concluded that the rhizospheres of young maize plants seemed to select for *Ascomycetes* belonging to the order *Pleosporales*, while different members of the *Ascomycetes*, as well as basidiomycetous yeasts were detected in the rhizospheres of senescent maize plants. A group of filamentous fungi commonly associated with the rhizosphere and known to form mutualistic symbioses with plants is mycorrhizal fungi (Huang and Germida 2002). These fungi may facilitate uptake of up to 80% of the phosphorous and 25% of the nitrogen requirements of the host plant (Marschner and Dell 1994). In turn the plant provides photosynthate for the fungus. This nutrient exchange takes place via specialized fungal structures intimately associated with host root cells. However, a wide diversity of other microbes also occur in the rhizosphere; some are known to fix N_2, some are known to degrade complex organic compounds, thus participating in soil mineralization processes, while the ecological role of others has not been fully established yet.

A variety of both ascomycetous and basidiomycetous yeasts were isolated from the rhizosphere (Table 11.2). If the redox potential in the rhizosphere should decrease as a result of water logging (Huang and Germida 2002) some of these yeasts will still able to grow as result of a fermentative metabolism. It was found that, similar to the microbial populations in general, larger yeast populations normally exist in the rhizosphere than in the bulk soil away from the roots (Moawad et al. 1986). Root exudates containing a variety of potential yeast carbon and nitrogen sources (Table 11.3) may contribute to the growth and maintenance of yeast populations on or near the roots. As the chemical profiles of these exudates differ with plant species, genotypes and growth conditions (Fan et al. 2001) it may be assumed that these differences impact on the species composition of the yeast community in the rhizosphere. Figure 11.2 illustrates a yeast microcolony on the rhizoplane of a sorghum seedling growing in washed sand containing no carbon sources other than those provided by the plant. Figure 11.3 illustrates the relative abundance of yeasts in the rhizosphere and in the bulk soil away from the roots.

Although the biological interactions of the rhizosphere yeasts are largely unknown, it can be assumed that similar interactions would occur in the rhizosphere as discussed before. It was found that inoculation of legumes with viable *S. cerevisiae* increased nodulation and arbuscular mycorrhizal (AM) fungal colonization (Singh et al. 1991). Later, hyphal growth of the AM fungus *Glomus intraradices* colonizing cucumber roots was found to be enhanced by the presence of baker's dry yeast

Table 11.2 Some yeasts species that were found to occur in the rhizosphere during a number of surveys

Species according to Kurtzman and Fell (1998a)	Original identification during survey	Reference	F[a]
Bullera species	*Bullera* species	De Azeredo et al. (1998)	–
Candida azyma	*Candida azyma*	De Azeredo et al. (1998)	–
Candida krusei	*Candida krusei*	Kvasnikov et al. (1975)	+
Candida maltosa	*Candida maltosa*	De Azeredo et al. (1998)	+
Cryptococcus albidus	*Cryptococcus albidus/diffluens*	Kvasnikov et al. (1975); Moawad et al. (1986); de Azeredo et al. (1998)	–
Cryptococcus curvatus	*Candida curvata*	Moawad et al. (1986)	–
Cryptococcus humicolus	*Candida humicola*	Moawad et al. (1986)	–
Cryptococcus laurentii	*Cryptococcus laurentii*	Kvasnikov et al. (1975); de Azeredo et al. (1998)	–
Debaryomyces hansenii	*Debaryomyces hansenii/kloeckeri*; *Torulopsis famata*	Moawad et al. (1986); de Azeredo et al. (1998)	v
Debaryomyces polymorphus	*Debaryomyces phaffii/cantarelli*	Kvasnikov et al. (1975)	+
Debaryomyces vanrijiae	*Debaryomyces vanriji*	Kvasnikov et al. (1975)	v
Fellomyces species	*Fellomyces* species	De Azeredo et al. (1998)	–
Hanseniaspora uvarum	*Hanseniaspora apiculata*	Kvasnikov et al. (1975)	+
Leucosporidium scottii	*Leucosporidium scottii*	De Azeredo et al. (1998)	–
Metschnikowia pulcherrima	*Metschnikowia pulcherrima*	Kvasnikov et al. (1975)	+
Pichia guilliermondii	*Pichia guilliermondii*	De Azeredo et al. (1998)	+
Rhodotorula glutinis	*Rhodotorula glutinis*	Moawad et al. (1986); de Azeredo et al. (1998)	–
Rhodotorula minuta	*Rhodotorula minuta*	De Azeredo et al. (1998)	–
Rhodotorula mucilaginosa	*Rhodotorula mucilaginosa*	De Azeredo et al. (1998)	–
Torulaspora delbreuckii	*Torulaspora delbreuckii*	De Azeredo et al. (1998)	+
Tremella mesenterica	*Tremella mesenterica*	De Azeredo et al. (1998)	–
Trichosporon cutaneum	*Trichosporon cutaneum*	Kvasnikov et al. (1975)	–
Saccharomyces cerevisiae	*Saccharomyces cerevisiae*	De Azeredo et al. (1998)	+
Williopsis californica	*Hansenula californica*	Kvasnikov et al. (1975)	+
Williopsis saturnus	*Hansenula saturnus*	Kvasnikov et al. (1975)	+

[a]Ability of species to ferment carbohydrates according to Kurtzman and Fell (1998b); + positive, – negative, *v* variable

Table 11.3 Some components of root exudates that were found during different surveys (Campbell et al. 1997; Fan et al. 2001; Huang and Germida 2002)

Amino acids	Carboxylic acids	Carbohydrates	Phenolic acids	Mucilage	From sloughed-off cells
Alanine γ-Aminobutyric acid Asparagine Aspartic acid Glutamic acid Glutamine Glycine Isoleucine Leucine Phenylalanine Proline Serine Threonine Valine	Acetic acid *trans*-Aconitic acid Butyric acid Citric acid 2-Deoxymugineic acid Formic acid Fumaric acid Lactic acid Malic acid Malonic acid Oxalic acid Propionic acid Pyruvic acid Succinic acid Tartaric acid	Arabinose D-Fructose D-Galactose D-Glucose Maltose D-Raffinose Ribose L-Rhamnose Sucrose Trehalose Xylose	Caffeic acid Chlorogenic acid Coumaric acid Ferulic acid Hydroxyl benzoic acid Protocatechuic acid	Polygalacturonic acids	Plant cell lysates

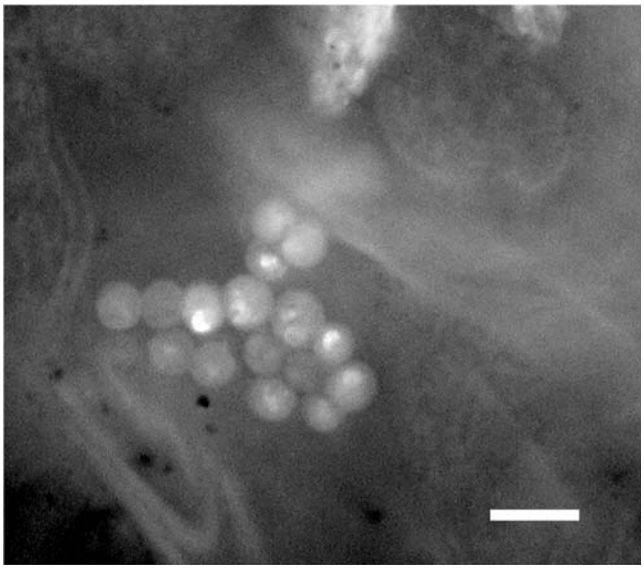

Fig. 11.2. Epifluorescence micrograph of yeast microcolony on the rhizoplane of a 2-week-old sorghum seedling growing in a medium of washed sand with no additional carbon source. Yeasts were visualized after staining with the fluorescent probes FUN 1 and Calcofluor White M2R (both from Molecular Probes). FUN 1 is a yeast-viability stain, marking intravacuolar structures of metabolically active cells. Calcofluor is a UV-excitable dye which labels yeast cell walls. The *bar* represents 10 μm. (Photograph; L. Joubert, Department of Microbiology, University of Stellenbosch)

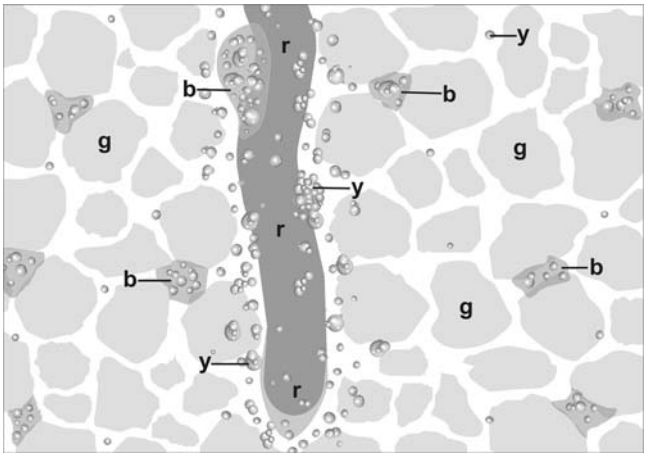

Fig. 11.3. A simplified illustration demonstrating only the distribution of soil yeasts (y) in relation to a plant root (*r*), omitting the remainder of the soil microbial community. More yeasts are found in the rhizosphere close to the root than further away from the plant in the bulk soil, where nutrients are less readily available. Yeasts in the latter nutrient-poor region are usually able to form biofilms (*b*) enabling them to sequester nutrients. These EPS-producing yeasts play a role in soil aggregate formation by binding soil particles (*g*) together

(Ravnskov et al. 1999). Uptake of phosphorous by the AM fungus, however, was unaffected by the yeast. When the ascomycetous yeast *Y. lipolytica* and *Glomus deserticola*, both entrapped in alginate gel, were used as an inoculum for tomato plants growing in soil microcosms enriched with rock phosphate, the yeast cells stimulated the level of mycorrhizal-root colonization (Vassilev et al.2001).

The basidiomycetous soil yeasts were also found to enhance AM fungal growth (Fracchia et al. 2003). *R. mucilaginosa* stimulated AM hyphal lengthening during in vitro spore germination of *Glomus mosseae* and *Gigaspora rosea*. This stimulatory effect on AM hyphal lengthening was also observed when exudates of the yeast produced in liquid culture were added to the spores. In addition, in glasshouse experiments it was found that these exudates increased AM colonization of soybean by *G. mosseae* and red clover by *G. rosea*. These results on the stimulatory effects of yeast exudates on AM fungi were later confirmed when the effects of *C. laurentii, R. mucilaginosa* and *S. kunashirensis* were studied on *G. mosseae* in similar in vitro and glasshouse trials (Sampedro et al. 2004). It was found that the presence of the yeasts, or their soluble and volatile exudates, stimulated the percentage spore germination and hyphal growth of the AM fungus. The percentage root lengths colonized by this AM fungus and plant dry matter of soybean were increased only when the soil yeasts were inoculated prior to the AM fungus.

Recently it was found that basidiomycetous yeasts, such as *Cryptococcus* and *Dioszegia* species, occur on AM fungal spores in soil, as well as on roots colonized with these fungi (Renker et al. 2004). The type of interaction that exists between these yeasts and mycorrhizal fungi is still unknown. Other yeasts isolated from the rhizosphere, i.e. *Candida valida*, *R. glutinis* and *Trichosporon asahii*, were found to be antagonistic towards growth of the fungal root pathogen *Rhizoctonia solani* (El-Tarabily 2004). The antagonistic effect of *C. valida* was ascribed to β-1,3-glucanase activity, of *R. glutinis* to the production of inhibitory volatiles and of *T. asahii* to the production of diffusible antifungal metabolites. It was also found that these yeasts were able to colonize sugar beet roots and protect the seedlings and mature plants from *R. solani* diseases in glasshouse trails. Interestingly, the three yeasts exerted a synergistic effect on disease suppression and promoted plant growth. The latter was ascribed to the production of indole-3-acetic acid and gibberellic acid by the yeast isolates used in the study. The study indicated that yeasts have the potential to be used as biological fertilizers.

11.7 Conclusion

Studies on yeast interactions were conducted on only a minute fraction of the vast diversity of soil yeasts. It can therefore be anticipated that when more yeast species are discovered and studied, more types of interactions will be uncovered and the role of yeasts in the soil ecosystem will be further elucidated. A challenge however, will be to determine the relative contribution of soil yeasts to ecological processes known to involve a wide range of soil organisms. Also, the fate of individual strains or species within a natural soil ecosystem needs to be studied. New technological advances in the field of rDNA sequence analyses of the total soil community DNA (Hunt et al. 2004), as well as fluorescence in situ hybridization (Spear et al. 1999),

make future studies of soil yeasts in their natural habitats an exciting possibility. Considering the enormous potential effect of soil yeasts on, for example crop performance, further investigation into this yet largely unexplored realm is expedient.

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Chapter 12

Yeast Biodiversity in Freshwater, Marine and Deep-Sea Environments

TAKAHIKO NAGAHAMA

Extremobiosphere Research Center, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2–15 Natsushima-cho, Yokosuka 237-0061, Japan (e-mail: nagahama@jamstec.go.jp, Tel.: +81-46-867-9698, Fax: +81-46-867-9715)

12.1 Introduction

Many investigators have reported a variety of yeasts in substrates such as water, sediment, plants, animals and other organic matter in aquatic environments, including rivers (Spencer et al. 1970, 1974; Simard and Blackwood 1971a, b; Kvasnikov et al. 1982; Sláviková and Vadkertiová 1997; Libkind et al. 2003), lakes and ponds (van Uden and Ahearn 1963; Spencer et al. 1974; Kwasniewska 1988; Sláviková et al. 1992; Sláviková and Vadkertiová 1995; Boguslawska-Was and Dabrowski 2001; Lahav et al. 2002; Libkind et al. 2004), estuaries, coasts, and mangrove areas (Goto et al. 1974a; Hagler and Mendonça-Hagler 1979; Hagler and Mendonça-Hagler 1981; Boiron and Agis 1982; Hagler et al. 1982, 1993; de Araujo et al. 1995; Soares et al. 1997; Gadanho and Sampaio 2004), oceans and the deep sea (Goto et al. 1974b; Yamasato et al. 1974; Fell 1976; Kohlmeyer and Kohlmeyer 1979; Hernandez-Saavedra et al. 1992; Nagahama et al. 1999, 2001b, 2003b; Gadanho et al. 2003). The accomplishments before 1987 have been expatiated upon in some reviews (Kriss 1963; Kriss et al. 1967; Morris 1968, 1975; van Uden and Fell 1968; Fell 1976; Hagler and Hagler 1978; Kohlmeyer and Kohlmeyer 1979; Sieburth 1979; Hagler and Ahearn 1987). In this paper, I will try to summarize the advances in the ecological and taxonomical study of aquatic yeasts during the last 2 decades, and will place in that context our investigation of yeasts taken from deep-sea floors in the Pacific Ocean.

12.2 Sampling and Isolation

The sampling methods for yeast isolation do not differ fundamentally from those used for bacteria. However, because the frequency of yeasts in natural aquatic environments is lower than that of bacteria, especially in places with low nutrient conditions, a higher volume of samples is required. Sterile bottles, boxes or containers have been used in various samplings from such accessible sites as the surface or

shallow regions of marine and freshwater environments. Whereas this sampling method is relatively easy to implement, sampling from offshore and deep-sea regions involves higher expenses and more complicated equipment. For water sampling, some known samplers are the Nansen bottle, the Niskin sampler and the van Dorn sampler. In order to avoid contamination, sterile samplers, such as the Niskin biosampler (or some modified form of it), can be used, as formerly discussed by Fell (1976). The J-Z sampler (or some modified form of it) has also been used frequently in bacterial studies. Yeasts in aquatic sediments have been studied less (Hagler and Ahearn 1987). The sampling of deep-water sediments has normally been performed with core samplers, though there is the risk of contamination. We have developed some samplers for the aseptic sampling of deep-sea sediments. A primitive version of this sterile sediment sampler is shown in Fig. 12.1 (Ikemoto and Kyo 1993); it can be incorporated into a manned or an unmanned submersible and can be handled by the manipulators (Fig. 12.1). However, the amount of sediment obtained through this method (maximum 50 ml) is too small to allow the isolation of yeasts, because this sampler was originally designed for bacterial sampling. Later, a second version was produced which could sample 10 times as large a volume as the primitive version; however, it was too heavy and bulky. The more advanced systems were capable of sampling water (Jannasch and Wirsen 1977; Jannasch et al. 1982) or sediment (Kyo et al. 1991) while retaining the hydrostatic pressure and low temperature characteristic of deep-sea environments, but their sampling capacity was very low.

The isolation procedure varies depending on yeast density, the volume and shape of the source, and the source itself (water, sediment, plant or animal material). Yeast cells in water samples were mostly filtrated through membranes and then used for isolation, because of the low number of yeast cells, especially from open and deep-sea waters. The solid sources, such as plants, animals and sediments from polluted

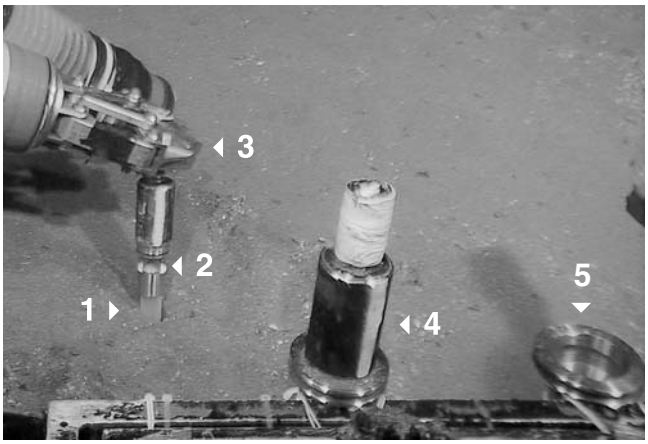

Fig. 12.1. Sterile sediment samplers. Each *numbered arrow* indicates the following: *1* a 50-ml polypropylene tube for retrieving sediment; *2* the entry point for sediment; *3* a manipulator of the submersible; *4* a sediment sampler in a sealed outer bracket; *5* an outer bracket

environments, which usually carry denser yeast populations, can be applied to the agar plate media directly or after being broken into pieces. These samples may be washed with water, and then the suspended cells in the water can be collected by filtration. Instead of filtration, organic contents, including yeast cells, have also been collected from water by using a precipitant (Sláviková and Vadkertiová 1995, 1997). When the sampling volume is too low owing to the capacity of the sampler, the sample can be enriched, but it should be noted that enrichment often changes the proportion of the yeast population and occasionally leads to a disappearance of the minority species or the slower-growing species. On the other hand, some enrichment media used for isolating specific yeast species from marine environments have been reported (Yanagida et al. 2002).

Because our ecological knowledge of the whole range of yeasts is very limited and many niches in aquatic environments have remained unexplored, it may be worth considering to attempt various new isolation media and conditions. The selection of media for isolating yeast strains from aquatic environments is important. Nevertheless, the media currently used are not so different from those used for terrestrial yeasts, even though aquatic environments generally contain lower organic nutrient concentrations than terrestrial environments. Most investigators prefer organic media mainly consisting of malt extract, yeast extract, peptone and glucose (Hagler and Ahearn 1987). Gadanho and colleagues (Gadanho et al. 2003; Gadanho and Sampaio 2004) employed media with MYP (0.7% malt extract, 0.05% yeast extract, 0.25% soytone) agar, broth, and no added glucose (Bandoni 1972). We also tried different media, but now we usually use five kinds of agar: yeast extract–malt extract (YM) agar, tenfold diluted YM agar, potato–dextrose agar, corn-meal agar and marine agar (all Difco), with 0.01% chloramphenicol. A new species of the genus *Cryptococcus* (Nagahama et al. 2003b) and a possibly an undescribed species of the genus *Dipodascus* (unpublished data) have been obtained as a result of cultivation in media unusual for yeast isolation, such as corn-meal and marine agar 2216. Seawater or sodium chloride was usually added, although this is not essential for the reproduction of most yeast species. But a marine yeast species which requires seawater for reproduction has been reported (Tong and Miao 1999). The temperature and pH of the media are often adjusted to prevent the growth of bacteria and filamentous fungi as competitors against the yeasts, as reviewed in many articles (Morris 1968, 1975; Fell 1976; Hagler and Hagler 1978; Hagler and Ahearn 1987). Recent investigators prefer to add chloramphenicol as an antibiotic, in concentrations sufficient to suppress the coexistent bacterial population (Sláviková and Vadkertiová 1995; Nagahama et al. 2001b, 2003b; Gadanho et al. 2003; Gadanho and Sampaio 2004); another method is acidification of the medium (Hagler et al. 1993; de Araujo et al. 1995; Soares et al. 1997; Libkind et al. 2003).

12.3 Identification and DNA Detection

Yeasts occurring in marine and other aquatic environments in high concentrations often comprised basidiomycetous yeasts. This made identification troublesome, owing to the unstable reactions in the assimilation tests and the lack of morphological information on the sexual reproduction of many basidiomycetes. But such

problems have been overcome with the progress of molecular taxonomy in the last 2 decades. The efficiency of using nucleotide sequences to identify marine yeasts was demonstrated in early reports (Fell and Kurtzman 1990; Fell et al. 1992). The extensive application of nucleotide sequences from the D1/D2 region of the large subunit of the ribosomal RNA gene (26S rDNA) and from the internal transcribed spacer (ITS) regions between the 18S rDNA, 5.8S rDNA and 26S rDNA domains vastly facilitated the identification of yeasts, which led to the discovery of novel yeast species (Kurtzman and Robnett 1998; Fell et al. 2000; Scorzetti et al. 2002). Combined analyses with other molecules may further improve the phylogenetic reliability of identifications within a taxonomic group if the issue is unresolved based on the D1/D2 or/and ITS sequences (Kurtzman and Robnett 2003). Molecular taxonomic identification has the advantage of giving scalable genetic distances, as opposed to conventional identification based on morphological and physiological characteristics. Conserved gene sequences such as 18S rDNA, 26S rDNA, translation elongation factor 1-α (EF-1α), or RNA polymerase II (RPB2) are useful for the inference of higher relationships, whereas the rapidly substituted molecules, such as ITS or intergenic spacers (IGS) between 26S rDNA and 18S rDNA, can be used to estimate the relationships between closely related species or the intraspecific variation between strains with different geographic origins. This method can successfully discriminate aquatic species or strains from terrestrials, the two never having been distinguishable phenotypically.

In contrast, small- or single-molecule sequence data are prone to give wrong or ambiguous results. In this, morphological, physiological, biochemical and molecular fingerprinting analyses, which are often called polyphasic taxonomic approaches (Vandamme et al. 1996), can lend added support. In such analyses, fingerprinting techniques such as those based on restriction fragment length polymorphisms (RFLPs) (Mantynen et al. 1999; Montes et al. 1999; Villa-Carvajal et al. 2004) and microsatellite-primed PCR (Gadanho et al. 2003; Libkind et al. 2003) are often performed prior to sequencing, with the aim of avoiding sequencing redundancy and minimizing the expenditure of reagents. These procedures should be considered if it is observed that identical strains tend to appear repeatedly in numerous isolates.

The traditional isolation approaches mentioned before depended on cultivation in the laboratory, and it was often not possible to grow and isolate the organisms in proportions reflecting their natural communities. On the other hand, a complete DNA-sequence database for yeasts enables the investigation of the microbial community and the identification of component species by means of direct DNA extraction from environmental samples. This cultivation-independent analysis has successfully detected uncultured prokaryotic microorganisms (Ward et al. 1990) and fungi in the soil and the rhizosphere (Crowe 2002; Anderson and Cairney 2004). In contrast to the microorganisms mentioned before, the direct analysis of yeasts from sources in marine and other aquatic environments is just beginning (Gadanho and Sampaio 2004), probably because uncultivable yeasts were not expected to be found in these environments. In the study of Gadanho and Sampaio, fingerprinting was performed by means of temperature-gradient gel electrophoresis and clone sequencing of the fungal PCR amplicon of 26S rRNA, from the DNA samples extracted directly or via enrichment from the estuarine water in Portugal. These approaches

may assist the discovery of new marine or other aquatic yeasts as well as their ecological niches.

Some new inexpensive techniques for examining the specific abundance of yeast species have been reported. In order to detect rapidly and specifically such clinically important species as *Candidas*, quantitative PCR has been utilized (Brinkman et al. 2003; Filion et al. 2003). A hybridization macroarray assay has been developed for the rapid identification of yeasts occurring frequently in marine environments (Kiesling et al. 2002).

12.4 Yeast Distribution and Biodiversity in Aquatic Environments

12.4.1 Cell Density

Water is common as a source to isolate yeasts from either freshwater or marine environments (Kohlmeyer and Kohlmeyer 1979). This may indicate that the main interest of many investigators has been water pollution. Yeast populations in fresh water are denser than in marine water (Hagler and Ahearn 1987), but the density varies with the substrate and the pollution level. Yeast densities are known to be below 10 cells/l in open ocean water and below 100 cells/l in unpolluted lakes and coasts. Lower yeast counts have been observed in an inaccessible lake in Patagonia, Argentina (Libkind et al. 2003). The exceptional increase of the cell counts in oceanic water is often associated with increased concentrations of invertebrates or plankton blooms, and may exceed 3×10^3/l (Fell 1976). The total yeast count usually increases with increasing pollution, and occasionally exceeds 2×10^8 cells/l (in sewage). A great variation in the number of yeast cells with depth was observed in the Gulf Stream (Fell and van Uden 1963). The number of yeasts in seawater above the Álvares Cabral Trench averaged about 5.4 cells/l and varied with depth (Gadanho et al. 2003).

In sediments, cell counts above 100/g have been reported in polluted areas (Hagler and Ahearn 1987). The yeast-cell frequency in sediment depends on the type of sediment (Fell et al. 1960). In our research of deep-sea floors, no yeast colony has been discovered so far in sandy sediments, except in hydrothermal vent areas. Hagler and colleagues have extensively surveyed the various substrates in the polluted estuaries of Sepetiba Bay, Rio de Janeiro, Brazil. The yeast population at the beach was 3.7×10^3 most probable number (MPN) cells/l in water, whereas the density in mud sediments was about 20 times higher, and that in shrimp intestines was more than 400 times higher than that in water, at the same site (Pagnocca et al. 1989). The average MPN count in invertebrates was about 37 times higher than that in water (de Araujo et al. 1995). In intertidal estuary sediments, the MPN counts were about 20–30 times higher than in the water (Soares et al. 1997). In contrast, some researchers reported that there were fewer yeast cells in invertebrates than in the surrounding sediments (Volz et al. 1974; Hagler and Ahearn 1987). It is possible that the obligatory localization of yeast vegetation in/on invertebrates depends on a certain association between the species and the environmental situation. Yeast counts in the water of mangrove bromeliads were from 10^4 to 10^5 cells/l (Hagler et al. 1993).

12.4.2 Fresh Water

Perfectly natural and unpolluted aquatic sources are almost impossible to find on Earth, and sampling while avoiding as far as possible the effects of human activity is difficult and usually expensive. In the past 2 decades, yeast communities in freshwater environments have been studied mostly in association with polluted water. Studies on freshwater yeasts have been focused mostly on their application as organic pollution indicators. Freshwater rivers and lakes in Europe, recreational lakes (Sláviková et al. 1992), fish ponds (Sláviková and Vadkertiová 1995), rivers (Sláviková and Vadkertiová 1997) and lagoons (Boguslawska-Was and Dabrowski 2001) were found to host mainly the genera *Candida*, *Cryptococcus*, *Pichia* (*Hansenula*) and *Rhodotorula*. Some species in these genera are regarded as bioindicators of the level of pollution (Dynowska 1997). Common species are *Aureobasidium pullulans*, *Cryptococcus albidus*, *Cr. laurentii*, *Debaryomyces hansenii* (*C. famata*) and *Rhodotorula mucilaginosa* (*Rh. rubra*) (Table 12.1). The black yeast

Table 12.1 Twenty species frequently isolated in four studies at European freshwater sites

Phylum	Species	Frequency (%)			
		Origins[a]			
Ascomycota	*Aureobasidium pullulans*	17.5	27.8	8.0	1.1
	Saccharomyces cerevisiae	–	2.0	17.3	7.8
	Pichia anomala	14.7	6.3	1.7	–
	Issatchenkia orientalis/ Candida krusei	11.2	2.2	6.6	–
	Debaryomyces hansenii/ Candida famata	0.6	0.9	1.4	11.5
	Pichia fermentans/Candida lambica	7.5	0.7	4.5	–
	Pichia guilliermondii/Candida guilliermondii	11.2	1.3	–	–
	Pichia burtonii	4.2	3.4	1.4	–
	Candida maltosa	–	–	8.0	–
	Geotrichum capitatum	–	–	6.9	–
	Candida sake	–	2.3	–	4.1
	Galactomyces geotrichum/ Geotrichum candidum	3.3	2.5	–	–
Basidiomycota	*Rhodotorula glutinis*	8.8	–	7.3	36.8
	Sporobolomyces roseus	–	22.9	–	–
	Cryptococcus albidus	0.6	3.6	5.2	6.8
	Cryptococcus laurentii	0.4	4.5	3.8	4.9
	Rhodotorula mucilaginosa/ Rhodotorula rubra	4.8	1.1	1.4	6.2
	Trichosporon cutaneum	–	5.2	3.5	4.0
	Cystofilobasidium capitatum	–	–	7.3	–
	Rhodotorula minuta	0.7	0.5	5.2	–

[a]*A* Sláviková et al. (1992), *B* Sláviková and Vadkertiová (1995), *C* Sláviková and Vadkertiová (1997), *D* Boguslawska-Was and Dabrowski (2001)

A. pullulans is an opportunistic pathogen (Koppang et al. 1991; Girardi et al. 1993; Redondo-Bellon et al. 1997), and accounts for the highest population in the total yeast population of recreational lakes (56–76%) and fishponds (38–48%) during autumn. It has only been found in water (Boguslawska-Was and Dabrowski 2001) and is considered to enter aquatic zones with plants, flowers, fruits, etc. A significant incidence of the black yeasts in lake water has been reported globally, including lake St. Clair, Canada (Kwasniewska 1988), and Lagoa Olhos d'Água, a lake on the karstic plateau of Lagoa Santa, Brazil (Rosa et al. 1995). *Cr. albidus*, *Cr. laurentii*, *D. hansenii* and *Rh. mucilaginosa* were found to be small, stable components at the European sites. The majority of ascomycetous yeasts, such as *Candida* spp. and *Pichia* spp., tend to be prevalent in summer. These are considered to be of human origin in recreational lakes (Sláviková et al. 1992), and may be related to the increase in phytoplanktons during summer in eutrophicated waters. The species relatively specific to studies in these European areas were *Bullera alba*, *Galactomyces geotrichum* (*Geotrichum candidum*), *Pichia burtonii* and *Lachancea* (*Saccharomyces*) *kluyveri*, although their proportions were low.

The increased numbers of red yeasts in autumn, including the ballistospore-forming *Sporobolomyces* spp. (Sláviková and Vadkertiová 1995, 1997), may be affected by the microbial effluents from the phyllosphere carried by the fallen leaves of terrestrial plants. *Rh. minuta* and *Rh. mucilaginosa* were found in relatively low proportions in almost all of the studies, whereas *Rh. glutinis* and *Sporobolomyces roseus* unexpectedly increased in their specific area (Table 12.1) and season (Sláviková and Vadkertiová 1995). It has been reported that red yeasts are found in higher proportions in the total yeast population of clean water, as compared with polluted water (Hagler and Ahearn 1987), but the response to pollution may depend on the red yeast species involved.

Rh. mucilaginosa was one of two dominant species found in high-salinity conditions in chemical wastewater evaporation ponds (Lahav et al. 2002). This species was prevalent also in many of the oligotrophic lakes in northern Patagonia, Argentina (Libkind et al. 2003), but it was not found in ultra-oligotrophic lakes with little exposure to human activity, and its niches were mainly occupied by some ballistosporogenous species comprising the undescribed yeasts *Sporidiobolus longiusculus* and *Sporobolomyces patagonicus* (Libkind et al. 2005). In the same series of oligotrophic lakes, members of the *Occultifur* lineage (Fig. 12.2), *Rh. minuta*, *Rh. slooffiae* and *Rh. pinicola*, along with ballistosporogenous yeasts such as *Sporidiobolus salmonicolor*, *S. ruberrimus* and *S. roseus*, have also been isolated.

Red yeasts have also been isolated from the deep groundwater (Ekendahl et al. 2003) and identified as *Rh. minuta* on the basis of 18S rDNA phylogeny and the physiological properties. In our study, these phylogenetic positions were reconsidered, and the strains J1, J2 and J3 were subsequently revealed to be different from *Rh. minuta* (Fig. 12.2). Strains J1 and J3 were found to be related to *Rh. slooffiae*, whereas the classification of strain J2 was unclear owing to low bootstrap reliability. Nevertheless, all three strains were at a remarkable distance from their closest relatives, and seemed to be new species. Unlike the type strain of *Rh. slooffiae*, strains J1 and J3, which cannot grow above 23°C, were found to be psychrophilic. Strain J2 had a unique physiology in that the optimum growth temperature was 4°C, although the maximum was 30°C.

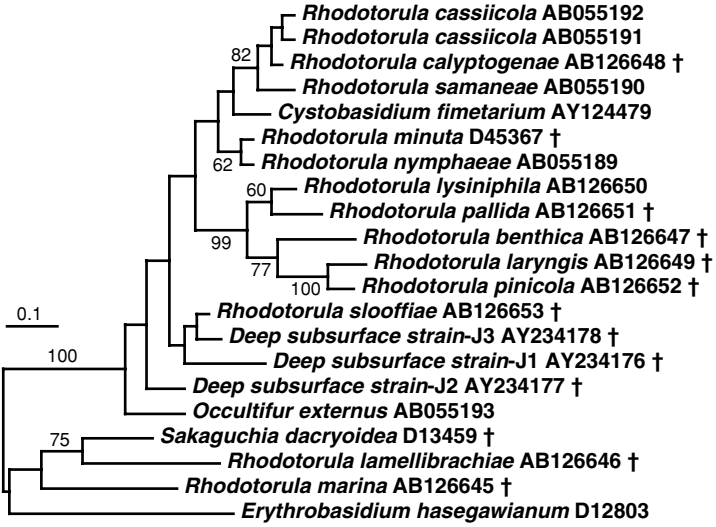

Fig. 12.2. The phylogenetic relationship within the *Occultifur* lineage and the positions of the red yeasts isolated from deep groundwater, based on the 18S ribosomal DNA (*rDNA*) sequences. The maximum likelihood tree was calculated by using the Bayesian phylogenetic inference on the program MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with the GTR+I+G model. The *numbers* on the branches are estimates of a posteriori probabilities. A *dagger* indicates species that have been isolated from aquatic environments

12.4.3 Estuaries and Mangrove Areas

Yeast communities of polluted estuary and mangrove ecosystems in subtropical marine environments were found to exhibit extreme diversity. The species diversity of basidiomycetous yeasts in these communities was less than or not notably different from that in other aquatic regions, but the ascomycetous yeasts comprised a remarkably greater number of species in these ecosystems than in other aquatic environments (Hagler and Mendonça-Hagler 1981; Hagler et al. 1982, 1993; Pagnocca et al. 1989; de Araujo et al. 1995; Soares et al. 1997). But highly dominant species tended to be absent, because many of the ascomycetous yeast species were represented by only one or a few isolates. Many of these species were considered to be allochthonous (de Araujo et al. 1995). Basidiomycetous yeasts were generally less frequent than ascomycetous yeasts in the polluted aquatic environments (Hagler and Ahearn 1987). The average proportion of basidiomycetous yeasts in the total yeast population in some studies conducted at Sepetiba Bay was 12.6% (Pagnocca et al. 1989; Hagler et al. 1993; de Araujo et al. 1995; Soares et al. 1997). The higher proportion of basidiomycetes in water (12.0%) as compared with sediment (3.8%) or invertebrates (3.5%) at same sites (Pagnocca et al. 1989) could be due to the fact that basidiomycetous yeasts are mostly oxidative and frequently occur on the water surface. *C. intermedia*, *D. hansenii*, *Issatchenkia occidentalis* (*C. sorbosa*), *P. guillier-*

mondii and *P. membranifaciens* (*C. valida*) were the ubiquitous ascomycetous species in the yeast studies conducted at Sepetiba Bay. The comprehension of a widely diverse ecology comprising numerous species, such as the yeast community in the subtropical mangrove ecosystem, may be impeded by the uncertain identities of the species owing to the ambiguous taxonomic results of phenotypic characterization.

Kluyveromyces aestuarii was found to be associated with detrital feeding invertebrates and sediments within specific mangrove areas (de Araujo et al. 1995; Soares et al. 1997). Previously, this species was presumed to be an obligate marine species (Kohlmeyer and Kohlmeyer 1979; Lachance 1998), and the other strains were isolated from shallow sediments of South Florida and the Bahamas and from the mangrove brackish area of the Everglades in Florida and the seawater from Torres Strait, Australia. *K. aestuarii* is phylogenetically placed in the *K. marxianus* lineage, genus *Kluyveromyces* nom. cons. (Kurtzman 2003) or the genus "*Zygofabospora*" (Naumov and Naumova 2002), and is ecologically similar to some members of the lineages *K. lactis* and *K. nonfermentans*. The "aquatic" strains of *K. lactis* (Naumova et al. 2004), which were isolated from rhizosphere sediments in the marine marshlands (Meyer et al. 1971), were suggested to be evidence of the possible evolution of the mangrove-inhabiting species *K. aestuarii* from the marshland-inhabiting species *K. lactis* (de Araujo et al. 1995; Soares et al. 1997). But the ITS phylogeny of the genus *Kluyveromyces* (Fig. 12.3) did not support the theory of direct evolution from *K. lactis* to *K. aestuarii*, whereas *K. aestuarii* obviously shared a common ancestor with *K. nonfermentans*, which was isolated from sediments and invertebrates in deep-sea environments. *K. nonfermentans* may have lost its fermentative ability as a consequence of adapting to oligotrophic oceanic environments.

Plant-associated yeasts on bromeliads in mangrove areas are distinct from those typical of polluted areas, and comprise a larger number of species and isolates with basidiomycetous affinities (Hagler et al. 1993). Some human-associated species, such as *C. parapsilosis* and *C. tropicalis*, and the prevalent species in polluted water, such as *Hanseniaspora uvarum* (*Kloeckera apiculata*), *I. orientalis* (*C. krusei*) and *P. anomala*, were absent, although they were common in other sources from the mangrove ecosystem.

Yeasts are prevalent in salt marshes or mangrove ecosystems where the yeasts play an important role in the detrital food web (Meyers et al. 1975), and they might be a food source for some marine invertebrates and zooplanktons. The fungus-growing behavior of the marine snail on the marine marsh grass was recently reported (Silliman and Newell 2003). In contrast, the infection of the freshwater prawns *Macrobrachium rosenbergii* by *C. sake* (Lu et al. 1998) and *Metschnikowia bicuspidata* (Chen et al. 2003) is known. In *Spartina* grass, in addition to *K. lactis*, *P. spartinae* has been previously found as the prevalent species in the outer- or intra-culm (fistulous stalk) cells and tissues, but PCR-based analysis of an ascomycetous community colonizing decaying *Spartina alterniflora* blades in the salt marsh was not able detect these two yeasts or any other yeast clones (Buchan et al. 2002). Further extensive research could reveal the ecological roles of these yeasts and their interaction with the other organisms of the salt marsh.

The yeast community from the estuary in Portugal, investigated with the help of fungal DNA clones, suggested the presence of some species known to be prevalent

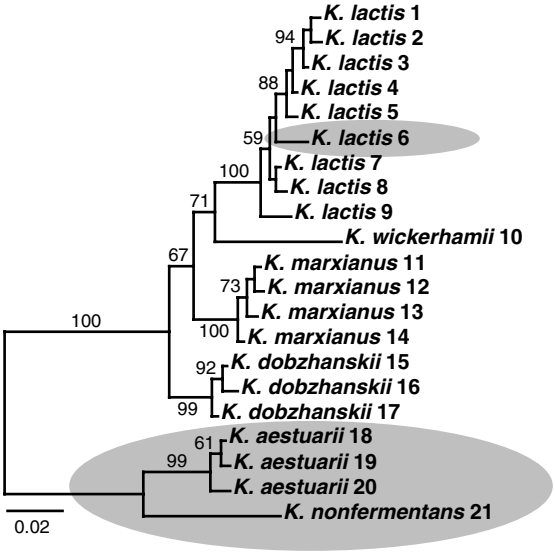

Fig. 12.3. The phylogenetic relationship of the genus *Kluyveromyces*, as inferred from internal transcribed spacer and the 5.8S rDNA sequences. The tree was drawn as for Fig. 12.2. The *numbers* on the branches are estimates of a posteriori probabilities. The marine species are in the *gray oval*. The accession numbers for the sequences (see the number after the name of the species) are as follows: *1* IFO 1090, AB011515; *2* JCM 6846, AB011516; *3* CECT 1121, AJ401702; CECT11401, AJ401718; CECT10366, AJ401708; CECT10364, AJ401707; CECT10361, AJ401706; CECT11395, AJ401715; CECT1961, AJ401704; CECT11401, AJ401718; *4* CECT 1122, AJ401703; CECT10669 AJ401710; CECT11380, AJ401713; CECT11394, AJ401714; CECT11396, AJ401716; *5* JCM 9563, AB011517; *6* CBS 6170, AY338967; CBS6171, AY628330; *7* UWO79-169, AY628331; CECT10390, AJ401709; CECT11337, AJ401711; UCD61-200, AY623808; CECT11340, AJ401712; UWO79-169, AY628331; UWO80-45, AY623809; *8* UWO79-127, AY626022; UWO80-12, AY626023; *9* UCD 69-8, AY338968; DV30, AY338969; *10* IFO 1675, AB011521; *11* IFO 10005, AB011518; JCM 1630, AB011519; CECT1123, AJ401692; CECT1031, AJ401694; CECT1058, AJ401698; CECT1066, AJ401699; *12* JCM 2188, AB011520; *13* CECT 1048, AJ401700; *14* CECT 1442, AJ401693; CECT1035, AJ401695; CECT1036, AJ401696; CECT1058, AJ401697; CECT1138, AJ401701; *15* IFO 10603, AB011514; NRRL Y-1974, AY046215; CECT 1952, AJ401719; *16* CBS 210, AJ229068; *17* CECT 10177, AJ401720; CECT 10180, AJ401721; CECT 10187, AJ401722; *18* NRRL YB-4510, AY046210; *19* NRRL Y-4510, U09324; *20* IFO 10597, AB011513; *21* JCM 10227, AB011507; JCM 10230, AB011508; JCM 10231, AB011509; JCM 10234, AB011510; JCM 10236, AB011512; JCM 10232, AB012264

in polluted aquatic environments, and six yeast species were regarded as putative undescribed species (Gadanho and Sampaio 2004). From among these yeast species, all five ascomycetous yeasts were relatively closely related to species which have been reported to occur in aquatic environments, e.g., *C. inconspicua*, *C. intermedia*, *D. hansenii* and *P. guilliermondii*, whereas the only basidiomycetous yeast, which, interestingly, was considered to be uncultured, was found to be related to *Cr. longus*, which had previously not been reported to be aquatic.

12.4.4 Offshore and Deep-Sea Environments

There have been a few yeast studies conducted in oceanic regions in the last 2 decades. This is probably because there has been little expectation of obtaining new findings in this area, and, in addition, the expenses for offshore and oceanic sampling are considerable. In an extensive study of yeasts in oceanic regions (Fell 1976), the yeast communities appeared to be constituted of the ubiquitous species and species restricted geographically, hydrographically and biologically. Among the ascomycetous yeasts, the halotolerant species *D. hansenii* was a typical ubiquitous species in oceanic regions as well as in other aquatic environments. Among the basidiomycetous yeasts, some species of *Cryptococcus*, *Rhodotorula*, *Sporobolomyces* and their teleomorphs were found to be widespread across various oceanic regions. Generally, basidiomycetous yeasts often account for the overwhelming majority of the total yeast population in oligotrophic oceanic water. *Candida* spp. also occur, but at lower frequencies than in the inshore or polluted freshwater regions, and the occurring species are also different in these habitats. Some of the *Candida* species appeared only in the oceanic regions around Antarctica, and, along with *Leucosporidium* spp. and *Sympodiomyces parvus*, they are known to be psychrophilic. They are probably autochthonous marine species (Lachance and Starmer 1998). *Metschnikowia* spp. are known to be associated with seawater, fresh water, invertebrates, fish and algae. The phylogenetic relationship (Fig. 12.4) shows that *M. australis*, *M. bicuspidata* var. *bicuspidata*, *M. bicuspidata* var. *chathamia*, *M. krissii* and *M. zobellii*, all of which are prevalent in marine environments, are monophyletic, whereas the occasionally aquatic species *M. reukaufii* and *M. pulcherrima* are distant, as previously reported (Mendonça-Hagler et al. 1993). The latter two are usually found to associate with terrestrials such as flowers, fruits and insects, like almost all of the other *Metschnikowia* species. The monophyly of the marine species suggests that their divergence has evolved in the course of association with marine environments.

The ubiquitous species in various marine habitats are usually regarded as being allochthonous. Especially because many basidiomycetous yeasts are often found to associate with the phyllosphere of terrestrial plants, the marine occurrences were considered to be run-offs from the phylloplane (Hagler and Ahearn 1987; Lachance and Starmer 1998). The yeasts of the ballistosporogenous genera *Sporobolomyces* and *Bullera* and their teleomorphs are typical inhabitants of the phylloplane. During a survey of yeasts in the Pacific Ocean off Mexico, yeasts of the genus *Sporobolomyces* (and *Bullera*) were the most commonly encountered (Hernandez-Saavedra et al. 1992). Interestingly, the frequencies increased with increasing distance from the coastline and increasing depth. The yeasts of these genera were also found in benthic invertebrates collected from deep-sea floors in the Pacific Ocean off Japan (Nagahama et al. 2001b). These facts may indicate that ballistosporogenous yeasts are not effluents from terrestrial plant foliages but are indigenous to the sea.

In a study of yeasts in the seawater of the Atlantic Ocean off Faro in the south of Portugal (Gadanho et al. 2003), however, few ballistosporogenous yeasts were found, and mainly basidiomycetous yeasts were isolated. *Rhodosporidium babjevae*

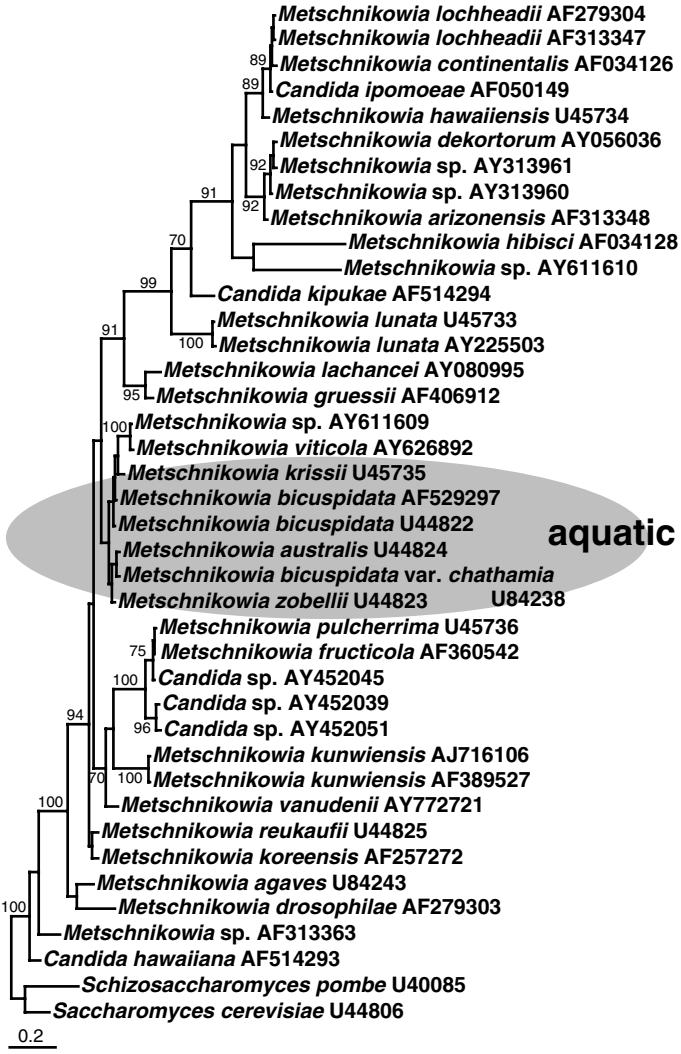

Fig. 12.4. A 26S rDNA D1/D2-based tree for the genus *Metschnikowia* and its relatives. The tree was drawn as for Fig. 12.2. The *numbers* on the branches are estimates of a posteriori probabilities. The species associated with aquatic environments are in the *gray oval*

and *R. diobovatum*, the two possible species previously identified as *Rh. glutinis*, and *Sakaguchia dacryoides* and *Pseudozyma aphidis* were frequent among the basidiomycetous yeasts. Notably, an ustilaginomycetous yeast, *P. aphidis*, was widely distributed in the area studied, but its ecological behavior in the sea is still unknown. As in the case of the *Rh. glutinis* species complex, a species previously identified as *Cr. laurentii* also occurred, and was found to be involved with undescribed species.

From quantitative surveys of microbiota in deep-sea sediment, abundance of yeast-like cells has been reported (Burnett 1981; Alongi 1987, 1992). During a survey of the yeast distribution in deep-sea environments around the Pacific Ocean, a number of yeasts have been isolated (Nagahama et al. 1999, 2001a, b, 2003a, b). Our most frequently visited site is around a cold seep at a depth of about 880–1,200 m near Hatsushima Island, Sagami Bay. At this site, giant white clams (*Calyptogena* spp.) and tubeworms (*Lamellibrachia* spp.), which inhabit the area, were collected. Suruga Bay (380–2,500 m), the Japan Trench (4,500–7,500 m) and Iheya Ridge (990–1,400 m) have been investigated a few times. The other sites (Kagoshima Bay, 220–260 m; the Mariana Trench, about 11,000 m; the Palau–Yap Trench, 3,700–6,500 m; the Manus Basin, 1,600–1,900 m) have only been visited once (Fig. 12.5). Iheya Ridge and the Manus Basin have biologically fertile spots owing to the hydrothermal vent ecosystem, and tubeworms (*Lamellibrachia* spp.) were collected at the former site.

R. sphaerocarpum, *Williopsis saturnus* and *C. pseudolambica* were found to be frequent species in a total account of all of the sites (Table 12.2), but their distributions were limited mostly to the sediments of Suruga Bay and Kagoshima Bay. *D. hansenii* occurred only in the sediments of Sagami Bay and Suruga Bay, although it had been known to be the commonest ascomycete in marine waters (Hagler and Ahearn 1987). Almost all ascomycetous yeasts were isolated only from sediments. The only exception was *K. nonfermentans*, which was common to both sediments and benthic invertebrates, whereas its distribution was limited to Sagami Bay and Suruga Bay. In contrast, *R. diobovatum* and *Rh. mucilaginosa* were widely prevalent in the various locations and sources.

The frequency of occurrence of each corresponding phylogenetic taxon is obviously different according to the source and geographical origin (Fig. 12.6). The ascomycetous yeasts constituted the majority of the total yeast population in the sediments of Sagami Bay, Suruga Bay and Kagoshima Bay, sites which are relatively inshore (5–20 km) near urban and industrial areas and where the deep-sea floors are affected by the human activities. But *P. anomala* and *H. uvarum*, which are known as the pollution-associated species, did not appear. Species in the *Erythrobasidium* clade have been isolated mostly from the benthic invertebrates, and the exceptional isolates from the sediments of the Manus Basin are considered to give clues about the hydrothermal ecosystems. Many of these species belong to the *Occultifur* lineage (Fig. 12.2), and some were found to be undescribed species (Nagahama et al. 2001a, 2003a). The association with animals is probably favorable for yeasts, owing to the abundance of nutrients (Hagler and Ahearn 1987). The reasons why species associated with animals are limited are unknown so far. Hymenomycetous species, mostly assigned to the genus *Cryptococcus*, were localized in the Japan Trench, Sagami Bay and Suruga Bay, and did not appear farther southwest. Species of Sporidiobolales were present at all of the sites.

However, there is poor evidence for physiological adaptation. Yeasts with an optimum growth under elevated hydrostatic pressure condition have not been reported, whereas many yeast species from both terrestrial and marine origins are moderately pressure-tolerant (ZoBell and Johnson 1949; Yamasato et al. 1974). Especially, the carotenogenic basidiomycetous yeasts such as *Rhodotorula* and *Rhodosporidium* were reported as psychro- and pressure-tolerant (Davenport 1980). A *Rhodotorula* species

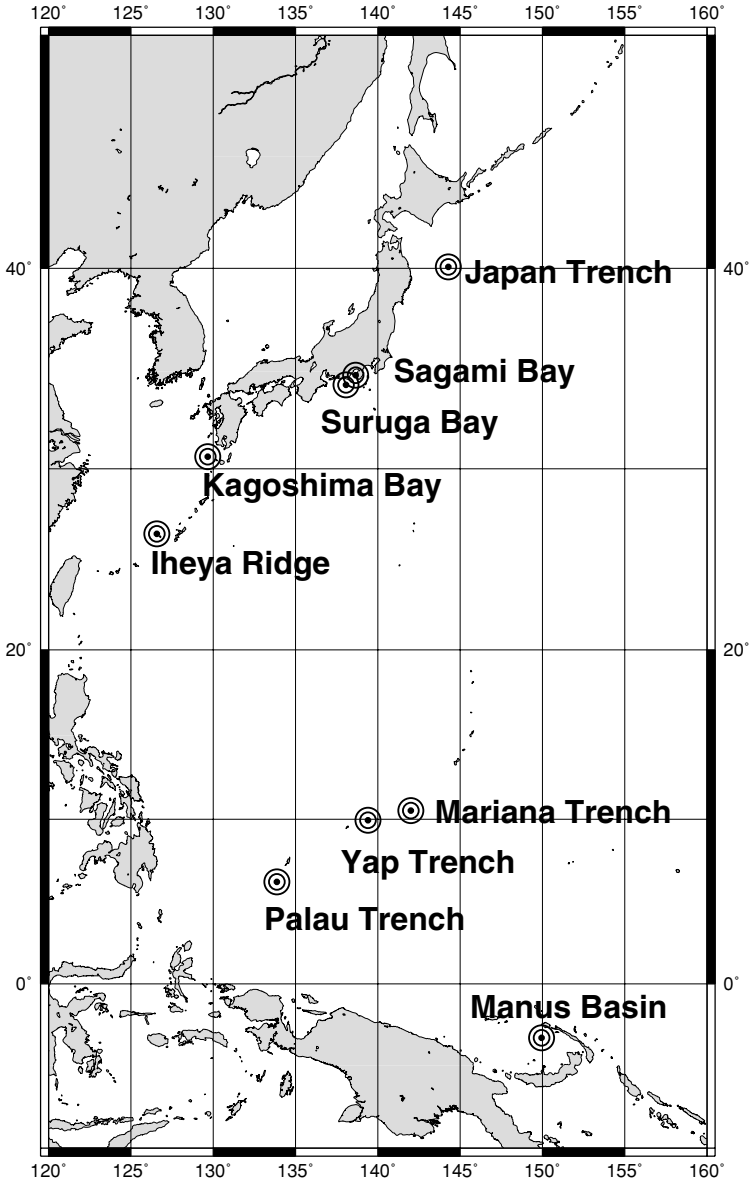

Fig. 12.5. Sampling sites in the Pacific Ocean

showed that growth at 20 MPa (equivalent to 2,000-m depth) was not significantly different as compared with that at 0.1 MPa and growth at 40 MPa was reduced to 20–30% (Lorenz and Molitoris 1997). But it should be noted that the growths were observed at a remarkably high temperature (27˚C). In our study, the yeasts isolated from seafloors deeper than 4,000 m did not grow well under hydrostatic pressures

Table 12.2 The yeast species and the number of isolates from sediment and invertebrates collected from deep-sea floors around the northwest Pacific Ocean

Species[a]	Sediment	Invertebrates	Total ($n \geq 2$)
Rhodosporidium sphaerocarpum	33	–	33
Williopsis saturnus var. *saturnus*	30	–	30
Candida pseudolambica	27	–	27
Debaryomyces hansenii var. *hansenii*	25	–	25
Rhodosporidium diobovatum	16	9	25
Kluyveromyces nonfermentans	15	4	19
Rhodotorula benthica	10	7	17
Rhodotorula mucilaginosa	9	2	11
Candida vartiovaarae	6	–	6
Cryptococcus gastricus	6	–	6
Debaryomyces hansenii var. *fabryii*	5	–	5
Kondoa aeria	4	1	5
Rhodotorula lamellibrachiae like	1	3	4
Rhodotorula minuta	–	4	4
Candida melibiosica	3	–	3
Williopsis californica	3	–	3
Aureobasidium pullulans	3	–	3
Cryptococcus albidus	3	–	3
Cryptococcus laurentii like	2	1	3
Rhodosporidium toruloides like	3	–	3
Sporobolomyces salmonicolor	–	3	3
Candida boidinii	2	–	2
Candida sake	2	–	2
Pichia pinus like	2	–	2
Sarcinomyces petricola	2	–	2
Cryptococcus victoriae	2	–	2
Trichosporon pullulans	2	–	2
Rhodotorula dairenensis	2	–	2
Rhodotorula dairenensis like	2	–	2
Sporidiobolus pararoseus	1	1	2
Rhodotorula calyptogenae	–	2	2

[a]"Like" represents a similar but not identical species with greater than 1% differences in the comparisons of D1//D2 26S ribosomal DNA or internal transcribed spacer sequences.

corresponding to the sources at which they were collected (2–4°C, more than 40 MPa). But this result may also be due to the specifications of our compressed incubation system, which allows sharp pressure changes and insufficient oxygen supply. Psychrophilic strains have not been found so far in the deep sea, but many isolates were psychrotolerant and grew well at less than 4°C (unpublished data).

12.5 The Impact of Molecular Taxonomic Approaches on the Ecological Studies of Yeasts in Marine and Other Aquatic Environments

It is still difficult to give a convincing answer to an old question about marine yeasts: "Are there indigenous marine yeasts?" (Kohlmeyer and Kohlmeyer 1979). Even if

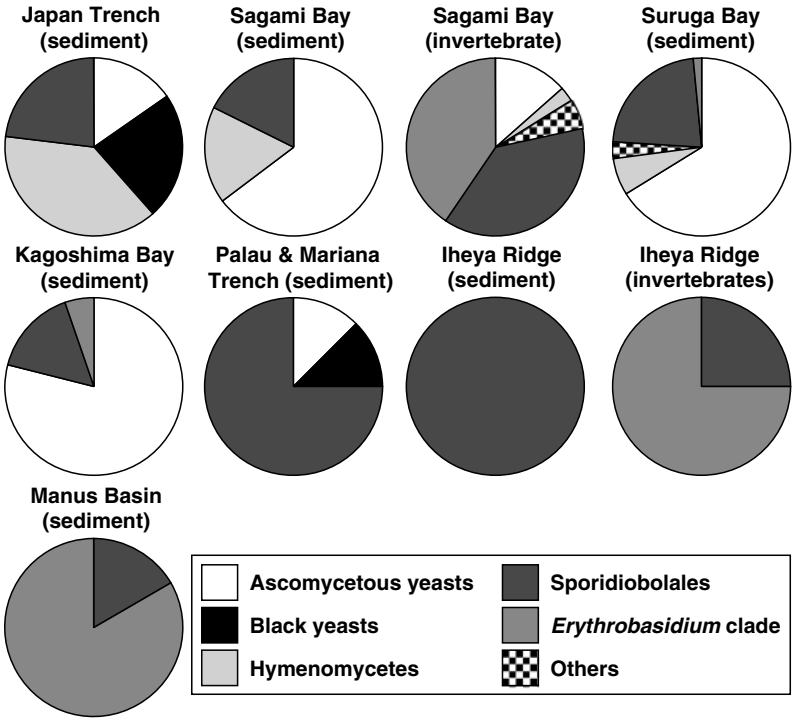

Fig. 12.6. The frequency of occurrence of each phylogenetic taxon by geographical origin and source

there is evidence that there are, there is another question: "Which are indigenous?" In general, the physiological properties of yeasts do not give clues as to whether they are marine species or terrestrial species. Although some marine species may be resistant to higher NaCl concentrations than related terrestrial species, almost all yeast species can grow well in media with NaCl concentrations exceeding those normally present in the sea (Kohlmeyer and Kohlmeyer 1979). Few yeast species with a physiological dependence on NaCl or other seawater components have been reported. Yeast concentrations in marine environments are known to decrease with increasing depth and increasing distance from land. But this tendency could be explained in two ways: one is the availability of nutrients coming from land, and the other is frequent encounters with terrestrial yeasts (Sieburth 1979). Thus, physiological characterization cannot discriminate between marine and terrestrial yeasts.

Many of the yeast species found in aquatic environments are considered to be allochthonous (Lachance and Starmer 1998). The number of yeast species prevalent in marine environments is limited, but each species of marine yeast is very similar to its terrestrial counterpart. Only a few of the isolated species have been found to be restricted to marine environments. These physiological and taxonomical observations could imply that most of the marine species are terrestrial contaminants.

Yeasts are known to be the dominant fungi in the open ocean, though the bacterial population is hundreds of times denser than the yeast population, according to counts of colony-forming units (Sieburth 1979). Because most natural seawater contains amounts of organic carbon nutrients insufficient for yeast-cell reproduction, many marine and other aquatic environments may be used by the yeasts as transit areas only. The candidates for autochthonous marine yeasts are not thought to be ubiquitous; their occurrence is thought to be restricted to specific geographical latitudes or to associations with certain macroorganisms, as in the case of the psychrophilic species from the circumpolar area or *Metschnikowia* spp.

Many of the yeast species that have a widespread distribution across aquatic environments often pose identification problems, owing to their ambiguous phenotypic characteristics. However, this problem has been overcome through the development of phylogenetic molecular identification. The aquatic strains that had been considered phenotypically identical to or indistinguishable from terrestrial strains can now be segregated genetically. Evidence of such imperceptible differentiation is offered by rapidly substituted molecules such as ITS or IGS. Thus, the aquatic strains of the species *K. lactis* were revealed to be differentiated as the variety level, by means of RFLP fingerprinting of the PCR replicons in IGS and the phylogenetic relationships based on ITS sequences (Naumova et al. 2004).

Red yeasts may also provide clues as to the differentiation of aquatic yeasts from terrestrials. These basidiomycetous yeasts are characterized as having the ability to produce carotenoids or carotenoid-like compounds, and are found in higher proportions in the total yeast population of clean freshwater than of open-sea water. The species *Rh. glutinis*, *Rh. minuta* and *Rh. mucilaginosa* are the predominant red yeasts in aquatic environments, although each has been considered to be multitypical. Notably, many strains previously identified as *Rh. glutinis* may genetically differ from the type strain of this species, and may be anamorphs of species in the genus *Rhodosporidium* or may belong to a novel species. The marine isolates related to the three *Rhodotorula* species have been found as undescribed species (Gadanho and Sampaio 2002; Nagahama et al. 2003a). The Sporidiobolales, which include *Rh. glutinis* and *Rh mucilaginosa*, and the *Occultifur* lineage, which includes *Rh. minuta* (Fig. 12.2), comprise many species which often are associated with aquatic sources.

Cr. laurentii is one of the commonest basidiomycetous yeasts in aquatic environments, but some aquatic strains previously identified as belonging to this species are possibly different from this type strain, as in the case of the *Rhodotorula* species mentioned before. Some new combinations have been recently reported in the *Cr. laurentii* species complex. *Cr. peneaus* is one of these, and has been isolated from the surface of shrimp. More new aquatic species may be recognized on the basis of further taxonomic studies of this complex. The widespread yeasts occurring across various ecological niches often form varieties or species complexes, and the identification of these yeasts based on traditional taxonomical approaches is difficult. A reexamination of aquatic strains previously classified into certain species is worth considering.

In contrast, *C. austromarina*, known as a psychrophilic marine species inhabiting the Antarctic Ocean, was regarded as a synonym of a mesophilic species, *C. sake*, because of the identical D1/D2 26S rDNA sequence. Because *C. sake* is widely

distributed in oceanic and other aquatic regions, *C. austromarina* is considered to be a variety indigenous to the Antarctic region. This conspecificity suggests the possibility that the psychro-adaptation occurred over a short period of time. Their genetic differentiation could be determined through further comparisons of rapidly substituted genes.

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Chapter 13

Phylloplane Yeasts

Á. Fonseca and J. Inácio

Centro de Recursos Microbiológicos (CREM), S.A. Biotecnologia, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Quinta da Torre, 2829-516 Caparica, Portugal (e-mail: amrf@fct.unl.pt, Tel.: +351-21-2948500, Fax: +351-21-2948530)

Abbreviations

BC	Ballistoconidiogenic
CFU	Colony forming units
DGGE	Denaturing gradient gel electrophoresis
EPS	Extracellular polysaccharide
FISH	Fluorescence in situ hybridization
rDNA	Ribosomal DNA
SEM	Scanning electron microscopy
SFM	Spore-fall method

13.1 The Phylloplane as a Microbial Habitat

Plant surfaces have been recognized as an important habitat for microorganisms for over a century. Different zones along the plant axis provide a multitude of topographical features, sources of nutrients and water, and a range of microclimatic conditions for correspondingly diverse communities of microbes, which in turn establish varied relationships with their hosts (Andrews and Harris 2000). These associations range from relatively inconsequential or transient (unspecific or ephemeral epiphytic saprophytes) to substantial or permanent (epiphytic residents, endophytes or pathogens). Phytopathogens have long been identified and studied owing to the economic impact of the diseases they cause on agricultural crops (e.g. Leben 1965; Morris 2001) but for many years much less was known about the identity or properties of the numerous saprophytic microbes that inhabit plant surfaces. However, the last few decades have witnessed a renewed interest in microbial epiphytes that apparently play important roles in nutrient cycling or in modulating population sizes of deleterious microbes, and some are being exploited as biological control agents for disease or frost control (Windels and Lindow 1985; Fokkema 1991; Andrews 1992; Lindow and Leveau 2002). Two main physical environments (or vertical stratification zones) for microbial colonization are distinguished: the aboveground surfaces, often referred to as the phyllosphere or the phylloplane, and the belowground

surfaces, termed the rhizosphere or rhizoplane (Diem 1973; Fokkema and Schippers 1986; Andrews and Harris 2000). In this chapter we will focus exclusively on the aerial surfaces of plants and in particular on the leaves. We have chosen the term phylloplane to refer to leaf surfaces (Last and Price 1969), although other authors advocate the use of the term phyllosphere (Fokkema 1991; Morris 2001; Lindow and Brandl 2003) originally proposed by Last (1955) and Ruinen (1956).[1] Our rationale was that the latter also comprises the internal leaf tissue that may be colonized by endophytes, which are not the object of this review.[2]

Plant leaves constitute one of the largest terrestrial microbial habitats and the total surface area available for colonization has been estimated to be about $2–6\times10^8$ km^2 (Morris 2001; Lindow and Brandl 2003). Leaf surfaces have been studied as habitats for microbial epiphytes since the 1950s and phyllosphere microbial ecology has been the theme of a regular series of scientific meetings since the early 1970s. The proceedings of those meetings summarize the progress made in understanding the nature of leaf habitats, their microbial colonists, and the interactions among microbes and between microbes and plants (Morris 2001, and references therein). The epiphytic (non-phytopathogenic) microbial communities of leaves are very diverse and their best-studied components have been bacteria and fungi, including yeasts (Andrews and Harris 2000; Hirano and Upper 2000; Morris 2001; Lindow and Brandl 2003). Bacteria are generally the earliest colonists of leaves and may be the most numerous, often being found in densities up to 10^7 cells/cm^2 (approximately 10^8 cells/g) of leaf under favourable environmental conditions, such as when high relative humidity or free water is present (Hirano and Upper 2000; Morris 2001; Lindow and Brandl 2003). Filamentous fungi are often considered transient inhabitants of leaf surfaces, being present predominantly as spores, whereas rapidly sporulating hyphomycetes, dimorphic species such as *Aureobasidium pullulans*, and yeasts appear to colonize this habitat more actively (Pennycook and Newhook 1981; Fokkema and Schippers 1986; Andrews and Harris 2000; Lindow and Brandl 2003). Some epiphytic mycelial fungi appear to be particularly active in leaf litter decomposition following leaf fall, at least in its initial stages (Mishra and Dickinson 1981; Osono 2002). However, extensive hyphal growth on healthy, intact, non-senescent leaves is relatively rare (except of course for biotrophic plant pathogens).

From a microbe's perspective the phylloplane is a continuously fluctuating physical environment both spatially and temporally, and may thus be considered an extreme environment (Hirano and Upper 2000; Morris 2001). Exposed to the atmosphere and the sun, leaf surfaces and, hence, their resident microbes are subjected to changes in aspects of microclimate such as temperature, relative humidity, wind speed, radiation, and others that occur on different time scales, from a few minutes to months. Water availability, for instance, is one of the most dynamic parameters of the phylloplane environment: condensation (dew, fog) and impaction of water

[1] It should be noted that in some texts the term phylloplane is used to refer to any aerial plant surface, including bark or fruits (Andrews and Harris 2000).

[2] Yeast endophytes in leaves have been reported by, for example, Larran et al. (2001) and Pirttilä et al. (2003).

droplets (rainfall) on leaf surfaces alternates with evaporation and run-off of water in cycles that are generally diurnal. The property that makes the phylloplane a harsh environment is not the extremes to which it is exposed, but the frequent, repeated, rapid shifts in those very different conditions, any one of which may be considered stressful to at least some of the prospective microbial colonists.

Cuticle composition and topographic features (stomata, trichomes, veins, etc.) are also highly variable both within a leaf and among different plant species (Baker 1971; Hallam and Juniper 1971) and may influence the composition and distribution of phylloplane communities (Kinkel 1997). Besides topography and microclimatic conditions, the nature and the size of epiphytic microbial populations are also shaped by the availability of nutrients and in this respect the phylloplane is generally considered oligotrophic (Andrews and Harris 2000). There are multiple possible origins of nutrients on the phylloplane. These can be endogenous and include diffusion of compounds out of the leaf tissue from hydathodes, trichomes or fissures in the cuticle accompanying weathering or injury, or exogenous, such as nutrients in guttation fluids or compounds contained in debris, pollen and honeydew, or originating in other organisms (microbes or insects). Molecules leached from plant leaves include a variety of organic and inorganic compounds, such as sugars, organic acids, amino acids, methanol and various salts (Blakeman 1971; Tukey 1971; Morris 2001). The abundance of such nutrients varies with plant species, leaf age and growing conditions. Exogenous nutrient sources, such as aphid honeydew and pollen, have been associated with dramatic increases in the microbial carrying capacities of some leaves (Diem 1974; Fokkema et al. 1983; Stadler and Müller 1996). However, in the common absence of such significant but ephemeral nutrient sources, plant leaves are still usually colonized by large microbial populations. Nutrients released to the leaf surface should thus be adequate to support microbial growth and depletion of sugars on the phylloplane by bacteria and yeasts has been demonstrated (Dik et al. 1991; Leveau and Lindow 2001). This suggests that epiphytic microorganisms normally compete for a limited amount of nutrients, which in turn determine the microbial carrying capacity of the leaf. Depending on the system studied, carbon compounds alone or both carbon and nitrogen compounds were shown to be limiting factors for bacterial and yeast populations on leaves (Bashi and Fokkema 1977; Mercier and Lindow 2000). Antimicrobial compounds may also leach to leaf surfaces and were found to affect the composition of phylloplane communities (Irvine et al. 1978; Fokkema 1991).

All the factors just discussed taken together account for the extraordinary plasticity and diversity of phylloplane microbial populations. Some of those aspects will be discussed in more detail in the following sections, with particular emphasis on yeasts.

13.2 Methods for Detection, Enumeration and Identification of Epiphytic Microorganisms

Several methods have been used for the detection, isolation and/or enumeration of microorganisms from the phylloplane, including yeasts (reviewed by Beech and Davenport 1971; Drahos 1991; Jacques and Morris 1995). Standard direct detection

methods, mainly in studies before the 1980s, were based on optical or scanning electron microscopy (SEM) of the leaf surface or corresponding leaf impressions (e.g. in nail varnish pellicles or acetate adhesive tape). These methods are generally quite laborious, there are difficulties in detecting small yeast cells or small cell densities against the background, and observations may be hindered by topographical features of the leaf surface. Some authors employed leaf clearing and staining procedures (Lindsey and Pugh 1976; McBride and Hayes 1977; Andrews and Kenerley 1978), which can alter the position of the epiphytic microorganisms on the leaf surface, therefore cancelling out some of the advantages of direct observation. Moreover, it is not usually possible to distinguish between different species with similar cell morphologies, or between viable, viable but non-culturable and dead cells. This is one possible explanation for the common finding that microscopy-based quantification of phylloplane microorganisms gives rise to higher counts than cultivation-based methods (Babjeva and Sadykov 1980; Mishra and Dickinson 1981). About one third of the 35 studies listed in Table 13.1 employed the direct observation of leaves and/or leaf impressions with optical microscopy, usually in complement to some other cultivation-based method. Five studies used SEM (Table 13.1). The main targets for the majority of these studies were filamentous fungi. In some cases yeasts were only detected using cultural methods, but not by direct microscopy (Hogg and Hudson 1966; Lindsey and Pugh 1976).

Enumeration of microbial populations associated with leaves relies almost exclusively on indirect methods, involving culture of the organisms on artificial agar media (Table 13.1). About two thirds of the studies in Table 13.1 employed methods based on the plating of leaf washings (or of leaf macerates) onto selected media for the isolation and enumeration of phylloplane microbial communities. These methods may underestimate the occurrence of non-spore-forming hyphal fungi and overestimate the spore-forming ones, but are generally considered appropriate for yeast quantification (Fokkema 1991). Leaves are usually cut into small pieces and submerged in a washing solution (e.g. water or Ringer solution, with or without low concentrations of a surfactant to aid dislodging of cells), which is then shaken more or less vigorously for a certain length of time (e.g. with the aid of a vortex, rotary shaker or sonicator). The resulting suspension is then serially diluted and aliquots are plated onto appropriate media. Several additives can be added to the culture medium, enabling the selective isolation of specific microorganisms (e.g. an antibacterial antibiotic such as chloramphenicol is normally used to prevent bacterial growth for isolation of fungi or yeasts). Isolation and quantification of yeasts from leaves is sometimes difficult owing to the growth of rapidly expanding moulds that overgrow yeast colonies. There is no single methodology or medium that inhibits one type of organism without affecting the other. For example, Azeredo et al. (1998) compared two agar media for yeast isolation from sugarcane leaf washings: yeast extract -malt extract and yeast nitrogen base with 1% glucose (both media supplemented with chloramphenicol to prevent bacterial growth). They claimed that the latter medium was superior owing to a lesser expansion of mould colonies. According to our own experience, however, yeast colonies develop poorly on this medium and are thus difficult to distinguish from one another. Some authors advocate the use of media supplemented with Rose Bengal (Pennycook and Newhook

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Lolium perenne (H); *Trifolium repens* (H); *Festuca arundinacea* (H)	New Zealand, C	*Cr. laurentii, Rh. glutinis, Rh. ingeniosa, Rh. marina, Rh. mucilaginosa, Rh. graminis, S. salmonicolor* (*Sp. odorus*); minor: *B. alba, Cr. albidus, Cr. diffluens, Cr. luteolus* (10^4–10^5 CFU/g)	A: PLW, B: P	Seasonal dynamics and species diversity (yeasts) on leaves of pasture plants; densities higher in summer and autumn and on senescent leaves; proportion of red yeasts lowest in winter (6%) and highest in late autumn (92%); soil yeasts rarely found on leaves; species composition independent of site	di Menna (1959, 1971)
Triticum aestivum (G)	England, C	*Sporobolomyces*	A: SFM	Seasonal dynamics (fungi); numbers of colonies increased with leaf age and were maximal when leaves died (while still attached)	Last (1955)
	Scotland, C	*Cr. albidus, Cr. laurentii, Cr. macerans, Rh. glutinis, Rh. ingeniosa, Sp. roseus, T. cutaneum*	A: PLW; PLM (w/washed leaves)	Seasonal dynamics and species diversity (fungi and yeasts); densities increased from early June to September	Flannigan and Campbell (1977)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Hordeum (G)	France, C	Pink yeasts (up to 10^4 CFU/cm^2)	A: PLW	Seasonal dynamics; densities increase from spring to autumn; bacteria always dominant; higher densities on plants in the centre of the cropping area than at the fringes	Diem (1967, cited in Last and Price 1969)
Beta vulgaris (D;H)	The Netherlands, C	*Cr. albidus*, *Cr. laurentii*, *Sp. roseus*	A: PLW	Seasonal dynamics (fungi and yeasts); densities increase from spring to summer but decrease in autumn; yeast numbers higher than those of other fungi	Kerling (1958, cited in Last and Price 1969)
	England, C	White and pink yeasts (10^6 CFU/g dry weight)	A: PLM, B: S	Short-term community dynamics (emphasis on bacteria); bacterial densities fluctuated: increased overnight and declined during daylight hours; yeast densities constant throughout the study	Thompson et al. (1995)

Pisum sativum (D;H)	England, C	Yeasts (up to 10^4 CFU/cm^2)	A: PLW, OMLI	Fungal dynamics with leaf age; yeast counts on plates lower than direct counts; yeast cells outnumbered fungal propagules; yeast-like cells increased sharply in senescent leaves	Dickinson (1967, cited in Last and Price 1969)
Typha latifolia (E;H)	England, C	*Sp. roseus*	A: PWL, SFM	Seasonal dynamics (fungi); *Sp. roseus* in mid-summer and on senescent leaves (only by SFM)	Pugh and Mulder (1971)
Oxalis acetosella (E;H)	Russia, D	*Cr. albidus*, *Cr. flavus*, *Cr. laurentii*, *Cy. capitatum*, *Le. scottii*, *Rh. fujisanensis*, *Rh. glutinis*, *Sp. roseus* (10^3–10^5 CFU/g)	A: PLM, B: P	Seasonal dynamics and species diversity (yeasts); densities and species richness higher in autumn and lower in spring; yeasts classified into 27 species (19 basidiomycetous species) belonging to 12 genera	Glushakova and Chernov (2004)
Various herbs/ bushes (tundra)	Russia, E	*Cr. laurentii*, *Mr. frigida*, *Le. scottii*, *Rh. glutinis* (10^5–10^6 CFU/g)	A: PLW, B: P	Species diversity (yeasts); yeasts dominate mycobiota and are present in high densities only on plants or litter (not in soil); some psycrophilic species	Chernov (1985)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Various plants (e.g. *Allium*, *Brassica*, *Malus*, *Tilia*, *Trifolium*)	Russia, D	*C. krusei*, *C. tropicalis*, *Cr. albidus* var. *albidus*, *Cr. albidus* var. *diffluens*, *Cr. laurentii*, *M. pulcherrima*, *Rh. glutinis* var. *glutinis*, *Rh. glutinis* var. *dairenensis*, *Rh. mucilaginosa*	A: PLW; LI, B: P	Yeast in rhizosphere and phyllosphere (agricultural plants vs. non-cultivated plants); isolation with and without pre-enrichment yielded 46 and 23 species, respectively; highest densities in August; basidiomycetous yeasts dominant; *Cryptococcus* and *Thichosporon* more abundant on agricultural plants	Kvasnikov et al. (1975)
Betula (D;T) and *Phleum* (G)	Russia, D	*Cr. albidus*, *Cr. laurentii*, *Cr. macerans*, *Rh. glutinis*, *Rh. rubra*, *Sp. pararoseus*, *T. pullulans* (minor: *Sp. roseus*, *Cy. capitatum*) (4–72×10^3 CFU/dm^2 – *Phleum*; 8–90×10^3 CFU/dm^2 – *Betula*)	A: PLW; LI; OML, B: P	Seasonal dynamics and species diversity (yeasts); direct counts 3–5 times higher than plate counts; 300 isolates (13 species and 7 genera); yeast densities higher in autumn	Babjeva and Sadykov (1980)

Fagus sylvatica D;T	England, C	*B. alba, Sp. roseus* and *Tilletiopsis minor*	A: PWL; SFM; OML	Seasonal dynamics (mainly filamentous fungi); yeasts detected right after unfolding and persisted thereafter (only by SFM)	Hogg and Hudson (1966)
Malus spp. (D;T)	New Zealand, C	*Cr. laurentii*, *Rh. ingeniosa* (buds and young leaves); *Sp roseus*, *Rh. rubra*, *Cr. laurentii*, *B. alba* and *U. pyricola* (expanding leaves) (10^4–10^5 CFU/cm^2)	A: PLM; SFM	Seasonal dynamics and species diversity (bacteria and fungi); densities increased throughout spring and early summer but decreased during autumn; densities increased with leaf age; comparison of buds, rosette leaves and shoot leaves	Pennycook and Newhook (1981)
	England, C	White (*Cr. laurentii*) and pink yeasts (*Sp. roseus* and *Rhodotorula*) (10^6 CFU/g)	A: PLW; PLM; SFM	Seasonal dynamics and species diversity (bacteria and fungi); effect of fungicides; bacteria and yeasts more numerous than that of moulds; densities increased from May to November on unsprayed trees	Hislop and Cox (1969)
	USA, C;D	*Sporobolomyces*, *Rhodotorula* and *Cryptococcus* (10^6 CFU/g)	A: PLW; LI; SFM; OML; SEM	Effect of pesticides on seasonal dynamics of non-target phylloplane microbiota; bacteria more severely affected than fungi; densities increased from May to September	Andrews and Kenerley (1978)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Malus spp.—*cont'd*	USA, C;D	*Sporobolomyces*, *Rhodotorula* and *Cryptococcus* (10^5 CFU/g – dormant buds)	A: PLW; SEM	Seasonal dynamics (bacteria and fungi) in buds and young leaves; after unfolding, densities decrease on expanding young leaves (yeasts lagged slightly)	Andrews and Kenerley (1980)
Acer pseudoplatanus (D;T)	England, C	*Sporobolomyces*	A: SFM; SEM	Seasonal dynamics (fungi); densities increase from May/June to August/September; *Sporobolomyces* present predominantly on upper leaf surface, mainly on veins (only by SFM)	Pugh and Buckley (1971)
Acer platanoides (D;T) and *Tilia platyphyllos* (D;T)	Scotland, C	Yeasts (*Acer platanoides*) (10^1–10^3 cells/cm^2 – optical microscopy; 10^1–10^2 CFU/cm^2 – leaf washings). White and pink yeasts (*Tilia platyphyllos*)	A: PLW; PWL; OMLI	Seasonal dynamics (fungi); effect of inhibitory substances from leaves (viz. gallic acid); yeast densities low throughout the season, with increase in September; increased fungal growth observed later in the season –	Irvine et al. (1978)

				coincident with decline in inhibitory activity of leaf washings	
Acer platanoides (D;T)	Scotland, C	Yeasts (*Sp. roseus* more abundant)	A: OMLI	Seasonal dynamics (fungi); low densities in early summer and maximum values during October and November	Breeze and Dix (1981)
Larix decidua (D;T)	Scotland, C	*Rh. glutinis*, *Sp. roseus*, *Cryptococcus* and *Torulopsis*(?) (10^4 CFU/g)	A: PLM; OMLI, B: P	Seasonal dynamics (bacteria and fungi); densities increase from June to November; leaf age is a more important factor for population development than weather or airborne inocula	McBride and Hayes (1977)
Ilex aquifolium (E;T)	England, C	Yeasts (10^4–10^5 cells/cm^2 – optical microscopy; 10–10^4 CFU/cm^2 – leaf washings)	A: PLW; PWL; OMLI; SEM	Seasonal dynamics (fungi); densities higher in autumn and early winter, and on older leaves; upper and lower leaf surfaces with similar yeast population size; litter appeared to provide a refuge for phylloplane yeasts during winter	Mishra and Dickinson (1981)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Various plants (spruce, alder and birch forests)	Russia, D	*Cr. albidus*, *Cr. laurentii*, *Le. scottii*, *Rh. glutinis*, *Rh. minuta*, *Rh. mucilaginosa*, *Sp. roseus* (up to 10^6 CFU/g)	A: PLW	Variation of species composition (yeasts) with substrate: plant leaves, leaf litter or soil; higher densities on plant surfaces; species composition differs with sample type (vertical stratification)	Maksimova and Chernov (2004)
Camellia sinensis (E;B) *Vicia faba* (D;B)	Sri Lanka, A England, C	*S. salmonicolor*, *Rh. mucilaginosa* and *Cryptococcus* sp. (*Camelia sinensis*); *Sporidiobolus* sp. and *B. alba* (*Vicia faba*)	A: PLW	Sensitivity of phylloplane yeasts to UV-B radiation; vulnerability to UV-B correlated with radiation doses of natural habitat; tolerance to UV-B may be related to pigmentation	Gunasekera et al. (1997)
Agrostis palustris (H;G)	USA, C	White (>95% – *Cryptococcus*) and pink yeasts (10^4–10^5 CFU/cm^2)	A: PLW	Seasonal dynamics (yeasts); effect of fungicides; densities higher in autumn; fungicide resistance widespread among phylloplane yeasts	Buck and Burpee (2002)
Various gramineous plants (steppe)	Russia, B;C	*Cr. diffluens*, *Cr. laurentii*, *Rh. glutinis*, *Sp. roseus* (*A. pullulans* dominant)	A: PLW, B: P	Variation of species composition (yeasts) with substrate: plant	Vinovarova and Babjeva (1987)

		(10^5–10^6 CFU/g)		surfaces, leaf litter or soil; higher densities on plant surfaces; species composition differs with sample type (vertical stratification)	
Hippophae rhamnoides (B)	Gibraltar, B;C	*Sporobolomyces*	A: PWL; SFM; OML; OMLI; SEM	Spatial distribution of fungi on leaves; higher densities associated with trichomes and midrib; yeasts isolated only by SFM, more frequent on upper leaf surfaces	Lindsey and Pugh (1976)
Various plants	Canary Islands, B;C	*D. hansenii*, *Cr. albidus*, *Cr. laurentii*, *Rh. glutinis*, *Rh. mucilaginosa*, *Pseudozyma* sp.	A: PLW, B: P	Species diversity and biochemical activities (yeasts); 44 isolates able to degrade and assimilate many high and lowmolecular weight plant constituents	Middelhoven (1997)
Various plants (subtropical deserts)	Southwest Asia, B	*Bullera* spp., *Cr. albidus, Cr. laurentii* (minor), *U. puniceus* (green leaves: $<10^3$ CFU/g; dead leaves: 10^5–10^6 CFU/g)	A: PLW, B: P	Species diversity (yeasts) on green leaves, dead leaves and soil; yeasts present only on plant surfaces	Chernov et al. (1997)

Continues

Table 13.1 Studies of phylloplane microbial ecology that included or focused on yeasts—*cont'd*

Plant(s)[a]	Geography, climate[b]	Dominant yeast genera or species (densities)	Methods[c]	Main subject of study and summary of conclusions	Reference
Acer monspes-sulanum (D;T), *Quercus faginea* (D;T), *Cistus albidus* (E;B), *Pistacia lentiscus* (E;B) and *Osyris quadripartita* (E;B)	Portugal, C	*Cr.* cf. *laurentii*, *E.* cf. *hasegawianum*, *L. inositophila*, *Rh. bacarum*, *Rh.* cf. *sloffiae, Sp. coprosmae*, *Sp.* cf. *gracilis*; putative spp. nov.: *Cryptococcus*, *Filobasidium*, *Rhodotorula* and *Sporobolomyces* (*Sp.* cf. *roseus*, *Sp. ruberrimus,* and *Tilletiopsis* spp. – SFM) (10^1–10^2 CFU/cm^2 – *Osyris*, *Pistacia*; 10^2–10^4 CFU/cm^2 – *Cistus*, *Acer*, *Quercus*)	A: PLW; SFM, B: P; MI	Seasonal dynamics and species diversity (yeasts); total counts and species richness varied according to plant species, season and sampling site; densities increased from spring to autumn, notably on deciduous trees; apparent specificity of some yeast species for *Cistus albidus* leaves (e.g. *Cryptococcus* sp. nov.)	Inácio et al. (2002); Inácio (2003)
Mangifera indica (E;T)	South Africa, C	*Cryptococcus* spp., *Sp. roseus* (minor: *Candida*, *Kluyveromyces*, *Rhodotorula*, *Torulopsis*, *Trichosporon*) (10^4–10^5 CFU/cm^2)	A: PLW; LI, B: P	Seasonal dynamics and species diversity (bacteria and fungi); densities higher in winter and spring; densities and species diversity increased with leaf age	de Jager et al. (2001)

Various plants	Indonesia, Suriname and Ivory Coast, A	*Cr. laurentii*, *Cr. luteolus*, *Rh. glutinis*, *Rh. graminis*, *Rh. mucilaginosa*, *Rh. rubra*, *S. ruineniae* (minor: *Candida*, *Rh. bogoriensis*, *Rh. diffluens*, *Rh. foliorum*, *Rh. javanica*, *Sp. roseus*)	A: PL; LI, B: P	Species diversity (yeasts); 65 strains representing 22 species (predominance of basidiomycetous yeasts)	Ruinen (1963)
Ananas comosus (E)	Brazil, A	*Cr. flavus*, *Cr. laurentii*, *Rh. minuta*, *Rh. rubra*, *Rhodotorula* sp. (10^3–10^4 CFU/cm^2)	A: PLW, B: P	Species diversity (yeasts); comparison of leaves, flowers, fruits and soil; yeast counts higher during flowering	Robbs et al. (1989)
Saccharum officinarum (G)	Brazil, A	*Cr. albidus*, *Cr. laurentii*, *Rh. mucilaginosa*, *Sp. roseus*, *Trichosporon* sp. (minor: *Bullera* sp., *D. hansenii*, *Di. hungarica*, *Rh. minuta*) (10^7 CFU/g dry weight)	A: PLW, B: P	Species diversity (yeasts); comparison of leaves, stems and rhizosphere; predominance of basidiomycetous yeasts on leaves	Azeredo et al. (1998)

[a]Plant type: *T* tree, *H* herb, *G* grass, *B* bush; *D* deciduous, *E* evergreen
[b]Köppen climate classification system: *A* tropical moist climates; *B* dry climates; *C* moist mid-latitude climates with mild winters; *D* moist mid-latitude climates with cold winters; *E* polar climates
[c]*A* detection and/or isolation methods: *OML* optical microscopy directly on leaves; *OMLI* optical microscopy on leaf impressions; *SEM* scanning electronic microscopy; *PLW* plating of leaf washings; *PWL* plating of washed leaves; *PL* plating of leaves; *PLM* plating of leaf macerates; *LI* leaf impressions onto solid media; *SFM* spore-fall method. *B* identification methods: *S* simplified; *P* phenotypic (morphology, physiology); *MI* molecular identification

1981; Inácio et al. 2002) or incubation at lower temperatures (e.g. 5°C, Maksimova and Chernov 2004) to delay mould growth. The utilization of lower incubation temperatures may however select for psychrophilic yeast species (Beech and Davenport 1971). Rose Bengal and other dyes have the disadvantage of being taken up by some yeasts, thus inhibiting their growth or endowing their colonies with a pinkish tinge that can be mistaken for the carotenoid pigments of true "pink" yeasts. Instead of agar media some authors advocate the utilization of artificial substrates that mimic leaf nutritional conditions, such as a wax-based medium amended with nutrients (McCormack et al. 1994a). Counting and isolation of single colonies for subsequent characterization are usually done by selecting randomly from the plates or by picking colonies of each morphological type. Two major limitations of this procedure are that unrelated organisms may display similar colony morphologies and that fast-growing moulds may hamper the enumeration and isolation of other, more fastidious, organisms. Moreover, microorganisms from populations in numerical minority (less than 1% of the dominant species numbers) will most probably go undetected (Flannigan and Campbell 1977). All of the indirect methods mentioned previously are labour-intensive, time-consuming and have the inherent limitations of culture-dependent methods. It is often difficult to compare results from different studies owing to different sampling strategies, washing procedures, culture media and incubation conditions (Jacques and Morris 1995). For example, Azeredo et al. (1998) showed that although the two different washing methods used (sonication and agitation for 30 min on a rotary shaker) gave similar total yeast counts, they resulted in different proportions of the isolated yeast species. The second most used cultivation-based method for yeast isolation from the phylloplane was the spore-fall method (SFM), which is largely specific for ballistoconidiogenic (BC) yeasts (see also Sect. 13.4). Leaf samples are fixed under the lid of a Petri dish, suspended above the agar surface, and colonies develop from discharged spores that fall on the culture medium (Nakase and Takashima 1993). Only a few studies aimed mainly at epiphytic filamentous fungi used isolation methodologies based on the plating of leaves, with or without previous washing, or on leaf impressions onto agar media (Table 13.1).

In recent years several molecular methods have been used for studying the ecology of phylloplane microorganisms without the need of cultivation steps. The main target organisms have been bacteria, but in a few cases yeasts and dimorphic fungi were also included. For example, Yang et al. (2001) used denaturing gradient gel electrophoresis (DGGE) of DNA fragments amplified with universal primers for ribosomal DNA (rDNA) regions, followed by cloning and sequencing of the dominant amplicons, to study bacterial populations in the phyllosphere of *Citrus sinensis* (orange). Among several organisms detected, some DNA bands were ascribed to the ubiquitous epiphytic fungus *A. pullulans*. Bramwell et al. (1995) compared a range of DNA extraction protocols for microbial cells on leaf samples for this type of study. A different molecular technique, fluorescence in situ hybridization (FISH), has been used successfully in the direct detection and identification of microorganisms in their natural environments (e.g. Moter and Göbel 2000). The rationale underlying FISH is that, under appropriate hybridization conditions, the annealing between complementary sequences in the oligonucleotide fluorochrome-labelled probe and target molecules (usually rRNA sequences) can be detected by fluorescence microscopy (or flow

cytometry) in intact cells. Despite some methodological problems that arise when working with whole leaf samples or the respective washings (e.g. background fluorescence of chlorophyll of leaf tissues or topographical structures like trichomes), FISH has already been used for detecting and/or enumerating *A. pullulans* on apple leaves (Li et al. 1997; Spear et al. 1999; Andrews et al. 2002), *Taphrina deformans* on peach leaves (Tavares et al. 2004), and *Cryptococcus* sp. nov. and other yeasts on leaves of different Mediterranean plant species (Inácio 2003).

The motto 'no ecology without taxonomy' has often been applied to microbial ecology (Phaff and Starmer 1987; Fokkema 1991) and pertains to the crucial issue of accurate identifications of phylloplane microbes. Yeast identifications in the studies published until the late 1990s were based mainly on more or less extensive phenotypic characterizations (see Table 13.1) and in many cases can be merely considered tentative. However, the last decade has witnessed remarkable improvements in yeast (and fungal) systematics as a consequence of the so-called molecular revolution (Kurtzman and Fell 1998). In fact only the full implementation of molecular methods facilitates fast and unambiguous identifications, which are now possible owing mainly to the comprehensive rDNA sequence databases made available by the work of Kurtzman and Robnett (1998) for ascomycetous yeasts and of Fell et al. (2000) and Scorzetti et al. (2002) for basidiomycetous yeasts. The impact of these developments is especially significant in the ecology of epiphytic yeasts in view of the many changes that took place in the classification system of basidiomycetous yeasts in recent years (Fell et al. 2001; Sampaio 2004; see Sect. 3). Therefore, it cannot be ruled out that the apparent ubiquity of various epiphytic yeasts, irrespective of host plant and/or locality, stemming from many studies is a result of inaccurate identifications (see Sect. 13.4). In fact yeast species deemed to be ubiquitous phylloplane colonists have been shown to be taxonomically heterogeneous on the basis of molecular phylogenetic analyses only in the last 4 years: *Cr. albidus* (Fonseca et al. 2000; Sugita et al. 2001); *Cr. laurentii* (Sugita et al. 2000; Takashima et al. 2003); *Rhodotorula minuta* (Fell et al. 2000); *Sporobolomyces roseus* (Bai et al. 2002; Fell et al. 2002). A different picture of the composition of epiphytic yeast communities is indeed already emerging from the results of recent studies that used molecular identification methods (Inácio 2003; Prakitchaiwattana et al. 2004).

13.3 Plant Surfaces as Yeast Habitats

The occurrence of yeasts in association with plants has been acknowledged for many years (Lund 1954; Last and Price 1969; Phaff et al. 1978; Phaff and Starmer 1987). Their ability to utilize a plethora of organic compounds through respiratory processes greatly expands the range of ecological niches that they may colonize beyond sugar-rich substrates such as fermenting fruit juices. Many compounds, such as pentoses, polyols, organic acids, aromatic compounds and amino acids, which specific yeasts can assimilate, are either products of plant metabolism or products of other microorganisms from precursors in plants (Phaff et al. 1978). Examples of plant habitats that have been extensively investigated for their yeast inhabitants include the nectar of flowers (Golonka 2002; Herzberg 2004), tree exudates or slime fluxes (Phaff and Starmer 1987) and the necrotic tissues of cacti (Starmer et al.

1991). The yeast communities found therein and whose composition was specific for each type of habitat were generally dominated by ascomycetous species and insects were identified as the major vectors for the introduction and/or dispersal of those yeasts (Phaff and Starmer 1987; Babjeva and Chernov 1995). In contrast, communities found on plant surfaces such as leaves, flowers (excluding nectaries), immature or intact fruits and bark were dominated by basidiomycetous yeasts and the species composition of those communities was generally considered more uniform (Last and Price 1969; Phaff and Starmer 1987; Babjeva and Chernov 1995). The microenvironments that involve aerial organs of plants such as stems, fruits or flowers are analogous to those found on the phylloplane and it is thus not surprising to find parallels in the dynamics and composition of the respective yeast communities (Last and Price 1969; Buck et al. 1998). The distinctive nature of epiphytic yeasts received further support from comparisons with the communities found in the rhizosphere, ensuing from the early work of di Menna with pasture plants in New Zealand (reviewed in Carmo-Sousa 1969 and Last and Price 1969) and later confirmed by other workers (Kvasnikov et al. 1975; Fokkema and Schippers 1986; Maksimova and Chernov 2004). The results of those studies showed that although basidiomycetous yeasts were also dominant, the species found in soils near the roots of plants (e.g. *Cr. albidus*, *Cr. diffluens*, *Cr. humicola*, *Cr. curvatus*; di Menna 1959) did not coincide with those isolated from the aerial surfaces of the same plants (*Cr. laurentii*, *Rh. ingeniosa*, *Rh. graminis*, *Rh. mucilaginosa*, *Sp. roseus*; di Menna 1959). Another relevant observation by di Menna was that while the composition of the soil yeast communities varied with soil type but not with season, the phyllosphere populations changed with season but not with locality or plant (see Sect. 13.5). Similar conclusions ensued from the work carried out at the Department of Soil Biology of the State University of Moscow by Babjeva and co-workers since the 1980s (Babjeva and Chernov 1995). A reasonable corollary from these observations is that basidiomycetous yeasts seem to have distinct niches in the same geographic location despite displaying apparently similar phenotypes (strictly aerobic and generally oligotrophic). Another observation that points to the distinctive nature of epiphytic yeasts stemmed from studies of the communities found on the surfaces of fruits. Work by different authors revised in Last and Price (1969) and Phaff and Starmer (1987) suggested the frequent occurrence of important shifts in the yeasts populations present on intact fruits (generally dominated by basidiomycetous yeasts) when compared with those found on damaged or fermenting fruits (dominated by ascomycetous and/or fermenting yeasts). This trend was nicely demonstrated in a recent study of the yeast communities on the surface of grapes by Prakitchaiwattana et al. (2004), who found species of *Hanseniaspora* and *Metschnikowia* only on damaged, ripe grapes, whereas undamaged berries in different ripening stages were populated by basidiomycetous yeasts (besides *A. pullulans*). Some of these topics will be further discussed in the following sections.

Before proceeding we want to mention an apparently minor issue but one of relevance to the ensuing discussions on the nature and dynamics of phylloplane yeasts. It pertains to the euascomycete *A. pullulans*. This dimorphic fungus, sometimes inappropriately called yeast-like, is apparently ubiquitous on aerial plant surfaces in temperate or subtropical regions worldwide (Andrews et al. 1994) and has been

reported as the dominant fungal epiphyte of intact fruits (apple, Beech and Davenport 1970; pear, Chand-Goyal and Spotts 1996; grapes, Prakitchaiwattana et al. 2004) and leaves of trees such as *Acer platanoides* (Norway maple, Breeze and Dix 1981), *llex aquifolium* (holly, Mishra and Dickinson 1981), *Fagus sylvatica* (beech, Hogg and Hudson 1966), *Malus domestica* (apple, Andrews and Kenerley 1978) and *Mangifera indica* (mango, de Jager et al. 2001). In some studies *A. pullulans* was even considered a member of the yeast community (Breeze and Dix 1981; de Jager et al. 2001) but in this review we will focus mainly on the yeast taxa dealt with in Kurtzman and Fell (1998).

13.4 Diversity of Phylloplane Yeasts

Yeasts have been recognized as important phylloplane colonists since the classic publications by Last (1955), di Menna (1959) and Ruinen (1961). These and other early studies were reviewed in Last and Price (1969) and Phaff and Starmer (1987). We have summarized in Table 13.1 the results of those studies, as well as others not covered in the mentioned reviews or published more recently. The studies were grouped according to plant type and/or to the climatic characteristics of the sampling site. Whenever possible we have tried to update species names according to current notions on yeast classification (Kurtzman and Fell 1998; Sampaio 2004). Methods used for yeast isolation and identification are also indicated since they have a major influence on the results obtained (see Sect. 2).

The most obvious trend that emerges from the analysis of Table 13.1 is the clear dominance of basidiomycetous yeasts on leaves, a feature common to most aerial plant surfaces (see Sect. 3). Phylloplane communities usually comprise deeply pigmented species belonging to the genera *Rhodotorula* and *Sporobolomyces* (collectively referred to as 'pink yeasts' in many studies) and non-pigmented *Cryptococcus* species ('white yeasts') (Hislop and Cox 1969; McBride and Hayes 1977; Fokkema et al. 1979; McCormack et al. 1994b). In some cases, however, ascomycetous yeasts were reported as important leaf colonists (Phaff and Starmer 1987; Middelhoven 1997; Azeredo et al. 1998; de Jager et al. 2001). Attempts to draw inferences on distribution patterns of phylloplane yeast species from published data are hampered by the inherent limitations of the conventional methodologies used for strain identification in all but one of the studies listed in Table 13.1. In some cases identifications were admittedly tentative because the authors were essentially interested in unravelling the dynamics of the phylloplane populations (Hislop and Cox 1969; McBride and Hayes 1977; Irvine et al. 1978; Fokkema et al. 1979; de Jager et al. 2001). Some authors acknowledged the difficulties in achieving accurate identifications on the basis of phenotypic properties and noted the heterogeneity of certain yeast species (Hislop and Cox 1969; Flannigan and Campbell 1977; Azeredo et al. 1998). Comparison of the results of those studies is also constrained by the use of diverse isolation methods by different authors, an illustrative example being the use of the SFM specific for recovery of BC yeasts (Last 1955; Hogg and Hudson 1966; Lindsey and Pugh 1976; Pugh and Buckley 1971; Pugh and Mulder 1971). In fact, members of the genera *Bullera*, *Sporobolomyces* or *Tilletiopsis* are thought to be especially adapted to this kind of habitat owing to the production of forcibly ejected

conidia that ensure their efficient dispersal and are thus commonly isolated from leaves (Nakase 2000; Sampaio 2004). However, the SFM is not amenable to quantification (Pennycook and Newhook 1981; Fokkema 1991) and it is therefore not possible in those studies to estimate the relative size of BC yeast populations within the phylloplane communities. These yeasts will be dealt with separately at the end of this section.

Another general trend observed in studies that employed plating of leaf washings for yeast isolation is that populations are commonly dominated by relatively few abundant species (Table 13.1), a situation also observed for other microbial epiphytes (Andrews 1991). Nevertheless, minor species may account for a significant fraction of species richness on the phylloplane, especially in samples with large yeast counts, as demonstrated in some studies (Inácio et al. 2002; Maksimova and Chernov 2004) and many novel taxa were discovered among them (Nakase 2000; Inácio 2003). Amongst the dominant species, *Cr. laurentii*, *Cr. albidus* (less abundant than *Cr. laurentii* and not as widespread), *Rh. glutinis*, *Rh. minuta*, *Rh. mucilaginosa* and *Sp. roseus* appear to be prevalent regardless of plant type or geography (Table 13.1). However, as pointed out in Sect. 13.2, the majority of these species were recently found to be genetically heterogeneous and their apparent ubiquity thus needs revaluation. Whereas the presence of the previously mentioned species seems to be essentially independent of plant type and geography, their relative abundance varies not only with plant type but also with season (see also Sect. 13.5). For instance, non-pigmented *Cryptococcus* spp. (mainly *Cr. laurentii*) dominated phylloplane communities on pasture plants and other herbaceous plants (di Menna 1971; Buck and Burpee 2002; Glushakova and Chernov 2004) but pigmented yeasts (namely *Rh. glutinis* and/or *Sp. roseus*) were prevalent on barley and wheat (Diem 1967, cited in Last and Price 1969; Flannigan and Campbell 1977; Southwell et al. 1999) and possibly on conifers (McBride and Hayes 1977). Deeply pigmented *Rhodotorula* and *Sporobolomyces* spp. were also dominant on xerophytic shrubs when compared with other Mediterranean plant species sampled simultaneously (Inácio et al. 2002; Inácio 2003). Another trend that emerged from surveys on multiple plants was that yeast densities and species richness were consistently lower on leaves of xerophytic shrubs and coniferous trees than on those of hydrophytic or mesophytic herbs and deciduous trees (Inácio et al. 2002; Maksimova and Chernov 2004). On the other hand, some species appear to be restricted to specific biocoenoses or geographic regions: *Rh. ingeniosa* on pasture plants and apple leaves in New Zealand (di Menna 1971; Pennycook and Newhook 1981); *Rh. fujisanensis*, *Cystofilobasidium capitatum* and *Leucosporidium scottii* in subboreal forests in Russia, mainly on senescent leaves (Glushakova and Chernov 2004; Maksimova and Chernov 2004); *Rh. bacarum* as well as a few *Rhodotorula* and *Sporobolomyces* spp. belonging to the Erythrobasidium lineage (*sensu* Fell et al. 2000) on Mediterranean plants in Portugal (Inácio et al. 2002; Inácio 2003). Associations of particular yeast species to specific plants appear to be rare (Andrews 1991) but one such case was reported by Inácio and co-workers, who found consistently significant numbers of a putative novel *Cryptococcus* species with orange colonies on the leaves of the evergreen shrub *Cistus albidus* (grey-leaved cistus or white-leaf rock rose) but not on four other plant species sampled at the same sites for two consecutive years (Inácio et al. 2002; Inácio 2003).

Ascomycetous yeasts are usually rare on the phylloplane but the species *Debaryomyces hansenii* was found with high frequency on plants from the Canary Islands (Middelhoven 1997) and on sugarcane in Brazil (Azeredo et al. 1998) and was also reported to occur on leaves of forest plants in Russia (Babjeva et al. 1999; Glushakova and Chernov 2004; Maksimova and Chernov 2004). Yeasts related to the archiascomycete genus *Taphrina* and classified in *Lalaria* (Inácio et al. 2004) were found in high densities on Mediterranean plants in Portugal (Inácio et al. 2002; Inácio 2003). Putative *Lalaria* spp. were also reported on forest trees in Russia by Babjeva and co-workers (Babjeva and Reshetova 1998; Babjeva et al. 1999). This is an unexpected finding since members of the dimorphic phytopathogenic genus *Taphrina* are rarely isolated from plant material other than infected tissues of the host (Kramer 1987). Inácio et al. (2004) claim that their *Lalaria* isolates may represent genuine phylloplane inhabitants that have lost the parasitic stage altogether. The relatively rare occurrence of ascomycetous yeasts on the phylloplane is also evidenced by comparing the composition of epiphytic yeasts on fruits and leaves of the same plant species (Beech and Davenport 1970; Robbs et al. 1989). For example, on apple fruit skin (Beech and Davenport 1970; Bizeau et al. 1989) species of *Hanseniaspora* and *Metschnikowia* are commonly present together with the basidiomycetous species (and *Aureobasidium*) that are also found on the leaves, on which the former are absent (Pennycook and Newhook 1981). The unusual finding of species of *Torulaspora* and *Kluyveromyces* on elm trees in California was probably due to the sugary syrup secreted by its leaves (Phaff and Starmer 1987).

A different approach to the ecology of epiphytic yeasts has been undertaken by Babjeva and co-workers: instead of focusing on specific substrates (i.e. plant species) they have chosen to analyse the spatial structure and biogeography of yeast populations obtained from large numbers of samples in plant–soil systems from different geographic regions (Babjeva and Chernov 1995; Maksimova and Chernov 2004). They distinguished three yeast complexes occupying different substrates that correspond to a vertical stratification of each type of ecosystem studied (tundra, forest, steppe and desert): the epiphytous complex, i.e. the yeasts that occur on living, green aboveground plant parts (mainly the phylloplane); the litter complex, i.e. the yeasts that are present on senescent leaves and leaf litter; and the soil complex, i.e. the species inhabiting mineral soil horizons. The dominant species of the first complex, consisting mainly of *Cr. laurentii* and *Sp. roseus*, were regarded as non-geographic (similar species composition in different zones). However, species richness and minor species varied from tundra to desert ecosystems. Higher yeast densities and species richness were found in forest biotopes for which some differences in community composition were noted depending on the plant type (hydrophytic vs. xerophytic, conifer vs. broad leaf) or forest type (spruce, alder or birch) (Maksimova and Chernov 2004).

As mentioned previously, BC yeasts in general and the species *Sp. roseus* in particular are frequently associated with leaf surfaces and were already reported in the early phylloplane work by Last (1955), Kerling (1958, cited in Last and Price 1969) and di Menna (1959) (Table 13.1). Production of ballistoconidia provides a clearly efficient means for dispersal of those yeasts and appears to be stimulated by the nutrient-poor conditions found on leaf surfaces. In some studies the SFM was used

as the sole method for yeast isolation and therefore only that type of yeast was recovered from the phylloplane (Table 13.1). The majority of recognized species in the genera *Bensingtonia*, *Bullera* and *Sporobolomyces* were in fact isolated from phylloplane yeast surveys employing the SFM and were conducted mainly by researchers in Asia (Nakase 2000). However, it should be noted that BC yeasts may occur in very different habitats, such as seawater (Sampaio 2004), and that the SFM may yield non-BC yeasts (Inácio 2003; Inácio et al. 2004). The ubiquitous phylloplane species *Sp. roseus* was thought to have a worldwide distribution (Last and Price 1969) but may actually be more abundant in temperate regions (climate types C and D; Köppen's system). Babjeva and co-workers found *Sp. roseus* in high frequencies as a member of the epiphytous complex in tundra, forest and steppe ecosystems in Russia but not in desert biotopes (Babjeva and Chernov 1995). This species was dominant on steppe plants but in the different forest types studied it was never found on mosses despite being isolated from the phylloplane of all other herbaceous and ligneous plants (broad-leaved or conifers) sampled (Maksimova and Chernov 2004). *Sp. roseus* was also found on Mediterranean plants in Portugal but only using the SFM and was seldom detected on plates inoculated with leaf washings (Inácio et al. 2002; Inácio 2003). In an overview of the occurrence of BC yeasts in the Asia–Pacific region, Nakase (2000) claims that there is no correlation between the presence of each yeast species and the respective host plant but that there appear to be some geographic patterns. Once again *Sp. roseus* was apparently most frequently isolated in temperate and continental regions (climate types C and D) but not in the tropics (climate type A). According to the same author this pattern is shared by less frequent species such as *Udeniomyces pyricola*, *Sp. inositophilus*, *Sp. sasicola* and *Bensingtonia naganoensis*. Conversely, species that were isolated exclusively in the tropics include *Sporidiobolus ruineniae* and particular species in the genera *Kockovaella*, *Sporobolomyces* (namely those having hydrogenated CoQ10) and *Bullera*. Species such as *Bullera alba*, *B. crocea*, *B. variabilis*, *S. pararoseus* and *S. salmonicolor* were found in all the surveys reviewed by Nakase (2000) independent of climatic region.

13.5 Population Dynamics on the Phylloplane: Variation in Space and Time

One unifying feature of phylloplane microbial populations is their variability across a wide range of spatial and temporal scales (Kinkel 1997; Andrews and Harris 2000; Morris 2001). Some information is available about the patterns of epiphytic microbial community dynamics, at least for some of its components, but elucidation of the processes that generate those patterns is limited (Kinkel 1997). The dynamics of microbial populations on leaves are a function of four processes: immigration (I), emigration (E), growth (G) and death (D). The contribution of each process to the variability in phylloplane community sizes and composition is likely to differ markedly over time and for different organisms. Some studies dealing with changes in numbers of given species in the phyllosphere over time and with patterns of their localization on leaves focused mainly on bacteria (Kinkel 1991; Jacques et al. 1995; Hirano and Upper 2000) or fungi (Andrews et al. 1987, 2002; Kinkel et al. 1989) but

comparatively less is known about yeasts. However, some of the general trends observed may be extrapolated for different epiphytic microbes (Kinkel 1997).

The significance of the atmosphere as both a source (I) and sink (E) for phylloplane populations has been acknowledged by many authors (Andrews 1991; Kinkel 1997). However, despite a substantial body of literature on airborne propagules and their significance to dispersal of epiphytic microbes (yeasts: Last and Price 1969) there have been few attempts to correspond shifts in air inocula with specific changes in phylloplane populations and there is little quantitative information on the dynamics of those airborne cells in relation to immigration and emigration of microbes on individual leaves (Andrews et al. 1987). Emigration from leaves occurs as a function of active dispersal mechanisms generated by rain, water movement or wind. Immigration to leaves occurs by impaction of particles onto the leaf surface, gravity settling or sedimentation, or rain-splash dispersal to the leaf surface (Kinkel 1997). The uniformity in occurrence (but not necessarily population size; see later) of the dominant yeast species on leaves of different plants in the same geographic area (di Menna 1971; Babjeva and Sadykov 1980; Inácio et al. 2002; Maksimova and Chernov 2004) suggests that immigration is a quantitatively significant process in the build-up of phylloplane populations. Within-leaf processes of growth and death are a function of leaf age, season and plant species but are also influenced by the physical environment: growth of bacteria, filamentous fungi or yeasts on leaf surfaces is generally more significant when temperatures are moderate and moisture levels are high (fungi/yeasts: Bashi and Fokkema 1977; Inácio et al. 2002); death of microbes on plant surfaces is enhanced under conditions of intense UV radiation, high temperatures, low relative humidity and/or low availability of free moisture (Kinkel 1997). However, the presence of protected sites on leaf surfaces and the survival mechanisms of many microbial epiphytes allow individuals within the population to persist despite the prevalence of conditions non-conducive to growth or survival. In summary, differences in populations of microbial epiphytes among leaves of different plant species (and over time) may be initiated as a function of immigration and emigration, and the subsequent effects of differential growth and death are likely to act to further distinguish leaves as habitats (Kinkel 1997). Significant variations in relative sizes of populations of different yeast species on different plants in the same geographic area were demonstrated in the studies by Inácio et al. (2002) and Maksimova and Chernov (2004).

13.5.1 Patterns in Space

Microbial populations are unevenly distributed across individual leaf surfaces. Aggregated populations have been noted for bacteria (Morris et al. 1997), yeasts (Bashi and Fokkema 1976; Babjeva and Sadykov 1980) and filamentous fungi (Pugh and Buckley 1971; Mishra and Dickinson 1981). Aggregation of microbial cells is often correlated with specific structures on the leaf surface (leaf veins, trichomes, stomates, fungal hyphae, pollen grains) or may be a function of their existence within biofilms or within an extracellular matrix on the leaf surface (Kinkel 1997; Andrews and Harris 2000). The importance of discrete sites conducive to microbial growth and survival suggests that population densities are likely to be correspondingly

aggregated. Such patterns have been recently revealed in a study of the distribution of *A. pullulans* on the apple leaf surface using FISH (Andrews et al. 2002). The authors found that most of the leaf surface was not colonized and that there was a highly patchy occupancy of leaf space by *A. pullulans* often related to landmarks, especially the midvein. The reasons for this pattern in the distribution of microcolonies were not elucidated, but physiological and anatomical evidence suggests that veins may be zones of nutrient enrichment (Andrews et al. 2002). Veinal distribution patterns had already been shown for other phylloplane microbes (Kinkel 1997) and in particular for BC yeasts in studies that used the SFM (Last and Price 1969; Lindsey and Pugh 1976) and were confirmed by optical microscopy for other yeasts (Babjeva and Sadykov 1980) and by SEM for fungi in general (Pugh and Buckley 1971). Association of fungi with trichomes was revealed also using SEM on leaves of *Hyppophae rhamnoides* (sea buckthorn, Lindsey and Pugh 1976) and of apple (Andrews and Kenerley 1978). Inácio et al. (2002) suggested that the dense trichome cover on the leaves of the evergreen shrub *Cistus albidus* may have accounted for the high densities and species diversity of fungi on this plant when compared with those observed on other plants sampled concurrently. Andrews et al. (2002) also found that leaves were rarely seen with natural occurring wounds in early summer but that such damage (almost exclusively in the interveinal areas) increased over the course of a growing season. The authors claim that natural wounds accounted overall for an increasing fraction of the total *A. pullulans* population on leaves. If confirmed for other leaf–microbe systems, wounding, and the consequent increased leaching of nutrients, may contribute significantly to well-known but unexplained phenomena such as seasonal (see later) and leaf-to-leaf variations in microbial counts.

Epiphytic bacterial populations tend to be log-normally distributed even among identical leaves, as well as among small leaf segments (Kinkel 1997; Andrews and Harris 2000). Evidence based on colony forming units (CFU) counts on plates inoculated with washings from wheat leaves suggested that phylloplane yeasts and filamentous fungi were normally as opposed to log-normally distributed (Fokkema and Schippers 1986). However, more recent studies using improved plate count methods (Woody et al. 2003) or FISH (Inácio 2003) suggest that *A. pullulans* on apple leaves and *Cryptococcus* sp. nov. on *Cistus albidus* leaves, respectively, followed log-normal distributions.

Microbial populations tend to be higher on the lower (abaxial) than the upper (adaxial) leaf surface (Kinkel 1997), presumably owing to more favourable nutrient, microhabitat or microenvironmental settings, but this pattern possibly varies with plant type. Available data for yeasts are not consistent. Pugh and Buckley (1971) reported that *Sporobolomyces* evaluated with the SFM was present predominantly on adaxial surfaces and the same trend was found by Last and Deighton (1965, cited in Pugh and Buckley 1971) on elm leaves, by Lindsey and Pugh (1976) on leaves of *Hyppophae rhamnoides* and by Pennycook and Newhook (1981) on apple leaves. The opposite situation was found on leaves of chrysanthemum and rowan but no significant differences between the two surfaces were detected for barley (Last and Deighton 1965, cited in Pugh and Buckley 1971). Pennycook and Newhook (1981) claim that evidence for the distribution of *Sp. roseus* based on spore-fall data must be

treated with caution, since they found that spore discharge rates do not necessary correlate with population size (see Sect. 4). Moreover, using a maceration-dilution plating method they found that the yeast population on the abaxial surface was approximately twice as dense as that on the adaxial surface at all sampling dates (Pennycook and Newhook 1981). However, Andrews and Kenerley (1978) found no consistent differences between yeast counts on adaxial or abaxial apple leaf surfaces using a leaf-imprinting method. On the other hand, Mishra and Dickinson (1981) examined impression films taken from green *Ilex aquifolium* leaves of different ages and showed that the abaxial surfaces usually supported larger numbers of yeasts than adaxial surfaces, the differences between surfaces being most pronounced on younger leaves. Breeze and Dix (1981) observed the opposite situation for *A. pullulans* on the leaves of *Acer platanoides*, using the same technique. These apparent contradictory results could be due to different leaf topography of each surface (e.g. dense trichome cover of abaxial vs. adaxial surface of apple leaves; Andrews et al. 2002), which may have gone unnoticed and was not taken into account in most studies.

Variability in phylloplane population sizes among leaves is sometimes correlated with leaf position, especially height in the canopy, but varies with plant species (Kinkel 1997). For yeasts limited evidence suggests that the largest populations generally occurred on leaves from the basal portion of shoots (Mishra and Dickinson 1981) and on leaves closer to the ground (Andrews et al. 1980). Densities of phylloplane microbes are also determined by plant position in the field as shown in studies with barley (Diem 1967, cited in Last and Price 1969) and apple (Bakker et al. 2002).

13.5.2 Patterns in Time

The processes that govern the dynamics of epiphytic microbial populations depend on many intrinsic (e.g. abundance and composition of plant exudates) and extrinsic (e.g. temperature, humidity, solar radiation) factors, which in turn undergo significant seasonal and ontogenetic changes, thus causing pronounced temporal shifts in the species composition of phylloplane communities. Although short-term variations have been studied by some authors, they appear to be more significant for bacteria than fungi (including yeasts) (Thompson et al. 1995; Kinkel 1997). More importantly, the largest body of evidence comes from the numerous studies on seasonal dynamics of epiphytic populations and concerns long-term patterns (Kinkel 1997; Andrews and Harris 2000). The most consistent trends observed in those studies were (1) a general increase in population sizes over time (i.e. with leaf age and/or season) and (2) a seasonal succession in bacterial and fungal populations, featuring an early prominence of bacteria, followed by yeasts, and eventually filamentous fungi as leaves mature and senesce. These features also emerged from a significant number of detailed studies that focused on or included yeasts, listed in Table 13.1. Absolute values of yeast densities reported in different studies are difficult to compare owing to utilization of different methods of isolation and enumeration. However, there appear to be some significant differences in the maximum capacity of leaves depending on plant type and/or climate: values of 10^3–10^4 CFU/cm^2 (equivalent to approximately 10^4–10^5 CFU/g) are common on leaves of herbs or

deciduous trees in temperate climates but values as high as 10^6 CFU/cm^2 (approximately 10^7 CFU/g) were found in some cases (Table 13.1). Yeast population densities estimated from plate counts often exceeded those of filamentous fungi and this trend was corroborated by estimates from direct counts (Hogg and Hudson 1966; Dickinson 1967, cited in Last and Price 1969; McBride and Hayes 1977; Breeze and Dix 1981; Pennycook and Newhook 1981).

Studies that evaluated the dynamics of yeast populations and provided evidence for the increase of population sizes with leaf age focused on different plant types: a deciduous conifer (larch, McBride and Hayes 1977), a deciduous broad-leaved tree (apple, Pennycook and Newhook 1981) and two evergreen trees (holly, Mishra and Dickinson 1981; mango, de Jager et al. 2001). Other studies demonstrated similar trends but yeasts were evaluated solely by the SFM (Last 1955; Hogg and Hudson 1966; Pugh and Mulder 1971). The general increase in phylloplane populations was attributed to immigration processes, which should be more pronounced in the initial stages of leaf development, and to growth at the later stages mainly due to increased leaching of nutrients onto the leaf surface (see Sect. 5.1). Inoculation experiments on cereals provided circumstantial evidence that the limited nutrient availability on young leaves is a most probable cause for the relatively poor development of yeasts on non-senescing leaves (Bashi and Fokkema 1977). The fact that older leaves are more conducive to microbial growth has also been attributed to changes in surface properties, namely increasing wettability, i.e. lower hydrophobicity (Mishra and Dickinson 1981; Buck and Andrews 1999a), or to diminishing concentrations of antimicrobial compounds (Irvine et al. 1978). The interplay of the previously mentioned processes is evidenced in the thorough study of the mycobiota of apple buds and leaves by Pennycook and Newhook (1981): on newly unfurled rosette leaves the phylloplane yeasts were similar to those in unopened buds, but within 5 days some of the components began to disappear and new ones took their place; there was a sharp initial decline in population densities followed by a more gradual increase; the authors claim that the initial decrease was partly caused by the loss of those species of the bud microflora which were ill-adapted for survival on exposed leaf surfaces (D) and partly to dilution of the populations by the rapid increase in the surface area of each lamina during its expansion, the subsequent increase in population densities being due to multiplication of the surviving species (e.g. *A. pullulans*, *Cr. laurentii*, *Rh. ingeniosa*, *Rh. mucilaginosa*) of the bud microflora (G), and to deposition and establishment of new species (e.g. *Sp. roseus*) derived from the air spora (I). The presence of yeasts (and other microbes) inside leaf buds as possible sources of leaf inocula was confirmed by Andrews and Kenerley (1980). The prominence of yeasts on senescent leaves and on leaf litter in the initial stages of decomposition was demonstrated by Dickinson (1967, cited in Last and Price 1969), di Menna (1971), Mishra and Dickinson (1981) and Maksimova and Chernov (2004). The latter authors also noted important shifts in the yeast community composition on leaf litter of subboreal forests in Russia with the clear dominance of species that were minor components on green leaves, namely *Rh. fujisanensis*, *Cy. capitatum* and *Le. scottii*.

Several studies evaluated the seasonal dynamics of size and composition of phylloplane fungal and/or yeast communities on different plant types – pasture herbs,

di Menna (1959, 1971); wheat, Flannigan and Campbell (1977); wood sorrel, Glushakova and Chernov (2004); apple, Pennycook and Newhook (1981); larch, McBride and Hayes (1977); Mediterranean plants (Inácio et al. 2002) – while others focused mainly on population sizes (maple, Irvine et al. 1978; holly, Mishra and Dickinson 1981; mango, de Jager et al. 2001). A continuous increase in population size from spring to autumn/winter was the general rule but a significant decrease in autumn was observed on beet (Kerling 1958, cited in Last and Price 1969), apple (Hislop and Cox 1969; Pennycook and Newhook 1981) and wheat (Flannigan and Campbell 1977). A notable exception was the finding of higher densities of yeasts and fungi in winter and spring on mango leaves by de Jager et al. (2001), which the authors attributed to the winter flowering of this species that released large amounts of pollen (see Sect. 13.1). The increase in population size later in the growing season may be due to the factors mentioned before for leaf age but some authors also suggest the positive impact of the more conducive environmental factors that prevail in autumn, such as higher humidity and milder temperatures (Breeze and Dix 1981; Kinkel 1997). An almost universal trend in many of the studies mentioned before was that higher yeast densities were associated with larger numbers of species being recovered (species richness) (Inácio et al. 2002; Maksimova and Chernov 2004). However, important shifts in the composition of phylloplane yeast communities with season were reported in studies that evaluated the dynamics of species diversity in more detail: apple (Pennycook and Newhook 1981), wood sorrel (Glushakova and Chernov 2004), pasture plants (di Menna 1959, 1971). The most notable trend was the predominance of deeply pigmented *Rhodotorula* and/or *Sporobolomyces* spp. during the summer months that was attributed exclusively to environmental parameters such as prevailing temperatures, moisture levels and/or daily duration and intensity of sun exposure to which those yeasts are presumably better adapted. Conversely, *Cryptoccocus* spp., namely *Cr. laurentii*, dominated during the colder, more humid months (spring and late autumn/winter) when overall yeast populations were relatively sparse.

13.6 What are the Makings of a 'Phylloplane Yeast'?

Microbial phylloplane colonists (i.e. the residents as opposed to the transients) are presumably endowed with suitable phenotypes for survival and/or growth in their particular surface habitats. The adaptations shared by the diverse microbial colonists of a given region have been referred to as niche-specific traits. Some of these attributes include fast growth rates, the ability to compete for nutrients and to withstand periods of drought or intense light, varying nutrient levels, osmotic conditions and temperatures (Andrews and Harris 2000; Morris 2001; Lindow and Brandl 2003). Many fitness traits are intuitive and have been sustained experimentally but others are not, or are actually counterintuitive (Lindow 1991; Andrews and Buck 2002). Some of those traits have been elucidated for bacteria using modern molecular genetic tools and include production of extracellular polysaccharide (EPS), production of surfactants that modify surface properties such as wettability or production of compounds that stimulate release of nutrients (Lindow and Brandl 2003). Those methodological approaches have not yet been applied to fungi or yeasts

and available data on fitness traits in the latter organisms are mostly circumstantial. Is it possible then to propose a preliminary definition for 'phylloplane yeast'? In the absence of solid evidence we chose to offer some speculative thoughts that are intended merely as potentially useful hints for future studies.

Dominant phylloplane yeast colonists are mostly of basidiomycetous affinity. Phenotypic traits associated with these yeasts include a strictly respiratory metabolism, oligotrophic nutrition and production of ballistoconidia, and these characteristics seem most adequate for growth or dispersal on leaf surfaces (Last and Price 1969). However, basidiomycetous yeasts are also prevalent in quite different habitats, such as soil or aquatic environments (Sampaio 2004), suggesting that those traits are not unique to phylloplane colonists. Another interesting observation is that basidiomycetous phylloplane isolates are usually anamorphic yeast stages, whereas their teleomorphic filamentous counterparts are rarely found on leaves (Inácio 2003; Maksimova and Chernov 2004). An obvious if rather naive corollary is that unicellular growth forms are better suited for phylloplane colonization owing to more efficient nutrient uptake and dispersal, whereas a mycelial growth habit is better suited for invasion of leaf tissues. The latter situation occurs most likely during leaf decomposition and it concurs with the finding on leaf litter of filamentous yeast taxa (e.g. *Cy. capitatum*, *Trichosporon pullulans*) not present on green leaves (Maksimova and Chernov 2004).

A more tangible fitness trait, common to many phylloplane yeasts, is the production of EPS capsules (Bashi and Fokkema 1976; Babjeva and Sadykov 1980; Glushakova and Chernov 2004) mainly by members of *Cryptococcus* but also by some *Rhodotorula* spp. (Golubev 1991). EPS has already been shown to confer improved fitness to phylloplane bacteria (see earlier) and the importance of biofilms in bacterial colonization of surfaces is widely recognized (Andrews and Harris 2000). Experimental evidence for yeasts suggests that the role of capsules in providing increased fitness for survival and growth on natural substrates pertains to two environmental factors that are particularly significant on the phylloplane: water and nutrient stresses (Golubev 1991). On one hand capsules appear to act as cellular buffer systems preventing too rapid a loss of water and providing for efficient rehydration following periods of drought (Golubev 1991, and references therein). On the other hand capsular material was shown to bind both ionic and non-ionic nutrients providing for higher growth rates of encapsulated yeast cells versus non-capsulated (or hypocapsulated) variants on nutrient-poor media (Golubev 1991). Improved capacity for growth of encapsulated yeasts in oligotrophic conditions was also demonstrated by Kimura et al. (1998). Conversely, nutrient-poor media, particularly with low nitrogen content (high C-to-N ratio), which parallel the situation normally encountered on leaf surfaces, were shown to stimulate EPS production by yeasts (Golubev 1991). The presence of capsules confers yeast colonies with a mucous texture on solid media as was already noted by Ruinen (1963) in her pioneering studies of phylloplane yeasts. However, she attributed the observed mucilage to lipid production by the yeasts. Interestingly, lipid production was later confirmed in studies by Ruinen and other authors mainly by *Rhodotorula* spp. (e.g. *Rh. bogoriensis*, *Rh. graminis*) that do not produce considerable amounts of EPS (Hunter and Rose 1971). The significance of these compounds for leaf colonization has not been deter-

mined but they might well function as biosurfactants in a manner similar to that of equivalent compounds produced by phylloplane bacteria (see earlier). Bashi and Fokkema (1976) had already suggested that the mucilage secreted by *Sp. roseus* cells on barley leaves seemed to impregnate (but not dissolve) the wax layer of the leaf cuticle, thus changing surface properties (viz. wettability).

As previously mentioned oligotrophy appears to be a suitable trait for growth on the generally nutrient-poor leaf surfaces. Yeast species often found on the phylloplane (but also in soils) have been shown to be particularly fit to grow in media with extremely low concentrations of nutrients (Vishniac 1982; Kimura et al. 1998). This feature has been attributed to the exceptionally high affinity uptake systems for sugars and amino acids found in those yeasts (Kimura et al. 1998). A low nitrogen requirement by phylloplane yeasts was already noted by Ruinen (1963) and di Menna (1959) and although their findings have not been confirmed in later studies they clearly deserve to be further investigated. Ruinen's phylloplane isolates were actually obtained on nitrogen-deficient media which she used for isolation of nitrogen-fixing bacteria (Ruinen 1963). Oligotrophy is thought to provide an advantage in competition for nutrients on the phylloplane and oligotrophic microbes are likely to outcompete copiotrophic ones (Andrews and Harris 2000). This trait could explain preliminary results of co-inoculation of *A. pullulans* and *Tremella foliacea* (soil isolate) on an artificial wax substrate that was claimed to mimic the leaf surface (McCormack et al. 1994a).

Tolerance to UV radiation by means of efficient DNA repair systems and/or abundant production of pigments by both fungi and bacteria is thought to be a fitness trait for phylloplane colonists (Pugh and Buckley 1971; Lindow 1991; Moody et al. 1999; Morris 2001). Some solid evidence exists for bacteria (Lindow and Brandl 2003) but available data for yeasts are manifestly insufficient to prove a direct and major role of pigments in enhancing survival on the phylloplane (Pugh and Buckley 1971; Gunasekera et al. 1997). Nevertheless, it is noteworthy that a large majority of yeasts found on the phylloplane (notably deeply pigmented *Rhodotorula* and/or *Sporobolomyces* spp.) produce potentially photoprotective compounds, namely carotenoids, and that these yeasts occurred in higher frequencies in the summer months (see Sect. 13.5). A new class of UV-inducible compounds (mycosporines) was recently found in a number of basidiomycetous yeast species from freshwater lakes (Libkind et al. 2004), some of which are also found on the phylloplane (e.g. *Cr. laurentii*, *Rh. minuta*). It should be emphasized that mycosporines as well as some carotenoids (e.g. β-carotene) are not restricted to 'pink' yeasts but are also present in some of the non-pigmented (i.e. 'white') yeasts commonly found on the phylloplane (namely *Cryptococcus* spp.).

Production of hydrolytic enzymes (cutinases, pectinases, etc.) has been suggested to enhance the capacity of phylloplane colonists to actively extract nutrients from the plant tissue (Kinkel 1991; Morris 2001) but available evidence suggests that microbial epiphytes grow essentially at the expense of compounds that leach passively to the leaf surface (Mercier and Lindow 2000; Leveau and Lindow 2001). Conversely, production of hydrolytic enzymes is demonstrably essential for the efficient invasion of leaf tissues by many phytopathogenic bacteria and fungi (St. Leger et al. 1997; Hirano and Upper 2000). Nevertheless, some authors have

emphasized the biodegradative abilities of microbial epiphytes. In the case of yeasts, Ruinen (1963) found that the majority of phylloplane isolates produced extracellular lipases and she later claimed that those yeasts were able to degrade leaf cuticles (Ruinen 1966). However, later studies gave rise to conflicting results (Bashi and Fokkema 1976; McBride 1972, cited in Buck and Andrews 1999b). Middelhoven (1997) assayed many biochemical activities by the phylloplane yeast isolates he had obtained and found that the large majority were lipolytic and proteolytic and a considerable fraction of the basidiomycetous isolates were able to hydrolyse xylan and pectin (but not cellulose) and/or to assimilate phenolic compounds. Inácio (2003) also tested for different enzymatic activities (e.g. proteases, lipases, pectinases, xilanases) among a set of representative strains from a survey of yeasts on the phylloplane of Mediterranean plants. He detected significant extracellular hydrolytic abilities in *Cryptococcus* spp. belonging to the Filobasidiales (proteases, lipases, pectinases, cellulases and/or xilanases) and by some members of the Ustilaginomycetes (proteases and/or lipases); however, those were not the dominant species on the phylloplane of the sampled plants. Nevertheless, proteolytic, amylolytic and/or cellulotytic activities were detected in some strains of *Cryptococcus* spp. in the Tremellales and strong lipolytic activity was observed by some members of the Sporidiobolales, namely *Sp*. cf. *roseus* (in agreement with the results of Ruinen 1963), these yeasts being more prevalent on the same plants. It is not known if the observed activities are strain-specific and restricted to phylloplane isolates of each species or if they endow those yeasts with any competitive advantage on leaf surfaces.

Adhesion of cells to leaves is an intuitive pre-requisite for successful colonization of the phylloplane in analogy to what is known for other surface-inhabiting microbes, notably human and plant pathogens (Andrews and Harris 2000). However, available evidence for epiphytic bacteria and yeasts points to a more complex situation. Several publications by Buck and Andrews (reviewed in Andrews and Buck 2002) describe experiments aimed at unravelling the role and the mechanism of adhesion of the yeast *R. toruloides* to barley leaves. Their major findings were that although attachment of cells to the leaf could be demonstrated, especially by actively growing cells, this attachment was transient and the majority of cells could be easily removed by agitation. The authors concluded that adhesive ability does not appear to play a major role in leaf colonization and that instead efficient dispersal (i.e. emigration) and rapid recolonization (i.e. growth) may be more important attributes of a well-adapted phylloplane colonist (i.e. a resident). Transient adhesion should, however, be important to maintain a reservoir of cells that would expand quickly when nutrient availability and environmental conditions are conducive for growth allowing them to outcompete their less fit neighbours. However, rather surprisingly the yeast species chosen for this study is not a prevalent phylloplane colonist (the strain used most extensively was a soil isolate) and reports on the more or less ease of removal of yeasts, namely *Sp. roseus*, from leaf surfaces are contradictory (di Menna 1959; Preece and Dickinson 1971; Bashi and Fokkema 1976; Buck and Andrews 1999b).

Although the phenotypes cited here may be found in yeasts in a wide range of habitats (e.g. soil), many of the yeasts in these habitats are apparently incapable of growing and/or surviving on the phylloplane (see Sect. 13.3). Likewise, a large

number of the yeasts adapted to the phylloplane survive poorly in aquatic or soil ecosystems, for example. However, it seems clear that the key phenotypes essential for the fitness of phylloplane yeasts have not yet been fully elucidated.

13.7 Future Directions in Ecological Studies of Epiphytic Yeasts

In the previous sections we have tried to give a brief overview of the knowledge accumulated in recent years on the ecology of phylloplane microorganisms, with an obvious emphasis on yeasts. In spite of the considerable number of studies covering the topic many issues remain unresolved, including the defining characteristics of a phylloplane yeast (Sect. 13.6) and a more precise rendering of the diversity of epiphytic yeast populations on many different types of plants and climatic regions (Sect. 13.4). A better understanding of phyllosphere microbiology is important not only from a fundamental perspective, for leaf-inhabiting microbes are a fundamental component of terrestrial ecosystems and thus contribute to the nutrient cycling processes that occur therein, but also for their recognized potential as biocontrol agents or as sources of bioactive compounds of biotechnological interest (e.g. Fokkema 1991; Lindow and Leveau 2002). Here we provide some additional suggestions for future research efforts (see also Andrews 1991).

Firstly, sampling strategies and the methods used for isolation, enumeration and identification of phylloplane yeasts should be thoughtfully planned according to research goals. Aspects to be considered include the intensity of sampling (one plant vs. multiple plants, frequency of leaf collection, examination of other aerial organs for comparative purposes, etc.), the choice of protocols for removal of yeast cells from leaves and of adequate culture media and incubation conditions, etc. (see Sect. 13.2). A range of recently developed culture-independent methods have found application in microbial ecology (e.g. DGGE or temperature gradient gel electrophoresis, direct DNA extraction followed by cloning and sequencing) but only a few studies with phyllosphere microorganisms have made use of those methods (see Sect. 13.2); however, these methods have their own limitations and rather than replacing traditional isolation methods they should be used concurrently (Prakitchaiwattana et al. 2004). Sequence data available in public databases facilitate rapid and accurate identifications and it is imperative that such data be used in new surveys of phylloplane yeasts or that isolates from previous studies be re-identified using molecular approaches (see Sect. 13.4). Only then can phylloplane yeast communities be accurately appraised and their distribution patterns determined, which will in turn enable unravelling of possible associations with specific plants, biotopes and/or geography. Those patterns have already been disclosed for other yeast habitats such as tree exudates (Lachance et al. 1982), ephemeral flowers (Lachance et al. 2001) and cacti (Starmer et al. 2003). The resolution power of certain molecular typing methods provides the means to further discriminate distinct populations within a species and to test hypothesis about their turnover on leaves or the biogeography of ubiquitous phylloplane inhabitants such as *A. pullulans*.

The issue of population dynamics on the phylloplane remains vastly unexplored especially with respect to yeasts (see Sect. 13.5). Molecular genetics tools offer the possibility of examining the relative contributions of the processes of immigration,

growth and emigration to the development of microbial communities on leaf surfaces, but such studies have not yet been carried out with yeasts. The possible role of insects in the immigration and/or emigration processes of phylloplane yeasts has not been investigated, which is somewhat surprising given the well-known involvement of insect vectors in other yeast habitats (Starmer et al. 1991; Lachance et al. 2001). It is therefore desirable to assemble multidisciplinary teams for ecological surveys of phylloplane yeasts, which should involve the collaborative efforts of yeast biologists, entomologists and botanists. Knowledge of the topography, biochemistry and physiology of leaves is also important to understand the role of the leaf in shaping both the spatial and temporal patterns of its epiphytic microbial populations.

Another issue that deserves being looked into is the possible existence of yeast–plant interactions through molecular signalling mechanisms which might provide clues on why some yeast species are found on the phylloplane of certain plants and others are not. Although only a few yeast species were found to infect plants (Phaff and Starmer 1987) it is worth emphasizing in this respect the close phylogenetic relationship between some of the yeasts found on plant surfaces and dimorphic plant parasites (e.g. *Lalaria*/*Taphrina*; *Rh. bacarum*/*Microstroma* spp.; *Pseudozyma*/*Ustilago*). Likewise, interactions between the different members of the epiphytic communities have not been consistently explored and since bacteria constitute normally the early leaf colonists it would be most interesting to determine whether or not yeasts depend on those bacteria for their own colonization of leaf surfaces. Ruinen (1963) suggested that the common presence of nitrogen-fixing bacteria on the phylloplane might be beneficial for the ensuing colonization by yeasts. Some phylloplane yeasts have been found to produce antimicrobial compounds (McCormack et al. 1994b) but their ecological significance is not known. Moreover some yeasts with the ability to antagonize plant pathogenic fungi by producing lipids with fungicidal activity and that are being used as biocontrol agents may also be found on plant surfaces (Avis and Bélanger 2002).

Finally, it was not possible to identify with certainty which of the characteristics displayed by the yeasts commonly isolated from leaf surfaces define the required fitness traits for successful colonization of the phylloplane (see Sect. 13.6). For instance, while production of ballistoconidia is an obvious advantage as an efficient means of dispersal and is found in many yeasts inhabiting leaf surfaces, it is apparently not a pre-requisite for many other equally prominent phylloplane species (see Sect. 13.4). It would therefore be most interesting to uncover the selective pressures for the conservation of this phenotypic trait as well as its genetic determinants since it is well-known that BC yeasts tend to lose this capacity upon maintenance on nutrient-rich artificial culture media and are phylogenetically intertwined with non-BC taxa (Nakase 2000). Other frequent putative fitness traits of phylloplane yeasts are the production of EPS capsules and of photoprotective compounds. However, conclusive evidence of their relevance for yeast survival and/or growth on leaves is still lacking. The same applies to the adhesiveness of cells onto leaf surfaces which seemed like a good candidate to explain the observed differences between residents and transients in phylloplane colonization.

It seems clear that there are still many significant gaps in our understanding of the nature, dynamics and ecological role of phylloplane yeast populations. We are

maybe at the proverbial tip of the iceberg in accessing some of those topics and this appears to be the right time to make good use of the plethora of DNA-based methods and of molecular genetics tools already available for that purpose. In this context it seems fitting to quote Andrews (1991): "Renewed efforts at an experimental [i.e. hypothesis-testing] as opposed to a descriptive approach, integration of relevant advances from other disciplines, and recognition of the interplay among theory, experimentation, and observation, will be the essential ingredients for expanding our knowledge of phyllosphere ecology in the coming decades."

Acknowledgements

The authors are grateful to Andrey Yurkov and I.Y. Chernov (Moscow State University) for providing some publications by Russian authors and for assistance in their translation from Russian.

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Chapter 14

Yeast and Invertebrate Associations

PHILIP F. GANTER

Department of Biology, Tennessee State University, 3500 John Merritt Blvd, Nashville, TN 37209, USA
(e-mail: pganter@tnstate.edu)

14.1 Introduction

The genesis of this review occurred at the 23rd International Special Symposium on Yeasts. The meeting was excellent, but, as I listened to the presentations, I was taken by the realization that most of presentations dealing with interactions between yeasts and their environments, especially presentations that could be thought of as applied science, focused on yeast–substrate interactions. This has been noted previously (do Carmo-Sousa 1969). Because the talks and posters presented interesting new data and insights, I did not think this a situation in need of correction but I did feel that yeast–animal interactions were a bit underrepresented. In several instances, yeast–animal interactions could have offered alternative explanations for the data or might have suggested answers to questions generated by the data. What caught my attention was that, in discussing their data, only one of the presenters made specific reference to animals as a possible alternative. Recalling this impression gave me a goal for this review. What I would like to accomplish is to present an argument that yeast–animal interactions are common, understudied, and a necessary part of understanding yeast ecology and evolution.

I will not review situations where yeasts are known to be animal parasites or pathogens. However, many interactions are not well understood and the nature of relationships is not fixed but is subject to evolutionary change. This sometimes made it difficult to set a proper boundary for the review and I chose to be inclusive rather than exclusive. It is organized according to the animals that are involved in the interactions. This decision was made because the field of study of yeast–animal interactions is organized, to the degree one can say that it is organized, in this fashion. The result is that ecological and evolutionary ideas may be revisited within separate sections. The alternative, organizing according to the evolutionary or ecological ideas presented, would mean revisiting animal groups instead of ecological concepts. More importantly, organizing the review by concept would give the impression that some overall scheme for animal–yeast interactions exists at this time. I believe that it would be misleading to imply conceptual unity beyond some broad generalizations. Animal–yeast interactions are varied, from mutualistic endosymbioses to simple

phoresy, from pairs of interacting animal and yeast species to interacting animal and yeast communities. So, if I am correct in my belief that much more remains to be discovered about yeast–animal interactions than we currently know, presenting the field as a coherent set of relationships might be premature.

Eliminating pathogenic and parasitic interactions has other ramifications. Vertebrate associations receive little attention here owing to a paucity of information about nonpathogenic interactions, although yeasts can be isolated from vertebrate guts (do Carmo-Sousa 1969; Abranches et al. 1998) and at least one yeast, *Cyniclomyces guttulatus* (Phaff and Miller 1998), is an endocytobiont. The scope of relationships reviewed here potentially involves fungivory, mutualism, commensalism, or amensalism (no interaction, simply co-occurrence). Other categories, such as competition or predation, are not likely. Symbiosis, another descriptor of interactions, does not seem to have a universally agreed upon definition. Some use it broadly for any relationship in which two organisms spend significant time in contact (parasites and pathogens may then be symbionts) and some authors restrict it to mutualistic or commensal interactions (the caveat about contact applying). Here, I will attempt to use the more restricted meaning when either observational or experimental evidence reasonably justifies the implication of a positive interaction.

Some broad generalizations are useful to state at the outset. When considered a subset of all fungus–animal interactions, there seems to be little that is unique to yeast–animal interactions. Most relationships are based on yeasts as a food for the animal and the animal as a vector for the yeasts. What is interesting is the widespread nature of the association. Table 14.1 is a reasonably complete and up-to-date list of yeasts associated with beetles found in wood and mushrooms. It is over 200-species long. For the vast majority of instances, we do not know if the association is happenstance or significant. I suspect that most are significant but do not involve obligate pairwise interactions between one species of yeast and one species of insect. If true, this would mean that most yeast–animal relationships must be studied in multispecies assemblages if the nature of the relationship is to be fully understood. The number of possible iterations becomes large, especially if variation in environmental factors, which may change the nature of yeast–animal interactions, are considered. It is a daunting task but there are also positive sides to the situation. Many necessary techniques have been perfected (obtaining axenic insects and pure cultures of yeasts, efficient means of differential yeast population counts, detection of spatial inhomogeneities with differential dye labeling, etc.) that might be applied to experiments on the dynamics of yeast–animal relationships. Both yeast and animal systematics are improving to the point that comparative phylogenetics may be used to generate a subset of testable hypotheses from a much larger set of possible hypotheses. Coalescence theory promises insights into basic ecological parameters (e.g., effective population size, dispersal patterns, and prevalence of recombination) and historical aspects of population biology (e.g., recent bottlenecks and founder events).

There have been several excellent reviews of the associations between yeast and animals, often as part of a more general review of yeast habitats (do Carmo-Sousa 1969; Phaff et al. 1978; Phaff and Starmer 1987). Here, I will focus, when possible, on recent additions to the field. Some terminological standards should be set at the

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Candida diddensiae	1																				30
C. oregonensis	1						1														50
C. (Torulopsis) nitratophila	1						1														30, 32, 64
Cryptococcus albidus var. *diffluens*	1																				30
Cr. curvatus (*C. curvata*)	1																				32, 64
Cr. magnus	1																				30
Cr. skinneri	1																				32
Cuniculitrema polymorpha	1																				58
Debaryomyces hansenii	1																				30
Dipodascus aggregatus	1																				42
Myxozyma melibiosi (*T. melibiosum*)	1																				2
Pichia (*Hansenula*) *bimundalis*	1	1																			32, 43
P. (*H.*) *capsulata*	1						1														32
P. haplophila	1																				32
P. (*H.*) *holstii* (*C. silvicola*)	1	1			1																30, 32, 21
P. pini	1	1																			30, 32
P. (*E.*) *scolyti*	1	1																			32, 43
P. tolentana	1	1									1										30, 43
Rhodotorula aurantiaca (*crocea*)	1																				2
Rh. (*C.*) *hylophila*	1			1																	13, 30
Sporobolomyces (*Bullera*) *tsugae*	1																				2
Sp. singularis	1																				32

Continues

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Tsuchiyaea (*Sterigmatomyces*) *wingfieldii*	1																			33	
Ascoidea hylecoeti		1																			39
C. arabinofermentans		1																			47
C. ernobii		1											1								50, 21
C. homilentoma		1																			17
C. litsaeae		1																			60
C. melibiosica		1																			50
C. piceae		1																			57
C. ponderosae		1																			60
C. quercitrusa		1																			50
C. quercuum		1																			21
C. rugosa		1																			21
C. silvanorum		1		1																	13, 21
C. tenuis		1			1	1	1														2, 21
C. ulmi		1																			56
C. wickerhamii		1																			50
C. wyomingensis		1									1										57
Clavispora (*Candida*) *lusitaniae*		1																			21
Cr. humicolus		1																			21
Cr. laurentii		1																			21
D. vanrijiae var. *yarrowii*		1																			51
Issatchenkia scutulata var. *exigua*		1																			46
Ogataea (*P.*) *glucozyma*		1																			43
O. (*P.*) *henricii*		1																			43
O. (*P.*) *kodamae*		1																			32
O. polymorpha (*P. angusta, H. polymorpha*)		1																			43

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
P. (*H.*) *americana*		1																			2, 32, 43
P. amylophila		1																			32
P. bovis		1																			21
P. canadensis (*Hansenula wingei*)		1																			43
P. euphorbiae		1																			20
P. (*H.*) *euphorbiaphila*		1																			18
P. hampshirensis		1																			31
P. heimii		1																			32
P. japonica		1																			31
P. media		1																			43
P. membranifaciens (*C. valida*)		1																			21
P. mexicana (*C. entomoea, C. terebra*)		1																			15, 21
P. meyerae		1																			19
P. mississippiensis		1																			3
P. nonfermentans		1																			21
P. pastoris		1																			43
P. philodendri		1																			21
P. ramenicola		1																			57
P. segobiensis		1			1	1															32, 43, 62
Stephanoascus smithiae		1																			54
Torulaspora delbruckii		1									1										44
Trichosporon cutaneum		1								1											2, 21
Williopsis californica		1																			45
Zygosaccharomyces bisporus		1																			21

Continues

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Z. rouxii		1																			21
Zygozyma oligophaga		1																			53
Ambrosiozyma (*P.*) *ambrosiae*				1																	9, 14
A. (*P.*) *cicatricosa*				1																	8, 14
A. (*E.*) *monospora*				1																	14
A. philentoma				1																	52
A. (*E.*) *platypodis*			1	1																	14
Ascoidea africana				1																	39
C. berthetii				1							1										32
C. (*T.*) *dendrica*				1	1																12
C. dendronema				1																	13
C. entomophila				1																	13
C. (*T.*) *insectalens*				1	1																12
C. insectorum				1																	15
C. maritima				1							1										25, 34, 50
C. mycetangii				1																	56
C. (*T.*) *nemodendra*				1																	12
C. (*T.*) *silvatica*				1																	12
C. trypodendroni				1																	48
Dipodascus ambrosiae				1																	42
Fellomyces (*Sterigmatomyces*) *polyborus*				1																	5, 48
O. (*P.*) (*H.*) *philodendri*				1		1															5
P. (*H.*) *dryadoides*				1																	7
P. (H.) sydowiorum (*C. nitrativorans*)				1	1	1															4, 15
P. acaciae				1	1	1	1														1
Rh. (*C.*) *philyla*				1																	12
Saccharomycopsis synnaedendra				1																	11

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Zygozyma smithiae				1																	36
C. ergastensis					1																32, 61
C. parapsilosis var. intermedia					1				1												50
C. rhagii					1	1	1														2
C. shehatae var. *inesctosa*					1		1														32,35,62
Schizosaccharomyces fibuligera					1	1															2, 32
?P. onychis (*P. xylopsoci*) – lost culture						1															10
C. (*T.*) *torresii*						1															32
C. fennica (*Trichosporon melibiosaceum*)						1															5
Cr. amyloentus (*C. amylolenta*)						1															15
P. (*H.*) *anomala*						1															32
P. burtonii (*Trichosporon behrendii*)						1								1							32, 63
P. (*H.*) *ciferrii*						1															32
P. stipitis					1					1					1					1	2
Bullera dendrophila							1														6
C. insectamans							1														15
C. naeodendra							1														16
C. silvicultrix							1														15, 32
D. vanrijiae var. *vanrijiae*							1														32
P. (*C.*) *guilliermondii*							1					1	1								32
T. behrendii							1														32
Blastobotrys elegans-like								1													32
C. ambrosiae-like								1				1				1					64
C. pyralidae-like								1				1									64
Malassezia restricta				1				1				1									64

Continues

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
Stephanoasaus farinosus-like								1				1									64
C. athensensis									1			1				1	1				67
Candida sp.									1			1									64
T. scarabaeorum										1											65
Ascoidea corymbosa											1										39
A. rubescens											1										39
Blastobotrys capitulata											1										41
C. ambrosiae											1										59
C. ancudensis											1										28
C. antillancae											1										22
C. bertae											1										23
C. castrensis											1										22
C. chilensis											1										50
C. coipomoensis											1										29, 34
C. drimydis (same rDNA as *C. ancudensis*)											1										25
C. freyschussii											1										50
C. hungarica											1										61
C. ingens											1										34
C. laureliae											1										29
C. llanquihuensis											1										29
C. novakii											1										37
C. oleophila											1										26, 34
C. ontarioensis											1										48
C. parapsilosis											1										29, 34
C. peoriaensis											1										60
C. petrohuansis (same rDNA as *C. ancudensis*)											1										27
C. pini											1										50
C. pyralidae											1										59

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
C. railenensis											1										23, 24, 34
C. santjacobensis											1										28
C. savonica											1										50
C. shehatae var. *lignosa*											1										50
C. shehatae var. *shehatae*											1										50
C. sophiae-reginae											1										26
C. tammaniensis											1										48
C. tepae											1										50
C. xylopsoci											1										59
Cephaloascus fragrans											1										40
Cr. huempii											1										25
D. carsonii											1										51
Leucosporidium scolyti											1										24, 34
Metschnikowia pulcherrima											1										27, 34
O. (*P.*) *dorogensis*											1										61
O. (*P.*) *methylivora*											1										43
O. (*P.*) *zsoltii*											1										61
P. castillae											1										43, 48
P. pilisensis											1										61
Rh. futronensis											1										24, 34
Rh. nothofagi											1										24, 34
C. anneliseae												1									66
C. atakaporum												1									66
C. bokatorum												1									66
C. bolitotheri												1									66
C. bribrorum												1									66
C. chickasaworum												1									66
C. choctaworum												1									66
C. elateridarum												1						1			67
C. emberorum												1									66

Continues

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
C. fermentati												1		1							63
C. guaymorum												1									66
C. kruisii												1									50
C. kunorum												1									66
C. maxii												1									66
C. panamericana												1									34, 66
C. schatavii												1									50
C. smithsonii												1				1			1		67
C. taliae												1									66
C. terraborum												1									66
C. wounanorum												1									66
C. yuchorum												1									66
D polymorphus												1									51
Dipodascus armillariae												1									42
Dipodascus macrosporus (actually from a myxomycete)												1									42
Endomyces cortinarii (parasite of basidiocarp)												1									38
E. decipiens (parasite of basidiocarp)												1									38
E. polyporicola (parasite of basidiocarp)												1									38
E. scopularum (parasite of basidiocarp)												1									38
Kluyveromyces dobzhanskii												1									49
K. lactis var. drosophilarum												1									49
K. thermotolerans												1									49
K. waltii												1									49

Table 14.1 Yeasts associated with beetles or beetle larval substrates (not including flowers)—*cont'd*

Yeast	Scolytinae	Bark Beetle Frass and Galleries	Platypodinae	Ambrosia Beetles	Cerambycidae	Bostrichidae	Bupresidae	Tenebrionidae	Curculionidae	Scarabaeidae	From Decaying, Insect-infested Wood	Basidiocarps and Assoc. Beetles	Anobiidae	*Hypothenemus hampei*	Lucanidae	Erotylidae	Cucujoidae	Elatridae	Endomychidae	Passalidae	Source[a]
O. (*P.*) *minuta* var. *minuta*												1									43
Saccharomyces dairenensis												1									55
S. farinosus												1									54
C. karawaiewii													1								50
C. xestobii													1								50

[a] *1* van der Walt (1966), *2* do Carmo-Sousa (1969), *3* Pignal (1970), *4* Scott and van der Walt (1970a), *5* Scott and van der Walt (1970b), *6* van der Walt and Scott (1970), *7* Scott and van der Walt (1971a), *8* Scott and van der Walt (1971b), *9* van der Walt and Scott (1971a), *10* van der Walt and Scott (1971b), *11* van der Walt and Scott (1971c), *12* van der Walt et al. (1971a, *13* van der Walt et al. (1971b), *14* van der Walt (1972), *15* van der Walt et al. (1972), *16* van der Walt et al. (1973), *17* van der Walt and Nakase (1973), *18* van der Walt (1982a), *19* van der Walt (1982b), *20* van der Walt and Opperman (1983), *21* Bridges et al. (1984), *22* Ramierez and González (1984e), *23* Ramierez and González (1984a), *24* Ramierez and González (1984b), *25* Ramierez and González (1984c), *26* Ramierez and González (1984d), *27* Ramierez and González (1984f), *28* Ramierez and González (1984g), *29* Ramierez and González (1984h), *30* Leufvén and Nehls (1986), *31* Kurtzman (1987), *32* Phaff and Starmer (1987), *33* van der Walt et al. (1987), *34* Ramierez (1988), *35* Kurtzman (1990), *36* van der Walt et al. (1990), *37* Péter et al. (1997), *38* de Hoog (1998a), *39* de Hoog (1998b), *40* de Hoog and Kurtzman (1998), *41* de Hoog and Smith (1998), *42* de Hoog et al. (1998), *43* Kurtzman (1998a), *44* Kurtzman (1998b), *45* Kurtzman (1998c), *46* Kurtzman (1998d), *47* Kurtzman and Dien (1998), *48* Kurtzman and Robnett (1998b), *49* Lachance (1998), *50* Meyer et al. (1998), *51* Nakase et al. (1998), *52* Smith (1998a), *53* Smith (1998b), *54* Smith and de Hoog (1998), *55* Vaughn-Martini and Martini (1998), *56* Kurtzman (2000a), *57* Kurtzman (2000b), *58* Kirschner et al. (2001), *59* Kurtzman (2001a), *60* Kurtzman (2001b), *61* Péter et al. (2003), *62* Suh et al. (2003), *63* Vega et al. (2003), *64* Zhang et al. (2003), *65* Middelhoven et al. (2004), *66* Suh et al. (2004b), *67* Suh and Blackwell (2004c)

outset. The literature has some overlapping terms and terms with multiple meanings and I will try to use only one when multiples are available. A member of a symbiotic relationship can be either a symbiote or a symbiont and I will prefer symbiont. The term is more often applied to the microbe than the animal, which is commonly called the host. Mycetocytes are specialized animal cells that host endocytobionts (Jones et al. 1999 for the latter term). Many authors make no distinction based on the nature of the microbe but others use bacteriocyte when bacteria are resident and confine mycetocyte to situations when the resident is a fungus. I will use the term in the narrower sense. Association and interaction are not totally overlapping terms

in this review and the distinction is important. An association means that co-occurrence has been established. The use of "interaction" implies that some functional relationship has been established or that observational data strongly implies a functional relationship. Phoresy is the dispersal of small organisms, seeds, or spores by animals. Some authors use the term only for a commensal relationship and others use it no matter what sort of relationship exists between the carrier and the organism carried, which is how I will use it. Mycangia are external pockets on arthropods that hold microbes during dispersal by the animal. They may be almost anywhere on the host and range from simple depressions to more elaborate structures that may nourish the fungus or bacterium. "Mycetangia" looks confusingly similar to mycangia but refers to an organ or a cluster of cells within an organ that house an endocytobiont inside the body. Finally, several abbreviations occur more than once: LSU and SSU for large and small subunit plus ITS for internal transcribed spacer (of the ribosomal RNA gene); and YLS for a yeast-like symbiont.

14.2 Beetles

Most yeast–beetle interactions involve beetles that live on plants (although the interactions of yeasts and beetles associated with mushrooms discussed in the last part of this section are a significant exception). Beetles are closely associated with plants and Farrell (1998) has suggested that this association (especially with angiosperms) is a major cause of beetle diversity. Beetles use all parts of plants (see flower-associated beetles in Sect. 14.2.5) but a significant part of beetle diversity is due to the many beetles that use wood as a refuge, a nest, and a source of food. They feed on all parts of the woody stem: sap, phloem, cambial tissue, bark, wood, and the fungi that digest wood or attack the plant's living tissues (Lawrence 1989). The fossil record indicates they have done so for a very long time (Kirejtshuk 2003). Eating wood involves digestion of lignified cellulose and overcoming the toxins found there (Eriksson et al. 1990). (Farrell et al. 2001 point out that fallen logs are often protected by toxins, perhaps because mature trees are a combination of living and dead tissue and so have an interest in what eats dead wood.) Digestion and detoxification are often accomplished through symbioses between beetles and microbes that range from endocytobiosis to agricultural associations (Mueller et al. 1998; Farrell et al. 2001; Mueller and Gerardo 2002). Historically, most attention has been paid to xylophagic (or xylophagy-related) yeast–beetle interactions. It is my intention to discuss recent additions to this rich literature (see Buchner 1965; Phaff and Starmer 1987; Lawrence and Milner 1996 for previous reviews) and to cover some recent work on associations between floricolous yeasts and beetles and on yeasts and beetles from basidiocarps.

There are several feeding strategies used by beetles that feed on woody stems (Berryman 1989). Many Nitidulid beetles feed on fermenting plant sap and yeasts are among the microbes responsible for sap fermentation. Production of low molecular-weight volatile compounds by yeasts attracts nitidulid beetles (Nout and Bartelt 1998). Volatile production by bacteria growing on the same substrates as the yeasts did not attract the insect. Some nitidulids live in flowers and are associated with yeasts that feed on nectar and pollen. Many Scolytid beetles feed on the living tis-

sues beneath the bark. These bark beetles are often associated with euascomycete phytopathogens in the *Ophiostoma* and *Ceratocystis* clades as well as yeast and filamentous basidiomycetes. Infestation often leads to the rapid death of the plant as volatiles produced by attacking organisms attract more beetles to the attack. The relationship between the fungi and the beetles involves transport for the fungi and both facilitation of the attack (the fungi can block resin or latex channels from flooding beetle galleries) and nutrition for the beetles. Other Scolytid (as well as Platypodid) beetles, collectively termed ambrosia beetles, bore into the xylem of living trees where they feed on fungi that line their tunnels (galleries). However, most of the xylem eaten by beetles is eaten by beetles that bore into dead branches or trunks. Passalid beetles form multigeneration colonies in dead wood and are associated with yeasts able to digest xylose, a major component of lignocellulose. In addition, species from other beetle families also feed on xylem, including members of the Anobiid, Cerambycid, Buprestid, Bostrichid, and Scarabaeid beetle families. For many of these families, xylophagy is believed to be the ancestral feeding mode. Yeast or yeast-like endosymbionts have been isolated from the guts of many of them (Table 14.1).

In addition to the xylophagic beetles, fungi (including yeasts) are associated with other beetles and other beetle feeding modes. Recent efforts have identified yeasts associated with flower beetles and with beetles that feed on basidiocarps. Owing to the number of species that have been isolated from beetles, beetle frass, galleries, or rotting wood infested with beetles, they are summarized in Table 14.1, which does not include those found in association with flowers, which are summarized in Table 14.2.

14.2.1 Bark Beetles

The feeding strategies of Scolytid beetles have been summarized by Berryman (1989). There are three basic types: saprophagy (Scolytids that feed in rotting logs), phytophagy (bark beetles), and mycetophagy (ambrosia beetles). Berryman views saprophagy as the ancestral state. Saprophagous Scolytids feed on the fungi in the wood and use fermentation products to locate fallen wood. Perhaps because this group of beetles is not economically important, they remain understudied and specific associations among the fungi and beetles in this group have not been investigated. However, the use of fermentation products to locate suitable resources was probably an important preadaptation for both the phytophagous and mycetophagous life styles that followed. Both bark and ambrosia beetles use pheromones that are often wood components modified by fungal symbionts. Bark beetles use both aggregation (verbenol from pinene, Berryman 1989) and avoidance pheromones (verbenone from verbenol; Hunt and Borden 1990; Paine et al. 1997), so that adults are recruited to the tree during early stages of infestations and discouraged from joining the attack during later stages. Colonial living is characteristic of Scolytid beetles and Berryman believes that the use of fungal products to locate wood leads to colony formation, which he views as a preadaptation for both the phytophagous and mycetophagous life styles that followed. Bark beetles, the phytophagous group, are either colonial and virulent or solitary and far less virulent

Table 14.2 Recently described species associated with flowers and flower beetles

Clade	**Species**[a]	**Host plant**	**Insect**	**Source**[b]
First *Metschnikowia*	*M. continentalis*	*Ipomoea*	*Conotelus*	D, P
	M. borealis	Convolvulaceae	*Conotelus*	D, P
	M. hibisci	*Ipomoea, Hibiscus*	*Conotelus, Aethina*	D
	M. lochheadii	various	*Conotelus*	K
	M. hawaiiensis	*Ipomoea*	*Conotelus*	A
	M. santaceciliae	*Ipomoea*	*Conotelus*	O
	C. ipomoeae	Convolvulaceae	*Conotelus*	B
	M. lochheadii-like	*M. tuberosa*	*Conotelus*	L
	M. hawaiiensis-like		Clerid beetles	L
Second *Metschnikowia*	*M. dekortorum*	Ipomoea	*Conotelus*	M
	M. arizonensis	*Opuntia*	*Carpophilus*	M
	C. kipukae	*Ipomoea*	*Conotelus*	O
Third *Metschnikowia*	*M. agaves*	*Agave tequilana* var. *azul*	No beetles	
	C. hawaiiana	*Ipomoea*	*Conotelus*	O
Other *Metschnikowia*	*M. reukaufii*	Convolvulaceae	No beetles	
	M. koreensis	*Ipomea, Lilium*		I
	C. tolerans	*Ipomoea, Hibiscus*	*Conotelus, Aethina*	F
	C. kunwiensis	*Ipomea, Helleborus*	Bees	N
	M. gruessii-like	Convolvulaceae	No beetles	L
	C. tolerans-like	Various	Beetles	L
Kodamaea	*K. anthophila*	*Ipomoea, Hibiscus*	*Conotelus, Aethina*	G
	K. kakaduensis	*Ipomoea, Hibiscus*	*Aethina*	F
	K. nitidulidarum	Cactus flowers	Nitidulid beetles	G
	C. restingae	Cactus flowers	Nitidulid and other beetles	G
Wickerhamiella	*W. occidentalis*	Convolvulaceae	Conotelus	*C*
	W. lipophila	*Ipomoea*	*Conotelus*	C, H
	W. australiensis	*Ipomoea, Hibiscus*	*Conotelus, Aethina*	C
	W. cacticola	Cactus flowers	Nitidulid and other beetles	C
	C. drosophilae	*Ipomoea*	*Conotelus*	C
	C. sorbophila-like	Various	*Conotelus*	L

Starmerella (associated with bees)	*S. bombicola*	Various	*Conotelus*	E
	C. tilneyi	Various	*Conotelus*	J
	C. powellii	Various	*Conotelus*	J
	C. powellii-like	Various	*Conotelus*	L
	C. floricola-like	*Ipomoea*	*Conotelus*	L
	C. geochares-like		*Aethina*	L
Other clades	*C. cleridarum*		Clerid beetles	J
	C. nanaspora-like	Various	Beetles	L
	Saccharomycopsis sp.	*Hibiscus*	Beetle	L
	Sporopachydermia sp.	Various	Beetles	L
	Fermentative yeast-like mold	*Ipomoea, Hibiscus*	*Conotelus, Aethina*	L
Various basidiomycetes				L

[a]*C.* represents *Candida*; other genera as in clade name
[b]*A* Lachance et al. (1990), *B* Lachance et al. (1998a), *C* Lachance et al. (1998c), *D* Lachance et al. (1998b), *E* Rosa and Lachance (1998), *F* Lachance et al. (1999), *G* Rosa et al. (1999a), *H* Lachance et al. (2000), *I* Hong et al. (2001), *J* Lachance et al. (2001a), *K* Lachance et al. (2001b), *L* Lachance et al. (2001d), *M* Lachance and Bowles (2002), *N* Hong et al. (2003), *O* Lachance et al. (2003), *P* Marinoni and Lachance (2004)

(Berryman 1989; Paine et al. 1997). The mechanism of tree death depends on the impact of the beetles, including their tendency to aggregate and to girdle the tree as they burrow tunnels, and the effect of the fungi, which attack the sapwood (many are blue-staining) and can range from deadly to mildly detrimental to the tree (Paine et al. 1997). The specificity of the relationship between bark beetle and fungi is low, with most beetle species associated with different pathogenic fungi at different times and places or on different host species. However, it is clear that the beetles exploit the fungi as food, as a means of reducing the toxicity of the tree, and as a means of overcoming the physical defenses of the tree, primarily by blocking resin or latex secretion that are primary defenses and by responding to the induced defenses (cell wall alterations, additional toxins, etc.) provoked by the attack on the tree (Paine et al. 1997; Farrell et al. 2001). However, the association may also involve cost to the beetles. In laboratory experiments, *Roptrocerus xylophagorum* and *Spathius pallidus*, Hymenopteran parasitoids, were attracted to odors from loblolly pine, *Pinus taeda* infected with strains of *Ophiostoma* spp. (Sullivan and Berisford 2004). Data from the field were less clear, as the fungus alone was not sufficient to explain long-range host location.

Fungi may benefit from the beetles' burrowing and from physiological changes their presence provokes in the tree's tissues but they clearly benefit from vectoring by the beetles. Many fungi have been isolated from the external surface of the beetles (Paine et al. 1997) but the clearest indication that the beetles vector fungi from their galleries is the presence of special cuticular structures, mycangia, that transport fungi, including yeasts (Whitney and Harris 1970). Mycangia are a diverse set of structures (including pits on various body parts such as the head and feeding appendages, grooves on the pronotum, and patches of setae that can hold spores and cells between the shafts) that are often secretory, producing waxes, fatty acids, and amino acids. The type of mycangia does not appear to be a diagnostic trait in Scolytid taxonomy (Berryman 1989). Mycangia are also found in the ambrosia beetles, in nonxylophagous Scolytids (Morales-Ramos et al. 2000), and even in other insect orders (Vasiliauskas and Stenlid 1999).

Bark-beetle-associated fungi include dimorphic *Euascomycetes* and *Hemiascomycetes*. The euascomycete species were once thought to belong to a single taxonomic group but recent DNA sequence analysis has not supported monophyly. The *Ophiostoma* and *Ceratocystis* clades are related perithecal euascomycete clades that contain plant pathogens and species associated with bark beetles (Spatafora and Blackwell 1994). They include numerous anamorphs, mostly in the genera *Ambrosiella*, *Leptographium* and *Pesotum*. Some have obligate associations with beetles and are transported in mycangia, while others have facultative associations and are vectored on the beetles mouthparts and body surfaces. Other teleomorphic genera (*Pyxidiophora*, *Kathistes*, and *Subbaromyces*), once included with *Ophiostoma* and *Ceratocystis*, are only distantly related (Blackwell and Jones 1997; Hausner et al. 2000). They have peritheca and are dispersed by beetles, but the former characteristic may be the result of convergent evolution due to their association with beetles.

It has long been unclear if many of the anamorphic and teleomorphic species in these clades deserve specific status or if the generic and specific designations repre-

sented monophyletic groupings. Recent molecular data have confirmed that there are ambiguities in the current classification. SSU ribosomal DNA (rDNA) sequence analysis has shown *Ambrosiella* to be polyphyletic, with some species related to *Ceratocystis* and others to *Ophiostoma*, which may be two separate clades (Cassar and Blackwell 1996; Rollins et al. 2001). Hausner et al. (2000) determined that neither *Leptographium* nor *Pesotum* were monophyletic. Problems can extend to specific designations, also. Using a multigene (rDNA, actin, β-tubulin, and transcription elongation factor 1α) and multistrain approach to the relatedness of a cluster of species physiologically and ecologically similar to *Ophiostoma clavigerum*, Lim et al. (2004) demonstrated that one (*L. terebrantis*) of the five species examined was paraphyletic in the combined tree and it was not clear if this would have been resolved with the addition of more loci to the analysis. Using a separate locus (rDNA ITS), ambiguous results were also obtained for a clade of teleomorph species related to *O. stenoceras* (de Beer et al. 2003). Although strains of *O. stenoceras* from Colombia, Kenya, Uruguay, and South Africa were included, there was no intraspecific variation linked to geographic origin and the sequences of both *O. albidum* and *O. ponderosae* were indistinguishable from those of *O. stenoceras*. The specific status was also ambiguous for the other two members of this clade, *O. narcissi* and *O. abietinum*. There was some sequence variability among *O. narcissi* strains and the species is paraphyletic as the *O. abiethinum* strains lie within the *O. narcissi* clade. It appears that, independent of the sequences studied, closely related species of *Ophiostoma* and its anamorphs need careful analysis to distinguish among species and physiological variants.

Yeasts are commonly isolated from bark beetles (Table 14.1). In an extensive sampling of *Dendroctonus ponderosae,* Six (2003) found yeasts in 80% of the mycangia (n=224) and was able to isolate them from the surface of 90% of the beetles (n=256) sampled in western USA. Unfortunately, the yeasts were only enumerated and not identified. Some *Ophiostoma* species isolated from bark beetles appear to be vectored both on the beetle's surface and in mycangia, while others appear not to survive well on the surface and show greater dependence on dispersal in mycangia (Six 2003). In a quantitative study of yeasts associated with a bark beetle, *Ips typographus*, Leufvén and Nehls (1986) found several species of yeasts on or in the beetle. Some species were present throughout the beetle's life cycle (Table 14.1), but *Pichia holstii* and *Candida diddensiae* outgrew the other species by several orders of magnitude as the galleries were excavated, eggs laid, and larvae reared. Gallery initiation corresponded to the lowest yeast population sizes, perhaps owing to the release of resin during this phase. Older galleries also did not support large yeast populations, perhaps owing to resource depletion. Because yeasts were isolated from whole-beetle homogenates, it was impossible to separate those on the surface from those in the gut and so it is not known if the beetles fed on yeasts. *C. diddensiae* (as well as two other less common species) is able to convert *cis*-verbenol (an important component of the beetle's aggregation pheromone) into verbenone (Leufvén and Nehls 1984). Bridges et al. (1984) reared wild-caught beetles in pine bolts brought into the laboratory. They sampled the bacteria, fungi, and yeasts from the larval chambers excavated by the wild-caught beetle's progeny. Fungi were absent from all substrates (frass and phloem in the vicinity of the egg niche, larval mines and chambers) at the

egg and young larvae stages. Whenever significant populations of fungi were present, all four substrates were dominated by yeasts [28 species in all, dominated by *Pichia pini*, *C. tenuis*, *P. capsulata*, and *Ogataea polymorpha* (*P. angusta*) – including three isolates from two basidiomycetous species]. Bacteria were present at all times, but at low numbers. Thus, yeasts may be of greater importance to larval development than isolations done from the portions of galleries occupied by the adults would suggest.

Yeasts recently isolated from beetles or beetle frass include a clade of anamorphic strains related to *C. tanzawaensis*. Kurtzman (2001b) isolated six new species, three from insect frass or tunnels (*C. ambrosiae*, *C. pyralidae*, and *C. xylopsoci*), one from soil (*C. canberraensis*, later isolated from beetle gut contents), and two from trees (*C. prunicola* and *C. caryicola*). The clade is only distantly related to a sexual genus (*Lodderomyces*). The collections were done from widely spaced sites (Illinois, USA; Natal, South Africa; and Canberra, Australia) and no beetles were examined, so it is not known if this represents a major clade of beetle symbionts but the data would indicate a speciose clade that has been undersampled in the past. Dimorphic basidiomycetes are not commonly isolated from bark beetles. Two species of *Fibulobasidium* were isolated near galleries and are probably associated with the beetles (Bandoni 1998). There is little doubt about a beetle association for *Atractocolax pulvinatus* as it was isolated from bark beetles (Kirschner et al. 1999). Kirschner et al. (2001) found a second, dimorphic basidiomycete, *Cuniculitrema polymorpha*, on bark beetles. It is very similar (physiological profile, LSU rDNA sequence, and DNA–DNA homology) to *Sterigmatosporidium polymorphum* and Kirschner et al. believe *Sterigmatosporidium* to be an anamorphic genus and *Cuniculitrema* its teleomorph. Little is known about the role these fungi might have in the bark-beetle system but it is suggested that *C. polymorpha* may be a mycoparasite owing to the presence of haustoria.

Stevens (1986) studied the biogeography of wood-boring beetles in the Scolytidae, Buprestidae, Cerambycidae, and Curculionidae in eastern North America. He found that the number of beetle species using dead wood depended on the size of the geographic range of the plant that supplied the wood. His data supported the hypothesis that plant species with greater ranges supported more beetle species because habitat diversity increased as the plant's range increased. However, Stevens was not able to suggest what sort of habitat diversity increased with plant range. His consideration of habitat diversity was confined to edaphic factors and did not stray into habitat differences related to the numbers or types of fungal species inhabiting the wood. Stevens mentions that beetle species that inhabit a plant's wood may be inexplicably missing from parts of the plant's range. Data on the distribution of the beetle's fungal associates may be pertinent in such cases.

There are cases of yeasts associated with Scolytid beetles that do not bore wood. An example is the coffee bean borer, *Hypothenemus hampei. P. burtonii* and *C. fermentati* were isolated from the internal organs of the beetle (Vega et al. 2003). Since caffeine is one of the better known insecticidal plant secondary compounds, Vega et al. (2003) investigated one of the yeast species for its ability to detoxify the beetle's diet. In this case, not only does *P. burtonii* not detoxify the beetle's diet, it suffers reduced spore production in the presence of the toxin at levels commiserate with those in coffee beans (*C. fermentati* was not tested). While this study failed to

identify any potential value of the yeasts to the beetle, it does underline the need to be cautious in inferring more to an association between yeasts and insects than has been experimentally demonstrated. Vega et al. suggest that the yeasts may be an important source of nutrition, but there is a stronger case for *Fusarium solani* as the beetle's fungus of choice, as the yeast provides ergosterol to the beetle (Morales-Ramos et al. 2000).

14.2.2 Ambrosia Beetles

There are over 3,400 species of ambrosia beetles. Ambrosial associations appear to have arisen at least 6 times within the Scolytid lineage (which includes the Platypodinae) (Farrell et al. 2001). There are several differences between bark and ambrosia beetles. Both adult and larval bark beetles tunnel but most ambrosia beetles have a form of parental care in which the adults tunnel, maintain the tunnel fungi, and feed the larvae, which may be solitary or aggregated in special chambers. Several clades of ambrosia beetles have developed sib-mating. The largest clade (1,300 species) has taken sib-mating to the extreme, abandoning outcrossing and developing flightless, dwarf males and haplodiploidy, perhaps as a means of controlling brood sex ratios (Jordal et al. 2002). Inbreeding may be a means of increasing the rate of establishment of successful colonies (Jordal et al. 2001). This suite of characters appears to be successful, as beetles from the haplodiploid clade dominate the wood-boring-beetle community in tropical lowlands (Jordal et al. 2000). Ambrosia beetles also respond to aggregation chemicals, but colonization by ambrosia beetles does not normally result in the death of the tree. These beetles form obligate mutualisms with vertically transmitted dimorphic fungi that are seen by entomologists as "domesticates" cultivated by diligent arthropod agriculturalists. Insect agriculturalists (including beetles, termites, and attine ants) can be important, even dominant, members of terrestrial herbivorous or macrodecomposer communities (Farrell et al. 2001; Mueller and Gerardo 2002). Some Lymexylid beetles have a life history that is similar to the ambrosia beetles. Adult *Melittomma* and *Elateroides* do not bore galleries but lay eggs coated with *Ascoidea* spp. on bark. The larvae bore tunnels that become lined with yeasts that are eaten by the larvae (Francke-Grosmann 1967).

In addition to the primary (ambrosial) fungus, yeasts (Table 14.1) and euascomycete fungi grow in ambrosial beetle galleries and can be isolated from mycangia of adults, although at lower numbers than ambrosial fungi. Batra (1966) demonstrated that these fungi could support the growth and development of ambrosia beetles. Most of the yeasts and fungi tested could not support all of the beetles tested and the proportion of successful larvae was lower for larvae reared on auxiliary fungi than for those reared on primary (ambrosia) fungi, so it appears that agricultural monocultures are potentially harmful to the beetles. The identification of an association between a yeast community and the beetles complicates the situation but raises many interesting questions about interactions between beetles, between the beetles and the fungi, and between beetle species.

Many of the fungi associated with ambrosia beetles are dimorphic and grow as hyphae in the beetle's galleries, including some of the *Hemiascomycetes*. However,

the literature is split on how they are vectored. Batra (1966) believed them to be vectored as yeast-like cells and was able to show that the transformation from hyphal to yeast forms occurred in the mycangia. Numerous subsequent authors have described the mycangia as specialized for fungal spore transport. Related to the transport issue is the means by which the beetles influence which species of fungi are present in their galleries. There is evidence that the adult beetles are responsible for eliminating airborne fungal contaminants from the system (Batra 1966). How this is accomplished is not known, although some beetles produce exocrine secretions with antiyeast activity (Gross et al. 1998). If the adults could potentially eliminate auxiliary yeasts from their galleries, then the continued presence of yeasts may indicate that they have a role to play in the ambrosia fungus–beetle system.

Several recent discoveries have enlarged the group of yeasts associated with ambrosia beetles. *C. trypodendroni*, an anamorph related to *C. insectorum* (on the basis of rDNA sequence data), has been isolated from *Trypodendron lineatum*, an ambrosia beetle (Kurtzman and Robnett 1998b). As it is in a poorly resolved clade, the relationship with *C. insectorum* is somewhat unclear, but the clade is rich with other ambrosia beetle and bark beetle yeasts, including another yeast described at the same time from insect frass in an insect tunnel in a dead oak, *C. tammaniensis*. Kurtzman and Robnett described a third species from frass in spruce trees, *C. ontarioensis*. This species partial LSU rDNA sequence was not very similar to any known yeast sequence (*C. entomophila* was most similar, but still 18% divergent). *C. mycetangii* and *C. ulmi* are also both beetle-associated, the former with an ambrosia beetle and the latter from insect frass (Kurtzman 2000a). It is not known if the newly described yeasts are functionally associated with beetles.

14.2.3 Anobiid Beetles

Anobiidae, a family in the Polyphaga, are related to powder-post (Lyctidae) and twig boring beetles (Bostrichidae). Xylophagy is thought to be the ancestral feeding habit of the family (Jones et al. 1999) although some species have become adapted to anthropogenic habitats. Two such species, *Lasioderma serricorne* (the cigarette beetle) and *Stegobium paniceum* (the drugstore beetle) have become pests. Both harbor fugal endocytobionts in the cecal cells between the midgut and the foregut. The YLS supply nutrients (vitamins and sterols, Pant and Fraenkel 1954; Pant et al. 1960; Buchner 1965; Nasir and Noda 2003) and detoxify various substances for their hosts (Dowd 1989, Shen and Dowd 1991). Unlike Homopteran YLS (see later), Anobiid YLS are culturable and have been the object of much systematic attention. They were first thought to be *Candida* (= *Torulopsis*) yeast but were later removed from the *Hemiascomycetes* and placed in the *Taphrinales* as *Symbiotaphrina kochii* and *S. buchneri* (van der Walt 1961; Gams and von Arx 1980). Using SSU rDNA, Noda and Kodama (1996) were able to confirm that the two were separate, related lineages (on the basis of sequence differences and the presence of five group I introns in the SSU locus of *S. buchneri*). However, their analysis removed these fungi from the *Taphrinales* and placed them in the paraphyletic *Discomycetes*. Sequence comparison of *ERG5* (a locus in the ergosterol synthesis pathway) from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *C. albicans* with the locus from beetle

and planthopper YLS clearly supports this conclusion (Noda and Koizumi 2003) although it does not help resolve their placement beyond that.

In addition to *S. buchneri* and *S. kochii*, there are other YLS found in Anobiid beetles and still classified as *Candida* species. *C. ernobii*, *C. karawaiewii*, and *C. xestobii* are all endocytobionts of Anobiid beetles (Jones et al. 1999). Other beetle families host YLS. Cerambycid beetles, only distantly related to Anobiids, are also xylophagous and harbor culturable YLS described as *Candida (C. rhagii*, *C. shehatae* var. *insectosa*, *C. ergatensis*, *C. parapsilosis* var. *intermedia*, *Rhodotorula glutinis,* and *C. tenuis)*. Except for *Rh. glutinis*, all of these are *Hemiascomycetes*, on the basis of both LSU and SSU rDNA sequences (Jones et al. 1999). For those species where analysis of their LSU sequences is possible (too few SSU sequences are available to allow a comparable analysis using SSU sequences), some Cerambycid YLS are siblings of teleomorphic species (*C. rhagii* and *P. heimii*, *C. xestobii*, and *P. guilliermondii*, Kurtzman and Robnett 1997). Some questions remain. LSU sequences indicate that *C. ernobii* and *C. karawaiewii* are conspecifics and related to *P. holstii* (Meyer et al. 1998). However, SSU sequence analysis indicates that they are not the same species (Jones et al. 1999). As Jones et al. (1999) point out, the origins of beetle YLS in both the *Euascomycetes* and the *Hemiascomycetes* are an interesting case of multiple, independent origins and convergent evolution occurring within (in the case of the Anobiids) a single host family. Further comparisons of YLS and their nonsymbiotic relatives might shed light on the adaptive characteristics (and preadaptations) for successful association with beetles.

14.2.4 Other Xylophagous Beetles

Beetles in the Passalidae are subsocial and live in multigeneration colonies in wood undergoing degradation by white-rot fungi. Larvae and adults feed on adult fecal pellets plastered onto tunnel walls by the adults. Coprophagy is a possible means of providing the microbes time and the proper conditions for further digestion of refractory food items such as lignocellulose. Suh et al. (2003) have isolated yeasts from the guts of two members of the family, *Odontotaenius disjunctus* and *Verres sternbergianus*, one common in temperate North America and the other from Central America. Two undescribed varieties were present and both were able to ferment xylose, possibly beneficial to both yeast and beetle. Xylose fermentation is characteristic of the clade that includes these yeasts and *P. segobiensis* and *P. stipitis*. It was not clear whether these lineages were separate species or varieties of *P. stipitis*. Both have sequences and physiologies that differ slightly from those of *P. stipitis*. One lineage had been identified previously as a *Euascomycete*, *Enteroramus dimorphus* (Lichtwardt et al. 1999), owing to its hyphal growth (all members of this clade are capable of hyphal growth).

Scarab beetles have also been found to harbor yeasts. The green June beetle, *Cotinis nitidia*, has no yeast in its gut during the larval or pupal stages but adults develop a diverse yeast flora that is vectored to new food items (Vishniac and Johnson 1990). The yeasts include *Aureobasidium pullulans*, *C. guilliermondii*, *C. krusei*, *C. sake*, *C. tropicalis*, *Cryptococcus albidus*, *Debaryomyces hansenii*, *Hanseniaspora uvarum*, *Rh. glutinis*, *Rh. rubra*, and *Trichosporon cutaneum*. The xylophagous scarabs, *Pachnoda*

ephippiata and the rose chafer *P. marginata*, have bacterial endosymbionts that contribute to their ability to digest plant fibers, but have not been sampled for yeasts (Cazemier et al. 1997; Egert et al. 2003; Lemke et al. 2003). The gut flora has been implicated in the beetle's ability to digest plant fibers (Cazemier et al. 1997). It should be noted that other insects (*Schistocerca gregaria*, the Cerambycid *Psacothea hilaris*, and the Phasmid, *Eurycanta calcarata*) are able to digest wood without the aid of microbes, so a microbial association is not necessary for this mode of feeding (Cazemier et al. 1997; Scrivener et al. 1997).

14.2.5 Flower Beetles

Flowers are visited by many insects, including beetles from several families. Recent efforts by Lachance and colleagues have isolated numerous species of yeasts either from floricolous beetles (mostly Nitidulids) or from the flowers with which they are associated. Rather than list individual instances, a table of recently described species is provided (Table 14.2).

The beetle–flower–yeast system is dominated by ascomycetous yeasts from the *Saccharomycetales*, which account for about 90% of the strains isolated from beetles (Lachance et al. 2001d). Many of the basidiomycetes and nonsaccharomycetales *Ascomycetes* from the system were either widely distributed (and, so, show little habitat specificity), dispersed through the air (*Aureobasidium*), or so rare that their occurrence was probably serendipitous. Lachance et al. (2001d) include many undescribed species, which have been included in Table 14.2. The table also includes species from flowers that are related to those from beetles with the exception of flower strains associated with bees, which are discussed in the section on Hymenoptera. There is a second set of yeasts from flowers (*Wickerhamiella australiensis*, *W. occidentalis*, *Metschnikowia santaceciliae*, *C. drosophilae*, *C. lipophila*, and *C. tolerans*) that have been isolated from *Drosophila* that lay eggs in the open flower, which they inoculate with yeast, and the larvae develop on the fermenting flower tissue (Lachance et al. 1998c, 1999, 2003). *M. santaceciliae* has been isolated from both beetles and flies, and so it included in Table 14.2. Cladograms based on partial LSU rDNA sequences show that flower-beetle-associated yeast cluster into several unrelated clades within the *Saccharomycetales* (Lachance et al. 2001d, or many of the references cited in Table 14.1) and Table 14.1 is organized by these clades. Each includes a single sporogenous genus, which gives the clade its name, and one or more asexual species (as defined by rDNA sequence divergence).

The *Metschnikowia* clade in Table 14.2 has been subdivided with information extracted from the several cladograms published for these species. The systematics of this clade is not completely understood. Hybridization experiments with auxotrophs showed that, like many sexual eukaryotes, some hybridization and horizontal gene transfer is possible between some species but that there is significant reproductive isolation among sexual *Metschnikowia* species (Marinoni and Lachance 2004). Some *Metschnikowia* species have several insertions in the region of the 26S rDNA used for phylogenetic studies (Hong et al. 2003). As the presence and sequences of the insertions can vary among species, it is not apparent whether or not to include this information in phylogenic analyses. Although the composition of subclades within

Metschnikowia are often well supported (over 70% bootstrap support), basal support for connecting the subclades is often weak. This impression is further strengthened by the cladogram (on the basis of the D1/D2 loop region of the LSU rDNA) presented by Suh et al. (2004a) as part of their description of *M. chrysoperlae* from lacewings. This species and two closely related anamorphs (*C. picachoensis* and *C. pimensis*) belong to a well-supported clade with *M. pulcherrima*, *M. fructicola*, and three undescribed species. However, much of the genus is part of a large polytomy consisting of well-supported subclades (including those in Table 14.1 and others). This ambiguity may indicate that the genus needs to be split or that species with transitional sequences have not yet been sampled. It is difficult to assess the degree of completeness of the known *Metschnikowia* sequences (indeed, this is difficult to know for any lineage). Lachance et al. (2001d) concluded that the biogeography of these yeasts depends on location and beetle, which are confounded (*Aethina* replaces *Conotelus* as the beetle species most often sampled in Australia and the South Pacific, except in Hawaii, where both are introduced). While their efforts have been extremely wide ranging (Table 14.1 includes samples from North America, Costa Rica, Brazil, Australia, Hawaii, Korea, and several South Pacific islands), there are many gaps (Europe, most of Asia, and Africa), which means that there may be systematic gaps in the current set of known species.

Sampling gaps or not, the conclusion that yeast species in the flower–beetle–yeast system tend to occur in restricted locales and that the distribution of the yeast is related to the distribution of both host plants and vectors is well supported. Within each clade, the teleomorphic species tends to have a wider distribution than the anamorphic species (Table 1 in Lachance et al. 2001d) but no species is truly ubiquitous throughout the system. This situation is similar to that in the cactus–*Drosophila*–yeast system. Both have several clades of related species. Some cactus–*Drosophila*–yeast system clades (such as the *Starmera* and *Phaffomyces* clades) conform to the plant–beetle–yeast model of geographic subdivision. However, some species (*P. cactophila* and *C. sonorensis*) seem to be ubiquitous, a situation without parallel in the flower–yeast–beetle system. This may be an historical artifact. Many of the species in the cactus–*Drosophila*–yeast system were described before sequencing became routine. Recent data from that system support the plant–beetle–yeast model. *Sporopachydermia cereana* was once listed among the ubiquitous members of the system but isolations were often reported as "*S. cereana* complex" owing to physiological variation. It is now known that this "complex" is composed of at least ten species, most with very restricted distributions (Lachance et al. 2001c). *C. sonorensis*, an exclusively asexual lineage, is genetically variable with a strong correlation between genetic variation and geography (Ganter et al. 2004). New collections from the Caribbean and South America indicate that *P. cactophila* may also have regional variants, some of which are heterothallic, in contrast to the widely distributed homothallic form (Ganter, Rosa, and Cardinali, unpublished data).

The occurrence of asexual lineages may be related to the means of yeast dispersal. Yeast species vectored by insects disperse to new habitat when their vectors do. Some asporogenous species may represent hybrid opportunists well adapted to very local situations as sometimes happens in plants (Grant and Grant 1971, 1980). Animal vectoring may produce founder events with interesting ramifications for

heterothallic species that occur as haploids. Lachance et al. (2001b) suggest that the Hawaiian species *C. kipukae* and *C. hawaiiana* may be stranded mating types of undiscovered *Metschnikowia* species not native to Hawaii. Only one of the mating types of *M. lochheadii* has been introduced into Hawaii, where it is among the most commonly isolated species from beetles and, more rarely, *Drosophila*. This species is hard to distinguish from some congenerics on the basis of physiology, but it is reproductively isolated (Lachance et al. 2001b). Hawaii has other *Metschnikowia* species, *M. reukaufii* and *M. hawaiiensis*. The mechanism of reproductive isolation from *M. reukaufii* has not been reported, but *M. lochheadii* is partially isolated from *M. hawaiiensis* (Lachance et al. 2001b). Only mating between the h+ mating type of *M. lochheadii* and h– of *M. hawaiiensis* produces sterile asci. Since the mating type of *M. lochheadii* found on Hawaii is h+ and it is ecologically isolated from *M. hawaiiensis*, hybridization is not expected in this instance, although other opportunities exist for hybridization between the invader and native *Metschnikowia* species (Lachance, personal communication). However, Lachance et al. (2001b) also point out that some asexual species (e.g. *C. ipomoeae*, a member of the *Metschnikowia* clade, and *C. azyma*, a member of the *Wickerhamiella* clade) are among the most frequently isolated yeasts from the flower–beetle–yeast system and have wide distributions that are not consistent with expectations for hybrid opportunists. Asexuality may be favored by more than one set of circumstances.

An unresolved question in this system is the degree to which beetles, yeasts, and flower are interdependent. The system is open in the sense that many animals visit the flowers, the beetles are not pollinators, and we do not know what the beetles eat. Whether or not the *Metschnikowia* clade is rightly subdivided, the apparent clustering of related lineages within this environment suggests that both yeast and beetle have specialized to some degree. That the presence of the beetle is valuable to the yeast has been demonstrated through experiments that exclude the beetles from flowers (Lachance et al. 2001d). With no beetles, no beetle-associated yeast colonizes the flowers. What is not yet demonstrated is the influence the yeasts have on the beetles. Nout and Bartelt (1998) found that *Carpophilus humeralis*, a member of a Nitidulid genus that includes some flower-associated species, was attracted to microbial fermentation of corn, its favorite substrate. The most attractive fermenters were all yeasts. Flower yeasts have been isolated from the frass of beetles with regularity owing to the method used to sample yeasts from beetles, so they at least pass through beetle guts. However, there is no direct evidence at this time on the nutritional value of yeasts to flower beetles.

14.2.6 Beetles Associated with Mushrooms

Our understanding of the yeast community associated with mushroom-associated beetles is undergoing a bit of a revolution. Suh, Blackwell and colleagues have embarked on a program to isolate yeasts from basidiocarps. As of their most recent publication, they have 650 isolates from beetles in 26 families. Many of the isolates represent undescribed species. One example is a relatively small clade including *C.* (*P.*) *guilliermondii* and *C. xestobii*, known from xylophagous beetles, *C. fermentati* a previously described species Suh and Blackwell (2004a) isolated from mush-

room beetles, and three new species: *C. smithsonii*, *C. athensensis*, and *C. elateridarum* (Suh and Blackwell 2004a). Although closely related, the yeast species seemed not to be associated with specific basidiomycete species, specific beetle hosts (six strains used to describe three new species were isolated from five beetle families), or specific places (*C. athensensis* was isolated in Panama and Georgia, USA). Recently they (Suh et al. 2004b) have described the largest clade from the collection: 164 isolates (30% of the total) in 17 species, one previously described (*C. ambrosiae*) and 16 new species, all anamorphic *Hemiascomycetes* related to *C. tanzawaensis* (*C. guaymorum*, *C. bokatorum*, *C. kunorum*, *C. terraborum*, *C. emberorum*, *C. wounanorum*, *C. yuchorum*, *C. chickasaworum*, *C. choctaworum*, *C. bolitotheri*, *C. atakaporum*, *C. panamericana*, *C. bribrorum*, *C. maxii*, *C. anneliseae*, and *C. taliae*). The collection locales varied from Vermont and the southeastern USA to Panama. The isolates in this clade were collected from the gut contents of members of 11 beetle families, although 85% came from Erotylidae and Tenebrionidae, with some of the new species possibly associated with particular beetle species. This clade has just been recently expanded from a single isolation from moss in Japan by the addition of six species from trees and beetle frass (see Sect. 2.1 and Kurtzman 2001b).

Considering that basidiocarps have been previously sampled for yeasts, Suh et al.'s work constitutes a remarkable demonstration that animal-associated yeasts are undersampled. The situation may be even more diverse than standard sampling methods will uncover. For some time, bacteriologists have amplified DNA regions useful for assessing biodiversity from environmental samples in search of lineages that do not grow under standard culture conditions. Comparisons between sequence diversity amplified from cultures and from environmental samples lead to the conclusion that only a small percentage of bacterial species are culturable (Torsvik et al. 1990; Kemp and Aller 2004). Environmental PCR has its pitfalls (Tanner et al. 1998; Qiu et al. 2001; Speksnijder et al. 2001) and is only beginning to be applied to yeast studies but has considerable potential (Lipson et al. 2002; Renker et al. 2004), especially in the area of yeast–animal interactions, where it is known that some YLS are not culturable. Using rDNA sequence amplification, Suh and Blackwell (2004b) report that as many as 150 new yeast species may be awaiting discovery in the guts of beetles. Zhang et al. (2003), sampling beetles collected from basidiocarps and rotting wood, found that there was less than expected overlap between the yeasts isolated from the beetles' guts and rDNA fragments amplified from their guts. Six sequences similar to known *Hemiascomycetes* sequences, five sequences from the Pezizomycotina, and two *Basidiomycetous* sequences were detected. Of these, only five were greater than 96% matches to known species and only one was a described yeast, *P. stipitis*. Only three of the six yeast sequences amplified from the gut matched sequences amplified from colonies isolated at the same time. Seventeen of the sequences from isolates did not occur in the sequences amplified directly from the gut. Considering that isolations are reasonable proof of the yeast's presence and the sensitivity of the PCR method, it is unexpected that so few of the isolated species' sequences were detected in the gut amplifications. Since both samples (isolation of viable colonies and direct amplification of gut contents) were small, it is difficult to know if the differences in the results are systematic and represent bias in the direct amplification method or are simply due to chance.

14.3 Termites

Most biologists associate termites with cellulose digestion mediated by the presence of protistans in the insect's gut. Study of the details of lignocellulose digestion and the distribution of microbes in the various sections of the highly differentiated termite gut have, for some termites, reduced the importance of the microbe–insect mutualism and raised questions about the role of nonprotistan microbes in both carbon (Wenzel et al. 2002; Ohkuma 2003) and nitrogen metabolism (Potrikus and Breznak 1977, 1980). Termites produce their own cellulose-degrading enzymes and the higher termites (75% of termite species) lack the protistan symbionts long thought to be a necessary component for digestion in termites (Slaytor 1992; Varma et al. 1994; Tholen et al. 1997; Tokuda et al. 2004). Some higher termites acquire cellulases from their fungal gardens (Martin and Martin 1978). Although prokaryotic and fungal symbionts have long been known from termites, the true complexity of the system is only now being dissected. Yeasts are a member of the system, and there role is still not understood. Fungi have been isolated and identified from termite mounds many times. Hendee (1935, cited in Prillinger et al. 1996) found dozens of genera of fungi associated with the lower termites and there has been considerable interest in the association between *Termitomyces*, a mycelial basidiomycete genus, and some higher termites in the Macrotermitinae (Aanen et al. 2002; Rouland-Lefevre and Bignell 2002; Ohkuma 2003). Fungus-cultivating termites can be the dominant macrofaunal decomposer in some tropical biomes (Abe et al. 2000), giving this association widespread ecological importance. A second association between termites and *Laboulbeniales* is known (Blackwell and Kimbrough 1976a, b; Rossi and Blackwell 1986; Blackwell 1994). The relationship is one of parasitism or, given the lack of impact on the host, perhaps a form of phoretic commensalism.

Yeasts have not received the attention of other termite-associated fungi. Boidin and associates collected *Trichosporon*, *Candida* (including *Torulopsis*), and *Saccharomyces* from African termites (cited in do Carmo-Sousa 1969). This promising start was not immediately followed up. In an extensive review of termite gut flora, Breznak (1982) did not discuss yeasts except to mention the utility of yeast extract in media used to cultivate some of the first protistan symbionts to be isolated. Subsequent reviews of termite microbes involved in lignocellulose digestion by Breznak and Brune (1994) and Varma et al. (1994) do not mention yeasts at all (although Varma et al. do use the term in their abstract). In 1996, Prillinger et al. isolated strains from six species of lower termite and a cockroach (as a sister group to the termites). They subjected the yeast isolates to an array of methodologies (randomly amplified polymorphism DNA, physiological profile, ultrastructure, cell wall constituents, ubiquinones, and 18s rDNA sequences) in order to develop useful taxonomic characters. Using this multifaceted approach, they isolated strains that could be grouped into 12 ascomycetous species from the lower termites (one basidiomycete isolate, probably from the genus *Trichosporon*, was collected). Three of the species were tentatively assigned to the *Lipomycetaceae*. Although the physiologies of the remaining ascomycetous species were similar to *Debaryomyces* species, the sequence data led Prillinger et al. (1996) to remove them from the *Hemiascomycetes* and place them in the *Ophiostoma* – *Sporothrix* clade within the *Ophiostomatales*

(see Blackwell and Jones 1997 for a discussion of the phylogeny of insect-associated *Ascomycetes*). It is notable that Prillinger et al. (1996) did not find a single isolate that they could identify as a member of a known species.

Prillinger et al. (1996) did not investigate the nature of the interaction between the yeast they isolated and their putative hosts except to conclude that the relationship was a stable symbiosis because they were able to isolate members of the same clades from a variety of termite species collected from widely dispersed locales. Using what appear to be some of the strains isolated by Prillinger et al. (1996), Schäfer et al. (1996) tested them and a larger set of bacterial isolates for their ability to produce a set of four enzymes (α-L-arabinofuranosidase, β-D-galactosidase, 1,4-β-xylanase, and β-D-xylosidase) that play a part in the digestion of hemicellulose. Twelve strains (from five of six termite species) had positive activity for at least one of the enzymes, although no strain had all four. From these results, Schäfer et al. concluded that yeast enzymatic activity may play a role in termite nutrition.

14.4 Ants

Although the association between fungi and ants is well established, evidence for a yeast–ant relationship remains more equivocal. Fungus-farming ants in the tribe Attini are associated with mycelial basidiomycetous fungi. The relationship has arisen independently at least three times from within the *Agaricales* (Chapela et al. 1994; Mueller et al. 1998; Mueller and Gerardo 2002). Fungi from this clade cultivated by members of the genus *Cyphomyrmex* grow as yeasts. It is not known if the switch to the yeast-like form is adaptive or is an outcome of chance and the particular details of this association. The yeast strains are not sexual and seem to be propagated by transfer from ant colony to daughter colony by the ants themselves (vertical transfer). Some horizontal transfer does occur (Green et al. 2002). While confirming that the yeasts cultivated by *Cyphomyrmex* species did form a single, well-supported clade within the larger ant-farmed clade, Mueller et al. (1998) found that ant colonies traded yeast cultivars, sometimes with ants from other species, and that members of this yeast clade could be collected from natural substrates outside of the nest. Indeed, the strain not isolated from an ant nest was sampled from a basidiocarp and did not grow as a yeast. This implies that the yeast habit is a consequence of the association with the ants and that adoption of the yeast growth form occurs when a free-living fungus is recruited by the ants.

Other yeasts have been observed in the gardens of attine ants that cultivate mycelial fungi (Craven et al. 1970), although the yeasts present were not identified. Several species of yeasts were isolated from *Atta sexdens rubropilosa* colonies that were maintained in a laboratory on *Eucalyptus albus* leaves collected from nature. *C. apis*, *C. colliculosa*, *C. famata*, *C. homilentoma*, *C. guilliermondii*, *C. robusta*, *C. sake*, *Cr. aerius*, *Cr. albidus*, *Cr. laurentii*, *Cr. haglerorum* (a new species), *Rh. aurantiaca*, *Rh. glutinis*, *Sympodiomyces attinorum* (another new species), *Sporobolomyces roseus*, *Tremella foliacea*, *T. jirovecii,* and several variant physiologies perhaps representing undescribed species were all present in the fungal garden but not on the leaves. *Hemiascomycetes* constituted 71% of the 84 isolates from the gardens, waste piles, surface of ants, or colony floors (Carreiro et al. 1997, 2004; Middelhoven et al.

2003). Quite different results were obtained from an attempt to isolate yeasts from gardens in the wild. A search of 14 colonies of *Acromyrmex octospinosus* (another Attine species) detected no *Hemiascomycetes* rDNA sequences among 41 fungal sequences amplified from workers (van Borm et al. 2002). The absence of yeasts from some gardens may not be due to chance. Fungi other than cultivated species have been isolated from nests in the wild and the laboratory, including both commensal and parasitic (*Escovopsis*) species (Currie et al. 1999). The ants have behaviors (weeding and grooming) and chemicals that may control contaminants. The chemical defenses include both those produced by the ants (metapleural and maxillary glands) and by cultivated *Actinomycetes* bacteria that produce toxins effective against the parasite (Currie 2001). However, the effectiveness of these defenses has not been tested, as the dynamics of the fungal populations are unknown.

Hemiascomycetes have been found in association with other ants. Zacchi and Vaughn-Martini (2002) isolated *Rh. mucilaginosa* and *P. guilliermondii* multiple times from the body fluid of *Iridomyrmex humilis* in Italy. *D. polymorphus* (*D. cantarellii*) and *D. vanrijiae* (*D. formicarius*) have been isolated from *Formica rufa* in eastern Europe (Golubev and Bab'eva 1972; Sláviková and Kocková-Kratochvilová 1980). In Texas, USA, Ba et al. (2000) found 13 yeast species in samples from the nest of the invasive red fire ant, *Solenopsis invicta*, and from the soil in the area of the nest but most yeast species were equitably distributed between brood chamber (the site of the nest samples) and surrounding soil. *C. parapsilosis* and *C. lipolytica* were strongly associated with the nest and these two species had previously been isolated from the ants themselves (Ba and Phillips 1996). There was some indication that *D. hansenii* was also associated with the ants and that *Cr. terreus*, *C. vini*, *Rh. minuta*, and *Rh. rubra* were excluded from the nests. The nature of the interaction (whether positive or negative) between any of the yeasts and the ant is not known.

A possible role for yeasts in ant nutrition has been suggested in a recent study of carpenter ants and yeasts. Carpenter ants, such as *Camponotus vicinus,* are wood-dwellers, although they are not xylophagous. Workers of *C. vicinus* ingest a liquid diet, which can include honeydew. The infrabuccal pocket, located just before the crop, filters large particles from the food stream but not microbes. Mankowski and Morrell (2004) isolated over 150 strains of yeasts from ant guts, their nest (including frass), and surrounding soil. Of the 18 taxa identified, six were found in the infrabuccal pocket. *D. polymorphus* comprised ten of the 17 isolates from the ant guts. No other species occurred more than twice. To see if the presence of the yeasts has any impact on the ant, Mankowski and Morrell (2004) fed workers (initially cleared of yeasts) on an artificial diet that had been previously shown to be adequate for workers of this species. Some of the workers were given access to *D. polymorphus*, while others were not. Those with yeasts in their diet were significantly heavier after 12 weeks. This, plus Ba and Phillips' (1996) observation that colonies with yeasts were more vigorous, suggests that yeasts can be important sources of nutrition for some ants.

14.5 Other Soil-Associated and Xylophagous Arthropods

Byzov and his associates have studied the interaction between yeasts and diplopods (*Pachyiulus flavipes*, *Glomeris connexa*, *Leptoiulus polonicus*, and *Megaphilium pro-*

jectum). Comparison of yeasts from the fore-, mid-, and hindguts with those from the surrounding soil exposed significant differences in both species composition and live yeast counts (Byzov et al. 1993). Yeasts in the litter that comprises the myriapod's food (numerous species were present and differed among the litter types sampled) were only rarely found in the midgut (Byzov et al. 1993). The hindgut and feces contained high densities of yeasts, mainly *D. hansenii*, *Torulaspora delbrueckii*, and *Zygowilliopsis californica*, but these were not found in the soil samples. Digestion of yeast species is selective. Midgut fluid is able to rapidly kill and enzymatically break the cell wall of most species commonly found in the soil. Those yeasts associated with the hindgut were either resistant to the effects of the midgut fluid or were killed and digested at a much slower rate (Byzov et al. 1998a, b). The common species in the hindgut were also able to use the insect's nitrogenous waste (uric acid) as their sole nitrogen source (Byzov et al. 1993). Byzov et al. (1998a) proposed that the soil microbes (fungi and bacteria) are the main foods of diplopods and that the hindgut flora (yeasts and bacteria) are mutualistic symbionts. The latter claim is open to some doubt in that they have only speculated on the benefits accruing to the animal. The hindgut flora clearly benefits as it grows on materials derived from the millipede, which also provides the habitat. Byzov et al. (1998a) argue that the flora provides benefits to the insect by becoming food when antiperistaltic movement carries cells from the hindgut into the midgut. The hindgut yeasts are typically found only in the posterior portions of the midgut and are not found in the foregut, which contains litter-associated yeasts, or the anterior portions of the midgut, which contains few yeasts. Those hindgut species that are susceptible to digestion may, in this fashion, contribute to the insect's nutrition but the contribution may only be a small portion of the insect's diet. The second benefit, detoxification of uric acid, was not measured in vivo and we do not know if the diplopods actually derive any benefit. The situation may be similar to that of cockroaches, which recycle nitrogenous waste through their bacterial microflora (Cochran 1985; Cruden and Markovetz 1987). Thus, while the symbiosis between yeasts and diplopods has been amply demonstrated, the prima fascie case for a mutualistic symbiosis, while reasonable, awaits experimental examination.

Cockroaches are soil-associated insects that receive attention here more for their potential as objects of study than for what is known about the yeasts associated with them. Although much is known about the rich bacterial community found in the guts of human-associated cockroaches (Cruden and Markovetz 1987), they have yielded only a single species of yeast, *Kluyveromyces blattae* (Henninger and Windisch 1976, from Prillinger et al. 1996), and the relationship between yeast and cockroach has not been explored. The outlook for yeast associations is brighter for the wood-eating cockroaches. Their gut microflora is similar to that of the lower (protistan-associated) termites and Prillinger et al. (1996) discovered a species of yeast from a wood-eating cockroach (*Cr. punctulatus*). While they could not assign a generic name to the three isolates they obtained, they did ascertain that the strains were similar to some yeasts isolated from termites and all three belonged to the same clade in the *Endomycetales*. Nothing is known about the interaction between the yeast and the cockroach but it remains a promising avenue for research. In addition to the cockroach-termite clade, xylophagy has been demonstrated in a Scarabaeidid

beetle, *Oryctes nasicornis* (Bayon 1980; Bayon and Mathelin 1980). Unfortunately, no attempt has been made to isolate the gut microbes so we do not know if yeasts are present in the system. One last note on xylophagy and yeast. Xylophagy is not the exclusive domain of insects. Nelson et al. (1999) report that a xylophagous catfish in the genus *Panaque* has a gut microflora capable of lysing cellulose and of sustaining itself on artificial media with cellulose as the sole carbon source. The identification work done on the microbes was minimal and no effort was made to isolate yeast. However, nonpathogenic yeasts are associated with fish guts (Andlid et al. 1995) and it would not be unexpected to find them present in *Panaque*.

In addition to termites and ants, there are several other soil-associated arthropod clades, including some Orthopera, Collembola, Thysanura, Diplopods, Diptera, Coleoptera, and Hymenoptera, that have known microbial associations. Yeasts have been identified only from a few of these groups, but that may simply result from a failure to look. The effort put into collecting yeasts from these sources has not been sufficient to eliminate yeasts from membership in any of these gut floras. *D. mycophilus* is a novel species isolated from an Oniscoid isopod (Thanh et al. 2002). Woodlice and pillbugs are common litter organisms long noted for consumption of rotting vegetation and may harbor more undescribed species.

14.6 Neuroptera

Lacewing larvae and adults are useful agents for biological control of aphids and other agricultural pests, including some mites and whiteflies. As such, their biology has received attention, first from agricultural entomologists and, more recently, yeast biologists. *Chrysoperla* (= *Chrysopa*, "golden pearls" for their golden eyes) is a large genus from which several biological control agents have come, including *C. rufilabris* and *C. carnea* (commonly used in the USA). Larvae can be purchased packaged in honeycombs of 500 cells, each with a single larva (they are cannibals). Investigation of *C. carnea* uncovered an unexpected interaction among aphids, yeasts, and lacewing adults. The feeding habits of *C. carnea* are unusual in that adults are not predaceous. Instead of eating the aphids, they feed on the honeydew extruded out of the anus of the aphid (Hagen and Tassan 1972). The honeydew is not a complete diet for the adults (it is deficient in several essential amino acids and lipids). Hagan and coworkers determined that endosymbiotic yeasts were able to make up any dietary deficiencies and observed that the standard laboratory food for rearing the adults consists of honey and *Saccharomyces cerevisiae* (Thompson 1999; Winterton 1999)

Hagan and Tassan (1972) found the yeasts in the crop and larger trachea of the adults but they could not find any in the predaceous larvae. Phaff and Starmer (1987) conclude that the adults must acquire yeasts through feeding or trophallaxis. The yeast population of the crop can grow until the adult deposits compact spheres of yeasts, which can be seen where the adults are ovipositing. Recently, there was a report that vertical transmission is possible and that the protocol for cleansing the adults of yeasts used by Hagan is not adequate (Gibson and Hunter 2003). Suh et al. (2004a) isolated yeasts from eggs, although it is not clear if the yeasts came from the surface or were internal. Although these observations establish the possibility of vertical transmission they would not exclude horizontal transmission as an important

dispersal process. Woolfolk and Inglis (2003) found no yeast present in the guts of newly eclosed *Chrysoperla* adults from larvae raised on laboratory food. The eggs were obtained from a mass-rearing facility. These data raise questions about vertical transmission but do not settle the question of transmission in nature. No work has been done to establish whether or not the appropriate yeasts are available from nonanimal sources in the adults' range (honeydew, plant surfaces, etc.) or if trophallaxis is common enough to ensure an adult has a complete diet. Seven of 24 adults sampled by Woolfolk and Inglis (2003) had no yeast, so adults can manage without the yeast for at least part of their adulthood. Clearly, more work needs to be done before we will know how yeast populations are established and maintained in this system and what effect they have on lacewings.

The distribution of yeasts in time and space was not random in Woolfolk and Inglis's study (2003). They sampled 24 *C. rufilabris* adults from two sites in Mississippi, USA, five times between October 2000 and June 2001. Fewer yeasts were collected in the cooler months, per capita colony-forming units were consistently lower at one site, and most yeasts were found in the diverticulum, with populations declining along the gut (the fore-, mid-, and hindguts were also sampled). Almost 90% of the isolates were assigned to *M. pulcherrima*. Isolates from other species were never recovered from the diverticulum and, in the other regions of the gut, almost always when no *M. pulcherrima* was present. These patterns hint at interesting ecological interactions among the yeast species but this awaits investigation.

The diversity of yeasts from lacewings follows a common trajectory: first a single species similar to, but not identical to, a published species is found and subsequent work redefines and adds to the initial discovery. Hagen and Tassan (1972) isolated a single species of yeast, which they felt was a *Torulopsis* (= *Candida*) species. Phaff and Starmer (1987) reported that they isolated only a single yeast from several *Chrysoperla* adults and that its physiological profile was roughly similar to *T. multisgemmis* (= *C. multigemmis*) but that its G+C value indicated that it was a new species. Woolfolk and Inglis (2003) originally divided their more than 750 isolates into five groups, two basidiomycetous and three ascomycetous, but analysis of rDNA sequences caused them to divide one basidiomycete group into two known species (*Cr. victoriae* and *Cr. luteolus*) and assign the other basidiomycete group to a new species, which they did not describe. Their sequence analysis suggested that the unknown species' sister taxon was *Bullera oryzae,* with which its physiological profile was consistent, but with which the new species shared only 63% sequence similarity. The three ascomycete groups' sequences were identical. Comparison with known sequences indicated that the ascomycete isolates from Mississippi formed a well-supported (bootstrap support) clade with *M. pulcherrima* as its sister group (also a well-supported clade). Owing to the physiological similarities between *M. pulcherrima* and the *Chrysoperla* isolates (and the presence of needlelike ascospores), they accepted them as members of the described species, although with reservations. Indeed, a very similar situation in their cladogram of basidiomycete isolates (Woolfolk and Inglis 2003, compare Figs. 5, 7) was used to suggest that the sister group to *B. oryzae* was a new species. I do not mean to suggest that Woolfolk and Inglis were being inconsistent but to remark on the difficulty of assigning isolates to species, especially when working with an undersampled group of yeasts.

The diversity of yeasts associated with lacewings has recently increased. Suh et al. (2004a) isolated 14 strains from *C. carnea* and *C. comanche* (two strains from eggs and the rest from adults). All were ascomycetes and the partial sequence of the LSU (from the D1/D2 loop region), SSU, and ITS region rDNA was determined for each strain. Three unique LSU sequences were found. The sequence differences corresponded to differences in physiological profile, so three new species related to *M. pulcherrima* were described: *M. chrysoperlae*, *C. picachoensis*, and *C. pimensis*. Comparison of the rDNA sequences from the strains isolated by Woolfolk and Inglis with those isolated by Suh placed the isolates identified as *M. pulcherrima* by Woolfolk and Inglis in *M. chrysoperlae* and matings of strains from both studies produced asci and needle-shaped ascospores. However, there are several physiological differences between the two groups, so there may be significant genetic differences below the specific level that are related to geography (Suh et al. collected in Arizona, USA). The two asexual species might never have been recognized without sequence data. Although there were significant differences among their rDNA sequences, there was very little difference in their physiologies. Sequence data place them in a separate clade that is the sister to several described and undescribed *Metschnikowia* species and which includes *M. chrysoperlae*. We can only speculate at this time on the origin of the asexual lineages and how they are maintained. It would be very interesting to know the age of the asexual lineages and if the asexuals are found with each other or with related *Metschnikowia* species.

14.7 Homoptera

A convincing case for mutualism between insect and yeast has been made for the interaction between Homoptera and their endosymbionts (in this case, intracellular YLS). All phloem-feeding Homoptera are believed to have mutualistic interactions with symbiotic microbes, most of which are bacteria (Wilkinson and Ishikawa 2001). YLS are found in planthoppers and some aphids. Some endosymbionts are found in special enlarged insect cells (bacteriocytes or mycetocytes for their respective symbionts). Aphid endosymbionts (various species of *Buchnera*) in bacteriocytes are referred to as primary symbionts. Secondary endosymbionts are sometimes found in aphid and planthopper fatbodies or hemolymph (but not intracellularly). Most aphid endocytobionts are bacteria, but a single clade of eusocial aphids from southeast Asia has no primary endosymbiont. Instead, they have YLS that are not intracellular (Fukatsu and Ishikawa 1992; Suh et al. 2001). They are found in the hemocoel and intracellular lacuna in the fat body, like secondary bacterial endosymbionts (Houk and Griffiths 1980; Lee and Hou 1987; Baumann et al. 1995; Douglas 1998). Because the association between aphids and bacteria is thought to predate the founding of the aphid clade (Munson et al. 1991), the loss of bacteria and acquisition of YLS is a derived state. It is not known if this happened more than once (Fukatsu and Ishikawa 1992). This is not the only time a loss of endosymbiotic bacteria is thought to have taken place. In those instances that have been examined, planthoppers that have abandoned phloem feeding in favor of piercing and eating individual plant cells have also lost their bacterial endosymbionts (Douglas 1998; Wilkinson and Ishikawa 2001).

Yeast-like endosymbionts of the rice planthoppers, *Nilaparvata lugens*, *Sogatella furcifera*, and *Laodelphax striatellus*, are obligate mutualists and are inherited vertically as a clump of cells within the egg called the symbiote ball (Lee and Hou 1987). Although the symbiont has not been cultured in vitro, YLS cells can be isolated by density-gradient centrifugation and protein and molecular work done on the extracted cells. This technique has allowed investigation of their taxonomic affiliations without isolation in pure culture. Noda and Kawahara (1995) determined that the symbiont genome sizes and electrokaryotypes were consistent with those of other ascomycetes. On the basis of the sequence of a portion of the 18s rDNA, Noda et al. (1995) concluded that the symbionts isolated from the rice planthoppers represented three different lineages (species determinations were impossible to make and the authors did not attempt to do so) that formed a single, well-supported clade. Analysis of a set of fungi chosen to represent all higher fungi convincingly placed the planthopper YLS clade in the *Euascomycetes*. A second sequence comparison with a set of species chosen to represent *Euascomycetes* placed all three symbiont sequences as a single clade within the *Pyrenomycetes*, in the order *Hypocreales* (*Sphaeriales*, reported by Noda et al. 1995, has been invalidated) with *Hypomyces chrysospermus* as the sister group. However, the distance between the three symbionts groups and *H. chrysospermus* was large enough to suggest that the sister group might actually be another *Euascomycetes* lineage. Using more strains and both LSU and SSU rDNA sequences, Suh et al. (2001) were able to confirm that the three planthopper YLS strains formed a well-supported clade within the *Clavicipitaceae*, one of three families in the *Hypocreales*, with *Cordycepioideus bisporus* as the most probable sister lineage. This represents an independently evolved adoption of a yeast-like morphology as the planthopper YLS lineage is not closely related the YLS from Anobiid beetles (Noda and Kodama 1996) nor any of the fungal lineages associated with beetles. A fourth sequence from an aphid YLS was also part of the clade, suggesting either a common YLS ancestor in the insect's common ancestor, horizontal transmission among planthoppers and aphids, or an unknown source of potential endosymbionts available to both groups (see later).

Phloem is rich in carbohydrates but amino acid poor. The role of the endosymbiont, whether bacteria or YLS, seems primarily to be to supply essential amino acids (Wilkinson and Ishikawa 2001). This has been extensively documented for some aphids and is reviewed by Baumann et al. (1995) and Douglas (1998). Wilkinson and Ishikawa (2001) were able to demonstrate similar effects of symbiont loss in an aphid (*Acyrthosiphon pisum*) and a planthopper (*N. lugens*). Both insects developed more slowly and were underweight without symbionts. However, the effect was less pronounced for the planthopper. This result is in agreement with the work of Lee and Hou (1987). *N. lugens* adults in which the number of symbiotic YLS had been reduced by heat treatment did not suffer increased mortality or shorter lives so the YLS seem not to have had a direct effect on adult survivorship in their study. However, heat-treated female planthoppers laid fewer eggs so it appears that the amino acids supplied by the YLS boost fertility. Of interest in the Wilkinson and Ishikawa (2001) study was the production of what they refer to as the "metabolic signature" of symbiont loss in both insects despite their phylogenetic distance from one another and the difference in their symbionts. *Acyrthosiphon*

pisum contains a bacterium (*Buchnera* sp.) and *N. lugens* a YLS. Asymbiontic adults of both insects had a decreased total protein percentage while at the same time an increased concentration of amino acids in their tissues when compared with conspecifics reared with symbionts. Both effects are seen as a direct outcome of the loss of essential amino acids from the insect's diets. Once again, the effect was not as large for the planthopper.

A third part of the "metabolic signature" in both insects without symbionts was the imbalance between essential and nonessential amino acids found in both Homopterans (Wilkinson and Ishikawa 2001). Asparagine and glutamine were predominant in the aphid, while alanine and glutamate were relatively abundant in the planthopper tissues. The use of the term "essential" is only conditionally true in this case. *N. lugens* nymphs with their normal symbionts were able to develop into adults no matter which amino acid was eliminated from their diets (Koyama 1985), which leads to the conclusion that the symbionts can supply whatever amino acids might be essential to the insect. The source of the nitrogen for amino acid synthesis is both the plant and the insect. *N. lugens* produces uric acid as its nitrogenous waste but does not excrete it. Instead, it appears to be metabolized by its YLS (Sasaki et al. 1996), thus recycling the waste and eliminating the need for excretion. On high-nitrogen diets, rice planthoppers can store waste in their tissues as uric acid. Under conditions of nitrogen stress, the uric acid is mobilized by uricase (urate oxidase) from their YLS (Hongoh and Ishikawa 1997; Hongoh et al. 2000). There may be a second source of nitrogen for the YLS. The pea aphid produces ammonia plus excess asparagine and glutamine as its nitrogenous recycling materials (Sasaki and Ishikawa 1995; Douglas 1998). Its bacterial symbionts metabolize all three. Wilkinson and Ishikawa's (2001) discovery of excess alanine and glutamate in asymbiontic planthoppers suggests that these amino acids are also part of its nitrogen recovery system, although Douglas (1998) feels that more work is needed before amino acid recycling is unequivocally established. Sasaki et al. (1996) offer an interesting explanation for the lack of uric acid production in the aphids. Recycling ammonia and amino acids requires fewer steps than the production of uric acid and, so, is more efficient. Planthoppers (and the cockroaches and termites with bacterial endosymbionts that recycle their uric acid) may not take advantage of the streamlining of the recycling process as aphids do because they all lay eggs, which may require nitrogen waste storage in the form of uric acid crystals. Aphids, with their viviparous development, may have no life history stage that needs to store waste and can, therefore, streamline the recycling by simply not producing uric acid. Cockroaches and some termites (under some conditions) also produce uric acid and do not excrete it but recycle it (Cochran 1985). The systems for recycling differ in the two groups but bacteria are involved in both (gut bacteria in the Reticulitermes; fat-body mycetocytes in the cockroaches).

The YLS of aphids is related to that of planthoppers (Fukatsu et al. 1994; Suh et al. 2001). The role of the YLS in aphids is not understood at this time. Aphids, generally, do not produce uric acid and analysis of the uricase gene sequences from aphids, planthoppers, and related, free-living fungi showed that, while present, some of the aphid genes had mutations that rendered them incapable of coding functional enzymes (Hongoh and Ishikawa 2000). Hongoh and Ishikawa (2000) tested one

species of YLS-associated aphid (which Fukatsu and Ishikawa 1992 identified as *Astegopteryx styraci*, Hongoh and Ishikawa 2000 named it *Tuberaphis styraci*, and Suh et al. (2001) subsequently called it *Hamiltonaphis styraci*) that lacked a functional uricase gene and determined that it produced no uric acid nor any endogenous uricase. However, some of the aphid sequences did indicate that a functional gene product was possible, although the product's function was not clear. Phylogenetic analysis of the sequences showed that the sequence of a functional aphid YLS uricase gene was closest to the sequence of the gene from free-living fungi, which were included as an outgroup. This puts the uricase genes in planthopper YLS in a derived position with respect to some aphid YLS genes. This supports the hypothesis that YLS were associated with aphids before planthoppers and implicates horizontal transfer as the means by which planthoppers acquired their endosymbionts.

Protein synthesis is not the only area of nutrition affected by the presence of YLS. Phytophagous insects depend on phytosterols for the production of their own sterol requirements. The exceptions to this may be insects with eukaryotic intracellular symbionts (bacteria do not seem to produce sterols – Douglas 1998). Noda et al. (1979) have made a case for YLS as the source of sterols for the rice planthoppers, *N. lugens, S. furcifera*, and *L. striatellus*. The phloem of rice contains β-sitosterol and a much lower concentration of campesterol. The sterols found in *L. striatellus* are primarily cholesterol and 24-methylenecholseterol with a small amount of β-sitosterol. Although the concentrations are not as expected, this situation might be explained as the outcome of the conversion of campesterol into cholesterol because 24-methylenecholesterol is an intermediary in the process. However, asymbiontic *L. striatellus* have negligible concentrations of 24-methylenecholesterol in their tissues and a reduced concentration of cholesterol, even though their plant diet is unchanged. The precursor of 24-methylenecholesterol is ergosta-5,7,24(28)-trienol. When planthoppers are maintained on artificial diets lacking sterols, the concentration of 24-methylenecholesterol in the insect increases, implicating YLS as the source of its precursor, since the insect cannot synthesize the precursor itself (Wetzel et al. 1992). This suggests that the source of cholesterol in the planthopper is its YLS. Noda et al. (1979) present other data suggesting that the YLS are the source of sterols in their host insects. Two other YLS-associated planthoppers on rice (*N. lugens* and *S. furcifera*) also have 24-methylenecholesterol in their tissues. A fourth rice planthopper, *Nephotettix cincticeps*, which is host to bacterial endosymbionts but not YLS, does not have detectable levels of 24-methylenecholesterol in its tissues, even though its natural plant diet is the same as the other three. It appears that the YLS, although the source of ergosta-5,7,24(28)-trienol, cannot metabolize it themselves (Noda and Koizumi 2003). It is an intermediate in the ergosterol synthetic pathway in yeast and is normally converted into ergosta-5,7,22,24(28)-tetraenol, the last precursor before ergosterol. However, planthopper YLS lack a functional gene for sterol C-22 desaturase, the enzyme responsible for the conversion.

The role of YLS in planthopper biology is not confined to nutrition. The fungi are not only passed on to offspring in the egg; they are a requisite part of normal planthopper development (Schwemmler 1974; Noda et al. 1979) in at least one

species, *N. lugens*. Embryos from which YLS had been eliminated or without YLS in their symbiote ball (because the eggs were laid by heat-treated females) failed to develop. They did not undergo blastokinesis or dorsal closure and the entire abdomen was missing (Lee and Hou 1987). The mechanism by which YLS affect planthopper development appears to be through direct supply of proteins needed for normal development. Eggs receive their YLS before yolk production and without YLS were deficient in more than one yolk protein (Lee and Hou 1987). Whether or not the YLS supply essential nutrients to the planthopper, the role of the YLS in the development of *N. lugens* makes the relationship with YLS obligatory.

Yeasts are associated with other members of the Homoptera but less is known about the nature of the association. Zacchi and Vaughn-Martini (2002) isolated seven yeast species from an olive tree scale insect, *Saissetia oleae*. Isolations were all from the internal organs, hemolymph, or the gut. A surprising number were basidiomycetous (*Bullera variabilis*, *S. roseus*, *Leucosporidium scottii*, and two undescribed species, one *Cryptococcus* and one *Rhodotorula*). They did not report serendipitous isolations but only those that occurred 10 times or more in their study. However, no species was present in all scale samples and no interactions between yeasts and insect were documented, although subsequent work determined that the number of yeasts in the body cavity fell as the insects developed (Zacchi and Vaughn-Martini 2003). Zacchi and Vaughn-Martini felt that the failure of the populations to grow lessened the likelihood that they were parasitic.

There are many unanswered and interesting questions pertaining to the evolution and ecology of YLS–insect associations. We do not know why different Homopteran lineages rely on different microbes. The answer to questions about the origins and effects of endosymbionts is likely to be found by studying variation in microbe, insect, and plant at the population level, but the few studies at this level have focused on bacterial endosymbionts. Baumann et al. (1995) and Douglas (1998) believe that YLS have replaced bacterial endosymbionts in some aphids, but no reason is suggested for how or why the replacement occurred. Ferrari et al. (2004) found that the presence of different secondary (facultative) bacterial endosymbionts in the pea aphid correlated both with differences in host plant utilization within that species and with resistance of the aphids to attack by different parasitoids, suggesting that the patterns of occurrence of facultative bacterial symbionts in Homopterans are not solely the outcome of chance. Chen and colleagues (Chen and Purcell 1997; Chen et al. 2000) demonstrated the possibility of horizontal transmission of endosymbiotic bacteria and that acquisition of facultative endosymbionts could profoundly affect both longevity and fecundity of their hosts. They believe that horizontal transmission has not been observed in the field owing to strong selection against it. Oliver et al. (2003) demonstrated that secondary bacterial endosymbionts were responsible for resistance to a Hymenopteran parasitoid. The facultative symbionts were fatal to the developing larvae of the parasitoid. We do not know if YLS can be horizontally transmitted under normal conditions (see later) or what the effects of transmission are.

Often these questions are studied through analysis of phylogenies, i.e., as history. Since the microbes are endocytobionts and (in the case of YLS) do not grow in pure culture, the switch from bacteria to YLS in the Cerataphidini has been viewed as a

single, historical event amenable to comparative study through phylogenetic analysis. If the microbes can only survive in an aphid and are vertically transmitted, then it is reasonable to assume there are no sources of the endocytobiont outside of its host. No mechanisms for horizontal transmission under such a scenario are known. However, recent data suggest that the assumption of no external sources might be wrong. Pea aphids without symbionts survived on bean plants but did so because their symbionts were replaced by a diverse set of microbes picked up from the environment, including all of the known YLS from planthoppers and aphids (Nakabachi et al. 2003). These remarkable results may mean that the relationship between the aphid and *Buchnera* sp., its primary symbiont, is not uniquely evolved, but that *Buchnera* is the competitive winner for the pea aphid mycetocyte niche. It also means that the presence of *Buchnera* or YLS may not be the result of an historical event but is the result of an ongoing dynamic process amenable to direct experimental study.

14.8 Bees and Wasps

Associations between yeasts and ants have already been discussed in the section on soil-associated arthropods. Here I will examine Hymenoptera other than ants. Bees are, of course, associated with flowers and it is in this context that most of the yeasts associated with bees have been isolated. A study of honeybee-associated yeasts in California isolated close to 400 strains from both pollen- and nectar-collecting bee guts (Phaff and Starmer 1987). Over 20 yeast species were isolated sufficiently often that Phaff and Starmer felt they could not be considered merely incidental. There were strong seasonal differences in the yeasts present in the guts. The number of isolations from pollen-collecting bees was negatively correlated with number in nectar-collecting bees, probably reflecting variation in flower types and abundance. Their interpretation was that the majority of yeasts were associated with the flowers on which the bees had been foraging and had no special relationship with the bees.

Yeasts associated with bees have been reported from numerous sources. *Debaryomyces robertsiae* (originally *Pichia*, van der Walt 1959) was collected from the pollen used to feed larvae of a bumblebee, *Xylocopa caffra,* in South Africa. Zacchi and Vaughn-Martini (2002) reported *C. bombicola*, *S. roseus*, and *P. guilliermondii* were commonly isolated from the body fluid of *Andrena* bees that feed on nectar and pollen. In an extensive survey of yeasts associated with seven *Bombus* species (*B. terrestris*, *B. lucorum*, *B. cryptarum*, *B. lapidarius*, *B. pascuorum*, *B pratorum*, and *B. hortorum*) and the flowers that they visit, Brysch-Hetzberg (2004) found that similar ascomycete species predominated in both flowers and bees. The ascomycetes were able to tolerate high sugar concentrations while the basidiomycetes were not. *M. reukaufii* and *M. gruessii* were the numerical dominants in both bee and flower, although they were not to be found in queens emerging from hibernation, leaving their means of overwintering still unknown. Brysch-Hetzberg suggests that two characteristics are most responsible for this domination: rapid growth on concentrated sugars and a pseudohyphal morphology ("airplane cell configurations") that favors dispersal on insect setae. *C. kunwiensis* and *C. bombi*, less commonly isolated species, were present in the hibernating queens and may be more dependent on their

association with bees than the numerically dominant species in the system. Other osmotolerant species (*D. hansenii*, *D. maramus*, *M. pulcherrima*, *C. rancensis*, and *Zygosaccharomyces rouxii*), including a new species, *C. bombiphila* (Brysch-Herzberg and Lachance 2004), were also present in small numbers.

Although described by several authors over decades, many yeasts isolated from bees form a distinct clade within the *Ascomycotina* (Lachance et al. 2001d; Stratford et al. 2002; Teixeira et al. 2003). The only teleomorphs so far identified in this clade belong to the genus *Starmerella* (Rosa and Lachance 1998; Teixeira et al. 2003). Several anamorphic species related to *Starmerella* (on the basis of rDNA sequence similarity) have also been described from bees or wasps, including *C. apicola* from bees (Hajsig 1958); *C. bombi* from bumblebees (Montrocher 1967), *C. batistae* from solitary-nesting digger bees (Rosa et al. 1999b), *C. powellii* and *C. tilneyi* from bees (Lachance et al. 2001a), *C. bombicola* from bumblebee honey and alfalfa leaf-cutter bees (Spencer et al. 1970; Inglis et al. 1993; Rosa and Lachance 1998), *C. magnoliae* (Deak and Beuchat 1993; Gilliam 1997), *C. apis* (Barnett et al. 2000), and, most recently, *C. davenportii* from a dead bee found next to a tap on a sugar-syrup tank in a Dutch soft drink bottling plant (Stratford et al. 2002). This list is only suggestive of the diversity of bee-associated yeast within this clade. Lachance et al. (2001d) report *C. etchellsii*, *C. ipomoeae* and three undescribed taxa related to *C. etchellsii* from bees. *Starmerella bombicola* is usually isolated as a haploid and Rosa and Lachance (1998) suggest that many of the anamorphic species might be unmated teleomorphs.

Stratford et al. (2002) and Lachance et al. (2001d) believe that the relatedness of bee-associated yeast species is not a chance occurrence but indicative of a functional relationship between yeasts and bees. Lachance et al. (2001d) note that yeasts isolated from flower beetles and bees rarely overlap. However, the degree to which the yeasts and the bees are associated remains somewhat uncertain at this time. The phylogenetic evidence is somewhat mixed. Lachance et al. (2001d) collected (from bees) *C. glabrata*, *C. azyma*, and four undescribed taxa that do not belong to the "bee" clade. *C. bombicola* was initially described from honey in a bee nest (Spencer et al. 1970), but it mates with the type species of *Starmerella* (Rosa and Lachance 1998), which was collected from flowers (*Calystegia sepium*) and floricolous beetles (*Conotelus* sp.), not bees. Two other members of the clade, *C. powellii* and *C. tilneyi*, were described from both beetles and bees in Costa Rica. Most of the floricolous *Metschnikowia* species are associated with beetles or *Drosophila* (Lachance et al. 2001d) but *M. reukaufii* and *M. gruessii* were commonly collected from bumblebees (Brysch-Herzberg 2004). *C. kunwiensis*, a recently described anamorphic species associated with bumble bees in Germany (Hong et al. 2003), is also a member of the *Metschnikowia* clade. Although the phylogenetic evidence is suggestive of a functional association among bees and yeasts, experimental data on the interaction are needed.

Data on the functional relationships between yeasts and bees are scarce. One of the methods used to collect yeasts from bees, allowing the bees to deposit yeasts as they walk over the surface of agar plates, provides evidence that the bees vector the yeasts to a new habitat. Exclusion experiments from flowers associated with beetles indicate that insects are obligate vectors for flower yeasts (Lachance et al. 2001d).

However, the walking method does not elucidate where the yeasts are found in the bees, which might help clarify the utility of the yeasts to the bees. Some studies report yeasts from the surface of the insect (Hong et al. 2003), while others have found yeasts in the guts of workers but not larvae (Gilliam 1997; Teixeira et al. 2003). Honeybees can digest yeasts, which has long been an ingredient in artificial bee diets (Peng et al. 1984). Gilliam (1997) found a diversity of yeast species associated with pollen in the flower but noted that fewer species were found in the corbicular pollen (pollen carried in the pollen baskets on the bee's legs) of *Apis mellifera* and only a single species, *C. magnoliae,* in bee bread made from corbicular pollen. She concluded that the bees are using yeasts to process pollen before it is suitable as food. Teixeira et al. (2003) also found a single yeast associated with bee bread, although it was *Starmerella meliponinorum,* a relative of *C. magnoliae*. Rosa et al. (1999b) found a new member of the "bee" clade, *C. batistae*, and an undescribed yeast-like *Mucor* species in the pollen provisions and larvae from nests of two species of solitary bee. The suggestion is that the microbes are part of the nutrition of the larvae but more work needs to be done. Pupae had a lower incidence of yeasts than larvae and adults did not deposit any of the nest species on agar plates, although they did deposit other yeasts, which leaves open the question of how the nests were provisioned with microbes.

There has been little direct work on yeast in bee nutrition. Fermentation has been suspected as the means of transforming pollen into bee bread (Haydak 1958; Gilliam 1997) but it is not clear if yeasts or bacteria (or both) are responsible. The honey and bee bread yeast populations studied by Teixeira et al. (2003) were large enough to suggest that yeast growth had taken place in situ. Pain and Maugenet (1966, cited in Gilliam 1997) found that pollen fermented by *Lactobacillus* alone produced an unacceptable bee bread and suggested that yeasts are a necessary part of the process. At the present, yeasts are implicated in bee nutrition but have not been shown to play an important role.

There may be additional roles for yeasts in bee life histories. Some species of bees are aggressive invaders that displace native bees (Goulson 2003). Interactions between microbes, plants, and bees have not been specifically studied, so it is not known if floral microbes play a role in the invasion or if they are affected by the outcome of the invasion. Only scattered studies suggest that yeast might be involved. There are indications that flower yeasts can affect pollination in some plants (Eisikowitch et al. 1990a, b), suggesting that a change in the floral yeast community can have consequences for organisms that do not directly interact with the yeasts. Different species of bees are associated with different species of yeasts (Teixeira et al. 2003) and there may even be partitioning of the flower into distinct microbial communities (pollen versus nectar) and the bee's body as means of transport (body versus head and proboscis, Hong et al. 2003). Thus, a change might occur in the microbial community when one bee species replaces another. The implications for plant and animal ecology are important and deserve attention.

Yeasts may have negative as well as positive effects on bees. Stressed adult honeybees have increased yeast populations, although it is not known if the increase mitigates or worsens the situation (Gilliam 1973; Gilliam et al. 1974, 1977). Caged honeybees had more yeasts than wild adults. Streptomycin administered in their

diet also increased yeast populations in adult bees, suggesting that bacteria are responsible for keeping yeast population sizes low in adult honeybees. Yeasts were rarely isolated from healthy honeybee broods or queens (Gilliam and Prest 1977, 1987). It does not appear that all bees are stressed by the presence of yeasts. Teixeira et al. (2003) found yeasts in healthy adult *Tetragonisca angustula*, a stingless bee, and in their bee bread, bee glue, and garbage pellets.

Siricid wood wasps have symbiotic, species-specific associations with basidiomycetous fungi in the genus *Amylostereum* (Thomsen and Koch 1999; Vasiliauskas and Stenlid 1999; Slippers et al. 2002). The life history of these wasps is not unlike that of ambrosia beetles (including mycangial transport of the fungi) except that only larvae bore into wood. Presumably, their fungal symbionts have a relationship with the wasp that is similar to that of ambrosia fungi and beetles. Even wasps in the family without mycangia will only lay eggs in wood already infected with the fungus (Fukuda and Hijii 1997). Few yeasts have been isolated from wasps. In addition to *C. davenportii,* described earlier, *C. guilliermondii* has been isolated from fig wasps, *Blastophaga psenes* (summarized in Phaff and Starmer 1987). The wasp introduces the yeast and a bacterial species into figs, where their numbers increase but only to moderate levels (and, hence, do not spoil the fruit for human consumption). The presence of the yeasts did seem to increase the fig's attractiveness to various *Drosophila* species, which, in the process of ovipositing on the fig, brought a diverse yeast community. Eventually, beetles (*Carpophilus* sp.) were also attracted and brought another set of yeasts. The yeast community that developed was typical of that found in many fermenting fruits, including apiculate yeasts, *C. stellata*, and *C. krusei*.

14.9 Other Insects and Invertebrates

A new and interesting group of hemiascomycetous species has recently been described from the surface of nematodes in the genus *Panagrellus* living in beetle galleries, acid rots of grapes, and angiosperm slime fluxes. The isolates all belong to the genus *Ascobotryozyma* or its anamorph, *Botryozyma* (Kerrigan et al. 2004). All species described so far are oligotrophic and have determinate thallus growth. They are dimorphic, growing as yeast in pure culture on agar plates and as thalli on nematodes. The thallus begins with a branched basal cell that acts as a holdfast. A single cell buds from the basal cell's apex and multiple cells can bud from this second cell, with the first being another apical bud and others lateral to this. Three-celled vegetative propagules bud from above the first two apically budded cells. Conjugation can occur between any two cells from different thalli excluding the basal cell. Asci form as lateral buds and produce four lunate spores that are released from the ascus, which remains attached to the cell from which it buds. Strains propagated in the laboratory lose sexual reproduction, whether maintained on nematodes or not (Kerrigan et al. 2001).

Four species are known: *A. americana* on *Panagrellus dubius*, a nematode living in the galleries of the poplar borer, *Saperda calcarata*, in *Populus*; *A. cognata* from the same nematode in *Salix* and *Populus* galleries made by the bark beetles *Cryptorhynchus lapathi* and *S. calcarata*; *B. muscatilis* from *Panagrellus dubius* living

in *Populus* slime fluxes visited by beetles (*Carpophilus lugubris* and *Cryptarcha ampla*) and various Diptera; and *B. nematodophila* from *Panagrellus zymosiphilus* living in grapes and vectored by *Drosophila* (Kerrigan et al. 2001, 2004; Kerrigan and Rogers 2003). The first three are all from the northwestern USA and the last is from northern Italy. Superficially, the life history of *Ascobotryozyma* appears similar to that of the *Laboulbeniales* and its allies (Blackwell 1994). However, systematic data based on rDNA sequences places the *Ascobotryozyma* in the *Hemiascomycetes* (Kerrigan et al. 2004) and the *Laboulbeniales* outside (Blackwell and Jones 1997). This is an interesting case of convergent evolution (the *Trichomycetes* should also be mentioned here; Lichtwardt et al. 1999, 2001) to take advantage of animals as both a place to live and a means to reach fresh habitat.

Ascobotryozyma appears to be a commensal in that it attaches to the outside of the nematode and does not penetrate the cuticle. The nematodes can be successfully maintained with yeast as their only food, but they do not seem to eat the commensal. The nematodes can be covered with the fungi. Beyond observing that nematodes are able to feed and reproduce in culture with yeast attached, nothing is known of the impact the yeast has on the fitness of its host. This will remain a question until we know more about the yeasts, their hosts, and the plant system to which both belong. The role of the insect (beetle, *Drosophila*, or other) in this system may still be prominent. These nematodes require a vector, which may be beetles and *Drosophila* that visit slime fluxes and rotting acidic fruit or the beetles that drill galleries.

14.10 Drosophilids

Wherever *Drosophila* oviposit and feed, yeasts have been found if sampling has been done. Fly substrates include flowers, rotting fruits, mushrooms, soft rots, leaf litter, and tree bark, exudates, and fluxes. (See Sect. 2.5 for a list of some recently isolated *Drosophila*-associated yeasts from flowers.) Some unusual larval substrates have not been sampled for yeasts, such as spider egg cases (Heed 1968) or kleptoparasitic fly hosts (Sivinski et al. 1999). Even in some unsampled situations, there is reason to expect that yeasts are present. Three Drosophilidae, representing separate lineages, lay their eggs on land crabs. *Drosophila carcinophila* oviposits and feeds on the land crab, *Gecarcinus ruricola*, found on islands in the Caribbean basin. *Drosophila endobranchia* oviposits and feeds on *G. ruricola* and *G. lateralis* also in the Caribbean but does not co-occur with *D. carcinophila*. On Christmas Island (the one in the Indian Ocean), *Lissocephala powelli* oviposits on at least four of the resident species of land crab but pupates and feeds elsewhere. At first, it was felt that the flies were attracted to the crab's food, not the crabs themselves, but Carson (1967, 1974) observed that the *Drosophila* adults rarely left the crabs, even when he encouraged them to do so. In addition, when active on the crab, they were clustered near not only the mouth or feeding appendages as expected but also near the gill chambers and nephric grooves. These latter structures are filament-lined grooves in the carapace below the excretory pores. The hairs in the groove are "replete with an abundant, whitish, wet, caseous material which usually showed nematodes as well as mites" (Carson 1967). Both larvae and adult *D. carcinophila* feed on this substance, which might be predicted to

contain at least one species of yeast from its description. Carson felt that the material was microbial and that it might be there as a means of removing toxins from the crab's liquid waste, which exits the excretory pore, flows along the groove, and is (partially) reabsorbed at the end of the groove. Land crabs are adapted to dry conditions and wastewater recycling might be beneficial under such conditions. Larvae of the other fly species also fed in the gill chambers where microbial growth was also observed. It would be of interest to know if yeasts are present in all three cases and if they are also derived from separate lineages.

Except for a single exception (*Drosophila* endosymbiotic yeast, discussed later), yeasts and *Drosophila* have important but diffuse or indeterminate (with respect to the species involved) relationships. Indeed, all of the major *Drosophila* breeding habitats are also home to other animals, including beetles, other Diptera, nematodes, and mites. This does not lessen the importance of the relationship, as there is no evidence that animal-associated yeasts arrive at a new substrate without being vectored there (Gilbert 1980; Fogleman and Foster 1989; Lachance et al. 2001d) and most plant resources are poor-quality nutrition and can be improved for the fly by addition of yeasts (Sang 1978; Begon 1982).

The yeasts associated with *Drosophila* have been most recently reviewed by Begon (1982) and by Phaff and Starmer (1987) and I will briefly summarize their conclusions and concentrate on work done since or not covered in these reviews. Begon (1982) reviewed the qualities of yeasts as food and concluded that yeast species differ in composition and quality and that these differences depend on both the yeast and the environment in which the yeast grows. Laboratory experiments demonstrated that different *Drosophila* species and even genotypes within species grew and eclosed at different rates on different yeasts (Brito da Cunha et al. 1951, and works discussed therein). Begon further concluded that these differences might be the basis upon which the niches of regional *Drosophila* faunas were kept separate (Oakeshott et al. 1989; Morais et al. 1995b).

Supportive evidence for yeasts as a partitioned resource is habitat-dependent. The first samples of yeasts associated with *Drosophila* were yeasts associated with "domestic" species (*D. melanogaster* and *D. simulans*) attracted to fermenting vegetation in human dwellings. These carried in their crops a different set of yeasts from those found in the "wild" flies attracted to fermenting banana baits or captured in the wild. In addition, yeasts isolated from crops of adult wild flies often differed from yeasts found in the rotting vegetation where larvae fed and where it was at first presumed that the adults fed; thus, larval and adult yeast diets seemed to differ (Carson et al. 1956; Begon 1982; Phaff and Starmer 1987). Adult crop contents did not support a significant degree of dietary specialization and did not support resource partitioning. However, this conclusion was based on data from temperate woodland flies. As more habitats were sampled, partitioning gained greater support. Flies that feed on mushrooms, figs, or cactus rots had less variation between larval and adult yeast and each habitat was associated with different sets of yeasts (Phaff et al. 1978; Begon 1982; Starmer 1981b). This was also the case for fruit-breeding African *Drosophila* and Brazilian *Drosophila* (Begon 1982; Rosa et al. 1995), where adult diets corresponded to larval substrate yeast communities.

Pattern analysis of more recent species occurrence data has also supported a role for yeasts in the distribution of co-occurring flies. Fruit-breeding flies exploited a patchy yeast environment in different ways during the succession of yeasts and flies on rotting amapa (*Parahancornia amapa*) in a seasonally flooded (igapo) forest in Brazil, a condition which may have promoted coexistence of the *Drosophila* species (Morais et al. 1995a). Lachance et al. (1995) revisited the association between yeasts and *Drosophila* for temperate woodland flies. They included isolates from several *Drosophila* species in both the western and the eastern USA and based their comparisons on both species presence/absence data and on differences in combined yeast physiological profiles. They were able to distinguish among *Drosophila* species on the basis of either the yeast species isolated from them or the yeast's physiological profiles.

The inverse of the proposition in the preceeding paragraph, that yeast species differentiate their niches on the basis of their associations with *Drosophila* species, is harder to support. Cactophilic yeasts, in general, will grow on tissue from any cactus species (author's unpublished data) but analysis of their distribution supports the notion that cactus chemistry is an important distributional determinant (Starmer 1981a) and adult cactophilic *Drosophila* carry yeasts not found in their larval environment (Ganter et al. 1986). However, factors such as yeast–yeast interactions and yeast–insect interactions cannot be ruled out. Yeast species can have both positive and negative effects on the growth rates and maximal population sizes of co-occurring yeasts (Starmer and Fogleman 1986; Ganter and Starmer 1992). Yeast–yeast interactions are affected by the presence of fly larvae (Starmer and Fogleman 1986) and both may be affected by host tissue composition (Starmer and Aberdeen 1990; Lachance and Pang 1997). Separating these three factors is difficult and it may prove that the interactions among them are as important as any direct effect.

14.10.1 Cactophilic *Drosophila* and Yeast

There has been extensive investigation of the association between yeast and both larval and adult *Drosophila* that inhabit cacti. Cactus-breeding *Drosophila* lay eggs and feed in soft rots of cactus stems or closely associated habitats (most notably, *D. mettleri* breeds in soil soaked by outflow from persistent soft rots in giant columnar cacti such as saguaro, *Carnegia gigantea*). Each species oviposits in a small subset of host plants, often just one (Fellows and Heed 1972). The soft-rot pockets are also home to other insects plus a microbiota of bacteria, molds, and yeasts. Bacteria arrive first through aerial transport and are followed by insect-vectored yeasts about 2 days later (Fogleman and Foster 1989). Yeasts associated with this habitat are mostly specialists in this habitat and tend to be oligotrophic (Heed et al. 1976) but do not come from a single or even a small number of clades (Starmer et al. 2003). The cactus stem–*Drosophila*–yeast community is distinct from surrounding yeast communities associated with *Drosophila* (Ganter et al. 1986; Rosa et al. 1995) and there is almost no overlap among the yeasts found in cactus stems and those in fruits or flowers on the same plant, although insects are found in all three.

Since the first descriptions of cactophilic yeast, the number of species restricted to the cactus soft-rot habitat has steadily increased, often through recognition that cactus isolates had been misidentified owing to the small number of positive physiological tests and their resemblance to known species. The trend through time has been to recognize the cactus isolates as separate species. *P. cactophila,* the most commonly isolated species, was originally identified as *P. membranifaciens*. *P. heedii* and *P. deserticola* are specific to cactus stems. *Starmera* and *Phaffomyces*, small genera once classified as related groups of *Pichia* species, are cactus-specific. Some of what were once though to be regional variants of species are now known to be separate species. Originally, *Sporopachydermia cereana* (as its anamorph *Cr. cereanus*) was isolated from several cacti in the Sonoran Desert. As more collecting was done, variation in physiological profile and morphology led to awareness that more than one species was present. Isolates were referred to for a time as "*S. cereana* complex" members. This complex has yielded ten species up to now (Lachance et al. 2001c) and recent collections may increase the total (Lachance, unpublished data).

Some isolates once identified as cactophilic strains of species that also occur in other habitats are now known to be cactophilic specialists related to species in other habitats. *Candida ingens* from cactus is now known to be *Dipodascus starmeri*. *Hansenula polymorpha* from cactus has been recognized as a new species but it is not yet described (Naumov et al. 1997). *P. kluyveri*, which does occur in both fruit and cactus habitats, has varieties that occur only in cacti (Phaff et al. 1988). There are several unresolved situations. *C. sonorensis* shows surprising genetic variation and some lineages within this asexual species may have diverged enough to deserve specific status. *P. pseudocactophila* is a phenocopy of *P. cactophila* except that is restricted to columnar cacti in the Sonoran Desert. *P. norvegensis* is isolated from cacti in the new world with modest regularity but is rarely found anywhere else. The species was originally described as a rare human pathogen (as an anamorph) and the type strain is from a human vagina. It would not be surprising if the cactus strains were a separate species. Some cactophilic species are found only in particular hosts or are restricted geographically or both (Starmer et al. 1990). For species distributed widely on multiple hosts, the abundance may vary among host types. This variation means that the yeast community of each host differs in its proportional species composition (Starmer et al. 1990; Rosa et al. 1994) and these differences are relatively stable over both distance (Barker et al. 1987) and over time (Latham 1998).

14.10.2 Yeast as Food for *Drosophila*

Drosophila development is affected by the species of yeasts available as food for larvae (Starmer and Aberdeen 1990) and bicultures of yeasts are generally better food than monocultures (Starmer and Fogleman 1986; Starmer and Aberdeen 1990), although the biculture effect is not universal. Biculture effects (on development time or survivorship) were observed only when yeasts grew on their usual host tissue. The effect was clearest for host tissue that was poorest in nutrients and could be erased by supplementing the tissue with yeast extract, suggesting that the biculture effect might be due to nutrient complementation.

Both *Drosophila* larvae and adults prefer to feed on particular yeast species when offered a choice between pure cultures of species (Cooper 1960). Cooper worked

with temperate-forest flies and noted that larvae demonstrated stronger preferences than adults and that larval preferences did not always agree with those of adults. These observations are in accord with the lack of correspondence between yeast species isolated from the adults and those found in the larval breeding sites (discussed earlier). Larvae tended to prefer those species that best supported their development. Recent work has confirmed earlier observations that adult and larval cactus-breeding *Drosophila* have preferences for particular yeast species as food (Morais et al. 1994). Larvae of *D. mojavensis* preferred *P. cactophila* in laboratory tests and this yeast was commoner in their guts than in the cactus rots where they fed (Fogleman et al. 1981, 1982). *P. cactophila* is the most commonly isolated yeast from this habitat but preference and abundance do not always correlate. *C. sonorensis*, the second commonest species, was avoided by the larvae and was underrepresented in their guts (Fogleman et al. 1981).

Because the relationship between cactophilic *Drosophila* and yeasts is diffuse, interactions between any two species may change when other factors change. Data on the development of *D. mojavensis* will illustrate this. *Stenocereus* spp. tissue will not support *D. mojavensis* development although this is the fly's preferred larval habitat throughout most of its range (Starmer 1982b). The addition of yeasts will rectify the problem but the details involve the role of yeasts both as food and as an influence on the larval environment. Yeasts are far richer sources of organic nitrogen than cactus tissue (Starmer and Aberdeen 1990) and contain essential vitamins and lipids for the flies (Sang 1978). But other aspects of yeast activity are also important to the development of the fly. Most of the yeasts characteristic of *Stenocereus* rots are able to utilize uric acid or urea as a nitrogen source. Both waste products are toxic to larvae and the yeasts may improve the environment by lowering waste concentrations, although this has not been tested in the cactophilic community. Cacti differ in their stem chemistry and Starmer (1982b) was able to show that particular yeast species may be beneficial to a species of *Drosophila* under the right conditions. *Stenocereus thurberi* tissue (Organpipe, one of the preferred larval substrates of *D. mojavensis*) contains triterpene glycosides and unusual lipids that can slow both yeast and fly growth. The yeast community characteristic of this host includes yeasts that secrete extracellular lipases (*Dipodascus starmeri* and *P. mexicana*). *D. starmeri* is able to both resist the harmful effect of the lipids and utilize them as a sole carbon source. The lipolytic yeasts support *Drosophila* development both by detoxifying the tissue and by serving as food. Separating the two effects is difficult but it is clear that both effects are present and that rots with lipolytic yeast improve the quality of the habitat for *D. mojavensis* feeding on *S. thurberi*. In other parts of its range, *D. mojavensis* larvae feed on cacti with tissue that has more simple sugars and lacks most of the secondary chemicals present in *Stenocereus* spp. In these hosts, the relationship between lipolytic yeast and the fly is not as important.

Another of the problems in the study of yeasts as food in natural situations is the determination of the relative contribution of different yeast species to fly nutrition when multiple yeast species are present. Markow et al. (2000) have shown that stable isotope ratios (δ^{13}C and δ^{15}N) can be used in combination to identify cactus species and that a change of diet (in this case, yeast substrate and not yeast species) is detectable in the fly over a period of 24 h. If yeast species are distinguishable with

this technique, it might be adapted to allow direct comparison of the contribution of different yeast species to both larval and adult nutrition.

Yeasts may play a significant indirect role in *Drosophila* nutrition through their production and assimilation of low molecular weight volatiles. Some *Drosophila* adults are able to assimilate small volatile molecules directly from the atmosphere (Starmer et al. 1977; van Herrewege and David 1978). A variety of volatiles, including methanol, acetic acid, and acetaldehyde, can produce the effect (Jefferson and Aguirre 1980; Parsons 1980, 1989). This may be especially important for species that have little access to a sugar-rich substrate, such as cactophilic flies (Starmer et al. 1977). Early studies concentrated on longevity experiments in which the adults were exposed to water vapor alone or water supplemented with low concentrations of volatiles (higher concentrations are toxic). These results raised questions about the mechanism by which longevity was increased and the relevance of the effect to the fitness of the flies, although Starmer et al. (1977) observed that the ovarioles were retained when *D. mojavensis* fed on atmospheric ethanol. Etges and Klassen (1989) confirmed that ethanol was a food resource for *D. mojavensis* and that the increase in life span is not due to physiological changes brought on by starvation. Ethanol increased longevity but also increased the metabolic rate and fecundity, effects inconsistent with starvation. Radiolabeling demonstrated that adults incorporate ethanol vapors into their tissues (Etges and Klassen 1989). Volatiles are also implicated in resource location and partitioning (Fogleman 1982; Oakeshott et al. 1982).

Volatile production varies with substrate and microflora, but cactophilic yeasts can produce acetaldehyde, acetic acid, propionic acid, methanol, ethanol, ethyl acetate, isobutyl alcohol, isobutyric acid, *n*-butyric acid, and isoamyl alcohol (Fogleman 1982; Barker et al. 1994). Other volatiles found in cactus rots include acetone and 1-propanol and 2-propanol, possibly owing to untested yeast's abilities or bacterial fermentation (Fogleman 1982; Starmer et al. 1986). Most of these compounds can also be metabolized by one or more cactophilic species. For this reason, interactions among volatiles, yeasts, and *Drosophila* are expected to be complex and have not, at this time, been adequately studied. Starmer et al. found that 2-propanol addition led to larger flies when the yeast present in the system could assimilate the volatile (in this case, *C. sonorensis* and *S. cereana*) but not when the yeast was unable to assimilate it. Interestingly, only *D. mojavensis* feeding on its preferred host, *Stenocereus gummosus*, benefited from 2-propanol. *D. arizonae* and *D. buzzatii* did not benefit but they both fed on *Opuntia* tissue in the experiment, which contains more simple sugar than does *S. gummosus* and this difference may have mitigated potential benefits from the presence of the volatile. Ganter et al. (1989) demonstrated that the longevity of *D. mojavensis* was increased by the presence of methanol and a yeast that could assimilate it, *C. sonorensis*, but there was no effect if other species of yeasts that could not assimilate the volatile were substituted. Yeast was inoculated onto agar supplemented with *S. gummosus* powder (no other nutrients were included) and the flies had access to the yeast during the experiment. Greater longevity was not observed for either *D. melanogaster* or *D. buzzatii*. The former is not a cactus breeder, while *D. buzzatii* is but it does not utilize *S. gummosus*.

Although the relationship between *Drosophila* and yeasts is diffuse, it is similar in some ways to fungal–insect interactions that are more specific. Although some vari-

ation existed, slime-flux breeders in a desert climate (where availability of alternative feeding sited may be limited, Begon 1982) carried yeasts similar to those found in their larval breeding sites (Ganter et al. 1986). For cactus breeding flies, the correlation was even closer (Ganter et al. 1986; Ganter 1988). In both cases, the host plant was of greater importance to the yeast found in the larval substrate than was locality (also see Barker et al. 1983, 1984, 1987). Cactophilic *Drosophila* vector a subset of yeasts present in the breeding site: those that are strong producers of volatiles, principally ethyl acetate (Ganter 1988). These are also the same species that cactus-breeding fly larvae preferred to eat (Fogleman 1982; Fogleman et al. 1982; Vacek et al. 1985). The larval preference may also be linked to production of ethyl acetate or other small volatile molecules by the yeasts (Fogleman 1982). *Drosophila* adults and larvae are found in younger cactus rots, where a yeast flora from other sources is less likely to be present (Starmer 1982a; Starmer et al. 1987; Ganter 1988). The result is that, when cactus-breeding adults arrive at a suitable rot, they deposit both eggs and the yeasts that will provision the rot for their offspring. Although not as species-specific as other instances, this too should be considered a case of insect agriculture (Mueller et al. 1998; Farrell et al. 2001; Mueller and Gerardo 2002).

There is yet another possible role of yeasts in the diet of *Drosophila* that goes beyond simply providing nutrition. Some *Drosophila* larvae and pupae develop more slowly and with greater mortality in the presence of sunlight, a situation which can be improved with the addition of yeasts to their diet (Bruins et al. 1991). The reason for the harmful effect of light is unknown, but may be linked to the production of oxygen radicals when light interacts with porphyrins. The yeasts may supply antioxidants that palliate the effect of the light.

14.10.3 Yeast and *Drosophila* Oviposition

Drosophila oviposition behavior depends on the microbiota found on their larval substrates but the exact form of the preference is linked to habitat and may vary with the successional stage of the rot. Most fruit-breeding *Drosophila* prefer to oviposit on yeast-colonized substrates over those where bacteria or molds predominate (Oakeshott et al. 1989). This is not true of *D. mojavensis,* which prefers to lay eggs on early-stage cactus rots. *D. mojavensis* was attracted to its favorite host tissue only after the addition of bacteria associated with the formation of the rot but was not attracted to mixtures of volatiles characteristic of later rot stages (Fogleman 1982). Not all cactophilic species show an oviposition preference for substrates without yeasts. Vacek et al. (1985) found that adult *D. buzzatii*, a cactus-breeder, preferred to feed and oviposit on the same species of yeast (*C. sonorensis*, *P. cactophila*, *P. barkeri*, *Phaffomyces opuntiae*, and *Clavispora opuntiae*) and, except for *Ph. opuntiae,* these were the most commonly isolated yeasts from their oviposition substrate (*O. stricta*). Bacteria from cactus soft rots were present and were one of the possible choices but were rejected. Oviposition preferences are variable among isolines of *D. buzzatii* and there is heritable genetic variation for the character (*P. cactophila* was once again the most preferred species) (Barker and Starmer 1999). Establishing the cause for such preferences is difficult as yeast–yeast interactions and preferences for yeast change with different combinations of yeast species (Barker 1992; Barker et al. 1994).

14.10.4 Yeast and *Drosophila* reproduction

Many studies of *Drosophila* life history have sought to understand the importance of trade-offs in the evolution of traits such as time to maturity, adult size, lifespan, fecundity, and egg size. An example of this is the potential trade-off in energy and resource allocation between metabolic (growth, maintenance, and storage) and reproductive functions. Yeasts in the diet of flies have been shown to directly affect egg production (by at least an order of magnitude) (Chippindale et al. 1993, 1997; Simmons and Bradley 1997) and to have a direct impact on the importance of such trade-offs for the fly (Leroi et al. 1994).

Male size is often an indicator of mating success for *Drosophil*a (Partridge et al. 1987), including *D. buzzatii* (Santos et al. 1988, 1992). However, the size of axenic *D. buzzatii* males had no effect on their reproductive success (Norry and Vilardi 1996). When the same strains of flies were allowed to feed on live yeast from their larval substrate (*O. vulgaris* soft rots), size-related sexual selection was observed. Males with wider faces were preferred if the face was contaminated with live yeast. Yeasts are transmitted between sexes during courtship (Starmer et al. 1988). The reason for the preference was not obvious so this effect cannot be labeled as a nuptial gift and there was no indication that the effect varied with yeast species. Some *Drosophila* males present their mates with a nuptial gift of a drop of fluid, rich in yeasts, regurgitated from the crop. Nuptial gifts are varied and their function may also be varied (Vahed 1998). Some gifts have potential interactions with associated microbes, such as the gift of stored nitrogen waste products by male Blattid cockroaches. Steele (1986b) found that *Drosophila* in the *obscura* species group produced a drop and, if the drop were of sufficient quality, the female would remain still while feeding long enough for the male to successfully mount her. Females kept on a low-quality diet that were fed a nuptial meal were more fecund than those that had no nuptial meal, so the meal may benefit both parents (Steele 1986a).

Drosophila mate choices can also be affected by the composition of cuticular hydrocarbons, which may function both in selection of a conspecific mate and in species recognition. In a study of both intraspecific and interspecific variation in hydrocarbon makeup, Stennett and Etges (1997) found that food type could significantly alter the composition of the cactus-breeding flies, *D. mojavensis* and *D. arizonae*. The relevance of this finding to this review is that they varied both the substrate and microbial components of the larval food (laboratory food with an uncontrolled microbial flora versus cactus tissue seeded with a subset of yeast species commonly isolated from it). As yeasts can supply insects with a significant portion of their lipid intake, it is not clear which component of the diets was responsible for their results. Yeasts may play an indirect role in mate choice and speciation by supplying the flies with a varied assortment of lipids in their diets.

14.10.5 Yeast Endosymbionts in *Drosophila*

One of the oldest known instances of endosymbiotic yeast involves *Drosophila* and *Coccidiascus legeri* (Chatton 1913; Lushbaugh et al. 1976). This is the only known *Drosophila* endosymbiont, which has been observed to produce ascospores but has

never been cultured. The means of transmission is not known, although, as it resides within gut epithelial cells and *Drosophila* larvae have been observed to feed on the carcasses of adults (Gregg et al. 1990), feeding is thought to play a role. The most basic question is about the nature of the relationship between yeast and fly. It has been found in temperate-woodland *Drosophila* (Ebbert et al. 2003) but it occurs in less than 4% of adults. This is consistent with a parasitic relationship. However, Ebbert et al. (2003) found that, in contrast to another member of the *Drosophila* gut flora (a Trypanosomatid flagellate), the yeast affects its host's life history in potentially beneficial ways. In laboratory experiments, infected fly larvae were more likely to eclose and did so an entire day earlier than uninfected individuals. The mechanism for this is not known but this does suggest a positive effect of the yeast on the fly. Most *Drosophila* biologists assume that flies that linger in the larval stage and expose themselves to predation and mischance are lowering their fitness and so, since no other habitat other than the gut epithelium is known for the yeast, the relationship appears mutualistic. At this time, it is not possible to reconcile the prevalence data with the experimental data.

14.11 Other Diptera

There are scattered reports of yeasts associated with Dipterans other than Drosophilids. Zacchi and Vaughn-Martini (2002) isolated *P. guilliermondii* from the guts of Heteromyzidae larvae that feed on truffles. *C. tropicalis* has been implicated as the causative agent in an outbreak of fermentation spoilage of ripening figs in Brazil. Gomes et al. (2003) believe that the fruit fly *Zaprionus indianus*, a member of the Drosophilidae and recently arrived in Brazil, is the sole vector of this yeast in this system. *Phorbia phrenione* is the principal pollinator of *Epichloë typhina*, a Clavicept endophytic parasite. The fly transfers conidia to a stroma of the opposite mating type before formation of perithecia (Bultman and White 1988). Perhaps haploid sexual yeasts rely on animal dispersal to bring mating types together as a part of their reproductive biology. Cranefly larvae (*Tipula abdominalis*) feed on leaf litter. Their hindgut is enlarged and home to microbes that may be important to the fly's nutrition (Breznak 1982).

14.12 Conclusions

The diversity of yeasts associated with animals may be severely underreported. It is hard to cite evidence that something has not happened, but there are hints that the previous is true from patterns existing in the current literature. Some habitats are understudied in general and when yeasts are reported from them, it is hard to tell how important they might be in that habitat. Yeasts from deep-sea tube worms and clams are an example of this (Nagahama et al. 2003). Collecting effort has not been uniform. The percentage of ambrosia and bark beetle yeasts known from South Africa is unexpectedly high (Table 14.1), perhaps because bark beetle yeast diversity is unusual in South Africa or the systematic search for these yeasts contributed by J.P. van der Walt and associates has not been duplicated in other regions. A similar observation may be made for the yeasts from decaying wood, which seem to reach a

diversity peak in Chile (Table 14.1, most described by C. Ramirez and A. Gonzalez), but this may also be a sampling artifact. Many of the yeasts identified from these two regions have not been collected elsewhere and one can only speculate that other species may fill these niches in other places. There are also more positive indications where habitats have only recently come under systematic scrutiny (the flower- and mushroom-beetle yeast communities) yet have proven to be very speciose and again the species have strong geographic associations. Although small, the nematode-associated *Ascobotryozyma* seems to be a potentially speciose clade, given that all recognized species have been sampled from a small geographic area and from only two species of Nematoda – itself one of the most speciose animal lineages known. One can argue (successfully) that part of the recent increase in yeast diversity is due to the application of molecular systematic techniques as predicted by Kurtzman (in Kurtzman 1994, Kurtzman and Robnett 1998a; and other sources). However, the techniques have thrown light on the diversity, not created it. What should interest us are the ecological and evolutionary processes that have created it. For the yeasts covered in this review, both insect associations and limited distributions seem to encourage diversification. These causative factors can be seen as interdependent because relying on animals as vectors may limit yeast dispersal range even as it increases dispersal success. That geography is important to the description of a yeast species has not always been accepted, but the previously given evidence is convincing that, for at least some habitats, it is essential.

Historically, much interest has been focused on substrate–yeast interactions rather than vector–yeast interactions. It was felt that different substrates would have the major effect on yeast evolution and that the vector's effect would not have much explanatory power. This is obviously not the case for mutualistic, endosymbiotic interactions, but these were seen as exceptional cases and not as central to a general understanding of yeast evolution or ecology. However, evidence is slowly building for the importance of animal–yeast interactions in yeast evolution. There are two important factors so far identified. First is the fundamental importance of dispersal. Animal-vectored yeasts seem to have populated the biosphere as many localized species, closely tied to geography and local conditions. This population structure is much closer to the population structure of the animals that vector them than to microbes with spores or dispersal morphs light enough and resistant enough to depend on air and dust to vector them. The model of wind-dispersed bacteria (Howard et al. 1985), where the genetic variation across a continent can be mirrored in the variation among strains from a single apple, or fungi (Gosselin et al. 1996), simply does not fit many yeasts.

The second factor is the importance of mutualisms that have diffuse, variable memberships rather than mutualisms between specific pairs of species. These mutualisms often involve communities of yeasts and animals. This makes them harder to detect and study but not less important in yeast evolution. An excellent example of the impact of diffuse mutualisms is the cactus–*Drosophila*–yeast system. While cacti are important in yeast evolution, they chiefly supply necrotic tissue to the system. Cactus rots are not initiated by the organisms that live in them (Fogleman and Foster 1989) and there is no evidence at this time that the cacti respond beyond limiting the amount of tissue available to the rot community. The opportunity for

coevolution seems limited. The *Drosophila*, while not the only vector in the system, are probably the most important vectors and are dependent on yeast for their nutrition. Thus, the probability of coevolution between fly and yeast seems more likely than cactus–yeast coevolution. However, at this time, we are much more likely to ask how variation in yeast genotype or phenotype relates to plant–yeast interactions than to animal–yeast interactions. Upon finding that there is variation among regional variants of a yeast species, are the differences more likely to be tied to adaptation to different hosts or to different vectors? Of course, the only answer is that both are vital to the yeast's success and both deserve study.

When is it reasonable to define an interaction as mutualistic? Yeasts can be animal parasites, commensals, or mutualists. The clearest cases for mutualism come from endocytobionts. That there is a cost to the host in terms of resource inherent in the presence of endocytobionts seems incontestable. Thus, the nature of the interaction depends on the benefit-to-cost ratio. Evolutionary costs and benefits must be measured in terms of fitness. What the drain of resources by the microbe costs the host in terms of fitness may be returned to the host in some benefit (production of essential molecules, detoxification, resistance to parasites) that equals or exceeds the cost. Unfortunately, costs are almost always measured in units (calories, millimoles of of nutrient, etc.) different from those used for measuring benefits so that they are not directly comparable. Fitness (or a component if fitness if both cost and benefit affect it) allows direct comparison but it is much more difficult to measure.

The advantage of considering animal–yeast interactions from an evolutionary perspective is that it opens up interesting lines of inquiry. When acquisition of an endosymbiont allows the host to exploit a new niche, there is a strong prima facie case that the relation is mutualistic. Phloem-feeding Homoptera cannot sustain themselves on plant sap without the presence of their endocytobionts. From an evolutionary perspective, questions arise about this relationship based on conflicts between hosts, between endosymbionts, and between host and symbiont. These conflicts complicate assessment of fitness for species involved in an endocytobiosis even when the likelihood of mutualism is high. There are asymmetries that need clarification if the system is to be understood beyond establishing that the presence of the microbe is necessary for exploitation of the niche. Does the host seek to limit the cost of and maximize the benefit from the symbiont cells, as though the host were the owner of the means of production and the endocytobiont only a worker in the factory? Do the symbiont cells cooperate with the host's limitations so that the host's, and therefore the microbe's, fitness is maximized or does conflict between symbiont varieties eliminate cooperation with the host beyond the minimum needed for host success? Once an endocytobiotic relationship is established, is the system open to invasion by other microbes and under what conditions does this lead to coexistence in the host versus replacement of one endocytobiont with another? (Jones et al. 1999; Suh et al. 2001) How are resources allocated in hosts with multiple endocytobionts, such as many Homoptera and Coleoptera? Is the host in charge or do the various endosymbionts compete? Is only one endocytobiont a mutualist while the rest exist as free-loading commensals, supplying only enough benefit to the host to cancel the cost of their presence? Horizontal transfer seems integral to the origins of the relationship between host and microbe and unculturable endocytobionts can still be present in an organism's environment

(Nakabachi et al. 2003) but there are few models for how and under what conditions horizontal transmission is replaced by vertical transmission. For established symbioses, it is necessary to know if horizontal transfer is possible and, if so, how likely must it be before it affects both host and endocytobiont? Considerations such as these make it difficult to assume that symbioses are mutualistic without experimental confirmation but the answers to the questions they pose are necessary if we are to fully understand animal–yeast interactions.

Note Added in Proof

Since completing the submitted draft of this chapter, several new insect-associated yeasts have been described. *Candida leandrae* was collected from a Drosophilid captured on the Hawaiian Islands (the LSU sequence places it in the *Kodamaea* clade near *C. restingae*) (Ruivo et al. 2004). *Metschnikowia hamakuensis, M. kamakouana,* and *M. mauinuiana* are all associated with nitidulid beetles on the same group of islands (Lachance et al. 2005). *Saturnispora hagleri* and *Geotrichum silvicola* were isolated from *Drosophila* captured in Brazil's Atlantic rainforest, although the latter was also isolated from silkworm caterpillars in India (Morais et al. 2005, Pimenta et al. 2005). The same forests also yielded *Candida riodocensis* and *Candida cellae,* two species isolated from bees and belonging to the Starmerella clade (Pimentel et al. 2005). *Komagataella phaffii* has been described from trees and sap fluxes (Kurtzman 2005) but its LSU sequence is identical to the *Pichia pastoris* strains collected from *Drosophila brooksae* by Ganter et al. (1986). Association with sap fluxes and the earlier misidentification argue for this being an insect-associated yeast. The variety of *Candida guilliermondii* isolated from fig wasps as been elevated to specific status as *C. carpophila* (Vaughan-Martini et al. 2005). Based on LSU sequence identity, this species also includes strains previously identified as *C. xestobii*.

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Chapter 15

Yeasts in Extreme Environments

PETER RASPOR AND JURE ZUPAN

Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia (e-mail: peter.raspor@bf.uni-lj.si)

15.1 Introduction: a Definition of Niches Where Yeasts Are Able to Tolerate Harsh Surroundings

As stated by Rothschild and Mancinelli (2001) it is still fact that where there is liquid water on Earth, virtually no matter what the physical conditions, there is life. What we previously thought of as insurmountable physical and chemical barriers to life, we now see as yet another niche harbouring "extremophiles". An organism that thrives in an extreme environment is an extremophile; in more than one extreme it is a polyextremophile.

Extreme environments include different parameters (Rothschild and Mancinelli 2001):

-Physical extremes: temperature, radiation, pressure, gravity or vacuum

-Geochemical extremes: desiccation, salinity, pH, oxygen species or redox potential

It could be argued that extremophiles should include organisms thriving in biological extremes (for example, nutritional extremes, and extremes of population density, parasites and prey). Although all hyperthermophiles are members of the Archaea and Bacteria, eukaryotes are common among the psychrophiles, acidophiles, alkaliphiles, piezophiles, xerophiles and halophiles (which respectively thrive at low temperatures, low pH, high pH, and under extremes of pressure, desiccation and salinity) (Rothschild and Mancinelli 2001).

Although these characterizations seem straightforward, three philosophical issues need further exploration. First, what is "extreme"? Perhaps extreme is in the eyes of the beholder. It is clear that to a thermophile that dies at 21°C and a piezophile that finds atmospheric pressure extreme what determines an extremophily is based on definitions that are perhaps anthropocentric. Second, there are ecological aspects to determine a definition of extremo*tolerant* and extremo*phile* organisms. Besides, there is a very thin line between these two terms. Third, it has to be defined if an extremophile has to show its extreme physiology during all life stages, and under all conditions (Rothschild and Mancinelli 2001).

Life requires an input of energy, but must also be able to control energy flow. Redox chemistry is universal. As life is based on organic chemistry, such chemistry

must be allowed to operate. An extremophile must either live within these parameters, or guard against the outside world in order to maintain these conditions intracellularly (Rothschild and Mancinelli 2001).

Extremophilic microorganisms capable of developing in extreme environments have recently attracted considerable attention because of the challenge that their discovery has posed to our current notions of the limits of life and its possible origin, but, most importantly, because of their biotechnological potential (Boekhout and Phaff 2003; Gónzalez-Toril et al. 2003). In this paper we focus on yeasts that are able to survive in harsh surroundings and are therefore potentially useful in biotechnological applications.

15.1.1 Dry Environments

Water possesses many properties that seem to make it the essential solvent for life. It has high melting and boiling points with a wide temperature range over which it remains liquid, and a high dielectric constant important for its solvent action. Water expands near its freezing point, and it forms hydrogen bonds. No other compound possesses all of these traits. Thus, water limitation is an extreme environment (Rothschild and Mancinelli 2001). Water availability not only depends on the water content of an environment, that is, how moist or dry a solid microbial habitat may be, but is also a function of the concentration of solutes such as salts, sugars, or other substances that are dissolved in water. This is because dissolved substances have an affinity for water, which makes the water associated with solutes unavailable to organisms, so the salt or sugar solution can, in effect, be considered analogous to a dry environment (Madigan et al. 1997).

15.1.1.1 Low Water Activity

Water availability is generally expressed in physical terms such as water activity (a_w). a_w is defined as the ratio of the vapour pressure of the air in equilibrium with a substance or solution to the vapour pressure at the same temperature of pure water (Madigan et al. 1997). So, low a_w value defines an environment where water is in unavailable state for unadapted organisms. Such conditions can be found in freezing areas where water molecules form ice. Low a_w is also a characteristic feature for environments of high salinity [salt lake (a_w=0.75), salt fish, seawater, salami] and for environments of high concentrations of sugars [cereals, candy, dried fruit (a_w=0.7)] (Madigan et al. 1997). Limitation of water in the latter case is not meant in a way of a different state of water, as it is for ice, but the law of osmosis controls its availability.

15.1.1.1.1 Cold Environments

Much of the Earth's surface (over 80% of the total biosphere) experiences fairly low temperatures. The oceans, which make up over half of the Earth's surface, have an average temperature of 5°C, and the depths of the open oceans have constant temperatures of about 1–3°C. Vast land areas of the Arctic and Antarctic are permanently frozen or are unfrozen for only a few weeks in summer (Madigan et al. 1997;

Cavicchioli and Torsten 2000). These natural environments also include cold soils, in and on ice in polar or alpine regions, polar and alpine lakes, and sediments, caves, plants and cold-blooded animals (e.g., Antarctic fish). Artificial sources include many refrigeration appliances and much refrigeration equipment (Cavicchioli and Torsten 2000).

Cold environments have been successfully colonized by numerous organisms, in particular bacteria, yeasts, unicellular algae and fungi (Gerday et al. 2000). Temperature creates a series of challenges, from the structural devastation wrought by ice crystals at one extreme, to the denaturation of biomolecules at the other. At low temperatures with nucleation, water freezes. The resulting ice crystals can rip cell membranes, and solution chemistry stops in the absence of liquid water. Freezing of intracellular water is almost invariably lethal (Rothschild and Mancinelli 2001).

Active microbial growth in extremely cold environments is under the influence of ice formation and consequently of little biologically available liquid water. Thus, water activity (rather than extremely low temperatures) in habitats such as snow, sea ice and glacier ice is the dominant factor in the external chemistry that influences microbial activity. During freezing and binding of water in ice crystals, ions are expelled and the ion concentration in the remaining liquid water increases (Gunde-Cimerman et al. 2003).

The coldest, driest places on Earth are the dry valleys of Antarctica. The primary inhabitants for both hot and cold deserts are cyanobacteria, algae and fungi that live a few millimetres beneath the sandstone rock surface (Rothschild and Mancinelli 2001). Antarctica is a remote continent whose ice-free desert regions are subject to a unique combination of extreme environmental stresses (see Chap. 16). Paramount amongst these is the scarcity of water for the integrity of biomolecules and metabolic activity (biochemical processes) of living organisms. Precipitation is minimal, so transient summer melt water is frequently the sole source of moisture. Precipitation itself is in the form of snow whose ice crystals mainly sublime before they can melt under the influence of solar heating of the substratum. Little moisture enters the microhabitats of these desert ecosystems. There are frequently high concentrations of salts in the scarce melt water, resulting in surface evaporite habitats. These, by their osmotic effects in the microbial microhabitat, make the accessibility of water for metabolism energetically demanding. When desiccation becomes extreme, water replacement molecules, such as the sugar trehalose, are also essential to conserve the ultrastructure of fundamental biomolecules. These include membrane proteins associated with energetic sequences and photosynthetic systems, all of which require spatial organization. They also protect the tertiary structure of enzymes and the nucleic acids, DNA and RNA (Wynn-Williams and Edwards 2000).

In addition to desiccation stress and low-temperature constraints, the Antarctic also has the most extreme UVB flux on the Earth because of the ozone hole. UVB damages a variety of vital biomolecules. Not only does it cause mutation in DNA but it also disrupts proteins, including the lipoproteins of cell membranes and organelles. Experiments on the extraterrestrial environment of low Earth orbit are also valuable for augmenting environmental research in Antarctica, especially the effects of maximal ozone depletion with the biological consequences of its concomitant maximal UVB transmission (Wynn-Williams and Edwards 2000).

15.1.1.1.2 Hypersaline Environments

Organisms live within a range of salinities, from essentially distilled water to saturated salt solutions. Osmophily refers to the osmotic aspects of life at high salt concentrations, especially turgor pressure, cellular dehydration and desiccation. Halophily refers to the ionic requirements for life at high salt concentrations. Although these phenomena are physiologically distinct, they are environmentally linked. Thus, a halophile must cope with osmotic stress (Rothschild and Mancinelli 2001).

The definition of a hypersaline environment is one that possesses a salt concentration greater than that of seawater (3.5% w/v). For water-containing environments, the salt composition depends greatly on the historical development of the habitat, and the environments are normally described as thalassohaline (marine derived) or athalassohaline (the chemical composition is mainly determined by geological, geographical, and topographical parameters, for example, Great Salt Lake in Utah and the Dead Sea). In addition to lakes formed by evaporation in moderate climate conditions, hypersaline Antarctic lakes have been formed from the effects of frost and dryness in this environment. Antarctic and moderate-temperature soils may also contain salinities between 10 and 20% (w/v) (Cavicchioli and Torsten 2000).

15.1.1.1.3 Environments with High Concentrations of Sugars

A typical environment which has extremely high concentrations of sugars is honey. Microorganisms in honey may influence quality or safety. Owing to the natural properties of honey and control measures in the honey industry, honey is a product with minimal types and levels of microbes. Microbes of concern in post-harvest handling are those that are commonly found in honey (i.e., yeasts and spore-forming bacteria), those that indicate the sanitary or commercial quality of honey (i.e., coliforms and yeasts), and those that under certain conditions could cause human illness (Snowdon and Cliverb 1996).

15.1.1.2 Low Humidity

The relative humidity (RH) limit for functioning of the most resistant forms of life is near 70%. Data of the last few years show that the specificity of the role of water in living systems consists not only in its participation in forming the structures of biopolymers and membranes, but also in forming the environment for biochemical reactions. Water because of the complex character of its influence as stabilizing and as loosening factors determines the equilibrium of forces within such structures and its sensitivity to the regulation. The capability of functioning and reproducing as the necessary conditions for the existence of life is determined by requirements of regulation but not of durability or chemical resistance of structures, which have limited character. At low RH the interaction of water with the polar groups plays the basic role, but increase of the RH to 30% and more leads to a new effect – the competition of water for hydrogen bonds within the macromolecules of biopolymers and between the lipid molecules that form such bonds. This is the necessary condition for forming the native biological structures. This also determines the possibility of the enzyme reactions in the solid phase, the efficiency of photooxidation of cytochrome

in the photosynthesis chain and the lability of structures of the proteins and DNA. The next effect is determined by the appearance of the free water with high dielectric constant at a RH more than 60%, which makes weaker charge interactions and leads to lateral mobility within membranes. This water also causes the hydrophobic interaction, which stabilizes the structure of biopolymers and a set of other important processes for life. Any of these factors are necessary for functioning of the living systems that determine the previously mentioned limit of the RH for active life (Aksyonov 2002).

15.1.2 Acidic Environments

pH is defined as $-\log_{10}[H^+]$. Biological processes tend to occur towards the middle range of the pH spectrum, and intracellular and environmental pH often fall in this range (for example, the pH of sea water is approximately 8.2). However, in principle, pH can be high, such as in soda lakes or drying ponds, or as low as 0 ($[H^+]=1$ M) and below. Proteins denature at exceptionally low pH (Rothschild and Mancinelli 2001).

Environments with low pH includes volcanic soils and waters, acid mine drainages, acid soils, vinegar, gastric fluids and fruit juices. (Madigan et al. 1997). The majority of extremely acidic environments are associated with the mining of metals and coal (Cavicchioli and Torsten 2000). Acid-mine drainage water (e.g., effluent of a uranium ore mine) is an example of such an acidic environment and is an interesting model to obtain a better understanding of life in extreme acidic conditions and to elucidate the basis of the survival and role, if any, of acidotolerant yeasts. In these two cases tolerance to metals as well as several physiological parameters at acidic pH are important (de Silóniz et al. 2002b).

The microbial processes that produce the acidic environment are a result of dissimilatory oxidation of sulfide minerals, including iron, copper, lead and zinc sulfides. This process can be written as $Me^{2+}S^{2-}$ (insoluble metal complex)$\rightarrow Me^{2}+SO_4^{2-}$, where Me represents a cation metal. As a result of the extremely low pH in these environments, and due to the geochemistry of the mining sites, cationic metals (e.g., Fe^{2+}, Zn^{2+}, Cu^{2+} and Al^{2+}) and metalloid elements (e.g., arsenic) are solubilized; this process is referred to as microbial ore leaching (Cavicchioli and Torsten 2000).

The Tinto river (Huelva, southwestern Spain) is an environment where the pH remains low and rather constant year-round (mean 2.3), regardless of the temperature or the river flow. This is the consequence of the buffer effect produced by the presence of high concentrations of ferric iron along the river:

$$Fe^{3+}+H_2O \leftrightarrow Fe(OH)^{2+}+H^+$$
$$Fe(OH)^{2+}+H_2O \leftrightarrow Fe(OH)_2^{+}+H^+$$
$$Fe(OH)_2^{+} +H_2O \leftrightarrow Fe(OH)_3+H^+$$

When the river is diluted by tributaries or rain, ferric hydroxide is formed and protons are released, thus maintaining the acidic pH of the system. During the summer, when extremely high temperatures are reached, evaporation is increased, but the pH is maintained constant because the reverse reaction operates: ferric hydroxide precipitates dissolve, consuming protons (Gónzalez-Toril et al. 2003).

15.1.3 Environments with High Concentrations of Metals

Industrialization has long been accepted as a hallmark of civilization. However, the fact remains that industrial emanations have been adversely affecting the environment. Municipal water-treatment facilities in most developing countries, at present, are not equipped to remove traces of heavy metals; consequently every consumer is exposed to unknown quantities of pollutants in the water they consume. The main sources of heavy-metal pollution are mining, milling and surface finishing industries, which discharge a variety of toxic metals such as Cd, Cu, Ni, Co, Zn and Pb. They can be found regularly in municipal wastewaters and, in consequence, in sewage sludge and compost (de Silóniz et al. 2002a; Malik 2004).

15.1.4 Xenobiotic Environments

Xenobiotics are chemically synthesized compounds that have never existed naturally. Some of the most widely distributed xenobiotcs are the pesticides, which are common components of toxic wastes (Madigan et al. 1997). The most important and common pollutants among organochlorine pesticides are dichlorodiphenyltrichloroethane and its metabolites (DDTs), polychlorinated biphenyls (PCBs), hexachlorocyclohexane isomers (HCHs), chlordane-related compounds (CHLs), hexachlorobenzene (HCB), cyclodienes, dieldrin, etc. A great number of industries, such as textile, paper and pulp, printing, iron-steel, coke, petroleum, pesticide, paint, solvent, pharmaceutical and wood-preserving chemical, consume large volumes of water, and organic-based chemicals. These chemicals show a great difference in chemical composition, molecular weight, toxicity, etc. Effluents of these industries may also contain undesired quantities of these pollutants and need to be treated (Aksu 2004).

Another source of environmental pollution by xenobiotics is trinitrotoluene or TNT. Since the environmental conditions in explosive-polluted soils and waters are typically aerobic or microaerobic, the models of TNT transformation can reflect many real situations in such anthropogenic ecological niches where the microbial cells, growing on suitable accompanying substrates, can convert TNT into the early metabolites. The most important aspect of the environmental pollution with TNT and its metabolites is obviously the exposure of wildlife, food animals and humans to the toxic effects of these compounds. Presumably, the hydroxylamino derivatives of TNT, as the most toxic metabolites, may be responsible for the sharp reduction in both microbial numbers and population diversity in the petrochemical waste sludges exposed to TNT (Zaripov et al. 2002).

15.2 Yeasts in Extreme Habitats

It is difficult to determine a good definition of extremophilic yeast, so we shall limit our discussion of extremophiles to those yeasts that require or tolerate extreme environments for growth. Cavicchioli and Torsten (2000) also define an extremophile as an organism that is isolated from an extreme environment and often requires the extreme condition for growth. At the end of this definition they emphasize that,

again, "extreme" is anthropocentrically derived. While many organisms are able to tolerate extreme environments, true extremophiles exhibit optimal growth and function under extreme conditions.

15.2.1 Yeasts in Environments of Low Water Availability

15.2.1.1 Xerophilic Yeasts

Organisms that are able to grow in very dry environments (made dry by lack of water) are called xerophiles. This term is often mixed-up with osmophiles and halophiles, but the osmotic mechanism does not create the possibility to survive in very dry environments, because already at a RH of 50% the retention of water requires a pressure of about 1,000 atm. Investigations are trying to reveal the earlier unknown mechanism for retaining the free water. A study of more than 100 species and strains of yeasts, mosses and lichens by the NMR method has shown that the air-dry cells of species tolerant to water loss retain some of the mobile free water. In most cases the cells contain 1% or few percent of such water but some species of yeasts have been shown to contain about 30% of mobile water of the weight of air-dry biomasses. Such extreme ability is possessed by species of the genus *Cryptococcus* inhabiting high mountain deserts of the East Pamir, where sharp oscillations of temperature and humidity during a day are observed. The retention of water is evidently related to the ability of forming structures that are impermeable for rest water. The cells of *Cryptococcus* are known to have such a mechanism and therefore are able to survive subzero temperatures at night, keep the water collected through the night when the RH rises, and function during the day when temperatures become more suitable (but still extreme) for life. The polysaccharide capsule surrounding cells of *Cryptococcus* inhabiting high mountain deserts helps to keep the water intracellularly, while such a capsule is almost absent in similar species collected from Antarctica, where the diurnal oscillations of temperature do not have such a sharply marked character (Aksyonov 2002).

15.2.1.2 Psychrophilic Yeasts

Little is known about fungal diversity in extremely cold regions. In addition to low nutrient levels, low temperatures induce the formation of ice crystals and therefore also the creation of low water activity (a_w). These are the dominant factors in external chemistry that influence microbial biota in cold regions (Hirsch 1986; Gunde-Cimerman et al. 2003).

Organisms with low-temperature optima are called psychrophiles. A psychrophile can be defined as an organism with an optimal temperature for growth of 15°C, a maximum growth temperature below 20°C, and a minimal temperature for growth at 0°C or lower. Organisms that grow at 0°C but have optima of 20–40°C are called psychrotolerant (Madigan et al. 1997; Cavicchioli and Torsten 2000).

The lower temperature limit for growth of microorganisms is difficult to determine accurately. The problem is primarily a technical one since the amount of antifreeze substance required to be added to the media to prevent freezing is often

inhibitory to growth. Within this limitation, a low-temperature limit of between –5 and –7°C for growth of bacteria and yeasts has been reported (Larkin and Stokes 1968; Arthur and Watson 1976). Psychrophilic yeasts are of the genus *Candida*, and psychrotolerant members are mostly of the genera *Candida*, *Cryptococcus*, *Rhodotorula*, *Hanseniaspora* and *Saccharomyces* (Cavicchioli and Torsten 2000).

It has been reported that fungi can be isolated in concentrations from 6,000 to 7,000 CFU l^{-1} (where CFU is colony forming units) from melted sea ice and up to 13,000 CFU l^{-1} in melted glacier ice, while the majority of species represent yeasts. The dominant taxa are ascomycetous and basidiomycetous yeasts, melanized fungi, mainly represented by the genera *Cladosporium* and *Aureobasidium* plus different species of the genus *Penicillium*. The highest value of colony forming units per litre from melted ice was reported on media for moderate osmophiles – media with 20% glucose (a_w=0.941) and media with 5% NaCl (a_w=0.946). It shows an important connection between the environmental temperature and the a_w value (Gunde-Cimerman et al. 2003).

The most frequently isolated yeast species from Antarctic mosses were of the genus *Cryptococcus* – *Cr. albidus* and *Cr. laurentii* (Tosi et al. 2002). Several other species of the genus *Cryptococcus* were isolated from the Antarctic: *Cr. vishniacii*, *Cr. albidosimilis*, *Cr. antarcticus*, *Cr. friedmannii* and three new representatives of this genus, *Cr. adeliae* and *Cr. adeliensis*, xylanase producing basidiomycetous yeasts (Gomes et al. 2000; Petrescu et al. 2000; Scorzetti et al. 2000), and recently a novel psychrotolerant member of the hymenomycetous yeasts from Antarctica – *Cr. watticus* (Guffogg et al. 2004).

Mrakia frigida (formerly *Leucosporidium frigidum* and *Leucosporidium nivalis*) is classified as an obligate psychrophile since it is unable to grow at temperatures above 20°C (Arthur and Watson 1976; Barnett et al. 2000). *M. frigida*, which does not exist north of 62° S, has been subjected to much longer and more strenuous selective pressure than have other, more widely disseminated psychrophilic microorganisms, leading to adaptation of its metabolism to incessantly low temperatures (the temperature of Antarctic waters ranges from –2.2°C in shelf waters to 4°C in open waters, the average temperature being –1°C), and it can be considered as a representative source of cold enzymes (Turkiewicz et al. 2003). The growth of *M. frigida* at –0.5 and 17°C was essentially the same when either ethanol or glucose was used as the carbon source, except for a shorter lag phase with glucose as the substrate. The maximum cell yield was obtained at subzero temperatures, although the lag phase was considerably greater than at higher temperatures. Rapid growth occurred at 17°C; however, a slight increase in temperature to 18.5°C resulted in decreased growth. Strains of these yeasts have been reported to grow at –7°C (Larkin and Stokes 1968; Arthur and Watson 1976).

Barnett et al. (2000) reported 16 yeast species that are not able to grow at temperatures between 25 and 45°C: *Bullera huiaensis*, *B. hannae*, *Candida psychrophila*, *Cryptococcus aquaticus*, *Cr. consortionis*, *Cr. friedmanii*, *Cystofilobasidium capitatum*, *C. lari-marini*, *Fellomyces horovitziae*, *Leucosporidium antarcticum*, *M. frigida*, *Sporobolomyces coprosmicola*, *S. dracophylli*, *S. ruber*, *Tausonia pamirica* and *Udeniomyces puniceus*.

15.2.1.3 Halophilic Yeasts

Microbial life can be found over the whole range of salt concentrations from freshwater and marine biotopes to hypersaline environments with NaCl concentrations up to saturation. Organisms that require salt from 1–15% NaCl (Madigan et al. 1997) or above 0.2 M salt (Cavicchioli and Torsten 2000) for growth are called halophiles; those capable of growth in very salty environments [15–30% NaCl (Madigan et al. 1997) or 2.0–5.2 M (12–30%) NaCl with the optimum above 3.0 M (18%) NaCl (Cavicchioli and Torsten 2000)] are called extreme halophiles. Halophilic and halotolerant microorganisms are found in all three domains of life: Archaea, Bacteria, and Eucarya. Colonization of hypersaline environments such as salt lakes and salted food products by these microorganisms is often highly successful, and salt-loving and/or salt-tolerant microorganisms may reach high population densities in such ecosystems (Oren 1999).

A characteristic feature of the physiology of *Debaryomyces hansenii* is its resistance to NaCl. *D. hansenii*, a halophile yeast found in shallow sea waters and salty food products, grows optimally in 0.6 M of either NaCl or KCl, accumulating high concentrations of Na^+ or K^+ (Gonzalez-Hernandez et al. 2004). Since this yeast has been described as involved in spoilage, it is particularly important to understand the behaviour of the yeast when a high salt concentration is present together with other stress agents in the food environment. In general Na^+ is toxic to *Saccharomyces cerevisiae* but it improves the growth of *D. hansenii*. This is evident for growth at supraoptimal temperature. In contrast, K^+, a non-toxic cation, does not affect *S. cerevisiae*, but still improves growth of *D. hansenii*. The same pattern was found at high and low pH (Almagro et al. 2000). It has been reported that the presence of salt improves the performance of *D. hansenii* under normal conditions. From the results of work, Almagro et al. (2000) concluded that this improvement is more significant under stress conditions, contributing to protect the cells against those factors.

Gunde-Cimerman et al. (2000) presented data on fungal populations in a crystallization pond during the months of salt production, with salinity in the range 3–35% NaCl. The salterns as the natural ecological habitat of these yeasts represent an extreme and highly changeable environment (Kogej et al. 2004b). Among the black yeasts *Hortaea werneckii*, *Phaeotheca triangularis*, *Trimmatostroma salinum* and dimorphic fungus species *Aureobasidium pullulans* were detected with the highest frequency just before the peak of halite (NaCl) concentration. Since *H. werneckii*, *P triangularis* and *T. salinum* are not known outside saline environments, these results suggest that hypersaline water is their natural ecological niche (Gunde-Cimerman et al. 2000). *H. werneckii* is one of the most salt-tolerant species among all microorganisms and as such a highly appropriate model organism in which to study salt tolerance in eukaryotes (Petrovič et al. 2002).

Hypersaline water not only contains generally osmotolerant fungi, but also truly halophilic fungi. These taxa are all melanized and belong to a single order of the *Ascomycetes*, the *Dothideales*. They all have thick, melanized cell walls, slow, often meristematic growth and proliferation with endoconidiation. A similar morphology is observed with stone-inhabiting fungi, and this can thus be regarded as an

extremophilic ecotype. None of the known species of marine fungi were encountered; these fungi may therefore not be regarded as extremophilic, and belong to quite different orders of *Ascomycota*. Consequently the inhabitants of hypersaline waters are unlikely to have evolved from fungi living in seawater (Gunde-Cimerman et al. 2000).

The phylogenetic and physiological diversity among the halophiles suggest that halophily may have arisen more then once during evolution and is not a rarity. Because data from Mars missions suggest that Mars almost certainly had abundant liquid water on its surface at some time in the past it could have harboured some form of life. As Mars has lost its atmosphere it has not only become cold but also dry owing to water evaporation. As the water evaporated, the dissolved minerals became more concentrated and formed salty brine pockets. Therefore, if there was any life on Mars in water, there should have been some type of osmophile or halophile. Data regarding the survival of halophiles in permafrost, in evaporates and in freeze-thaw cycles suggest that these types of organisms may serve as a terrestrial analogue to the last vestiges of life on Mars (Mancinelli 2004).

15.2.1.4 Osmophilic Yeasts

Organisms able to live in environments high in sugar are called osmophiles (Madigan et al. 1997). Honey is a good example of such an environment; it is essentially water (average 17.2%) suspended in fructose (average 38.4%) and glucose (average 30.3%) (Snowdon and Cliverb 1996). These conditions make honey an appropriate environment for yeasts with osmophilic characteristic. Osmophilic yeasts use the pentosephosphate pathway. The regulation of the pentosephosphate pathway for osmophilic yeasts is not yet known (Burschäpers et al. 2002). They can grow under acidic conditions and are not inhibited by sucrose. Most samples of honey contain detectable levels of yeasts. Moreover, moulds and yeasts are the only microbes that have been reported to grow in honey. Certain bacteria will survive in honey but growth is unlikely. Although yeast counts in many honey samples are below 100 CFU g^{-1} (because of industry control efforts), yeasts can grow in honey to very high numbers (Snowdon and Cliverb 1996). An obligate osmophilic yeast that requires high sugar concentrations (10–20% glucose) for growth was identified in honey as *Saccharomyces bisporus* var. *mellis* (= *Zygosaccharomyces mellis*). Optimum growth for this strain was at 60% glucose (Munitis et al. 1976). There are many other osmophilic yeast genera that can be found in honey: *Debaryomyces*, *Hansenula*, *Lipomyces*, *Nematospora*, *Oosporidium*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces*, *Schwanniomyces*, *Trichosporan*, *Candida*, and *Zygosaccharomyces* (Snowdon and Cliverb 1996).

Osmophilic or sugar-tolerant yeasts are a problem in the honey industry, because they can grow even at the limited level of water available in ripe honey. As a result, osmophilic yeasts readily ferment honey. Standard industry practices control yeast growth. However, honey has antimicrobial properties that discourage the growth or persistence of many microorganisms. Typically, honey can be expected to contain low numbers and a limited variety of microbes (Snowdon and Cliverb 1996).

The osmotolerant yeast *Pichia sorbitophila* was found to differ from other yeast species, not only from the conventional ones (*S. cerevisiae*, *S. pombe*), but also from

those widely known as osmotolerant (*D. hansenii*, *Z. rouxii*). *P. sorbitophila* was able to survive extremely high extracellular concentrations of salts (e.g., saturated solution of KCl) and other osmolytes (70% glucitol), although it is not classified as halophilic (or osmophilic). *P. sorbitophila* assimilated a broad range of carbon and nitrogen sources with extreme effectiveness. On solid media, *P. sorbitophila* created colonies of variable shapes and sizes in relation to media composition, number of colonies on the plate and cultivation conditions. The colonies were able to produce long-distance signals between each other that resulted in growth inhibition of the facing parts of both colonies, but were not inhibited by colonies of other yeast species growing on the same plate. Though sometimes *P. sorbitophila* has been indicated as a synonym of *P. farinosa*, comparative physiological studies together with PCR amplification of *P. farinosa* DNA fragments homologous to known *P. sorbitophila* genes provided a strong indication that this strain should be classified as a separate species (Maresova and Sychrova 2003).

James and Stratford (2003) have studied the osmophilic characteristics and the resistance to preservatives of *Zygosaccharomyces lentus*. Strains of *Z. lentus* grew over a wide range of temperature (4–25°C) and pH 2.2–7.0. Growth at 4°C was significant. All *Z. lentus* strains grew in 60% w/v sugar and, consequently, are osmotolerant. *Z. lentus* strains are resistant to food preservatives. It was confirmed that *Z. lentus* is an important food spoilage organism potentially capable of growth in a wide range of food products, particularly low-pH, high-sugar foods and drinks. It is likely to be more significant than *Z. bailii* in the spoilage of chilled products (Steels et al. 1999). The *Zygosaccharomyces* genus contains some of the most osmotolerant organisms known, and most are capable of growth at very low pH or in environments high in acids; *Z. bailii* is significantly resistant to weak acid preservatives and *Z. rouxii* and *Z. mellis* are known to be extremely osmotolerant. Foods particularly at risk are therefore acidic and contain relatively high levels of sugar. These include fruit juices, soft drinks, juice concentrates and sugar syrups, candied fruit, jams and preserves, honey, tomato sauce, mayonnaise and wines (James and Stratford 2003).

15.2.2 Thermotolerant/Thermophilic Yeasts

This category is not so extreme as are the others, because there are no yeasts adapted to such high, extreme temperatures like species of bacteria and archaea are. Moreover, there are no eukaryotes in environments with temperatures above about 60°C. This most likely involves the stability of organellar membranes, which must remain fairly porous to permit passage of large molecules like ATP and RNA. It is likely that porous membranes such as these would be more temperature-labile than the typical lipid bilayers of prokaryotes (or lipid monolayers of some hyperthermophiles). Thus, above 60°C, the organelles of eukaryotes cannot survive and the only life forms observed are prokaryotes (Madigan et al. 1997). Here again it must be said that this classification of extreme conditions is artificial and was constructed with the eye of the beholder, while temperatures above 40°C (Arthur and Watson 1976) represent a niche for thermotolerant/thermophilic yeasts.

It is clear that the definition of the terms psychrophilic and thermophilic as applied to microorganisms is dependent not only on whether the organism is a

prokaryote or a eukaryote but also on the species under consideration. With the yeasts, the upper temperature limit is close to 46°C, which is, by definition, the lower limit for thermophilic organisms (Arthur and Watson 1976; Madigan et al. 1997).

Yeast *Arxiozyma telluris* (= *Kazachstania telluris*) is classified as an obligate thermophile since it exhibits a narrow growth temperature range of between 28 and 45°C. *A. telluris* showed a maximum cell yield at 37°C when grown on ethanol. At 25°C growth was extremely slow, and at 20°C no growth was observed, thus indicating that this species possesses a more thermophilic character than thermotolerant yeast *Candida parapsilosis*. The upper temperature limit for growth of *A. telluris* was close to 45°C (Arthur and Watson 1976).

Yeast strain Y94T, which is capable of growth at high temperature, was isolated from soil in Korea. Characteristics of the strain include asexual reproduction by multilateral budding, the absence of extracellular starch-like compounds, a negative diazonium blue B colour reaction, and the absence of arthrospores, ballistoconidia and ascospores; the strain can therefore be placed in the genus *Candida*. A maximum growth temperature of 50–51°C, along with certain other physiological characteristics, and a unique 26S ribosomal DNA partial sequence separate this strain from other ascomycetous yeasts. This new species was described as *Candida thermophila* (Shin et al. 2001).

Barnett et al. (2000) reported five yeast species that are clearly (all the tests are marked with +) able to grow to grow at 25, 30, 35, 37, 40, 42 and 45°C: *Arxula adeninivorans*, *C. blankii*, *C. freyschussii*, *Pichia mississippiensis* and *Sporopachydermia lactativora*.

15.2.3 Acidotolerant/Acidophilic Yeasts

Each organism has a pH range within which growth is possible and usually has a well-defined pH optimum. Organisms that live at low pH are called acidophiles. Cavicchioli and Torsten (2000) define acidophiles as organisms that have a pH optimum for growth at or below pH 3. This definition excludes microorganisms that are tolerant to pH below 3, but that have pH optima closer to neutrality. It should be emphasized that despite the requirements of a particular organism for a specific pH for growth, the optimal growth pH represents the pH of the extracellular environment only; the intracellular pH must remain near neutrality in order to prevent destruction of acid-labile macromolecules in the cell. In extreme acidophiles the intracellular pH may vary by 1–1.5 pH units from neutrality (Madigan et al. 1997). Members of *Candida*, *Cryptococcus*, *Hanseniaspora*, *Metschnikowia*, *Rhodotorula, Sporobolomyces* and some others have acidophilic behaviour (BioloMIC 2005).

In general, growth rates decrease with pH. However, remarkable differences were obtained between species. Whereas *S. cerevisiae* was unable to grow at pH lower than 2.5, the isolates (*C. sorbophila* and *Rh. mucilaginosa*) from the effluent grew at pH 2. In addition to pH tolerance, a slight acidophilic behaviour was also observed, especially in *C. sorbophila*. This strain showed an optimum pH value of 2.5–3.0, and was able to grow at pH 2 and pH 4.5 at very similar rates (de Silóniz et al. 2002b). There was a report of another acidophilic yeast species of the genus *Candida*. The natural

habitat of *C. slooffiae* (= *Kazachstania slooffiae*) was reported to be the extremely acid environment of the stomach of domestic animals, in particular the pig and the horse, and it is not difficult to conceive that it had adapted to this extreme environment in such a way as to preclude the necessity for conventional mitochondrial metabolism (Arthur and Watson 1976). *Rh. mucilaginosa* showed the highest rate of growth at pH 3. Apparently, respiration was less affected than growth by acidic pH: even the control strain of *S. cerevisiae* was able to respire at pH 1, a pH value at which it was not able to grow (de Silóniz et al. 2002b).

It is very difficult to define the precise role and importance of each organism as part of a dynamic population in this habitat, especially with respect to non-acidophilic microorganisms. As an approach to this point, mixed cultures of yeast and chemolithotrophic bacteria were utilized, and the effect on ferrous oxidation carried out by the bacteria was studied. Finally, an evaluation was carried out to determine if the excreted metabolites could support the growth of yeasts *Rh. mucilaginosa* and *C. sorbophila*. When the yeasts were inoculated together with the bacteria in the same medium, an unexpected inhibitory and even lethal effect on yeast population was detected. Most probably, the inhibitory agent could be some metabolite excreted to the medium by bacteria, while nutritional competition, oxygen limitation and ferric ion were excluded. This antagonism would explain why yeasts have not usually been isolated from active leaching heaps, but only from the effluents, where the number of such bacteria is usually inferior (de Silóniz et al. 2002b).

The Tinto river is an extreme environment with a constant acidic pH (mean 2.3), a high concentration of heavy metals and a remarkable level of microbial diversity (bacteria, archaea, photosynthetic and heterotrophic protists, yeast and filamentous fungi). The extreme conditions found in the river are the direct consequence of the active metabolism of chemolithotrophic microorganisms thriving in the rich polymetallic sulfides present in high concentrations in the Iberian Pyritic Belt. Some of these microorganisms are important in several processes of biohydrometallurgical interest: acid mine drainage, biomining and bioremediation (Gónzalez-Toril et al. 2003).

15.2.4 Yeasts Resistant to High Concentrations of Metals

Chromium plays an important role in yeast metabolism, where its form and concentration in the microenvironment and the macroenvironment of the yeasts are the crucial factors (Batič and Raspor 1998; Paš et al. 1999, 2004). Batič et al. (1996) tested 35 yeast species for their tolerance to zinc(II) and chromium(III). Both *Yarrowia lipolytica* and *Arthroascus javanensis* were placed in the groups of the highest measured tolerance to both Zn(II) (above 16 mM) and Cr(III) (above 6 mM). Moreover, *Y. lipolytica* showed the highest tolerance to Cr(III) among 35 yeast species (49 yeast strains) tested, but with very high absolute deviation, while *A. javanensis* was the third in the list but had a more reliable result. Most of the strains of the species *S. cerevisiae* were placed in the group of the highest tolerance to Zn(II). Moreover, a *S. cerevisiae* strain was reported to be the most tolerant to Zn(II) among the all yeast species. However, *S. cerevisiae* is often used for metal bioremediation (Malik 2004) and a mathematical model was developed which shows the

behaviour (viability/mortality) of Cr(VI)-tolerant organisms, where *S. cerevisiae* represented this group (Raspor et al. 1999) and *Schizosaccharomyces pombe* (Belagyi et al. 1999; Czakó-Vér et al. 1999). High tolerance to Zn and Cr was also observed in the strains of *S. roseus*, *K. starkey*, *K. thermotolerans*, *Schizoblastosporion starkeyi-henricii* and some others.

Manganese toxicity is the second most important growth-limiting factor (after Al toxicity) in acid soil. The resistance of microorganisms to the toxic effects of manganese ions is an important factor for survival in acid soil. It was confirmed that the acid-tolerant yeast *Rh. glutinis* is also resistant to very high concentrations of aluminium and manganese ions (Nguyen et al. 2001). Another species of this genus, *Rh. mucilaginosa*, isolated from the effluent of the mineral heap, exhibited a higher tolerance than *S. cerevisiae*, except for zinc, for which this *S. cerevisiae* and *Rh. mucilaginosa* presented the same tolerance (de Silóniz et al. 2002b). On the other hand, a process and a system for removal of metals from groundwater or from soil by bioreducing or bioaccumulating the metals (Cr, Mo, Co, Zn, Ni, Ca, Sr, Hg and Cu) using metal-tolerant *S. cerevisiae* have been reported (Krauter and Krauter 2002). Another Cu-resistant strain (*S. cerevisiae* SN41) was found to successfully remove Cu from wine during fermentation (Brandolini et al. 2002).

The bioaccumulation of heavy metals (cadmium, nickel, cobalt and zinc) and the effects of these metals on the production of metallothionein and metallothionein-like proteins (MT) in *Y. lipolytica* were studied by electrochemical methods. The highest concentrations of heavy metals were found in the cell wall and membrane debris, while the lowest concentrations were detected in the cytoplasm. Cadmium and nickel showed the most significant effect on the production of MT. Sensitivity of the yeast to heavy metals can be classified as follows: the first class – zinc – exhibited a weak effect on living cells; the second class – nickel and cobalt – showed a medium effect on yeast cells; the third class – cadmium – is very toxic for cells. Therefore, the tolerance of the yeast to Zn, Co, Ni and Cd decreases as follows: Zn>Co~Ni>> Cd (Strouhal et al. 2003).

15.2.5 Xenobiotic Environments (Man-Made Xenoenvironments)

The organisms that are able to metabolize pesticides and herbicides are fairly diverse, including genera of both bacteria and fungi (Madigan et al. 1997). Yeasts such as *Saccharomyces* sp., *Candida* sp., and *Hansenula* sp. have been reported as capable of transforming a nitroaromatic hydrocarbon, TNT. Comparative toxicological assessment of TNT, isomeric monohydroxylaminodinitrotoluenes (HADNT), and hydride-Meisenheimer complex of TNT (H-TNT) chemical standards revealed that HADNTs are the most toxic compounds. The lowest mortality rates were observed with the supernatants of *Candida* sp. AN-L13, which converts TNT to H-TNT (Zaripov et al. 2002).

Unlike most of these microbes that are of terrestrial origin, the *Y. lipolytica* strain that Jain et al. (2004) have reported is a marine yeast transforming TNT. In addition to the transformation of TNT into products that can be utilized by other microbes, *Y. lipolytica* has the genetic make-up to degrade other environmental pollutants, such as triglycerides and aliphatic hydrocarbons.

Recek and Raspor (1999) reported that *Pichia ciferrii* could be used for reducing the chemical oxygen demand (COD) load of wastewater from ergot alkaloid production. This study is described more in detail in the "Bioremediation" section.

Although yeasts *K. marxianus* and *C. zeylanoides* have been explored in the context of aerobic biological processes for the removal of dyes from textile effluents (Aksu 2004), biochemical oxidation suffers from significant limitations since more dyestuffs found in the commercial market have been intentionally designed to be resistant to aerobic microbial degradation. Therefore, a wide variety of microorganisms including bacteria, fungi and yeasts are used for the biosorption of a broad range of dyes. Textile dyes vary greatly in their chemistries, and therefore their interactions with microorganisms depend on the chemical structure of a particular dye, the specific chemistry of the microbial biomass and characteristics of the dye solution or wastewater. Depending on the dye and the species of yeast used different binding capacities have been observed (Table 15.1).

15.2.6 Combined Extreme Environments

15.2.6.1 Low a_w – a Connection Between Cold and Hypersaline Environments

Halophilic and xerophilic fungi have been isolated from rocks and natural hypersaline environments. It can be speculated whether these species share some features with those isolated from ice. Whereas salinity creates both ionic and osmotic stress drought, low temperature per se cause osmotic stress. Freezing leads to cellular dehydration owing to reduced water absorption and conduction, whereas high salinity causes the same effects owing to osmotic imbalances. Compatible solutes are known to accumulate in response to certain physical stresses, such as desiccation and high salinity, but have so far been ignored with regard to protection against freezing temperatures. Since protection against dehydration damage is correlated with intracellular accumulation of compatible solutes, an increase in the amount of

Table 15.1 Data on the biosorption of dyes by various yeasts (Aksu 2004)

Yeast	Biosorption of dye
Candida spp.: *C. lipolytica*, *C. membranifaciens*, *C. guilliermondii*, *C. tropicalis*, *C. utilis*	Remazol Blue
Candida rugosa	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Cryptococcuss heveanensis	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Dekkera bruxellensis	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Kluyveromyces marxianus	Remazol Black B, Remazol Turquoise Blue, Remazol Red, Remazol Golden Yellow, Cibacron Orange, Remazol Blue
Kluyveromyces waltii	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Pichia carsonii	Reactive Blue 19, Reactive Black 5, Sulfur Black 1
Saccharomyces cerevisiae	Remazol Blue, Reactive Blue 19
Schizosaccharomyces pombe	Remazol Blue

non-freezable water may arise through the production of such solutes as low molecular weight sugar alcohols (polyols) or sugars (trehalose) (Gunde-Cimerman et al. 2003). Presumably low temperature and the presence of the salts are antagonistic in their effects on the synthesis of appropriate lipids for adaptation to each of these situations and this was clearly shown in the study of the effects of salts on *D. hansenii* and *S. cerevisiae* under stress conditions (Almagro et al. 2000).

Gunde-Cimerman et al. (2003) confirmed in their study that by using low-water-activity media for the isolations of halophilic/xerophilic fungi from hypersaline waters, higher CFU numbers than previously reported from ice samples can be expected to be obtained. This is also supported by the fact that the main groups of isolated fungi that were isolated in Kongsfjorden appear as well in solar salterns of the Mediterranean coast (Gunde-Cimerman et al. 2000).

A high proportion of melanin-producing microorganisms are known to be associated with environmentally stressed areas such as hot and cold deserts, alpine regions and the upper biosphere. In addition, melanins are known to confer protection to UV radiation, temperature extremes and desiccation, and they also provide an osmotic role (Kogej et al. 2004b). Some yeast species associated with extreme environments also produce pigments other than melanin. *Rhodotorula*, *Rhodosporidium*, *Cryptococcus*, *Cystofilobasidium*, *Sporidiobolus* and *Sporobolomyces* form red, orange and pink carotenoid pigments that do not diffuse into agar, while *Metschnikowia pulcherrima* forms diffusible pigment pullcherim (Bab'eva and Reshetova 1997; Raspor et al. 2000b).

Considering a low *a*w, made by a high concentration of salts or sugars, Barnett et al. (2000) reported 25 yeast species that are able to grow at 10 and 16% NaCl and 50 and 60% glucose (with fully positive results for these tests). The majority of the species belong to the genus *Candida* while the others are *A. adeninivorans*, *A. terrestris*, *D. melissophilus*, *D. nepalensis*, *D. robertsiae*, *D. udenii*, *P. acaciae*, *P. triangularis*, *Rh. acuta*, *Sterigmatomyces elviae* and *Sympodiomycopsis paphiopedili*.

15.2.6.2 Deep Igneous Rock Aquifers – High Salts, High Pressure, Anaerobic Environment

The diversity of prokaryotes in the groundwater deep below the surface of the Baltic Sea at the Äspö Hard Rock Laboratory (HRL) in southeast Sweden is well documented. In addition, there is some evidence that eukaryotes, too, are present in the deep groundwater at this site, although their origins are uncertain. It is interesting that all the eukaryotic strains isolated from Äspö groundwater between 201 and 444 m below sea level were fungi. Yeast strains isolated were most likely strains of the identified species *Rh. minuta* and of a new species of *Cryptococcus*. In addition, cultures related to *Rhodotorula* and *Cryptococcus*, which were also isolated in this study, were found in the deep-sea environment and even in sea-floor sediments. Scanning and transmission electron microscopy demonstrated that the strains possessed morphological characteristics typical for yeast, although they were relatively small, with an average length of 3 μm. Enumeration through direct counting and most probable number methods showed low numbers of fungi, between 0.01 and 1 cells ml^{-1}, at some sites. Studies revealed that the strains grew within a pH range of 4–10, between temperatures of 4 and 25–30°C, and

in NaCl concentrations from 0 to 70 g l^{-1}. These growth parameters suggest a degree of adaptation to the groundwater at Äspö HRL. Despite the fact that these eukaryotic microorganisms may be transient members of the deep biosphere microbial community, many of the observations of this study suggest that they are capable of growing in this extreme environment (Ekendahl et. al. 2003).

Red yeasts are commonest among yeasts isolated from the deep sea. Nagahama et al. (2001) isolated 99 yeast strains, including 40 red yeasts, from benthic animals and sediments collected from the deep-sea floor in various areas in the northwest Pacific Ocean. Comparing the yeast isolates from animals and sediments collected from shallow locations, they found the proportion of red yeasts differed considerably, comprising 81.5 and 10.6% of the isolates from animals and sediments, respectively. All of the red yeast isolates belonged to the genera *Rhodotorula* and *Sporobolomyces*. On the basis of morphological and physiological characteristics, the isolates were identified as *Rh. aurantiaca*, *Rh. glutinis*, *Rh. minuta* and *Rh. mucilaginosa* of the genus *Rhodotorula*, and *S. salmonicolor* and *S. shibatanus* of the genus *Sporobolomyces*. Only *Rh. glutinis* and *Rh. mucilaginosa* were isolated from sediments. All of the others were isolated from animal sources. Some strains assigned to known species on the basis of phenotypic features should be regarded as new species as suggested by the results of molecular analysis. Yeast isolates having ascomycetous affinities included members of the genera *Candida*, *Debaryomyces*, *Kluyveromyces*, *Saccharomyces* and *Williopsis*. Basidiomycetous yeasts were more frequently isolated from sediments from deeper regions (64.0%) than from sediments collected at depths of less than 2,000 m (there were no basidiomycetous yeasts other than *Rhodotorula*). This may be due to a difference in the amount of organic debris in the sediments or the difference in hydrostatic pressure at the sampling points. Red yeasts comprise a higher proportion of total yeasts in clean water than in polluted water. The other basidiomycetous yeasts isolated from sediments of deeper regions were members of the genus *Cryptococcus* (Nagahama et al. 2001).

The proportions of basidiomycetous yeasts and red yeasts among the total yeast isolates from benthic organisms were 88.5 and 80.8%, much larger than in the case of yeasts from sediments. The differences in the proportion of red yeasts recovered from the animals and the sediments collected from almost the same locations and depths suggest that the occurrence of red yeasts in the deep sea is strongly limited by the nutrient conditions of their habitats. Microbes associated with macroorganisms in deep-sea environments presumably are exposed to favourable conditions with a stable source of nutrients, whereas in deep-sea sediments there is less organic debris available to be utilized by yeasts than in sediments in shallow regions. The other basidiomycetous yeasts isolated from the clams were members of the genera *Cryptococcus* and *Pseudozyma* (Nagahama et al. 2001) (see also Chap. 12).

A large number of yeasts can utilize hydrocarbon compounds as sole sources of carbon and energy, for example, species of *Rhodotorula* and *Candida*. However, fungi must outcompete bacteria, a large obstacle, because prokaryotes are extremely successful in this environment. One may suspect that even though carbon sources should not be limiting, selective advantages such as secretion of digestive enzymes may prove essential for yeasts to survive in the deep biosphere. Perhaps the largest problem for subsurface yeasts is the absence of oxygen in the groundwater. If the

Äspö strains are aerobic, it may be that they produce spores under oxygen-limiting conditions, an occurrence which should be favoured under stressful environmental conditions. Therefore, oxygen limitation does not exclude the existence of yeasts in the deep subsurface but may curb their ability to grow (Ekendahl et al. 2003).

15.2.6.3 Association of Bacteria and Yeasts in Extreme Environments

Rikhvanov et al. (1999) studied the association of bacteria and yeasts in hot springs and they indicated that yeasts may exist in hot springs in association with bacteria and that this may influence the resistance of the yeasts to extreme temperatures. The thermophilic bacterium *Bacillus* sp. was isolated in association with the yeast *D. vanrijiae* from hot springs at 46°C. It was shown that the bacterium excreted thiamine into the culture broth, which not only promoted *D. vanrijiae* growth in mixed culture but also increased the maximal temperature for yeast growth.

15.2.7 A Competition for the Title

Some species of genera *Arxula*, *Candida*, *Cryptococcus* and *Rhodotorula* can be clearly marked as "extreme yeasts". These genera seem to be the most adapted yeast taxa to extreme environments. *A. adeniniovorans* is able to grow at high temperatures (45°C) and low a_w values (growth at 16% NaCl and at 60% glucose) (Barnett et al. 2000). Strains of the genus *Rhodotorula* have been reported from deep igneous rock aquifers – an environment of high salts, high pressure and anaerobiosis (Ekendahl et. al. 2003) – from sediments collected at depths of less than 2,000 m, where there were no basidiomycetous yeasts other than *Rhodotorula* (Nagahama et al. 2001), from honey (a_w=0.65) (Snowdon and Cliverb 1996) and a strain was reported to be resistant to high concentrations of metals (Nguyen et al. 2001; de Silóniz et al. 2002b). Moreover, red yeast isolated from the acidic water of Kusatsu hot spring could grow in an acidic medium of pH 1.5 and was identified as *Rh. glutinis*. The acid-tolerant yeast also showed strong resistance to both aluminium and manganese ions (Nguyen et al. 2001).

Such extreme features can also be found among the species of the the genus *Cryptococcus*. The most frequently isolated yeast species from Antarctic mosses were of the genus *Cryptococcus* (Scorzetti et al. 2000; Tosi et al. 2002; Guffogg et al. 2004): species of this genus inhabit high mountain deserts of the East Pamir – an environment of sharp oscillations of temperature and humidity during the day (Aksyonov 2002), they are capable of biosorption of dyes (Aksu 2004) and were isolated from deep-sea environment like species of the genus *Rhodotorula* mentioned before (Nagahama et al. 2001; Ekendahl et. al. 2003).

15.3 Mechanisms of Yeast Stress Responses and Tolerance to Harsh Conditions – Modes of Survival in Extreme Environments

It is critical for an organism to maintain function, and the easiest approach to achieve this is to keep the external environment out. If it is impossible to keep the environment out, evolutionary responses entail protective mechanisms, altering

physiology or enhancing repair capabilities. Research has focused so far on three key classes of biomolecules: nucleic acids, membrane lipids and proteins. For nucleic acids, function and structure are linked inextricably. DNA is especially vulnerable to high temperature, radiation, oxidative damage and desiccation. This can lead either to convergence or to multiple ways to solve the problem of living in a particular environment. Understanding the alternatives by using extremophiles on Earth as a sample should help us understand evolutionary processes on Earth, predict them elsewhere, and be useful in commercial exploitation of extremophiles (Rothschild and Mancinelli 2001) (See also Chap. 9).

15.3.1 Adaptation to Low Water Activity

15.3.1.1 Compatible Solutes

When an organism grows in a medium with a low water activity, it can obtain water from its environment only by increasing its internal solute concentration. An increase in internal solute concentration can be accomplished by either pumping inorganic ions into the cell from the environment or synthesizing or concentrating an organic solute. The solute used inside the cell for adjustment of cytoplasmic water activity must be non-inhibitory to biochemical processes within the cell; such compounds are called compatible solutes. The concentration of compatible solutes in the cell is a function of the level of external solutes, and in each organism the maximal amount of compatible solute(s) made or that can be accumulated is a genetically directed characteristic; this results in different organisms tolerating different ranges of water potential (Madigan et al. 1997). Compatible solutes detected in halophilic and halotolerant microorganisms include polyols such as glycerol and arabitol, sugars and sugar derivatives (sucrose, trehalose, glucosylglycerol), amino acids and derivatives, and quaternary amines such as glycine betaine. Compatible solutes are typically low molecular weight compounds, soluble at high concentrations in water, and either uncharged or zwitterionic at the physiological pH (Oren 1999).

The energetic cost of producing organic osmotic solutes is huge. An organism living in 4 M salt (by no means the highest salt concentration allowing the growth of many species that exclude salt from their cytoplasm) may contain close to 8 M organic solute in its cytoplasm to maintain osmotic equilibrium, with the exact value depending on the activity coefficients of the ions and the organic solutes involved. It is thus clear that the strategy of accumulating osmotic solutes is energetically very costly, the more so when larger organic molecules such as disaccharides (12 carbon atoms) are to provide osmotic balance. The use of smaller molecules such as glycerol (three carbons) or glycine betaine or ectoine (five and six carbons, respectively) is less energy consuming. The amount of energy needed for the production of the osmotic solutes may thus greatly exceed the energy requirement for the biosynthesis of proteins, nucleic acids, cell walls, etc. The total amount of glucose required for the formation of structural cell material may be thus 4.4 times as much as for a non-halophilic microorganism (Oren 1999).

It is obvious that of all known compatible solutes, glycerol is the simplest and energetically cheapest to produce. Its solubility in molar terms exceeds that of all

other solutes, since it is miscible with water at all ratios. Moreover, even at extremely high concentrations (4 M and more) it still supports excellent activity of intracellular enzymes. Most biological membranes are highly permeable to glycerol, and therefore a specially adapted membrane structure is needed to keep the glycerol produced within the cell. Glycerol seems to be restricted to the domain Eucarya, in particular to yeasts (Oren 1999).

Xerophilic yeasts produce mainly glycerol as a compatible solute (Madigan et al. 1997). In studies of a response to environmental changes in temperature (30–44°C) and osmotic pressure (resulting from concentrations of NaCl in the media ranging from 0 to 1 M NaCl) accumulation of trehalose and glycerol production in *S. cerevisiae* was observed (Carvalheiro et al. 1999). NaCl was found to be more important than temperature in glycerol production but the combination of the two factors still encloses a considerable synergistic effect. NaCl contributes with an effect fivefold that of temperature on both responses. Increase in trehalose concentrations can be regarded as part of thermotolerance and osmotolerance mechanisms. It was shown that both temperature and NaCl contributed in an interactive manner towards the accumulation of trehalose. The presence of both factors leads to a more effective synthesis of trehalose than each one per se. In fact, independent of the type of shock, the ratio between the parameters of produced glycerol and the parameters of the intracellular trehalose concentration for each stress type was similar (Carvalheiro et al. 1999).

The evolution of the denomination of these compounds went from osmotic effectors or osmolytes to compensatory compounds. These compounds osmotically compensate for the decrease in ion concentration. Their increase in concentration thus permits the maintenance of the ion concentration at a steady level, avoiding the disrupting effects of their increase in amount on cell macromolecular structures. In this regulation system, the organic solutes would "chemically compensate" for the disrupting effects of ions on macromolecular structures in the initial transient stress conditions. After acclimation, they would osmotically compensate for the osmotic gap left by maintenance of the ions at their normal level. In short, a major characteristic of the compensatory solutes is that they are preferentially excluded from the protein surface and its immediate hydration sphere. This exclusion appears to stabilize folded protein structures. It also promotes subunit assembly and salting-out of protein (Gilles 1997).

To sum up these studies, it is actually clear that the control of the level of the different compensatory solutes cannot be related to a single major mechanism. It appears that changes in the activity of different processes – catabolism, de novo synthesis, efflux, influx, equilibrium between macromolecular components and their constitutive organic solutes – can be implicated depending on the solute considered and on the conditions in which the cells are. Often also, control of the amount of a solute implicates more than one of these processes. A priori, control can be exerted in two different ways: directly, by one or several "modulating factors" acting on target systems – transporters, channels and/or enzymes (such an "allosteric" modulation would lead to a change in the kinetics characteristics of the target system), or indirectly, by regulation of expression of genes coding for the transporters, channels and/or enzymes implicated. In this case, there would be a change in the concentration

and/or in the kinetics characteristics of the target system. Also one system is not a priori exclusive of the other (Gilles 1997).

15.3.1.2 Cold Shock and Cold Acclimation

Despite the strong negative effect of low temperatures on biochemical reactions, these organisms breed, grow and move at rates similar to those achieved by closely related species living in temperate environments. They have therefore developed various adaptations in the form of finely tuned structural changes at the level of, for example, their membranes, constitutive proteins and enzymes, enabling them to compensate for the deleterious effects of low temperature (Gerday et al. 2000).

The yeast cell with its wealth of membrane organization, together with its rapid growth, reproduction, and relative genetic simplicity, is an attractive system in which to study the mechanism of thermal adaptation in eucaryotic microorganisms (Arthur and Watson 1976).

The factors controlling an organism's minimum growth temperatures are not clear. The cytoplasmic membrane must be in a fluid state for proper functioning. There are indications that the minimum temperature of an organism results from "freezing" of the cytoplasmic membrane so it no longer functions properly in nutrient transport. This explanation is supported by experiments in which the minimum temperature for an organism is altered to some extent by adjustments in membrane lipid composition. Studies on the composition of cytoplasmic membranes from psychrophiles have shown them to contain a higher content of unsaturated fatty acids, which help to maintain a semifluid state of the membrane at low temperatures (Madigan et al. 1997). Marked variations in both phospholipid composition and the degree of fatty acid unsaturation of cold- and warm-adapted yeasts indicate that the ability to alter these cellular components may constitute an adaptation mechanism to suit a particular environment. Considerable interspecific variations in phospholipid distributions were observed in the strains examined. *M. frigida* was chosen as representative of the psychrophilic strains and was found to contain a greater amount of phosphatidyl ethanolamine than did any of the other species. The formation of hydrogen bonds between the amino groups of phosphatidyl ethanolamine and the polar water molecules may serve to retard ice formation at subzero temperatures (Arthur and Watson 1976).

The extent of such ability may be an important controlling factor which determines the growth temperature limits of the microorganisms, the lower limit being that at which the membrane lipids solidify and the upper limit being that at which the membrane lipids melt. The membrane lipid composition of the different yeasts conforms well to this concept. There is a direct correlation between temperature adaptation and the degree of membrane lipid unsaturation: the lower the temperature, the greater the degree of fatty acid unsaturation. The high C18:2 (melting point, −5°C) and C18:3 (melting point, −11°C) content of the psychrophilic yeasts would permit the cell membranes to remain in a sufficiently fluid state to allow unimpaired functioning of metabolic processes even at subzero temperatures. On the basis of the melting points of the major fatty acids present, it could be proposed that the psychrophilic yeasts would be able to grow at temperatures as low at −10°C

(Arthur and Watson 1976). Also the concentration of dissolved oxygen may be particularly important in regulating the degree of lipid unsaturation at various temperatures since (1) the solubility of oxygen decreases with temperature, and (2) oxygen is obligatory in reactions involving the fatty acid desaturase enzymes of yeasts (Arthur and Watson 1976; Cavicchioli and Torsten 2000).

In studies of stress tolerance Deegenaars and Watson (1997) identified the presence of heat-shock proteins (HSPs) 90, 70 and 60 in protein extracts from *C. psychrophila*. These HSPs seem to be involved in adaptative reactions to thermal stress changes. However, the absence of strong induction of these HSPs upon a heat shock in this study argues against their role in the heat-shock-induced thermotolerance observed in the Antarctic psychrophilic yeast. On the other hand, the psychrophilic yeast showed the presence of a strongly heat shock inducible protein of about 110k Da. It may well be that this protein plays a role in thermotolerance in psychrophilic yeasts, similar to that of HSP104 in mesophilic species. In *S. cerevisiae*, temperature-shock-inducible protein 1 (TIP1) is a major cold-shock protein; it is targeted to the outside of the plasma membrane and appears to be heavily glycosylated with *O*-mannose, therefore invoking a role for TIP1 in membrane protection during low-temperature adaptation (Cavicchioli and Torsten 2000).

Psychrophiles produce enzymes that function optimally in the cold and that are often denaturated or otherwise inactivated at even very moderate temperatures (Madigan et al. 1997). Clearly, the strategy of adaptation is unique to each enzyme. Decreased levels of prolyl and arginyl residues and increased levels of glycyl residues could be involved (Gerday et al. 2000). The presence of a highly polar motif within the enzyme's N-terminal fragment can be considered as an indirect proof of the structural flexibility of LAP2 (the first reported extracellular subtilase of a psychrophilic yeast *Leucosporidium*), and this motif has not been detected in molecules of mesophilic representatives of the subfamily C of the subtilase clan (Turkiewicz et al. 2003). The stability of the cold-adapted enzyme can also be lowered by a general weakening of the intramolecular interactions, increased interactions with the solvent, weakening of the interdomain or intersubunit interactions and a decrease in the cation or anion interactions. All of these factors give rise to an increase in the overall or local flexibility of the molecular structure. A better accessibility of the catalytic cavity can also improve the accommodation of the substrate, giving rise to a higher specific activity at low temperatures (Gerday et al. 2000).

It was said that the membrane system and well-adapted proteins are crucial for surviving in very cold or very hot environments, but there was a report of a yeast species of the genus *Cryptococcus* which can survive in high mountain deserts of the East Pamir, where sharp oscillations of temperature and humidity during the day are observed (Aksyonov 2002). Therefore, the organism must act as a psychrophile and a thermophile in a period of 1 day. Adaptation through saturation and unsaturation of cell membrane phospholipids is therefore less likely, so other adaptation mechanisms must be involved. Characteristic for the genus *Cryptococcus* is a wide range of consumable carbon compounds (Fell and Statzell-Tallman 1998), formation of chlamidospores and very thick capsules, which protect against extreme environmental influences (Chernov 1997). These are the features that can assure survival in such harsh surroundings.

15.3.1.3 Resistance to High Salinity

Progressive salinization of irrigated land is one of the main problems for agriculture worldwide. This trend necessitates research into the development of genetically engineered crop plants with greater salt tolerance. An important step in this direction is to understand the mechanisms of salt tolerance in those eukaryotic organisms that are salt-tolerant by nature.

Two fundamentally different strategies exist within the microbial world that enable microorganisms to cope with the osmotic stress inherent to the presence of high salt concentrations.

1. Cells may maintain high intracellular salt concentrations, osmotically at least equivalent to the external concentrations (the "salt-in" strategy). All intracellular systems should then be adapted to the presence of high salt concentrations. This option is used by a few specialized groups of prokaryotes only.
2. (ii) Cells may maintain low salt concentrations within their cytoplasm (the "compatible-solute" strategy). The osmotic pressure of the medium is then balanced by organic compatible solutes. No special adaptation of the intracellular systems is required (Oren 1999).

Since biological membranes are permeable to water, cells cannot maintain a water activity of their cytoplasm higher than that of the surrounding brine, because this would lead to a rapid loss of water to the environment. Therefore, any microorganism living at high salt concentrations may be expected to keep its cytoplasm at least isoosmotic with the extracellular environment. Build-up of a turgor pressure requires a hyperosmotic cytoplasm. With the possible exception of the halophilic archaea of the order *Halobacteriales*, all halophilic microorganisms maintain a turgor pressure (Oren 1999).

Salt tolerance in eukaryotic microorganisms has been mainly studied in the yeast *S. cerevisiae*. This is probably not the best model organism since it is a rather salt-sensitive yeast which tolerates only moderate salt concentrations. To understand the mechanisms involved in salt tolerance, *D. hansenii* has been used as an eukaryotic model. Although Almagro et al. (2001) reported the existence of Na^+ efflux systems in *D. hansenii* – Ena proteins which seem to play an important role in maintaining balanced levels of intracellular cations, ensuring the ionic homeostasis of the cell – it has been shown that *D. hansenii* grows optimally in 0.6 M of either NaCl or KCl, accumulating high concentrations of Na^+ or K^+. In contrast to the statement that the "salt-in" strategy is restricted to a few specialized groups of prokaryotes only (Oren 1999), it can be concluded that Na^+ in *D. hansenii* is not excluded, but, instead, its metabolic systems must be resistant to high salt concentrations (Gonzalez-Hernandez et al. 2004). Moreover, it was shown that Na^+ even improves the performance of *D. hansenii* under different stress conditions (Almagro et al. 2000). Presumably that tolerance to salt can be conferred by gene transference; a proteomic approach to the study of salt stress responses in *D. hansenii* has been started and spots specifically regulated by the presence of high salt in the growth medium have been identified. It is planned to evaluate new unknown mechanisms together with the contribution of well-known processes involved in salt tolerance: production and

accumulation of compatible solutes, ion transport and enzyme sensitivity (Ramos 2004).

H. werneckii was isolated from solar salterns as one of the predominant species of a group of halophilic and halotolerant melanized yeast-like fungi. It can grow at salinities ranging from 0% to saturated solution of NaCl. As a response to high salinity, it activates a high osmolarity glycerol (HOG) signalling pathway that results in activation of glycerol-3-phosphate dehydrogenase gene expression and consequently in cytosolic glycerol accumulation (Petrovic et al. 2002; Lenassi et al. 2004). In a study of the cation quantities in the halophilic *H. werneckii* and halotolerant *A. pullulans,* Kogej et al. (2004a) showed that the sodium and potassium contents were relatively low in fully adapted cells of *H. werneckii* and *A. pullulans* in comparison to those for *D. hansenii*, which is known to accumulate high concentrations of Na^+ or K^+ in the cell (Gonzalez-Hernandez et al. 2004). These results show that accumulation of sodium and potassium ions does not contribute to osmoadaptation of *H. werneckii* or *A. pullulans*. The accumulation of glycerol seems to be more significant for their survival in a hypersaline environment. Kogej et al. (2004a) assume these yeasts have efficient ion pumps, which probably enable them to keep their cation contents at a low level even with high NaCl concentrations.

Petrovič et al. (2002) proposed a model by which the glycerol accumulation, the use of alternative energy production mechanisms, such as the glycerophosphate shuttle, the isovaleryl-CoA dehydrogenase/electron transferring flavoprotein: ubiquinone oxidoreductase system, or the Na^+ and/or the H^+ electrochemical potentials, and the post-transcriptional regulation enable *H. werneckii* to thrive at NaCl concentrations unparalleled by other eukaryotic species. *H. werneckii* is able to keep extracellular glycerol concentrations efficiently low and independent of salt concentration between 0 and 17% NaCl, but unchanged intracellular glycerol concentration between 10 and 25% NaCl points to the presence of (an)other compatible solute(s) in the cells of *H. werneckii* grown at salinities above 10% NaCl. At salinities higher than 17%, the extracellular concentration of glycerol started to increase. This effect correlates with the diminished growth of *H. werneckii* at salinities above 17% NaCl. As in *S. cerevisiae*, glycerol biosynthesis in *H. werneckii* is regulated mainly through transcriptional regulation of glycerol-3-phosphate dehydrogenase. Since glycerol presumably becomes one of the most abundant molecules produced by the cells, relatively less metabolic flux remains for the late stages of glycolysis and presumably also for the citric acid cycle. Increased expression of SOL5, a putative aconitase gene, at 17% NaCl and 25% NaCl, could also be related to this phenomenon. When the citric acid cycle becomes less important, a cell could gain enough ATP through alternative electron donors or it could make use of Na^+ and/or H^+ electrochemical potentials across the plasma membrane. Petrovič et al. (2002) describe that genes for SOL1, SOL2, SOL4p and SOL8 could be part of this scenario.

Additionally, Lanišnik Rižner et al. (2001) reported an increase of 17β-hydroxysteroid dehydrogenases (17β-HSD) activity in *H. werneckii* in the presence of salt. The enzyme activity at 17% NaCl was 13 times higher than that at 5% NaCl. They proposed that 17β-HSD could be a part of a detoxification mechanism in *S. pombe* and *C. albicans* and different types of 17β-HSDs might have evolved because of

different needs for detoxification in different environments. These enzymes are responsible for reversible interconversions of biologically active 17-hydroxy and inactive 17-keto steroids. Influencing their activity with altering NaCl concentrations is therefore an interesting feature for biotechnological applications in steroid production.

Another phenomenon, which contributes to better understanding of eukaryotic adaptations, enabling life in extremely saline environments has also been observed in research on lipids in halophilic microorganisms. Lipid saturation (increase of oleic acid and decrease of linoleic acid) was shown to be important for salt tolerance in *Z. rouxii* cells under a high-NaCl condition (Yoshikawa et al. 1995). Gostinčar et al. (2004) showed that the fatty acid composition of the *H. werneckii* cells is at least partially regulated through the transcription control of genes, encoding three important fatty-acid-modifying enzymes: elongase and two different types of desaturase. Expression of all three observed genes rose at downshift, while it decreased at increased salinity (upshift).

15.3.1.4 Osmotic Adaptation in Yeast

High glucose concentrations increase extracellular osmotic pressure, and as a consequence, osmotolerant yeasts accumulate glycerol as a compatible solute (Sahoo and Agarwal 2001). Yeast cells possess robust systems for osmotic adaptation. Central to the response to high osmolarity is the HOG pathway, one of the best-explored mitogen-activated protein (MAP) kinase pathways. This pathway controls via different transcription factors the expression of more than 150 genes. In addition, osmotic responses are also controlled by protein kinase A via a general stress response pathway and by presently unknown signalling systems. The HOG pathway partially controls expression of genes encoding enzymes in glycerol production. Upon hypoosmotic shock, yeast cells transiently stimulate another MAP kinase pathway, the so-called protein kinase C (PKC) pathway, which appears to orchestrate the assembly of the cell surface and the cell wall. In addition, yeast cells show signs of a regulated volume decrease by rapidly exporting glycerol through Fps1p. This unusual major intrinsic protein (MIP) channel is gated by osmotic changes and thereby plays a key role in controlling the intracellular osmolyte content. Yeast cells also possess two aquaporins, Aqy1p and Aqy2p. The production of both proteins is strictly regulated, suggesting that these water channels play very specific roles in yeast physiology (Hohmann 2002).

Osmotic hypersensitivity is manifested as cellular death at magnitudes of osmotic stress that can support growth. All strains of *S. cerevisiae* display the osmotic hypersensitivity phenomenon in qualitative terms, while the quantitative values differ. It was proposed that the growth rate does not dictate the level of osmotic hypersensitivity of *S. cerevisiae*. The phenomenon is physiological and not genetic because cells that do survive and form colonies under conditions of high stress will exhibit the same growth-related osmotic hypersensitivity if inoculated into fresh basal medium. The osmotic hypersensitivitiy phenotype is not related to any of the strictly growth linked functions in the cell, like DNA replication or biosynthesis. The phenomenon might be valid for many if not all species of yeast and is not restricted to specific

examples. Osmotic shock resistance seems not to be restricted to non-proliferating cells (Blomberg 1997).

15.3.2 Heat-Shock in Yeasts

The membrane lipid composition of the thermophilic yeasts is distinguished by the high percentage (30–40%) of saturated fatty acid, as compared with the mesophilic and psychrophilic yeasts. The latter contained approximately 90% unsaturated fatty acid, 55% of which was linolenic acid, $C_{\alpha\text{-}18:3}$. Changes in phospholipid composition in relation to temperature were also noted. The respiratory-deficient thermophile *C. slooffiae* was characterized by the absence of cardiolipin (sensitivity 0.1 μg of phosphorus) and cytochrome aa_3. The absence of conventional mitochondrial structures in this thermophilic microorganism is tentatively suggested, although low concentrations of cytochromes b, c and c_1 were detected by low-temperature spectroscopy. On the other hand, the respiratory competent thermophile, *T. bovina* (= *Kazachstania bovina*), was characterized by a high cardiolipin (25% of the total phospholipid) and cytochrome aa_3 content (1 nmol mg^{-1} of mitochondrial protein). Low-temperature spectra showed the presence of one b-type cytochrome in the thermophilic yeasts, two b-type cytochromes in the mesophilic yeasts and three b-type cytochromes in the psychrophilic yeasts. It was concluded that knowledge of the properties of the biological membrane is fundamental to understand the ability of a microorganism to grow and reproduce in different temperature environments (Arthur and Watson 1976).

On the other hand, Swan and Watson (1997) showed that there was no obvious relationship among membrane fatty acyl composition, membrane fluidity and stress tolerance in the yeast strains examined. They suggest that protein denaturation may be responsible for the observed effect of elevated temperature on membrane fluidity and viability – a decrease in membrane fluidity following thermal treatment, which coincided with a reduction in cell viability. They also suggest that the thermotolerant state of heat-shocked cells and cells entering the stationary phase may be associated with increased protein stability.

For walled cells such as yeast, a heat stress does not provoke any measurable variation of viable cell volume, while spheroplasts are rapidly affected by an increase in temperature of the medium that induced firstly a volume increase and secondly cell bursting. This constant volume over time could be explained by the protective role of the cell wall that mechanically maintains the plasma membrane. This could be explained by the sensitivity of the plasma membrane or of the cell cytoskeleton to heat. If the cytoplasmic membrane is maintained by a wall, as for yeast, the main envelope pressure resistance can then be assumed by the wall, which is still resistant to pressure in spite of the heat stress. For walled cells, the entry of water is prevented by the wall that mechanically balances the turgor pressure through the exertion of an overpressure on the plasma membrane (Gervais et al. 2003). Someone can speculate which composition of the cell wall structure enables the highest turgor pressure: the thicker β-glucan and additional α-glucan structure of ascomycetous yeasts or the thinner but lamellare β-glucan structure of basidiomycetous yeasts. A contribution to this knowledge was made by Nguyen et al. (2001), who studied

the physiological adaptation to influences of extreme environments in *Rh. glutinis* discussed in the following section.

15.3.3 Characteristics of Acidophilic Behaviour of Yeasts

Acidophiles keep their internal pH close to neutral. As a result, extreme acidophiles have a large chemical proton gradient across the membrane. Proton movement into the cell is minimized by an intracellular net positive charge and as a result the cells have a positive inside-membrane potential. This is caused by amino acid side chains of proteins and phosphorylated groups of nucleic acids and metabolic intermediates, acting as titratable groups. In fact, the low intracellular pH leads to protonation of titratable groups and produces a net intracellular positive charge (Cavicchioli and Torsten 2000).

The strength of an acid is defined by its dissociation constant (pK_a) – at this pH value the dissociated and undissociated forms of the acid are in equal amounts. The lower the pH, the greater the proportion of the acid in the undissociated form, which is membrane-permeable and therefore it can enter the cell. Once inside the cell, weak acids dissociate because of higher interior pH and become toxic, which ultimately inhibits cell growth owing to the acidification of the cell interior (Beales 2004). In yeast there are approximately 20 multidrug resistance (MDR) genes underlying tolerance to toxic compounds that are all involved in membrane transport. The yeast Mdr proteins are generally referred to as pleiotropic drug resistance (Pdr) proteins. They are composed of three major classes: the ATP-binding cassette (ABC) superfamily, such as Pdr12p, the major facilitator superfamily (MFS) and transcription factors, such as PDR1. Pdr12p is membrane-localized and its role is involved in weak organic acid resistance. HSP26 and HSP30 also seem to contribute to the establishment of a new level of cellular homeostasis in weak-acid adapted cells, latter by moderating the activity of the proton-pumping plasma membrane ATPase, which can consume about a half of the total cellular ATP produced. However, it is speculated that the net effect could be that cells (e.g., *Z. bailii*) effectively extrude the antimicrobial weak acid from the cytoplasm (Brul et al. 2003; Beales 2004).

In the study of de Silóniz et al. (2002b) important differences were found among the three strains in relation to changes in specific qO2 as a function of external pH. The respirometric results obtained over the pH range studied (respiratory quotient approximately 1.0), reflect an oxidative metabolism of glucose in *C. sorbophila* and *Rh. mucilaginosa*, as could be expected from the negative Crabtree character of these yeasts. On the other hand, a different effect of acid on the yeast cells could be observed when qO2 and growth rate results were considered together. Oxygen uptake by *Rh. mucilaginosa* was only slightly affected at low pH. Nevertheless, the bulk of energy obtained is probably needed to support the viability of cells between pH 2.5 and 1.0, and, in consequence, cells grow slowly or not at all. In contrast, at optimal pH (3.0–2.5), *C. sorbophila* was able to maintain low qO2 and slightly greater growth rates than at higher pH (4.5), and with very similar kinetics of glucose consumption.

Red yeast *Rh. glutinis* shows a high level of physiological adaptation to influences of extreme environments. Electron microscope observations (scanning electron

microscopy and transmission electron microscopy) showed that the cell envelope became wrinkled and thick as the pH values of the media became lower. The cell membrane grown at pH 1.5 was about 4 times as thick as that grown at pH 6.0. Furthermore, it was found that the densities of the cell membranes decreased in the acidic media. It was suggested that the change of the cell envelope plays an important role in the acid tolerance. Cellular proteins at pH 1.5 appeared to be different from those at pH 6.0 and the amounts of phospholipids and non-phospholipids increased and decreased under low pH conditions, respectively. The results suggested that the increase of phospholipid content prevented the penetration of excess protons. As the amounts of phosphatidylcholine and phosphatidylethanolamine increased slightly and the amount of the total phospholipids increased markedly at low pH compared with those at high pH, there is a possibility of the presence of phospholipids other than phosphatidylcholine and phosphatidylethanolamine. It was demonstrated that *Rh. glutinis* is capable of lowering the acidity of a low-pH medium and that it is also capable of neutralizing acidic media. As the neutralization occurred in the stationary phase, it could be thought that the neutralization depended on not only the alkaline materials derived from the cells but also the enzyme(s) capable of reducing metal ions (Nguyen et al. 2001).

15.3.4 Resistance to High Concentration of Metals

Yeast defence mechanisms to high concentrations of metal include bioaccumulation and the precipitation/chelation of the metal at the cell wall (biosorption), which prevents the metal from reaching sensitive intracellular components. Yeast tolerance to high metal concentrations is dependent on cell wall characteristics, which can regulate the number of cations that are able to reach the cell membrane and cytoplasm (Raspor et al. 2000a). The cell wall characteristics are determined by its structure and the distribution of homopolysaccharides (mannans and glucans), single saccharides and acid components (which can be good binding agents). Considering the growth of yeast it was observed that the hyphal type of growth of yeasts other than *Saccharomyces* has a better tolerance capacity to chromium in the environment than normal budding (Batič et al. 1996; Batič and Raspor 2000). Metal ion uptake in yeasts is known to involve an initial rapid biosorption of metal ions to negatively charged sites on the cell wall followed by a slower, energy-dependent entry into the cell (Raspor et al. 2003). Both the outer mannan–protein layer of the yeast cell wall as well as the inner glucan–chitin layer play important role in heavy-metal accumulation. A majority of intracellular metals become bound to polyphosphate granules localized in and near the vacuoles or may also get detoxified via binding to specific low molecular weight proteins, namely, metallothioneins and phytochelatins. Thus, it appears that in case of yeast and microalgae as well, most of the metals are accumulated intracellularly (Malik 2004).

Accumulation of cadmium and nickel in *Y. lipolytica* CCM 4510 was higher than that of cobalt and zinc, i.e., cobalt and zinc were less bonded on yeast biomass in comparison with cadmium or nickel. Moreover, cadmium and nickel were not detected on the surface of the cell wall, but mainly they interacted with the cell wall and membrane debris. The high level of cadmium and nickel in the cell wall and

membrane debris is probably due to the interaction of heavy metals with carboxylic groups, which are dominant functional groups in the cell wall. These carboxylic groups descended from peptides would be the potential sites for binding of heavy metals, while the binding process is not exactly described. The incorporation of heavy metals into individual cell compartments of *Y. lipolytica* decreases in the following order: (1) cell surface, Co>Zn » Cd, Ni; (2) cell wall and membrane debris, Cd, Ni» Zn>Co; and (3) cytoplasm of the yeast cell, Zn, Co>Cd» Ni (Strouhal et al. 2003). As an example, the distribution of accumulated chromium in yeast cells is shown in Fig. 15.1.

MT are an important class of eukaryotic stress-responsive proteins whose biosynthesis is induced by a variety of environmental and physiological stresses including metal sequestration and oxidative stress (de Silóniz et al. 2002a; Strouhal et al. 2003). The differences in MT levels in the yeast cell treated with heavy metals may arise owing to the variety of physiological-biochemical changes that take place during the growth process and the ageing of cells. Though the toxic metals (Cd, Ni) induce biosynthesis of MT more significantly than essential metals (Co, Zn), it was observed that when cytoplasmic concentrations of essential metals (Co, Zn) were high, the amount of MT was lower than that for toxic metals. Therefore it was supposed that the cell wall is the first protective barrier to prevent penetration of heavy metals into the cell (Strouhal et al. 2003).

De Silóniz et al. (2002a) compared the cell morphology of yeast *P. guilliermondii* between control cultures without copper and cultures with different concentrations of copper. They observed a shift to filamentous forms and no unicellular forms, and a change in colour to green in the copper cultures. A mixture of yeast-like and filamentous cells was always observed in the absence of copper, with the yeast cells

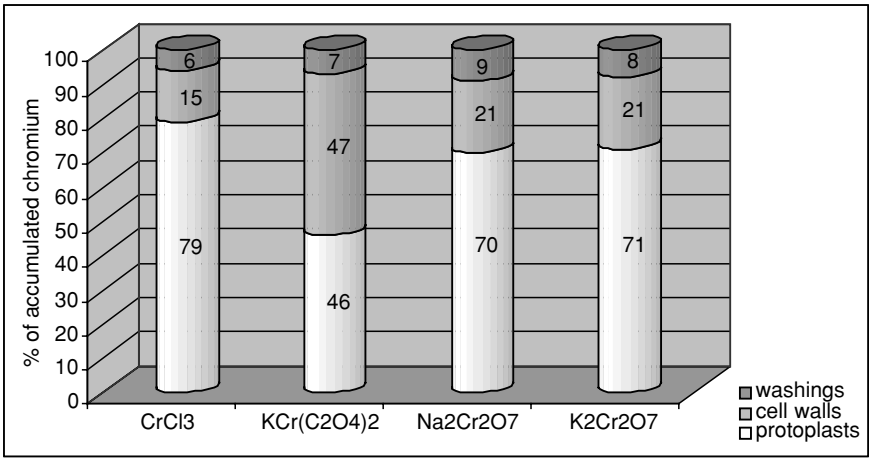

Fig. 15.1. Distribution of accumulated chromium in yeast cells, expressed as a percentage of Cr in cell walls, protoplasts and washings, after 12-h cultivation in the presence of 1 mM Cr(III) [as $CrCl_3$ or $KCr(C_2O_4)_2$] and 20 μM Cr(VI) (as $K_2Cr_2O_7$ or $Na_2Cr_2O_7$) (Paš et al. 2004)

being predominant. The addition of copper increased the proportion of filamentous forms, and this could be considered a primary response to copper toxicity. When the cells were adapted by being trained to grow at high concentrations of copper, only yeast-like forms developed. In this sense, a long-term response to copper toxicity could be responsible for the appearance here of yeast-like cells. Independent of the degree of stress, it seems that a process of bioaccumulation may occur in the presence of copper in both types of cells. These results indicate that unadapted cells growing in the presence of the metal must spend energy on the mechanisms involved in protecting the yeast cell from the toxic effect of copper. However, the mechanism of adaptation to copper in *P. guilliermondii* could be considered a system of all or nothing, i.e., once the mechanism of adaptation to high concentrations of copper is developed, the growth is stable and the concentration of copper has no influence on the metabolic parameters. The results of uptake indicate and confirm the microscopic observation that the resistance mechanism is a sequestration of copper, rather than a mechanism of avoidance. Consequently, it is possible that the metallothionein-mediated mechanism could be responsible for metal sequestration in *P. guilliermondii*. From the point of view of bioremediation, this mechanism contributes to the interest and feasibility of application of *P. guilliermondii* in such processes (de Silóniz et al. 2002a).

Generaly we relate mechanisms for metal toxicity to to cell stress response (Jamnik and Raspor 2003). There are few studies which consider this issue in more detail regarding genotoxicity (Plaper et al. 2002) and oxidative stress (Fujs et al. 2005).

15.3.5 Physiological Make-Up of Yeasts to Degrade Xenobiotic Compounds

Frequently, xenobiotic metabolism involves a combination of P450 and phase II enzymes. Yeast expression makes it possible to include at will some particular phase II activities, such as epoxide hydrolase activity (Pompon et al. 1997). A marine strain of *Y. lipolytica* has a set of enzymes for the bioconversions of nitroaromatic compounds. Very often, non-specific nitroreductases bring about these tranformations. The presence of glucose could preferentially modulate the choice of the reaction to the ring reduction and in the absence of glucose the nitro groups are preferentially reduced to amino derivates (Jain et al. 2004). Zaripov et al. (2002) reported that no strains analogous to *Candida* sp. AN-L13, which performs a practically unidirected reduction of the aromatic ring, were found among other strains in their collection. Such microorganisms are also unknown in the literature. Interestingly, the taxonomic affiliation and the type of metabolism (fermentative vs. respiratory) of yeasts correlated with the pathway of TNT degradation.

15.4 Biotechnological Potential of Yeasts from Extreme Habitats

Extremophiles have provided data that are basic to molecular biology, including information on protein folding. Enzymes from extremophiles have potential in multiple areas, either by using the enzymes themselves, or by using them as sources of

ideas to modify mesophile-derived enzymes. Human health may benefit from extremophiles indirectly through biotechnology and bioremediation. Antifreeze proteins show potential as cryoprotectants of frozen organs. It is now widely accepted that these microorganisms provide a valuable resource not only for exploitation in novel biotechnological processes but also as models for investigating how biomolecules are stabilized when subjected to extreme conditions (Herbert 1992; Rothschild and Mancinelli 2001). However, extremophilic yeasts can be used in many applications since yeasts are the most important and the most extensively used microorganisms in industry (Madigan et al. 1997) (see also Chap. 22).

15.4.1 Bioremediation

In modern society, an increasing number of hazardous organic compounds are being discharged into the environment. Most are degraded or detoxificated by physical, chemical and biological treatments before being released into the environment. Although the biological treatments are a removal process for some organic compounds, their products of biodegradation may also be hazardous. Moreover, some non-degradable compounds discharged into the environment along with the treated compounds can cause problems because they usually come back to human beings through several channels, such as bioaccumulation. Bioaccumulation is defined as the accumulation of pollutants by actively growing cells by metabolism-independent, temperature-independent and metabolism-dependent mechanism steps. Although bioaccumulation of dyes by yeasts was accomplished, there are significant practical limitations regarding the inhibition of cell growth at high dye concentrations and the requirement of metabolic energy externally provided. So there is a need to find alternative treatment methods that are effective in removing dyes and organic molecules that are not biodegradable from large volumes of effluents and are low in cost, such as biosorption. The term "Biosorption" is used to indicate a number of metabolism-independent processes (physical and chemical adsorption, electrostatic interaction, ion exchange, complexation, chelation and microprecipitation) taking place essentially in the cell wall rather than oxidation through anaerobic or aerobic metabolism (biodegradation). The main attractions of biosorption are high selectivity and efficiency, cost effectiveness and good removal performance. Biosorption is also becoming a promising alternative to replace or supplement the present removal processes of organic pollutants from wastewaters. Among these pollutants, dyes, phenolics and pesticides have recently been of great concern because of their extreme toxicity and/or persistency in the environment (Aksu 2004).

The use of dead microbial cells in biosorption is more advantageous for water treatment in that dead organisms are not affected by toxic wastes, they do not require a continuous supply of nutrients and they can be regenerated and reused for many cycles. Dead cells may be stored or used for extended periods at room temperature without putrefaction occurring. Their operation is easy and their regeneration is simple. Moreover, dead cells have been shown to accumulate pollutants to the same or to a greater extent than growing or resting cells. The mechanism of binding by inactivated biomass may depend on the chemical nature of the pollutant (species, size, ionic charge), the type of biomass, its preparation and its specific

surface properties and environmental conditions (pH, temperature, ionic strength, existence of competing organic or inorganic ligands in solution). As hydrophobic organic pollutants show a high tendency to accumulate onto microbial cells or sludge, the microbial biomass could be used as an adsorbent of biological origin for the removal of very low concentration hazardous organics from the wastewater (Aksu 2004).

Dönmez (2002) and Aksu and Dönmez (2003) reported the biosorption capacities and rates of nine yeast species (*S. cerevisiae*, *S. pombe*, *K. marxianus*, *Candida* sp., *C. tropicalis*, *C. lipolytica*, *C. utilis*, *C. guilliermendii* and *C. membranifaciens*) for Remazol Blue reactive dye from aqueous solutions. The yeasts studied were found to be more effective for concentrating Remazol Blue dye at different capacities according to the dye concentration. They explained the differences between yeast species for dye binding capacity in terms of the properties of the yeast (e.g., structure, functional groups, surface area and morphological differences depending on the yeast division, genera and species). They proposed that cell walls of yeasts contain polysaccharides as basic building blocks, which have ion-exchange properties, and also proteins and lipids and therefore offer a host of functional groups capable of binding dye molecules. These functional groups such as amino, carboxylic, sulfydryl, phosphate and thiol groups differ in their affinity and specificity for dye binding. Among the nine yeast species tested, *C. lipolytica* exhibited the highest dye uptake capacity (Aksu 2004).

Pharmaceutical wastewater remaining after ergot alkaloid production has a high COD load to sugar compounds, metabolic products of fungus *Claviceps* and organic solvents content (COD is a standard test that measures the amount of organic matter in wastewater that can be oxidized by a very strong chemical oxidant). The cost of discharging the wastewater into a wastewater treatment plant is forcing the pharmaceutical industry to pretreat it. With the aim to develop a more efficient system for wastewater treatment the isolation of a spontaneous population of microorganisms from a wastewater collecting tank was performed. Isolated strains of yeasts were identified as *P. ciferrii* and the results showed that the COD reduction in this case was significantly higher (75–80% in 60 h) in comparison with a previous selection among 60 yeast strains which showed that only three of them were able to grow in wastewater with a COD reduction efficiency of 60–64%. It was found that *P. ciferrii* could be used for reducing the COD load of wastewater from ergot alkaloid production (Recek and Raspor 1999; Recek et al. 1999, 2002).

Heavy-metal pollution represents an important environmental problem owing to the toxic effects of metals, and their accumulation throughout the food chain leads to serious ecological and health problems. Biotechnological approaches that are designed to cover such niches have, therefore, received a great deal of attention in recent years. At times, when pure biosorptive metal removal is not feasible, application of a judicious consortium of growing metal-resistant cells can ensure better removal through a combination of bioprecipitation, biosorption and continuous metabolic uptake of metals after physical adsorption. Such an approach may lead to simultaneous removal of toxic metals, organic loads and other inorganic impurities, as well as allow optimization through development of resistant species. However, the sensitivity of living cells to extremes of pH or high metal concentrations and the

need to furnish metabolic energy are some of the major constraints of employing growing cells for bioremediation (Malik 2004).

In order to overcome the problem, heavy-metal ions are commonly removed from wastewaters by chemical precipitation, ion-exchange or reverse osmosis processes. Such techniques can be very expensive and may have several disadvantages, therefore development of cost-effective alternatives, such as biosorption, has become an intensive area of exploitation over the past decade. Non-viable biomass has several advantages for metal biosorption from solution, especially as there is no requirement for maintenance and nutrition (de Silóniz et al. 2002a).

Some microorganisms are able to grow in extreme concentrations of different heavy metals in the environment. Yeasts have a great potential for removal and uptake of metals from the environment as well as for controlling environmental pollution (Batič et al. 1996).

Copper is one of the most abundant toxic heavy metals in municipal wastewaters and, in consequence, in sewage sludge and compost. The ability of a strain of the yeast *P. guilliermondii*, which was isolated from sewage sludge, to eliminate copper has been reported. It was found that raising the concentrations of copper affected both the morphology and the physiological parameters of the viable yeast, and it is thought that a process of bioaccumulation may be involved in its copper uptake. The growth rate of unadapted cells decreased with increasing concentrations of copper, mainly owing to a decrease in the biomass yield. The cells could be adapted by training them with increasing copper concentrations up to 317.7 mg l^{-1}. This adaptation was an all-or-nothing process: once the cells had adapted, the biomass yield, the metabolic flux and consequently the growth rate were constant and independent of the external copper concentration (de Silóniz et al. 2002a).

The acid-tolerant yeast *Rh. glutinis* showed strong resistance to both aluminium and manganese ions. It was found that *Rh. glutinis* R-l has the ability to adapt to an acidic environment by changing the components of its cell membrane and the shape of its cell envelope, and that the old cells can restore sterile environments such as acidic soil. It took only 5 days to reach neutral pH and the neutralization of the acidified culture could be repeated at least five times. An acidic medium (pH 3.0) containing these ions (100 mM) was shifted to neutral pH by long-term cultivation of the red yeast, suggesting the potential of using this yeast in the bioremediation of acidic soil containing these ions at a high level (Nguyen et al. 2001).

The microorganisms converting TNT quantitatively to the reactive metabolites can be useful for their immobilization through the detoxifying interaction with the soil components, such as humic compounds. Complex environmental contaminations such as explosives and petroleum hydrocarbons are not uncommon and in this regard the strain *Candida* sp. AN-L13, which was isolated earlier as one of the dominant microorganisms from oil-polluted peat bogs (Langepas, western Siberia, Russia), deserves special attention. In addition to its ability to perform the initial TNT conversion step, it is able to utilize crude oil and several individual aliphatic and aromatic hydrocarbons. This strain, as well as other microorganisms with comparable metabolic capabilities, is very interesting not only for academic research but it also possesses vast potential for bioremediation of areas with complex contaminations (Zaripov et al. 2002).

Another yeast was found to be useful for bioremediation purposes, like TNT bioconversion and oil degradation. The ability of a tropical marine strain of *Y. lipolytica* to transform TNT into products such as 2,4-DNT which in turn could be metabolized by other microbes has implications in the use of this yeast in the bioremediation of TNT-polluted marine environments (Jain et al. 2004). Oil contamination of soil and water is found frequently. Accidents are likely to occur in the form of pipeline leaks, train derailments, ship wreckages, storage tank ruptures, transport accidents, etc. Diesel oil, a distillate fraction of crude oil, is one of the major pollutants of soil and groundwater near petrol stations (Margesin and Schinner 1997). Among a number of biotechnological applications, *Y. lipolytica* is used in the bioremediation of soils contaminated by petroleum products (Strouhal et al. 2003). The effect of temperature on oil degradation by this psychrotrophic yeast in liquid culture and in soil was studied by Margesin and Schinner (1997). *Y. lipolytica* showed its capabilities for oil degradation, but the results demonstrated that bioaugmentation is not helpful in the case of oil pollution of alpine soil. The subsoil investigated harbours hydrocarbon-degrading indigenous soil microorganisms that are able to metabolize diesel oil at low temperature more effectively than the psychrotrophic oil-degrading microorganisms introduced. This yeast can be used for remediation of the environment contaminated by various pollutants, including heavy metals, and can grow in the presence of hydrocarbons. The *Y. lipolytica* cells can bind large amounts of heavy metals and produce specific protein metallothionein as a protective agent. This approach offers the possibility to apply the yeast in bioremediation of soil and water contaminated by petroleum products in connection with heavy metals (Strouhal et al. 2003).

15.4.2 "Cold enzymes"

Psychrophilic (cold-adapted) organisms and their products have potential applications in a broad range of industrial, agricultural and medical processes. Relative to this undisputed potential, psychrophiles and their products are under-utilized in biotechnology; however, recent advances, particularly with cold-active enzymes, have heralded rapid growth for this burgeoning field. Psychrophilic enzymes have two properties that have the most obvious biotechnological application: a high specific activity at low and moderate temperatures, and they are inactivated easily by a moderate increase in temperature (Margesin and Schinner 1994; Cavicchioli et al. 2002). These properties can be extremely useful in various applications; these enzymes are both innovative and invaluable. Using X-ray crystallography, these properties are beginning to become understood, and the rules governing their adaptation to cold appear to be relatively diverse. The application of these enzymes offers considerable potential to the biotechnology industry, for example, in the detergent and food industries, for the production of fine chemicals and in bioremediation processes. It is likely that the potential value of cold-adapted enzymes is greater, in view of the diverse capabilities of these enzymes in comparison with the annual market for thermostable enzymes (Gerday et al. 2000). There is a project to examine enzymes from Antarctica, some of which may have commercial potential. Enzymes arising from this work and some of the more obvious applications include α-amylase (used in breadmaking, textiles, brewing and detergents), cellulase (used in textiles and the pulp and paper industries, detergent additives), β-galactosidase (which

eliminates lactose from milk), lipase (used in detergents and flavourings), pectinases (in the fruit juice industry), proteases (used in detergents, meat tenderizing and baking processes), xylanase (baking processes) and enzymes in brewing and wine industries, cheese manufacturing and animal feed. The use of psychrophilic enzymes can be advantageous not only for their high specific activity, thereby reducing the amount of enzyme needed, but also for their easy inactivation. Cold-adapted enzymes offer economic benefits also through energy savings: they negate the requirement for expensive heating steps, function in cold environments and during the winter season provide increased reaction yields, accommodate a high level of stereospecificity, minimize undesirable chemical reactions that can occur at higher temperatures and exhibit thermal lability for rapidly and easily inactivating the enzyme when required (Gerday et al. 2000; Cavicchioli et al. 2002).

There is an industrial trend to treat foodstuffs under mild conditions in order to avoid spoilage and changes in taste and nutritional value at ambient temperatures. Therefore, cold-active enzymes are attractive for the processing of foods. Nakagawa et al. (2002) isolated a psychrophilic basidiomycetous yeast *C. capitatum* strain PPY-1, which could grow on pectin at 5°C. This is the first report of a psychrophilic yeast utilizing pectin as a sole carbon source. Moreover, it seems that the strain produced cold-active enzymes that degrade pectin, although the pectin-depolymerizing enzymes consisted of isozymes. On the basis of these facts, this strain and its pectin-degrading enzymes may be applicable to the food industry (Nakagawa et al. 2002).

Turkiewicz et al. (2003) first reported the extracellular serine proteinase of a *Leucosporidium* strain. The Antarctic enzyme is a newly found subtilase of the clan SB (family S8 of subtilisin and subtilases) and belongs to the group of so-called cold enzymes. This enzyme was termed LAP2 and is the first reported extracellular subtilase of a psychrophilic yeast. The proteinase LAP2 is specific towards synthetic substrates of chymotrypsin and subtilisin.

Ecologically, one of the important substrates is xylan, which is, after cellulose, the most abundant renewable polysaccharide on Earth (Scorzetti et al. 2000). Gomes et al. (2000) discovered another cold-enzyme.This was the first report on the production as well as on the properties of thermolabile xylanase produced by another Antarctic yeast *Cryptococcus adeliae* that exhibits optimal growth at low temperature. In the range from 0 to 20°C, the cold-adapted xylanase displays a lower activation energy and a higher catalytic efficiency. All these observations suggest a less compact, more flexible molecular structure (Petrescu et al. 2000). The cold-adapted xylanase can be used in dough fermentation, protoplast formation and in the wine and juice industry (Cavicchioli et al. 2002).

15.4.3 Glycerol Production

Maintaining high extracellular osmotic pressure is necessary for glycerol accumulation in either batch or continuous mode of operation. Multistage continuous culture techniques seem particularly suitable for possible enhancement of glycerol accumulation since the glucose concentration could be controlled at a relatively high level in the early bioreactors to induce enzyme formation and improve the activity of the enzyme for glycerol synthesis. Higher glycerol yield based on glucose consumed and higher productivity were also obtained with the increase of the initial glucose

concentration. The requirement for relatively high initial glucose concentration was due to the desirable high extracellular osmotic pressure to promote glycerol accumulation. The final glycerol concentration, glycerol yield and productivity could be improved by either raising the feed glucose concentration or employing sodium chloride as a regulator of osmotic pressure. However, the addition of NaCl to media is disadvantageous to downstream processing of product. Therefore, further studies involved higher feed glucose concentrations. A high extracelullar osmotic pressure could result in the efficient metabolic pathway of glycerol that inhibited other alternative metabolic pathways. The results for the effects of feed glucose concentration on continuous fermentation process showed that maintaining the high osmotic pressure in the early stages was necessary for improvements of glycerol yield, glycerol concentration and productivity. It was expected that productivity might be further improved if a multistage cascade bioreactor with cell recycling was used to avoid the disadvantages of low biomass concentration under high osmotic pressure and high dilution rates (Liu et al. 2002).

Trehalose and glycerol may act as protectors inducing a high tolerance to a range of stresses which appear in the production and industrial utilization of yeast. Trehalose is a technologically important parameter for baker's yeast performance as the accumulation of this compound strengthens the strain against stress conditions, namely increasing thermotolerance and storage stability (Carvalheiro et al. 1999). Glycerol production by osmophilic yeasts has a significant potential for large-scale production. The initial glucose concentration, the carbon-to-nitrogen ratio and the aeration rate significantly affected cell growth and glycerol production by osmophilic/halophilic yeast *C. magnoliae* I2B, while the variation of the medium pH in the range 3.5–5.0 had little influence on glycerol production. The availability of oxygen was suggested to be the most important factor controlling cell growth, glucose uptake and yield of glycerol (Sahoo and Agarwal 2001).

There are also some biotechnological applications that cannot be placed in one of the previously described categories, but which are very important in the fields of industry or ecology. Burschäpers et al. (2002) reported the production of sugar alcohols with the osmophilic yeast *Moniliella tomentosa* var. *pollinis* (= *Moniliella pollinis*). This process was conducted as a batch and fed-batch operation in a stirred tank. Kourkoutas et al. (2002) presented continuous wine fermentation using an alcohol-resistant psychrophilic *S. cerevisiae* AXAZ-1 immobilized on apple cuts at different temperatures. This technique was found to be suitable for continuous wine fermentation at temperatures between 5 and 15°C. The application of two halotolerant yeast species *C. versatilis* and *Z. rouxii* in soy-sauce production has been optimized. Immobilization of these two yeasts considerably decreases the total time required for the flavour development in soy-sauce processes. Especially, the application of immobilized salt-tolerant yeasts in a continuous bioreactor proved to be very effective by reducing the processes time by about 90%. This reduction seemed to be caused by the 10–100-fold higher concentration of yeast cells in the immobilized-cell process compared with the concentration in the conventional process. For immobilization of cells poly(ethylene oxide) rather than alginate gel seems to be suitable for use in soy-sauce processes (van der Sluis et al. 2001). Applications for various industrial processes and biotechnology using extremophilic yeasts are summed up in Table 15.2.

Table 15.2 Potential of some extremophilic yeasts for various industrial processes and biotechnology

Source	Industrial process	Advantages	Yeasts	Source
Psychrophiles				
Lipases, neutral proteases	Cheese maturation, dairy production	Stable at low temperatures	*Candida antarctica*	Rothschild and Mancinelli (2001); Cavicchioli et al. (2002)
Glucoamylase	Starch hydrolysis	Stable at low temperatures	*C. antarctica* CBS 6678, *Cryptococcus flavus*	Rothschild and Mancinelli (2001); Cavicchioli et al. (2002); Wanderley et al. (2004)
Proteases, amylases, lipases, cellulases	Degradation of polymers in detergents	Improved performance of detergent	*C. rugosa*, *Yarrowia lipolytica*	Ferrer et al. (2001); Fickers (2005)
Microorganisms	Bioremediation – reduction of oil spills	Works efficiently in cold waters	*Y. lipolytica*	Rothschild and Mancinelli (2001); Margesin and Schinner (1997)
Dehydrogenases	Biosensors, biotransformations		*C. tropicalis*	Takamizawa et al. (2000)
Polyunsaturated fatty acids	Pharmaceuticals, food additives, dietary supplements		*Saccharomyces kluyveri*	Oura and Kaiwara (2004)
Protease	Contact-lens cleaning solutions, meat tenderizing		*Pichia pastoris*	Cavicchioli and Torsten (2000); Sinha et al. (2005)
Subtilase			*Leucosporidium antarcticum*	Turkiewicz et al. (2003)
Ice nucleating proteins	Artificial snow, ice cream, other freezing applications in the food industry		*S. cerevisiae*	Cavicchioli and Torsten (2000); Hwang et al. (2001)
Antifreeze proteins and antifreeze glycoproteins	Binding to ice crystals and inhibiting ice crystal growth		*P. pastoris*	Li et al. (2001)
β-Galactosidase	Lactose hydrolysis in milk products		*Kluyveromyces lactis*	Cavicchioli and Torsten (2000); Becerra et al. (2004)

Continues

Table 15.2 Potential of some extremophilic yeasts for various industrial processes and biotechnology—*cont'd*

Source	Industrial process	Advantages	Yeasts	Source
Pectinase	Food industry	Treatment of foodstuffs under mild conditions in order to avoid spoilage and changes in taste and nutritional value at ambient temperatures	*Cystofilobasidium capitatum*, *Cyst. lari-marini*, *Cr. macerans*, *Cr. aquaticus*	Nakagawa et al. (2002); Birgisson et al. (2003)
Xylanase	Dough fermentation, protoplast formation and wine and juice industry	Lower activation energy and a higher catalytic efficiency	*Cr. adeliae*	Gomes (2000); Petrescu et al. (2000)
Microorganisms	Continuous wine fermentation		*S. cerevisiae*	Kourkoutas et al. (2002)
Halophiles				
Carotene	Food colouring	Inexpensive to produce	*Rhodotorula mucilaginosa*	Libkind et al. (2004)
Glucoamylase gene	Fermentation of starch		*Arxula adeninivorans*	Wartmann and Kunze (2000)
Glycerol, compatible solutes	Protein and cell protectants in a variety of industrial uses, pharmaceuticals	Inexpensive to produce	*C. magnoliae*	Rothschild and Mancinelli (2001); Sahoo and Agarwal (2001)
Membranes	Surfactants for pharmaceuticals		*C. bombicola*	Shepherd et al. (1995); Guilmanov et al. (2002)
Lipids	Liposomes for drug delivery and cosmetic packaging			Cavicchioli and Torsten (2000)
Microorganisms	Waste transformation and degradation		*C. utilis*	Cavicchioli and Torsten (2000); Zheng et al. (2005)
Microorganisms	Flavour development in soy-sauce processes		*C. versatilis*, *Zygosaccharomyces rouxii*	van der Sluis et al. (2001)

Eukaryotic homologues	Cancer detection, screening antitumor drugs			Cavicchioli and Torsten (2000)
Osmophiles				
Glycerol	Cosmetic, paint, tobacco, food, and pharmaceutical industries		*C. krusei*	Zhang et al. (2002)
Sugar alcohols	Production of sugar alcohols in stirred tank (batch and fed-batch operation)		*Moniliella tomentosa var. pollinis*	Burschäpers et al. (2002)
Genes phbA, phbB and phbC	Production of polyhydroxy-alkanoates		*A. adeninivorans*	Terentiev et al. (2004)
Acidophiles				
Microorganisms	Organic acids and flavour		*C. versatilis*, *Z. rouxii*	van der Sluis et al. (2001)
Alkaliphiles				
Cellulases, proteases, amylases, lipases	Degradation of polymers in detergents	Stable at high pH	Many	Rothschild and Mancinelli (2001)
Alkaline phosphatase	Molecular biology		*S. cerevisiae*	Cavicchioli and Torsten (2000)
Others				
Viable cells	Bioremediation of TNT polluted marine environments		*Y. lipolytica*	Jain et al. (2004)
			Candida sp. AN-L13	Zaripov et al. (2002)
Viable cells	Bioremediation of acidic soil containing aluminium and manganese ions at a high level		*Rh. glutinis*	Nguyen et al. (2001)
Viable cells	Copper elimination from wastewaters, sewage sludge and compost by bioaccumulation		*P. guilliermondii*	de Silóniz et al. (2002a)

Continues

Table 15.2 Potential of some extremophilic yeasts for various industrial processes and biotechnology—*cont'd*

Source	Industrial process	Advantages	Yeasts	Source
Viable cells	Metal bioremediation		*S. cerevisiae*	Stoll and Duncan (1997); Malik (2004)
Viable or dead cells	Biosorption of dyes from aqueous solutions		*S. cerevisiae*, *Schizosaccharomyces pombe*, *K. marxianus*, *Candida* sp., *C. tropicalis*, *C. lipolytica*, *C. utilis*, *C. guilliermondii*, *C. membranifaciens*	Dönmez (2002); Aksu and Dönmez (2003); Aksu (2004)
Viable cells	Aerobic biological processes for the removal of dyes from textile effluents		*K. marxianus*, *C. zeylanoides*	Aksu (2004)

15.5 Conclusion

Discussing extreme environments it is difficult to draw the line as to what is extreme in terms of organisms, since this conclusion is mainly based on human standards of extremes. Certainly, we put in the extreme group of microbes what we consider to be extreme according to the standards and knowledge from known data for physiological behaviour of organisms. We still do not use energy consumption as a regular indicator to measure extreme surroundings in specifically selected universal biomarkers, which we could apply in this process. We believe that in the future of "omics" we will develop suitable energy markers which will bring us a new toll for clustering organisms in terms of extremeness. This will be connected to stress response as well, and some traditional stress mechanisms will probably become universal markers for stress sensing in extreme environments. Among other issues we see cultivation and isolation of particular species from natural milieu as very important and should be carefully considered. In particular we should also focus on "viable but not cultivable" phenomena since organisms from this group deserve special attention Owing to their covered activity and consequently we will see development in this direction as well.

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Chapter 16

Yeast Biodiversity in the Antarctic

HELEN S. VISHNIAC

Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK 74078, USA
(e-mail: hsvishi@okstate.edu)

16.1 Introduction

Yeast biodiversity in the Antarctic should be expected to be low. Biodiversity may be rich when an area provides varied habitats and/or when varied functions are assumable in a community but is richest when the organic energy supplies are large enough to support specializations in habitat utilization and community function. Primary productivity on the Antarctic continent is very low. It is inhibited by low temperatures and the resulting low availability of liquid water as well as by comparatively low insolation. Yeasts are saprophytes for which any other than a simple degradative role would be difficult in the Antarctic. Despite this, a variety of yeast species have been reported from Antarctic sources, and new species continue to be described. But it is impractical to apply biodiversity indices to Antarctic yeasts. Biodiversity indices require accurate identification of some systematic unit, typically species, and of the indigenicity of the populations identified. Both of these requisites are problematic in the Antarctic.

The problem of identification can be illustrated by the example of *Cryptococcus albidus*, a valid species with a readily available type culture to which other isolates can be compared. "*Cr. albidus*" isolates from continental Antarctica have been reported by di Menna (1960, 1966), Soneda (1961), Babyeva and Golubev (1969), Cameron et al. (1971), Artamonova and Krasnilnikov (1972), Atlas et al. (1978), Abysov et al. (1983), del Frate and Caretta (1990), and Baublis et al. (1991). Subsequent to these reports, Fonseca et al. (2000) used the currently popular method of sequencing the D1/D2 areas of ribosomal DNA (rDNA) to establish eight new species, elevate two former varieties to specific rank, and reinstate two synonyms in specific rank. In the apparent absence of current cultures, these Antarctic isolates cannot be assigned to any of the 13 resulting species, yet their accurate identification might well result in significant changes in any formal diversity index. While species described since the advent of such sequencing are stable for the nonce, many isolates have been ascribed to older species which have not been subjected to such broad examination as the *Cr. albidus* clade. Earlier attributions to such species as

Cr. laurentii (Sugita et al. 2000; Takashima et al. 2003) and *Rhodotorula minuta* (Fell et al. 2000), subsequently shown to lack homogeneity, cannot be taken at face value. Unidentifiable Antarctic isolates attributed to *Cr. albidus*, *Cr. laurentii* (di Menna 1960, 1966; Tubaki 1961; Babyeva and Golubev 1969; Atlas et al. 1978), and *Rh. minuta* (di Menna 1966) will not be further referred to; unreliable or tentative attributions to other species have been omitted (with one exception) from the tables. The practice of depositing voucher cultures in culture collections and the availability of sequencing at reasonable cost has already begun to obviate this problem. Sequencing of the D2 area alone suffices to distinguish most of the currently valid basidiomycetous yeast species – this is available commercially for submitted cultures so that the collecting laboratory need not be equipped to do more than the usual (now much expanded) phenotypic tests. The ecologists' need for accurate identification has made too much of the older literature useless.

Indigenicity is best indicated by the ability to reproduce in the habitat from which a microbe is isolated. Reproduction is fairly easy to observe in macrobiota, but somewhat more difficult in the case of microbiota. As applied to the Antarctic, this criterion is particularly important because microbes enter the region carried by wind, ocean currents, birds, humans, and other animals. Yeasts were reported as "a minor component" of airspora by Meyer (1962), have been isolated from a mummified penguin (Baublis et al. 1991), bird dung, and skua's nests (see later), and are common around field camps (see later). Such yeasts may survive for long periods under cold and dry conditions without being able to reproduce and might actually outnumber any natives in a given sample. Dehydrated baker's yeast cached at Cape Evans by the Scott Expedition contained both viable *Saccharomyces cerevisiae* and *Rh. pallida* after 50 years (Meyer et al. 1962).

Direct evidence of reproduction is possible. The insertion of sterile glass slides and pedoscopes into Antarctic soil has allowed essentially direct observation of microbial reproduction (Vishniac and Mainzer 1973; Uydess and Vishniac 1976) although no yeasts were seen. Wynn-Williams (1980, 1982) used laboratory manipulations of peat cores from Signy Island as microcosms to demonstrate effects on the seasonal reproduction of native yeasts there. Both of these methods rely on the presence of sufficiently large populations to make their way to the limited surfaces in the first method and to produce significant plate counts (i.e., from 30 to 300 cfu ml^{-1} of menstruum for spread plates) in the case of microcosms.

When such evidence is not available, less reliable indicators may suggest indigenicity without proving it – the possession of adaptations to life in a cold, arid, and energy-poor environment, such evidences of establishment as high population density with dominance in the community and enough genetic variation to imply a center of origin, persistence in the environment, or simply failure to occur elsewhere. Of course, failure to occur elsewhere may reflect only the failure of investigators' time and/or funds or the youth of a new species, while reports of extra-Antarctic occurrences may not need mean establishment in another venue, particularly when a single isolate is being reported. The number of isolations over the years may indicate dormant survival of individuals rather than establishment of a population. Soil culture has been a useful low-technology method of storing yeast isolates for many years; the author's personal collection has revived well after up to 11 years in storage.

Large populations are unusual in energy-poor habitats and in any case these yeasts have not been given the same intense investigation as, say, the origin of the human species. Adaptation is certainly expected of microbes growing in any habitat, but the absence of adaptation to extreme cold, aridity, and starvation need not indicate the tourist rather than the native.

Antarctica does provide less extreme habitats as well as those which caused it for a time to be considered a Martian analogue. The cold is moderated as one proceeds north (with major allowance for elevation and minor allowances for aspect, shading, and radiation received), yet the soil at Lake Vanda in the Ross Desert reached a maximum of 16.4°C and a 90-day austral summer mean of 3.9°C when the maximum at Marble Point on the continental margin was 13.9°C and the 90-day mean was 0.1°C. (To be sure, Lake Vanda experienced 30 freeze–thaw cycles rather than the 10–15 cycles of Marble Point and has frost-free surface soil for around 30 days rather than the 50 days of Marble Point.) The "Asgard Range" (presumably at a higher elevation than Lake Vanda in the Transantarctic Mountains) had no time when the soil surface was continuously above 0°C for several days (Campbell and Claridge 2000). Less extensive observations than the continuous monitoring of Campbell and Claridge have recorded higher temperatures as isolated effects of insolation, though none higher than the surface soil temperatures around active volcanoes where the soil and vegetation surface ranged from 41.5 to 43.4°C, with warm ground around fumaroles reaching 50°C (Bargagli et al. 1996). The highlands of the Ross Desert are nearly barren on the surface but the continental margin and the islands within the Antarctic Ocean are home to a flora of mosses, liverworts, algae, cyanobacteria, and lichens though phanerogams are found only on the Palmer Peninsula and islands of such latitude. (Detailed description of this flora is available from the Australian Antarctic Division. Search for "Antarctic flora" on the net.) The desert highlands are quite arid, but glacial melt streams are seasonally wet and drain into lakes which, like the streams, support cyanobacteria, algae, and the occasional moss. The Antarctic Ocean is productive enough to support large populations of krill.

Yeast biodiversity in the Antarctic has to be described under some disadvantage of taxonomic uncertainty and with recognition of habitat variation and the nature of possible adaptations.

16.2 Psychrophilic Yeasts in Antarctica

The most consistent characteristic of Antarctic habitats is relatively low average temperatures. It is accordingly not surprising that Babyeva and Golubev (1969) isolated more yeasts at 5°C than at "moderate... (20–30°C)" temperatures. Forty percent of their 63 isolates were designated "obligate psychrophiles", failing to grow above 20°C. A caveat – Artamonova and Krasilnikov (1972) remarked that for microbiota of all kingdoms "Antarctic psychrophiles adapt quickly to room temperature", a remark which leads one to suspect the original description. Indigenous yeasts are nonetheless expected to be psychrophiles, psychrophiles which may have to deal with frequent freeze–thaw cycles and with the possibility of temperature excursions above T_{max} as well as far below freezing.

Psychrophily, though literally "cold-loving", has been most usefully defined as failing to grow at 25°C. Maximum growth temperatures are usually available, while minimum growth temperatures (T_{min}) are often not determined – indeed, may be impossible to determine for psychrophilic yeasts because of the toxicity of the compounds required to prevent media from freezing (Larkin and Stokes 1968). The lowest temperatures at which currently recognized Antarctic psychrophiles are known to grow are –3°C for *Cr. vishniacii* (Vishniac and Hempfling 1979a) and below –7 C for *Mrakia frigida* (Fell et al.) (as *Candida frigida*, *C. gelida*, and *C. nivalis*) (Larkin and Stokes 1968).

Antarctic strains of psychrophilic yeasts of known T_{max} are listed by species in Table 16.1. This list contains a presently unreliable identification: that of *Rh. aurantiaca*. At the time the two strains listed were identified this species was heterogeneous (Inacio and Fonseca 2004). The isolate A19 of Sabri et al. (2000) is apparently available at the Mycotheque of the University of Louvain-la-Neuve, Belgium (registration number 40,267), but does not have a deposited rDNA sequence and differs in T_{max} from the better known mesophilic strains [T_{max}>25°C, V30°C,V35°C, <37°C (Barnett et al. 2000), >25°C, <30°C (Inacio and Fonseca 2004)]. These strains were included because, while their identification is presently uncertain, there is some prospect of confirming the identity at least one of them.

Four of the listed species – *C. psychrophila*, *Cr. vishniacii*, *Leucosporidium antarcticum*, and *M. frigida* – probably are obligate psychrophiles since they were first described (though not necessarily by the same epithets) over 35 years ago yet no mesophilic strains have surfaced. *L. antarcticum* has been isolated in Germany, from a mature willow catkin near Neubrandenburg, but the temperature of cultivation was not mentioned (Kockova-Kratochvilova et al. 1972). Mesophilic strains are known for *Cr. victoriae*, which was the dominant species in soil from Nome, Alaska (personal observation); apparently minor occurrences were recorded in Portuguese seawater (Gadanho et al. 2003), Thuringian roots (Renker et al. 2004), and Mississippian lacewing guts (Woolfolk and Inglis 2004). Some 27 % of isolates from a soil sample from Providenya (Russian Far East) were identified as *Cr. watticus* or a very close relative with T_{max} ranging from below 25°C up to 30°C or above (personal observation). *Cr. antarcticus* isolates with T_{max}>25°C represented significant proportions of yeasts in soils from Iceland (14% at one site, 20% at another) and 6% of isolates from Providenya (personal observation). *Dioszegia hungarica* Zsolt (Barnett et al. 2000 as *Cr. hungaricus*) and *L. scottii* have well-known mesophilic strains (Barnett et al. 2000). With some reservations and explanations required, mesophilic strains also exist for *C. sake* and possibly for *Cr. friedmannii. C.* (*Torulopsis*) *austromarina* was synonymized with *C. sake* on the basis of the identity of the D1/D2 regions of rDNA during the only detailed correlation of differences in these sequences with nuclear DNA (nDNA)/nDNA similarities (Kurtzman and Robnett 1998) but without a comparison of the nDNAs of these particular species. The case for synonymy is its probability; sequence identity can accompany a 46% nDNA reassociation, indicating lack of conspecificity, but all the other examples of sequence identity in Table 2 of Kurtzman and Robnett's paper show conspecificity of the tested strains. The reason for doubting this synonymy is the very wide range of T_{max} which would then appear in *C. sake*, the 15 degrees between 22° C

Table 16.1 Psychrophilic yeasts from Antarctic sites

Species	Substrate and site	T_{max}	Reference	Reported as
Candida psychrophila	Penguin dung, Cape Royds	>17°C, <20°C	Goto et al. (1969)	*Torulopsis*
Candida sake	Seawater, Antarctic Ocean	18–21°C	Fell and Hunter (1974)	*Torulopsis austromarina*
Cryptococcus antarcticus	Damp soil and dry drainage channel, University Valley	>15°C, <20° to <25°C	Vishniac and Kurtzman (1992)	
Cryptococcus friedmannii	Rock with cryptoendolithic lichen	>20°C, <25°C	Vishniac (1985)	
Cryptococcus nyarrowii	Snow petrel carnage, soil and lichen	22°C	Thomas-Hall and Watson (2002)	
Cryptococcus statzelliae	Soil, Lichen Valley, Vestfold Hills	22°C	Thomas-Hall et al. (2002)	
Cryptococcus victoriae	Moss, lichen, soil, Granite Harbour	<20°C	Montes et al. (1999)	
	Soil, Lichen Valley, Vestfold Hills	<25C	Thomas-Hall et al. (2002)	
Cryptococcus vishniacii	Soil, arid highlands, Ross Desert	>15°C, <23–25°C	Vishniac and Hempfling (1979a)	
Cryptococcus watticus	Soil,Vestfold Hills; stromatolite and shell, Watts Lake	>20°C, weak 25°C, <30°C	Guffogg et al. (2004)	
	Soil, Edmonson Point	>15 to >22°C, <23°C	Tosi et al. (2005)	
Dioszegia hungarica	Taylor Valley melt stream	< room temperature (~23°C max)	Personal observation	
Leucosporidium antarcticum	Seawater NE end of Palmer Peninsula	>12°C, weak 17°C, <19°C	Fell et al. (1969)	
	Taylor Valley melt stream	< room temperature (~23°C max)	Personal observation	
Leucosporidium scottii	Soil ± algae, Granite Harbour, Wright Valley, Ross Island areas	"~15°C"	di Menna (1960,1966)	*Candida scottii*
	Moss, penguin rookery, Haswell Island	<20°C	Babyeva and Golubev (1969)	*Candida scottii*
	Mosses, Cape Evans; lakeside algae, Lake Fryxell	>15°C, <20°C	Goto et al. (1969)	*Candida scottii*
Mrakia frigida	Soil ± photosynthesizers, "Ross Dependency"	"~20°C"	di Menna (1966)	*Candida frigida, gelida, nivalis*
	Antarctic snow	>20°C, <25°C	Sinclair and Stokes (1965)	*Candida* sp. P216

(Fell and Hunter 1974) and 37^0 C (Barnett et al. 2000), a range which exceeds the normal (Vidal-Leira et al. 1979; van Uden 1984). *Cr. friedmannii* is at the least closely related to *Cr. saitoi*, a species proposed (Fonseca et al. 2000) largely on the basis of differences from *Cr. friedmannii* in D1/D2 rDNA sequences of an extent not generally accepted as contraindicating conspecificity, even when internal transcribed spacer sequences (Scorzetti et al. 2002) are also considered. If *Cr. saitoi* does not have sufficiently low nDNA similarity to *Cr. friedmannii* to complete its establishment as a valid species, its T_{max} of "approximately 30°C" would disqualify *Cr. friedmannii* as obligately psychrophilic. *Cr. nyarrowii* and *Cr. statzelliae* have been too recently described for any definite decision about the obligate nature of their psychrophily.

The D1/D2 large subunit rDNA based phylogeny of the species listed in Table 16.1 suggests that psychrophily has arisen independently several times when mesophilic ancestors became adapted to Antarctic habitats. *C. psychrophila* and *C. sake* (in which psychrophily is confined to the former *T. austromarina*) are both in the *Debaryomyces*/*Lodderomyces* clade of ascomycetous yeasts, but in distant branches of this clade (Kurtzman and Robnett 1998). Of the basidiomycetous species, *Cr. antarcticus, Cr. friedmannii*, and *Cr. vishniacii* have all been placed in neighboring branches of the *Albidus* clade of *Filobasidiales*, *Hymenomycetes* (Fonseca et al. 2000). Each species is there most closely associated with mesophilic species. *Cr. nyarrowii* (Thomas-Hall and Watson 2002) and *Cr. watticu*s (Guffogg et al. 2004) were described as most closely related to each other and then to *Holtermannia corniformis* in the *Tremellales*, *Hymenomycetes* (T_{max}>25°C, <30°C). *Cr. statzelliae* appears in the otherwise borderline (T_{max}>20°C, V25°C, <30°C) *Dioszegia* clade of the *Tremellales* (Thomas-Hall et al. 2002) including *Dioszegia hungarica. Cr. victoriae* sits in yet another tremellalean lineage as near neighbor to *Cr. dimennae* and *Bullera globispora* (Thomas-Hall et al. 2002), both with T_{max}>25°C. The genus *Leucosporidium* contains one mesophilic species (Barnett et al. 2000); the psychrophilic species are separately embedded among mesophiles in the *Microbotryum* clade of the *Urediniomycetes* (Fell et al. 2000). *Mrakia* is a monospecific genus grouping most closely with the psychrophile *Cr. aquaticus* in the *Cystofilobasidiales*, *Hymenomycetes* (Fell et al. 2000), an order with varied T_{max}.

16.3 Effects of Temperature on Antarctic Psychrophiles

Life in a cold habitat requires a complex suite of adaptations; life in or on Antarctic soils is further complicated by inconstancy of habitat temperatures. Even on continental Antarctica, excursions in both directions occur. Soil surface temperature at a site in Taylor Valley (Ross Desert) reached 23°C under insolation during the austral summer (Cameron 1974) and 26°C at another desert site (Cameron 1971). Even greater temperatures need not result in damage to Antarctic psychrophiles. Van Uden et al. (1968) pointed out that the rate of thermal death for two strains of *M. frigida* (as *C. frigida* and *C. nivalis*) was dissociated from growth rates, unlike the death rates of eight mesophilic yeasts; the psychrophiles died in roughly the same temperature range as the mesophiles. More recently Deegenaars and Watson (1997) reported that "essentially 100%" of stationary-phase *C. psychrophila* survived a

60-min exposure to 35°C. Cameron noted that "for approximately three to four weeks during midsummer, many Antarctic soils are subjected to diurnal freeze–thaw cycles" (Cameron 1971). While the psychrophiles obviously survive these conditions, studies of freeze–thaw resistance have been largely vitiated by the unnatural conditions under which they were done, for example, that which concluded that a psychrophilic yeast (*M. frigida* as *L. stokesii*) was less resistant to freeze–thaw stress than a mesophile (*C. utilis*, anamorph of *Pichia jadinii*) even when the growth stage and the rate of cooling below –5°C were considered (Meyer et al. 1975). Such studies are more realistically conducted in microcosms, as when indigenous (unidentified) yeasts appeared in greater biomass after four and seven freeze–thaw cycles of Signy Island peat cores (Wynn-Williams 1982).

The role of unsaturated fatty acids in maintaining membrane function at varying low temperatures has long been recognized throughout the biological world. The unsaturation index calculated by Watson's formula [percentage monoenes + 2(percentage dienes) +3(percentage trienes)/100] (Watson et al. 1976) increased with decreasing temperature in *M. frigida* (as *L. frigidum*, *L. gelidum*, *L. nivalis*, in Watson et al. 1976) and in *C. psychrophila* (as *Torulopsis*) and the psychrophilic strain of *C. sake* (*T. austromarina*) (Watson 1980). Comparable data for *Cr. antarcticus*, *Cr. statzelliae*, *Cr. nyarrowii*, and *L. scottii* are shown in Table 16.2. Table 16.2 illustrates the dependence of unsaturation on temperature and the high degree of unsaturation of psychrophiles, but should not be used for precise comparisons, as the index is affected by medium and growth phase as well as temperature. Mesophilic yeasts do, however, tend to have similar index numbers when grown at suboptimal temperatures. *C. lipolytica* (anamorph of *Yarrowia lipolytica*), when grown at 10°C, had index numbers of 1.07 and 1.27 (calculated from Kates and Baxter 1962). *C. saitoana* (as *T. candida*), a mesophilic yeast grown under the same conditions as the *C. psychrophila* and *C. sake* psychrophiles and showing the same pattern of increasing unsaturation with decreasing growth temperature, produced a more similar unsaturation index at 15°C (1.42), though not the same distribution of fatty acids (Watson 1980). (The distribution of individual fatty acids tends to be species-specific.)

When *M. frigida* (as *L. stokesii*) attempted to grow at 23°C (a temperature barely above T_{max}), increased cell size, irregular wall growth, and the production of anucleate and aseptate buds ensued (Silver and Sinclair 1979). This was not lethal; downshift to 15°C restored normal morphology. The mechanisms underlying these defects were not entirely clear but failure of DNA synthesis at 23°C was considered to account for the anucleate condition of the buds, while the persistence of RNA synthesis (impaired at 25°C) and protein synthesis (unaffected at either 23 or 25°C) accounted for the increase in cell size (Silver et al. 1977). While inappropriately fluid membranes do leak in this species (studied as *C. nivalis*) at 25°C (Nash and Sinclair 1968), other systems begin to fail as well. Ninety percent of polypeptide synthesis activity in a cell-free ribosome preparation was lost after 5 min at 35°C and polypeptide synthesis had vanished entirely at 40°C, though only 60% of charged soluble RNA (sRNA) binding activity was lost at the latter temperature. Thirty minutes at 35°C abolished the activity of three essential aminoacyl–sRNA synthetases, and greatly diminished the activity of three more, though five escaped injury (Nash et al.

Table 16.2 Fatty acid unsaturation in Antarctic psychrophilic yeasts

Strain	Growth temperature (°C)	Unsaturation index	Reference
Candida psychrophila CBS 5956	0	1.78	Watson (1980)
	1	1.37	
Candida sake CBS 6179	0	1.87	
	15	1.44	
Cryptococcus antarcticus CBS 7687	14	1.33	Vishniac and Kurtzmann (1992)
Cryptococcus antarcticus CBS 7688	14	1.46	
Cryptococcus antarcticus CBS 7689	14	1.18	
Cryptococcus antarcticus CBS 7690	14	1.42	
Cryptococcus nyarrowii CBS 8804	6	1.39	Thomas-Hall (personal communication)
	15	1.34	
Cryptococcus nyarrowii CBS 8805	6	1.55	
	15	1.38	
Cryptococus statzelliae CBS 8925, 8926	15	~1.31	Thomas-Hall et al. (2002)
Leucosporidium scottii AL25	10	1.71	Kates and Baxter (1962)
Leucosporidium scottii 5AAP2		1.69	
Mrakia frigida CBS 5270	8	1.93	Watson (1980)
	19	1.32	
Mrakia frigida CBS 5272	8	1.5	
	18	1.41	
Mrakia frigida CBS 5266	8	1.82	
	18	0.91	

1969). Thirty minutes at 35°C also inactivated another thermolabile enzyme, pyruvate decarboxylase (Grant et al. 1968).

Increased cell size and leaking were also noted by Sabri et al. (2001) in *Rh. aurantiaca* A19 (a psychrophilic strain) grown at 18°C. They ascribed the leaking to a toxic accumulation of myristoyl-cofactor A (CoA), which was in turn ascribed to characteristics of a long chain acyl-CoA thioesterase in this strain. Substrate affinity and catalytic efficiency were optimal below 5°C and decreased dramatically with increases in temperature, corresponding to the buildup of myristoyl-CoA.

The question of the structural differences which made these enzymes inefficient or thermolabile and, indeed, why some enzymes (but not all) need to be so closely tailored to certain temperature ranges has not been examined in any of these Antarctic psychrophiles. It has been suggested that the catalytic efficiency required to maintain adequate metabolic rates at low temperatures requires methods of obtaining conformational flexibility which render the thermolabile enzymes unstable at higher temperatures – a trade-off (Feller and Gerday 1997). These authors adduced, among other examples, the xylanases of a pair of mesophilic cryptococci of the *Albidus* clade – *Cr. adeliensis* (as *Cryptococcus* TAE85), which has a cold-adapted enzyme, and *Cr. albidosimilis* (as *Cr. albidus*), which does not. (A note to clarify the identity of these yeasts: *Cr. adeliensis* CBS 8351, ATCC 201412, has also been referred to as *Cr. albidus* TAE85, and *Cr. adeliae* – a nomen nudum; ATCC 34633, referred to as *Cr. albidus* by Feller and Gerday, was identified as *Cr. albidosimilis* by Scorzetti et al. 2000.) The catalytic efficiency (K_{cat}/K_m) of the cold-adapted and thermolabile xylanase of *Cr. adeliensis* was nearly 4 times higher at 4°C (largely because of an increase in K_{cat}). Comparison of their molecular structures indicated that the cold-adapted enzyme had less compact hydrophobic packing, lost one salt bridge, and destabilized the macrodipoles of the helices (Petrescu et al. 2000). These are changes which are considered to increase both flexibility and thermolability (Feller and Gerday 1997).

Exponential phase cells do not have the thermotolerance of stationary-phase cells. Less than 0.1% of the *C. psychrophila* cells which survived a 60-min exposure to 35°C would have survived had they been in the exponential phase (Deegenaars and Watson 1997). Although unsaturated fatty acids are typically more abundant in stationary-phase cells, membrane fatty acid composition and membrane fluidity are unrelated to thermotolerance in *Saccharomyces cerevisiae* (Swan and Watson 1997) – this point has not been studied in psychrophiles. Thermotolerance can, however, be induced. Psychrophilic yeasts, like most living things, have a buffer for sudden and stressing raises in temperature – the heat shock proteins (hsps). *C. psychrophila* cells grown at 15°C acquire induced thermotolerance after mild heat shocks for 30 min at 25°C or 3 h at 20°C, with the latter and better pretreatment providing roughly 90% survival after 2 h at 35°C. Cells with induced thermotolerance contained two intrinsic hsps (hsps 60 and 90) and three induced hsps: hsp 70 and novel heat-shock-inducible proteins at 80 and 110 kDA (Deegenaars and Watson 1997). The 110-kDa protein was also inducible in *M. frigida* (as *M. stokesii*, *M. frigida*, and *M. gelida*) but not in *L. antarcticum*. Mesophilic strains of *L. scottii* and *L. fellii,* lacking hsp 60, also contained hsp 104, a protein lacking in the psychrophiles but considered important for thermotolerance in *Saccharomyces cerevisiae* (Deegenaars and Watson 1998).

16.4 Other Niche Determinants for Psychrophilic Yeasts

The variety of isolate sources listed in the second column of Table 16.1 implies the presence of more than one series of habitat types in Antarctica, suggesting that factors other than temperature may define the niches of Antarctic psychrophiles. Do isolate sources describe ecological niches or simply the coincidence of investigators and accidental yeast occurrences? The list of taxonomically approved isolations in Table 16.1 is short and would be little longer if T_{max} for each isolate were not required; it does not provide enough instances to directly derive niche definitions. The isolations of *C. psychrophila* and *Cr. friedmannii* (but see the taxonomic discussion above) certainly do not warrant even speculation. The factors suggested are highly correlated – water and organic energy availability form a geographically irregular cline in Antarctic soils, from the arid highlands of the Dry Valleys (Ross Desert) in the Transantarctic Mountains to the better watered and vegetated glacial melt streams and lakes and the continental margin and finally the embracing Antarctic Ocean. The distribution of significant populations more or less follows this cline.

The arid highlands of the Dry Valleys are the habitat of *Cr. vishniacii*. This species, though occurring at a population density of less than one microcolony per gram of soil and sometimes failing to occur in samples producing single mesophilic isolates, dominated overall in soil samples from the arid highlands (Vishniac and Hempfling 1979a, b; and, under a variety of synonyms, Klingler and Vishniac 1988a). Many soil samples from this area failed to yield any yeasts, a failure attributable to the high salt content typical of Antarctic soils (Klingler and Vishniac 1988b; Vishniac and Klingler 1988). This salt content had been modified, presumably by occasional snow melt, in the samples in which *Cr. vishniacii* was found. *Cr. vishniacii* is xerotolerant but not halotolerant or osmotolerant. None of the cryptococci have been reported to be halo- or osmotolerant, though a model cryptococcus, *Cr. albidus*, successfully competed with other soil microbiota only under conditions of low water potential (Vishniac 1995). The ability of *Cr. vishniacii* to survive solely on the meager deposits of substrates from the exfoliation of cryptoendolithic lichens (Friedmann and Weed 1987) and the ablation of organic matter from the Taylor Valley lakes (Lake Chad and Lake Hoare) (Parker et al. 1989) indicates that this species is also oligotrophic.

The melt streams of glaciers in the Dry Valleys provide both water and, in season, a varied population of photosynthesizing algae and cyanobacteria as energy sources. The soil adjacent to melt streams and lakesides also support a varied flora as well as a quite limited fauna. Di Menna (1966) collected soil samples between the glaciers of the "Ross Dependency." Since the majority of the samples contained visible algae, lichens, or mosses and many of those lacking visible producers were moist with melt water, it is not surprising that she recorded yeast populations far greater than those of the arid highlands. Six samples taken between Nimrod and Beardmore Glaciers, lacking visible or culturable producers, contained 12–40 cfu g^{-1} of soil; moist soil containing producers contained 10^4 (Koettlitz Glacier, Campbell-Mawson Glaciers) to more than 10^5 cfu g^{-1} (Mawson-Koettlitz Glaciers).

A dry melt stream in University Valley was the home of *Cr. antarcticus*. Although the population density of *Cr. antarcticus* was low, persistence over 2 years

and the isolation of four biotypes strongly suggest a center of population in this habitat. *Cr. antarcticus* is more halotolerant and less oligotrophic than *Cr. vishniacii* (Vishniac and Kurtzman 1992). An active melt stream in Taylor Valley produced yeasts with larger populations and greater diversity. The aqueous phase of a sample contained 70 cfu ml^{-1}, the solid phase 32.26 microcolonies per gram of which some 70% were identified as *Dioszegia hungarica,* the remainder comprised *L. antarcticum* (Vishniac and Klingler 1988, personal observation of density and *Leucosporidium* sp. identification) and two isolates tentatively identified (i.e., not confirmed by sequencing) as *M. frigida* (as *C. curiosa*) and *Rh. foliorum* (as *C. foliorum*). *L. antarcticum* appears to be an aquatic yeast, since it was originally described from a large population (46 isolates) collected at four oceanic sites off of Joinville Island, at the tip of the Palmer Penninsula, from ocean water at –0.19 to –1.70°C at the time of collection (Fell et al. 1969). *L. antarcticum* has also been reported from a peat bog in Russia (Golubev et al. 1981 – cited from Summerbell 1983) and from a willow catkin in northeast Mecklenburg (Germany) (Kockova-Kratochvilova et al. 1972). *Dioszegia hungarica*, named for its geographic origin (Zsolt 1957 – cited from Barnett et al. 2000), includes strains with T_{max}>25°C and is well known from substrates other than water – soil, cereals, and flowers at a number of sites outside of Antarctica.

The Vestfold Hills best represent yeast diversity on the continental margin. Davis Station provided three recently described species – *Cr. nyarrowii*, *Cr. statzelliae*, and *Cr. watticus*, species whose niche is difficult to ascertain, as well as novel isolates of *Cr. victoriae*. Their descriptions provide insufficient evidence for declaring this habitat a center of population, mentioning only two isolates of each species and being recent for species recognition in other venues. *Cr. watticus* does occur at another site on the continental margin (Tosi et al. 2005) and at sites in the Russian Far East (Yttygran Island), Alaska (Nome), and Iceland (personal observation). Another obligate psychrophile, *M. frigida*, appears widespread in the northern hemisphere, in soil from Greenland (di Menna 1966) and high-latitude Russian regions [Babyeva et al. 1976; Babyeva and Azieva 1980 (unavailable papers cited from Summerbell 1983); Polyakova et al. 200l], and in Nome and Yttygran Island (personal observation). The other obligately psychrophilic species have been unsuccessfully searched for, using appropriate methods, in Russian Far Eastern, Alaskan, and Icelandic soils by the author as well as by the observers cited. *Cr. watticus*, and *M. frigida* are therefore considered to form significant populations only in relatively rich habitats, as are the sometimes mesophilic *Cr. victoriae* and *L. scottii. Cr. victoriae* has been reported from three widely separated locations outside of the Antarctic and was the dominant yeast species in a well-watered and densely vegetated soil sample from Nome (Alaska) (personal observation). The wide distribution of *L. scottii* in boreal and temperate areas was both reviewed and investigated by Summerbell (1983).

The Antarctic Ocean is the home of the psychrophilic strain of *C. sake* (i.e., the former *T. austromarina*). It is well represented there. A total of 85 strains were isolated from two sectors of the Antarctic Ocean with temperatures of 2.06–3.59°C at the time of collection. As it has not been reported from elsewhere, it may be considered a marine yeast.

16.5 Mesophilic Yeasts in Antarctica

The much longer list of Antarctic mesophilic isolates is given in Table 16.3. This table includes some isolates which were described with T_{max}<25°C; the species to which they are attributed all have T_{max}>25°C according to Barnett et al. (2000). All of the listed species except the recently described *Cr. adeliensis* occur outside of Antarctica, even *Cr. albidosimilis,* which was isolated from sparrow plumage in the Czech republic (Scorzetti et al. 2000) and *Pseudozyma antarctica*, which was isolated from rice in Japan by Ito et al. (1974) as *Trichosporon oryzae*. Tubaki (1961), one of the earlier investigators of Antarctic yeasts, dismissed his mesophilic isolates as "not of the Antarctic". Table 16.3 does omit isolates from the food cache of the Scott Expedition as obviously "not of the Antarctic", but does include others which were quite possibly imported. The situation is ambiguous for several reasons: some mesophiles are psychrotolerant; the border between psychrophily and mesophily is artificial (T_{max} is a continuous variable); and the coastal areas include active volcanoes at which the soil temperature can reach 50°C (Bargagli et al. 1996). The coastal areas are heterogeneous but in general are relatively rich in plant and animal life; their soils are lower in salinity. The soils at Casey Station (on the coast of eastern Antarctica) have mean total organic carbon contents ranging from 5 to 45 mg g^{-1} in the surface layer (Beyer et al. 2004a), including compounds comparable to those found in other podzols (with the exception of lignin and its derivatives; mosses do not produce lignin) (Beyer et al. 2004b).

It may be possible for mesophiles to colonize the milder areas. The soils of volcanic areas have not been adequately explored. The sites from which the isolates of Table 16.3 were obtained are almost all in areas with the higher photosynthetic productivity that accompanies increased water availability. Investigators have naturally preferred to search readily available sites in the vicinity of the coastal bases and moister sites in the justified hope of obtaining greater results. Reports of mesophile presence become more meaningful as survival of yeast cells becomes less assured; zymophagous invertebrates also inhabit the less arid and better vegetated areas. Protozoa and nematodes are capable of outstripping yeast reproduction at 1.5°C. Protozoa studied in a peat core from Signy Island (held at 1.5°C) consumed 4.3 yeast cells per amoeba per day, nematodes 8.8 yeast cells per nematode per minute (Wynn-Williams 1983). Taking all of these factors, and proximity to human activity, into account, we can group these isolates in order of the probability that they are "of the Antarctic."

The most probable candidates for indigenicity are *Guehomyces pullulans* and *Cr. adeliensis. Guehomyces pullulans* [a comb. nov. for *Trichosporon pullulans* (Fell and Scorzetti 2004)], a common soil yeast, includes psychrophilic strains (Barnett et al. 2000). Such psychrophilic strains constituted 24% of yeasts in a soil sample taken near Vatnajokul (Iceland) (personal observation). An Arctic isolate (Grise Fjord, Northwest Territory, Canada) was highly psychrotolerant, capable of growth at a k (generations/time in hours) value of 0.020 at 0°C (Julseth and Inniss 1990b) (k=0.231 at the "optimal" temperature of 21°C; growth was inhibited at T_{max}=30°C with k=0.005). One wonders why di Menna (1966) found only a single isolate at Scott Base, one out of the 126 Antarctic soil samples she examined, but three in the

Table 16.3 Antarctic mesophilic yeasts

Species	Substrate and site	T_{max}	Reference	Reported as
Bulleromyces albus	Moss-bed soil, Schirmacher Oasis	>25°C, V30°C, <35°C[a]	Ray et al. (1989)	*Bullera*
Candida albicans	Soil adjacent to camp liquid waste barrel, Taylor Valley	>25°C, V30– 45°C[a]	Baublis et al. (1991)	
Candida parapsilosis	Ice tunnel, South Pole base	>37°C, V40–42°C, <45°C[a]	Jacobs et al. (1964)	
Candida saitoana	Soil containing photosynthesizers, "Ross Dependency"	>30°C, V35–42°C, <45°C[a]	di Menna (1966)	*Torulopsis candida*
	Moss, penguin rookery, Haswell Island	> "room temperature"	Babyeva and Golubev (1969)	
Candida sake	Algae and shore soil, Lake Bonney; penguin dung, Cape Royds	33°C	Goto et al. (1969)	*Candida australis*
Clavispora lusitaniae	Soil adjacent to camp liquid waste barrel, Taylor Valley	>42°C, V45°C[a]	Baublis et al. (1991)	*Candida lusitaniae*
Cryptococus adeliensis	Decaying algae in ice, near Terre Adelie	>25°C, w30°C	Scorzetti et al. (2000)	
Cryptococus albidus	Soil, Edmonson Point	>25°C, <30°C	Tosi et al. (2005)[b]	
Cryptococcus albidosimilis	Soil, Linnaeus Terrace (Mt. Oliver/Wright Valley)	>25°C, slow 30°C, <35°C	Vishniac and Kurtzman (1992)	
Cryptococcus diffluens	Soil, Wright Valley	> "room temperature"	di Menna (1960)	
	Moss, penguin rookery, Haswell Island and sites (± lichen) Molodozhnaya Station	> "room temperature"	Babyeva and Golubev (1969)	
Cryptococcus humicola	Water, Lake Vanda, lakeshore soil	>25°C, V30–37°C, <40°C[a]	Goto et al. (1969)	*Candida*
	Soil, Schirmacher Oasis	>25°C, <37°C	Ray et al. (1989)	*Candida*
Cryptococcus luteolus	Pool edge, Lake Vanda; soil ± photosynthesizers, "Ross Dependency"	>25°C, V30– 35°C, <37°C[a]	di Menna (1960, 1966)	
	Soil, The Strand Moraines, McMurdo Station		Atlas et al. (1978)	

Continues

Table 16.3 Antarctic mesophilic yeasts—*cont'd*

Species	Substrate and site	T_{max}	Reference	Reported as
Cryptococus macerans	Soil + photosynthesizers, "Ross Dependency"	>25°C, <30°C[a]	di Menna (1966)	*Rhodotorula*
Debaryomyces hansenii	Soil, Showa Base	>30°C	Soneda (1961)	*Torulopsis famata*
	Soil, East Ongul Island and Antarctic continent	>25°C, V30–40°C, <42°C[a]	Tubaki (1961)	*T. famata*
	Soil + photosynthesizers, "Ross Dependency"		di Menna (1966)	*T. famata*, *Debaryomyces koeckeri*, *D. subglobosus*
	Moss, penguin rookery, Haswell Island	> "room temperature"	Babyeva and Golubev (1969)	*T. famata*
	Soil from skua's nest, Schirmacher Oasis	>25°C, V30–40°C, <42°C[a]	Ray et al. (1989)	
Guehomyces pullulans	Soil + photosynthesizers, Scott Base	>20°C, V25°C, <30°C[a]	di Menna (1966)	*Trichosporon*
Issatchenkia orientalis	Soil adjacent to camp liquid waste barrel, Taylor Valley	>40°C, V42,45°C[a]	Baublis et al. (1991)	*Candida krusei*
Pseudozyma antarctica	Sediment Lake Vanda	>30°C, <35°C	Goto et al. (1969)	*Sporobolomyces antarcticus*
Rhodosporidium sphaerocarpum	Antarctic Ocean near Palmer Peninsula and Archipelago	30°C	Newell and Fell (1970)	
Rhodosporidium toruloides	Water, Lake Vanda	>30°C, <35°C	Goto et al. (1969)	*Rh. glutinis* var. *refusa*
Rhodotorula aurantiaca	Moss	<20°C	Babyeva and Golubev (1969)	
	Accepted T_{max} range for *Rh. aurantiaca*	>25°C, V30,35°C, <37°C[a]	Barnett et al. (2000)	
Rhodotorula diffluens	Water and sediment, Lake Miers, Lake Vanda	~25°C	Goto et al. (1969)	*Candida*

Rhodotorula glutinis	Moss, penguin rookery, Haswell Island and lichen sites Molodozhnaya Station	>"room temperature"	Babyeva and Golubev (1969)	
Rhodotorula graminis	Soil, Wright Valley and soil with photosynthesizers, "Ross Dependency"	>20°C	di Menna (1960, 1966)	
	Soil, Asgard Range	>30°C, <35°C[a]	Atlas et al. (1978)	
Rhodotorula laryngis	S. Onofri isolate, soil, Edmonson Point	>25°C, <30°C	Tosi et al. (2005)	
Rhodotorula marina	Soils with algae between Campbell and Mawson glaciers	>30°C, <35°C[a]	di Menna (1966)	
Rhodotorula minuta	Water, Lake Miers and Lake Vanda	30°C	Goto et al. (1969) ‡	*Rh. texensis*
Rhodotorula mucilaginosa	Soil, "the continent" and West Ongul Island	>25°C, <30°C	Soneda (1961)	
	Soil + photosynthesizers, "Ross Dependency"	>30°C, V35–37°C, <40°C[a]	di Menna (1966)	
	Moss, penguin rookery, Haswell Island; sites (+ lichen) Molodozhnaya Station	> "room temperature"	Babyeva and Golubev (1969)	
	Water and soil, Lake Vanda, Lake Miers	33°C	Goto et al. (1969)	*Rh. rubra*
	Soil, Victoria Valley	>30°C, V35–37°C, <40°C[a]	Atlas et al. (1978)	
	Lake shore, lake sediment and under rock, Schirmacher Oasis	>25°C, <37°C	Ray et al. (1989)	
	Soil adjacent to camp liquid waste barrel, Taylor Valley	>30°C, V35–37°C, <40°C[a]	Baublis et al. (1991)	*Rh. rubra*
Rhodotorula pallida	Soil + photosynthesizers, "Ross Dependency"	> "room temperature"	di Menna (1966)	
Sporidiobolus johnsonii	Soil, Ross Desert	>25°C	Vishniac and Hempfling (1979b)	*Sporobolomyces holsaticus*
Sporidiobolus pararoseus	Soil and lichens, vicinity of Molodozhnaya Station	> "room temperature"	Babyeva and Golubev (1969)	*Sporobolomyces pararoseus*

Continues

Table 16.3 Antarctic mesophilic yeasts—*cont'd*

Species	Substrate and site	T_{max}	Reference	Reported as
Sporidiobolus salmonicolor	Soil, Wright Valley	>30°C, V35–37°C, <40°C[a]	di Menna (1960)	*Sporobolomyces odorus*
	Soil, Marble Point and Brown Peninsula	>30°C, V35–37°C, <40°C[a]	Atlas et al. (1978)	*Sporobolomyces*
Sporobolomyces roseus	Lakeshore sediment and lichen, vicinity of Molodozhnaya Station	> "room temperature"	Babyeva and Golubev (1969)	
	Soil, Ross Desert	>25°C	Vishniac and Hempfling (1979b)	
Stephanoascus ciferrii	Microbial mat, Lake Hoare	>35°C, V37–45°C[a]	Baublis et al. (1991)	*Candida ciferrii*
Torulaspora delbrueckii	Soil, Victoria Land	>30°C, V35–40°C, <42°C[a]	del Frate and Caretta (1990)	
Trichosporon cutaneum	Soil, Showa Base	>25°C, <30°C	Soneda (1961)	
	Soil, Showa Base	>30°C, V35°C, <37°C[a]	Tubaki (1961)	
	Soil adjacent to camp liquid waste barrel, Taylor Valley		Baublis et al. (1991)	*Trichosporon beigelii*
Trichosporon moniliiforme	Lake water, Lake Vanda	>30°C, <35°C	Goto et al. (1969)	*Tr. cutaneum* var. *antarcticum*

V response is variable (strain-dependent)
[a] T_{max} taken from Barnett et al. (2000)
[b] Other reports of these species are considered unreliable (see text).

eight Greenland soil samples she investigated. The T_{max} of di Menna's isolate was not reported. The Canadian strain produced 11 or 12 inducible hsps (depending on the method of shocking) (Berg et al. 1987; Julseth and Inniss 1990a); it was also the first yeast which evinced the induction of 10–26 cold-shock proteins (csps) (again depending upon the method of shocking) (Julseth and Inniss 1990b). These proteins act as buffers; they do not extend growth beyond the previously determined range of temperatures. The hsps significantly extended survival at 45°C, (but not 50°C); the csps were induced by suddenly switching cells from near optimal temperatures to 5°C, a temperature well within the normal growth range of this isolate. Yet buffers may be very useful when sudden changes in insolation occur.

Cr. adeliensis also grows (weakly) at 30°C. The single known isolate grew better than the Czech isolate of *Cr. albidosimilis* at 4°C, a situation that was reversed at 28°C (Scorzetti et al. 2000). In another contrast to *Cr. albidosimilis*, the xylanase of *Cr. adeliensis* was cold-adapted (vide supra). *Cr. adeliensis* was isolated from a relatively rich source of organic energy. These factors, taken together, make *Cr. adeliensis* a possible Antarctic native.

The least probable candidates for indigenicity are those associated with field camps, are fermentative (presumably copiotrophs), or have T_{max} well above 30°C. When considering human contamination it is important to remember that a "base" is not a field camp; a base is a geographic entitlement which contains at least the equivalent of a field camp but is not limited to its immediate vicinity. Isolates from a base need not therefore be suspect. Rookeries may also be centers of imported propagules but it is premature to declare every yeast isolated there an unsuccessful immigrant. Of the yeasts isolated from soil adjacent to the field camp liquid-waste barrel by Baublis et al. (1991) *C. albicans*, *Clavispora lusitaniae* (as *C. lusitaniae*), and *Issatchenkia orientalis* (as *C. krusei*) fail on all three criteria. *Trichosporon cutaneum* is a "rather rare species"(Gueho et al. 1992), a superficial pathogen of warm-blooded animals (Gueho et al. 1994) and so surely a result of human presence. *Saccharomyces cerevisiae* and *Stephanoascus ciferrii* (as *C. ciferrii*), isolated from "benthos box core strata" by the same group may not have been contaminated by human presence, but still fail on one or two counts. The work of Meyer et al. (1962) on the refuse of the Scott Expedition demonstrates that ascomycetous yeasts entering Antarctica by whatever means could have a lengthy survival. The *C. parapsilosis* in the South Pole ice tunnel (Jacobs et al. 1964) fails on the same criteria as *C. albicans*.

We are left with a still considerable list of species which may or may not have been able to colonize. Many of these species were reported after the appearance of only one or two isolates, lending an adventitious aspect to their occurrence in Antarctica. A single cell may easily divide during the manipulations required for isolation. Population densities strongly hinting at establishment were reported for *Rhodosporidium sphaerocarpum* and *Rh. mucilaginosa. Rhodosporidium sphaerocarpum* has a mesophilic T_{max}, but 37 isolates were obtained from the 15 associated sites in the Antarctic Ocean, from water temperatures of −1.68 to 1.33°C (Newell and Fell 1970). The seven reports of *Rh. mucilaginosa* from Antarctic areas did not always make it clear how many isolates came from each site (an annoying omission in several other reports as well) but Babyeva and Golubev (1969) isolated 15 strains

from a moss collected in the penguin rookery on Haswell Island where the only other species yielding more than one or two isolates was a psychrophilic strain of *Rh. aurantiaca* (three isolates). While T_{min} is not recorded for Babyeva and Golubev's Antarctic strain, the strains of *Rh. mucilaginosa* isolated by Goto et al. (1969) were declared to be capable of growth at 0°C. *Debaryomyces hansenii* turned up at five locations, but never in quantity; this species is ascosporogenous and distributed so widely that it would be remarkable if it did not turn up in Antarctica. Had isolates of the remaining species simply not met their consumers yet?

16.6 Conclusion

Any assessment of yeast biodiversity in the Antarctic must remain incomplete at this time. The Antarctic provides a variety of habitats, some of which have never been adequately examined: the warm volcanic soils; the streams whose photosynthetic flora are meticulously catalogued, but the yeasts barely so; the phanerogams of the Palmer Peninsula. Antarctica is open to any yeast that can survive the various means of transport, but the absence of experimental evidence of the inability of putatively allochthonous yeasts to colonize leaves the position of many isolates ambiguous. It does appear that yeast biodiversity correlates with the availability of water and organic sources of energy. The arid highlands of the Ross Desert provide a unique habitat which contains a single species adapted to this habitat and which is unknown elsewhere – *Cr. vishniacii*, the eponym of a scientist who met with a fatal accident there – while the streams and margins of the continent contain six or more species of psychrophiles, many of which have also been found in Arctic or near-Arctic regions with temperatures approaching (at least seasonally) the cold of the Antarctic margins and with more varied vegetation.

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Yeast Biodiversity in Tropical Forests of Asia

Takashi Nakase, Sasitorn Jindamorakot, Somjit Am-in, Wanchern Potacharoen and Morakot Tanticharoen

NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, 2-5-8 Kazusakamatari, Kisaraza, Chiba 292–0818, Japan (e-mail: nakase-takashi@nite.go.jp)

17.1 Introduction

It is well known that the tropical region of Asia, especially southeastern Asian countries, has a long history of utilizing microorganisms for the production of foods and beverages. A number of unique fermented foods and beverages have been produced and consumed in this region. Quite naturally, the investigation of yeasts associated with these fermented products, including starters for fermentation, were started in the early stage of the history of microbiology of Asia by European and Japanese researchers. However, different from mushrooms and filamentous fungi, yeasts living in the natural environment such as forests were not studied until recent years. The recent studies from the viewpoint of biodiversity revealed the richness in the species diversity of yeasts in tropical forests though such studies have just started and further extensive studies are required. This chapter discusses yeasts found in forests of tropical and subtropical regions of Asia. The irreducible minimum of yeasts associated with fermented products is also discussed if it is necessary for comparing them with those associated with forests.

17.2 Yeasts in Tropical Forests of Asia

17.2.1 Yeasts Found in Forests of Thailand

Among tropical Asian countries, yeasts have been most extensively studied in Thailand. However, the study of yeasts living in the natural environment has just started. Yeasts are a group of microorganisms that require more intense study. Currently some 150 species have been recorded in Thailand, from fermented products and substrates in the natural environments.

Generally speaking, yeasts found in fermented foods and related substrates are not rich in diversity. Ascomycetous yeasts are dominant in these substrates and species are common to other countries. Saito et al. (1983) isolated 386 strains of yeasts from 54 samples of fermented foods and related substrates and identified

them as 21 species in 11 genera. *Saccharomyces cerevisiae* was the dominant species and occupied 30.6% of the isolates and was found in 39.4% of samples, followed by *Issatchenkia orientalis* (anamorph *Candida krusei*) (28.2%), *Hanseniaspora valbyensis* (9.8%), *C. tropicalis* (5.4%), *Pichia membranifaciens* (4.7%), *P. ohmeri* (4.4%) and *Saccharomycopsis fibuligera* (3.9%). The remaining 14 species occupied 1.8–0.3% of the isolates. In this study, all of the isolates belong to known species. However, yeasts were identified by keys devised by Barnett et al. (1979) in this study so that some misidentification might be inevitable.

Saccharomycopsis fibuligera is a unique species found in fermented foods in southeastern Asian countries. This species has strong amylolytic activity and shows alcoholic fermentation, and is often found in various kinds of starters in this region. Limtong et al. (2002) reported that this species is contained in most of the *loog-pang* solid starter for fermentation in Thailand.

Suzuki et al. (1987) studied 80 strains of yeasts that were isolated from fermented foods and related substrates in 1984 by several Thai researchers. They identified these yeasts as 17 species in nine genera. *I. orientalis* is the dominant species and occupied 42.5% of the isolates, followed by *Saccharomyces cerevisiae* (11.3%), *I. occidentalis* (anamorph *C. sorbosa*) (10.0%) and *C. tropicalis* (6.3%). In this study, a strain was found to represent a new species and described as *C. stellimalicola* (Suzuki et al. 1994). In these two studies on yeasts associated with fermented foods and related substrates, yeast species were common to those found in fermented foods in other countries, not only in southeastern Asian countries but also in European countries and in Japan. Further, more than 80% of the isolates belong to species that are commonly found in these two studies.

Jindamorakot (2000) studied yeasts associated with fermented foods and related substrates using high-salt-content media. She isolated 137 strains and identified 123 of them as 33 known species. *I. orientalis* is the dominant species and occupied 27.0% of the isolates, followed by *S. cerevisiae* (8.8%), *C. parapsilosis* (7.3%) and *C. glabrata* (5.8%). In this study, 57.6% of the yeasts belonged to species commonly found in studies by Saito et al. (1983) and/or by Suzuki et al. (1987), in spite of the use of high-salt-content media for isolation. Two strains from fermented soybean and dried salted squid were identified as *Citeromyces matritensis* in this study. However, further detailed studies revealed that the two strains represented a new species. They were described as *Citeromyces siamensis* (Nagatsuka et al. 2002). This is the second species of this genus. Jindamorakot assigned the remaining 12 strains to the genera *Debaryomyces*, *Saccharomyces* and *Candida* but she could not identify them as any known species of these genera. Probably, these strains represent hitherto undescribed species.

It is concluded that yeasts associated with fermented foods and related substrates in Thailand are not rich in biodiversity and that most of them belong to already described species that are common to other southeastern Asian countries, European countries and Japan.

In contrast to fermented foods and related substrates, yeasts found in the natural environment are rich in biodiversity and many undescribed species have been found so far as discussed later.

Basidiomycetous yeasts, especially ballistoconidium-forming yeasts, have been extensively studied in Thailand. In 1987 and 1990, isolation studies of this kind of

yeast were carried out as a joint study of Thailand Institute of Scientific and Technological Research (TISTR) and Japan Collection of Microorganisms (JCM).

In 1987, 42 samples of various plants were collected in the forests, fields and rice fields and from the roadsides in the western suburb of Bangkok and near Ayutthaya, and also in the urban areas of Bangkok, including plant leaves collected in several markets. Yeasts were isolated from these samples at 23 and 30°C by the ballistoconidium-fall method (Nakase and Takashima 1993). Sixty-three strains of ballistoconidiogenous yeasts were isolated from 20 samples (50%) examined. The frequency of isolation reached 81.2% when samples were collected in the suburbs of Bangkok and Ayutthaya but it was very low (15%) when samples were collected in the urban areas of Bangkok, including markets (Nakase et al. 2001).

Sixty-three isolates were identified as 16 species in the genera *Bullera*, *Kockovaella*, *Bensingtonia*, *Sporidiobolus*/*Sporobolomyces* and *Tilletiopsis*. Three strains produced non-ballistoconidiogenous stalked conidia and budding yeast cells in addition to ballistoconidia. This kind of conidiogenesis has not been found in hymenomycetous ballistoconidium-forming yeasts so far; therefore, a new genus *Kockovaella* was proposed for these strains. Two species, *K. imperatae* and *K. thailandica,* were included in the genus (Nakase et al. 1991). In addition to new species of *Kockovaella*, *Sporobolomyces nylandii* and *S. vermiculatus* were also described (Takashima and Nakase 2000). Three strains out of six of yeast-like fungi were assigned to *Tilletiopsis*. They were found to represent three new species, *T. derxii*, *T. oryzicola* and *T. penniseti* (Takashima and Nakase 2001a).

In 1990, 73 ballistoconidiogenous yeast strains were isolated from 33 plant materials (82.5%) out of 40 collected in forests, grasslands and rice fields along the southeastern seacoast from Bangkok to Pattaya. The isolation of yeasts was carried out at 25°C. These yeasts were identified as 13 species in the genera *Bullera*, *Kockovaella*, *Bensingtonia* and *Sporidiobolus*/*Sporobolomyces. Bensingtonia musae* (Takashima et al. 1995), *Bullera penniseticola*, *K. sacchari* (Takashima and Nakase 1998), *Sporobolomyces blumeae* and *S. poonsookiae* (Takashima and Nakase 2000) were described as new species. In this isolation study, *Tilletiopsis* strains were not isolated though they are commonly found in the samples.

A total of 136 strains isolated in 1987 and 1990 were identified as 21 species, 105 strains (77.2%) as nine known species and 31 strains (22.8%) as 12 undescribed species (Nakase et al. 2001). Eight of them were commonly isolated in 1987 and 1990, but the remaining 13 species were isolated in either year. In the isolation study of 1987, *Sporobolomyces shibatanus* (teleomorph *Sporidiobolus pararoseus*) was the dominant species and was found in 21.4% of plants examined, followed by *B. sinensis* (19.0%), *B. crocea* (16.7%) and *S. salmonicolor* (11.9%). In the isolation study in 1990, the most frequently isolated species was *B. sinensis* and was found in 52.5% of plant samples examined, followed by *Sporobolomyces shibatanus* (30.0%), *Sporidiobolus ruineniae* (17.5%) and *Sporobolomyces poonsookiae* (15.0%). *Sporobolomyces roseus*, the most frequently encountered ballistoconidium-forming species in the temperate zones, was not found in these studies.

In addition to the ballistoconidiogenous yeasts just mentioned yeast colonies excreting black pigment were found from several samples of 1987 and 1990. These colonies are assumed to be anamorphs of *Entyloma* (Boekhout, personal communication).

Among the ballistoconidium-forming yeasts isolated in 1987 and 1990, a strain had Q-9 as the major component of ubiquinone and was described as *Bensingtonia musae* (Takashima et al. 1995). The remaining yeasts had Q-10 and were assigned to the genera *Bullera*, *Kockovaella*, *Sporidiobolus*, *Sporobolomyces* and *Tilletiopsis* (Nakase et al. 2001). No yeast was found to have Q-10 (H_2) as the major ubiquinone.

In the studies carried out in 1987, two incubation temperatures, 23 and 30°C, were used for the isolation of yeasts. Twelve species were isolated at 23°C, eight were isolated at 30°C and four were isolated at both temperatures. Apparently, the use of 23°C is better than 30°C to recover a variety of yeast species. It is interesting that several undescribed species of ballistoconidiogenous yeasts found in Thailand have relatively low maximum growth temperatures below 30°C in spite of the high atmospheric temperatures during the isolation study (Nakase et al., unpublished). We examined the maximum growth temperatures of yeasts isolated in Thailand and Japan and found that there is no relationship between the maximum temperature of yeasts and the atmospheric temperature of the places where the yeasts were isolated, as discussed later.

Further isolation studies of ballistoconidium-forming yeasts were carried out in the project named "Asian Network on Microbial Research" supported by the Science and Technology Agency of the Japanese government. In 1996, Fungsin isolated 175 strains of yeasts from plants collected in a protected tropical rain forest in Sakaerat, Nakhon Ratchasima Province, northeastern Thailand, by the ballistoconidium-fall method (Fungsin 2003). After confirming the ballistoconidium-forming ability, he assigned 151 strains to the genera *Bullera* (51 strains), *Dioszegia* (two strains), *Kockovaella* (four strains), *Bensingtonia* (ten strains), *Rhodotorula* (six strains), *Sporidiobolus* (two strains), *Sporobolomyces* (57 strains) and *Tilletiopsis* (18 strains) as shown in Table 17.1. He identified 141 of them as 47 species and a variety, 14 known species, 33 undescribed species and an undescribed variety. Among the 141 strains identified, 106 (75.2%) belonged to undescribed species or an undescribed variety. The frequency of isolation of the undescribed taxa was more than 3 times that of those from plants in Bangkok and along the seacoast from Bangkok to Pattaya (Nakase et al. 2001). This result clearly suggests that ballistoconidium-forming yeasts associated with plants in protected forests of Thailand are rich in biodiversity and numerous unknown species live in these substrates.

Among the undescribed species just mentioned, six were described as new species in the genera *Bensingtonia*, *Kockovaella* and *Bullera*, i.e., *Bensingtonia thailandica* (Fungsin et al. 2001), *K. barringtoniae* (Fungin et al. 2002a), *B. arundinariae* (Fungsin et. al. 2002b), *B. siamensis*, *B. panici* (Fungsin et al. 2003a) and *B. sakaeratica* (Fungsin et al. 2003b). Fungsin named two more new species, *B. lagerstroemiae* and *B. nakhonratchasimensis*, but they have not yet been validly described (Fungsin 2003). The two undescribed *Bullera* species were located in the *Trichosporonales* clade in the phylogenetic trees based on the 18S ribosomal DNA (rDNA) and D1/D2 domain of 26S rDNA sequences though they did not produce arthroconidia, a characteristic feature of the *Trichosporon*. Further, he found that one strain (TY-213) belonged to *Sporobolomyces* by its morphology of ballistoconidia but is located in *Ustilaginomycetes* and not in *Urediniomycetes* in the phylogenetic tree based on 18S rDNA sequences. He presumed that this strain may represent a new genus of ustilaginomycetous yeasts.

Table 17.1 Ballistoconidium-forming yeasts found in Thailand (Fungsin 2003)

Species	No. of strains		Species	No. of strains	
Hymenomycetous yeasts			Urediniomycetous yeasts		
Bullera	51		*Bensingtonia*	10	
B. arundinariae		1	*B. thailandica*		10
B. boninensis		3	*Rhodotorula*	6	
B. coprosmaensis		8	*R. marina*		5
B. hannae		1	*R. bogoriensis*		1
B. lagerstroemiae		1	*Sporidiobolus*	2	
B. panici		1	*S. ruineniae*		2
B. sakaeratica		2	*Sporobolomyces*	57	
B. schimicola		1	*S. odoratus*		4
B. siamensis		3	*S. vermiculatus*		1
B. sinensis		4	*Sporobolomyces* spp., Q-10[b]		33
B. variabilis		3	*Sporobolomyces* spp., Q-10(H_2)[c]		19
B. nakhonratchasimensis		6	Ustilaginomycetous yeasts		
Bullera spp.[a]		17	*Sporobolomyces*	1	
Dioszegia	2		*Sporobolomyces sp.* TY-213		1
D. zsoltii var. *thailandica*		2	*Tilletiopsis*	18	
Kockovaella	4		*Tilletiopsis* spp.		18
K. barrintoniae		1			
K. sacchari		1			
K. thailandica		1			
Kockovaella sp.		1	Total		151

[a]Seven species are recognized.
[b]Twelve species are recognized.
[c]Two species are recognized.

In this isolation study, Fungsin (2003) reported that yeasts with Q-10 were recovered from all of the samples examined and occupied 80.9% of isolates, yeasts with Q-10(H_2) were found in 57.7% of plant samples and occupied 12.6% of the total isolates and yeasts with Q-9 were recovered from 26.9% of samples and occupied 6.6% of the isolates. This result is quite different from the studies in 1987 and 1990 on plants collected in urban and suburban areas of Bangkok and the southeastern seacoast from Bangkok to Pattaya where no yeasts with Q-10(H_2) and only one strain of yeast with Q-9 were isolated (Nakase et al. 2001). Yeasts with Q-10(H_2) are known to be rare in the temperate zones but rich in the tropical and subtropical regions (Nakase 2000). Fungsin's data suggested the richness of yeast diversity in protected forests of Thailand.

According to Fungsin (2003), ballistoconidium-forming strains of *Rhodotorula marina* and *R. bogoriensis* were isolated from a forest in Sakaerat. Apparently, ballistoconidium-forming ability has no phylogenetic value though their morphology has a certain taxonomic value (Nakase 2000). However, this property is very useful for the selective isolation of basidiomycetous yeasts from substrates in the natural environment. Generally speaking, basidiomycetous yeasts grow slower than ascomycetous yeasts and are often suppressed on isolation media by the latter yeasts.

In addition to the basidiomycetous yeasts mentioned before, Prillinger et al. (1997) found a new stalked conidium-forming yeast from a lichen in Thailand and described it as *Fellomyces thailandicus*.

The study of ascomycetous yeasts in the natural environment followed the study of basidiomycetous yeasts. Limtong and coworkers carried out extensive studies on the thermotolerant methylotrophic yeasts and found that these kinds of yeasts were widely distributed in the natural environment of Thailand. Three of them were described as new species of the genera *Pichia* and *Candida*, *P. siamensis*, *C. krabiensis* and *C. sithepensis* (Limtong et al. 2004).

Recently, Nakase and coworkers carried out extensive isolation studies of yeasts from substrates collected in the natural environment of Thailand using the direct streaking technique and enrichment methods using yeast extract–malt extract agar supplemented with 100 ppm chloramphenicol and 0.2% sodium propionate. They sequenced the D1/D2 domain of 26S rDNA of 283 yeast isolates from insect frass, mosses, flowers, wild mushrooms and several other substrates. One hundred and ninety-four strains belonged to ascomycetous yeasts and the remaining 89 belonged to basidiomycetous yeasts (unpublished). These yeasts were tentatively identified on the basis of the D1/D2 sequences by consulting a guideline of Kurtzman and Robnett (1998), namely, strains having a nucleotide difference of zero to one are conspecific, those having a nucleotide difference of two to three are conspecific or sister species of earlier described species and those having a nucleotide difference of four or more are different species. In the case of known yeasts, morphological and physiological properties were also compared.

On the basis of the above mentioned standard, 194 strains of ascomycetous yeasts were classified into 134 species. Ninety-six strains were identified as 41 known species (Table 17.2). In this study, yeast isolates having the same D1/D2 sequences as yeasts whose sequences were already registered in DNA data banks were dealt with as known species because they were already found in places other than Thailand. The 41 known species just mentioned include such seven species consisting of ten strains (Table 17.2). *C. tropicalis* (nine strains) is the most frequently isolated and is followed by *S. kluyveri* (eight strains), *S. cerevisiae* (five strains), *Metschnikowia koreensis* (five strains) and *Debaryomyces nepalensis* (four strains).

Eighty-four ascomycetous strains were found to represent 79 undescribed species and the remaining 14 strains showed two to three nucleotide differences from known species. The latter 14 strains are considered to represent 14 different species but have not yet been identified. Probably, some of them are conspecific with known species and some others represent undescribed species. Further molecular studies such as DNA–DNA reassociation experiment are required for the identification of these 14 species.

Eighty-nine strains (31.4%) included in the basidiomycetous yeasts were classified into 53 species, 17 known species (41 strains), 22 undescribed species (31 strains), and 14 not yet identified species (17 strains). The latter 14 species showed two to three nucleotide differences from known species. Probably, several of them belong to known species and some others represent undescribed species. As shown in Table 17.3, *Cryptococcus heveanensis* (eight strains) is the most frequently isolated and is followed by *Exobasidium vexans* (four strains), *Sporobolomyces* sp. (four strains) and *Trichosporon asahii* (four strains).

Table 17.2 Known species of ascomycetous yeasts isolated from substrates in the natural environment of Thailand

Species	No. of strains	Species	No. of strains
Ambrosiozyma monospora	2	*Hanseniaspora guilliermondii*	1
Aureobasidium pullulans	2	*H. opuntiae*	3
Blastobotrys capitulata	1	*H. vineae*	1
Candida diversa	3	*Hanseniaspora* sp. CBS 8772	1
C. fukuyamaensis	3	*Kloeckera lindneri*	1
C. gotoi	2	*Kluyveromyces lactis*	2
C. leandrae	1	*Kodamaea ohmeri*	3
C. natalensis	1	*Metschnikowia koreensis*	5
C. palmae	1	*Pichia nakazawae* var. *akitaensis*	2
C. parapsilosis	3	*P. stipitis*	2
C. rancensis	1	*P. sydowiorum*	2
C. sithepensis	1	*Pichia* sp. UWO(PS)99-305.1	1
C. tropicalis	9	*Saccharomyces cerevisiae*	5
Candida sp. NRRL Y-17456	4	*S. kluyveri*	8
Candida sp. UWO(PS)00-147.3	1	*S. unisporus*	2
Debaryomyces polymorphus var. *africanus*	1	*Stephanoascus smithiae*	3
		Torulaspora delbrueckii	1
D. nepalensis	4	*Torulaspora* sp. IFO 11061	1
D. polymorphus	3	*Williopsis saturnus* var. *mrakii*	1
D. vanrijiae var. *yarrowii*	3	*W. saturnus* var. *subsufficiens*	1
Debaryomyces sp. NRRL-7804	1	*Zygosaccharomyces* sp. IFO 11070	1
Geotrichum fragrans	2		
		Total 41 species	96

Species whose D1/D2 sequences are registered at DNA Data Banks are dealt with as known species (Nakase et al., unpublished).

Several undescribed species found in these isolation studies were described as new species, *C. easanensis*, *C. pattaniensis* and *C. nakhonratchasimensis* (Jindamorakot et al. 2004), but the majority of them are still under study.

Among 101 undescribed species found in this study, 93 species (92.1%) comprised one strain and the remaining eight species comprised two to five strains. Further, among 28 species of not yet identified species, 27 (96.4%) comprised one strain and the remaining one species comprised three and four strains. This is quite a contrast to known species where two or more strains up to nine were isolated for 32 species (55.2%) and 27 species (44.8%) comprised one strain (Tables 17.2, 17.3). This fact clearly indicates that species diversity of yeasts is extremely rich in forests of Thailand and a vast number of unknown yeasts live there. This is quite different from yeasts associated with fermented foods. Further extensive studies are required to clarify the yeasts living in forests of Thailand.

Table 17.3 Known species of basidiomycetous yeasts isolated from substrates in the natural environment of Thailand (Nakase et al., unpublished)

Species	No. of strains	Species	No. of strains
Bullera dendrophila	1	*Rhodotorula nothofagi*	1
Bullera sinensis	2	*Sporidiobolus ruineniae*	3
Cryptococcus heveanensis	8	*Sporobolomyces bannaensis*	1
Cryptococcus sp. CBS 8372	1	*S. odoratus*	3
C. humicola	2	*S. poonsookiae*	1
C. laurentii	3	*Sporobolomyces* sp. TY-241, etc.[a]	4
Exobasidium vexans	4	*Tilletiopsis* sp. TY-235[a]	1
Rhodosporidium paludigenum	1	*Trichosporon asahii*	4
Rhodosporidium toruloides	1		

Species whose D1/D2 sequences were registered at DNA Data Banks were dealt with as known species.
[a]These two species are the same as those reported by Fungsin (2003).

17.2.2 Yeasts Found in Forests of Indonesia

In Indonesia, like other southeastern Asian countries, yeast research started in the fairly early stage of microbial studies of traditional fermented products, foods, beverages and starters of fermentation. The study of yeasts living in forests and other natural environments started some 40 years ago. Deinema (1961) isolated yeasts for the study of lipid production. In this study, a new yeast species was found from the surface of leaves of the flowering shrub *Randia melleifera* (Rubiaceae) in Bogor and was described as *C. bogoriensis*. This *Candida* species showed basidiomycete affinity so it was transferred to the genus *Rhodotorula*, as *R. bogoriensis* by von Arx and Weijman (1979).

Ruinen (1963) isolated yeasts living on leaves of trees and shrubs collected in several tropical countries, Indonesia in Asia, Surinam in South America and Ivory Coast in Africa, and studied lipid production from these isolates. In Indonesia, leaf samples were collected from Bogor Botanical Garden, Bogor, Java Island. These Indonesian yeasts were identified as *Hansenula anomala* var. *sphaerica*, *H. anomala* var. *heteromorpha*, *Sporobolomyces salmonicolor*, *S. roseus*, *Cryptococcus luteolus*, *C. guilliermondii*, *C. bogoriensis*, *R. glutinis*, *R. graminis*, *R. rubra* and *Pullularia* (*Aureobasidium*) sp. In addition to these species, two new species, *C. javanica* and *C. foliorum*, and a new variety, *C. bogoriensis* var. *lipolytica*, were found from these isolates. Van Uden and Buckley (1970) did not accept the latter variety. It is now regarded as a synonym of *R. bogoriensis* (Fell and Statzell-Tallman 1998). Since the two new *Candida* species demonstrated basidiomycetous nature, they were transferred to the genus *Rhodotorula*, an urediniomycetous genus, as *R. javanica* and *R. foliorum* (Weijman et al. 1988; von Arx and Weijman 1979). These basidiomycetous yeasts isolated in Indonesia are rich in cellular lipids.

Among the yeasts just mentioned, Ruinen (1963) discussed the properties of six strains of *Sporobolomyces salmonicolor*. She stated that "the presence in these strains of a buckled mycelium and with chlamydospores would make the identification with the genus *Sporidiobolus* even more plausible." Holzschu et al. (1981) validated this species as *Sporidiobolus ruinenii,* and it is now listed in *The yeasts, a taxonomic study, 4th edn* as *Sporidiobolus ruineniae* (Statzell-Tallman and Fell 1998).

In 1996, Haryono et al. collected 40 plant leaves in the suburb of Yogyakarta, Java Island, and isolated more than 300 strains of yeasts by the ballistoconidium-fall method. They examined the ballistoconidium-forming ability of 70 strains of them isolated from Kaliurang area, 900–1,000 m above sea level, and found that 61 strains were ballistoconidiogenous. These strains were assigned to the genera *Bullera* (11 strains), *Bensingtonia* (14 strains) and *Sporobolomyces* (36 strains) (Haryono et al. 1998). Among the strains assigned to *Sporobolomyces*, eight strains possessed mono-saturated ubiquinone, Q-10(H_2), as the major component of ubiquinones. They differed in the internal transcribed spacer region sequences from the known Q-10(H_2)-containing species, *Erythrobasidium hasegawianum* and *Sporobolomyces elongateus*, and were divided into two groups on the basis of their physiological characteristics (Haryono et al. 1999).

Tropical fruits are considered as good habitats for yeasts. Sjamsuridazal and Gandjar (1994) studied yeasts associated with overripe fruits collected in Jakarta and vicinities of Java Island and isolated 17 yeast strains. Fifteen strains were identified as *Kloeckera apiculata*, *Kluyveromyces marxianus*, *K. blattae, K. waltii*, *C. oleophila*, *C. tropicalis*, *P. burtonii*, *P. humboldtii*, *P. acaciae*, *P. etchelsii*, *P. pijperi*, *P. strassburgensis* and *Trichosporon cutaneum*. The respective species comprise one strain except for *K. marxianus* and *P. humboldtii*, for which four and two strains were isolated, respectively. The remaining two strains were dealt with as *Dekkera* sp. and *Pichia* sp. Further, Oetari et al. (1999) studied 25 strains of yeasts isolated from 21 samples of 17 banana cultivars. The yeasts isolated mostly belonged to *Pichia* followed by *Saccharomyces*. Nineteen strains of *Pichia* were identidied as *P. amethionina* (one strain), *P. chambardii* (one strain), *P. farinosa* (one strain), *P. kluyveri* (one strain) and *P. membranifaciens* (seven strains). Three *Saccharomyces* strains found in three banana cultivars were identified as *S. cerevisiae* (two strains) and *S. kluyveri* (one strain). The remaining three strains were identified as *Hanseniaspora uvarum, K. marxianus* var. *wikenii* and *Zygosaccharomyces rouxii*. In their studies, samples were collected from traditional markets around Jakarta, Depok and Bogor, Java Island, but not from orchards or forests. However, yeast flora is supposed to be similar to those of banana in the orchards or forests.

Naruki et al. (1999) isolated 109 strains of sugar-tolerant yeasts from several plants in the natural environment together with foods and fruits and their products and studied sugar alcohol production. On the basis of the morphological and physiological characteristics described in *The yeasts, a taxonomic study, 3rd edn* (van der Walt and Yarrow 1984), they predicted the taxonomic positions of 74 strains. The predicted species include, for example, *S. cerevisiae*, *S. kluyveri*, *S. capensis*, *S. steineri*, *D. polymorphus*, *D. hansenii*, *H. anomala* (*P. anomala*), *P. guilliermondii*, *P. ohmeri*, *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *C. haemulonii*, *C. magnoliae*, *C. catenulata*, *C. insectorum*, *C. famata*, *C. pelliculosa*, *C. sorboxylosa*, *Schwanniomyces*

occidentalis, *Schizosaccharomyces pombe* and *Zygosaccharomyces fermentati*. Several strains were identified at generic level. This is an excellent study from the viewpoint of applied microbiology. However, it is doubtful whether their identification is correct because they reported the isolation of *Malassezia* species. It is not easy to isolate yeasts of the genus *Malassezia* from the natural environment using ordinary isolation media for yeasts because they require fatty acids for growth (Ahearn and Simmons 1998). It is assumed that most of the species were correctly predicted but some might have been mispredicted.

Yeast biodiversity in the forests of Indonesia is assumed to be rich as well as in those of Thailand. However, further detailed isolation studies and the identification based on molecular methods are required to clarify the yeasts living in forests of Indonesia. Probably, many undescribed species will be found in there.

17.2.3 Yeasts in Tropical Forests of Vietnam

Luong et al. (1999) isolated 151 strains of yeasts from 20 samples of plants collected in a tropical rain forest of Cuc Phuong National Park, Ninh Binh Province, Vietnam, using the ballistoconidium-fall method. One hundred and twenty-one of them produced ballistoconidia. They carried out taxonomic studies of 85 strains and assigned them to the genera *Bullera* (39 strains), *Kockovaella* (five strains), *Sporobolomyces* (39 strains) and *Tilletiopsis* (two strains). Among strains assigned to *Sporobolomyces*, 34 have Q-10 and the remaining five have Q-10(H_2) as the major component of ubiquinones.

All five strains of *Kockovaella* belonged to hitherto undescribed species and were described as four new species, *K. calophylli* (one strain), *K. cucphuongensis* (two strains), *K. litseae* (one strain) and *K. vietnamensis* (one strain) (Luong et al. 2000). According to Luong (personal communication), two new species have been found among *Bullera* strains so far. It is assumed that the ballistoconidium-forming yeasts found in Cuc Phuong National Park included many undescribed species as easily suggested by the fact mentioned before that all of *Kockovaella* isolates represented new species.

17.3 Yeasts in the Subtropical Forests of Asia

17.3.1 Yeasts in Forests of Taiwan

The study of yeasts in Taiwan started about 70 years ago but the study is related to the alcoholic fermentation such as the isolation of *S. formosensis* (= *S. cerevisiae*), a powerful alcoholic fermenter from molasses, and yeasts living in the natural environment were not studied until recent years.

Lee et al. (1994) described a new species, *Arthroascus fermentans*, from soil in orchards. Later, this species was transferred to the genus *Saccharomycopsis* (Kurtzman and Robnett 1995).

In 1997, Nakase and coworkers isolated 154 strains of yeasts from plants collected in a protected subtropical rain forest in Fu-Shan Experimental Forest, Taiwan Forestry Research Institute, located in northeastern Taiwan, by an improved

ballistoconidium-fall method (Nakase and Takashima 1993). One hundred and forty-six of them produced ballistoconidia. These ballistoconidium-forming yeasts were assigned to the genera *Bullera* (90 strains), *Dioszegia* (one strain), *Kockovaella* (one strain) and *Sporobolomyces* (54 strains) though the identification is not yet complete (Table 17.4). Eight new species were described from these isolates, *B. taiwanensis*, *B. formosensis* (Nakase et al. 2002), *Sporobolomyces magnisporus* (Nakase et al. 2003), *B. begoniae*, *B. setariae* (Nakase et al. 2004a), *B. melastomae*, *B. formosana* (Nakase et al. 2004b) and *S. fushanensis* (Nakase et al. 2005). In addition to new species just mentioned, at least four new species have been recognized so far, three of *Sporobolomyces* and one of *Kockovaella. Sporobolomyces roseus*, the commonest ballistoconidium-forming species in the temperate zones, is a minor species in Taiwan (Table 17.4). The identification of this species needs to be confirmed because *S. roseus* is heterogeneous and consists of several different species (Fell et al. 2000; Bai et al. 2002b). Therefore, this species is tentatively dealt with as *S. roseus* complex (Table 17.4).

B. formosensis is located in *Trichosporonales* clade in the phylogenetic tree on the basis of the 18S rDNA sequences though this species does not produce arthroconidia. Probably, this is the second ballistoconidiogenous yeast in this clade because

Table 17.4 Ballistoconidium-forming yeasts found in a subtropical rain forest in Taiwan

Species	No. of strains	References
Bullera	90	
B. taiwanensis[a]	1	Nakase et al. (2002)
B. formosensis[a]	1	Nakase et al. (2002)
B. begoniae[a]	1	Nakase et al. (2004a)
B. setariae[a]	1	Nakase et al. (2004a)
B. melastomae[a]	1	Nakase et al. (2004b)
B. formosana[a]	1	Nakase et al. (2004b)
B. coprosmaensis	1	
B. oryzae	1	
B. sinensis	8	
B. variabilis complex	16	
B. sinensis complex	18	
Bullera spp.	40	
Dioszegia	1	
D. zsoltii	1	
Kockovaella	1	
Kockovaella sp.	1	
Sporobolomyces	54	
S. magnisporus[a]	2	Nakase et al. (2003)
S. fushanensis[a]	1	Nakase et al. (2005)
S. shibatanus complex	27	
S. roseus complex	3	
Sporobolomyces spp.	21	
Total	146	

[a]New species found in Taiwan (Nakase et al., unpublished)

Sporobolomyces albidus described by Ramirez Gomez (1957) is assumed to be the first ballistoconidium-forming yeast in this clade. For many years *S. albidus* had been dealt with as a synonym of *C. humicola* (= *Cryptococcus humicola*) because no one observed ballistoconidia after the description of this species. Takashima et al. (2001b) reported that this yeast is located in *Trichosporonales* in the phylogenetic tree on the basis of 18S rDNA sequences and gave it a new name, *Cryptococcus ramirezgomezianus*. Nakase et al. (2002) stated that *B. formosensis* produces bilaterally symmetrical ballistoconidia as reported for *Sporobolomyces albidus* (Ramirez Gomez 1957).

It is assumed that many unknown species of ascomycetous yeasts live in forests of Taiwan as well as ballistoconidium-forming yeasts. However, such reports have not been published so far.

17.3.2 Yeasts in Forests of Yunnan, China

In 1996, Bai and coworkers isolated 670 strains of ballistoconidium-forming yeasts from 43 semidried leaf samples collected in subtropical forests of Yunnan Province, China, by the ballistoconidium-fall method. They selected 109 strains and characterized them morphologically, physiologically and chemotaxonomically, and recognized 25 groups. Among these groups they carried out detailed studies on five groups including 16 strains that form symmetric ballistoconidia and contain xylose in the whole cell hydrolysates. One strain was identified as *B. mrakii*, seven strains as *Bulleromyces albus* and the remaining eight strains in three groups represented three undescribed species of the genus *Bullera* (Bai et al. 1999). The latter strains were described as *B. kunmingensis* (Bai et al. 2001a), *Dioszegia zsoltii* including two varieties, *D. zsoltii* var. *zsoltii* (four strains) and *D. zsoltii* var. *yunnanensis* (two strains) (Bai et al. 2002a) and *B. anomala* (Bai et al. 2003). They studied another strains isolated from plants in Yunnan Province and described additional three new species, *B. pseudovariabilis* (Bai et al. 2003), *Sporobolomyces phaffii* (Bai et al. 2002b) and *S. yunnanensis* (Bai et al. 2001c). *B. pseudovariabilis* is a second species of the *B. variabilis* cluster and *S. yunnanensis* is a Q-10(H_2)-containing species in the *Erythrobasidium* cluster. Three strains of *Sporobolomyces roseus* were also isolated in Yunnan Province (Bai et al. 2002b). As formerly stated, this species is very common in the temperate zones but is rare in the tropical regions.

17.3.3 Yeasts in Forests of the Ogasawara Islands in the Pacific Ocean

The Ogasawara (Bonin) Islands are isolated islands in the subtropical region of the Pacific Ocean about 1,000 km from Tokyo, Japan, and are known to have a unique flora of higher plants. In the autumn of 1994, we received various plant samples collected in these islands from T. Sato, and isolated yeasts by the ballistoconidium-fall method. The number of yeast colonies that appeared on the agar plates was less than 1% of the number from plants collected from other regions of Asia including the tropics, subtropics and the temperate zone. According to T. Sato, the plant samples were not stored in good condition and some of them were infected by molds. Furthermore, the islands are small and plants are greatly affected by the strong wind

from the sea that results in a high salt concentration on the surface of plants. In spite of the small number of colonies, we found that the species diversity of yeasts is very high in these islands. A total of 199 strains were isolated from 24 samples (66.7%) of 36 examined. One hundred and ninety-seven of them produced ballistoconidia. These strains were assigned to seven genera on the basis of the morphology of ballistoconidia, the presence of xylose in the cell hydrolysates and the ubiquinone types (Table 17.5).

Forty-six strains of the genus *Bullera* isolated from ten samples (27.8%) were identified as six species (Table 17.5). Three of them were described as new species, *B. boninensis*, *B. schimicola* and *B. waltii* (Sugita et al. 1999). *Bullera* sp. 1 and 2 seem to represent new species and *Bullera* sp. 3 is closely related to *B. variabilis*, a taxonomically heterogeneous species comprising several different species (Bai et al. 2001b).

Sixteen strains isolated from three samples (8.3%) represented three new species of *Kockovaella* and were described as *K. machilophila*, *K. phaffii* and *K. schimae* (Gibas et al. 1998). Two strains of *Bensingtonia*, which were isolated from the same sample, was found to be a new species and was named *B. sakaguchii* (Sugita et al. 1997).

One hundred and thirty-three strains were assigned to the genus *Sporobolomyces* in which 103 strains possessed Q-10 and the remaining 30 strains

Table 17.5 Basidiomycetous yeasts found on the plants of the Ogasawara Islands

Species	No. of strains	Frequency of isolation (%)[a]	Species	No. of strains	Frequency of isolation (%)[a]
Hymenomycetous yeasts			Urediniomycetous yeasts		
Bullera	46	27.8	*Bensingtonia*	2	2.7
B. boninensis	16	8.3	*B. sakaguchii*	2	2.7
B. schimicola	1	2.7	*Bannoa*, Q-10(H_2)	6	11.1
B. waltii	5	13.9	*B. hahajimensis*	6	11.1
Bullera sp. 1	1	2.7	*Sporobolomyces*, Q-10(H_2)	24	27.8
Bullera sp. 2	1	2.7	*S. ogasawarensis*	19	16.7
Bullera sp. 3	2	5.6	*S. bischofiae*	1	2.7
Kockovaella	16	8.3	*S. syzygii*	4	5.6
K. machilophila	1	2.7	*Sporobolomyces*, Q-10	103	52.8
K. phaffii	14	5.6	*Sporobolomyces* spp.	103	52.8
K. scimae	1	2.7			
Fellomyces	2	2.7	Subtotal	135	
F. ogasawarensis	1	2.7			
F. distylii	1	2.7			
Subtotal	64		Total	199	66.7

[a]Percentage of plant samples from which corresponding yeasts were isolated

were found to possess Q-10(H_2) as the major component of ubiquinones. The latter 30 strains were found in ten samples (27.8%) of plants examined. The Q-10(H_2)-containing strains constituted a cluster which was close to the cluster where *Erythrobasidium hasegawianum* and *Sporobolomyces elongatus*, known species of Q-10(H_2)-containing yeasts, are located. On the basis of a DNA–DNA hybridization experiment, these strains were found to represent four different species, including a species demonstrating a sexual stage. *Bannoa hahajimensis* was proposed for this teleomorphic species (Hamamoto et al. 2002). *Bannoa* is the second genus for Q-10(H_2)-containing basidiomycetous yeasts. Three new species, *Sporobolomyces ogasawarensis*, *S. syzygii* and *S. bischofiae*, were proposed for Q-10(H_2)-containing anamorphic species.

In addition to ballistoconidium-forming yeasts, two strains of non-ballistoconidiogenous, stalked conidium-forming yeasts were also isolated in the phyllosphere of the Ogasawara Islands. These yeasts represented two different new species and were named *Fellomyces distylii* and *F. ogasawarensis* (Hamamoto et al. 1998).

17.4 Growth Temperature of Yeasts from the Tropics and the Temperate Zone of Asia

It is quite natural to assume that yeasts living in the tropical region have higher growth temperatures than those living in the temperate zones. Probably, however, this assumption seems to be erroneous. In the isolation study of ballistoconidium-forming yeasts in 1987, as mentioned before, we found that 23°C is better than 30°C to recover a variety of yeast species though the atmospheric temperatures was 24–34°C during the period of the isolation studies.

We examined the maximum growth temperatures of 1,111 yeast strains, 889 ascomycetous yeasts and 222 basidiomycetous yeasts, isolated in Thailand and Japan as representatives of the tropics and the temperate zones, by incubating them at an interval of 1°C using water baths or metal block baths whose temperatures were accurately controlled. Regardless of their locality, the average value of the maximum growth temperatures of ascomycetous yeasts was between 36 and 37°C (Table 17.6); those of the basidiomycetous yeasts were between 30 and 31°C in yeasts isolated in Thailand and between 31 and 32°C in yeasts isolated in Japan. The isolation temperatures of these yeasts were 23, 25 and 30°C in Thailand and 17 or 25°C in Japan. In Japan, 14.2% of basidiomycetous yeasts have their maximum growth temperatures below 25°C. These psychorophilic yeasts were isolated by incubation at 17°C. Psychrophilic yeasts have not been isolated in Thailand so far but we cannot discuss the psychrophiles in Thailand at present because psychrophilic yeasts cannot grow at the temperatures used for the isolation of Thai yeasts. Further study is required in this respect using low isolation temperatures below 20°C and samples collected in mountainous areas of northern Thailand.

It is concluded that yeasts living in the tropics have similar growth temperatures to those in the temperate zones. The growth temperatures of yeasts depend on the species and ascomycetous yeasts have higher growth temperatures than basidiomycetous yeasts. However, the number of basidiomycetous yeasts sampled is not enough to compare the temperature relationships of yeasts in Thailand and Japan.

Table 17.6 Maximum growth temperatures of yeasts isolated in Thailand and Japan (Nakase et al., unpublished)

Maximum growth temperature (°C)	Ascomycetous yeasts				Basidiomycetous yeasts			
	Thailand		Japan		Thailand		Japan	
	No. of strains	Cumulative %	No. of strains	Cumulative %	No. of strains	Cumulative %	No. of strains	Cumulative %
22–23							1	0.6
23–24							20	12.4
24–25							2	14.2
25–26							1	14.8
26–27							7	19.1
27–28					5	8.2	12	26.6
28–29					10	24.6	11	33.4
29–30					13	45.9	11	40.2
30–31	1	0.5	18	2.6	4	52.5	6	43.9
31–32	4	2.5	23	6.0	7	62.3	13	52.0
32–33	8	6.4	42	12.1	7	73.8	14	60.7
33–34	12	12.3	46	18.8	5	82.0	10	66.9
34–35	17	20.7	97	32.9	2	85.3	16	76.8
35–36	40	40.4	72	43.4	6	95.1	11	83.6
36–37	28	54.2	84	55.6	1	96.7	5	86.7
37–38	19	63.6	34	60.6			17	97.3
38–39	31	78.9	86	73.1				
39–40	27	92.2	41	79.1			2	98.5
40–41	7	95.6	32	83.8				
41–42	8	99.5	41	89.8			1	99.1
42–43			39	95.5	1	98.3		
43–44	1	100	9	96.8	1	99.9	1	99.7
44–45			22	100				
Total	203		686		61		161	

Apparently, further studies are required to confirm the temperature relationship between yeasts of the two countries.

17.5 Conclusion

Since long ago, Asian people have utilized microorganisms for human beings and produced various kinds of fermented foods and beverages. Quite naturally, the study of yeasts associated with fermented products preceded the study of yeasts living in the natural environment. It has been reported that yeasts found in fermented products and related substrates such as raw materials of fermentation are poor in biodiversity and most yeast species associated with such products of tropical Asia are common to other countries in the temperate zones. In recent years, yeasts associated with plant leaves, especially ballistoconidium-forming yeasts, a group of basidiomycetous yeasts, have been extensively studied in tropical and subtropical Asia. More recently ascomycetous yeasts in Asian forests, especially those in Thailand, have been studied using molecular methods. In contrast to yeasts in fermented products, yeasts in the forests are rich in biodiversity and many undescribed species were found from various substrates of tropical Asian forests. However, only some of these unknown species have been described as new taxa so far.

Yeast species having Q-10(H_2) have long been considered to be rare among yeasts. Only two strains belonging to two species, *Erythrobasidium hasegawianum* and *Sporobolomyces elongatus*, were reported to have this ubiquinone homologue as the major component (Yamada et al. 1973; Nakase and Suzuki 1986). The former was isolated from an old culture of beer yeast in Philadelphia, USA. (Sugiyama and Hamamoto 1998) and the latter was from a leaf of *Callistemon viminalis* in Australia (Shivas and Rodrigues de Miranda 1983). The recent isolation studies of ballistoconidium-forming yeasts, however, proved that Q-10(H_2)-containing ballistoconidium-forming yeasts are widely distributed in the tropical and subtropical regions in Asia – Thailand (Fungsin 2003), Indonesia (Haryono et al. 1999), Vietnam (Luong et al. 1999) – and the Ogasawara Islands in the Pacific Ocean (Nakase 2000).

The diversity of molecular species of ubiquinones is a good example of rich yeast biodiversity of tropical forests of Asia. It is assumed that many new groups of yeasts will be found in tropical forests of Asia in the near future. It should be emphasized that studies of yeasts in tropical forests of Asia has just started and further extensive studies are required to clarify the whole profile of yeasts in forests of this region. This kind of study must be done for the progress of yeast systematics and for the utilization of useful functions of yeasts for the welfare of humans. A fruitful achievement is expected from this kind of yeast research.

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Yeast Communities in Tropical Rain Forests in Brazil and other South American Ecosystems

Paula B. Morais[1], Fernando C. Pagnocca[2] and Carlos A. Rosa[3]

[1]*Laboratorio de Microbiologia Ambiental e Biotecnologia, Campus Universitário de Palmas, Universidade Federal do Tocantins (LAMBIO – UFT), ALCNO 14 NS15, Bloco II, s.08, Palmas, TO, 77020-220, Brazil*
(e-mail: moraispb@uft.edu.br)
[2]*Centro de Estudos de Insetos Sociais, CEIS, C.P. 199, Universidade Estadual Paulista, Rio Claro, SP, 13506-900, Brazil*
(e-mail: pagnocca@rc.unesp.br)
[3]*Departamento de Microbiologia, ICB, C.P. 486, Universidade Federal de Minas Gerais, Belo Horizonte, MG, 31270-901, Brazil*
(e-mail: carlrosa@icb.ufmg.br)

18.1 Introduction

Yeast ecology has a strong emphasis on the description of novel taxa of yeasts colonizing a substrate. Studies on the structure and functioning of yeast communities are rare and based on a multitude of methodological approaches and different strategies of collection used by different researchers. Among fungi, yeasts are immotile, predominantly unicellular organisms that are functionally heterotrophic colonizers of sugary substrates. Yeasts are known as specialized organisms with limited physiological characteristics (Lachance and Starmer 1986; Phaff and Starmer 1987). Ecological studies aim to understand colonization and distribution of yeasts in different ecosystems and substrates (Phaff 1990), and have shown that the majority of yeast species and groups have specialized habitats. It is possible to isolate yeasts typical of different natural substrates in the geographical areas where they occur (Phaff and Starmer 1987).

The commonest difficulty in studies of yeast ecology in natural ecosystems is to define a yeast community. Möbius (1877) made the first attempts to describe an ecological community when describing oyster banks in marine ecosystems, which he called biocenosis. According to Odum (1994) communities are defined as groups of different species living together in a geographic area, or else to say in space and time defined altogether. Brewer (1988) describes communities as systems composed of species populations bound by coactions. Guilds are defined as groups of species that share a resource in a community. Lachance and Starmer (1986) consider a yeast community as a multidimensional array, the elements of which are its component species or the characteristics of these species. Differing from bacterial communities,

yeast communities present physiological homogeneity since very few yeast species have highly specialized nutritional requirements or high degrees of tolerance to extreme physicochemical conditions. Such homogeneity normally precludes the description of yeast communities as complex networks of nutritional specialists (Lachance and Starmer 1986). In an attempt to solve the problem of characterization of yeast communities, Lachance and Starmer (1986) offer a strategy based on physiological profiles of yeast assemblages. Physiological profiles are vectors of the proportions of yeasts, in a particular community, which give a positive response on each of a series of physiological tests and are considered to be sometimes more informative, as multivariate descriptors, than vectors of yeast taxa frequencies. Lachance and Starmer (1986) calculated the mean responses of all described yeast species to 33 selected physiological tests and revealed that some physiological attributes are characterized by high responses while others are comparatively low. They estimated the probability that each yeast in a random collection will be positive for each attribute Y. According to them, if in an actual collection of yeasts, the number of isolates positive for the attribute Y departs substantially from the expected mean, then ecological specificity may be significant.

In this chapter, we will consider yeast communities as guilds of taxonomically defined groups of species having similar functional/physiological attributes in a geographically limited habitat. The environment of a yeast community may be viewed as a multidimensional geometric space following Hutchinson's (1958, 1959) model and that Brewer (1988) defines as a biotope – the specific set of environmental conditions under which an individual species or community exists. Sometimes restricted to conditions of the physical environment, its dimensions would include physical factors, as well as biotic factors such as predation, competition, or food. The habitat of a yeast species is generally well defined in most studies, specially when studying yeasts associated with trees and other plant substrates, but it is less clear when dealing with yeasts associated with animals, that would include mammals and insects, mostly. There are many reports of yeasts isolated from flowers, tree saps and fruits of angiosperms (Miller and Mrak 1953; Shehata et al. 1955; Carson et al. 1956; Phaff and Knapp 1956; de Camargo and Phaff 1957; da Cunha et al. 1957; Prada and Pagnocca 1997; Lachance et al. 2001, 2003) and visiting insects, mainly drosophilids (da Cunha et al. 1957; Morais et al. 1992a, 1995a, b, 1996; Morais and Rosa 2000), and a few have studied the yeasts associated with mushrooms (Ramirez Gomes 1957).

18.2 Specificity of Association Between Yeasts and Substrates

The distribution and specificity of yeast communities depend mainly on the composition of nutrients present in the substrate, the presence of inhibitory compounds and the vectors that utilize those substrates as feeding and breeding niches (Gilbert 1980; Phaff and Starmer1987; Ganter 1988; Starmer et al. 1988). Yeasts have a nutritional dependence on the substrate, and different substrates bring up different assemblages of yeasts. It is well accepted that yeast ecology is more than just a more or less random distribution of these organisms in substrates where sugars are present at detectable levels. Plants that serve as habitats for large yeast communities are

often sites of ecological interactions involving other organisms and the substrate itself (Phaff and Starmer 1987).

Starmer (1981) demonstrated that the physiological attributes of yeasts are useful indicators of habitat characteristics, but a few studies apply the other approach: to use the biochemical characteristics of habitats to understand the associated yeast communities. Extensive knowledge of yeast habitat characteristics, such as sugar and water content and composition of fruits, tree exudates, nectar and pollen, would be of help in defining habitat specificity. Nevertheless, those efforts were found solely in the works of Starmer (1981) when looking for explanations of the specificity of interactions between *Drosophila* and yeasts. One would argue that is difficult to find information on the chemical composition of most natural substrates in forests, and limitations could be due to this scarcity. There is certainly a need for an interdisciplinary approach to the study of the relations between yeasts and specific substrates, and to fill the missing information on substrate chemistry.

18.3 Vectors of Yeasts

Yeasts are immotile organisms, which need to be carried by vectors. This association makes yeasts dependent on vectors, mainly insects, and probably they benefit from yeasts. A great number of studies have isolated yeasts associated with insects, especially *Drosophila*. Gilbert (1980) and Starmer et al. (1988) showed that these flies are important vectors of yeasts, which are important food sources for adult and larval stages of the flies (Begon 1982). *Drosophila* species show specific relation to certain species or groups of species of yeasts, usually owing to substrate composition or to biogeographic distribution of insects (Starmer et al. 1990). Lachance et al. (2001) discuss the role of nitidulid beetles in the distribution of yeasts in different biogeographical areas. Nevertheless, most studies on forests in Brazil have focused only on drosophilids, and efforts must be made to search for other associations that may be as important as the association between *Drosophila* and yeasts.

18.4 Forests as Ecosystems for Yeast Communities

Forest ecosystems are an attractive site for the collection of yeasts, and various researchers have made efforts to study the yeast diversity in those forests. Today, forests occupy approximately one third of Earth's land area, account for over two thirds of the leaf area of land plants, and contain about 70% of the carbon present in living things of terrestrial and aquatic habitats. Tropical forests are characterized by the greatest diversity of species. They occur near the equator, within the area bounded by latitudes 23.5° N and 23.5° S. One of the major characteristics of tropical forests is their distinct seasonality: winter is absent, and only two seasons are present (rainy and dry). The length of daylight is 12 h and varies little. The temperature is on average 20–25° C and varies little throughout the year: the average temperatures of the three warmest and the three coldest months do not differ by more than 5°C. Precipitation is evenly distributed throughout the rainy period of the year, with annual rainfall exceeding 2,000 mm. The tree canopy in tropical forests is multilayered and continuous, allowing little light penetration and the flora is highly

diverse since one square kilometer may contain as many as 100 different tree species. The trees are 25–35-m tall, with buttressed trunks and shallow roots, mostly evergreen, with large, dark-green leaves. Plants such as orchids, bromeliads, vines (lianas), ferns, mosses, and palms are present in tropical forests. The fauna includes numerous birds, bats, other small mammals, and insects.

Theoretical ecology indicates that ecosystems such as forests are in fact a mosaic of patchy habitats for organisms – and one could even state that they represent mosaics of ecosystems for microorganisms. The vertical arrangement of forests, from soil, litter, tree stems, trunks and canopy, together with flowers and fruits in different stages of ripening represent different niches for colonization of yeasts. Because they have a sugary composition flowers and fruits are ephemeral substrates constantly visited by insects and other vectors of yeasts creating a diversity of abiotic and biotic interactions that would probably support a huge biodiversity of microorganisms.

Differing from temperate forests, in tropical forests different plant species present flowers and fruits in different periods of the year, a characteristic that would produce food and substrates for colonization of yeasts throughout the year.

Brazil is the largest country in South America. Approximately 2.3 million square kilometers – 27% of Brazil's total area of 8.5 million square kilometers – can be considered "frontier" forest. Brazil's frontier forests comprise 17% of the world's remaining frontiers, making it the third highest ranked country in terms of remaining frontier forest. Brazil has the third largest block of remaining frontier forest in the world and ranks first in plant biodiversity among frontier forest nations.

Brazil's geographic space presents a great diversity of climate types, physiognomy, soils, and vegetation. As early as the nineteenth century, C.F.P. Martius recognized five phytogeographic provinces (defined as geographic spaces containing endemism). Such endemisms reflect diverse environmental conditions that create geographical isolations and allowed the birth to a highly diverse biota. Ab'Saber (1977) classified those provinces as dominions based on morphoclimate and phytogeography. A dominion is a geographic space having subcontinental extension where morphoclimatic and phytogeographic characteristics prevail that are distinct from those in other areas. This means that in a particular dominion, different biomes can be found. For example in the Dominion Cerrado (savannas) we can find forest ecosystems representative of biomes other than savanna. In the dominion of Cerrado, savannas dominate, but fragments of dry (deciduous) forests, semideciduous forests, gallery forests and tropical rain forests are also found. The dominion Caatinga, dominated by dry vegetation, also presents different forest ecosystems, including dry and humid forests. This increases the impact of the rarity of studies on yeast from Brazil's forest ecosystems, since the studies until now have focused mainly on Atlantic rain forest and Amazon forest sites.

18.5 Yeast Studies in Atlantic Rain Forests

The Atlantic rain forest represents one of the areas of the planet with the greatest biodiversity (Guedes-Bruni and Lima 1997) and is considered the second most threatened tropical forest among 15 regions denominated as hot spots (areas with high biodiversity, elevated levels of endemism and great antropic pressure) (Myers

et al. 2000). Originally the Atlantic forest was the forest with the largest latitudinal extension of the planet, going from about 6 to 32° S. In those days it covered about 11% of the national territory. Today, however, the Atlantic forest covers only 4% of its original area (Aidar et al. 2001). The climate along its extension varies, ranging from superhumid in the south to tropically humid and semiarid in the northeast. The uneven relief of the coastal zone adds more variability to this ecosystem. In the valleys, the trees grow a lot, forming a dense canopy. At the coastlines this forest is less dense, owing to the frequent fall of trees. At the southern end the Atlantic forest gradually mixes with the forest of Araucarias. In the state of Rio de Janeiro, where it represented 97% of the original plant coverage, it is nowadays reduced to less than 10% as a result of the ever-increasing rate of reckless destruction (Guedes-Bruni and Lima 1996). The forest types mentioned before are different from the Amazonian Hylaea in many respects, especially in relation to the greater availability of light at the lower portions. This is due to the layered distribution of trees in often-steep mountains, which generates a discontinuous canopy, allowing the entrance of more light (Silva and Leitão Filho 1982; Shepherd 2002). The greater availability of light makes possible the existence of different plant species at different levels in the forest, greatly boasting its biodiversity and making it in one of the most complex ecosystems of the planet (Kricher 1999).

Studies of yeasts in the Brazilian Atlantic forest started in the late 1950s with the study of da Cunha et al. (1957), who streaked individual crops of 281 *Drosophila* captured in Serra da Cantareira Forest reserve and other forest sites in São Paulo on propionate–malt agar at 25°C. They isolated 417 strains, of which 391 were identified to species level. The other 186 isolates were probably new species that could not be identified at that time. Most *Drosophila* crops (69%) contained only one species of yeast, whereas 22.8% contained two species, 7.1% contained three species and 1.1% contained four species of yeasts. They found 43 different yeast species, with 79% isolated one to six times, 9.3% found 10–20 times, 9.3% isolated above 20–30 times, and 4.6% found around 40 times. *Kloeckera apiculata* was isolated 122 times, and it was considered to have a uniform distribution among *Drosophila* species. *Candida krusei* and *C. mycoderma* were consistently associated with the medio group of *Drosophila*, whereas *C. parapsilosis* var. *intermedia*, *C. brasilae* (undescribed yeast species that does not have a synonym in the literature), and *Pichia membranifaciens* were associated with *Drosophila bocainensis. P. fermentans* was clearly associated with *D. willistoni*. The study also concluded that food preferences were different among *Drosophila* flies, because *P. fermentans* comprised 20.6% of the yeasts isolated from crops of *D. willistoni*, and *P. membranifaciens* represented 19.6% of the yeasts from crops of *D. griseolineata* and 11.1% of *D. willistoni. C. krusei* was 24.2% of the total isolates from crops of *Drosophila* of the medio group and 14.3% of the yeasts from *D. willistoni* crops. *C. mycoderma* constituted 14.9% of the yeasts from crops of *D. bocainensis*, 7% of yeasts in crops of *Drosophila* of the medio group and 5% of yeasts in crops of *D. guaramuru*, and it was not isolated in other drosophilids. *C. brasilae* was frequent (12.3%) in crops of *D. bocainensis*, being rare or not existing in other drosophilids. *K. apiculata* was prevalent in all *Drosophila*, being dominant in crops of *D. paulistorum* (58.8%), *D. griseolineata* (43.5%), *D. guaramuru* (38.5%), *D. bocainensis* (32.4%), *D. willistoni* (22.2%) and medio group (16.7%).

In the same work, da Cunha et al. (1957) isolated 46 yeasts from ten species from fruits at the same sites where they collected *Drosophila. C. brasilae* and *K. apiculata* were prevalent species, and *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, *Cryptococcus luteolus*, *P. fermentans*, *Rhodotorula mucilaginosa* and *Saccharomyces acidifaciens* (= *Zygosaccharomyces bailii*) were also isolated. Among those, only three were not isolated from flies.

Morais et al. (1992a) studied the distribution and diversity of yeast species associated with *Drosophila* in three forest fragments in Rio de Janeiro. They isolated 557 cultures from the surface of 228 insects and 305 from crops of 286 flies collected at two sites in the Tijuca National Forest reserve and at one site of the fruit orchard at the Universidade Federal do Rio de Janeiro. The flies were attracted by means of baits and set to walk on yeast extract–malt extract agar (YMA) plates with chloramphenicol (100 mg/l) for 15 min, after which they were stored for identification. The other 286 flies were identified and crops dissected from them were streaked on the surface of YMA. Seventy-two species of yeasts were obtained after 3 days at room temperature. Apiculate yeasts, especially of the species *K. apis*, *K. japonica* and *K. javanica* were the most frequent isolates from *Drosophila* vectors, and *Aureobasidium pullulans, C. apicola*, *C. citrea*, *Debaryomyces vanrijiae*, *Geotrichum* spp., *P. beckii* and *Williopsis californica* were also found. This work could establish a specific association between yeast and *Drosophila* species. For example, *Kluyveromyces delphensis* was associated with the *D. willistoni* group, from which all but one of 34 strains were isolated. *D. vanrijiae* var. *yarrowi* was the predominant yeast associated with the *D. tripunctata* group. *K. javanica* was frequently isolated from the *D. melanogaster* group. Together with *P. beckii*, *D. vanrijiae* var. *yarrowi* was isolated from external parts of flies, and rarely from crops. *Saccharomyces cerevisiae* was represented by only three isolates. Two of these isolates were later shown to represent a new species, and they were described as *S. cariocanus* by Naumov et al. (2000). A few strains of *Pichia* and *Williopsis* were isolated from disturbed areas, but *P. angusta*, *P. beckii*, *P. carsonii*, *P. kluyveri*, *W. californica* and *W. saturnus* were represented by multiple isolates at primary forest sites and were associated with *D. guarani*, *D. tripunctata* and *D. willistoni groups*. With exception of *C. citrea* and *C. colliculosa*, the species of the genus *Candida* were restricted to primary forest sites. Basidiomycetous yeasts and their anamorphs were represented by only a few isolates. More extensive collections of insects would probably enlarge the list of yeast species.

In the same work, taxonomic questions were set, since differences were noted in physiological profiles between the type strains and those isolated from *Drosophila*. Some biotypes differed from the standard descriptions of species by growing at higher temperatures than expected. Among the apiculate yeasts, 68 strains of *K. apis* grew at 40°C, and 117 strains of *K. javanica* and four strains of *K. africana* grew at 37°C (Morais et al. 1992b). Strains of *C. citrea* grew at 37°C and strains of *C. berthetti*, *C. guilliermondii*, *P. acaciae* and an unidentified *Candida* species positive for the Diazonium Blue B (DBB) test grew at 43°C. Six strains were named under the epithet "like" that meant they did not fit a standard description and correspond to potential new species.

Morais et al. (1995a) isolated yeasts from a specific group of *Drosophila* in forests – the *fasciola* subgroup of the cactiphilic *repleta* group in the Tijuca National Forest

Park and Ilha Grande State Park in Rio de Janeiro. They isolated 61 yeast species vectored by 202 flies allowed to walk 8-12 h on YMA, and 38 species from crops of 205 flies that were dissected and streaked on YMA plates. A *P. membranifaciens* like species, *C. colliculosa* and *K. apiculata* were the most frequent yeasts, summarizing 35% of the isolates. The frequency of yeasts in male and female flies did not differed except for *K. apiculata*, which was present solely in females that also presented a higher diversity of associated yeast species. Taxonomic difficulties were encountered, and many strains did not follow the description of the type strains of species. Twenty-eight strains of *Candida* spp., nine strains of *Pichia* spp., six strains of *Saturnispora* sp. and 13 strains of *Sporopachydermia* spp. were probably new species, together with four isolates of a species similar to *C. blankii*, one isolate of a species similar to *C. buffoni*, five of a species similar to *C. colliculosa*, one isolate similar to *C. stellata*, 47 isolates similar to *C. valida*, 133 isolates similar to *P. membranifaciens* and one similar to *P. silvicola*, indicating that the diversity of yeasts in Atlantic rain forests in Brazil is almost unknown. The sequence of the D1/D2 domains of the large subunit ribosomal DNA (rDNA) of a *Saturnispora* strain showed that this yeast is a sister species of *S. dispora*. It is described as *S. hagleri* (Morais et al. 2005).

Morais et al. (1996) isolated 223 strains of 40 species of yeasts from 103 flies of the *D. quadrum* group in forests of Rio de Janeiro. The authors isolated 139 strains of 33 species in external parts of 82 flies that were allowed to walk on YMA plates, and 36 strains of 18 species of yeasts from 21 crops streaked on the surface of YMA plates after dissection. The prevalent yeast species associated with *D. quadrum* in the forest floor were *D. melissophilus* and *D. vanrijiae*, together with *C. guilliermondii* and *K. apis*, that are usually associated with flowers. Black yeasts, *C. antarctica* and *Rh. rubra* are typical of plant surfaces and were also frequent in samples. *C. diversa*, *C. insectamans*, *C. quercitrusa*, *P. membranifaciens* and its anamorph *C. valida* were also isolated. Yeasts belonging to this community did not significantly differ from the standard description of the species. The feeding niche of *D. quadrum* was probably limited to the forest floor and vicinities which this fly species visited and fed on yeasts colonizing fruits in late states of decomposition and flowers.

Yeast occurrence in bromeliad tank waters was studied at an Atlantic rain forest site in Rio de Janeiro (Araújo et al. 1998). Samples from *Nidularium procedurum* and *Quesnelia arvensis* were collected, and many yeast isolates found in these microhabitats were phenotypically similar to *S. cerevisiae*, and including a probable new species belonging to the *Saccharomyces sensu lato* group. Other isolated species were *C. famata*/*D. hansenii*, *C. intermedia*, *C. tropicalis*, *Cr. albidus*, *D. vanrijiae*, *Rh. mucilaginosa* and *W. saturnus*.

Abranches et al. (1998) recorded a high yeast diversity associated with some wild small mammals in a patchwork of habitats including cultivated fields, small Atlantic forest fragments, pasture and grasslands. The yeasts were isolated from fecal pellets from the traps in which the animals were caught. Fecal pellets from the marsupials *Didelphis marsupialis* (opossum, Didelphidae) and *Philander frenata* (Didelphidae) and from the rodents *Akondon cursor*, *Oryzomys* sp. and *Nectomys squamipes* (Akodontinii) were studied. The most frequent isolates were *P. membranifaciens*, *Issatchenkia orientalis* and its anamorph *C. krusei*, and *D. hansenii*. From 57 yeast species isolated, 21 were probably new species or biotypes. Most of the yeast species

isolated were typical of fruits. The high diversity of yeasts found in this work probably reflected the diversity of food items that these animals ate. The authors suggested that the fecal pellets of these animals could serve as an additional tool for assessing the diversity of yeasts in a given habitat.

A survey of the yeasts found in association with native fruits of a tropical rain forest in Brazil was made by Prada and Pagnocca (1997) at the Ecological Station of Juréia-Itatins, São Paulo, Brazil. During this study, 202 strains were isolated from ripe and/or unripe fruits from 20 representative angiosperms. Most (74%) of the isolates had ascomycetic affinity with *Candida* and *Kloeckera* (including its teleomorph *Hanseniaspora*) being the predominant genera, followed by *Cryptococcus*, *Sporobolomyces*, *Pichia*, *Bullera* and *Hansenula* (*Pichia*). *Candida* was the most frequently found genus (51%) with 30 species, whereas *K. apiculata* was the predominant species isolated (12.7%). The following species were identified in decreasing order of frequency: *C. valida*, *C. magnoliae* like, *C. versatilis* like, *P. kluyveri*, *C. boidinii* and *C. colliculosa*. Together, they represented 34.1% of the total number of isolates identified at the species level (n=126). It is important to emphasize that many of the strains isolated differed from the standard description of known species suggesting that they could represent new biotypes or even new species. Unfortunately at that time the methodology of sequencing of rDNA was not available for the authors and they could not confirm this hypothesis.

Similar research was made from 1999 through 2002 at a site close to the same ecosystem (Serra do Mar State Park, Picinguaba area). This area of around 47,000 ha is part of Ubatuba city, São Paulo state, on the border with Rio de Janeiro state. Three hundred and eleven flowers from 13 plant species, 72 fruits of six plant species and 75 water deposits in tanks of two bromeliad species were sampled in order to isolate the yeasts associated with them. Three hundred and twenty-six strains of yeasts were obtained, with 75.8% of them having ascomycetic affinity (Ruivo 2005). *Candida* was the predominant genus, followed by *Metschnikowia*, *Hanseniaspora*, *Bullera* and *Cryptococcus*. Among the ascomycetous yeasts and their anamorphs, 37 species were identified. *Hanseniaspora uvarum* was the prevalent species in flowers and fruits. These data are still being analyzed but some light has already been put on the richness of the yeast communities of this environment. Eight isolates of *C. leandrae*, a new ascomycetous yeast, were found in decaying fruits of *Leandra reversa* (Melastomataceae) (Ruivo et al. 2004). Additionally, another five new ascomycetous yeast species were found. Two of them, *C. bromeliacearum* and *C. ubatubensis*, were isolated from the bromeliad tank of *Canistropsis seidelii* (Bromeliaceae) (Ruivo et al. 2005a) and three others, *C. heliconiae, C. picinguabensis* and *C. sanpauloensis*, were isolated from *Heliconia velloziana* (Heliconiaceae) (Ruivo et al. 2005b). Some other strains did not fit well the standard description and some biotypes initially considered as *Candida* sp. A, B, C, D, E, F, G, H, I and *Debaryomyces* sp. A, B, C, among others, are still under investigation and it is possible that new yeasts species will be found.

Pimenta (2001) isolated 54 yeast species from different substrates at an Atlantic rain forest site (Rio Doce State Park) in Minas Gerais state in Brazil. Yeasts were isolated from fruits, mushrooms, plant exudates and *Drosophila* flies. Twenty-two species differed from known species in their physiological characteristics, and they

probably represent new yeast species. A new species of *Geotrichum*, *G. silvicola*, was described. This new species was associated with *Drosophila* flies (Pimenta et al. 2005).

18.6 Yeasts from Amazon Forest Sites

The Amazon Forest occupies the northern area of Brazil, embracing about 47% of the national territory. It is the largest forest formation of the planet, conditioned by the humid equatorial climate. It has a great variety of flora and fauna, living between dense forests and open fields. Three types of forest are common: dense forests on firm soil (*terra-firme*), periodic forested floodland (*várzea*) forests, and seasonally flooded (*igapó*) forest. The Amazon region attracted attention in the early 1950s with works of A.C. Batista focusing on yeasts in fruits, but an ecosystem approach was not done because fruits were collected in the markets of Manaus (Batista et al. 1961).

Mok et al. (1982) captured a total of 2,886 bats in the Amazon Basin of Brazil and processed these samples for the isolation of fungi. From the livers, spleens and lungs of 155 bats (5.4%), 186 fungal isolates of the genera *Candida* (123 isolates), *Trichosporon* (26 isolates), *Torulopsis* (*Candida*) (25 isolates), *Kluyveromyces* (11 isolates) and *Geotrichum* (one isolate) were recovered. Seven known pathogenic species were present: *C. parapsilosis*, *C. guilliermondii*, *C. albicans*, *C. stellatoidea (C. albicans)*, *C. pseudotropicalis*, *Trichosporon beigelii* (= *T. ovoides*), and *Torulopsis glabrata* (= *C. glabrata*). In another work, Mok et al. (1984) studied the ecology of pathogenic yeasts in Amazonian soils. The authors collected 1,949 soil samples from diverse geographical and ecological settings of the Brazilian Amazon basin, and analyzed them for the presence of nonkeratinophilic fungi by the indirect mouse inoculation procedure and for the presence of keratinophilic fungi by the hair bait technique. Two hundred and forty-one yeast and yeast-like isolates were obtained from 12% of the soil samples. *Candida* was the prevalent genus representing 89% of isolated species. *C. glabrata*, *C. famata* (= *D. hansenii*), *T. candida* (= *D. hansenii*), *C. guilliermondii* and *T. dattila* (= *C. dattila*) were the most frequent species. Forty-six isolates could not be classified and were composed of 25 *Torulopsis* isolates, 13 *Candida*, and eight nonascosporic white yeasts.

Morais et al. (1995b) presented a study focusing on fruits of *Parahancornia amapa* (*amapa*, Apocynaceae), *Anacardium giganteum* (*cajuí*, Anacardiaceae), *Helycostis* sp. (Moraceae), *Platonia insignis* (*bacuri*, Guttiferae) and *Clusia grandiflora* (*cebola da mata*, Clusiaceae) in two upland (*terra-firme*) forest sites near Belem and on Marajo Island, in Para. Yeasts were isolated from fallen fruits with different days of falling and counts were generally between 5×10^5 and 1×10^6 cfu/g, reaching 2×10^7 and 8×10^6 cfu/g. The most frequent species in amapa fruits were *K. apiculata*, which represented 16% of total isolates, and it presented the highest populations. *C. amapae*, *C. krusei*, *C. sorbosa* like complex, *C. fructus*, *C. sorboxylosa* like complex and *P. kluyveri* var. *kluyveri* were also frequent. These species constituted 50% of all isolates from amapa fruits. The yeasts *C. amapae* and *C. krusei* can be considered as consistently associated with amapa fruits, since they showed a high frequency in amapa fruits, but were not isolated from other fruit substrates at the same forest

sites. The most frequent species in *A. giganteum* fruits were *C. guilliermondii*, *K. apiculata* and *P. membranifaciens* like complex. In *Helycostis* fruits, *K. apiculata*, *K. apis* and *C. sorboxylosa*-complex were the most frequently isolated yeasts. In *Platonia insignis*, *K. apiculata* and *C. sorboxylosa* complex were commonly isolated. In *C. grandiflora* fruits, black yeasts, *Cr. humicola* and *K. apiculata* were frequently isolated.

In this study, the succession of yeasts colonizing 99 fallen fruits from amapa trees was verified from the first to the fourteenth day. Yeast succession was represented by 19 species, among which *C. amapae* and *C. krusei* were isolated throughout the course of succession in fallen fruits. *C. sorbosa* like, *K. apis* and *P. membranifaciens* like dominated the fruits soon after they had fallen, *C. guilliermondii*, *C. sorboxylosa* like and *K. apiculata* appeared during early ripening (1–2 days), whereas *C. fructus*, *C. insectamans*, *C. norvegensis*, *P. kluyveri*, *P. membranifaciens* and *P. pijperi* appeared after the third to tenth day after the fruits had fallen. Of these, *P. kluyveri* and *C. fructus* secreted killer toxins, which enabled these yeasts to persist longer. After day 8, the counts were lower and the yeast species were represented by *C. diversa*, *C. karawaiewii* and *C. quercitrusa*.

In the same work, Morais et al. (1995b) isolated 215 isolates of 18 species of yeasts from surface of 45 flies of the *D. willistoni* group, 26 flies of *D. malerkotliana* and 11 *D. sturtevanti* flies associated with fallen amapa fruits. The yeasts most frequently vectored by flies included *K. apiculata*, *P. membranifaciens* like complex and *C. citrea*. Sixteen species were associated with the *D. willistoni* group, with which a high diversity of yeast species were associated, although *P. membranifaciens* was dominant. Flies of *D. malerkotliana* were associated with seven yeast species, dominated by *K. apiculata*, and *D. sturtevanti* was associated with five species of yeasts, *C. sorboxylosa* and *K. apiculata* being the most frequent ones. Among male flies of the *D. willistoni* group, *C. insectamans* was isolated only from *D. willistoni*, showing a close association with this fly species. *D. tropicalis* was frequently associated with *C. norvegensis*. The authors also isolated yeasts from crops of 55 flies of the *D. willistoni* group, 20 *D. malerkotliana* flies and six *D. sturtevanti* flies. The most frequent isolates in crops of flies were *K. apiculata*, *C. insectamans* and *P. membranifaciens* like. *C. citrea*, frequently isolated from fly surfaces, was not isolated from crops. Twelve yeast species were isolated from crops of flies of the *D. willistoni* group, *K. apiculata* being dominant, together with *C. insectamans*, which was isolated solely from this fly group. The *P. membranifaciens* like complex was frequently isolated from crops of male flies of *D. paulistorum*, and it was rarely isolated from crops of females of this fly or from other groups. *C. norvegensis* was isolated only from crops of *D. tropicalis* males, together with *C. fructus*, and varieties of *C. sorboxylosa* complex, indicating diet specificity in flies of the group. Five species were isolated from *D. malerkotliana* crops, and *K. apiculata* was the dominant yeast. *K. apiculata* and *P. pijperi* were the only species isolated from crops of *D. sturtevanti*.

This work resulted in the description of a new species, *C. amapae* (Morais et al. 1994). D1/D2 sequences of the 26S subunit of the rDNA showed that this species is closely related to *Saccharomycopsis crataegensis* (Kurtzman and Robnett 1998). Eleven species were assigned as species similar to *C. blankii*, *C. deformans (Yarrowia lipolytica)*, *C. diddensiae*, *C. quercitrusa*, *C rugopelliculosa*, *C. sorbosa*, *C. sorboxy-*

losa, *C. terebra* (= *P. mexicana*), *P. kluyveri*, *P. membranifaciens*, *P. muscicola* and *P. sargentensis*. They are probably new yeast species that wait description.

Vital et al. (2002) isolated and identified 240 yeasts from soils of the Maraca Ecological Station, located on Maracá Island, in the Amazon state of Roraima, in the Brazilian Amazon. These isolates were grouped into 16 genera and 66 species, with 82% of the strains having ascomycetous affinity. Some isolates had an intermediate level of partial D1/D2 sequence similarity with known species, and represented new undescribed species.

18.7 Other Ecosystems in South America

Gonzalez et al. (1989) in a study of yeasts during the delignification and fungal transformation of wood into cattle feed in continental and island locations of rain forest of southern Chile isolated 327 yeast strains from 33 samples of wood from *Eucryphia cordifolia* (Eucyphiaceae), *Nothofagus dombey* (Nothofagaceae), *N. oblique* (Nothofagaceae), *Laurelia sempervirens* (Atherospermataceae, Laurales), *L. phillipiana* (Atherospermataceae, Laurales), *Drimys winteri* (Winteraceae) and *Myrceugenia planipes (*Myrtaceae) at different stages of degradation. Most representative yeasts belonged to 37 species whose occurrence was not influenced by the type of wood. The most abundant yeasts were *Schizoblastosporion chiloense*, *C. castrensis* and *Apiotrichum futronensis* (= *Rh. futronensis*). Several new species were described among the strains isolated in this study (Ramirez and Gonzalez 1984a–j). Different stages of decay by *Ganoderma applanatum* were dominated by different yeast species: *C. parapsilosis* and *C. railenensis* were dominant at the initial stage; *S. chiloense*, *Rh. rubra* and *C. sophiae-reginae* at the intermediate stage; *Citeromyces matritensis* at the advanced stage; and *C. bertae*, *Cr. albidus* var. *diffluens*, which was present during the four stages, and *C sake* prevailed at the final stage. The relative abundance of basidiomycetous yeasts was low at the initial stage of decay, but significantly higher at more advanced stages (40–50% of colonies), particularly in samples of wood decayed by the mushroom *Armillariella limonea*, where they were represented by *A. humicolum* (= *Cr. humicola*), *A. futronensis* and *A. eucryphiae* (= *Leucosporidium scottii*).

Spencer et al. (1992) isolated 127 strains of yeasts from spoiled fruits, oranges, lemons and mandarins from a shop in Tucuman, Argentina, that included *C. guilliermondii*, *C. famata*, *C. stellata* and *P. kluyveri*. They also isolated probable new species, including ten strains belonging to the *P. membranifaciens* group, 13 unidentified strains of *Candida* spp., 20 strains of *Kloeckera* spp., 31 strains of *Pichia* spp. and two strains of *Cryptococcus* spp.

The distribution of yeasts was studied in different plant substrata of sandy plains along the southeastern Brazilian coast (Rosa et al. 1995). These formations are classified under the broad category of "restinga" vegetation. Restinga ecosystems are located at the interface between marine and continental (Atlantic rain forests) environments. Plant species living in these ecosystems are adapted to stressful environmental conditions such as high solar radiation, nutrient deficiency, drought, salt spray and high winds (Lacerda et al. 1984). Yeast communities were sampled from the cacti *Cereus pernambucencis*, *Selenicereus rizzini*, *Opuntia vulgaris* and *Opuntia*

sp., from flowers of *Ipomoea pes-caprae* and *I. litoralis* (Convolvulaceae) and from extrafloral nectaries of *Senna australis* and *S. bicapsularis* (Leguminoseae). Fifty-seven yeast species were isolated in this study, and nineteen were possible new species. Three distinct groups were noted among the different yeast communities studied. Yeast communities from necrotic tissues of cacti were grouped together; a second group was formed by yeasts from cactus flowers; and the last group was formed by yeast of the other non-cactus substrata. The species identified as *C. domercqii* like was described as a new species of *Wickerhamiella*, *W. cacticola* (Lachance et al. 1998). The *P. ohmeri* like strain represented a new species, and it was described as *Kodamaea nitidulidarum* (Rosa et al. 1999). A yeast identified as *Pichia* sp. C in this work was described later as *C. restingae*, a species belonging to the *Kodamaea* clade (Rosa et al. 1999). These three species were associated with cactus flowers.

Flowers of *I. litoralis* and *I. pes-caprae* were collected within 100 m of the ocean and were subjected to salt spray. This may have influenced the high occurrence of species capable of growing in media containing 10% NaCl in these yeast communities. Most yeast species from *Ipomoea* flowers and extrafloral nectaries produced pigments, extracellular polysaccharides and utilized a wide array of carbon sources. These characteristics could be important adaptive traits to help these yeasts colonize the microhabitats occurring in the restinga ecosystems (Rosa et al. 1995).

Santos et al. (1996) isolated yeasts from flowers and fruits from cashew (*A. occidentalis)*, cajá (*Spondia lutea*) and umbu (*Spondia* sp.) in a semiarid region near Campina Grande and João Pessoa, state of Paraíba, Brazil. The most frequent yeasts in flowers of these three trees were basidiomycetous anamorphs and black yeasts. Green fruits of cajá and umbu had a high number of yeast species with prevalence of black yeasts, *Cr. laurentii*, *C. entomaea* like and *Candida* spp. Cajá ripe fruits had the highest yeast diversity, and *K. javanica*, *I. orientalis*, *C. entomaea* like and *I. terricola* as prevalent species.

Yeasts were isolated from exudates of algarrobo (*Prosopis* spp.) trees from nine sites in northwestern Argentina, three in the Chaco region, from pods of algarrobo and acacia, collected in the Quebrada de Cafayate (Spencer et al. 1996). This region is arid to semiarid. Among 379 yeast isolates from exudates of algarrobo most were identified as *Bullera variabilis*, *C. famata*, *Cr. albidus* and other *Cryptococcus* species, *D. hansenii*, *P. angusta* (*Ogataea polymorpha*), *P. ciferrii*, *P. farinosa* and *Torulaspora delbrueckii*. Other *Candida*, *Kluyveromyces* and *Pichia* species were also found. Most species were osmotolerant. The high sugar content of the exudates influenced the nature of the yeast species present. The pods of *Acacia* and Algarrobo contained *Cryptococcus* spp., *C. famata* and *C. ciferrii*. Half the species isolated from rotting cactus were *Cryptococcus* species. *P. membranifaciens* and *T. delbrueckii* were also isolated.

Trindade et al. (2002) isolated yeasts from four different tropical fruits: pitanga (*Eugenia uniflora*), mangaba (*Hancornia speciosa*), umbu (*Spondia tuberosa*) and acerola (*Malpighia glaba*). Fruits were collected in small farms in northeastern Brazil. The most frequent yeasts were *K. apis*, *Pseudozyma antarctica*, *Cr. laurentii* and several species of *Rhodotorula*. Frozen pulps of these fruits present *C. sorbosivorans*, *C. spandovensis*, *C. spandovensis* like, *P. membranifaciens* and *Schizosaccharomyces pombe* as

prevalent species. From 70 yeast species isolated in this study, 24 did not fit in any previously described species, and were probably new biotypes or new species. Trindade et al. (2004) showed that the species identified as *C. spandovensis* like was phylogenetically related to *C. spandovensis* and *C. sorbophila*, species belonging to the *Wickerhamiella* clade. This new species was described as *C. sergipensis*.

Currently, a study of yeasts associated with flowers of different plant species in the dominion of Cerrado is being done in middle northern Brazil. It included at this time 55 samples of tree exudates in *ipucas*, natural forest fragments in flooded plains that resulted in the isolation of 45 strains of yeasts which included a new methylotrophic yeast belonging to the genus *Ogataea*, *Ogataea falcaomoraisii* (Morais et al. 2004). The sampling effort included 86 flowers of *Tabebuia aurea* (Bignoniaceae), from which 125 yeast strains were isolated, including five new ascomycetous species that await description, and 106 flowers of *Ipomoea* spp., from which 187 strains were isolated that included yeasts such as *Metschnikowia continentalis*, *M. colocasiae*, a new species of *Metschnikowia, K. apiculata*, five new species of *Starmerella*, and *P. antarctica* (Morais and Rosa, unpublished).

18.8 Physiological Profiles of Yeast Communities in Tropical Rain Forests

Physiological characteristics and metabolic abilities of yeast communities show a pattern that accompanies the evolutionary history of the associated substrates and vectors (Starmer 1981; Lachance and Starmer 1982). Communities evolved from the colonization of foliar substrates and mushrooms, sites associated with yeasts presenting large physiological profiles, to colonization of flowers, fruits and specific substrates such as necrotic tissues, which are more specialized and evolutionarily more recent (Starmer 1981). According to Lachance and Starmer (1982), the physiological structure of yeast communities is a function of evolutionary divergence in tree taxa, as viewed by Dahlgreen (1980) and Hutchinson (1969). They showed a gradient from polytrophic communities associated with Betulaceae – trees deemed more "primitive" – at one end to the nutritionally selective yeasts linked to the Campanulaceae and the Myoporaceae – more "advanced" (Sporne 1980) – at the other end. It is probable that yeast communities are shaped in response to changes in nutrient composition of tree habitats, and that chemical breadth, rather than the absence of specific compounds, is the major determining factor.

A comparison was made by Morais et al. (1992a) of physiological profiles of yeasts associated with *Drosophila* from Atlantic rain forests and temperate forests (from Heed et al. 1976), which showed that most yeasts ferment glucose in both environments, but they differ in assimilation characteristics. Most yeast species associated with *Drosophila* in Atlantic rain forests were not unique but were similar to yeast communities associated with fruits and other fermentable substrates. The yeast biotypes assimilative abilities were mostly restricted to a few compounds that include glycerol, D-xylose, sucrose, cellobiose, mannitol and succinate. In contrast, yeasts from temperate American forests were largely assimilative of galactose, sucrose, maltose, trehalose, cellobiose, salicin, glycerol, mannitol and succinate. Those yeasts differed strongly from yeast profiles from other habitats such as cacti and associated *Drosophila* in

desert sites. The genera *Pichia* and *Hanseniaspora*/*Kloeckera* predominate among yeasts of flies from tropical forests and *Drosophila* in Brazil.

According to Morais et al. (1995b), the physiological abilities of the yeast community associated with amapa fruits were mostly restricted to the use of glycerol, cellobiose, D-lactate, salicin, and L-sorbose among 36 carbon compounds tested. The community is strongly fermentative (82% of isolates) and capable of growing at 37°C more than other tropical isolates. In Amazonian forests, a large variation of responses to temperature, including ability to grow at temperatures higher than that described for the species, was a general rule among yeasts, and Morais et al. (1995b) correlate this to the high daily variation in temperature (up to 6°C) in rainy forests of Brazil.

The different stages of deterioration of the amapa fruit should be regarded as a microhabitat mosaic in which different yeast species colonize different stages, in a successional pattern directed by the chemical composition of the substrate, and mainly by visitation of vectors attracted to it by yeasts. In the first stages the fruits were colonized by yeasts fermenting simple sugars and presenting restricted assimilation profiles, such as *C. sorbosa* like complex and *K. apis*, whereas later stages corresponded to colonization of yeasts with larger assimilative characteristics, such as *C. diversa*, *C. karawaiewii* and *C. quercitrusa*.

18.9 Ecological Interpretation of Yeast Diversity in Forests

Fruits, flowers and *Drosophila* flies provided a good estimate of yeast diversity in tropical ecosystems (Table 18.1). Yeast community structure and species diversity seems to reflect the degree of disturbance in tropical forests. Comparisons among habitats have shown that yeast diversity is higher in primary forest sites, probably as a result of more diversified food sources in primary than in disturbed areas. A higher diversity of food sources is also supported by the extensive physiological profiles of yeast communities associated with *Drosophila* species that prevails at primary sites (Morais et al. 1992a).

Morais et al. (1995b), studying yeasts associated with ripe Amazon fruits at two Amazon rain forest sites, detected a higher diversity at the more heterogeneous sites in protected areas with higher plant diversity than at homogenous sites of lower plant diversity. Yeast species composition and diversity could indicate habitat heterogeneity in tropical ecosystems, since environmental degradation and patch removal seem to extinguish populations and decrease diversity (Nee and May 1992).

18.10 Concluding Remarks

Many of the isolates in the studies of yeasts from forests in Brazil and other countries of South America varied from the descriptions of known species, confirming the need for further taxonomic studies of yeasts from tropical forests. We can conclude from the studies already made that South American forests are yet to be screened for yeasts. It was shown that yeast vectors such as drosophilids are an important sampling unit for further investigations, and will allow comparisons among yeasts communities from different ecosystems and biomes. There is a

Table 18.1 Comparison of yeast diversity associated with plants and insects in three different ecosystems in Brazil[a]

Yeast species	Sandy coastal plains of Rio de Janeiro		Atlantic rain forests, Rio de Janeiro			Amazonian rain forest	
	Flowers of *Ipomea* spp (*n*=91)[b]	Extrafloral nectaries of *Senna* spp (*n*=109)	*Drosophila fasciola* (*n*=399)	*Drosophila quadrum* (*n*=103)	*Drosophila* spp. (*n*=624)	*Parahancornia amapa* fruits (*n*=127)	*Drosophila* species (*n*=173)
Aureobasidium pullulans	40	38	19	8	21	6	
Candida amapae						29	
C. antarctica	55	25	4	1	2		
C. apicola	1		1	7	23		
C. apis				3	14		
C. berthetii					2		
C. bimundalis (= *Pichia bimundalis*)			1		1		
C. blankii like[c]			3	1		1	
C. boleticola			4		7		
C. citrea (= *P. nakasei*)			4		48	4	14
C. colliculosa			145	1	20		
C. colliculosa like			5				
C. datilla (*Lachancea thermotolerans*)			2				
C. deformans like						1	
C. dendrica			2			1	
C. diddensiae like						1	
C. diversa			7	5	1	18	8
C. fabianii (= *P. fabianii*)			1				
C. fructus			2	7	15	24	6
C. guilliermondii	2			16	2	16	
C. holmii (= *Kazachstania exigua*)						1	
C. humicola (Cr. humicola)					3		

Continues

Table 18.1 Comparison of yeast diversity associated with plants and insects in three different ecosystems in Brazil[a]—*cont'd*

Yeast species	Sandy coastal plains of Rio de Janeiro		Atlantic rain forests, Rio de Janeiro			Amazonian rain forest	
	Flowers of *Ipomea* spp (n=91)[b]	Extrafloral nectaries of *Senna* spp (n=109)	*Drosophila fasciola* (n=399)	*Drosophila quadrum* (n=103)	*Drosophila* spp. (n=624)	*Parahancornia amapa* fruits (n=127)	*Drosophila* species (n=173)
C. humicola like					1		
C. insectamans				1		10	30
C. intermedia				1			
C. karawaiewii			2			6	
C. karawaiewii like					1		
C. krissii like	2						
C. krusei			107			27	
C. lambica (= *P. fermentans*)			4	1	5		
C. lypolitica like						2	
C. magnoliae			1		2		
C. melinii (= *P. canadensis*)				2	11		
C. melinii like	1						
C. norvegensis			14		4	8	9
C. norvegensis like					3		
C. parapsilosis			3			8	
C. pelliculosa			3	3			
C. pulcherrima (= M. pulcherrima)					15		
C. quercitrusa				11	14		
C. reukafii like	12						
C. rugopelliculosa			1				
C. santamariae like	5						
C. sorbophila			1				
C. sorbosa			14				
C. sorbosa like	25	25			1	30	25

C. sorboxylosa like	17	17				75	17
C. sorboxylosa			40				
C. steatolytica (= Zygoascus hellenicus)	1						
C. stellata like			1				
C. tepae like					1		
C. terebra like							1
C. valida (P. membranifaciens)	1						
C. valida like			48				
Candida spp.	29	2	9		3		
Clavispora opuntiae			7				
Cryptococcus albidus				1			
Cr. albidus var. aerius		12					
Cr. albidus var. albidus		32					
Cr. flavus	11	4					
Cr. infirmominiatus (Cystofilobasidium infirmominiatum)					1		
Cr. humicolus				1		5	1
Cr. hungaricus		4	4	4			
Cr. laurentii	48	9					
Cr. luteolus		1		8			
Cr. macerans	1	15			2		
Cryptococcus sp.	1						
Debaryomyces melissophilus			2	17	7		
D. vanrijae var. yarrowii				28	25	10	
Geotrichum sp.			25	11	47	16	1
Hanseniaspora guilliermondii			16				
H. occidentalis			34		2	2	3
H. uvarum				2	8		
H. valbyensis					2		
H. vineae					1		
Issatchenckia occidentalis				2		1	
Kloeckera africana				1	10	3	
K. apiculata	2		84			128	150

Continues

Table 18.1 Comparison of yeast diversity associated with plants and insects in three different ecosystems in Brazil[a]—*cont'd*

Yeast species	Sandy coastal plains of Rio de Janeiro		Atlantic rain forests, Rio de Janeiro			Amazonian rain forest	
	Flowers of *Ipomea* spp (n=91)[b]	Extrafloral nectaries of *Senna* spp (n=109)	*Drosophila fasciola* (n=399)	*Drosophila quadrum* (n=103)	*Drosophila* spp. (n=624)	*Parahancornia amapa* fruits (n=127)	*Drosophila* species (n=173)
K. apis	29		75	18	145	42	2
K. corticis					3		
K. japonica				1	86	6	
K. javanica			23	1	117		
Kluyveromyces delphensis (= Nakaseomyces delphensis)					34		
K. marxianus var. drosophilarum			3	1	10		
K. marxianus var. marxianus					1		
K. thermotolerans (= L. thermotolerans)					2		
K. wickerhamii					5		
Nadsonia elongata like			5				
Pichia abadiae					1		
P. acaciae				9	1	1	2
P. angusta (O. polymorpha)					11		
P. anomala				1	1		
P. beckii				2	34		6
P. cactophila			13				
P. carsonii			2		5		
P. dryadoides					4		
P. etchelsii (= *D. etchelsii*)						7	
P. fermentans						7	1
P. holstii					4		
P. kluyveri			121	1	8	21	1

P. kluyveri like						35	18
P. lindneri (= *P. minuta*)				4			
P. membranifaciens like			133	10		53	73
P. muscicola like						1	
P. ohmeri (= *Kodamaea ohmeri*)	4						
P. pijperi						13	4
P. sargentensis like						3	
P. silvicola					2		
P. silvicola like			1				
P. toletana					1		
Pichia spp.			9				
Rhodotorula araucariae					1		
Rh. aurantiaca	17	1			1		
Rh. fujisanensis					1		
Rh. glutinis	48	38	2	4	1		
Rh. lactosa		1					
Rh. minuta	1	3			2		
Rh. rubra		5	1	17	2		
Saccharomyces cerevisiae					3		
Saccharomycodes ludwigii			1				
Saturnispora sp.			6				
Sporobolomyces roseus	24						
Sporopachydermia spp.			13				
Torulaspora delbrueckii			1	5	3		
T. globosa			5				
T. pretoriensis			7		3		
Tremella globispora	2						
Tremella spp.		5					
Williopsis californica					22		
W. saturnus					8		
Zygossaccharomyces rouxii			1				
Total yeast species	25	17	58	40	70	34	24

[a]Morais et al. (1992a, 1995a, b, 1996) and Rosa et al. (1995)
[b]Number of samples
[c]Probable new species similar in characteristics to the species indicated

significant amount of literature on Lepidoptera, Coleoptera and Hymenoptera, especially ants and bees that could guide a strategy for research of yeasts associated with insect guild and communities in different landscapes of the most threatened biomes. We need to make an effort to test methodologies applicable to interdisciplinary studies, such as field traps that could be employed to aseptic collections, collection procedures for yeast isolation less destructive to insects, and standard approaches to taxonomy, ecology and phylogeny of both groups.

Fruits and flowers are a very attractive sampling unit, since they are easy to collect and to handle in the laboratory, and many methodologies could be employed to isolate yeasts from those substrates. Also, phenological information on and the distribution of species in ecosystems and ecological characters are already known for many species of plants.

A map of known yeast communities in forests shows that most hot spots for biodiversity – also threatened ecosystems – are yet to be studied. A strategy focusing on biodiversity hot spots is important to direct efforts to understand yeast community structure and composition in forests, and that will result in magnification of yeast biodiversity in forests in South America.

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Chapter 19

The Biogeographic Diversity of Cactophilic Yeasts

William T. Starmer[1], Virginia Aberdeen[1] and Marc-André Lachance[2]

[1]*Department of Biology, Syracuse University, Syracuse, NY 13244, USA (e-mail: wstarmer@syr.edu; e-mail: vlaberde@syr.edu)*
[2]*Department of Biology, University of Western Ontario, London, ON, Canada N6A 5B7 (e-mail: lachance@uwo.ca)*

19.1 Introduction

Two commonly used measures of biological diversity are the number of unique species (species richness) and the uncertainty of species identity (diversity measured by H'; Pielou 1975). These metrics are affected by the availability of suitable habitats and are ultimately a function of speciation, extinction, and immigration patterns (Ricklefs 1987; Zobel 1997; Hubbell 2001). For this last function, it is generally thought that the size of an organism is important in its capacity to disperse and that very small organisms (microorganisms) freely disperse over large areas, usually as passive propagules in currents of air or water. In this situation the species in a local area are expected to be the same as species over larger, even global, expanses (Godfray and Lawton 2001). This "bugs are everywhere" hypothesis is, however, without foundation for many small organisms that have formed obligate relationships with vectors that constrain their dispersal patterns. Among the fungi, ascomycetous yeasts are often constrained by their vectors (Phaff and Starmer 1987). Two examples are the yeasts associated with drosophilids that feed and breed in the necrotic tissues of cacti (Barker and Starmer 1982) and yeast communities in blossoms of Convolvulaceae (morning glory, bindweeds) and *Hibiscus* species. This latter group has been reviewed and studied in a biogeographic context by Lachance et al. (2001b). They concluded that geographic factors may not act directly to determine yeast distributions but rather act indirectly through their insect vectors (i.e., Coleoptera) that distribute the yeasts to their flower–host resources. These insects have a major influence on the biogeographic diversity of flower-specific yeasts. The extensive records and wide ranging sampling that characterize studies of the flower-inhabiting yeasts make them a useful system for studying yeast ecology and insect–yeast interaction. The system is comparable to the cactophilic yeasts (Starmer and Fogleman 1986; Starmer et al. 1991).

The cactophilic yeasts have been collected and studied for over 30 years and provide insights into the origin of new species (Starmer et al. 1980), host and vector ecology (Ganter et al. 1986), community stability (Latham 1998), yeast diversification

(Lachance et al. 2000, 2001b), origin of yeast communities (Starmer et al. 2003), and the relationship of phylogeny to yeast community organization (Anderson et al. 2004). Only four dominant yeasts are found world-wide in the cactophilic yeast community [*Pichia cactophila*, *Candida sonorensis*, *Sporopachyderma cereana* (and related sister species) and *Clavispora opuntiae*]. Several species are very common (*Myxozyma mucilagina*, *S. amethionina* clade species, *Dipodascus starmeri*, *Pichia deserticola* and *Pichia kluyveri* v. *eremophila*). All of these species are cactus-specific; they are rarely recovered from other habitats such as sympatric fruit rots (Starmer et al. 1987) or slime fluxes of trees (Ganter et al. 1986). These species have diverse origins. They do not belong to a single cactus-yeast clade but evolved from distinct ancestors found in separate clades that show affinity to fruit-rot and tree-flux habitats (Starmer et al. 2003). Overall 80 species have been detected in decaying columnar-cactus stems or *Opuntia* cladode rots. In this chapter, we shall analyze the pattern of diversity for these 80 species found in cacti and discuss possible reasons for those patterns.

The biogeography of cactophilic yeasts has been described, compared and discussed in terms of host and geographic determinants of species and community distributions (Starmer et al. 1990). That analysis compared four community types (host cactus categories) across five semiarid regions of the southern USA, Mexico, northern Venezuela, as well as Caribbean and Bahamian islands. The analysis showed that yeast communities from the same host cactus type were more similar to one another across the five regions as compared with yeast communities from different host cactus types within regions. It thus appeared that the host plant had a larger influence on the diversity and the composition of the yeast community than geographic separation. However, this conclusion for community similarity was not a generality that extended to all species, where some geographic factors may be important to the diversification and speciation of some cactus specific species complexes (i.e., *Sporopachyderma* spp., Lachance et al. 2001b; *Phaffomyces* spp. Starmer et al. 2001; and *Starmera* spp. Starmer et al. 1990). Since the last review of the biogeography of the yeasts associated with cacti (Starmer et al. 1990) new collections and new species have been added to the database, and records from other continents (primarily Australia, where *Opuntia* cacti were introduced) have been incorporated. The additional data for Australian localities add another level to the determinants of biodiversity. This situation is unique because not only were the cacti introduced but the cactus yeasts (from the Americas) were also introduced when biological control was attempted in efforts to remove *Opuntia* from large geographic areas of eastern Australia (Starmer et al. 1987). In addition, the local yeasts already in Australia are expected to be significantly different from those on other continents. This difference in indigenous microbiota is expected to add significantly to the species richness and diversity in the rotting cladodes of *Opuntia* in Austrialia.

19.2 Methods

19.2.1 Measuring Diversity

Even though diversity can be quantified by many different metrics (for example, 24 measures of β diversity, Koleff et al. 2003), a useful method is to partition the global

diversity, γ, into diversity between and within levels of a hierarchy. The simplest partition is $\gamma = \alpha + \beta$, where α is the average diversity for all of the samples and β is the between-sample diversity or the difference between γ and α (Lande 1996; Loreau 2000). When several levels exist in a hierarchy, e.g., host plants, local areas, and regions such that n=3, then $\gamma = \alpha + \beta_1 + \beta_2 + \beta_3$. In this example α is the average diversity of the yeasts in the host plants, β_1 is the diversity between host plants, β_2 is the diversity between localities, β_3 is the diversity between regions, and γ is the total diversity.

The additive partition can be used for species richness S (defined as the total number of unique species in the entire collection), the Simpson index $d=1-\Sigma p_i^2$ or the Shannon–Weiner index $H' = \Sigma p_i \ln(p_i)$, where p_i is the proportional representation of each species in the level under consideration. This approach has been reviewed by Veech et al. (2002) and is becoming widely used by ecologists to understand the relationship between scale and biological diversity (Godfray and Lawton 2001). For an extensive discussion of β diversity see Vellend (2001). Veech et al. (2002) suggest converting the diversity components in the hierarchy into percentages or proportions of the total (γ) for comparison of the relative contribution to the total species richness (S), Simpson's index (d), or the Shannon–Weiner index (H'). We have followed their suggestion and present statistics for S and H'.

19.2.2 Hosts

Cactus hosts were categorized according to the systematic groupings outlined by Gibson and Nobel (1986) and conform to the listing given in Starmer et al. (1990). The major divisions we use in our analysis are given in bold in Table 19.1. They were chosen for study because they represent a nested taxonomic hierarchy and have adequate sample sizes.

19.2.3 Geography

The database used in this study includes records for 188 distinct collection localities in eight regions (listed in the following with the number of localities for each, see Fig. 19.1 for a map of the New World regions):

1. AU (44): Australia (Queensland and New South Wales)
2. CA (24): Caribbean Islands [Greater Antilles: Cuba, Cayman Islands, Jamaica, Navassa, Hispainola (Haiti and Dominican Republic); Lesser Antilles: Montserrat, US & British Virgin Islands, and Islas Los Roques]
3. FB (7): USA(Florida), Bahama Islands (Great Inagua and Conception Island)
4. HA (2): USA (Hawaii)
5. SM (25): Mexico (Chiapas, Guerrero, Hidalgo, Jalisco, Michoacan, Oaxaca, and Puebla), Honduras
6. SD (71): USA (Arizona and California), Mexico (Sinaloa and Sonora)
7. TX (5): USA (Texas)
8. VZ (10): Venezuela (northern)

Table 19.1 Cactus hosts sampled according to systematic groups. Those groups given in *bold* were used as categories for analysis. The number of yeast species and the number of plants sampled are in *parentheses*. Only the number of plants sampled is given for each genus

Family	Subfamily	Tribe	Subtribe	Genus
Cactaceae (80, 2649)	**Opuntioideae** (67, 1651), North America: (42, 950), introduced: (46, 701)			*Opuntia* (1602), *Nopalea* (49)
	Cactoideae (49, 998)	Cacteae		*Ferocactus* (13)
		Cereeae		*Cereus* (36), *Melocactus* (18)
		Hylocereeae		*Acanthocereus* (7)
		Leptocereeae		*Neoabbottia* (12)
		Pachycereeae	**Pachycereinae** (36, 335)	*Lophocereus* (80), *Carnegiea* (54), *Backebergia* (1), *Cephalocereus* (28), *Neobuxbaumia* (1), *Pachycereus* (37), *Pilosocereus* (134)
			Stenocereinae (38, 572)	*Stenocereus* (533), *Myrtillocactus* (28), *Escontria* (16)

The hierarchy available for cactus yeasts is easily viewed as a nested geographic set, starting from the global or continental level, which is divided into distinct regions, that are further divided into subregions composed of a group of localities in which individual rot pockets of plants are sampled to yield the incidence of yeast species. Thus, each yeast species has a plant, locality, subregion, region, and continental designation such that diversity can be viewed at all levels. Specific definitions of the levels for this study are as follows.

2.3.1 Plants

Individual plants are discrete. Even though a single plant can have multiple rot pockets, our collections have generally been limited to one sample per plant. The number of yeast species present in a plant-rot pocket is the species richness (S). The overall database does not allow calculations of p_i within a plant because the cell numbers for each species were not accurately estimated in all collections. As a consequence the diversity index H' could be calculated only for localities, subregions, regions, and continents. However, we do include the categories "within-plant" and "between

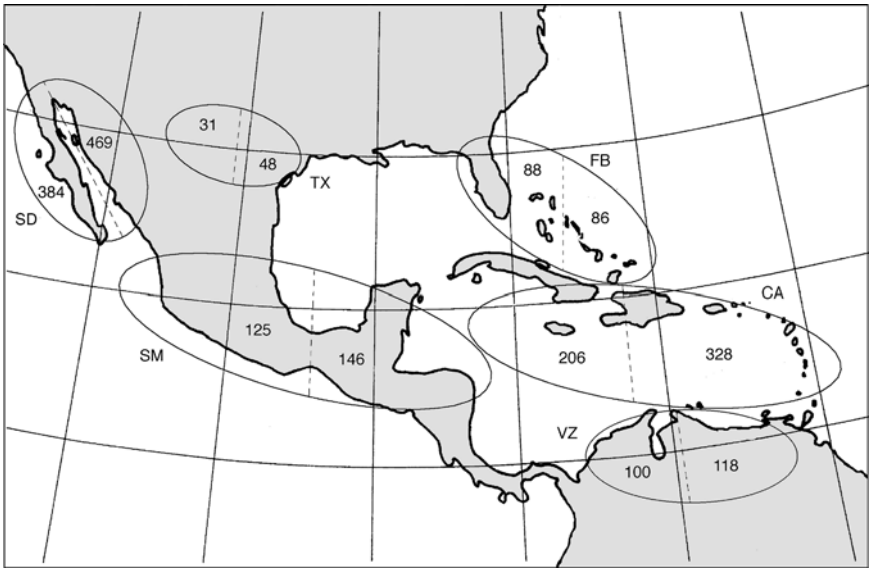

Fig. 19.1. Geographic areas where cactus necroses were collected. *Shaded oval areas* show the six distinct regions that were sampled (see Sect. 2.3 for the regional definitions). Each region is divided by a *dashed line* into subregions. The *numbers* within in each subregion are the number of plants sampled

plants" for studies of two *Stenocereus* species, *S. gummosus* (Pitaya agria) and *S. thurberi* (organpipe), for which detailed within-plant yeast-cell counts were made by selective-isolation methods (Starmer 1982; Fogleman and Starmer 1985). In order to eliminate noise from estimates of H', we excluded locations with small numbers of yeast isolates ($n<8$; e.g., a location with only one or a few plant samples).

2.3.2 Localities

Localities are somewhat subjective but were discrete. They range in size from a few square meters to several hectares. Separate localities were usually isolated in space by 10 km or more. Localities can be viewed as having more dispersal potential within than between.

2.3.3 Subregions

Subregional divisions of larger regions were mainly arbitrary but represented two more or less equal east–west contiguous partitions, except for Australia, where the division was north–south, and Hawaii, where no subregional division was warranted.

2.3.4 Regions

Regions were geographically distinct in the sense that dispersal between regions was severely limited by large expanses such as cactus-free terrain, bodies of water, or mountains.

2.3.5 Continents

Continental division was only possible for *Opuntia* hosts and is a "natural" versus "introduced" categorization of the *Opuntia* cacti. Those cacti in continental New World regions (North America, the Caribbean, the Bahamas, and northern Venezuela) were native, whereas those in Australia and Hawaii were introduced.

19.2.4 Yeasts

The localities were partitioned into eight regions and 15 subregions. Figure 19.1 shows the regional and subregional extent for North America. The number of plants sampled in each is shown for each subregion. The yeast taxa recovered from the entire collection and their frequency of isolation on a per plant basis are listed in Table 19.2 along with their isolation frequency in each of the eight regions.

19.3 Species Richness

Comparison of the proportion of diversity (*S*) explained as the scale of sampling increases shows a distinct trend for yeasts both from native columnar cacti and from *Opuntia* (Table 19.3, Fig. 19.2). The β diversity for species richness (*S*) between regions is over 50%. This differentiation at the highest level of division could have several causes that function to render the regions different from one another in their species composition: (1) the discontinuous geographic regions may severely limit the likelihood of dispersal from region to region, (2) allopatric speciation events result in differential community composition, (3) opportunistic local yeasts (non-cactus-specific) differ in the regions such that rare entry into the community causes the species richness to increase on pooling species from all regions, (4) cactus-specific species that were once widespread have become extinct in some regions but not all (i.e., relics), and (5) species from different regions are different but ecologically equivalent (Shmida and Wilson 1985).

19.3.1 Yeasts of Columnar Cacti

In order to evaluate the factors just listed we compared the species composition for the nature of species that were not shared among regions. Of the 49 species in the columnar community, 24 were unique (or essentially unique) to an individual region. In two instances the unique species were commonly recovered from cactus rots and also were closely related, i.e., *Phaffomyces antillensis* was found in the Caribbean region and the sister species *P. thermotolerans* in the Sonoran Desert; *Starmera caribaea* was exclusive to the Caribbean and *S. amethionina* in the Sonoran Desert.

Table 19.2 Number of each yeast species isolated from the entire collection and the eight geographic regions (see Sect. 2.3 for a description of each region). Yeast species listed as sp. "1", etc. are unknown or unidentified taxa

	Global	AU	CA	FB	HA	SM	SD	TX	VZ
Pichia cactophila	1,245	251	271	48	22	100	394	29	130
Candida sonorensis	862	314	106	54	19	35	220	54	60
Sporopachydermia cereana	592	45	108	29	2	54	282	26	46
Clavispora opuntiae	377	228	46	8	21	17	27	9	21
Myxozyma mucilagina	174	39	–	13	1	1	93	23	4
Starmera amethionina	166	27	–	–	1	–	125	13	–
Dipodascus starmeri	161	–	19	1	–	23	96	–	22
Pichia deserticola	140	–	47	–	–	15	70	8	–
Pichia kluyveri v. *eremophila*	119	–	–	–	–	10	76	33	–
Pichia mexicana	86	–	11	5	–	4	34	–	32
Pichia heedii	72	–	–	–	–	–	72	–	–
Pichia angusta	69	1	1	1	–	–	59	7	–
Cryptococcus albidus	67	30	3	–	1	5	21	2	5
Starmera caribaea	64	–	33	18	-	2	2	4	5
Candida boidinii	62	33	–	4	6	2	9	8	-
Pichia opuntiae	61	53	–	–	–	1	5	–	2
Pichia barkeri	57	41	8	4	–	4	–	–	–
Pichia kluyveri	48	15	12	8	3	3	6	–	1
Rhodotorula minuta	46	34	1	–	–	–	9	1	1
Pichia norvegensis	45	–	7	18	9	–	11	–	–
Cryptococcus laurentii	40	21	6	–	–	2	5	4	2
Pichia membranifaciens	39	4	–	1	–	2	1	–	31
Clavispora lusitaniae	32	–	–	–	–	29	3	–	–
Phaffomyces thermotolerans	26	–	–	–	–	–	26	–	–
Candida caseinolytica	26	–	–	–	–	1	24	1	–
Pichia pseudocactophila	25	–	–	–	–	5	20	–	-
Rhodotorula mucilaginosa	24	16	1	–	–	1	–	–	6
Debaryomyces hansenii	23	19	2	–	–	–	1	–	1
Kloeckera apiculata	22	11	3	–	3	–	–	5	–
Phaffomyces antillensis	20	–	20	–	–	–	–	–	–
Cryptococcus sp. "1"	15	–	–	–	–	–	–	–	15
Pichia guilliermondii	13	1	–	1	–	1	3	3	4
Kluyveromyces marxianus	13	–	–	–	–	–	12	1	–
Rhodotorula graminis	12	–	6	1	–	1	2	1	1
Williopsis californica	9	9	–	–	–	–	–	–	–
Rhodotorula glutinis	8	7	–	–	–	–	–	–	1
Cryptococcus macerans	8	8	–	–	–	–	–	–	–
Candida zeylanoides	8	7	1	–	–	–	–	–	–
Metschnikowia pulcherrima	7	7	–	–	–	–	–	–	–
Kloeckera apis	6	–	3	2	–	1	–	–	–
Cryptococcus luteolus	5	2	1	2	–	–	–	–	–
Candida sp. "2"	4	–	1	–	–	1	1	–	1
Candida vini	4	4	–	–	–	–	–	–	–
Candida orba	4	4	–	–	–	–	–	–	–
Trichosporon cutaneum	3	–	–	–	–	–	–	–	3
Rhodotorula fujisanensis	3	3	–	–	–	–	–	–	–
Pichia sp. "TM"	3	–	–	–	–	–	3	–	–
Candida viswanathii	3	3	–	–	–	–	–	–	–
Candida krissii	3	3	–	–	–	–	–	–	–
Yarrowia lipolytica	2	2	–	–	–	–	–	–	–
Rhodotorula aurantiaca	2	–	2	–	–	–	–	–	–

Continues

Table 19.2 Number of each yeast species isolated from the entire collection and the eight geographic regions (see Sect. 2.3 for a description of each region). Yeast species listed as sp. "1", etc. are unknown or unidentified taxa—*cont'd*

	Global	AU	CA	FB	HA	SM	SD	TX	VZ
Pichia onychis	2	–	1	–	–	–	1	–	–
Issatchenkia orientalis	2	–	–	1	–	–	1	–	–
Issatchenkia occidentalis	2	2	–	–	–	–	–	–	–
Cryptococcus skinneri	2	–	–	–	–	1	1	–	–
Pichia burtonii	1	1	–	–	–	–	–	–	–
Trichosporon sp. "2"	1	–	–	–	–	–	1	–	–
Trichosporon sp. "1"	1	1	–	–	–	–	–	–	–
Debaryomyces sp."1"	1	–	1	–	–	–	–	–	–
Rhodotorula sp. "2"	1	–	1	–	–	–	–	–	–
Rhodotorula sp. "1"	1	1	–	–	–	–	–	–	–
Rhodotorula marina	1	–	1	–	–	–	–	–	–
Rhodotorula auriculariae	1	1	–	–	–	–	–	–	–
Pichia strasburgensis	1	–	–	–	–	–	1	–	–
Pichia nakasei	1	1	–	–	–	–	–	–	–
Pichia glucozyma	1	1	–	–	–	–	–	–	–
Pichia farinosa	1	–	–	–	–	–	1	–	–
Kloeckera japonica	1	1	–	–	–	–	–	–	–
Dipodascus capitatus	1	1	–	–	–	–	–	–	–
Cryptococcus magnus	1	–	–	–	–	1	–	–	–
Cryptococcus infirmo-miniatus	1	1	–	–	–	–	–	–	–
Cryptococcus flavus	1	–	–	–	–	–	1	–	–
Candida sp. "3"	1	1	–	–	–	–	–	–	–
Candida sp. "1"	1	–	–	–	–	–	1	–	–
Candida vanderwaltii	1	1	–	–	–	–	–	–	–
Candida tropicalis	1	–	–	–	–	–	1	–	–
Candida tenuis "like"	1	1	–	–	–	–	–	–	–
Candida inconspicua	1	–	–	–	–	–	1	–	–
Candida diddensiae	1	1	–	–	–	–	–	–	–
Candida catenulata	1	–	1	–	–	–	–	–	–
Total	4,958	1,257	724	219	88	322	1,722	232	394

Both cases represent speciation events as sources of the overall species richness. Two other common, but unrelated cactus-specific yeasts, *Pichia heedii* and *Candida caseinolytica*, were region-specific (Sonoran Desert) and could represent old lineages that have only survived in the Sonoran Desert, i.e., relics. Alternately, they might be autochthonous members of other sympatric communities living in other plants but we have little evidence for this possibility (Ganter et al. 1986). *Pichia membranifaciens* was found frequently in cacti collected in Venezuela, occurring multiple times in different localities. This species is not cactus-specific and is found in a number of habitats, including fruit rots and tree fluxes. This may be a case where the species diversity is increased as a consequence of ecological equivalence. However, because the *P. membranifaciens* phenotype is convergent with a number of cactus-specific yeasts and because these strains are no longer available, it is not possible to verify their identity. They may in fact represent cryptic species similar to *P. membranifaciens*, as is known to be the case in recent studies of sap-flux yeasts in Costa Rica

Table 19.3 Species richness (*S*) and diversity (*H′*) for host categories in the geographic hierarchy. *Numbers* in *bold* are the percentage contribution of the total species richness or diversity. β diversity represents contributions of between-level components. α diversity is the within-sample diversity (see Sect. 2.1 for details on calculations)

	Global	Continental	Regional	Sub-Regional	Local	Plant	Within plant
Opuntioideae:*S*	67	44	22.13	16.60	5.78	1.94	–
β_c:β_r:β_{sr}:β_l:β_p:α	**34.3**	**32.6**	**8.2**	**16.2**	**5.7**	**2.9**	–
H′	2.68	2.505	2.189	2.08	1.669	–	–
β_c:β_r:β_{sr}:β_l:α	**6.5**	**11.8**	**4.1**	**15.3**	**62.3**	–	–
Cactaceae:S	–	56	26.17	19.83	7.93	1.95	–
β_r:β_{sr}:β_l: β_p:α	–	**53.3**	**11.3**	**21.3**	**10.7**	**3.5**	–
H′	–	2.642	2.299	2.193	1.705	–	–
β_r:β_{sr}:β_l: α	–	**13.0**	**4.0**	**18.5**	**64.5**	–	–
Opuntioideae:S	–	42	20.17	14.67	5.67	1.94	–
β_r:β_{sr}:β_l:β_p:α	–	**52.0**	**13.1**	**21.4**	**8.9**	**4.6**	–
H′	–	2.552	2.197	2.044	1.671	–	–
β_r: β_{sr}:β_l: α	–	**13.9**	**6.0**	**14.6**	**65.5**	–	–
Cactoideae:*S*	–	49	20.2	14.3	5.57	1.59	–
β_r:β_{sr}:β_l:β_p:α	–	**58.8**	**12.0**	**17.8**	**8.1**	**3.2**	–
H′	–	2.525	2.091	1.959	1.619	–	–
β_r:β_{sr}:β_l: α	–	**17.2**	**5.2**	**13.5**	**64.1**	–	–
Pachycereinae:*S*	–	36	13.6	9.89	3.81	1.34	–
β_r:β_{sr}:β_l:β_p:α	–	**62.2**	**10.3**	**16.9**	**6.9**	**3.7**	–
H′	–	2.567	2.005	1.820	1.501	–	–
β_r:β_{sr}:β_l: α	–	**21.9**	**7.2**	**12.4**	**58.5**	–	–
Stenocereinae:*S*	–	38	17.5	12.38	4.85	1.44	–
β_r:β_{sr}:β_l:β_p:α	–	**53.9**	**13.5**	**19.8**	**9.0**	**3.8**	–
H′	–	2.299	1.986	1.871	1.579	-	–
β_r:β_{sr}:β_l: α	–	**13.6**	**5.0**	**12.7**	**68.7**	-	–
Stenocereinae[a]							
H′	–	2.299	1.986	1.871	1.420	0.9145	0.7075
β_r:β_{sr}:β_l: β_p:β_w: α	–	**13.6**	**5.0**	**19.6**	**22.0**	**9.0**	**30.8**

[a]Local, plant, and within-plant categories are estimated from data of *Stenocereus gumosus* and *S. thurberi* collections in the Sonoran Desert that selectively isolated and recorded cell counts of yeast species within plants, while the other categories include *Stenocereus* species from all regions.

and *Clermontia* flowers in Hawaii (Lachance et al., unpublished). All other species (17) that were unique to one region were either single isolates (11) or only occurred in low numbers (6). These include basidiomycetes such as *Cryptococcus* and *Rhodotorula* species and ascomycetous species found commonly in fruit rots or tree fluxes (*Kloeckera* spp. and *Pichia* spp.). The overall assessment of the 24 unique yeasts is four are due to speciation, two are relics, one is uncertain and 17 are rare opportunistic non-cactus species.

19.3.2 Yeasts of *Opuntia* Cacti

A similar comparison of the 42 yeast species in the *Opuntia* yeast community showed that 11 were found in only one region. Eight were single isolates, of which two were normally specific to columnar cacti (i.e., *Pichia pseudocactophila* and

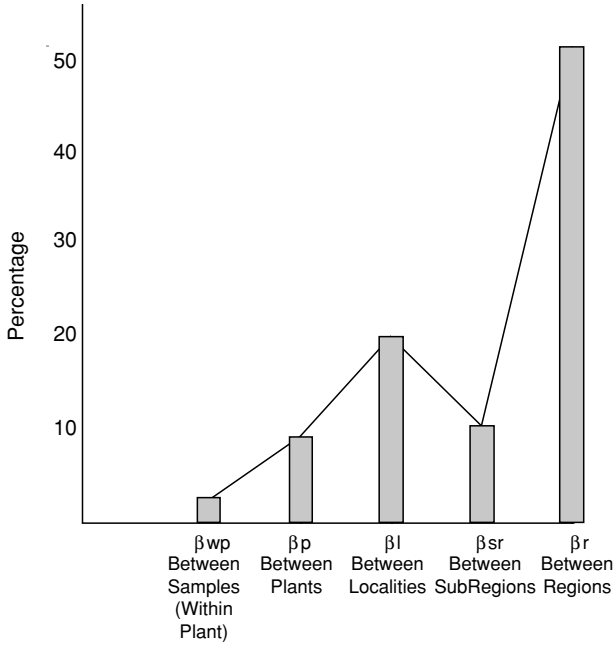

Fig. 19.2. Partition of species richness for yeasts in the Cactaceae (S=56) as a function of geographic level (Table 19.3)

P. thermotolerans) while the others were non-cactus yeasts usually associated with fruit or tree fluxes (*Kloeckera* spp., *P. membranifaciens*) or potentially airborne basidiomycetous species. These unique regional yeasts in *Opuntia* are mostly opportunistic and not products of speciation or extinction processes.

The global comparison of S for yeasts from *Opuntia* cladode rots shows a substantial increase in species richness when the Australian and Hawaiian yeast collections are included. Inspection of Table 19.2 shows that this increase is not due to replacement of one set of dominant cactus yeasts by another but is more likely a consequence of different rare indigenous yeasts in the local environments. Thirteen unique yeast species were recovered only once and only in Australia. A comparable number of single isolates were unique to the New World. Furthermore ten species found only in Australia had a low frequency of isolation (less than 10), whereas a number of common species in the New World have been found neither in Australia nor in Hawaii. These include seven species that were isolated between 161 and 45 times in the New World. Notable (Table 19.2) is that the top six cactophilic yeasts are found in Australia and other regions of the world (i.e., introduced and natural). It may be possible to trace the origin of the cactus-specific yeasts that found their way to Australia from the Americas during the campaign to eradicate the undesirable "prickly pear." However, because the introduction of yeasts and their vectors occurred a number of times (67 shipments of rotting plant material infected with

microorganisms and larval stages of insects) and from a number of different localities (59 from North America and eight from South America) in the New World (Dodd 1940; Starmer et al. 1988) multiple sources are likely.

19.3.3 Other Studies

Cactus rots have been investigated in Brazil by Rosa and colleagues (Morais et al. 1994; Rosa et al. 1995). Their species lists from columnar and *Opuntia* cacti contain the same dominant species we found in our surveys, with three to four additional unidentified or unknown *Candida* species collected in low numbers. It is likely that more extensive sampling of other localities in South America will increase the species richness, although the cactophilic yeast community clearly is very similar for both continents.

19.3.4 Biases Affecting Estimates of Species Richness

Among the factors that could bias our estimates of species richness are the methods that we use to detect yeasts in the individual rot pockets. Using general isolation media such as acidified yeast extract–malt extract agar or medium supplemented with chloramphenicol will necessarily cause one to overlook species present in low numbers, especially under conditions where the dominant yeasts are very numerous. We have shown this to be the case when we used selective isolation media to screen for rare yeasts and to obtain accurate counts of common yeasts (Starmer 1982; Fogleman and Starmer 1985; Latham 1998). However in those studies the yeasts that were revealed were still a subset of those found in the larger survey that only exposed those species with the greatest number of cells. Another factor that may prove important is the undetected diversification of taxa that we have called single species. For example, we expect the taxon *Pichia mexicana* to consist of a number of cryptic species. This was our initial finding when we identified the common dominant cactus yeast as *Pichia membranifaciens* in the mid 1970s (Heed et al. 1976; Starmer et al. 1976). Detailed study of host plant distribution, physiology, sexuality, GC content of the DNA, and DNA reassociations revealed several distinct species and complexes that were originally identified as *P. membranifaciens*. Among these were *Pichia cactophila*, *Pichia pseudocactophila*, *Pichia deserticola*, *Phaffomyces opuntiae, P. thermotolerans, Pichia eremophila,* and the *S. amethionina* complex, all of which have restricted physiologies that are convergent on a similar phenotype. In a like manner it is now recognized that *Sporopachydermia cereana* is a highly heterogeneous complex of species that show considerable geographic diversification (Lachance et al. 2001a).

19.4 Yeast Diversity (Shannon–Weiner)

The diversity index (H') has a very different pattern of change in the geographic hierarchy as compared with species richness (S). The most detailed comparison is available for yeasts found in the columnar cacti in the subtribe Stenocereinae (Table 19.3). In this case most of the variability occurs at three levels: within samples of

individual plants, between plants in a locality, and between localities in a region (Fig. 19.3). These three levels account for 72% of the diversity. Only a small amount of diversity remains for the other levels (i.e., within a plant or between samples, between subregions or between regions, Fig. 19.3). This result is explained by the following arguments.

Each sample of cactus tissue has about two to three species and if samples are taken repeatedly from the same rot they yield about the same number of cells of the same species, i.e. samples are homogeneous in the rot pocket and pooling them does not increase the diversity by much. However, each plant may represent a different inoculation history or may be at a different stage of the decay process. As a consequence there would be a large increase in diversity when plants from a single locality are pooled. Combining localities of a subregion also increases diversity but in this case the increase is likely due to changes in habitats and local species availability. This is reflected in the sharp increase in species richness (Fig. 19.2) seen for β diversity (between localities).

Comparison of the diversity index for categories where yeast cell numbers for each plant were not available gives no information for within-plant diversity. In these cases α diversity is for yeast species within localities. In all host categories the α diversity comprises most of the total diversity. The α partition accounts for

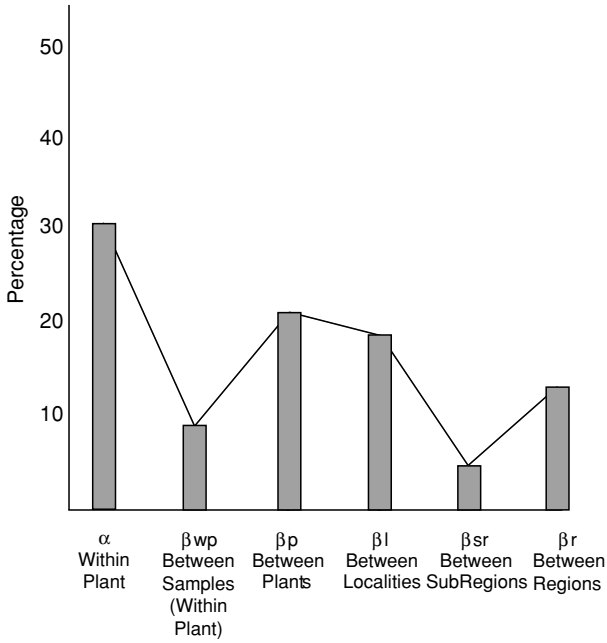

Fig. 19.3. Partition of species diversity for yeasts in the Stenocereinae (H'=2.299) as a function of geographic level (Table 19.3)

58.5–68.7% of the total diversity. This magnitude of increase in the metric likely reflects (as mentioned before) the inoculation history and the stage of the rotting process sampled in each locality. Likewise the increase (12.4–18.5%) for between-locality diversity is interpreted to be a function of the local species availability as a consequence of living in different habitats. There was little increase in diversity (4.0–7.2%) between subregions, whereas diversity between regions increase by 3 times as much (13.0–21.9%). The distribution of yeasts in the Stenocereinae would seem to argue in favor of the ubiquitous model of microbial diversity (Fenchel and Finlay 2004). However, it should be noted that these cacti are limited in distribution, such that the distinction between β and γ diversity would be expected to be less pronounced.

19.5 Conclusions

There is a striking difference when the geographic hierarchy is used to partition species richness (S) as contrasted to species diversity (H'). Most of the species richness is due to finding different relatively rare non-cactus-specific yeast species in different regions or continents. In this case the increase is most likely due to accidents, contaminants, or otherwise unusual circumstances and as such provides a "random" reason for an increase in biodiversity. Diversity as reflected in relative proportions of species and their uncertainty is influenced primarily by the number of cells (or abundance) of a small number of species (two or three) within a rotting sample (usually about 1 cm^3) of cactus tissue. Thus the α diversity, which almost always includes the core cactus-specific species, accounts for most of the information. This diversity metric has a biological meaning that reflects the salient factors mentioned in the "Introduction," i.e., habitat suitability, speciation, extinction, and immigration. Our previous work on habitat suitability and the insect (vector) yeast relationships has emphasized the likely mutualistic interactions among the core cactus yeasts and has shown experimentally that the cactus-yeast community is mutualistic with their vectors (Starmer et al. 1991). These relationships are not obligate but apparently strong enough to (1) maintain yeast communities that are stable over time and space (Latham 1998) and (2) restrict them to decaying cactus stem and cladode tissues (Ganter et al. 1986). These factors argue strongly that not all microorganisms are everywhere and that not all microorganisms freely and passively disperse to achieve world-wide distributions.

Acknowledgements

The following people all contributed to collecting the cactus samples that were used for isolating yeasts reviewed in this chapter: J.S.F. Barker, Jane Bowles, Denis Cornejo, Jim Fogleman, Antonio Fontdevila, Phil Ganter, Bill Heed, Bill Johnson, Bob Mangan, Marty Miller, Herman J. Phaff, Robert Metsger, Francesc Peris, Michal Polak, Ike Russell, and Jean Russell. We appreciate insightful discussion on biodiversity of cactus yeasts with Michael Anderson and Larry Wolf. Funding used to make many of the collections and related studies was provided by the NSF (USA) to W.T.S. and the NSERC (Canada) to M.A.L.

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Chapter 20

Black Yeasts and Meristematic Fungi: Ecology, Diversity and Identification

KATJA STERFLINGER

Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Austrian Center of Biological Resources and Applied Mycology, Muthgasse 18, 1190 Vienna, Austria
(e-mail: Katja.Sterflinger@boku.ac.at)

20.1 Black Yeasts, Meristematic, Microcolonial and Dematiaceous Fungi – a Disambiguation of Terms

For the morphological classification of the fungi discussed in this chapter three terms are used in the literature with overlapping meaning: black yeasts, meristematic fungi and microcolonial fungi (MCF). As will be described later, because of pleomorphic behavior the clear separation of fungi into one or the other group is impossible and fungi can just be clustered into one of those form groups according to their predominating morphological characteristics.

"Black yeast" is a *terminus technicus* subscribing a group of fungi that is quite heterogeneous from the taxonomic and phylogenetic point of view but having in common melanized cell walls and the formation of daughter cells by yeast-like multilateral or polar budding (Fig. 20.1). The resulting daughter cells may be encapsuled in a matrix of extracellular polymeric substances (EPS). Most black yeasts additionally exhibit mycelial growth and generate conidia from simple phialides, from phialides with collarettes, from annelated phialides, on rhachides or on undifferentiated conidiogenous cells (de Hoog and Hermanides-Nijhof 1977). Conidia may be unseptated or otherwise have up to three transversal septa. Also the formation of arthroconidia from fragmenting hyphae can occur in some genera. Only very few species, for example, *Phaeococcomyces exophialae*, do not form any hyphal states.

The term "meristematic fungi" was introduced by de Hoog and Hermanides-Nijhof (1977) for fungi that form aggregates of thick-walled, melanized cells enlarging and reproducing by isodiametrical division (Fig. 20.2). Propagules are liberated by breaking apart of aggregates as in the genus *Sarcinomyces* (Fig. 20.3) or by endogenous conidiogenesis with subsequent disruption of the mother cell wall as in the genus *Phaeotheca*. Some meristematic fungi might form blastic conidia from fairly undifferentiated cells, for example, in *Capnobotryella,* or even yeast-like budding cells as in *Hortaea werneckii*. Thus, some meristematic fungi can also be

Fig. 20.1. Hyphae, budding cells and annelidic conidiogenesis of the black yeast *Exophiala* sp. (2-week-old culture on 2% malt-extract agar, MEA, 21°C)

classified morphologically as black yeast and vice versa. A close phylogenetic relationship of both forms was suggested by de Hoog and Hermanides-Nijhof (1977) and was confirmed by molecular methods (Sterflinger et al. 1999).

A third term commonly used in the literature refers to the *in situ* growth pattern of the meristematic fungi and some black yeasts: MCF. MCF were first described by Staley et al. (1982) and the term characterizes fungi growing on mineral substrates –

Fig. 20.2. Species of *Coniosporium* form conidial chains; the secondary formation of longitudinal and transverse septa results in meristematic aggregates (2-week-old culture on 2% MEA, 21°C)

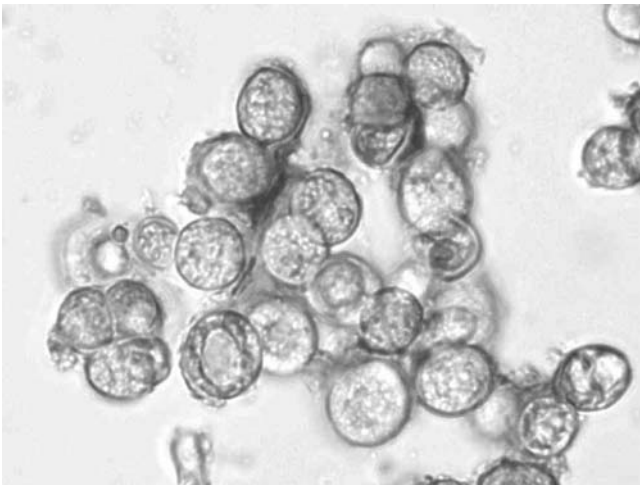

Fig. 20.3. Cell packages (*sarcina*) of *Sarcinomyces* sp. result from isodiametical division; solitary cells are released by breaking up of the common cell wall (2-week-old culture on 2% MEA, 21°C)

mostly rock but also glass or metal – and forming black cauliflower-like colonies consisting of densely aggregated thick-walled cells (Fig. 20.4). A single colony might be up to 1 mm in diameter and can be visible to the naked eye. Morphologically, colonies of MCF are absolutely indistinguishable from each other but after isolation onto suitable growth media they often differentiate into various morphologies, thus manifesting different genera and species (Sterflinger et al. 1999).

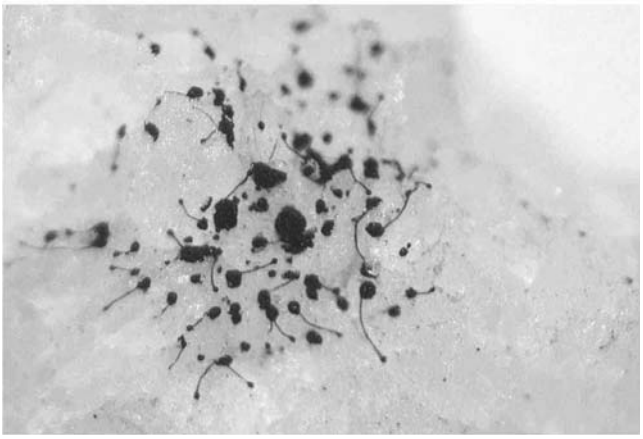

Fig. 20.4. Meristematic fungi form microcolonies with stolons and satellites on mineral surfaces; here on marble from Turkey

All black yeasts, meristematic fungi and MCF together with other darkly pigmented fungi are colloquially subsumed as so-called dematiaceous fungi. *Dematiaceae* stands for a highly artificial group the sole common feature of which is the pigmentation and thus the term should no longer be used as a taxonomic one. For practical reasons, however, it still has an significant impact for mycologists in routine diagnosis because the most important genera of melanized fungi are included in the keys "Dematiaceous Hyphomycetes" and "More Dematiaceous Hyphomycetes" (Ellis 1971, 1976).

20.2 Ecology of Black Yeasts and Meristematic Fungi

Black yeasts and MCF conquer extreme environments characterized by oligotrophic nutrient conditions, elevated temperatures, UV radiation, matrix and osmotic stress and combinations of these factors. The combined influence of these stress factors exerts a high selective pressure on the microbial community and as a consequence black yeasts and MCF are rarely found in complex microbial populations but solitary or in spatial association with comparably stress resistant organisms such as lichens and cyanobacteria in very special habitats.

Originally, darkly pigmented fungi with meristematic and/or yeast-like growth were described as inhabitants of living and dead plant material, including needles, leaves, bark, fruits and wood. While species of *Hormonema* and its teleomorph *Sydowia* are nearly exclusively found as opportunistic pathogens on plants – especially *Pinus* and *Prunus* – the closely related species *Aureobasidium pullulans* has a wide distribution on various materials, including plants, foodstuff, soil, textiles, metallic equipment, hypersaline water and rock (de Hoog et al. 1999). Also *Coniosporium* and *Trimmatostroma* are plant-associated, whereas single species of those genera are more restricted to discrete habitats: *Coniosporium perforans* does exclusively occur on rock (Sterflinger et al. 1999) and *Trimmatostroma salinum* was recorded only from hypersaline waters (Zalar et al. 1999). *Capnobotryella renispora* was detected on roof tiles and on *Sphagnum* (Hambleton et al. 2003), *Trimmatostroma abietis* from *Abies* and from sandstone (Butin et al. 1996), *H. werneckii* from rock, hypersaline water and as a causative agent of *Tinea nigra* on human skin. Additionally, lichenicolous fungi in the genera *Mycocalicium*, *Taeniolella*, *Trimmatostroma* (Ellis 1976) and *Intralichen* (Hawksworth and Cole 2002) form microcolonial colonies on and in lichen thalli.

Meristematic fungi are colonizers of bare rock surfaces in hot deserts and in semiarid climatic regions such as the Mediterranean (Staley et al. 1982; Gorbushina et al. 1993) but are also part of the cryptoendolithic community of Antarctica (Onofri and Friedman 1999). *Sarcinomyces petricola*, *Exophiala* species with close relationship to *Exophiala jeanselmei*, several *Coniosporium* species, strains of *Trimmatstroma* as well as *Phaeotheca* and *Phaeosclera* are very common rock inhabitants. Those fungi gained additional attention because they have a high destructive potential against historic buildings and works of art and are able to destroy even hard materials such as marble, sandstone and glass (Wollenzien et al. 1997; Sterflinger 2000). *H. werneckii* has its natural niche in saline to hypersaline water, in sea spray areas and is associated with salt efflorescence on walls. Together with

Trimmatostroma salinum, *Phaeotheca triangularis* and *Aureobasidium pullulans* it was isolated from hypersaline water of Slovenian salterns (Zalar et al. 1999).

Black yeasts and meristematic fungi are also found in human environments and as human pathogens or opportunists. Air conditioning systems with moisteners and humidifiers provide good living conditions for oligotrophic, stress-resistant fungi. The type strain of *P. triangularis* – a meristematic fungus with endogenous conidiogenesis – was isolated from an air conditioning system (de Hoog et al. 1997). Also strains of *Exophiala* and *Sarcinomyces* are frequently found in humidifiers as well as in bathing and sauna facilities (Matos et al. 2002). Especially black yeasts are of high clinical importance because they are causative agents of superficial and systemic mycosis. As mentioned before *H. werneckii* causes human *Tinea nigra* especially on the palms of the hands. Species of *Exophiala* are causative agents of chromoblastomycosis, phaeohyphomycosis and phaeohyphomycotic cysts and keratitis; they occasionally occur associated with pneumonia and sinusitis and they were isolated from subcutaneous infections (de Hoog et al. 2000). *E. dermatitidis* is of special importance because together with the closely related species *Cladophilalophora bantiana* and *Ramichloridium mackenziei* it causes lethal infections of the human brain (Horré and de Hoog 1999; Kantarcloglu and de Hoog 2004). *E. pisciphila* and *E. salmonis* infect fish, the latter causing the phenomenon of tumbling in salmon and trout owing to cerebral infections.

The habitats described seem to be quite different and widely apart at first sight but in fact rock and material surfaces, the phyllosphere and living mammalian tissue share some main ecological similarities, for example, raised temperature, osmotic stress, UV radiation and oxygenic action. Several properties are involved in stress tolerance of black yeasts and meristematic fungi:

20.2.1 Melanin, the All-Around Protective

One of the most important factors in stress resistance of black yeasts and meristematic fungi is the production of melanins and the incrustation of the cell walls with this high-molecular substances. In fungi, 3,4-dihydroxyphenylalanine (DOPA) and 1,8-dihydroxynaphtalene (DHN) melanin occur, while DHN seems to be predominant in meristematic fungi (Kogej et al. 2003). Melanins are not only responsible for the dark-green, brown and black color of the fungi but also for a number of properties helping the cells to survive environmental stress. Since in some habitats, for example, in Antarctica, UV radiation is the limiting factor for fungal diversity (Tosi et al. 2004), melanin together with some mycosporines (Volkmann et al. 2003) plays an important role as a UV-protection substance. However, as described by Langfelder et al. (2003), the relevance of fungal melanin is far beyond that. Wheeler and Bell (1986) described that melanins are not only UV-protective but also important for the penetration of host tissue in plants as well as in animal/human tissue. Melanin-deficient mutants are unable to generate appressorial turgor and thus lose their pathogenic potential. However, melanin alone does not define a plant or a human pathogenic fungus. Also fungi that are known for their capability to penetrate hard inorganic material mechanically are melanin-pigmented but otherwise nonpathogenic. Both DOPA as well as DHN melanin protect fungal cells against

osmotic stress, from reactive nitrogen species, from reactive oxygen, shelter them against phagocytosis in host tissue and inhibit tumor-necrosis factors (Langfelder et al. 2003). In human pathogenic black yeasts such as *E. dermatitidis* and *Cryptococcus neoformans* melanin is one of the most important virulence factors – albeit not the only one; in environmental and especially in rock-inhabiting fungi it is a major survival factor.

20.2.2 Morphological Adaptation

A general tendency in black yeasts and meristematic fungi seems to be that yeast-like stages are more associated with fungi inhabiting living tissue – mammalian as well as plant – and that meristematic/microcolonial growth is linked to bare material surfaces and to the highest demands towards stress resistance. Intermediate forms are also known: *E. dermatitidis* forms meristematic aggregates to survive low pH conditions in the digestive tract through which humans are invaded (Horré and de Hoog 1999). Generally, meristematic morphology is interpreted as a response to multiple stress factors supporting temperature tolerance and decreasing the rate and speed of desiccation by keeping the volume-to-surface ratio optimal (Wollenzien et al. 1997). Additionally, multilayered cell walls are developed as a response to raised temperature (Sterflinger and Krumbein 1995). As a consequence and in contrast to other fungi, only the spores or chlamydospores of which are stress-resistant, in colonies of meristematic fungi each vegetative cell is resistant and can additionally serve for propagation. In their natural habitat the development of spores or conidia was never observed for meristematic fungi; instead satellite colonies formed on stolons and colony fragments serve for propagation (Fig. 20.4). Moreover, the production of EPS in the form of loose slimes or dense capsules can be significant for survival under humid and hot conditions. *E. dermatitidis* forms slimy colonies and can grow up to 42°C, whereas the closely related species *Sarcinomyces phaemuriformis* and *E. mesophila* have their growth limit at 23 and 38°C, respectively (Matos et al. 2002). Since thermotolerance is a prerequisite for pathogenicity, a role of EPS in the virulence of *E. dermatitidis* was suggested by Yurlova and de Hoog (2002).

20.2.3 Desiccation Tolerance

As mentioned before modifications of the cell wall and the meristematic morphology are successful tasks in order to limit loss of intracellular water. However, in deserts and other extremely dry environments, for example, indoor environments, it is necessary to survive nearly complete dehydration in a dormant state. For this reason intracellular accumulation of the disaccharide trehalose is obligate in several rock-inhabiting fungi (Sterflinger 1998). Trehalose stabilizes the conformation of enzymes and lipid bilayers and plays a major role for so-called anhydrobiotic organisms capable of surviving complete dehydration. Taking together the oligotrophic situation in environments of black yeasts and meristematic fungi and the high carbon and energy demand caused by biosynthesis of trehalose, it can well be explained why the majority of those fungi have very low growth rates.

20.2.4 Temperature Tolerance

The growth of black yeasts and meristematic fungi from nature is limited to temperatures below 32°C, whereas phylogenetically closely related species that are causative agents of human chromoblastomycoses are able to grow at 37°C. The fact that none of the environmental species ever attracted attention as human pathogens indicates that the maximum growth temperature might play an important role as a virulence factor. Irrespective of that, conditions in the natural environment occasionally necessitate tolerance of temperatures significantly above the maximum growth temperatures. Sun-exposed surfaces reach temperatures up to 70°C and an additional, local temperature increase is caused by selective absorption of solar radiation by the black colonies themselves. For physiologically active and fully hydrated colonies the lethal temperature is between 35 and 75°C but dehydrated colonies withstand temperatures up to 120°C for at least 0.5 h and gain full growth activity after transfer to fresh medium (Sterflinger 1998). Thus, desiccation is a prerequisite to withstand temperature stress; similar results were reported for wood-inhabiting *Trimmatostroma* strains.

Cryophilic and cryotolerant meristematic fungi are described as important inhabitants of Antarctic rocks and thus conquer an ecological niche at the opposite end of the temperature scale. Especially in the *Friedmanniomyces* group strains can grow in temperature ranges from 0°C upwards. Amongst other features their cryotolerance is the reason why Antarctic fungi may be promising models to investigate exobiology under present Martian conditions (Onofri et al. 2004).

20.2.5 Osmotolerance

Although several species of black yeasts and MCF are osmotolerant, true halophilism seems to be rare and was merely found in *P. triangularis* (Zalar et al. 1999). In *H. werneckii*, however, halophilism is adaptive and the fungus can grow in up to 30% NaCl, which is almost the saturation point. Glycerol is the main compatible solute synthesized as a response to increased salinity (Sterflinger 1998). Because the spatial organization and density of melanin in the outer part of the cell wall is denser in cultures grown in increased salt concentration an osmoprotectant role of melanin was suggested by Kogej et al. (2001). Also alterations of the membrane properties seem to influence osmotolerance since the regulation of sterol biosynthesis in halophilic black yeasts was demonstrated to be different than in mesophiles (Petrovic et al. 1999).

20.2.6 Physiology

With respect to carbon and nitrogen both black yeasts and meristematic fungi have a fairly wide physiological spectrum; all strains known so far are restricted to an aerobic, oxidative metabolism; no fermentation of sugars was ever observed. The spectrum of sugars includes L and D forms of monosaccharides and disaccharides and sugar alcohols, a specialty of black yeasts is the usage of *meso*-erythritrol as a carbon source. Diagnostic differences can be the ability to assimilate sucrose, ribitol, rhamnose, sorbose and xylose. Nitrate, nitrite, ethylamine and complex proteins are used as nitrogen sources. Ethanol can be degraded by most species, whereas

the oxidation of methanol is rare. Several isolates from rock are able to degrade hydrocarbons as diesel oil, gasoline and kerosene; also the degradation of polycyclic aromatic hydrocarbons is observed in some rock inhabitants (Sterflinger, unpublished data). The wide spectrum of degradable carbon and nitrogen sources is an important prerequisite to conquer oligotrophic habitats where organic detritus and organic air pollutants that were carried by dust that has settled are the only nutrient sources available. In contrast to their mostly oligotrophic habitats the energy and nutrient demand for the synthesis of melanins, trehalose and polyols necessary for survival is comparatively high. Consequentially those fungi do not produce or excrete organic acids, biotechnological important secondary metabolites or any product of excessive metabolism.

20.3 Taxonomy and Phylogeny

As mentioned before the terms black yeast, meristematic fungus as well as dematiaceous fungus are applied for morphological groups and do not reflect natural phylogenetic relations. The identification of black yeasts and meristematic fungi is still a challenge for mycologists and hitherto we know that it is rather impossible to perform a reliable species identification based on morphology alone. Microscopic identification fails in species and sometimes even in genus determination because of two main facts:

1. Especially in meristematic fungi a lack of differentiation hampers the determination of their systematic position. The *in situ* morphology is nearly identical even in different genera and in most cases their black, cauliflower-like colonies do not allow differentiation (Fig. 20.2, 20.3, 20.4). In culture some genera switch to mycelial growth with meristematic conidiogenesis, such as *Coniosporium*, while others, such as *Phaeotheca*, remain meristematic even on different media. The conidiogenesis often takes place on undifferentiated hyphae without any discrete conidiogenous cell or conidiophore. Some genera, for example *Trimmatostroma*, form conidia that resemble hyphal fragments with secondary transformations such as thickening of cell walls and bending. Morphologically very similar meristematic fungi can be phylogenetically widely apart. On the basis of their morphological similarity, *S. phaeomuriformis* from human skin, *S. crustaceus* from conifer wood and *S. petricola* from rock were classified in a single genus, despite a large phylogenetic distance exceeding the ordinal level.
2. For black yeasts pleomorphic growth combined with variable morphologies of conidiogenesis leads to confusing taxonomic results. Black yeast genera such as *Exophiala* and its teleomorph *Capronia* are polymorphic with very fine differences in conidiogenesis that hamper the morphological identification even for routine adepts of the groups. A single species may exhibit morphologically widely different synanamorphs. Such a situation is known in the meristematic species *S. phaeomurifomis*, where some of the strains with molecular identity to the type strain were morphologically indistinguishable from the hyphal, annellidic species *E. dermatitidis* (Uijthof et al. 1994). In *Coniosporium apollinis* some strains exhibit a yeast-like growth pattern and other isolates display a meristematic growth pattern.

In order to overcome this confusion effort was put into the analysis of molecular data in order to be able to cluster morphologically divergent strains, to distinguish morphologically similar ones and to determine their phylogenetic positions. The first method introduced as routine was the restriction fragment length polymorphism (RFLP) analysis of the small ribosomal subunit (SSU). On the basis of the variations observed in SSU-RFLP patterns it was concluded that black yeasts and meristematic fungi are genotypically much more diverse than could be assumed from morphology or physiology (Uijthof and de Hoog 1995; Sterflinger and Gorbushina 1997).

Today, owing to emerging technical facilities, sequencing of DNA followed by homology search in the databanks of the European Bioinformatics Institute (http://www.ebi.ac.uk) and the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) by alignment and phylogenetic tree construction is the general praxis for resolving the taxonomy and phylogeny of black yeasts. However, for the detection of intraspecific variability and populations, DNA fingerprint methods still are valuable tools. For example, in *E. dermatitidis* seven populations were determined on the basis of random amplified polymorphic DNA patterns (RAPD). *E. dermatitidis* is the main neurotropic agent in east Asia and in Europe and it causes cystic fibrosis in the lung. Fingerprints showed that the pathogenic strains cluster in two main populations (Uijthof et al. 1994). Also within *E. jeanselmei* different populations were detected by RAPD which refer to different potentials of pathogenicity (Nucci et al. 2002). Intraspecific variability with three populations in *Aureobasidium pullulans* was analyzed using RFLP (Yurlova et al. 1995).

For the analysis of their position in fungal divisions DNA sequencing followed by alignment and phylogenetic analysis is necessary because teleomorphs are still nearly unknown for black yeasts and meristematic fungi. An exception is *Exophiala*, for which *Capronia* is known to be the teleomorph. Phylogenetic trees based on SSU sequencing showed that the black yeasts are phylogenetically far away from the class of Hemiascomycetes comprising the classical yeast genera *Saccharomyces*, *Candida* and *Pichia* (Haase et al. 1995). This result is confirmed by chemotaxonomical data since the cell walls of black yeasts and meristematic fungi contain chitin rather than mannose or glucose. With few exceptions both black yeasts and meristematic fungi are Euascomycetes (Fig. 20.5). In the Basidiomycetes *Trichosporonoides nigrescens*, *T. oedocephalis*, *Moniliella suaveolens* and the asexual state of *Ustilago maydis* are melanized and yeast-like; *Trichosporon asteroides* is the only fungus within the *Basidiomycetes* with meristematic conidiogenesis (de Hoog et al. 2000). Within the *Euascomycetes*, however, the melanized meristematic fungi are phylogenetically quite diverse. SSU-based phylogenetic trees by Spatafora et al. (1995) and Sterflinger et al. (1999) showed close relationships to the following three orders of *Ascomycetes* (Fig.20.5):

1. Chaetothyriales. Molecular data proved that the black yeasts within the Herpotrichiellaceae are an evolutionary hot spot with many genotypically divergent species in the genera *Exophiala*, *Phialophora* and the herpotrichiellaceous teleomorphs in *Capronia* (de Hoog et al. 2003). *Exophiala* is the main genus of the black yeasts; its synanamorph *Phaeococcomyces* does not form mycelia at all but remains completely yeast-like, *Phialophora* forms collarettes and *Rhinocladiella* is determined

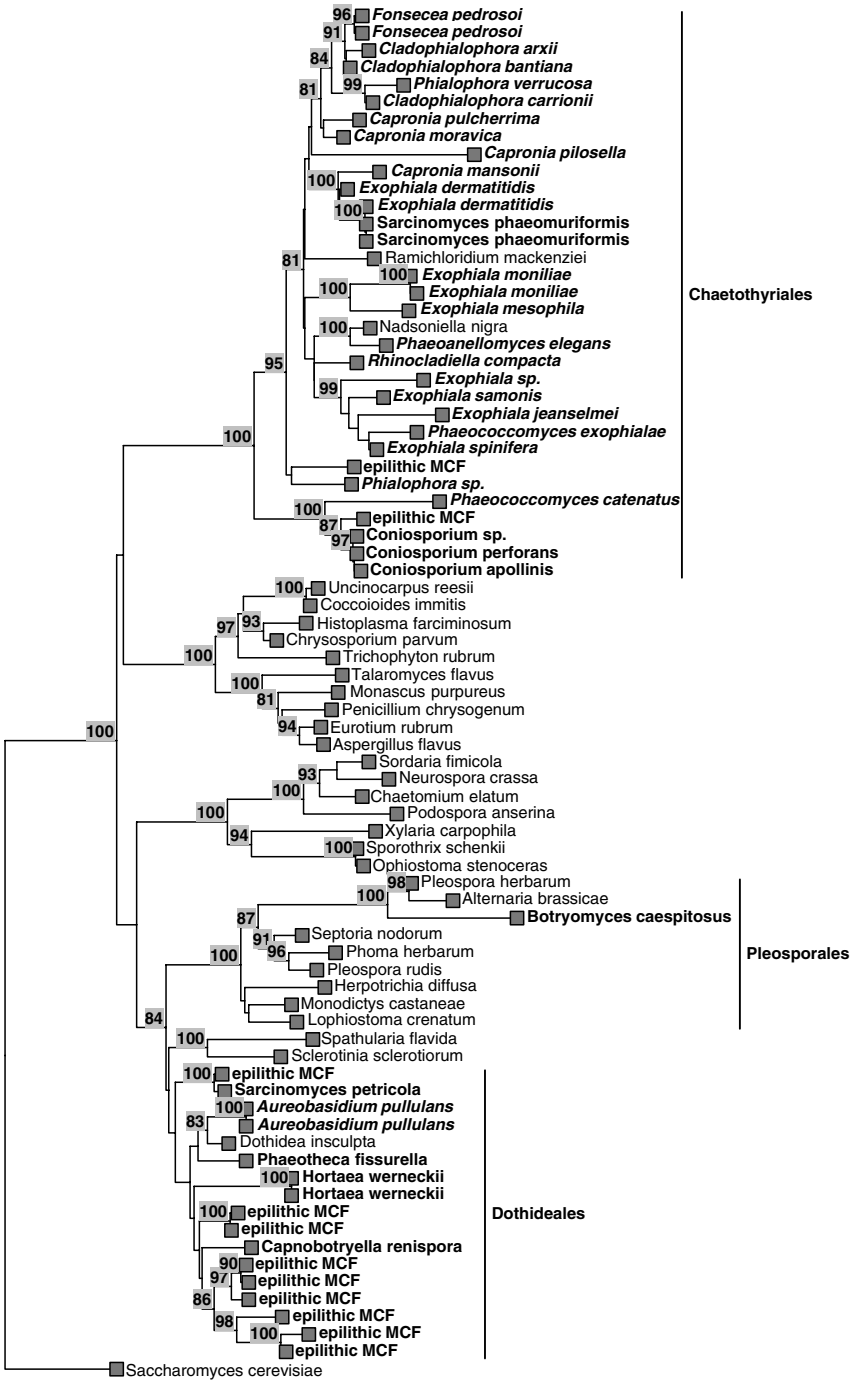

Fig. 20.5. Phylogenetic tree based on 1,700 positions of small subunit ribosomal DNA. Fungi with predominating black-yeast morphology are printed in *bold italics*; fungi with predominating merstematic growth are printed in *bold*. Bootstrap values were generated from 500 trees using the Felsenstein method

by sympodial conidiophores. In spite of this morphological classification, differences between species albeit constant are often difficult to perceive even for the mycologists specialized on this groups. Neurotropic species of *Cladophialophora*, *Ramichloridium* and *Exophiala* are distributed over the entire Herpotrichiellaceae. *S. phaeomuriformis* is known to be a meristematic member of the Herpotrichiellaceae (Haase et al. 1995), closely related to *E. dermatitidis* (Uijthof 1996) Also the agents of chromoblastomycosis, having meristematic tissue forms, are members of the family on the basis of molecular phylogeny (Haase et al. 1999).

2. In the order Dothideales several members are found which are meristematic during their whole life cycle (*Phaeotheca*, *Phaeosclera*, *Hyphospora*) or are able to produce meristematic synanamorphs (*Aureobasidium*, *Hortaea*). A further species found here is *Capnobotryella renispora*. 5.8S ribosomal DNA data suggest that several lichenicolous and lichen-associated meristematic fungi such as *Mycocalicium victoriae* are also members of the Dothideales (Sert and Sterflinger, unpubl. data). The SSU phylogenetic tree including new strains from Mediterranean habitats indicates that there are many species and genera with meristematic morphology that were hitherto unknown and will have to be described in the future (Fig. 20.5).
3. In the Pleosporales hitherto only one meristematic species was found. *Botryomyces caespitosus* is morphologically very similar to *S. phaeomuriformis* but can be distinguished by having pink colonies when young. Internal transcribed spacer (ITS) sequences suggest a close relationship of *Botryomyces caespitosus* to *Alternaria* (de Hoog et al. 2000).

Haase et al. (1995) suggested the 1,340–1,389 position in the SSU as a promising region for species specificity. However, the SSU turned out to be too conserved to resolve the enormous interspecies and intraspecies variability existing in black yeast species. For the species determination the ITS region 1 (ITS 1) evolved as suitable tool because this region is nearly identical within a single species of black yeasts (de Hoog et al. 2003).

Many of the meristematic fungi cannot be attributed to any of the known and sequenced orders of the Ascomycetes. Only few species of *Trimmatostroma* have hitherto been sequenced. *Trimmatostroma microsporum* has its anamorph in *Teratosphaeria* and an affiliation to Dothideales or Pleosporales has been discussed (Taylor and Crous 2000). *Taeniolella*, *Bispora*, *Lichenothelia*, *Scolecobasidium* and *Intralichen* have not been investigated at all in view of their genotypic consistency and phylogenetic position. The results obtained so far suggest that at least *Trimmatostroma* and *Taeniolella* are heterogeneous (Sert and Sterflinger, unpublished) and there is indication that these genera must also be regarded as mere form-taxa with phylogenetically separate species. The genus *Lichenothelia* for microcolonial nonlichenized rock inhabitants was introduced by Hawksworth (1981) and several species were described by Henssen (1987). However, for the whole genus only descriptions of the in situ morphology exist. It was later described by other authors that a wide diversity of fungal species can be camouflaged in situ by this microcolonial growth. Thus, the genus *Lichenothelia* must possibly be seen as an ecotaxonomic description of a habitat-specific growth pattern.

The overall phylogeny of black yeasts and meristematic fungi is a complex task and demands a polyphasic approach. While the sequencing of the SSU is suitable for placement into the right classes, ITS 1 is a taxonomic tool for species identification. The 5.8S and part of the ITS 2 region can close a gap between the species level (ITS 1) and higher taxonomic levels although the 5.8S gene sometimes indicates a higher heterogeneity than the ITS 1 region (de Hoog et al. 1999). Thus, the careful observation of morphology and life cycles as well as physiology is still an important tool for anamorph classification and for the understanding of generic placement. As described before assimilation patterns of black yeasts are broad and thus a physiological key can only be applied to very restricted groups, for example, for *Capronia* (Untereiner et al. 1999). New insights into phylogeny could possibly be derived from intergenic spacer regions, from the β-tubulin gene or from the translation elongation factor 1 that is used for identification of several hyphomycete genera. However, these genes have not yet been analyzed with respect to their suitability for taxonomy of black yeasts and meristematic fungi.

20.5 Working with Black Yeasts and Meristematic Fungi

Meristematic fungi and some black yeasts are characterized by slow growth rates. In nature the formation of new microcolonies may take several months, the single colony never exceeding more than 1 mm in diameter. In culture the maximum growth velocity of a single colony is 5 mm/week albeit some black yeasts may have faster growth rates in liquid cultures. Thus, on agar plates meristematic fungi are easily overgrown by fast-growing hyphomycetes. For this reasons the following points should be taken into account for the isolation and cultivation of meristematic fungi:

1. The isolation itself should be as selective as possible. Single colonies are best picked from the substratum using fine but hard needles – canulas are suitable tools – and transferred onto agar plates.
2. Meristematic fungi grow well on malt-extract agar but in order to limit growth of contaminants growth-inhibiting media should be used additionally. Dichlorane–rose bengal medium is suitable for this.
3. Isolation of those fungi needs patience and the incubation time may be up to 4 weeks until colonies become visible to the naked eye.

As described before several black yeasts and their close relatives are human pathogens. Some black yeasts belong to biological safety level 2 or even 3 (de Hoog et al. 2000) and thus while working with black yeasts all precautions necessary for these safety levels have to be followed carefully. This is of special importance if new unknown species are isolated from the environment or from clinical specimens.

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Chapter 21

Yeasts as Indicators of Environmental Quality

ALLEN N. HAGLER

Instituto de Microbiologia Prof. Paulo de Góes, CCS, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, 31941-590, Brazil
(e-mail: allenhagler@yahoo.com)

21.1 Introduction

Monitoring environmental quality is a growing concern as the increased human population taxes the Earth's resources to the limit and we need to better manage resources to allow sustainable development. Although invisible to us, microorganisms are largely responsible for the environmental conditions allowing our survival. Their populations respond quickly to environmental changes and some of them can be used to measure the quality of the environment relative to our needs. Yeasts are part of the invisible biodiversity that surrounds and even inhabits us (Kurtzman and Fell 1998; Lachance and Starmer 1998). Considering their wide distribution, ease of cultivation, well-developed taxonomy, and the amount they have been studied in the environment, it is surprising how little use has been made of them as indicators of environmental quality. Different yeast communities are associated with different habitats and population shifts of their component species should reflect conditions within the habitat. For this application they do not need to be pathogenic or otherwise disagreeable organisms themselves, but should have typical population levels in a habitat and change this level in response to the change of an important parameter of environmental quality. The presence of a species in a habitat other than its own can show the influence of one habitat on another.

Yeasts are largely unicellular immotile higher fungi including some that have important associations with humans. Whereas the filamentous fungi are more adapted to solid substrates, the mostly smaller and unicellular yeasts are more adapted to fluid substrates (Kornillowicz 1994). Their small size helps keep them suspended in their habitat and they are found associated with many industrial fermentations especially for production of alcoholic beverages. Much of the waste of the over six billion humans is discharged into the water, atmosphere and soil. So we should expect a response of yeast populations to nutrient enrichment in these natural substrates. Some yeast species are closely associated to man as an organism or his technological applications especially in the food industry. An obvious positive aspect of this association for humans is the domesticated species *Saccharomyces cerevisiae* used

extensively in industry especially in production of the bread and fermented beverages that are part of our everyday lives. But, there are also a few dozen species known to be opportunistic pathogens, most notably *Candida albicans* and *Cryptococcus neoformans* (Ahearn 1998).

About 1,000 different yeast species have been described, but it is typical that about half or more of the yeast species found in the communities of previously unstudied habitats do not fit any described species (Araujo et al. 1995; Hagler et al. 1997). This suggests that the total number of yeast species is much higher than those already known to exist. Most yeast species have been isolated only a few times and little is known about their function or distribution in nature. Many of these may be associated with specific microhabitats or regions that have received little study by zymologists and could have potential as indicators. Other species are ubiquitous in nature and in their associations with humans, making them more obvious targets for use as indicator organisms since a method based on them can be applied in diverse habitats. When a yeast species is consistently associated with a particular microhabitat, such as faeces of warm-blooded animals, it can indicate an influence of that source material in other segments of our lives. Also some yeast species may be typical of habitats in the pristine state and have decreased populations when that habitat is perturbed. These situations, combined with the ease of cultivation of most yeasts, make them good targets for application as indicator organisms. They can be detected and enumerated using selective culture media and also various molecular genetic methods that have been applied in studies of yeasts. Many of these same indicator organisms, and the methods used to detect and enumerate them, can also be applied to quality control of products such as foods. However, here we will look at them as indicators of the quality of the water, soil and air of our human scale macroenvironments.

21.2 Methods for Yeasts as Environmental Quality Indicators

The methods for applications of yeasts as indicators of environmental quality are specific applications of those available for yeast ecology and quality control of products. These subjects are covered in more detail in other chapters of this book.

21.2.1 Collection of Water, Air, and Soil from Natural Habitats

Aseptic methods should be used for collection of samples to avoid contamination with yeasts not actually present in the material under study. Because of their small size, the immediate environments of microbes are measured in millimeter dimensions or smaller, so minor differences in sampling strategy can have a dramatic influence on results. Careful selection of each specific sample site is essential so that the material collected will be representative of the study area. Samples taken near a point source of pollution are important, but should not be assumed to be typical of the general conditions of a habitat and could give the impression of the problem being more serious than it actually is. In pollution studies it is important to include uncontaminated sites as controls to determine background levels of the indicator organisms. Water samples can be taken from the surface by simply using sterile wide-

mouth screw-capped bottles or tubes of appropriate volume and taking care to avoid contamination (American Public Health Association 1998). Where water is relatively shallow and well mixed by currents or tidal action, surface samples are appropriate. However, surface samples may not be typical of a body of water especially under static conditions. Subsurface devices for sterile sampling are available, including the Zobell and Niskin samplers for deep water or simple mechanisms to open and close a sterile bottle at a few meters depth (Hagler and Ahearn 1987). Soil or intertidal beach sediment can be removed from different depths with a disinfected shovel or similar tool or taken as an intact core to preserve soil structure (Hagler et al. 1982). Air sampling is best done with an appropriate sampling device, like the Anderson sampler, and selecting the stages or sample fraction expected to have particles the size of fungal spores. It is possible to use particles settled out of the air by gravity directly on solid media or collected as dust, but such sampling will not be complete. Indirect measures of air quality can be done by sampling plant leaves although this is influenced by many factors, such as the type of plant and age of the leaves (Dowding 1987).

21.2.2 Preisolation Treatments

Environmental samples should be processed as soon as possible after collection, and maintaining them at low, but not freezing, temperatures can help preserve the yeast populations in them. Treatment of samples to more effectively extract, disperse and concentrate yeast cells is often ignored, but this is an important part of effective isolation and enumeration procedures (Martini et al. 1980). If counts are to be made by cultivation methods, cells associated with each other in colonies or on the substrate should be dispersed. The yeast cells should be separated or diluted from substances that can interfere with the detection of the target species of the assay. Sometimes the growth-inhibiting substances cannot be easily removed and diluting the sample may reduce their levels enough to allow isolation of yeasts. Sterile distilled water or protective solutions like 0.1% peptone or 0.85% NaCl can be used for this. Yeasts in aquatic samples may not be evenly distributed because of association with particles or because they are present as colonies. Also toxic compounds such as from algae or pollutants may be present in the samples and their removal should be considered in these procedures. Sedimentation of heavier particles, prefiltration that allows yeasts to pass but not larger fungi, or differential centrifugation has been used for this (Beech and Davenport 1971; Simard and Blackwood 1971a). Vigorous shaking is recommended as described in the APHA standard methods for analysis of water and wastewater by leaving a head space in the sample container and inverting vigorously 25 times (American Public Health Association 1975). Blending or sonification under aseptic conditions can be applied since yeasts have strong cell walls and are not easily disrupted (Martini et al. 1980; Santos et al. 1996). Sonification can also reduce the number of more delicate bacteria in the sample to facilitate yeast counts (Hassen et al. 2001). Aquatic sediment and soil samples are typically suspended in 10 vol of sterile water or an extraction solution that can contain substances like Tween detergents or peptone to facilitate dispersal and survival of the extracted yeasts. Vortexing or vigorous shaking by hand can be effective to extract

cells when sand particles are present to facilitate dispersal. In air sampling, prolonged exposure to strong air currents can cause drying of the medium surface of agar plates and can interfere with growth of samples. When the populations are too low to be detected or counted in the 0.1-ml volumes typically applied on spread plates, larger volumes can be concentrated by centrifugation (Slavikova et al. 1992) or on membrane filters (black filters with 0.8-μm pores are recommended) and these may be placed directly on culture media (American Public Health Association 1998). Preinoculation treatment can have a profound effect on the results of detection and enumeration of yeasts by cultivation methods.

21.2.3 Selective Culture Media for Monitoring Yeasts

All culture media are selective and their compositions will determine which of the yeasts extracted from the sample will be recovered. Selective and differential media may be employed for more specific isolation and presumptive identification of target organisms. Combinations of nutrients and conditions allowing the target organisms to grow are combined with substances and conditions inhibiting growth of nontarget organisms. Since bacteria are typically more numerous than yeasts in samples, their growth must be controlled if yeasts are to be cultivated successfully for study. This can be done by acidification of the medium, for example, to about 3.7–4.5 with HCl, and the use of antibiotics such as chloramphenicol at 200–400 mg/l (Buck 1975; Phaff et al. 1978). No single cultivation method should be expected to allow all the different yeast species in a sample to develop and incubation conditions like temperature can also select for different species (Buck 1975; Hagler et al. 1986). Buck et al. (1977) observed that some yeasts were isolated by enrichment technique that did not appear when plating methods were applied to the same samples. They also noted that total yeast counts, even at the restrictive temperature of 37°C to select for human-associated yeasts, may not provide useful information for quality control purposes and that application of certain species for monitoring requires development of selective media. After the yeasts have grown on solid media, distinctive colony morphology or indicator dyes can assist in presumptive identification of colonies that may be the targets of the assay. Examples are MCa medium to enumerate *C. albicans* (Buck and Bubucis 1978) and Chromagar *Candida* medium for isolation and presumptive identification of clinical yeasts (Odds 1993; Odds and Bernaerts 1994; Odds and Davidson 2000). Although these differential media with indicator dyes were intended for presumptive identification of a few pathogenic species in clinical samples, they can also help distinguish between different species appearing on isolation plates from other habitats and help increase the amount of diversity recovered in environmental studies. The development of methods to detect and count potential indicator groups is a key to further application of yeasts as environmental quality indicators.

21.2.4 Yeast Detection and Enumeration Methods

Agar plating methods can be in the form of streak plates to detect the presence of an indicator organism, or spread plates and pour plates to allow colony counts

(colony-forming units, CFU), but the results from these methods are mostly limited to the prevalent species making up a minimum of about 1% each the colonies obtained from samples. The 0.1-ml inoculum that can be applied for spread plates or the 1 ml inoculum for pour plates is a limiting factor for the sensitivity of plating methods. However, membrane filters or centrifugation can be used to concentrate yeasts from larger volumes, but keeping in mind there is a practical limit to the number of colonies that can be counted on a single plate or filter. At least some oligotrophic yeasts can be detected by cultivation on relatively low simple sugar content media like corn meal agar or yeast nitrogen base (YNB) with little or no added carbon source (Phaff et al. 1978). Most known yeasts grow at relatively high nutrient levels with glucose and reduced nitrogen sources but carbon source concentrations above about 2% produce large colonies and further limit the number of them that can fit on a single Petri dish. Larger sample volumes can be used in enrichment broth cultures using selective media to increase the sensitivity of detection if target species and populations can be estimated in them (Cooke et al. 1960; Buck et al. 1977; Hagler et al. 1986). This can be done by noting the presence or absence of the indicator organism in a single known volume (P/A test) or a series of measured volumes of inocula (typically done by decimal dilutions in an extinction dilution method) to yield an indicated number (IN) for approximation of the order of magnitude of the indicator population. When the IN is applied with multiple tubes, typically three or five at each sample volume (or dilution) tested, it can be used to determine the most probable number (MPN) for a more precise estimate of the population (American Public Health Association 1975). A method recommended for wastewaters by the American Public Health Association (1975, 1998) allows isolation and estimates of the yeast population in the presence of large populations of filamentous fungi. This approach is worthy of consideration since overgrowth by molds is a limiting factor in studies of yeasts in many habitats. In this enrichment method 15-ml samples are shaken with 135 ml of sterile water in a 250-ml Erlenmeyer flask at 120–150 rpm for 30 min. Two 250-ml Erlenmeyer flasks containing YNB with 1 and 20% glucose are each inoculated with 1 ml of sample and these are incubated at room temperature on a rotatory shaker at 120–150 oscillations per minute for at least 64 h. The flasks are then allowed to settle for 4–5 h and yeast cells will settle to the bottom leaving bacteria and filamentous fungi in suspension. A loopful of sediment is then removed from a tilted flask and streaked out on yeast extract–malt extract (YM) agar using three plates per flask. After 2–3 days' incubation reasonably isolated colonies that are morphologically different are picked from the plates to obtain pure cultures. The reciprocal of the highest positive dilution is the IN of the yeast population. These enrichment methods provide a low precision of counts but can be very sensitive since a single cell can be detected and the method can be made highly selective for a group of indicator organisms when the medium and cultivation scheme is well designed.

21.2.5 Cultivation-Independent Methods

Cultivation-independent methods have been applied using fungal specific primers in some habitats in which yeasts are among the prevalent fungal species. Cultivation-independent methods depend on efficient extraction of DNA without inclusion of

PCR-inhibiting substances. These methods combine PCR amplification of the DNA extracted and purified from samples with separation of the DNA from different species by methods like denaturing gradient gel electophoresis or in thermal gradient gel electrophoresis polyacrylamide gels (Gomes et al. 2003; Gadanho and Sampaio 2004; Prakitchaiwattana et al. 2004). They do offer an advantage in distinguishing between different prevalent species that produce colonies similar in morphology when cultivated and also in detecting species not growing on commonly used cultivation media, but are limited in sensitivity to prevalent species as is cultivation on solid media. Real-time PCR with specific primers for the indicator species would seem a very attractive approach for cultivation-independent monitoring of indicator organisms (Bleve et al. 2003; Brinkman et al. 2003). These molecular methods can be applied after enrichment to detect some lower population level species (Gadanho and Sampaio 2004). DNA hybridization methods such as microarrays using appropriate probes also have potential applications (Kim et al. 2004).

21.3 Applications of Yeasts as Environmental Quality Indicators

21.3.1 Yeast Counts as Quality Indicators in Aquatic Habitats

Populations of a few yeasts or less per liter are typical of clean water in oceans and pristine fresh waters. This contrasts with counts that can reach thousands per liter in eutrophic waters (Hagler and Ahearn 1987). Low populations of oligotrophs may exist as autochthones in low-nutrient pristine habitats, but they are expected to be at low population levels and not necessarily adapted to growth on the rich culture media normally used to isolate yeasts. Oceanic waters are a good example with typical yeast populations of a few cells per liter at best (Fell 1974).

Yeasts have been applied as indicators of sewage contamination and recreational water quality as a complement for the coliform and faecal *Streptococcus* counts used as indicators of recent fecal pollution. Some yeasts are part of the normal fecal flora of many animals, including humans (Ahearn 1998). Although yeasts present in feces are expected to be associated with the types of foods consumed, some opportunistic human pathogens, including *C. albicans*, are associated with warm-blooded animals and can be washed off the body during bathing in addition to being included in feces. Some of these are more resistant to chlorine treatment than most microbes, making them valuable as indicators for swimming pools or other chlorinated bathing waters (Engelbrecht et al. 1974). Methods for yeasts have been included for decades in the APHA standard methods (American Public Health Association 1975, 1998) and were based on the work of Cooke et al. (1960) and Cooke (1965). APHA recommends that in addition to noting yeasts appearing in pour plates with media for cultivation of fungi the enrichment procedure described before be used.

The yeasts associated with the feces of warm-blooded animals include various fermentative species and there is a higher proportion of fermentative yeasts in polluted than in clean water (Ahearn et al. 1968; Cooke et al. 1960; Wollett and Hedrick 1970; Wollett et al. 1970; Hagler and Mendonça-Hagler 1981). Selective methods have been suggested for human-associated yeasts based on incubation at an elevated temperature of 37°C (Buck 1975; Buck et al. 1977). However, this temperature was

not found sufficiently selective for tropical habitats and a method targeting especially *C. krusei* and *C. tropicalis* with 40 C incubation was suggested by Hagler et al. (1986). These two yeasts have been found to be especially associated with waters heavily polluted by domestic sewage (Cooke et al. 1960; Hagler and Mendonça-Hagler 1981). Hagler et al. (1986) made counts of fermentative yeasts with 1-ml volumes and decimal dilutions in aquatic yeast medium (2% glucose, 0.5% yeast extract, 0.5% ammonium sulfate, 0.2% monosodium phosphate, 400 mg/l chloramphenicol and pH adjusted to between 4.0 and 4.5 with HCl) with incubation under static conditions for 3 days. These MPN counts were found to correlate well with total yeast counts made using plating methods and also fecal coliform counts in polluted waters. That *C. krusei* and *C. tropicalis* are both highly typical of polluted water and associated sediments, both grow well at 43°C (a notably higher temperature than most yeasts) and both have strong fermentation, suggests that counts of fermentative yeasts at 43°C could be an effective method to monitor pollution by domestic wastes.

The use of total counts of "pink" carotenoid pigment producing yeasts was proposed by Simard (1971) and Simard and Blackwood (1971a, b) as a water quality indicator. Since they are easy to cultivate and their pigment formation allows presumptive identifications of colonies, the approach is attractive. Their method included a prefiltration of the sample through Whatman no. 1 filter paper to remove large particles and fungal hyphae before concentrating the yeasts on membrane filters. This method yielded higher yeast counts than was found in most other studies of yeasts in water. The preisolation filtration step may have eliminated some yeasts attached to particles or in filamentous form from the samples, but the elimination of a large portion of the molds that could overgrow and obscure yeast colonies on plates and possibly also disaggregating grouped yeasts should have increased the counts. Pink to red or orange pigmented yeasts are frequent in polluted waters at variable but often relatively high population levels. But, pigmented basidiomycetous yeasts can actually make up a higher portion of the yeast population of less polluted aquatic habitats (Hinzelin and Lectard 1978, 1979; Hagler and Mendonça-Hagler 1981). Spencer et al. (1974) noted that although pink yeasts were among the prevalent colonies from polluted freshwaters in Saskatchewan, Canada, their portion of the total yeast population varied from 0 to about 50%. A more recent study of carotenogenic yeasts isolated on membrane filters placed on acidified YM agar with 100mg/l chloramphenicol showed them to be present in temperate oligotrophic waters of glacial origin in Patagonia, Argentina (Libkind et al. 2003). Whereas *Rhodotorula muciliginosa* was present in the majority of the samples and made up about 50% of the isolates studied, this species was not detected in waters with very low anthropic influence. This suggests that if selective isolation media and more accurate identification now available were applied to allow monitoring of individual species of carotenoid-producing yeasts, they could serve as nonfecal pollution indicators at very low levels of contamination. The pink yeasts were among the first yeasts suggested as indicators of environmental quality and continue to show potential especially in more pristine environments.

The total yeast population is expected to have a stronger and more consistent general response to water pollution than pink yeasts, and has been suggested as an

indicator of the trophic state of aquatic environments (Rosa et al. 1990). *Trichosporon cutaneum* has been suggested as a fecal pollution indicator by Hinzelin and Lectard (1978) on the basis of a study of the Moselle River. By contrast, small tropical lakes in Minas Gerais, Brazil, were mostly populated by yeasts fluctuating between rainy and dry periods, in which constantly introduced transitory species dominated during rainy periods (Rosa et al. 1995; Morais et al. 1996). In contrast to the data from the temperate river, it was suggested that *T. cutaneum* is part of the indigenous aquatic microbiota of the dry period, meaning that the same species can indicate different things in different regions. Some apparently clean artificial lakes in rural areas of Slovakia had *C. krusei*, *C. lambica*, *C. tropicalis*, *C. guilliermondii*, *Pichia anomala*, *P. burtonii* and *Rh. glutinis* associated more with recreational areas having cottages, fishing and swimming areas (Slavikova et al. 1992). Yeast-like fungi were noted to have bioindicator properties in a Polish river because species including some potential pathogens, *C. albicans*, *P. guilliermondii*, *P. anomala*, *Rh. glutinis* and *T. beigelii*, were found in water with a high content of municipal sewage and altered chemical parameters, whereas *T. aquatile* was found in clean waters only (Dynowska 1997). Dabrowski et al. (1998) noted that in the Szezecin lagoon in Poland these species were not increased in their most polluted site although *Rh. glutinis* was frequent in all four stations in the study and *S. cerevisiae* had an unexplainable high frequency in one. The few yeasts isolated from lakes in a rural region of northwest Poland with low yeast populations were noted by Rózga et al. (1999) to not include *C. albicans* or the other commoner opportunistic pathogens except for *C. glabrata*. Although filamentous fungi can complicate enumeration of yeasts in polluted waters, counts of some *Rhodotorula* and *Trichosporon* species and also some fermentative species of the very diverse genera *Candida* and *Pichia* have been considered by authors from various regions as potential indicators of environmental quality.

C. albicans is an obvious target organism for pollution monitoring, but has not been isolated as frequently as expected on common yeast media in work with polluted water. This is apparently due to it being a poor competitor with other yeasts in mixed culture when growing on less selective culture media (Hagler and Ahearn 1987). This species is not comparable with coliforms or fecal streptococci as an indicator of recent fecal pollution, but it is an opportunistic pathogen present in feces and is also washed from body surfaces during bathing and can survive in natural waters (Valdez-Collazo et al. 1987). That makes it useful as a complement to counts of facal indicator bacteria in monitoring environmental quality. A culture medium for *C. albicans* based on YNB with maltose as a carbon source and cycloheximide to inhibit growth of many yeasts and other fungi was developed for membrane-filtered water samples (Buck and Bubucis 1978). With this methodology *C. albicans* was shown to be common in polluted waters and detectable in low pollution level samples. It could be used together with other media and germ tube formation to easily confirm the identity of this species (Cook and Schlitzer 1981). Application of this method in an extensive study of Lake Ontario bathing beaches showed a relation of *C. albicans* to elevated fecal pollution indicator levels observed in July and August in association with peak bather load at the beaches, although in only one instance the data suggested the beach was subjected to human fecal contamination (Sherry

et al. 1979). The application of a selective culture medium has allowed *C. albicans* counts to be applied as a valuable environmental quality monitoring tool for recreational quality of bathing waters and beach sands.

21.3.2 Specific Types of Industrial Contamination in Water

Industrial production strains like *S. cerevisiae* are not common in pristine habitats. If such yeasts are found in large populations it is clear evidence that pollution has occurred. In tropical aquatic habitats in south Florida (Ahearn et al. 1968) and also the North Paraiba River in Brazil (Oliveira 1990) large populations of *S. cerevisiae* were found present near sugar refineries. Yeasts responding to nutrients included in industrial wastes can also be useful. Xylose-assimilating yeasts of the *P. membranifaciens* clade, cellobiose-fermenting *Hanseniaspora uvarum*, sulfite-resistant *Trichosporon* strains and yeasts growing at 45°C were typical near pulp mills on Lake Champlain (Meyers et al. 1970). This contrasted with the low levels of yeasts and black yeasts dominated by *Rhodotorula*, *Cryptococcus* and *Aureobasidium* that were typical of most of Lake Champlain and are typical for pristine waters in general. These pulp mill waste associated yeasts were suggested as indicators of pulp mill residues in aquatic habitats.

Hydrocarbon degrading yeast populations can respond to pollution from petroleum sources. The species and densities of yeasts isolated from the North Sea before and after production of oil were different (Ahearn and Crow 1980). *Debaryomyces hansenii* was the prevalent species in both sets of samples, but after oil production the hydrocarbonoclastic yeast *C. guilliermondii* was commoner and the frequency of *A. pullulans* decreased. The species *C. lipolytica* (= *Yarrowia lipolytica*) has been suggested as an indicator of petroleum-related pollution in marine and estuarine environments. It uses relatively few carbon sources and is not of very common occurrence in water, but is able to grow in estuarine conditions, including the typical slightly alkaline pH that does not favor growth of most yeasts, and is a factor restricting many yeast species from marine habitats (Hagler and Mendonça-Hagler 1979). These characteristics make it a good target organism to monitor for petroleum pollution. We have observed a strong prevalence of *C. rugosa* in oxidation lagoons receiving petroleum refinery waste near mangrove ecosystems and also in intertidal estuarine sediments receiving high levels of urban sewage in Rio de Janeiro, suggesting it as another target species for environmental monitoring (unpublished data).

21.3.3 Yeasts in Aquatic Sediments

Also related to recreational use of water resources is the microbial quality of beach sand. This is of concern since the concentration of microbes in aquatic sediments is about 10 times higher than in water and most bathers actually spend much more time in contact with sand on the beach than they do in the water. Sediments in unpolluted estuarine environments have low yeast populations dominated by *Cryptococcus* and *Rhodotorula* species (Lazarus and Korburger 1974). More polluted sediments have higher levels of yeast populations in general and these

include a much more prevalent presence of fermentative ascomycetous species (Hagler et al. 1982; Soares et al. 1997). The use of *Candida* as an indicator to monitor beach sands has been suggested (Mendes et al. 1998). Studies of sands from marine recreational beaches in São Paulo, Brazil, have shown the presence of the opportunistic pathogen *C. albicans* (Sato et al. 2005). Also associated with littoral regions are filter-feeding aquatic invertebrates that can be sampled for their naturally accumulated microbes, including yeasts. Sampling mussels, for example, can allow increased sensitivity of yeast detection because they are concentrated about 10–100 times from those in the surrounding waters, and also reflect the quality of water at a site over longer periods of time during which the filtration by these animals occurred.

An example of a potential application of aquatic sediment yeasts as environmental quality indicators in aquatic sediments is the potential for combined use of *Kluyveromyces aestuarii* and *C. krusei* to indicate the environmental quality of mangrove ecosystems. Mangroves are typical of tropical estuaries and are important for the reproduction of many marine species, including species of economic importance (Mendonça-Hagler et al. 2001). Since coastal cities tend to be located in estuarine regions this type of habitat is frequently threatened by urban pollution and real-estate development. The species *K. aestuarii* has been found to be typical in or near mangrove regions (Fell et al. 1960; Fell 1961; Ahearn et al. 1968; Soares et al. 1997). It is found in association with marine and estuarine mangrove sediments under typical vegetation and within detritus-feeding animals, but not other animals and not in sediments a few meters outside the mangrove vegetation in adjacent tidal flats (Araujo et al. 1995; Araujo and Hagler 2005). It is rare or absent from more polluted mangrove areas in which *C. krusei* is typically a prevalent yeast species. It would seem an indicator system could be developed based on these two yeast species to monitor the environmental quality of mangrove ecosystems. For this application to be developed differential culture media or other means of accurate detection and population estimates of these species are needed and also environmental data to show the significance of their population levels relative to different levels of anthropic influence in mangrove systems.

21.3.4 Application of Yeasts as Indicators of Air Quality

There is considerable use of microbial counts in studies of air quality, but generally yeasts are noted only as colonies appearing on mycological culture media (Marchisio and Airaudi 2001; Shelton et al. 2002). Yeasts present in the air could be a direct threat to human health as a source of allergy-associated diseases. Unfortunately zymologists have not given much attention to isolation and identification of yeasts from air in spite of their conspicuous presence in many air samples. Ballistospore-forming yeast species are dispersed from plants and should be seen as normal components of air. By contrast, yeast species without such effective spores for air dispersal should be more expected to originate from material suspended during anthropic activities like harvesting or construction activities. As a result we should expect lower levels of yeasts like *Sporobolomyces* resulting from toxic chemicals in the air and a greater presence of other types of yeasts resulting from

suspension of dust from physical activities. Yeasts were included among the culturable fungi present in an extensive study using Anderson N6 samplers for air from 1,717 buildings and outdoors from different regions in the USA (Shelton et al. 2002). Although identification was limited to morphological characteristics in this work, the genera *Candida*, *Geotrichum*, *Rhodotorula*, *Sporobolomyces* and the black yeast *Aureobasidium* were specifically noted in addition to an unidentified yeast group after cultivation on Rose Bengal agar and malt extract agar. Median indoor fungal concentrations were about 80 CFU/m^3 but ranged from below detection levels to over 10,000 CFU/m^3. The median outdoor air fungal concentration was higher at about 500 CFU/m^3 but the range was similar. Fungal concentrations were highest in the fall and summer and indoor concentrations to outdoor concentrations did not vary substantially by season. Outdoor air is considered the dominant source for indoor fungi and median indoor fungal concentrations correlated with the corresponding outdoor concentrations in this study. Regional variations of fungal concentrations in air were substantial. The strength of the work by Shelton and colleagues was in the large number of samples allowing a significant statistical evaluation. It invites speculation that specific populations of yeasts in air could be more precise indicators of different kinds of contamination. For example, the opportunistic pathogen *Cr. neoformans* has been found associated with trees and deteriorating wood (Licea et al. 1999; Randhawa et al. 2001). Because of the relation between plant-associated yeasts and yeasts recovered from air it would seem advisable to target this species in air quality studies done with a more species specific medium and large sample volumes.

House dust settled from the air can be a practical measure of inside environmental quality. The yeast community structure was studied on indoor plants and house dust collected using a vacuum cleaner from 25 apartments in Moscow, Russia (Glushakova et al. 2004). This could be compared with data on yeast communities in other habitats of the region (Bab'eva and Chernov 1995). The yeasts found in house dust were not diverse and were mainly represented by epiphytic and eurybiotic basidiomycetous species that are most frequently found on live and dead plants in natural habitats. They noted the occurrence of the epiphytic basidiomycetous yeasts *Sporobolomyces roseus* in about 30% and *Rh. glutinis* in about 10% of the house dust samples, while the ascomycetous yeasts were dominated by *D. hansenii*. The abundance of the dominant yeast species varied greatly among the apartments. The mean abundance of yeasts on indoor plants was considerably lower than that on outdoor plants, whereas that in the soil of indoor flower pots was about the same as found in the upper horizons of soddy podzolic soils. The taxonomic composition of the epiphytic yeast communities of indoor plants and soil differed considerably from that of similar natural habitats and included opportunistic human pathogens in larger proportions. *Rh. muciliginosa* dominated in house dust and on the leaves of indoor plants. A previous study in Moscow had shown yeast species to be an important part of the fungi in house dust (Petrova et al. 2000). Glushakova et al. (2004) concluded that the anthropogenic yeast communities formed in domestic interiors differ from the respective natural communities in both abundance and species composition.

Yeasts have long been known to be significant in microbial phyllosphere communities (Phaff et al. 1978; Phaff and Starmer 1987). In spite of the importance

of this habitat, it has not received much attention from zymologists until recently. Extensive studies in the Asia–Pacific region have shown diverse balistosporous yeasts to be common in the phyloplane habitat (Nakase 2000). In our experience these yeasts were not frequently isolated from washing of plant surfaces, but when plant materials were suspended above the surface of culture media allowing ballistospores to be discharged and land on the surface, these yeasts were frequently isolated. Because of their prolonged exposure to air, it is reasonable to expect some effect of pollution on these communities. *Sporobolomyces* on leaves has been suggested and studied as a rough and indirect measure of air quality (Dowding 1987; Dowding and Richardson 1990). Ballistospore forming yeast counts were shown to have a relation with air pollution although the results were dependent on which types of leaves were sampled with differences expected depending on the species of plant, the age and position of the leaves and climatic conditions. A small study done in Costa Rica of the yeast community on leaves of the bromeliad *Tillandsia* is notable (Brighigna et al. 2000). This group of plants absorbs its nutrients through the leaves and seems to be especially prone to accumulating pollutants from the air to the extent that they have been used to sample for heavy metals in environmental monitoring. The *Tillandsia* leaves in city environments were noted to be different in appearance and a study of the yeast community also showed differences. Although the data were limited, yeasts were notably lacking on leaves from sites with air pollution accumulated on the leaves but were present on leaves from unpolluted sites. This approach seems promising especially in tropical regions where suitable leaves are more available throughout the year and especially if applied to epiphytes absorbing their nutrients from the air.

21.3.5 Anthropic Impacts on Forests

Measuring the yeast diversity of forests is a challenge because they represent a complex mosaic of many different types of microhabitats, including different plants, animals and soils (Phaff and Starmer 1987). Water running from a forest in pristine streams and rivers should include yeasts washed form the phylloplane, soils and other habitats included in the ecosystem of origin. Hagler et al. (1997) noted a high portion of diverse "atypical" yeasts many not fitting described species in streams running from uninhabited areas of the Tijuca Forest in Rio de Janeiro. This contrasted with higher levels of human-associated yeasts in the same stream as soon as these waters ran through populated areas. A similar situation was noted in the North American prairie in Saskatoon (Spencer et al. 1974). In neotropical forests many bromeliad species accumulate water in tanks formed by their rosettes of leaves. These bromeliad tanks maintain water for long periods of time and material degrading in them substitutes soil in providing nutrients for these plants. These small volumes of eutrophic waters attract many animal species to use their associated resources. They vector yeast species of various sources to these small but nonephemeral aquatic habitats spread throughout forests. The bromeliad tanks did have typical prevalent yeast species for the tank microhabitat independent of the plant species themselves and the ecosystem in which they were found. Ascomycesous yeasts including *C. intermedia, Debaryomyces* spp. and a *Saccharomyces* clade species similar to *Kazachstania martiniae* were typical in shaded tanks, but not in

unshaded plants that were dominated by basidiomycetous yeast species. However, the shaded tanks also had a diverse accumulation of yeast species present at lower population levels, as indicated by the lower isolation frequency that apparently reflected the ecosystem in which they were found (Hagler et al. 1993; Araujo et al. 1998). These should reflect the general yeast diversity of the forest habitats. Other types of forest microhabitat that can reflect surrounding yeast diversity are the fruit-associated populations of drosophilids or fruit flies involved in vectoring yeasts between degrading fruits. These fruits are natural yeast baits in the forest or other habitats and can accumulate different yeast populations typical of a site as they are vectored by the insects. The drosophilids can be attracted to fermenting banana bait in containers protected by sterile gauze and collected for analysis of associated yeasts they have collected from the habitat under study. Morais et al. (1992) found higher yeast diversity was associated with drosophilids from the least perturbed forests in and near the city of Rio de Janeiro, Brazil. The yeasts associated with degrading fruit change in time with an ecological succession involving different drosophilid vector species as well as changes in the fruit (Morais et al. 1995) so care must be taken to collect comparable samples. As studies of yeasts in forests yield more data we should expect to find some yeast species responding to anthropic influences and able to serve as environmental quality indicators for forests.

21.3.6 Soil Quality

Some species of yeasts have been known to be typical of soils for about as long as yeasts have been studied in natural habitats (di Menna 1957; Bab'eva and Chernov 1995). As with water, soils are expected to reflect yeast populations associated with various microhabitats. The upper soil layers have more degrading organic matter and eutrophic conditions supporting a wide diversity of species depending on specific conditions, whereas deeper layers have more oligotrophic conditions harboring yeasts like *Lipomyces* and *Cryptococcus* species at low population levels. Yeast isolations from soils are typically complicated by growth of many filamentous fungi that are more adapted to solid substrates than are yeast forms, and this has inhibited the enthusiasm of zymologists to study this important yeast habitat. Monitoring microbial populations following application of sewage or sewage sludge to soil for disposal is an obvious application. The potential of using yeasts of the genus *Candida* as an indicator for application of sewage sludge to soil has been suggested (Kacprzak and Stanczyk 2003) and yeasts with molds can be used to monitor composting of municipal solid wastes (Hassen et al. 2001). Application of enrichment methods and cultivation-independent methods should yield a more complete image of the yeast community in soil (Gomes et al. 2003). Focus on more specific groups of yeasts as eukaryotic indicator organisms for specific soil applications should yield useful results. Human-associated opportunistic pathogens could be as useful here as they have been in water. We have noted high levels of ascomycetous yeasts in soils of an experimental "organic" farm compared with uncultivated soils (unpublished results). Yeasts should be useful in monitoring the application of sewage sludge and organic fertilizers to soils and studies focused on more specific indicator species should yield positive results.

21.4 Conclusions

The use of yeasts to monitor water, air and soil quality of the environment has been limited. However, as the importance of environmental monitoring increases to allow better environmental management, yeasts should be considered more seriously for this task because of their ease of cultivation and our increasing knowledge of their distribution in nature.

Yeasts are amply but not uniformly distributed in nature and different habitats tend to harbor distinct yeast communities or guilds. Yeast species associated with man and other warm-blooded animals can be monitored to show their potential influence on environmental quality. Other yeasts can respond to habitat deterioration or enter the environment as part of industrial effluents. Yeasts have a large untapped potential to be employed as environmental quality indicators and community ecology data should be studied to identify potential environmental quality indicator yeasts.

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Chapter 22

Yeast Biodiversity and Biotechnology

PIETRO BUZZINI AND ANN VAUGHAN-MARTINI

Dipartimento di Biologia Vegetale e Biotecnologie Agroambientali, Sezione di Microbiologia Applicata & Industrial Yeasts Collection DBVPG, University of Perugia, Borgo XX Giugno, 74, 06121 Perugia, Italy
(email: pbuzzini@unipg.it; e-mail: avaughan@unipg.it)

22.1 Introduction

Since time immemorial fermented food and beverages have represented practical examples of yeast-associated biotechnology. Nevertheless, even though beer-, wine-, bread-, kefir- and koumiss-making technologies are centuries old, it was not until the mid 1800s that Pasteur demonstrated the essential role of microorganisms in these processes.

To most people yeasts are exemplified by the species *Saccharomyces cerevisiae* and with the production of alcoholic beverages. This is in spite of the fact that this domesticated microorganism represents only a fragment of the vast biodiversity and variegated biotechnological potential of the yeast world. In recent decades, in fact, studies of the metabolic diversity of so-called nonconventional yeasts (NCYs) have revealed innumerable promising biotechnological properties (Wolf et al. 2003).

Yeast biotechnology encompasses an enormous variety of processes involving the activity of yeast cells and metabolites which include fermented foods and beverages, chemicals and pharmaceuticals, as well as important agricultural and environmental interactions. The impact of yeast biotechnology has been extensively documented in a number of reviews (Burden and Eveleigh 1990; Demain et al. 1998; Walker 1998). While many products and molecules are commercially produced, other technologies are still confined to the laboratory and perhaps will eventually be developed into profitable ventures.

The present chapter will review current and past studies on yeast metabolic biodiversity for industrial, medical and environmental applications. In addition, future developments involving the biotechnological potentialities of these essential eukaryotes will be discussed.

22.2 Yeasts in Alcoholic Fermentation

While the ability of *S. cerevisiae* to produce alcoholic beverages is well documented and has been exploited for centuries, there is currently a world-wide interest in an

alternative use of ethanol of fermentative origin (bioethanol) as a partial or total gasoline substitute in internal combustion engines. Interest in bioethanol as a renewable, nonpolluting energy source has varied significantly during most of the last century mainly in response to oil prices. In the 1930s to 1940s, 75% of US production was by fermentation, while after World War II ethylene derived from petroleum and natural gas provided a less expensive source, and fermentative ethanol production declined rapidly (Burden and Eveleigh 1990; Walker 1998). Nevertheless, the oil crises of the 1970s and in recent years have generated renewed interest in bioethanol production.

Although bioethanol has normally been obtained through a batch process, the use of continuous fermentation and high-gravity systems give higher yields (Burden and Eveleigh 1990; Nagashima 1990). Long-term (6-month) continuous cultures have been carried out without substrate sterilization as the ethanol produced can control unwanted microbial contamination (Nagashima 1990). Higher yields have also been achieved by using more ethanol-tolerant strains or through the use of immobilized cell systems (Margaritis and Merchant 1984; Burden and Eveleigh 1990). A pilot-plant-scale 20,000 l operation using calcium alginate immobilized cells of *S. cerevisiae* operated continuously for several months (Nagashima 1990).

As mentioned before, the use of bioethanol is economical only when there is a low-cost supply of fermentable substrates. Since the 1980s some countries have considered this technology a means for transforming agricultural produce or for disposing of large amounts of wastes or by-products of agro-industrial origin. In some Latin American countries, particularly Brazil, interest in bioethanol is directed at obtaining economic independence from petroleum imports. As a result, in 1990 an ambitious scheme (National Alcohol Program of Brazil) was initiated for the production of one trillion liters per year; and Brazilian Gasohol (100% ethanol) from sugarcane became available throughout the country (Burden and Eveleigh 1990; Panek and Panek 1990). In the USA and Canada less ambitious programs for the production of ethanol from corn starch or spent sulfite liquor resulted in the production of over three billion liters per year in the 1990s (Murthagh 1986) and this has grown significantly in recent years to almost equal the Brazilian production levels (Ingledew and Bellissimia 2004).

Other interesting possibilities for bioethanol production, studied so far only on a laboratory scale, could be the fermentation of lignocellulosic hydrolysates, which constitute the most abundant renewable raw feedstock on Earth (Walker 1998; Zaldivar et al. 2001). The conversion of both cellulose and hemicellulose into bioethanol has been intensively studied (Chandrakant and Bisaria 1998). *Candida shehatae*, *Pichia stipitis* and *Pachysolen tannophilus* can ferment lignocellulosic hydrolysates containing cellobiose and xylose (Bashir and Lee 1994; Szczodrak and Fiedurek 1996). Innovative approaches for the utilization of hemicellulosic hydrolysates involving the simultaneous isomerization and fermentation of xylose or a simultaneous isomerization and cofermentation of a glucose/xylose mixture by *S. cerevisiae* in the presence of xylose isomerase (Chandrakant and Bisaria 2000) have been recently described.

An additional possibility since the 1980s is constituted by genetically engineered xylose fermenting strains of *S. cerevisiae*. In the 1990s, the first transformed strain

was able to ferment a mixture of glucose and xylose to ethanol thanks to a carefully designed xylose fermentation pathway with modified carbon flow dynamics (Ho et al. 1999). In addition, the recent development of a strain of *P. stipitis* able to give higher ethanol yields from lignocellulose hydrolysates could make the commercialization of bioethanol economically feasible for some applications (Jeffries and Jin 2004). Table 22.1 lists some alternative carbohydrate sources that have been proposed for bioethanol production using various yeast species.

In addition to ethanol, other industrially useful alcohols, including higher and polyhydric alcohols, can be obtained through yeast fermentation. Higher alcohols

Table 22.1 Yeast bioethanol production from different substrates

Scale	Substrate[a]	Species	Yield (g/l)	Reference
Industrial	Corn and grain	*Saccharomyces cerevisiae*	69	Maiorella (1985)
	Sugarcane molasses	*S. cerevisiae*	69	Maiorella (1985)
Laboratory: wild-type strains	Cane syrup	*Kluyveromyces marxianus*	80	Freer and Destroy (1982)
	Cellobiose	*Candida wickerhamii*	14	Fein et al. (1984)
		C. intermedia (*K. cellobiovorus*)	19	Morikawa et al. (1985)
	Cellulose (cellulase)	*K. marxianus*	10	Barron et al (1995)
	Dextrin	*S. cerevisiae*	80	Laluce et al. (1988)
	Starch	*S.* (*diastaticus*) *cerevisiae*	52	Debnath et al. (1990)
	Starch (amylase)	*K. marxianus*	15	Ward et al. (1995)
	Whey hydrolysate	*S. cerevisiae*	70	Bailey et al. (1982)
	Xylose	*C. shehatae*	6.6	Toivola et al. (1984)
		C. tenuis	6.4	Toivola et al. (1984)
		C. intermedia (*K. cellobiovorus*)	22	Morikawa et al. (1985)
		Pachysolen tannophilus	13.5	Slininger et al. (1982)
		Pichia stipitis	5.9	Toivola et al. (1984)
	Xylose (xylose isomerase)	*S. cerevisiae*	110	Gong et al. (1981)
	Xylulose	*C. shehatae*	17	Yu et al. (1995)
Laboratory: transformed strains	Starch	*S. cerevisiae*	1.3	Compagno et al. (1995)
	Lactose	*S. cerevisiae*	0.6	Compagno et al. (1995)
	Xylose	*Schizosaccharomyces pombe*	21	Chan et al. (1989)

[a]Substrate pretreatment in *parentheses*

("fusel oils" such as isobutyl alcohol, isoamyl alcohol, phenylethanol and isopropyl alcohol) are produced during amino acid metabolism and several of these compounds are important flavor ("bouquet") components in wines or spirits (Walker 1998).

Many yeasts produce various polyhydroxy alcohols such as glycerol, erytritol, mannitol, xylitol and arabinitol in relatively high amounts (Burden and Eveleigh 1990; Granstrom et al. 2001; Costenoble et al. 2003; Lee et al. 2003) which have a variety of commercial applications (Walker 1998). The first industrial-scale production of glycerol (a constituent for TNT) by yeasts was carried out in Germany during World War II (Burden and Eveleigh 1990). Today glycerol produced by *S. cerevisiae* is widely used as a base for synthetic resins, drugs, cosmetics and toothpaste (Walker 1998).

Yeast production of other polyhydroxy alcohols is still limited to the laboratory. Some processes under study include mannitol produced by *C. magnoliae* and by genetically engineered strains of *S. cerevisiae* (Costenoble et al. 2003; Lee et al. 2003), xylitol obtained from *C. guilliermondii* (Roberto et al. 1996; Sene et al. 2001a; Silva and Roberto 2001) and arabinitol produced by *C. entomaea* and *P. guilliermondii* (Saha and Bothhast 1996). All three of these polyhydroxy alcohols can be used as substitute sweeteners for confectionery, biscuits, soft drinks and pharmaceutical coatings (Burden and Eveleigh 1990; Walker 1998).

22.3 Yeasts as Producers of Single-Cell Protein

One of the first uses of yeasts as a source of single-cell protein (SCP) was during World War I when Germany used cells of *S. cerevisiae* as a meat substitute for human consumption. In more recent years, yeast SCP was mainly used as an animal feed additive in former socialist countries of eastern Europe (Burden and Eveleigh 1990).

During the 1960s to 1970s some companies developed pilot- or industrial-scale SCP production processes based on the use of *Yarrowia lipolytica* or some species of *Candida*, of the former genus *Hansenula* and of *Saccharomycopsis* for growth on *n*-alkanes (Solomons 1983; Burden and Eveleigh 1990). Although considerable money and effort were allocated to the development of an economically feasible process using petroleum-derived hydrocarbons, the industry never really took off for a number of economical and health-related reasons (Tuse 1984; Demain et al. 1998). Alternatively, a certain interest has been manifested towards using methanol as a pure, water-soluble substrate for SCP production (Tuse 1984; Burden and Eveleigh 1990).

Finally, various agro-industrial residues have been studied as possible substrates for SCP production. Starch from potatoes was proposed for the coculture of amylolytic (*Saccharomycopsis fibuligera*) and nonamylolytic (*C. utilis*) yeasts (Tubb 1986), while milk whey could be a substrate for lactose-positive yeasts (Burden and Eveleigh 1990). Additional investigations on SCP production from different feedstocks are reported in Table 22.2.

Table 22.2 Single-cell protein production from different feedstocks

Substrate	Species	Reference
Ethanol	*Pichia jadini* (*Candida utilis*)	Litchfield (1979)
Inulin	*Kluyveromyces marxianus*	Boze et al. (1995)
Lignocellulosic biomass	*C. shehatae*	Walker (1998)
	C. utilis	Litchfield (1979)
	P. stipitis	Walker (1998)
Methanol	Former *Hansenula* species	Litchfield (1979)
	P. pastoris	Boze et al. (1995)
Molasses	*Saccharomyces cerevisiae*	Litchfield (1979)
n-Alkanes	*Yarrowia lipolytica*	Litchfield (1979)
n-Paraffins	*Y. lipolytica*	Boze et al. (1995)
Starch	*Debaryomyces occidentalis* (*Schwanniomyces castellii*)	Boze et al. (1995)
Sulfite waste liquor	*P. jadini* (*C. utilis*)	Solomons (1983)
Whey	*K. fragilis*	Boze et al. (1995)

22.4 Yeasts as Producers of Industrially Relevant Molecules

22.4.1 Enzymes

Compared with certain fungi (e.g., *Aspergillus niger*) and bacteria (e.g., *Bacillus* spp.), yeasts are not particularly rich sources of industrially useful enzymes. Nevertheless, some species have been tested on the laboratory scale as enzyme producers for potential industrial exploitations (Bilinski and Stewart 1990; Burden and Eveleigh 1990; de Mot 1990; Guiraud and Galzy 1990; Ratledge and Tan 1990; Walker 1998). In recent years several large-scale investigations studying yeasts from various environments have been undertaken in the quest for novel enzymes (Ray et al. 1992; Abranches et al. 1997; Braga et al. 1998; Buzzini and Martini 2002). Some of these studies are listed in Table 22.3.

Yeast proteases have been extensively studied for application in the food and beverage industries (Nelson and Young 1986; Bilinski and Stewart 1990; Dizy and Bisson 2000; Poza et al. 2001). For a considerable period of time attention to amylolytic yeasts did not extend beyond *S. fibuligera* (Walker 1998). However, the potential of using amylases for candy and jam manufacture renewed interest in searching for additional starch-degrading species. As a result, several α-amylases, glucoamylases and cyclodextrinases produced by species of the genera *Saccharomyces*, *Candida*, *Filobasidium*, *Lipomyces* and *Schwanniomyces* have been purified and characterized (Wilson and Ingledew 1982; Burden and Eveleigh 1990; de Mot 1990; Demain et al. 1998; Walker 1998). In addition, the use of recombinant DNA techniques allowed for the cloning of glucoamylase genes from *Schwanniomyces* spp. and *S.* (*diastaticus*) *cerevisiae* and their introduction into brewer's yeasts (Walker 1998).

The most economically interesting application of yeast inulinases could be for the synthesis of bioethanol or of fructose for use as a sweetener (Guiraud and Galzy

Table 22.3 Some enzymes produced by yeast

Enzyme	Species	Reference
Amylase	*Schwanniomyces alluvius*	Wilson and Ingledew (1982)
Carboxypeptidase	*Saccharomyces cerevisiae*	Shiba et al. (1998)
α-Galactosidase	*S. pastorianus*	Church et al. (1980)
	Pichia guilliermondii	Church et al. (1980)
	Kluyveromyces lactis	Ward (1985)
Glucoamylase	*S.* (*diastaticus*) *cerevisiae*	Tubb (1986)
β-Glucosidase	*P. farinosa* (*Candida cacaoi*)	Drider et al. (1993)
	P. capsulata (*C. molischiana*)	Freer and Skory (1996)
	Torulaspora pretoriensis	Oda et al. (1993)
Invertase	*Rhodotorula glutinis*	Rubio et al. (2002)
	Torulaspora pretoriensis	Oda and Tonomura (1994)
Lipase	*Cryptococcus curvatus*	Hassan et al. (1994)
Polygalacturonase	*K. marxianus*	Serrat et al. (2002)
Protease	*C. caseinolytica*	Poza et al. (2001)
Xylose dehydrogenase	*C. guilliermondii*	Sene et al. (2001b)
Xylose reductase	*C. guilliermondii*	Sene et al. (2001b)

1990). This activity in *Kluyveromyces marxianus*, *C. salmanticensis* and *Debaryomyces polymorphus* has been extensively reviewed (Vandamme and Deryche 1983; Burden and Eveleigh 1990; Demain et al. 1998). In addition, several pectic enzymes from *C. norvegensis*, *Cryptococcus albidus*, *K. marxianus*, *S. cerevisiae* and *S. pastorianus* have been studied for potential applications (Blanco et al. 1999).

Lipases from *Y. lipolytica*, *Cr*. (*Candida*) *curvata*, *Y. lipolytica* (*C. deformans*), and *Rhodotorula glutinis* have been extensively investigated (Montet et al. 1985; Muderhwa et al. 1985; Ratledge and Tan 1990) for application in the oil and fat industries, in laundry detergents and in the food industry (Burden and Eveleigh 1990). Although similar to lipases, little attention has been devoted to yeast esterases as only *Y. lipolytica* and *C. guilliermondii* (Lloyd et al. 1971; Basaran and Hang 2000) have been studied. Finally, additional enzymatic activities have been found in yeast and yeast-like fungi such as cellulase from *Aureobasidium pullulans*, β-glucosidase from *Dekkera intermedia* and *C. intermedia* (*K. cellobiovorus*) (Morikawa et al. 1985), and phenylalanine ammonium lyase from *Cryptococcus* and *Trichosporon* spp. (Walker 1998).

In recent years, genetically modified yeasts with industrially relevant enzymatic activities have been developed. Lipase-overproducing mutants of *C. rugosa* and *Y. lipolytica*, as well as invertase-overproducing strains of *S. cerevisiae* have been recently proposed, and are under study (Ferrer et al. 2001; Fickers et al. 2003; Rossi-Alva and Rocha-Leao 2003).

22.4.2 Lipids

A small number of yeasts belonging to the genera *Candida*, *Cryptococcus*, *Endomycopsis*, some species of the former genus *Hansenula*, *Lipomyces*,

Rhodosporidium, *Rhodotorula*, *Trichosporon*, *Trigonopsis* and *Yarrowia* have been found to accumulate intracellular lipids as microdroplets. These cellular reserves, sometimes exceeding 70% of biomass weight, are composed almost exclusively of triacylglycerols, typically C_{18}-saturated and unsaturated (oleic, palmitic) and C_{16}-saturated (linoleic) fatty acids (Burden and Eveleigh 1990). Although lipid production has traditionally been done in batch culture (Burden and Eveleigh 1990; Ratledge and Tan 1990), the use of continuous culture systems has been reported (Papanikolaou and Aggelis 2002).

Additional classes of lipids are also produced by yeasts such as sophorolipids from *R.* (*C.*) *bogoriensis* and *C. bombicola* (Spencer et al. 1979; McCaffrey and Cooper 1995), cocoa butter-like lipids from *Cr. curvatus* and *Y. lipolytica* (Hassan et al. 1994; Papanikolaou et al. 2002a, 2003) and polyol fatty esters from *R. graminis* and *A. pullulans* (Spencer et al. 1979). Recent studies have also described the use of a genetically modified *S. cerevisiae* for the production of lipid-derived compounds (Dyer et al. 2002).

22.4.3 Carotenoids

Owing to their well-known antioxidant properties, carotenoids represent a valuable class of molecules for applications in the pharmaceutical, chemical, food and feed industries (Olson 1989; Krinsky 1994; Burton 1989; Nishino et al. 1999). *Phaffia rhodozyma* is a pigmented basidiomycetous yeast that was isolated in the early 1970s from the slime fluxes of some deciduous trees (Johnson et al. 1980). This yeast is used industrially for the production of astaxanthin, a pigment employed as a feed additive for salmonid fish grown in aquaculture (Tangeras and Slinde 1994). Studies of the astaxanthin biosynthetic pathway have resulted in the development of several overproducing mutants (Johnson and Schroeder 1995).

Although the occurrence of astaxanthin in yeasts appears to be confined to *Ph. rhodozyma*, additional species have been found which produce other carotenoids. Basidiomycetous yeasts belonging to the genera *Rhodotorula* and *Sporobolomyces*, along with their teleomorphic states *Rhodosporidium* and *Sporidiobolus*, produce carotenoids such as β-carotene, γ-carotene, torulene and torularhodin (Johnson and Schroeder 1995). The biotechnological potential of these pigmented yeasts has been extensively studied on the laboratory scale in recent years (Frengova et al. 1994; Buzzini and Martini 1999; Buzzini 2000, 2001; Bhosale and Gadre 2001a,b; Bhosale 2004). Finally, astaxanthin-, β-carotene- and lycopene-producing strains have been obtained by transforming *C. utilis* with pertinent bacterial genes (Miura et al. 1998a,b; Shimada et al. 1998).

22.4.4 Flavor compounds

Volatile organic compounds (VOCs) belong to several chemical classes (aldehydes, alcohols, esters, lactones, terpenes, sulfur compounds). These are characterized by having a low molecular weight, a high volatility and the ability to interact with olfactory receptors (Cheetham 1997). Despite their high number, only a few are currently exploited by the flavor industry for chemical, pharmaceutical, cosmetic or food and feed applications (Berger and Drawert 1987; Cheetham 1997).

Yeasts are well documented VOC producers (Torner et al. 1992; Romano et al. 1997; Martin et al. 2001; Spinnler et al. 2001; Rojas et al. 2001; Buzzini et al. 2003). Among these, volatile organic sulfur compounds (VOSCs), detected below parts per billion levels (Cuer et al. 1979; Cheetham 1997), are essential determinants of the aroma of some foods and beverages (Berger et al. 1999; Mestres et al. 2000; Martin et al. 2001; Spinnler et al. 2001). The production of some VOSCs (thiols, thioalcohols, thioesters and sulfides) has been observed in ascomycetous yeasts of the species *D. hansenii*, *Geotrichum candidum*, *K. lactis*, *S. cerevisiae* and *Y. lipolytica* (Berger et al. 1999; Spinnler et al. 2001). In addition, the production of terpenes from *Ambrosiozyma monospora* (Demain et al. 1998) and lactones from *Candida* spp., *K. lactis*, *Torulaspora delbrueckii*, *Sporobolomyces odorus* and *Y. lipolytica* has recently been observed (Endrizzi et al. 1996; Wache et al. 2001).

22.4.5 Vitamins

Although yeast cells have generally been considered a good source of vitamins, only riboflavin has been produced industrially since the 1930s from *Eremothecium* (*Ashbya*) *gossypii* and *E. ashbyi* (Vandamme 1992; Demain et al. 1998; Stahmann et al. 2000). Demain et al. (1998) reported that a patented riboflavin-overproducing strain of *D. hansenii* (*C. famata*) has recently been obtained by protoplast fusion and mutation.

The production of other vitamins from yeasts, studied until now only on a laboratory scale, include ascorbic acid and D-*erythro*-ascorbic acid from *Candida* spp., *Clavispora lusitaniae*, *Cr. terreus*, *Kluyveromyces* spp., *P. fermentans* and *S. cerevisiae* (Onofri et al. 1997; Demain et al. 1998; Hancock et al. 2000; Hancock and Viola 2002). In addition, ergosterol (a precursor of vitamin D2) has been obtained from strains of *S. cerevisiae* (Ratledge and Boulton 1985).

22.4.6 Organic Acids

Owing to a faster growth rate and easier cultivation than filamentous fungi, yeasts could potentially be employed for the production of some organic acids such as citric, α-ketoglutaric, itaconic or gluconic acids for the food and pharmaceutical industries (Burden and Eveleigh 1990). Citric acid synthesized by species such as *Y. lipolytica*, *C. zeylanoides, C. boidinii* and *C.* (*citrica*) *tropicalis* has been extensively studied (Tani 1984; Walker 1998; Papanikolaou et al. 2002b).

Other organic acids obtained from yeast and yeast-like organisms include gluconic acid from *Saccharomycopsis* spp. and from *A. pullulans* (Milsom and Meers 1985), fumaric acid from *C.* (*hydrocarbofumarica*) *blankii* (Sinkey 1983) and isocitric acid from *C.* (*brumptii*) *catenulata* (Atkinson and Mavituna 1983). Finally, Witte et al. (1989) obtained lactic acid from *K. thermotolerans*, while malic acid can be produced by *C. utlis*, *P. membranifaciens* and *A. pullulans* (Sinkey 1983).

22.4.7 Extracellular Polysaccharides

Yeast polysaccharides include β-(1→3)- and β-(1→6)-glucans, mannans, galactomannans and pseudonigeran (Barreto-Bergter and Gorin 1983), while glycopro-

teins are also produced as additional components of the cell wall (Burden and Eveleigh 1990). In spite of their viscosity and gelling properties, however, industrial application of these water-soluble polymers has so far been hampered because of their high sensitivity to salt, pH, shearing and heat (Kang and Cottrell 1979; Sutherland 1986; Burden and Eveleigh 1990). Additional extracellular polysaccharide (EPS) produced by yeasts include phosphomannans from *Pichia*, former *Hansenula* spp. and *Pachysolen* spp. (Walker 1998), and glycolipids from *Y. lipolytica* and *C. bombicola* (Burden and Eveleigh 1990; Walker 1998). Recently, an acidic heteropolysaccharide from *Rh. glutinis* composed of sugars (85%) and uronic acid (15%) was characterized (Cho et al. 2001).

The synthesis of pullulan, an α-glucan with α-(1→6)-linked maltotriose units, by the yeast-like organism *A. pullulans*, commercially employed in film-packaging of foods (Burden and Eveleigh 1990), has been well documented (Kang and Cottrell 1979). Also from that species, a novel glucan-like EPS characterized by having α-1, 4-D-, β-1,6-D- and β-1,3-D-glycosidic bonds has been obtained in recent years (Yurlova and de Hoog 1997).

22.4.8 Miscellaneous Compounds and Bioconversions

As early as 1970 it was known that yeasts produce amino acids, the most relevant examples being lysine (using 5-formyl-2-ketovaleric acid as a precursor) from *S. cerevisiae* and *C. utilis*, and phenylalanine from *Rh. rubra* (Walker 1998). Amino acid overproducing strains of *S. cerevisiae* and *P. angusta* (*Hansenula polymorpha*) have been obtained by mutation (Harder and Brooke 1990; Walker 1998) or by protoplast fusion (Brigidi et al. 1988).

Yeasts have also been studied as agents for a number of bioconversions (de Mot and Verachert 1984) such as ketoreductions, hydroxyl group oxidation, ester hydrolysis, hydrogenation of double bounds in steroids and phenol degradation (Demain et al. 1998). Some organic chemists have even used *S. cerevisiae* cells as an enantioselective catalytic agent (Kometani et al. 1996; Stewart et al. 1996). Additional studies on yeast bioconversions, some of which have been scaled up to the industrial level, are reported in Table 22.4.

22.5 Yeasts as Probiotics and as Biotherapeutic Agents

Since antiquity the health benefits of consuming fermented dairy products has been known. This became officially recognized by Metschnikoff, who at the end of the 1800s attributed the long life of Bulgarian peasants to their high intake of fermented milk products containing *Lactobacillus* species. Since then multiple health-promoting properties of so-called probiotic microorganisms have been suggested, such as modulation of the immune system, protection of the host from invading bacteria and viruses (and infection), assistance in digestion and prevention or alleviation of diarrhea (Ouwehand et al. 2002).

The term probiotic, popularized by Fuller in the 1980s (Fuller 1989), is derived from a Greek term "for life". Probiotic microbes are defined as those which after surviving the rigors of the human digestive system can compete with pathogens and

Table 22.4 Some bioconversions by yeast

Substrate	Product	Species	Reference
Cysteine–furfural	2-Furfurylthiol	*Saccharomyces cerevisiae*	Huynh-Ba et al. (2003)
Ethanol	Acetaldehyde	*Pichia angusta* (*Hansenula polymorpha*)	Moroz et al. (2000)
Ferulic acid	Vanillic acid + guaiacol	*Rhodotorula rubra*	Huang et al. (1993)
Fumaric acid	L-Malic acid	*Dipodascus magnusii*	Rosenberg et al. (1999)
Ketosulfone	*trans*-Hydroxysulfone	*Rh. rubra*	Lorraine et al. (1996)
L-Phenylalanine	2-Phenylethanol	*S. cerevisiae*	Stark et al. (2003)

assist in improving gut microbiota balance; or as living microorganisms which upon ingestion in adequate amounts confer health benefits on the host (Alvarez-Olmos and Oberhelman 2001). Such a definition does not comprise changes in intestinal microbiota or so-called "colonization"; however, as the probiotic organism can exert its effects locally or during transient passage through the gastrointestinal (GI) tract. While specific numbers are normally not mentioned in the definition, it is generally thought that at least 10^9 colony forming units (CFUs) need to be ingested per day for effective results.

Although the most traditionally employed probiotic microorganisms are members of the heterogeneous group of the lactic acid bacteria (LAB) (lactobacilli, enterococci), in recent years other bacterial groups (bifidobacteria) and even yeasts have been employed. The choice is often determined by the actual purpose for which the treatment is required.

The general requirements for a good probiotic include (1) resistance to intestinal enzymes, acid and bile so as to survive passage through the GI tract, (2) ability to adhere to intestinal mucosa in order to be active in pathogen exclusion and/or for enhancing healing of damaged mucosa, (3) a documented lack of pathogenicity and safety so as not to pose health risks for the consumer, and (4) positive technological properties including the possibility for large-scale production and strain stability with an acceptable shelf life (Ouwehand et al. 2002).

Today it is possible to find several commercially available supplements containing viable microorganisms with probiotic properties. These are sold either in lyophilized form or as part of fermented foods. The most commonly used are various LAB species, although some yeasts such as *K. lactis* (Bonekamp and Oosterom 1994) and more frequently *S. cerevisiae* or strains of that species referred to as "*S. boulardii*" have attracted attention.

A strain of *S.* (*boulardii*) *cerevisiae*, isolated from lychee fruit in Indochina, has been used for the prevention and treatment of antibiotic-associated diarrhea caused by *Clostridium difficile* since the 1950s (Nicod-Bertin and Panouse-Perrin 1984). It has been shown to be well suited as a treatment agent as it can quickly achieve high

concentrations and maintain constant levels in the colon. While it does not permanently colonize the organ, it can remain in the intestinal tract long enough to elicit benefits. In addition, since it is a yeast, it is not sensitive to antibacterial antibiotics and is therefore considered a "pharmaceutical probiotic" or "biotherapeutic agent" (BTA) (Surawicz et al. 1989; McFarland and Bernasconi 1993).

22.5.1 Mechanisms of Action of Yeast Probiotics

Although the mechanisms by which probiotics exert protective or therapeutic consequences is not fully understood, Fuller (1991) postulated four hypotheses regarding the possible effects of *S.* (*boulardii*) *cerevisiae* therapy:

1. Competition for nutrients. It has been demonstrated that probiotic bacteria prevent the colonization of pathogenic microorganisms (Ouwehand et al. 1999), although the actual mechanism of this activity has not been completely shown. It would appear logical that probiotic yeasts can also have this effect, and one of the hypotheses could be that the yeast consumes nutrients which are necessary for an overgrowth of undesirable germs.
2. Competition for adhesion receptors. Other studies have shown that *S.* (*boulardii*) *cerevisiae* can decrease in vitro attachment of *Entamoeba histolytica* trophozoites to erythrocytes (Rigothier et al. 1994).
3. Production of antitoxin and/or antireceptor substances. *S.* (*boulardii*) *cerevisiae* is probably responsible for a modification of the brush-border receptors (Czerucka et al. 1991), although it has been shown that mice are protected against the effects of *Cl. difficile* toxins only when the yeast is administered in a viable state prior to toxin introduction (Elmer and Corthier 1991). Studies have shown that treatment with living yeast cells reduces the quantities of free *Cl. difficile* toxins in the GI tract thanks to the production a 54-kDa protease which inhibits toxins A and B receptor binding and enterotoxicity (Castagliuolo et al. 1999). There is also a stimulation of intestinal membrane enzymes (hydrolases) as well as an endoluminal release of polyamines as a result of yeast intake which have antagonistic effects against overgrowth of pathogens in the intestine (Buts et al. 1986). More recent studies have shown that *S.* (*boulardii*) *cerevisiae* is also active against the cholera toxin in vitro (Czerucka and Rampal 2002).
4. Stimulation of immunity. Studies in mice have demonstrated that *S.* (*boulardii*) *cerevisiae* stimulates an increase in intestinal immunoglobin A secretion during a *Cl. difficile* toxin A challenge (Qamar et al. 2001); and also modulates proinflammatory cytokines (Rodrigues et al. 2000).

While a redundant number of studies have shown that the effectiveness of *S.* (*boulardii*) *cerevisiae* against the main causative agent of hospital diarrhea (*Cl. difficile)* is lost if it is not administered in a living state (Elmer and Corthier 1991; Pothoulakis et al. 1993; McFarland and Elmer 1995), other studies have shown that nonviable yeasts can have other effects. For example, a group from Japan showed that administration of mannan fractions from *S. cerevisiae* could have significant protective effects against intraperitoneal and intravenous infections by *Listeria*

monocytogenes and *Pseudomonas aeruginosa* in mice (Kobayashi et al. 1990). Another study showed that yeast glucans can have antitumoral activities (di Luzio et al. 1979; Sherwood et al. 1987).

The use of yeast as a probiotic is historically linked to animal feed. Surplus biomass from the fermentation industry, recycled as an additive to cattle, pig and poultry diets (Lyons et al. 1993), was shown to improve performance and product quality (Burnett and Neil 1977). This could be partly supported by a study showing that when live or autoclaved cells of *S. cerevisiae* were added to an *in vitro* coculture of rumen acetogenic and methanogenic prokaryotes, hydrogen utilization of the bacterium improved and methane emission by the methanogen diminished. It was hypothesized that the yeast in any state provides essential B vitamins and organic acids that can stimulate the acetogens.

Another investigation studied the stimulating effect of *S.* (*boulardii*) *cerevisiae* on LAB growth during yogurt production and their subsequent survival during shelf life (Lourens-Hattingh and Viljoen 2001). The results showed that the yeast was able to develop as a secondary microflora (reaching up to 10^6 CFU/ml) utilizing mainly lactic acid and galactose for growth. On the other hand, the addition to yeast in fruit yogurts presented problems of ethanol and gas production as a result of fructose and sucrose fermentation. Finally, a certain interest has been shown for other dairy-product-associated yeast species with specific enzymatic capabilities such as lipolytic or proteolytic activities (Jakobsen and Narvhus 1996).

22.5.2 Safety, Advantages and Disadvantages of Yeasts as Probiotics

A number of studies have demonstrated the general safety of oral administration of *S.* (*boulardii*) *cerevisiae* (McFarland et al. 1995; Bleichner et al. 1997). Nevertheless, disseminated fungemia, directly related to that strain, was found in hospitals where the BTA had been administered apparently having been transmitted through catheters (Piarroux et al. 1999; Cassone et al. 2003). Since this was observed almost exclusively in immunocompromised patients, this biotherapeutic is not recommended in those cases.

The future of probiotics and microbial BTAs depends upon further elucidation of basic mechanisms allowing scientists and clinicians to maximize their health benefits. This is very important since their use could offer an alternative to conventional antimicrobials to which many pathogenic microorganisms can develop resistance. Other advantages include low risk, relatively low production and formulation costs and the existence of multiple mechanisms of action. Finally, as stated earlier, yeasts represent the added advantage of inherently resisting the activity of antibacterials; and therefore can be used in parallel with these during therapies.

22.6 Yeasts for Biological Control of Postharvest Disease of Fruits and Grains

While rotting of stored fruits, vegetables and grains following harvest represents a significant loss to agriculture, controls through chemical treatment can introduce a

series of health and environmental risks. An alternative strategy for preventing fungal decay could be based on the surface treatment of the stored goods with antagonistic saprophytic yeasts. Studies have shown that molds exposed to cell-free extracts are not inhibited, but that effective protection appears to be the result of a competition for nutrients by living yeast cells (Spadaro and Gullino 2004). Strains of various yeast species, such as *C. oleophila*, *Cr. laurentii*, *D. hansenii*, *Metschnikowia pulcherrima*, *P. anomala* and *P. guilliermondii*, have been studied as biocontrol agents of fungal postharvest diseases of fruits and grains (Chalutz and Wilson 1990; Björnberg and Schnürer 1993; Filinow et al. 1996; Spadaro and Gullino 2004).

22.7 Yeasts as Bioremediation Agents

Although much interest for microbial bioremediation has been directed towards bacteria and filamentous fungi, some yeast species have been shown to be able to degrade synthetic pollutants originating from industrial activities. Some of the toxic molecules degraded by yeasts are reported in Table 22.5.

22.7.1 Yeasts for Cleaning Oil Spills

Hydrocarbon-assimilating species belonging to the genera *Candida*, *Clavispora*, *Debaryomyces*, *Leucosporidium*, *Lodderomyces*, *Metschnikowia*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, *Sporobolomyces*, *Stephanoascus*, *Trichosporon* and *Yarrowia* have been studied on a laboratory scale as possible microbial agents for the degradation of oil spills on land or water surfaces as a consequence of ecological disasters (Neujahr 1990; Demain et al. 1998).

Table 22.5 Biodegradation by yeast

Substrate	Species	Reference
4-Aminobutylphosphonate	*Kluyveromyces fragilis*	Ternan and McMullan (2000)
Benzonitrile	*Cryptococcus* spp.	Rezende et al. (2000)
Chlorobenzilate	*Rhodotorula gracilis*	Miyazaki et al. (1970)
Chloropropylate	*Rh. gracilis*	Miyazaki et al. (1970)
Diquat and paraquat	*Lipomyces starkeyi*	Demain et al. (1998)
Iminodiacetate	*K. marxianus*	Ternan and McMullan (2002)
Nitriles	*Candida guilliermondii*	Dias et al. (2001)
Nitrilotriacetate	*K. marxianus*	Ternan and McMullan (2002)
Phenols	*Trichosporon cutaneum*	Neujahr (1978)
Phthalic acid esters	*Saccharomyces cerevisiae*	Begum et al. (2003)
Plasticizers	*Rh. rubra*	Gartshore et al. (2003)
Tannin	*Zygosaccharomyces rouxii*	Demain et al. (1998)
Tetracyanonickelate (II)	*Cr. humicolus*	Kwon et al. (2002)

22.7.2 Yeasts as Biosorbents

Yeasts have also been studied as biosorbent agents of heavy or radioactive metals (Wenzl et al. 1990; Bustard et al. 1996; Omar et al. 1996; Singleton and Simmons 1996). Accordingly, innovative technologies have recently been investigated involving the use of *S. cerevisiae* as carriers in a crossflow microfiltration for the removal of heavy metal ions, Ni^{2+}, Cu^{2+} and Pb^{2+} (Bayhan et al. 2001).

22.7.3 Yeasts as Agents for the Degradation of Aromatic Compounds

A recent study showed that strains of *Aureobasidium*, *Rhodotorula* and *Trichosporon* isolated from industrial effluents were able to grow in the presence of phenol. Cell-free extracts from cultures grown on phenol exhibited catechol 1,2-dioxygenase and phenol hydroxylase activities, suggesting that catechol was oxidized by an *ortho*-type ring fission (Santos and Linardi 2001). Another study investigated the ability of a *T. cutaneum* strain to utilize various phenolic derivatives (resorcinol, 2.6-dinitrophenol. 3-nitrophenol, 4-nitrophenol and *m*-cresol) as sole carbon and energy sources. Results using yeast nitrogen base medium showed that all of these compounds except 4-nitrophenol were degraded. Resorcinol and 2,6-dinitrophenol were rapidly utilized, while 3-nitrophenol and *m*-cresol were only partly degraded (Aleksieva et al. 2002).

22.7.4 Yeast-Like Organisms for Dye Decolorization

The emission of synthetic dyes into the environment is causing significant ecological problems. Since many of these are poorly biodegradable, the treatments of choice tend to be physical or chemical, practices which often lead to the production of toxic substances. As a result, microbial approaches have been attempted as a more environmentally friendly solution to the problem. In a study carried out in Japan a strain of *Geotrichum candidum* able to decolorize 21 different azo and anthraquinone dyes was used (Kim et al. 1995). This broad spectrum of activity is due to the production of a novel extracellular peroxidase (DyP) of 60 kDa (Kim and Shoda 1999).

22.8 Improvement of Yeasts by Mutation, Protoplast Fusion and Recombinant DNA Technology

Despite some problems encountered with the appearance of unwanted secondary characteristics (Barney et al. 1980; Goodey et al. 1981), many properties of industrial relevance have been transferred from one yeast to another by protoplast fusion. Some of these include osmotolerance (Demain et al. 1998), ethanol tolerance (Legmann and Margalith 1983), flocculation (Panchal et al. 1982), lactose utilization (Farahnak et al. 1986) and killer activity (Young 1981; Bortol et al. 1986).

Many countries and international organizations devoted to food safety control are still reluctant to authorize the use of genetically modified organisms (GMOs) for food and beverage production, also in view of the fact that most consumers are hostile towards GMOs. In spite of these obstacles to their application outside of the

laboratory, there are many genetically modified yeast strains with improved characteristics of technological importance. Some examples which could be useful in the fermentation industry include: (1) the introduction of the *STA*2 glucoamylase gene from *S.* (*diastaticus*) *cerevisiae* enhances the ability of *S. cerevisiae* to ferment polysaccharides (Pretorius et al. 1986; Iserentant 1990), (2) the addition of an α-acetolactate decarboxylase gene from *Enterobacter aerogenes* allows the production of highly flavored beers by diacetyl-accumulating brewer's yeasts (Sone et al. 1988), and (3) the inclusion of a malolactic conversion encoding gene from *Lactobacillus delbrueckii* resulted in *S. cerevisiae* strains producing lower acidity and enhanced flavor in some wines (Demain et al. 1998; Pretorius 2000).

Finally, a strain of *S. cerevisiae* transformed with a plant α-glucosidase resulted in desirable properties. Fear of negative consumer response has so far prevented the commercial exploitation for bread making in the UK of an approved recombinant baker's yeast containing heterologous melibiase and maltose permease genes (Demain et al. 1998). Nevertheless, the future of this technology will inevitably include the commercial application of some of these strains when safety measures can be guaranteed, and when the consumer has been properly educated and assured. The use of recombinant DNA techniques for transforming non-*Saccharomyces* yeasts (NCYs) such as *Candida* spp., former *Hansenula* species, *Kluyveromyces* spp., *Pichia* and *Yarrowia* spp. has been extensively reviewed by Iserentant (1990).

22.9 Yeasts as Hosts for the Expression of Recombinant DNA

In spite of negative acceptance in the food industry, since the 1990s the possibility of expressing heterologous genes in yeasts for production of rare molecules of therapeutic value has represented a spectacular potential for the pharmaceutical industry. Although some products are now available on the market (Demain et al. 1998; Walker 1998), two principal reasons are responsible for the relatively low number of actual commercial applications. First of all, the factors inherent in heterologous gene expression may present significant obstacles to producing molecules with the appropriate structure and purity so as to insure biological activity as well as adequate safety guarantees. Secondly, scale-up to commercial production requires very large investments so as to meet both economic and regulatory criteria (Demain et al. 1998; Walker 1998).

The choice of a particular host for heterologous gene expression can be a very important element for the final outcome. Owing to their ease of cultivation and genetic manipulation, yeasts are the preferred hosts for introduction of genes encoding heterologous proteins. This is also due to their high product secreting ability, sometimes even in the glycosilated (active) form (Gellissen and Melber 1996; Walker 1998). Owing to its long history of use in industrial fermentations and its generally regarded as safe (GRAS) status, *S. cerevisiae* has been the most extensively studied host yeast. In fact, foreign genes encoding heterologous proteins are generally more accepted in pharmaceuticals and in food products for human consumption if synthesized by this species (Demain et al. 1998; Walker 1998). A considerable number of heterologous proteins of medical interest have been expressed in *S. cerevisiae* (de Wilde 1990; Gellissen and Melber 1996; Demain et al. 1998; Walker 1998).

The development of industrial-scale processes involving heterologous protein-producing *S. cerevisiae* strains has been reviewed by Mendoza-Vega et al. (1994).

Species other than *S. cerevisiae* such as *P. pastoris* and *P. angusta* (*H. polymorpha*) have received much attention for heterologous gene expression. Among the advantages that these methylotrophic yeasts display over *S. cerevisiae* is a higher protein expression efficiency (de Wilde 1990) and a reduced production of overglycosilated proteins (Demain et al. 1998; Walker 1998). Their ability to produce hormones, antigens and enzymes on an industrial or a laboratory scale has been reviewed by several authors (Gellissen et al. 1992; Subdery 1995). Some *P. angusta* (*H. polymorpha*) derived products are presently undergoing preclinical or clinical trials and are expected to reach the market in the near future (Gellissen and Melber 1996). Additional examples of studies focused on heterologous proteins expression in yeasts are reported in Table 22.6.

22.10 Conclusions

While *S. cerevisiae* will always be important for a wide variety of scientific, commercial and medical applications, it must be remembered that those who ignore the existence of the other 800-plus yeast species risk missing out on the benefits of an

Table 22.6 Some heterologous proteins expressed in yeast

Donor DNA	Protein expressed	Host Species	Reference
Viral			
Hepatitis B virus	Polymerase	*Pichia methanolica*	Choi et al. (2002)
Archea			
Pyrococcus furiosus	β-Glucosidase	*Saccharomyces cerevisiae*	Smith and Robinson (2002)
Bacteria			
Providencia rettgeri	Penicillin G amidase	*S. cerevisiae*	Sevo et al. (2002)
Sulfolobus solfataricus	β-Glucosidase	*S. cerevisiae*	Morana et al. (1995)
Thermomyces lanuginosus	β -1,4-Xylanase	*P. pastoris*	Damaso et al. (2003)
Thermus aquaticus	Aqualysin I	*P. pastoris*	Oledzka et al. (2003)
Protozoa			
Plasmodium falciparum	Malaria antigen	*S. cerevisiae*	Bathurst (1994)
Plant			
Cyamopsis tetragonoloba	α-Galactosidase	*Kluyveromyces lactis*	Hensing et al. (1995)
Human			
	α-Galactosidase A	*S. cerevisiae*	Chiba et al. (2002)
	Hepatitis B surface antigen	*S. cerevisiae*	McAleer et al. (1984)

enormous source of genetic and biotechnological biodiversity. As illustrated in this chapter, the so-called NCYs provide alternative biocatalysts to *S. cerevisiae* in applications that utilize a wide variety of inexpensive feedstocks.

Many genera of NCY have high osmotolerance to sugars and/or salts and significantly higher tolerance to organic acids such as acetate and lactate than *S. cerevisiae*. Although genetically engineered strains of that species are continuously being developed, many NCYs naturally exhibit significant tolerance to aromatic and phenolic compounds, ability to grow on methanol or *n*-alkanes and resistance to a wide variety of toxic compounds present in lignocellulosic hydrolysates or to commonly used food preservatives. The increased exploration of NCYs has fueled renewed research in their physiology, metabolism and genetics which will inevitably lead to a vast array of useful biotechnological and industrial applications in the years to come.

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In the last few decades more and more yeast habitats that were not investigated earlier, spanning cold climates to tropical regions and dry deserts to rainforests, have been explored. As a result, a large body of ecological data has been accumulated and the number of known yeast species has increased rapidly. This book provides an overview of the biodiversity of yeasts in different habitats. The recent advances achieved by the application of molecular biological methods in the field of yeast taxonomy and ecology are also incorporated in the book. Wherever possible, the interaction between yeasts and the surrounding environment is discussed.

ISBN 3-540-26100-1

9 783540 261001

springeronline.com

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The Yeast Handbook Volume 2

Volumes already published:

Volume 1: Biodiversity and Ecophysiology of Yeasts
2006, ISBN 3-540-26100-1

Volume 2: Yeasts in Food and Beverages
2006, ISBN 3-540-28388-9

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THE YEAST HANDBOOK

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Yeasts in Food and Beverages

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Yeasts in Food and Beverages

With 52 Figures and 30 Tables

Springer

Dr. Amparo Querol
CSIC
Institute of Agrochemistry and Food Technology
Department of Food Biotechnology
P.O. Box 73
46100 Burjassot (Valencia)
Spain

Professor Graham Fleet
Food Science and Technology
School of Chemical Engineering and Industrial Chemistry
The University of New South Wales
Sydney
NSW 2052
Australia

Cover pictures by courtesy of:
Vincent Robert, Centraalbureau voor Schimmelcultures (CBS), The Netherlands
Carlos Rosa, Universidade Federal de Minas Gerais, Brazil

ISBN-10 3-540-28388-9 Springer-Verlag Berlin Heidelberg New York
ISBN-13 987-3-540-28388-1 Springer-Verlag Berlin Heidelberg New York

Library of Congress Control Number: 2005934303

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Printed in Germany

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Editor: Dr. Sabine Schreck, Heidelberg
Desk Editor: Anette Lindqvist, Heidelberg
Production: SPI Publisher Services
Typesetting: SPI Publisher Services
Cover Design: Design & Production, Heidelberg

Printed on acid-free paper 39/3152-HM 5 4 3 2 1 0

Preface

As a group of microorganisms, yeasts have an enormous impact on food and beverage production. Scientific and technological understanding of their roles in this production began to emerge in the mid-1800s, starting with the pioneering studies of Pasteur in France and Hansen in Denmark on the microbiology of beer and wine fermentations. Since that time, researchers throughout the world have been engaged in a fascinating journey of discovery and development – learning about the great diversity of food and beverage commodities that are produced or impacted by yeast activity, about the diversity of yeast species associated with these activities, and about the diversity of biochemical, physiological and molecular mechanisms that underpin the many roles of yeasts in food and beverage production. Many excellent books have now been published on yeasts in food and beverage production, and it is reasonable to ask the question – why another book?

There are two different approaches to describe and understand the role of yeasts in food and beverage production. One approach is to focus on the commodity and the technology of its processing (e.g. wine fermentation, fermentation of bakery products), and this is the direction that most books on food and beverage yeasts have taken, to date. A second approach is to focus on the yeasts, themselves, and their biology in the context of food and beverage habitats. During the past 25 years, there have been major advances in understanding the basic biology of yeasts, and their cellular and molecular responses to environmental influences. We believe that there is sufficient knowledge and understanding, now, to present a book on food and beverage yeasts, which has a specific focus on the organisms, themselves, and their biology. It will fill a gap in current resources, integrating the technology of yeasts in food and beverage production with the latest understanding of their genomics. This book contains 13 chapters written by an international collection of contributors who are recognized authorities in their field. Chapter topics have been selected to demonstrate the broad significance of yeasts in food and beverage production, the diversity of yeast species involved, and the fundamental ecology, biochemistry, physiology and genomics of their activities.

The impact of yeasts on food and beverage production extends beyond the popular notions of bread, beer and wine fermentations by *Saccharomyces cerevisiae* (Chap. 1). We now know that they contribute to the fermentation of a broad range of other commodities where, in addition to *S. cerevisiae*, many other types of yeasts may work in concert with bacteria and filamentous fungi (Chaps. 2, 4). With increasing consumer demands for more natural foods, there is increasing interest in using microorganisms, including yeasts, as new sources of food ingredients and additives,

such as flavors, colors, antioxidants and vitamins (Chap. 10), and as novel agents for the biocontrol of food spoilage (Chap. 4). Although spoilage of foods and beverages by yeasts is well documented, new spoilage species and new strategies for their control have emerged in recent years (Chap. 11). Food safety and the linkage between diet and health are issues of major concern to modern consumers. Consequently, the public health significance of yeasts in foods and beverages is a topic of emerging interest where, in one context, yeasts could be novel probiotic species but, in other species circumstances, they could lead to infections and other adverse consumer responses (Chap. 12). The impact of yeasts on the quality and safety of foods and beverages is intimately linked to their biological activities. These activities are determined by physical and chemical properties of the ecosystem, and how yeasts respond according to their physiology, biochemistry and genetics. Drawing upon the latest advances in molecular biology and genomics, Chaps. 5, 6 and 8 describe how the different yeast species adapt, evolve, grow and function under the diversity of environmental conditions and stresses presented by food and beverage ecosystems. This fundamental knowledge and understanding, combined with new genomic technologies (Chap. 7), are providing the platform for genetic improvement strategies (Chap. 13), the development of novel bioreactor and biocatalytic technologies (Chap. 9) and new molecular methods for yeast identification and characterization (Chap. 3), that will underpin future innovation in food and beverage yeasts.

Amparo Querol
Graham Fleet
July 2005

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Chapter 1

The Commercial and Community Significance of Yeasts in Food and Beverage Production

Graham H. Fleet

Food Science and Technology, School of Chemical Engineering and Industrial Chemistry, The University of New South Wales, Sydney, NSW 2052, Australia (e-mail: g.fleet@unsw.edu.au, Tel.: +61-2-9385-5664, Fax: +61-2-93136635)

1.1 Introduction

The history of yeast association with human society is synonymous with the evolution of bread, beer and wine as global food and beverage commodities, originating some 5,000 years ago. The microbial science of these products commenced in the mid-1600s with the first observations of yeast cells being reported by Antonie van Leeuwenhoek (The Netherlands). The significance of these findings laid dormant until the classic studies of Pasteur (France) and Hansen (Denmark) during 1850–1900, which heralded the beginnings of the disciplines of microbiology and biochemistry. Subsequent studies by Guilliermond (France) and Kluyver (The Netherlands) in the early 1900s established yeasts as a unique group of microorganisms that had a major role in food and beverage production (Rose and Harrison 1969; Rose 1977). Since the 1950s, several classic texts have specifically highlighted the commercial and social significance of yeasts in foods and beverages (Cook 1958; Rose and Harrison 1970, 1993; Phaff et al. 1978; Skinner et al. 1980; Spencer and Spencer 1990; Reed and Nagodawithana 1991; Deak and Beuchat 1996; Boekhout and Robert 2003).

Today, the impact of yeasts on food and beverage production extends beyond the original and popular notions of bread, beer and wine fermentations by *Saccharomyces cerevisiae* (Table 1.1). In a positive context, they contribute to the fermentation of a broad range of other commodities, where various yeast species may work in concert with bacteria and filamentous fungi. Many valuable food ingredients and processing aids are now derived from yeasts. Some yeasts exhibit strong antifungal activity, enabling them to be exploited as novel agents in the biocontrol of food spoilage. The probiotic activity of some yeasts is another novel property that is attracting increasing interest. Unfortunately, there is also a darker side to yeast activity. Their ability to cause spoilage of many commodities, with major economic loss, is well known in many sectors of the food and beverage industries, while the public health significance of yeasts in foods and beverages is a topic of emerging concern. This chapter defines the scope and diversity of the many beneficial and

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
© Springer-Verlag Berlin Heidelberg 2006

Table 1.1 The commercial and community significance of yeasts in food and beverage production

- Production of fermented foods and beverages.
- Production of ingredients and additives for food processing.
- Spoilage of foods and beverages.
- Biocontrol of spoilage microorganisms.
- Probiotic and biotherapeutic agents.
- Source of food allergens.
- Source of opportunistic, pathogenic yeasts.

detrimental aspects of yeasts in foods and beverages. Subsequent chapters in this book will provide more detailed and specific coverage of these concepts.

1.2 The Informative Process

To effectively exploit and manage the growth and activities of yeasts in foods and beverages, a structured process of knowledge development and understanding is needed, the concepts of which are outlined in Table 1.2 (Fleet 1999). Obtaining this information is a challenging task, and requires the collaborative interaction of microbiologists, chemists, biochemists, molecular biologists, and food scientists. Application of molecular technologies to the detection and identification of yeasts in food and beverage ecosystems (Fernandez-Espinar et al., Chap. 3) and to determining the genetic bases of their biochemical and physiological responses to these habitats (Walker and van Dijck, Chap. 5; Barrio et al., Chap. 6; Bond and Blomberg, Chap. 7; Dickinson and Kruckeberg, Chap. 8) have greatly facilitated acquisition of this fundamental knowledge and understanding. Moreover, there is now a broad recognition and acceptance that many yeasts species other than *Saccharomyces cerevisiae* are intimately and significantly involved in food and beverage production Romano et al. (Chap. 2) and that their individual contributions can be moderated and impacted by interactions with bacteria and filamentous fungi (Viljoen, Chap. 4). For most commodities, the chain of knowledge linking yeast ecology, yeast activity, product chemistry and product quality is very incomplete. Wine could be singled out where most progress has been made in this context.

1.3 Production of Fermented Foods and Beverages

Most individuals, whether they have a scientific or nonscientific background, have a positive image of yeasts because of their well-known association with the production of bread, beer, wine and other alcoholic beverages. Some will know that there are differences between baker's yeast, brewer's yeast, wine yeast and distiller's yeast, and the more learned will know these as either *S. cerevisiae*, *S. bayanus*, or *S. pastorianus*, according to current taxonomic classifications (Vaughan-Martini and Martini 1998; Kurtzman 2003). However, there is increasing awareness that many species other than those of *Saccharomyces* make positive contributions to the fermentations of foods and beverages, and the diversity of these associations are described in Chap. 2.

Table 1.2 Information needed to exploit and manage yeasts in food and beverage production

- Taxonomic identity of species and strains that contaminate and colonize the food throughout the total chain of production and sale.
- Growth profiles of individual species and strains throughout the chain of production and sale.
- Physical location and spatial distribution of species within the product.
- Biochemical, physiological and molecular explanation of how yeasts colonize the product and change its chemical and physical properties.
- Impact of intrinsic, extrinsic and processing factors on yeast growth and metabolic activity in the product.
- Correlation between growth and activity of individual species/strains, and product quality and safety.

In addition to *S. cerevisiae* and *S. bayanus*, it is now well established that various species of *Hanseniaspora*, (*Kloeckera*), *Candida*, *Pichia*, *Metschnikowia*, *Kluyveromyces*, *Schizosaccharomyces* and *Issatchenkia* can make positive contributions to the fermentation of wine from grapes and cider from apples (Fleet 1998, 2003a; Pretorius 2000). *Dekkera* (*Brettanomyces*) species, in addition to *S. pastorianus* and *S. cerevisiae* are significant in the production of some styles of beer (Dufour et al. 2003), while *Schizosaccharomyces pombe* can be important in rum fermentations (Fahrasmane and Ganou-Parfait 1998).

Although the microbiology of dairy products is generally dominated by discussions of lactic acid bacteria, there is now substantial literature describing the important role of yeasts in flavour and texture development during the maturation stage of cheese production, and in the production of fermented milks such as kefir and koumiss (Fleet 1990; Frohlich-Wyder 2003). The most predominant and important species in these associations are *Debaryomyces hansenii*, *Yarrowia lipolytica*, *Kluyveromyces marxianus* and *S. cerevisiae*, but *Galactomyces geotrichum*, *Candida zeylanoides* and various *Pichia* species are also significant. In addition to lactic acid bacteria, micrococci and staphylococci, yeasts also play an important role in the fermentation of meat sausages and the maturation of hams. *D. hansenii*, other *Debaryomyces* species, *Y. lipolytica* and various *Candida* species are involved, and contribute to flavour and colour development in these products (Lucke 1998; Samelis and Sofos 2003).

Yeasts other than *S. cerevisiae* are found in the fermentation of various cereal products, including sourdough breads, where their activities impact on product flavour and rheology. Prominent contributors are *S. exiguus*, *C. humicola*/*C. milleri*, *Torulaspora delbrueckii*, various *Pichia* species and other species of *Candida* (e.g. *C. kruseil*/*Issatchenkia orientalis*) (Jenson 1998; Meroth et al. 2003; Hammes et al. 2005). Coffee beans and cocoa beans (chocolate) undergo natural, indigenous fermentations in the primary stages of their processing, where the growth and activities of a diversity of *Hanseniaspora*, *Candida*, *Pichia*, *Issatchenkia*, *Kluyveromyces* and *Saccharomyces* species have been reported. Essentially, these yeasts assist in degradation of bean pulp and contribute to the production of chocolate flavour precursors (Schwan and Wheals 2003, 2004). *Zygosaccharomyces rouxii*, *C. versatilis*, and *C. etchellsii* are important osmotolerant species that play a key role in soy sauce fermentation (Hanya and

Nakadai 2003). Finally, a vast range of traditional, fermented products are produced in Africa, Asia and Latin America, where, along with bacteria, a diversity of yeast species make important contributions (Steinkraus 1996; Nout 2003).

With very few exceptions (e.g. beer), most fermented foods and beverages involve a mixed ecology of yeasts, bacteria, filamentous fungi in some cases, and their viruses. Consequently, complex microbial interactions are likely to be involved (Chap. 4). The ultimate goal is to understand which species are important to product quality and process efficiency, and to develop operational parameters that maximize their positive contributions. Novel bioreactor technologies could be developed to improve process efficiency (Strehaiano et al., Chap. 9) and targets for genetic improvement of strains could be identified (Verstrepen et al., Chap. 13).

1.4 Yeasts as Sources of Ingredients and Additives for Food Processing

Because yeasts have a positive image with consumers, they are considered as a safe source of ingredients and additives for food processing (Demain et al. 1998). Preparations of baker's and brewer's yeasts have been available for many years as dietary, nutrient supplements because of their high contents of B vitamins, proteins, peptides, amino acids and trace minerals. Also, yeasts and are often considered as an alternative source of protein for human consumption (Peppler 1970; Harrison 1993). Many products are now derived from yeasts and, according to Stam et al. (1998), about 15–20% of the global industrial production of yeasts is used for this purpose. Abbas (Chap. 10) describes the production of antioxidants, aromas, flavours, colours and vitamins by yeasts. Other detailed accounts of these topics may be found in Halasz and Laszity (1991) and Reed and Nagodawithana (1991).

Flavour ingredients based on yeast extracts, yeast autolysates and dried yeast preparations represent the most commercially significant products extracted from yeasts, and are used extensively in the food industry as a source of savoury, roasted, nutty, cheesy, meaty and chicken flavours. In addition, some extracts are specifically enriched in their contents of glutamic acid and nucleotides that function as strong flavour enhancers (Dziezak 1987; Nagodawithana 1992; Kollar et al. 1992; Stam et al. 1998). While baker's and brewer's yeasts have been the traditional sources of these products, their diversity and functionality are being expanded by the use of other yeasts such as *C. utilis* (*Pichia jadinii*) and *K. marxianus* (Lukondeh et al. 2003). Yeasts are frequently mentioned as potential sources of high value aroma and flavour substances such as vanillin (*S. cerevisiae*, *Rhodotorula glutinis*), citronellol, linalool and geraniol (*K. marxianus*), and γ- and δ-decalactones (*Sporidiobolus sulmonicolor*, *Y. lipolytica*) (Hagedorn and Kaphammer 1994; Vandamme and Soetaert 2002).

The yeast cell wall, composed principally of β-(1→3) and β-(1→6)-glucans and mannoprotein, represents about 20–30% of the cell dry weight (Fleet 1991; Nguyen et al. 1998). The β-glucans have gelling, thickening and fat-sparing functional properties that offer a range of applications in food processing (Seeley 1977) and, moreover, they have been reported to have anticancer, (Bohn and Be Miller 1995) immunomodulating (Sandula et al. 1999) and cholesterol-lowering activities

(Bell et al. 1999). They also absorb mycotoxins and could offer a method for removing these substances from beverages such as wine (Yiannikouris et al. 2004; Bejaoui et al. 2004).

Food colorants such as astaxanthin and other carotenoid pigments (Lyons et al. 1993; Johnson and Schroeder 1995) and a diversity of vitamins (Reilly 1991; Sauer et al. 2004) can also be derived from yeasts.

1.5 Spoilage of Foods and Beverages by Yeasts

There is an extensive literature on yeasts as food and beverage spoilage agents and, no doubt, this reflects the enormous commercial and economic significance of this problem. Stratford (Chap. 11) gives an updated account of this topic. Earlier, comprehensive discussions include those of Ingram (1958), Walker (1970), Deak (1991), Fleet (1992), Tudor and Board (1993), Thomas (1993), Deak and Beuchat (1996) and Loureiro and Querol (1999). Yeast spoilage is a constant threat and widespread problem in the food and beverage industries that can only be managed by employing educated staff and implementing effective quality assurance programs.

Yeast spoilage is very predictable, principally occurring in those products where bacterial growth is either retarded or prevented by the intrinsic, extrinsic and processing that prevail. Without this competition, yeasts will grow and spoil the product. Typically, high-acid, low-pH foods, products with high sugar (e.g. more than 10% w/v) or high salt (more than 5% NaCl) content, and products preserved with weak organic acids (e.g. sorbic, benzoic, acetic) are prone to yeast spoilage. Fruits, fruit juices, and fruit drinks, fruit pulp, fruit juice concentrates, sugar and flavour syrups, confectionery products, alcoholic beverages, carbonated beverages, vegetable salads with acid dressings, salt- and acid-based sauces, fermented dairy products and fermented or cured (salted) meat products represent prime candidates for yeast spoilage (Walker 1970; Tudor and Board 1993; Deak and Beuchat 1996). Some yeasts (e.g. *Cryptococcus* and *Rhodotorula* spp.) grow better than bacteria at subfreezing temperatures, and will spoil frozen meat, poultry, seafood and other products stored for lengthy periods. Some high-fat, low-water-activity commodities such as margarine and butter can support the surface growth of yeasts (e.g. *Y. lipolytica*). While there is significant diversity in the yeast species associated with food and beverage spoilage, some specific associations are frequent and often predictable. For example, these include *Z. rouxii* in very high sugar products, *D. hansenii* in salted meat products, *Z. bailii* in products preserved with weak organic acids and *Y. lipolytica* in high-fat products. However, an open and enquiring outlook should be maintained, because new spoilage and food processing species may be present and await discovery – for example, *Z. lentus* (Steels et al. 1999) and *Tetrapisispora fleetii* (Kurtzman et al. 2004).

Controlling the growth and activity of spoilage yeasts requires good understanding of their physiology (Chap. 5), biochemistry (Chaps. 8, 9) and genetic responses (Chaps. 6, 7). Unfortunately, there remain large gaps in this knowledge, especially for yeasts other than *S. cerevisiae*. Factors affecting growth and survival, and being able to predict yeast response to these factors, are particularly important at the practical levels of quality control and assurance. For most yeasts, the growth and survival limits, and inactivation kinetics for basic technological parameters such as temperature,

pH, sugar concentration and salt concentration, are not well defined, and require more careful, systematic investigation (Praphailong and Fleet 1997; Betts et al. 2000). Also, new food and beverage processing technologies such as high hydrostatic pressure, exposure to low- and high-intensity electric fields, and treatment with novel antimicrobial plant extracts (Gould 2000) are emerging and new information on the growth, survival and inactivation responses of individual yeast species to these factors is needed. Finally, the ecological origin or source of spoilage yeasts remains a mystery for many species and requires further research. The first line of defense in controlling food spoilage by microorganisms is the prevention of contamination, but the importance of microbial ecology in quality assurance programs is often underestimated.

1.6 Yeasts as Biocontrol Agents

In Chap. 4, Viljoen notes that most food and beverage habitats present complex ecosystems where a diversity of microbial species and interactive responses are likely to occur and impact on product quality. These responses can be beneficial, antagonistic or neutral to individual species within the product. Antagonistic interactions have given rise to the concept of biocontrol, whereby one species could be deliberately exploited to inhibit the growth and survival of another, less desirable species. During the last 20 years, several yeast species that exhibit strong antagonistic activity against filamentous fungi have been discovered. These yeasts have been investigated as potential agents for the biocontrol of fungi that cause pre- and postharvest spoilage of fruits and vegetables (e.g. *Botrytis*, *Penicillium*, *Aspergillus*, *Rhizopus* spp.), thereby enabling a lesser use of chemical fungicides (Fleet 2003b; Punja and Utkhede 2003; Spadaro and Gullino 2004). *C. oleophila* and *Pseudozyma flocculosa* have been commercialized for such use, and other species with biocontrol potential include *Metschnikowia pulcherrima*, *P. guillierlmondii*, *C. sake*, *Sporobolomyces roseus*, *Aureobasidium pullulans* and various *Cryptococcus* species (Fleet 2003b). *P. anomala* has been well studied for its biocontrol of fungi that spoil cereal silages (Druvefors et al. 2002). Various mechanisms have been proposed to explain the antagonistic activity of yeasts towards other fungi, and these include production of killer toxins and other inhibitory proteins and peptides, competition for nutrients and space, production of fungal cell wall lytic glucanases and chitinases, production of toxic metabolites such as ethanol, acetaldehyde, ethyl acetate and fatty acids, and induction of fungal resistance or defense reactions within the plant (Punja and Utkhede 2003).

It should not be forgotten that some yeasts influence the growth and survival of other yeasts by the simple mechanisms of ethanol and killer toxin production (Shimizu 1993). These properties may also impact on bacteria and filamentous fungi (Fleet 1999, 2003a), and highlight the fact that yeasts probably have much greater potential as biocontrol agents than currently recognized. More research is needed on this topic. Ethanol and killer toxin production are significant properties in the selection and commercialization of yeasts for wine production (Degre 1993).

1.7 Public Health Significance of Yeasts in Foods and Beverages

With respect to the field of food safety, yeasts have an impeccably good record and this topic is discussed by Fleet and Roostita in Chap. 12. Unlike bacteria, viruses and some filamentous fungi, yeasts are rarely associated with outbreaks of foodborne gastroenteritis or other foodborne infections or intoxications. As part of normal, daily food consumption, humans are unknowingly and inadvertently ingesting large, viable populations of a diversity of yeast species without adverse impact on their health (e.g. yeasts in many cheeses, fermented and cured meats, fruits and fruit salads, home-brewed beer and wine). Nevertheless, an open mind and vigilance on yeasts and foodborne disease are required – several bacterial species (e.g. *Escherichia coli*) not considered to be serious foodborne pathogens 25 years ago, are now classified in the high risk category.

There is a significant body of "lay" and "alternative" literature that connects yeast presence in foods to the onset of a broad range of allergic and hypersensitive reactions in humans. Migraines, respiratory problems, chronic fatigue syndrome, dysfunctional gut syndrome, irritable bowel syndrome and gut dysbiosis are prominent among these disorders (Crook 1986; Eaton 2004). The linkage between human disorder, food and yeast is largely based on dietary observations – when the suspect food is removed from the diet, the disorder disappears, and returns when the food is reintroduced into the diet. The underlying mechanisms of the human response require systematic, scientific research and could reflect adverse reactions to the yeast cells themselves, or metabolites they have produced (e.g. proteins, biogenic amines, sulphur dioxide).

Unlike many bacteria and viruses, yeasts are not known as aggressive infectious pathogens. However, some yeast species fall into the category of opportunistic pathogens. *C. albicans* and *Cryptococcus neoformans* are prominent in this context, and cause a range of mucocutaneous, cutaneous, respiratory, central nervous, systemic and organ infections in humans (Hazen and Howell 2003). Usually, healthy, immunocompetent individuals are not at risk of such infections. Generally, individuals with weakened health and immune function are at greatest risk, and include cancer and AIDS patients, hospitalized patients and those undergoing treatments with immunosuppressive drugs, broad-spectrum bacterial antibiotics and radiochemotherapies. The increased frequency of such individuals in the community in recent years has lead to a significant increase in the reporting of yeast infections. Moreover, increasing numbers of yeast species, other than *C. albicans* and *Cryp. neoformans* have been associated with these infections and are now considered in the list of opportunistic pathogens (Hazen 1995; Hobson 2003; Georgiev 2003). These include yeast species that are frequently found in foods such as *C. krusei*/*I. orientalis*, *P. anomala*, *K. marxianus*, *S. cerevisiae* and various *Rhodotorula* species. Murphy and Kavanagh (1999) have drawn specific attention to the pathogenic potential of *S. cerevisiae*.

Epidemiological statistics suggest that many yeast infections in hospitalized patients originate from yeast contamination of catheters (Douglas 2003; Kojic and Daroviche 2004), and there is increasing concern that foods could be a significant

source of yeasts in the general hospital environment. It is also possible that foods could be a source of yeasts that colonize the intestinal tract, from where they translocate to the blood system, resulting in fungaemia and distribution to infect various organs (Cole et al. 1996). Greater understanding of the yeast ecology of the human gastrointestinal tract is needed.

1.8 Probiotic Yeasts

Probiotics are viable microorganisms that are beneficial to the host when consumed in appropriate quantities. Lactic acid bacteria are widely recognized as the main probiotic species but there is increasing interest in adding other organisms to the probiotic list, including yeasts (Klaenhammer 2001). Live *S. cerevisiae* preparations have been used as supplements to animal and poultry feeds for many years, and have been reported to improve the growth and health of these hosts (Lyons et al. 1993). Also, there is an expanding interest in using yeasts as probiotics in the aquaculture industry (Gatesoupe 1995). With respect to humans, *S. cerevisiae* var. *boulardii*, has been successfully used over the last 20 years as an oral, biotherapeutic agent to treat patients with severe cases of diarrhea and other gastrointestinal disorders (McFarland and Bernasconi 1993; Czerucka and Rampal 2002). The yeast colonizes the intestinal tract and, in this context, acts in a probiotic function. Research to establish its credentials as a probiotic agent that can be added to foods is still in progress (van der Aa Kuhle et al. 2005). However, there are significant concerns about its public health safety because of increasing reports of its association with cases of fungaemia (Cassone et al. 2003). Generally, the concept of using yeasts as human probiotics is at an early stage of development and further research is required. Apart from health benefit and safety issues, probiotic yeasts will also require certain technological properties for use in foods, namely, to remain viable in the food, not to grow in and spoil the food, and not to adversely affect sensory acceptability of the food (Heenan et al. 2004).

1.9 Future Prospects

As mentioned already, harnessing and exploiting the activities of yeasts in food and beverage production requires fundamental knowledge of their ecology, physiology, biochemistry and molecular biology. This knowledge provides the base for genetic improvement strategies (Chap. 13) and the discovery of novel bioreactor and biocatalytic technologies (Chap. 9) that are likely to drive the next generations of product and process development.

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Chapter 2

Taxonomic and Ecological Diversity of Food and Beverage Yeasts

PATRIZIA ROMANO[1], ANGELA CAPECE[1] AND LENE JESPERSEN[2]

[1]*Department of Biology, University of Basilicata, Viale Ateneo Lucano 10, 85100 Potenza, Italy (e-mail: pot2930@iperbole.bologna.it, Tel.: +39-971-205576, Fax: +39-971-205686)*
[2]*Department of Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30, 1958 Frederiksberg C, Denmark (e-mail: lj@kvl.dk, Tel.: +45-3528-3230, Fax: +45-3528-3214)*

2.1 Introduction

Fermentation has been used for several thousand years as an effective and low-cost resource to preserve the quality and safety of foods. Apart from this primary role, fermentation adds value and enhances nutritional quality and digestibility through biological enrichment, and provides dietary enrichment through aroma and flavour production and modifying textures of food substrates. All these changes are operated by microorganisms, which, naturally present (spontaneous fermentations) or added (inoculated fermentation) in raw materials, break down complex carbohydrates and proteins into more easily digestible elements.

Among the fermentation microorganisms, yeasts are undoubtedly the most important group of microorganisms that are exploited for commercial purposes. Yeasts used in food fermentation processes modify original materials organoleptically, physically and nutritionally and for this they have been used for millennia in bread-making and production of alcoholic beverages.

A diversity of fermented foods, which vary according to geographical area and cultural preference, are produced across the globe and the individual and peculiar characteristics which distinguish and typify each food are determined by the expression of the wide biodiversity of the fermenting microorganisms, such as yeasts. In Western countries yeasts are involved in the production of kefir from milk, beer from barley and hops, and wine from grapes, while in developing countries, where the lack of resources limits the use of techniques such as vitamin enrichment of food and the use of capital-intensive processes for food preservation, food fermentation contributes substantially to food security. In these countries there is a wide diffusion of traditional fermented foods, produced from both edible and inedible raw materials, based on local customs and generally consumed as dietary staples. Here we report the ecology and the role of yeasts involved in fermented food and beverages, focusing on the dominant species and their effect on the product quality, summarised in Table 2.1.

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
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Table 2.1 Distribution and principal activities of yeast species in fermented foods and beverages

More frequent yeast species	Origin (Food and beverage)	Major functions
Saccharomyces species	Wine, beer, sourdoughs, cider, sherry, cheese, indigenous fermented foods and beverages	Sugar fermentation Production of secondary metabolites Pectinase and glycosidasic activities Inhibitory effect on the growth of mycotoxin-producing moulds Degradation of some fractions of kasein CO_2 evolution Lipolytic, proteolytic and urease activities
Debaryomyces hansenii	Cheese, salami	Increase of pH Production of growth factors of importance for bacteria
Hanseniaspora (*Kloeckera*) species	Wine, cider, indigenous fermented foods and beverages	Proteolytic, glycosidasic and pectinolytic activities Production of secondary metabolites
Candida-fermenting species	Wine, sourdough, indigenous fermented foods and beverages	Proteolytic, glycosidasic and pectinolytic activities Production of secondary compounds Inhibitory effect on the growth of mycotoxin-producing moulds
Yarrowia lipolytica	Cheese, salami	Lypolytic, proteolytic and urease activities Reduction of fat rancidity

2.2 Yeasts in Dairy Products

For dairy products yeasts are mainly used in cheese production but may also be involved in the production of fermented milk products such as kefir. Yeasts are primarily used as single starter cultures but many products are still produced by backslopping or spontaneous fermentation. A mixture between the used starter culture and a dominant indigenous flora is also seen in many dairy products. Yeasts are in most cases used as secondary starter cultures in order to enhance the aroma production or to facilitate the growth of other microorganisms. Unfortunately, the functions of yeasts during cheese production and their influence on the cheese quality are in general poorly investigated. For cheese manufacturing *Debaryomyces hansenii*,

Saccharomyces cerevisiae and to some extent *Galactomyces geothricum* and *Yarrowia lipolytica* are the dominant yeast species (Jakobsen et al. 2002). But also other yeasts might be seen, such as *Kluyveromyces lactis*, which has been found to occur in high numbers in soft cheeses such as Camembert (Beresford et al. 2001).

The halophile yeast *D. hansenii* (perfect form of *Candida famata*) is a highly diverse yeast species as shown by phenotypic differences such as the ability to assimilate/ ferment different carbon compounds, differences in technological properties such as lipase and protease activity (Sørensen and Jakobsen 1997) and growth under different environmental conditions (Petersen et al. 2002). According to the present taxonomy *D. hansenii* is divided into two varieties, *D. hansenii* var. *fabryi* and *D. hansenii* var. *hansenii*. The two varieties can only be discriminated by different electrophoretic mobilities of their glucose-6-phosphate dehydrogenase and maximum temperatures for growth (Nakase et al. 1998). Apparently the predominant variety seen in cheese is *D. hansenii* var. *hansenii* (Petersen et al. 2001). The type strain (CBS767) of the variety *D. hansenii* var. *hansenii* has previously been reported to be haploid (van der Walt et al. 1977), whereas information on the ploidy of other strains belonging to the species *D. hansenii* is lacking.

D. hansenii is especially of importance during the production of surface-ripened cheeses such as Brick, Limburger, Port Salut, Taleggio, Tilsitter, Trappist, and the Danish Danbo cheese. The surface smear is found to consist of a mixed microbial population comprising both yeasts and bacteria. For these types of cheese the yeasts initiate the ripening by degradation of lactate, thereby increasing the pH on the cheese surface and allowing the growth of a more acid-sensitive bacterial population comprising, amongst others, *Brevibacterium linens* (Leclercq-Perlat et al. 1999; Petersen et al. 2001). Further, *D. hansenii* might produce growth factors of importance for the bacteria as well as aroma components and lipolytic and proteolytic enzymes that contribute to the ripening process (Jakobsen and Narvhus 1996). For the Danish cheese Danbo, the osmotolerant yeast *D. hansenii* has been found almost exclusively (Petersen et al. 2002), whereas in other types of surface-ripened cheeses, including cheeses such as Camembert and blue veined cheese, other yeasts such as *C. zeylanoides*, *Y. lipolytica* and *K. lactis* have been found (Eliskases-Lechner and Ginizinger 1995; Addis et al. 2001; Corsetti et al. 2001a). As mentioned, many of the yeasts occur as a positive part of the indigenous microbial population. However, the benefits of moving from spontaneous fermentations to controlled fermentations are many and therefore there seems to be growing interest in the use of *D. hansenii* as a purified starter culture. Consequently, the use of starter cultures with appropriate technological properties generates the need for fast and simple methods for identification of *D. hansenii* at strain level. Mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP), which was originally developed for differentiation of wine and beer strains of *Saccharomyces* spp., has been used for strain typing of *D. hansenii* (Petersen et al. 2001) and determination of chromosome length polymorphism (CLP) by pulsed-field gel electrophoresis (PFGE) has been used to clarify the genetic diversity of *D. hansenii* and for typing of dairy isolates (Petersen and Jespersen 2004). Figure 2.1 shows the chromosome profiles of 16 isolates of *D. hansenii* from Danish dairies producing surface-ripened cheeses. As seen from the figure, also at the genetic level *D. hansenii* shows an extensive degree of diversity.

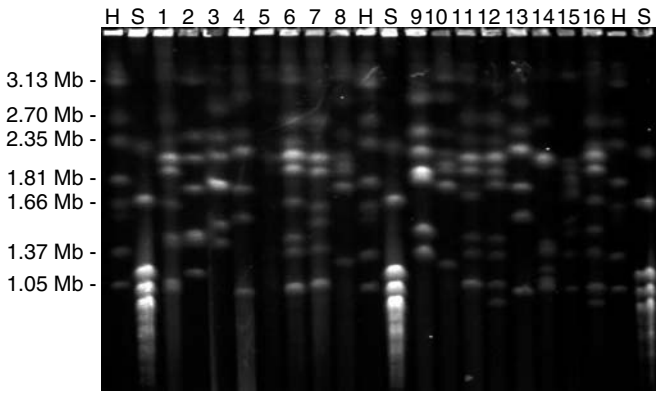

Fig. 2.1. Chromosome profiles of *Debaryomyces hansenii* isolated from Danish dairies producing surface-ripened cheeses. *H* marker "*Hansenula wingei*" (*Pichia canadensis*), *S* marker *Saccharomyces cerevisiae*, lanes 1–16 isolates of *D. hansenii* from brine and surface smear sampled from seven different Danish dairies

S. cerevisiae has been used as a starter culture in the production of, especially, Gorgonzola, but it apparently also occurs spontaneously, together with other yeasts, as an integral part of the microbial population of both blue veined cheeses and some types of soft cheese (Beresford et al. 2001; Hansen and Jakobsen 2001). On cheese substrate some strains of *S. cerevisiae* have been able to degrade α_{si}-kasein and β_{a2}-kasein as determined by capillary electrophoresis (Hansen and Jakobsen 2001). When used as a starter culture in Mycella, a Danish Gorgonzola-type cheese, *S. cerevisiae* was reported to stimulate sporulation and improve the mycelial growth of *Penicillium roqueforti*; however, the stimulation appeared to be strain-dependent (Hansen et al. 2001a). The use of *S. cerevisiae* as a starter culture further resulted in a softer texture and a significantly higher concentration of aroma compounds of the final cheese. The study also indicated the possibility of *S. cerevisiae* to compete with *D. hansenii* in the interior of the cheese.

Even though previously considered as a mould, *G. geotrichum* (perfect form of *Geotrichum candidum*) is now considered as a yeast species (de Hoog et al. 1998). Synonyms include former names like *Oidium lactis* and *Oospora lactis*, indicating the link to milk and dairy products. *G. geotrichum* is known as a starter culture for several types of mould-ripened cheeses, e.g. Camembert, surface-ripened cheeses and cheeses such as the French St. Albray where it is used in combination with other microorganisms such as *Penicillium camemberti* and *B. linens* (Molimard et al. 1995). On the other hand, *G. geotrichum* has also been shown to be a potential spoilage organism especially for blue veined cheeses. It has been shown to have antimicrobial activity and can, because of its rapid growth, effectively compete with other yeasts and moulds in cheeses (Jakobsen et al. 2002).

Y. lipolytica (perfect form of *C. lipolytica*) is often found in soft, blue veined and surface-ripened cheeses even though it is not used as a starter culture. *Y. lipolytica* is characterised by having a quite pronounced lipolytic and proteolytic activity (Guerzoni et al. 2001) that might be difficult to control if it is used as a starter culture

for cheese production. However, it has been reported to have a positive effect on the flavour of Raclette cheese as well as low-fat cheeses (Wyder et al. 1999; Jakobsen et al. 2002). It is rather sensitive to high NaCl concentrations, which might be a limitation for its growth in, for example, blue veined cheeses. Further *Y. lipolytica* is known to produce brownish pigments in cheeses.

For fermented milk, yeasts within the genera *Candida*, *Galactomyces*, *Kluyveromyces*, *Saccharomyces* and *Torulaspora* are generally used (Oberman and Libudzisz 1998). *G. geotrichum* is used as commercial starter culture in the production of *Villi*, a Scandinavian fermented milk product and *S. unisporus* and *K. marxianus* (*C. kefyr*) are used as commercial starter cultures in the production of milky kefir. *S. cerevisiae* has also been reported to be involved in the fermentation of a number of indigenous African fermented milk products known under names such as *amasi*, *nono* and *rob* (Okagbue and Bankole 1992; Gadaga et al. 2001; Abdelgadir et al. 2001).

2.3 Yeasts in Fermented Sausages

Fermented sausages could be classified as intermediate moisture meat products, in which the removal of available humidity is an effective strategy for inhibiting the growth of spoilage microorganisms. They represent foods prepared from raw materials, involving processes of fermentation and chemical curing. Fermentation is a crucial phase of the curing phase of sausages, since at this stage the principal physical, biochemical and microbiological transformations take place (Beriain et al. 1993). These changes are influenced by characteristics of the raw materials and process conditions, which influence the organoleptic properties of the final product, such as flavour, colour and texture, as well as its preservation and safety. The predominant microorganisms involved in the production of fermented meats are lactic acid bacteria, coagulase-negative staphylococci and micrococci, but also moulds and yeasts can be involved, reaching significant numbers and therefore playing a technological role. Yeasts are usually present in low numbers in fresh meat, but counts may increase during low-temperature storage and they may eventually dominate the microflora (Cook 1995).

The surface of dry fermented sausages is covered by a white superficial coat constituted by moulds and yeasts. Yeasts are naturally found on the hides of animals, from where they are easily spread to the fresh meat during slaughtering. The yeast presence is related to natural contaminating yeasts present in the meat (Dillon and Board 1991), in processing equipment and on workers' hands and aprons.

Studies on the yeast flora of fermented sausages are limited, compared with those focused on bacteria and, although yeasts do not form part of the starter cultures usually employed for salami processing, an increase in yeast number during ripening has been observed (Abunyewa et al. 2000). Yeast counts remain low during the fermentation stages when bacterial numbers increase, but their numbers rapidly increase during the maturation stage. The high number of yeasts observed during the later stages of maturation suggests that the yeasts play an important role in the ripening of salami. The progressive growth of lactic acid bacteria during the fermentation stages and yeasts during the ripening stages indicates competition between the different microorganisms for available substrates. However, the interaction between yeasts

and lactic acid bacteria at the later stages appears to be synergistic, since both populations continue to survive at high numbers with neither being inhibited by the other. The high salt concentration and, therefore, the low water activity, combined with the acidic environment, a typical characteristic of fermented sausage, favours the growth of certain yeast species since competing bacteria are repressed.

In the final products yeast cells have been detected in concentration up to 10^5 cfu/g (Encinas et al. 2000; Samelis et al. 1993), numbers that suggest a significant involvement of yeast activity in the salami production. According to Geisen et al. (1992) yeasts' requirements for oxygen restrict them to mainly growing near the surface of fermented sausages. In fact, Coppola et al. (2000) found 10^4 cfu/g yeast cells in samples from the core of Naples-type salami. This value remained almost unchanged until the end of the ripening period. In contrast, samples from the external part of the product reached the maximum level of 10^6 cfu/g after 3 weeks. Probably, higher oxygen availability in the external part of the product could facilitate the growth of these microorganisms.

Fermentative yeast species, however, can thrive under low oxygen conditions since they only require oxygen for production of cell wall constituents, such as sterols and fatty acids. The confirmation of this is also in the results of Gardini et al. (2001), who found increased yeast counts during the first few days of fermentation up to 10^5–10^6 cfu/g, whereas before casing yeast counts ranged from not detectable to about 10^4 cfu/g.

Although the initial flora present in the sausage emulsion and raw meat is extremely variable, strains of *D. hansenii* are frequently isolated during the fermentation and ripening stages. This species appeared to be the most frequent yeast species associated with the processing of salami: Abunyewa et al. (2000) found a frequency of this species of 20.37% of the total number of yeast strains isolated during a pilot scale production of commercial salami. Gardini et al. (2001) found *D. hansenii* and its anamorph *C. famata* with a frequency of 52% of the total isolates from production and ripening of typical salami in southern Italy. Other species frequently isolated are *Rhodotorula mucillaginosa* (15%), a typical air contaminant, described as a frequent food isolate, *Cryptococcus albidus* (10.18%), *Trichosporon beigelii* (*moniliiforme*) (9.26%), *Y. lipolytica* (13.89%), and *C. zeylanoides* and *D. occidentalis* both with about 5% (Abunyewa et al. 2000). Other species isolated, representing less than 5% of the total number of yeasts, are *C. haemulonii*, *C. gropengiesseri*, *D. polymorphus*, *D. vanrijiae*, *G. geotrichum*, *Pichia farinosa*, *P. philogaea*, *Rh. minuta*, *Sporobolomyces roseus*, *Sterigmatomyces halophilus*, and *Torulaspora delbrueckii*.

The incidence of the different yeast species varies depending on the salami processing. The species *C. gropengiesseri*, *P. farinosa*, *P. philogaea*, *Rh. minuta*, *Sp. roseus* and *St. halophilus* are isolated only during the fermentation stage, suggesting an inhibition of these yeast species due to the reducing water activity. Other species, such as *C. haemulonii*, *D. polymorphus*, *D. occidentalis* and *T. delbrueckii*, are not frequently isolated during processing, so they do not constitute the typical microflora of salami processing.

C. zeylanoides, *Cr. albidus*, *D. hansenii*, *Rh. mucillaginosa* and *Y. lipolytica* represent the species found most frequently. Parameters such as their tolerance to low temperatures, high salt concentrations and low pH levels could offer a fundamental ecological advantage for the growth of these species in fermented sausage. Different treatments during salami processing, such as smoking, variety of spices added and

method of ripening, could influence the yeast count. Encinas et al. (2000) found differences statistically significant in yeast counts between smoked and nonsmoked sausages. In particular, after the second stage of the manufacture, the mean counts in smoked sausages were lower than in nonsmoked ones. On the other hand, yeasts have been described as being affected by smoking and also by factors such as time and temperature (Leistner 1995). Other reports underline the influence of spices on yeasts, in particular a significant inhibitory effect of garlic on their growth (Ghamnnoum 1990). The addition of garlic powder strongly affects both *D. hansenii* and *C. utilis* (Olesen and Stahnke 2000), but *D. hansenii* is completely inhibited, whereas *C. utilis* seems to be more resistant. Therefore, dried garlic powder exerts at least a fungistatic potential, and perhaps also a fungicide potential.

Other compounds, such as sorbates, allowed by numerous legislations and frequently present in industrial sausages, seem to decrease the yeast counts, as a consequence of their known effect on the control of yeasts in food and drinks (Fleet 1990).

Also ripening conditions affect yeast growth and, for example, when an accelerated process is used, yeast counts decreased by 1 log unit/g (Encinas et al. 2000).

The role of yeasts in the manufacture of fermented sausage is mainly related to the development of colour (by removing the oxygen) and flavour, as a consequence of their ability to degrade peroxides, lipolytic activity and, to a lesser extent, proteolytic activity (Lücke 1985). Furthermore, yeasts protect sausages from the adverse effect of light. In fact, it is believed that yeasts delay rancidity and protect the red nitrosomyoglobin from breakdown by degrading peroxides and consuming oxygen, thus stabilising the appealing red colour of fermented sausages (Lücke and Hechelmann 1987), which is an indication that the product is fully cured.

Yeasts play an important role in sausage fermentation as well as in maturation of hams (Cook 1995) and bring about characteristic flavours and surface appearance. The contribution of these organisms to the typical aroma of the products is based on their primary and secondary metabolites and lipases and proteinases are key activities. Among the flavour products identified in dry sausages, the oxidation products of lipids account for about 60% of the total compounds which influence the flavour (Berdagué et al. 1993). The distinctive flavours of these products were found to be related to hydrolytic and oxidative changes occurring in the lipid fraction during ripening. The species *Y. lipolytica* has been found to be characterised by a higher lipolytic potential (Gardini et al. 2001), with isolates of this species exhibiting the capability to reduce the content of total free fatty acids after 6 days of incubation in a medium containing pork fat (5%).

The decrease of total free fatty acids content could be related to the activity of *Y. lipolytica*, which can further metabolise or oxidise unsaturated free fatty acids to flavour compounds (Ordoñez et al. 1999). Furthermore, lipases from isolates of *Y. lipolytica* favour at pH 5.5 the liberation of saturated free fatty acids rather than unsaturated ones. This tendency could have a positive effect by reducing the possibility of rancidity, which principally involves polyunsaturated free fatty acids.

D. hansenii and *C. zeylanoides* are able to reduce the fat rancidity of salami by hydrolysing lipids through lipolytic activity (Metiva et al. 1986), even if some authors (Sørensen 1997) report that the lipolytic activity is inhibited by low pH and low temperature. The anamorphic yeast *Cr. albidus* exhibits proteolytic activity

(Huerta et al. 1988) and may cause spoilage by hydrolisation of salami proteins. Most of these species have the ability to utilise organic acids produced by lactic acid bacteria (Besancon et al. 1992; Roostita and Fleet 1996), resulting in an increase of pH by the production of amines and ammonia, and thereby favouring the growth of spoilage bacteria. In contrast, data reported by other authors (Abunyewa et al. 2000) did not support the role of yeast to provide a means of spoilage by assimilating organic acid, such as lactic acid. It may be that the loss of water results in concentrating the acids and thus counterbalances their assimilation.

The introduction of starter cultures has become essential during industrial production of fermented sausages in order to shorten the ripening period, ensure colour development, enhance the flavour and improve product safety (Gardini et al. 2001). The inclusion of bacteria in starter cultures has frequently been investigated, while little attention has been focused on the role of yeasts in the fermentation of sausages. The use of commercially available starters, mainly constituted of lactic acid bacteria and micrococci, may also produce an impoverishment of flavour and aroma and a loss of peculiar organoleptic characteristics found in naturally fermented sausages.

Starting from the earliest studies on salami (Capriotti 1954), *D. hansenii* is the most commonly isolated yeast among the yeast population of fermented sausages. On the basis of this, *D. hansenii* has been used as a starter with positive effects on the development of a characteristic yeast flavour and stabilisation of the reddening reaction. *D. hansenii* and its imperfect form *C. famata* are now used in starter preparations and should be added to the sausage mixture at a concentration of 10^6 cfu/g (Hammes and Knauf 1994). The yeast *Y. lipolytica*, the perfect form of *C. lipolytica*, is also frequently isolated from fresh beef (Fung and Liang 1990; Dalton et al. 1984) and sausages (Viljoen et al. 1993). Owing to its lipolytic activity, *Y. lipolytica* could have significant technological interest (Sinigaglia et al. 1994) as a starter for the production of traditional dry fermented sausages by shortening the ripening time in relation to the evolution of the lipid fraction and also to protein breakdown, related to its elevated proteolytic activity.

2.4 Yeasts in Sourdough Breads

Bread-making, together with the making of wine, beer and sake, and the production of yogurt and cheese, is among the oldest biotechnology processes. Spontaneous souring with natural microflora and the use of special starter cultures are both used in sourdough bread baking. Sourdough is an important part of cereal fermentation and its preparation is based on traditional customs of each country: sourdough breads vary from a large number of different rye and flat breads to San Francisco sourdough bread made from wheat flour. In Italy, in which sourdough is used in more than 30% of bakery products, there are more than 200 different types of sourdough breads (INSOR 1995) (Corsetti et al. 2001b). Most of these breads are made by following old traditions and differ in the type of flour, other ingredients, type of sourdough, technology and shelf life. In all cases, the routine is to save a portion of the raw dough (or "leaven") from each batch, and this is then used to inoculate the next lot of dough. The leaven contains both yeasts and lactobacilli: in fact, sourdough could be considered a complex biological system which includes a mixed

microbial population mainly represented by yeasts and lactic acid bacteria. Since the mid-nineteenth century, yeast cultures have been available commercially and the use of inoculated fermentation has turned into common practice.

In doughs that are spontaneously fermented, the yeast population can range from 7×10^7 to 6.1×10^9 cfu/g of sourdoughs (Paramithiotis et al. 2000), while the ratio between yeast and lactic acid bacteria varies from 1:8 to 1:73. Gobbetti et al. (1994b) analysed the microflora composition of wheat sourdoughs from semi-industrial bakeries in an Italian region and reported an average ratio between lactic acid bacteria and yeasts of about 100:1, with the yeast count ranging from 3×10^4 to 5×10^7 cfu/g. These values are similar to results of other authors (Roecken and Voysey 1995) and seem to indicate that these products are really naturally fermented, without addition of commercial yeasts. In products from bakeries, where commercial yeasts are added to accelerate the leavening process, yeast populations are much higher than those of lactic acid bacteria.

The most frequent yeast species detected in sourdoughs are *S. cerevisiae*, *C. krusei*, *C. milleri*, *P. anomala*, *P. subpellicosa*, *S. exiguus*, *T. holmii* and *C. humilis* (Rossi 1996; Gullo et al. 2002).

In a study conducted on samples of homemade sourdoughs from small bakeries in Sicily (Italy) (Pulvirenti et al. 2004), among the wild yeast strains, identified by molecular techniques such as PCR/RFLP analysis of internal transcribed spacer regions, the dominant species was *S. cerevisiae*, followed by *C. milleri*, *C. humilis*, *S. exiguus* and *Issatchenkia orientalis*. Also Paramithiotis et al. (2000) analysed wild yeast strains isolated from traditional Greek wheat sourdoughs, both household and semi-industrial types, and reported *S. cerevisiae* as the predominant species in all the samples examined. Depending on the origin of the sample, *P. membranifaciens* or *Y. lipolytica* was found. When *S. cerevisiae* is associated with *P. membranifaciens* the ratio generally ranges from 1:0.5 to 1:0.31 with *S. cerevisiae* as predominant species. This is correlated to the capability of *S. cerevisiae* isolates to ferment all the sugars present in the dough, i.e. glucose, fructose, sucrose and maltose, whereas *P. membranifaciens* can only ferment glucose, but about 8 times slower than *S. cerevisiae* isolates.

The ratio between *S. cerevisiae* and *Y. lipoytica* is 1:0.14, with *S. cerevisiae* dominant. This is related to the absence of fermentative metabolism in *Y. lipolytica* isolates, while they only oxidise glucose. The occurrence of *S. cerevisiae* strains in every sourdough tested leads to the conclusion that this species represents the indigenous yeast microflora of traditional Greek sourdoughs, while *P. membranifaciens* and *Y. lipolytica* might represent an occasional microflora of these products. The frequent presence of *S. cerevisiae* in sourdoughs is reported by different authors (Ottogalli et al. 1996; Rossi 1996; Gobbetti et al. 1994a) and in many cases this species seems to constitute part of indigenous microflora, whereas in others its presence is related to the extensive use of this yeast in bakeries. The presence of *P. membranifaciens* strains in sourdough microflora is also mentioned by Rossi (1996) and it has been found in traditional Portuguese bread doughs (Almeida and Pais 1996). The data reported by Paramithiotis et al. (2000) represent the first evidence of the presence of *Y. lipolytica* in sourdoughs, a species mostly associated with meat and dairy products.

The numbers and the yeast species present in sourdough are influenced by the degree of yeast tolerance to the organic acid produced by lactic bacteria, particularly

lactic and acetic acids derived from homo- and heterofermentative bacteria. The yeasts *S. cerevisiae* and *S. exiguus*, very frequent species in sourdough, exhibit different behaviour for the resistance to these two acids: *S. cerevisiae* is very sensitive, while *S. exiguus* is very resistant. The sensitivity to these organic acids is related to the presence in sourdough of the undissociated form of acetic acid at low pH values (3.5–3.9). For this reason in some products, such as Italian Panettone obtained by a natural fermentation system, *S. exiguus* may be exclusively present during certain production phases, yielding satisfactory results (Rossi 1996).

However, technical parameters such as temperature, flour composition, degree of dough hydration, sodium chloride content and time between rebuildings are significant variables for the selection of microorganisms (Gobbetti et al. 1994a). Another critical point for the microbial development in sourdough is represented by the available sources of carbon (Lues et al. 1993; Gobbetti et al. 1994b). The available carbohydrates in wheat flour are (in decreasing order) maltose, sucrose, glucose and fructose, with some trisaccharides (maltotriose, raffinose). They may either increase during fermentation, as in the case of glucose, or decrease, as in the case of sucrose in the presence of yeasts possessing high invertase activity. This enzyme catalyses the hydrolysis of sucrose into glucose and fructose, thus increasing osmotic pressure. Therefore, baker's yeast strains with low invertase activity have been selected for sweet dough applications because they are suitable to elaborate dough containing low concentrations of sucrose (less than 8–10%). However, these strains do not show intrinsic osmotolerance and at the higher sugar contents the osmotic pressure inhibits their activity (Myers et al. 1997). The presence of *S. exiguus* in the dough is associated with a rapid consumption of soluble carbohydrates with the exception of maltose. In any case *S. cerevisiae* and *S. exiguus* are not in nutritional competition for maltose because *S. exiguus* does not metabolise maltose.

In bread-making many different functional properties have been defined for yeasts. The most important function of baker's yeasts is leavening (Paramithiotis et al. 2000), by producing CO_2 via the alcoholic fermentation of the sugars. In the initial stages of bread-making, the CO_2 produced stays in solution until the water phase becomes saturated. At this point, all further CO_2 diffuses into the existing gas cells produced during mixing. Increased production of CO_2 increases the dough volume, giving bread with characteristic a light, spongy texture. Baker's yeast also influences the development of the dough gluten structure, brought about by expansion of the dough owing to CO_2 production.

Furthermore, yeasts produce primary and secondary metabolites, such as alcohols, esters and carbonyl compounds which contribute to the development of the characteristic bread flavour (Damiani et al. 1996; Hansen and Hansen 1994; Martinez-Anaya 1996). Some of these compounds are volatile and are baked out of the bread. In addition, through their enzymatic activities, such as proteases, lecithinase, lipases, α-glucosidase, β-fructosidase and invertase, yeasts can affect not only the organoleptic characteristics, but also the overall appearance of the final product. In fact, these enzymatic activities have an influence on the dough stickiness and rheology, as well as on the crust colour, crumb texture and firmness of the bread (Antuna and Martinez-Anaya 1993; Collar et al. 1998).

The great potentiality of sourdough bread is related to the interaction between lactic acid bacteria and yeasts. Like other fermented foods produced by mixed microflora, the organoleptic, health and nutritional properties of this product depend on the cooperative activity of lactic acid bacteria and yeasts. In fact, each sourdough can be considered a microhabitat in which yeasts and lactic acid bacteria exist together in a dynamic equilibrium. The yeasts are responsible for the leavening process, while the bacteria determine the souring of the dough. The combined metabolic activity of these microorganisms leads to final products with particular sensorial properties and a prolonged shelf life (Gänzle et al. 1998).

Numerous pieces of research have reported the effect of the interaction between sourdough lactic acid bacteria and yeasts on the metabolism of carbohydrates, the production of CO_2 and other volatile compounds. The utilisation of soluble carbohydrates by lactic acid bacteria and, consequently, their energy yield, lactic and acetic acid production are greatly influenced by the associated yeasts and vary according to the type of sugars. In a continuous sourdough fermentation the association between *Lb. sanfranciscensis* and *S. cerevisiae* is optimal for producing acetic acid, while yeast extract does not produce the same effect (Vollmar and Meuser 1992). *Torulopsis holmii* has been found to improve dough acidification by *Lb. sanfranciscensis*, while *S. cerevisiae* enhances acid production by *Lb. sanfranciscensis* and *Lb. plantarum* (Spicher et al. 1982). The lack of competition between *Lb. sanfranciscensis* and *S. exiguus* for maltose is fundamental for the stability of this association in San Francisco bread (Gobbetti 1998). On the other hand, the lack of competition for the main carbon source seems to be one of the prerequisites for the stability of lactic acid bacteria/yeast association in food fermentation. When *Lb. plantarum* is associated with *S. cerevisiae* and *S. exiguus* in the presence of sucrose as a carbon source, cell yield and lactic acid production increase (Gobbetti et al. 1994b). The hydrolysis of sucrose by yeasts liberates glucose and fructose, which are then more rapidly depleted than the sucrose by lactic acid bacteria (Gobbetti 1998). Furthermore, yeasts hydrolyse sucrose about 200 times faster than the released hexoses which are fermented (Martinez-Anaya 1996), causing the rapid disappearance of sucrose during sourdough fermentations (Gobbetti 1998).

The relationship between yeasts and lactic acid bacteria seems to provide more favourable conditions for CO_2 production. Although yeast cell concentrations and the type of yeast are the major parameters determining gas production rates (Akdogan and Ozilgen 1992), the growth of lactic acid bacteria can influence the yeast leavening and CO_2 production (Gobbetti et al. 1995). The production of CO_2 by other yeasts, such as *S. exiguus*, is not comparable to the high gassing power of *S. cerevisiae*. Besides, compared with *S. cerevisiae* alone, the associative growth of *S. cerevisiae* and *Lb. sanfranciscensis* decreased to one third of the time necessary to reach the maximum production of CO_2 by the yeast. The same increase has also been observed with the associated growth of *S. exiguus* and *Lb. sanfranciscensis*. The associated growth of *Lb. plantarum* with *S. cerevisiae* caused an increase in CO_2 produced and improved the capacity of the dough to retain the gas.

The flavour of leavened baked goods is influenced by the raw materials (Hansen and Hansen 1994), sourdough fermentation, proofing, baking and by the starters. Even if the greatest number of compounds influencing aroma is formed during

baking, sourdough fermentation is essential for achieving an acceptable flavour. Each type of metabolism (heterolactic, homolactic and alcoholic) which characterises the sourdough fermentation is defined by typical volatile compounds (Damiani et al. 1996).

The differentiation is mainly related to 2-methyl-1-propanol and 2,3-methyl-1-butanol, the principal products of yeast fermentation, diacetyl, mainly produced with other carbonyls by homofermentative lactic acid bacteria, and ethyl acetate, mainly produced with some alcohols and carbonyls by heterofermentative lactic acid bacteria. The sourdough process starts with the association of *Lb. sanfranciscensis* and other homo- or heterofermentative lactic acid bacteria and/or *S. exiguus* characterised by a balanced profile. The sourdough produced with mixed starter composed by *Lb. sanfranciscensis*/*S. cerevisiae* has higher concentrations of yeast fermentation products (1-propanol, 2-methyl-1-propanol and 3 methyl-1-butanol) and fewer bacterial compounds (Damiani et al. 1996; Gobbetti et al. 1995). Annan et al. (2003) obtained similar results in a study aimed at comparing volatile compounds associated with Ghanaian maize dough samples prepared by spontaneous fermentation and by the use of added starter cultures. The starter cultures added were *Lb. fermentum*, *S. cerevisiae* and *C. krusei*. The amount of ethanol, which is the alcohol produced in the highest amounts, is higher in dough fermented spontaneously and with *S. cerevisiae* than with *C. krusei* or *Lb. fermentum*. The fusel alcohols 1-propanol, 2-methyl-1-propanol and 3-methyl-butanol are found in the highest amounts in fermentation with *S. cerevisiae*, while phenylethyl alcohol is found in the highest amounts in fermentations with *C. krusei*. Among esters, ethyl acetate, the most abundant ester formed, is generally found in higher amounts in fermentations with *S. cerevisiae*. Higher levels of acetic acid are formed in fermentations with *C. krusei* than with *S. cerevisiae*, which produces amounts not significantly different from those found in spontaneously fermented maize dough.

2.5 Yeasts in Grape Wines

Wine is a natural product resulting from several biochemical reactions, which start during ripening of the grapes and continue during harvesting, throughout the alcoholic fermentation, clarification and after bottling. Even if the grape must represents a complete growth medium, only a limited number of microbial species are able to grow as a consequence of its low pH values and high sugar content. Thus, of all the microorganisms present on the grapes, the yeasts are the principal agents of the biochemical transformation of grape must.

The variety and proportion of different yeasts in the must can depend on numerous factors, such as geographic location, climatic conditions, grape variety, physical damage caused by moulds, insects and birds and viticultural practices (Pretorius et al. 1999).

In traditional winemaking, spontaneous fermentation of grape juice is performed by a sequential development of different yeast species that originate from the grapes and the winery equipment. The initial population of yeasts in freshly extracted grape juice is 10^3–10^6 cfu/ml. *Hanseniaspora* (*Kloeckera*) spp. are often the predominant species on the surface of grape berries, accounting for roughly 50–75% of the total yeast population. Numerically less prevalent there are species

of *Candida* (e.g. *C. stellata* and *C. pulcherrima*), *Brettanomyces* (*B. anomalus* and *B. bruxellensis*), *Cryptococcus*, *Kluyveromyces*, *Metschnikowia* (*M. pulcherrima*, the perfect form of *C. pulcherrima*), *Pichia* (*P. membranifaciens*), the so-called film yeast, as well as species previously assigned to the *Hansenula* genus, e.g. *H. anomala* and the pink yeast *Rhodotorula* (*Rh. minuta*).

On immature grape berries a very low yeast population has been detected ($10–10^3$ cfu/ml) with the predominance of *Rhodotorula*, *Cryptococcus* and *Candida* species, along with the yeast-like fungus *Aureobasidium pullulans*. These species are also found on surface of ripe grapes, but at this stage *Hanseniaspora* and *Metschnikowia* species are mostly predominant. Damage to grapes increases the yeast population (above 10^6 cfu/ml), especially of the *Hanseniaspora*/*Kloeckera*, *Metschnikowia* and *Candida* species, as well as species of *Saccharomyces* and *Zygosaccharomyces* (Fleet et al. 2002).

The population of the main wine yeast *S. cerevisiae* in grape juice is initially very low: generally it reaches populations less than 10–100 cfu/g. The limited number of *S. cerevisiae* on grapes (Martini 1993) and the evidence that this yeast does not appear to be specifically associated with the vineyard or other natural substrates induced Martini et al. (1996) to conclude that grapes are not the primary source of *S. cerevisiae*, reported as strictly associated with the winery and fermentation plants; the few, if any, cells of *S. cerevisiae* resident on grapes should provide an extremely limited contribution to spontaneous must fermentation (Ciani et al. 2004).

Different conclusions have been reached by other authors who, even though recognising the exiguity of *S. cerevisiae* cells residing on grapes, sustain that vineyard-resident cells do play an important role in natural fermentation and the presence or absence of *S. cerevisiae* differs with each plant and grape cluster. Török et al. (1996) by using more elaborate isolation methods, classical genetic analysis and electrophoretic karyotyping of monosporic clones, demonstrated that the vineyard is the primary source for the wine yeasts and that the strains found on the grapes can be followed through the fermentation process.

While the fermentation process is occurring, the establishment of anaerobic conditions, the antimicrobial activity of sulphur dioxide added, the depletion of nutrients and the increasing levels of ethanol enlarge the selectivity of the medium. The non-*Saccharomyces* yeasts present in grape juice, such as *Hanseniaspora* (*Kloeckera*), *Candida*, *Pichia*, *Kluyveromyces* and *Metschnikowia* could proliferate to final populations of about $10^6–10^7$ cfu/ml, and started to decline by mid-fermentation, when the ethanol production by *S. cerevisiae* exceeds 5–7% (Heard and Fleet 1988; Gao and Fleet 1988). Ethanol production by *S. cerevisiae* is the major factor affecting the growth of non-*Saccharomyces* yeasts. However, *Hanseniaspora* and *Candida* species, when the fermentation is performed at temperatures lower than 15–20°C, decrease their sensitivity to ethanol, giving a significant contribution to wine flavour (Erten 2002).

During the latter stages of natural wine fermentation the strongly fermentative strains of the *Saccharomyces sensu stricto* group of wine yeasts, which are more ethanol-tolerant and more competitive for growth in media with high sugar content, become predominant ($10^7–10^8$ cfu/ml) and complete the fermentation. The *Saccharomyces sensu stricto* group is an evolutionary lineage, which is distinct from the species belonging to the *Saccharomyces sensu lato* group (Kurtzman and

Robnett 2003). Actually, the *sensu stricto* group comprises *S. cerevisiae*, *S. paradoxus*, *S. bayanus* and *S. pastorianus*, which are associated with wine fermentation, and three newly defined species, *S. cariocanus*, *S. mikatae* and *S. kudriavzevii* (Naumov et al. 2000). Apart from the well-known and recognised prevalence in winemaking of the species *S. cerevisiae*, *S. bayanus* plays a particular role in primary winemaking, especially when must fermentation takes place at low temperatures, and *S. pastorianus* is thought to originate from the hybrid cross between *S. cerevisiae* and *S. bayanus* (Tamai et al. 1998). In a recent work (Redžepović et al. 2002), in the indigenous population of *Saccharomyces sensu stricto* strains in Croatian vineyards, *S. paradoxus*, possessing potentially important oenological characteristics, occurs in much higher numbers than *S. cerevisiae*.

Other yeasts, such as species of *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, may also be present during the fermentation and ageing of the wine, and some of these yeasts influence adversely sensory quality. *Schizosacharomyces pombe*, *Zygosaccharomyces bailii* and *Zygosaccharomyces fermentati* are characterised by a high level of ethanol tolerance (more than 10%) and, although frequent in a winery environment, their contribution to grape must fermentation is rarely reported. The reason is probably related to their slower growth in comparison with that of other wine yeasts or to the production of inhibitory factors by other yeasts. These species could be very interesting in winemaking in consequence of their ability to utilise malic acid.

Numerous studies on grape juice fermentation reported that non-*Saccharomyces* species (especially *K. apiculata* and *C. stellata*) survive during fermentation at a significant level (Heard and Fleet 1988; Ciani 1997), but owing to their low ethanol tolerance these yeasts are generally undetectable at the end of fermentation.

The persistence of non-*Saccharomyces* yeasts during fermentation depends upon many factors, such as fermentation temperature, nutrient availability, inoculum strength of *Saccharomyces*, use and levels of sulphur dioxide and the quantity and identity of microorganisms initially present on the grapes (Fleet 2003).

The growth of non-*Saccharomyces* species affects both the kinetics of growth and the metabolism of *Saccharomyces* (Lema et al. 1996). These yeasts are capable of anaerobic as well as aerobic growth and may persist during the fermentation, competing with *Saccharomyces* for nutrients. In addition, non-*Saccharomyces* yeasts seem to be less tolerant to very low oxygen availability than *S. cerevisiae*. Therefore, removal of residual oxygen from fermenting grape juice by the vigorous growth of *S. cerevisiae* could contribute to the early death of non-*Saccharomyces* species (Hansen et al. 2001b). Other studies report that *K. apiculata* (*H. uvarum*) could strip the grape juice of thiamine and other micronutrients, leading to deficient growth of *S. cerevisiae* (Mortimer 2000). However, some non-*Saccharomyces* species, such as *K. apiculata* (*H. uvarum*) and *M. pulcherrima* possess a significant proteolytic activity (Charoenchai et al. 1997; Dizzy and Bisson 2000) and can generate amino acids useful for *S. cerevisiae*. The autolysis of these non-*Saccharomyces* yeasts (Hernawan and Fleet 1995) represents a possible source of nutrients for *S. cerevisiae*.

The presence of killer interactions represents another factor affecting yeast species and strain evolution during wine fermentation. The killer phenomenon is based on the secretion of polypeptide toxins which kill sensitive cells of their own

species and frequently those of other yeast species and genera. However, killer strains are immune to the toxin they produce.

A wide literature deals with the killer character in *S. cerevisiae* isolates from fermenting grape juice (Musmanno et al. 1999; Guriérrez et al. 2001). Killer strains of *S. cerevisiae* sometimes predominate at the end of the fermentation, suggesting that they take over the fermentation by asserting their killer activity. Killer strains have been found among wine isolates of *Candida*, *Pichia* and *Hanseniaspora* and some of these can exert their killer action against wine strains of *S. cerevisiae* (Fleet and Heard 1993).

It is expected that the inoculated cultures of *S. cerevisiae* will suppress indigenous microflora, both non-*Saccharomyces* and *S. cerevisiae* strains, thus dominating the fermentation.

The transformation of grape juice in wine is a biochemical process, in which the enzymes play a fundamental role, being the major force catalysing the numerous biotransformation reactions which characterise the complex event of winemaking. These enzymes originate from the grapes, from the indigenous microflora present on the grapes and from the microorganisms present during winemaking. Pectinase, proteases and glycosidases are some of the enzymes secreted by yeasts that are of interest in winemaking for their technological effects and their contribution to aroma formation. Pectinolytic enzymes cleave long pectin chains into shorter, more soluble chain segments that facilitate pressing of the grapes, contribute to the clarification of the musts, may increase extraction of the substances that contribute to colour and aroma and may enhance filtration of the wines. Enzymatic hydrolysis of the proteins into smaller, more soluble nitrogen-containing molecules (peptides and amino acids) facilitates the clarification and stabilisation of the musts and wines, also preventing incomplete fermentations due to a deficiency of assimilable nitrogen in the must. These enzymes also play a major role during the autolysis process in wines kept on yeast lees during ageing.

The aroma and flavour properties of the wine can be enhanced by glycosidases, which hydrolyse nonvolatile glycosidic precursors of the grapes. It must be underlined that yeasts involved in winemaking can be important producers of numerous enzymes (Esteve-Zarzoso et al. 1998). *S. cerevisiae*, the principal wine yeast, is not recognised as a significant producer of extracellular proteases, lipases or proteolytic enzymes, although a few strains have been reported to degrade polygalacturonate (McKay 1990; Gainvors et al. 1994). Various authors have reported glycosidase production by this species (Delcroix et al. 1994). Conversely, non-*Saccharomyces* yeasts, such as species of *Candida*, *Debaryomyces*, *Hanseniaspora* (*Kloeckera*), *Hansenula*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*, have been described as potential sources for the commercial production of enzymes, such as proteases, esterases, pectinases, lipases and glycosidases (Rosi et al. 1994; Saha and Bothast 1996; Charoenchai et al. 1997). Regarding wine production, it is now recognised that non-*Saccharomyces* wine species, occurring in the must during the early stages of vinification, contribute to the enzymatic reactions (Heard and Fleet 1986).

Proteolytic activity has been observed in strains of *C. pulcherrima*, *K. apiculata*, *P. anomala* and *P. membranifaciens* (Fernández et al. 2000).

The pectin esterase and polygalacturonase activities increase during grape ripening and are produced by non-*Saccharomyces* yeasts present in must. Fernández et al. (2000) analysed 182 non-*Saccharomyces* yeasts isolated from grape must and found that polygalacturonase was the enzyme most commonly found and was secreted by 45% of the yeasts analysed (*M. pulcherrima*, *B. clausenii*, *P. membranifaciens*, *K. thermotolerans*, *P. anomala*, *C. stellata*). Blanco et al. (1994) reported that at least 75% of oenological strains tested in their study possessed limited proteolytic activity. These results suggest that wine yeasts exert little influence on pectin composition of must/wine.

Difficulties in wine clarification and filtration can also arise from the presence of high molecular weight β-glucans produced by *Botrytis cinerea* in infected grapes. The action of glucanase enzymes can solve these problems. The presence of β-(1,3)-D-glucanases has been reported in many yeast species (Fleet 1991). These enzymes exert endo- and exo-activities and are constitutive glycoproteins. *S. cerevisiae* excretes several β-(1,3)-glucanases and endo-β-(1,3)-glucanases activity has been determined in dried yeasts used in winemaking (Canal-Llaubéres 1988).

Among glycosidase enzymes involved in flavour-releasing processes, β-D-glucosidase has been widely studied because of its widespread occurrence in plants and also in yeasts (Esteve-Zarzoso et al. 1998), such as in *H. vineae* (Vasserot et al. 1989) and in *Candida* species (Günata et al. 1990). A study conduced on 317 strains belonging to 20 wine yeast species indicated that yeast species of *Candida*, *Debaryomyces*, *Hanseniaspora* (*Kloeckera*), *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomycodes*, *Schizosaccharomyces* and *Zygosaccharomyces* genera possess β-glucosidase activities (Rosi et al. 1994). Saha and Bothast (1996) in a screening of 48 yeast strains of the genera *Candida*, *Kluyveromyces*, *Debaryomyces* and *Pichia* for the production of extracellular glucose-tolerant β-glucosidase activity found that all yeast strains tested produced extracellular β-glucosidase activity, but enzymes from only 15 yeasts showed very high glucose tolerance.

More recently β-D-xylosidase has become of interest in oenology because it is a component of the enzyme complex that degrades the xylan, the major hemicellulolytic component of plant cell walls, but there is a lack of information on β-D-xylosidase activity in wine yeasts. Manzanares et al. (1999) evaluated the β-xylosidase activity in 54 yeast strains belonging to the genera *Candida*, *Dekkera*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces* and *Zygosaccharomyces*, mainly isolated from grapes and wines. The β-xylosidase activity was only detected in eight yeast strains belonging to *H. osmophila*, *H. uvarum* and *P. anomala* species. With respect to the location of the enzyme activity, *P. anomala* strains exhibited extracellular, cell-wall-bound and intracellular β-xylosidase activities. *H. uvarum* strains showed the lowest level of β-xylosidase production, the majority of which was cell-wall-bound. High intracellular β-xylosidase activity was found in *H. osmophila* strains and no extracellular activity could be detected. Furthermore, β-xylosidase preparations from *P. anomala* and *H. uvarum* strains maintained their activities at pH and temperature values and at concentrations of glucose and ethanol typically found during winemaking processes, thus potentially allowing their application for the improvement of the aroma and flavour properties of wine. Recently, Capece et al. (2005)

by analysing numerous *H. uvarum* wine strains found several strains exhibiting β-glucosidase and β-xylosidase activities at a significant level.

Another fundamental parameter which differentiates the wines is the final bouquet, characteristic of each vine cultivar and produced by fermentation and transformation of the aroma during the ageing. The wine aroma appears mainly during yeast fermentation and among the more than 1,000 volatile compounds identified, more than 400 are produced by yeast.

Thus, the various yeast species/strains that develop during the fermentative process metabolise grape juice components to a wide range of volatile and non-volatile end-products that contribute to the aroma and flavour characteristics of the wine. The differences in the composition of the wines resulting from the different yeasts appear to be largely quantitative rather than qualitative: the fermentative products are usually identical, but the relative amounts are different. Therefore, conversion of grape sugars to alcohol and other end-products by a specific yeast population may yield wines with distinct organoleptic quality and the yeast species represents a prominent factor in determining the content of some by-products in wine. Strong polymorphism has been observed within the different wine species, with highly variable by-product formation, resulting as a species-specific pattern. Then, within each species, the level on the by-products is often an individual strain characteristic.

The growth of non-*Saccharomyces* species could be considered quantitatively significant and may produce secondary compounds affecting the analytical composition and quality of the wine. Thus, their potential to contribute to the fermentation of inoculated wines should not be underestimated. Zohre and Erten (2002) analysed the behaviour of *K. apiculata*, *C. pulcherrima* and *S. cerevisiae* with pure and mixed cultures in grape juice. The non-*Saccharomyces* yeasts in pure cultures exhibited very slow rates of utilisation of total sugars without completeness of fermentation in comparison with the performance obtained in mixed fermentation with *S. cerevisiae*. On the other hand, stuck or sluggish fermentations are reported in the literature in cases where *K. apiculata* and other non-*Saccharomyces* yeasts dominated in grape juice. The role of non-*Saccharomyces* yeasts in determining the organoleptic properties of the final product is strictly dependent on the strain and on the extent of its growth. Strains belonging to the species *K. apiculata* and *H. guilliermondii* have been extensively studied in relation to the formation of some fermentation products of oenological interest (such as glycerol, acetic acid, higher alcohols, acetaldehyde, acetoin and ethyl acetate), demonstrating that the amounts of these secondary compounds are strain-dependent (Ciani and Maccarelli 1998; Romano 2002).

Ethanol is the main volatile product of yeast metabolism, followed by diols, higher alcohols and esters. Ethanol determines the viscosity of the wine and acts as a fixer of aroma. Among non-*Saccharomyces* species, good fermentation power is exhibited by *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii* that could achieve 14–15% v/v of ethanol. *T. delbrueckii* shows a large variability of ethanol production. However, some strains are capable of producing considerable amounts of ethanol (13% v/v). A low fermentation power is characteristic of *C. stellata* and of apiculate wine yeasts (*Hanseniaspora*/*Kloeckera*).

Despite the low quantity of organic acids in the wine, they are sufficiently volatile to contribute to its aroma. The most important is acetic acid, whereas propanoic, butanoic and lactic acids are usually below the perception threshold. Acetic acid represents more than 90% of the volatile acid of wine (Henschke and Jiranek 1993; Radler 1993) and it is one of the most important by-products that negatively affect the analytical profile of the wine. In fact, by law its concentration limit in wine may not be higher than 1.0–1.5 g/l, depending on the country. Acetic acid becomes objectionable near its flavour threshold of 0.7–1.1 g/l and values between 0.2 and 0.7 g/l are considered optimal (Dubois 1994). Apiculate strains are well known to produce high concentrations of acetic acid and for this they have been considered for a long time as spoilage yeasts. The wine species of *Hanseniaspora*/*Kloeckera* usually produce acetic acid at elevated levels, in the range 0.7–3 g/l and above. In synthetic medium, strains of *K. apiculata* and *H. guilliermondii* exhibit great variability in acetic acid production, with strains producing amounts of this compound of less than 1 g/l (Romano et al. 1992). Strains of *K. apiculata* forming low amounts of acetic acid in fermentation of different grape musts have been described (Comi et al. 2001; Capece et al. 2005). As regards *S. cerevisiae* strains, acetic acid formation is affected by sugar concentration, pH, nitrogen and the high/low production varies depending on the strain involved in the fermentative process. Romano et al. (2003a) found that the production of acetic acid was the main variable for the differentiation of 115 wild *S. cerevisiae* strains isolated from Aglianico-Vulture wine (Southern Italy), with values ranging from 130 to 1,610 mg/l. Similar results were obtained by Paraggio and Fiore (2004), who analysed *S. cerevisiae* wild strains of different vine cultivar origin, and found a certain correlation between acetic acid production and strain origin. In particular, in both pieces of research *S. cerevisiae* strains isolated from Aglianico-Vulture were characterised by a high level of acetic acid production.

Two of the most important fermentation by-products affecting the "body" of the wine are succinic acid and glycerol. Succinic acid is the main acid produced by yeasts and its formation is strain-dependent. The non-*Saccharomyces* yeasts are usually higher producers of this acid than *S. cerevisiae*. High amounts of succinic acid are generally produced by *C. stellata* (Ciani 1997). Glycerol is quantitatively a very important wine constituent and contributes significantly to the sweetness and the body and fullness of the wine.

Another important, but not always desirable, secondary compound of wine fermentation is acetaldehyde, accounting for 90% of total aldehydes. In consequence of their low sensory threshold values, aldehydes are important to the aroma of the wine.

Acetaldehyde is the product of the decarboxylation of pyruvate during alcoholic fermentation. This compound plays an important role in the free/combined sulphur dioxide balance and its amount in wine is variable, ranging from 10 to 300 mg/l, depending on grape variety and yeasts involved in fermentation. Wines containing amounts of 500 mg/l are considered unmarketable. In fact, the high concentration of acetaldehyde may be sufficient to cause an undesirable oxidised taste in wines, especially in white wine, whereas in red wines it should be present in amounts up to 100 mg/l. The total aldehyde content varies with the type of yeast species/strain involved in the fermentative process. Some authors (Fleet and Heard 1993) reported that *S. cerevisiae* strains produce relatively high levels. The general behaviour recognised as

commoner in *H. uvarum*/*K. apiculata* strains is the production of acetaldehyde in amounts comparable to those in the case of *S. cerevisiae* (Ciani and Maccarelli 1998), with strains forming about 90 mg/l and strains producing about 200 mg/l. Romano et al. (1994) divided 86 *S. cerevisiae* strains into groups producing low, medium and high amounts of this compound. The low and high phenotypes also differed considerably in the production of other secondary compounds, such as acetic acid, acetoin and higher alcohols. The wide variability recorded among *H. uvarum*/*K. apiculata* strains (Romano 2002) leads to consider this by-product as having individual strain characteristics, as reported for *S. cerevisiae* strains (Romano et al. 2003a). Other data (Fleet and Heard 1993) indicate that non-*Saccharomyces* species, such as *K. apiculata*, *C. krusei*, *C. stellata*, *H. anomala* and *M. pulcherrima*, produce low levels (from undetectable amounts to 40 mg/l) of acetaldehyde. Among non-*Saccharomyces* species evaluated by Ciani (1997), only *T. delbrueckii* is a lower producer of acetaldehyde than *S. cerevisiae*, whereas other species (*C. stellata*, *S'codes ludwigii*, *H. uvarum*, *K. apiculata*) produce consistent amounts of this compound. Granchi et al. (2002) found that *H. osmophila* and *K. corticis* isolates produce this compound at significant concentrations, the anamorph form showing a greater variability among the isolates (from 40 to 100 mg/l).

Higher alcohols represent another important group of secondary products influencing the analytical profile of the wine. They are produced by the Ehrlich pathway in the presence of amino acids and from sugars via biosynthesis by yeasts during alcoholic fermentations. Isoamyl alcohol, active amyl alcohol, isobutyl alcohol and *n*-propanol are the principal higher alcohols. Small or enhanced amounts of these compounds contribute positively to wine quality, giving a certain complexity to wine bouquet, while high levels (above 500 mg/l) influence negatively wine aroma. Their overall presence in wine covers a wide range: from a concentration slightly lower than 100 mg/l to a concentration higher than 500 mg/l (Lambrechts and Pretorius 2000). These compounds are also important precursors for ester formation, and the esters of higher alcohols are associated with pleasant aroma. Higher alcohols are usually present at levels below their detection thresholds, but the presence of numerous wild yeasts may increase the concentration of these compounds until undesirable levels. Higher alcohol production appears to be a general characteristic of yeasts, although the amounts produced depend on cultural conditions, and also on yeast genus, species and strain. Amyl alcohols and isobutyl alcohol have been considered the most significant; the predominant one in commercial fermentations is isoamyl alcohol with a range of 50–300 mg/l. The production of higher alcohols by non-*Saccharomyces* yeasts is generally lower than for *S. cerevisiae*. Apiculate strains show a general pattern of low higher alcohol producers (Herraiz et al. 1990; Gil et al. 1996), even when a considerable variability was recorded among strains tested in different grape must (Comi et al. 2001). In particular, a great variability was found for active amyl alcohol (ranging from 6 to 110 mg/l) and isoamyl alcohol (from 13 to 132 mg/l). Tested in different grape musts, apiculate strains exhibit uniform behaviour for higher alcohol production, resulting generally in low producers. Despite the strain variability, the total amount of higher alcohols produced by apiculate yeasts is always at an acceptable level, thus representing a positive trait of this species, but not a selective strain parameter. Non-*Saccharomyces* yeasts of the early

fermentation phase produce low levels of active amyl alcohol, isobutyl alcohol and *n*-propanol, which are much below that of their flavour thresholds reported in the literature (Zohre and Erten 2002). Only isoamyl alcohol is produced at concentrations which could contribute to the sensory properties of wines.

Other compounds contributing to wine aroma are acetate esters, which impart a mostly pleasant smell. In fact, the fresh, fruit aroma of young wines derives in large part from the presence of the mixture of esters produced during fermentation. They are mainly produced by yeasts during alcoholic fermentation in a reaction between alcohols and acetylcoenzyme A (acetyl-CoA). Ethanol is the main alcohol in wine; therefore, ethyl acetate produced from ethanol and acetyl-CoA is the major ester formed by yeasts. This ester is always present in wines with concentrations below its high threshold taste level of 150 mg/l. At low level (less than 50 mg/l) it may be pleasant and adds to general fragrance complexity (called "fruit esters"), while at levels above 150 mg/l it yields a sour-vinegar off-flavour and can flaw the fragrance of wine. All apiculate yeasts form high amounts of ethyl acetate: Ciani and Picciotti (1995) reported that a strain of *H. uvarum* was able to produce about 380 mg/l, while Zohre and Erten (2002) found *K. apiculata* strains producing 580 mg/l of ethyl acetate. Also *C. pulcherrima* (anamorph of *M. pulcherrima*) strains produce large amounts of this ester (676 mg/l), whereas a lower amount (about 30 mg/l) was produced by the main wine yeast *S. cerevisiae* (Zohre and Erten 2002). In *K. corticis* and *H. osmophila* (Granchi et al. 2002) the average values found were always below the threshold taste level and were lower than the concentrations found with *K. apiculata*/*H. uvarum* strains.

Another secondary compound involved in the bouquet of wine is acetoin, which is present in amounts ranging from 2 to 32 mg/l. In contrast to *S. cerevisiae* wine strains, non-*Saccharomyces* species are high producers of acetoin (Romano et al. 1993, 2003b; Romano and Suzzi 1996), and only *T. delbrueckii* exhibits a low production of acetoin (Ciani 1997). A biometric study on acetoin production in *S. cerevisiae* (Romano et al. 1993) showed that low acetoin production is the dominant pattern of this species. *Hanseniaspora*/*Koeckera* species produce acetoin at concentrations higher than its threshold value (150 mg/l) (Ciani and Maccarelli 1998; Romano et al. 1998; Comi et al. 2001; Granchi et al. 2002). It is ascertained that apiculate yeasts produce high amounts of acetoin that, by means of acetoin reductase, is reduced by *Saccharomyces* wine yeasts to 2,3-butanediol, the second most abundant constituent of wine. High and low production levels of acetoin and 2,3-butenediol are exhibited constantly by each wine species with an inverse pattern (Romano et al. 2003c). Thus, strains of *H. uvarum*/*K. apiculata* always produce low amounts of 2,3-butanediol (from about 50 to 220 mg/l) (Romano et al. 1998) and high levels of acetoin, from about 50 to 250 mg/l. In particular, isolates of *K. corticis* were found as producers of traces of 2,3-butanediol (maximum amounts of about 0.1 g/l) (Granchi et al. 2002).

Phenolic substances can be very important to the taste, colour and odour of wines. Vinylphenols (4-vinylguaiacol, 4-vinylphenol) in white wines and ethylphenols (4-ethylguaiacol, 4-ethylphenol) in red wines are quantitatively the most significant volatile phenols, identified as classic components of wine aroma (Chatonnet et al. 1997). They are present at concentrations from 0 to 6,047 μg/l in wines and can

yield phenolic off-odours, often described as animal, stable, horse sweat, medical, and "elastoplast" when present above their threshold values. The phenolic off-flavours of red wines most often develop during ageing and especially in wines stored in old barrels. *Brettanomyes*/*Dekkera* species are reported to be involved in wine spoilage by production of these off-flavours (Egli and Henick-Kling 2001). In wine, these yeast species grow typically in low cell numbers, whereas after completion of the alcoholic and malolactic fermentation during the aging of wine in barrels, tanks and bottles, they can grow easily on traces of residual sugars. Only careful hygiene and proper sulphuring of wines and containers can prevent the development of these undesirable yeasts.

Other compounds related to yeast activity are sulphur compounds, which can make a significant contribution to wine flavour in consequence of their reactivity and extremely low threshold values (far below 0.002 ppb). The production of hydrogen sulphide by yeasts has been studied in detail, since its aroma is frequently detected during fermentation. This substance has an unpleasant aroma with a low sensory threshold (10–100 μg/l); amounts above these values cause an off-flavour similar to that of rotten eggs and high amounts of hydrogen sulphide can also lead to the formation of other undesirable volatile sulphur compounds. The production of this compound varies with the strain of *S. cerevisiae*, with some strains producing amounts exceeding 1 mg/l. Rauhut et al. (1996) tested the ability of several commercial wine yeasts to produce volatile S compounds, demonstrating that *S. cerevisiae* strains differ in their capacity to synthesise S compounds. In particular, yeasts are able to form sulphite during wine fermentation so it is very unlikely that SO_2-free wine could be produced. The formation of SO_2 by *S. cerevisiae* is a strain characteristic; in fact this species produces sulphite in the range 10–30 mg/l (Romano and Suzzi 1993). Some strains, called "SO_2-forming yeasts", produce sulphite in amounts exceeding 100 mg/l. Such sulphite, defined "biological sulphite", is of enological interest because it binds to acetaldehyde and other compounds, contributing to unacceptably high levels of SO_2 in wines. In fact, the selection programmes of wine strains consider this aspect and starter cultures are normally tested for their capability to produce SO_2. However, some of the high sulphite producing strains possess a rarely encountered character, defined "stabilising power", which affects the chemical and biochemical stability of the wine in a similar way to the addition of SO_2 to the must, but the precise mechanism has not yet been completely explained.

As a consequence of the wide and extensive reports, wine quality appears to be the direct consequence of the typical yeast microflora which develops during the fermentation. The synergistic interaction among different yeast strains and their effect on wine sensory properties remain to be fully investigated: yeast combinations of different *S. cerevisiae* strains, and possibly *S. cerevisiae* with selected non-*Saccharomyces* strains, might be used to enhance the profile to produce unique-flavour wines. With the important contribution of non-*Saccharomyces* yeasts now fully realised, interest should also be focused on this group, which contains a wide variety of yeasts that have been shown to produce a diverse array of extracellular enzymes compared with *S. cerevisiae*. Actually, there is growing demand to differentiate, among the fermentative yeast flora, autochthonous strains with typical oenological traits which could be considered representative of a particular oenological region. These strains are

better adapted to the different wine-producing regions of the world with their respective grape varietals, viticultural practices and winemaking techniques. As the importance of *S. cerevisiae* in winemaking is long established, the use of commercial strains of yeast cultures in fermentation is becoming one of the commonest practices in order to ensure a reproducible product and to reduce the risk of wine spoilage. However, this practice can determine a progressive substitution of local microflora and a consequent reduction or lack of some desirable and typical organoleptic characteristics of natural or spontaneous alcoholic fermentation.

2.6 Yeasts in Brewing

Two types of *Saccharomyces* yeasts are involved in beer fermentation: ale yeasts (also known as top-fermenting yeasts) and lager yeasts (also known as bottom-fermenting yeasts) (Hammond 1993). Traditionally ale and lager yeasts are differentiated by their ability to ferment melibiose as lager yeasts, in contrast to ale yeasts, produce the extracellular enzyme melibiase (α-galactosidase) and therefore are able to ferment melibiose (Stewart et al. 1984). Further, ale yeasts are able to grow at 37°C, while this is not the case for lager yeasts, which in contrast grow better at lower temperature than ale yeasts. Ale yeasts have, since the last century, been classified as *S. cerevisiae*, whereas lager yeasts have been known under a variety of names such as *S. carlsbergensis*, *S. uvarum* and *S. cerevisiae*. Both yeast species belong to the closely related *Saccharomyces sensu stricto* species all having relatively uniform karyotypes consisting of 16 chromosomes (Hansen and Piskur 2003). Even though closely related, the development of molecular typing techniques has revealed several genetic differences between ale and lager brewing yeasts (Jespersen et al. 2000; Tornai-Lehoczki and Dlauchy 2000) and according to recent classifications, lager yeasts are now considered to belong to *S. pastorianus* (Vaughan-Martini and Martini 1998) even though they are often still referred to as *S. carlsbergensis* (Børsting et al. 1997). It appears to be generally accepted that lager yeasts are allopolyploid and contain parts of two divergent genomes (Kielland-Brandt et al. 1995; Casaregola et al. 2001), one from *S. cerevisiae* and one from another *Saccharomyces* species, most likely *S. bayanus* (Tamai et al. 1998, 2000; Kodama et al. 2001) or a specific strain of *S. monacensis* (Børsting et al. 1997; Joubert et al. 2000), which according to recent taxonomic keys now also belongs to *S. pastorianus* (Vaughan-Martini and Martini 1998). The hybridisation theory is supported by the fact that analysis of individual genes in many lager yeast strains reveals at least two copies of each gene, one closely related to the equivalent gene in *S. cerevisiae* and one that shows a higher degree of divergence. Also a large segment of *S. cerevisiae* DNA on chromosome XVI failed to hybridise to genomic DNA from different lager yeast strains, suggesting that this region may have diverged significantly or that it is absent in the lager yeast strains.

The brewing industry has a long tradition of the use of single starter cultures of brewing yeast based on single cell cultures. Worldwide, up to 1,000 different brewing yeast cultures have been described. The brewing yeast strains vary in their technological properties, including aroma production, rate and degree of attenuation, flocculation, oxygen requirement and reproduction (Hammond 1993; Dufour et al. 2003). As fermentation of carbohydrates in the wort leading to the formation of aroma

components and ethanol is the key event of brewing, the rate and extent of attenuation and the formation of aroma components are in focus when selecting the right strain of brewing yeast. The spectrum of carbohydrates in wort normally comprises sucrose, glucose, fructose, maltose and maltotriose together with some dextrins (D'Amore et al. 1989). However, the use of adjuncts is widespread and will significantly influence the carbohydrate composition of the wort. During brewing fermentation maltose is the most dominant carbon source. Brewing yeast strains have been shown to vary in their ability to utilise maltose and genotypic variations in their number of maltose transporter genes have been reported (Jespersen et al. 1999). The primary yeast metabolites are ethanol and CO_2, which both have an inhibitory effect on the yeast. Secondary metabolites serve mainly as flavour components. Among the most important groups of secondary metabolites are esters, fusel alcohols, aldehydes (acetaldehyde), organic acids, fatty acids as well as vicinal diketones (diacetyl and 2,3-pentanedione) and some sulphur components (hydrogen sulphide and sulphur dioxide). Different strains of brewing yeasts vary significantly in their formation of aroma compounds especially in the formation of esters (Verstrepen et al. 2003).

Flocculation is a cell-wall-mediated phenomenon characteristic of late exponential or stationary phase cells. Interactions between protein, phosphomannan and calcium seem to be involved in the cross-link of the yeast cells (Speers et al. 1992a, b; Hammond 1993). Besides being linked to the genotype of the yeast strain and the surface properties of the yeast cells, flocculation is influenced by fermentation conditions such as temperature and carbon availability. Flocculation is of technical importance for the brewing process as it permits the separation of the yeast from the beer and allows the reuse of the cropped yeast. The yeast is cropped at certain intervals at the later stages of the fermentation and will often be in a poor physiological condition owing to exposure to high levels of ethanol and various other toxic metabolites as well as physical stress. Owing to the anaerobic conditions the yeast will suffer from depletion of sterols and unsaturated fatty acids. Replacement of the yeast culture after a number of generations, often eight to ten, is considered as good practice in order to avoid contaminations and too high a proportion of dead or damaged cells.

Even though *Saccharomyces* yeasts have been shown to be able to utilise sterols and unsaturated fatty acids from the surrounding media (Ness et al. 1998), the brewing wort does not contain sufficient amounts of especially sterols and therefore the presence of free oxygen is required in order to ensure satisfactory yeast proliferation. The amount of oxygen required is dependent on several factors such as wort composition, wort gravity, pitching rate, yeast handling, the physiological condition of the pitching yeast culture including the intracellular pool of sterol esters and finally the brewing strain used. Different strains of lager brewing yeasts have, on the basis of determination of the rate of attenuation at different wort oxygenation levels, been shown to vary in their oxygen requirement (Jakobsen and Thorne 1980). The reason for the variation in oxygen requirement between different strains of brewing lager yeast appears not to be known despite the fact that the oxygen requirement is of great importance for the brewing industry. Also the ability of the yeast strain to cope with hypoxic stress conditions appears to be important during industrial fermentations (Higgins et al. 2003).

Yeasts other than *Saccharomyces* spp. may be involved in the brewing of some special brands of beer such as the Belgian Lambic and Gueuze beers. These brands of beer are mostly fermented spontaneously and include both enterobacteria, *Pediococcus* spp. and yeasts (Dufour et al. 2003; Hansen and Piskur 2003). At the later stages of fermentation yeasts such as *Dekkera* spp. (perfect form of *Brettanomyces* spp.) are involved.

Yeasts occurring as contaminants are in the context of brewing defined as "wild yeasts" and in practice they are distinguished from cultures of brewing yeasts by their ability to grow on a number of well-defined selective substrates (Jespersen and Jakobsen 1996; van der Aa Kühle and Jespersen 1998). Brewing contaminants are traditionally divided into *Saccharomyces* and non-*Saccharomyces* yeasts (Jespersen and Jakobsen 1996), of which *Saccharomyces* spp. in general are considered to be the most hazardous (van der Aa Kühle and Jespersen 1998). The majority of the *Saccharomyces* brewing contaminants detected belong to *S. cerevisiae* but other *Saccharomyces* spp. have also been reported (Jespersen et al. 2000). Infections with these yeasts typically cause phenolic off-flavours and superattenuation of the final beer. The production of phenolic off-flavours is due to the ability of these wild yeasts to decarboxylate different phenolic acids such as ferulic acids and *trans*-cinnamic acids, resulting in the formation of 4-vinylguaiacol (Coghe et al. 2004). Superattenuation is due to the production and secretion of glycoamylases with starch-debranching activity which enable the wild yeasts to use dextrins normally not fermented by the culture yeast (Röcken and Schulte 1986). Infections by *Saccharomyces* wild yeasts can be very difficult to detect owing to their physiological and biochemical similarities with the culture yeast. However different differential techniques have been developed (Jespersen et al. 1993; van der Aa Kühle and Jespersen 1998). Once isolated, the *Saccharomyces* wild yeasts can often be distinguished from lager yeasts by cell morphology and spore formation as *S. cerevisiae* normally rather easily forms spores on sporulation media, which is generally not the case for lager yeasts (Ingledew and Casey 1982). The most important non-*Saccharomyces* wild yeasts are *P. membranifaciens* and *P. anomala* (perfect name of *C. pelliculosa* and formerly known as *H. anomala*) as well as a number of species belonging to such different genera as *Brettanomyces*, *Candida*, *Debaryomyces*, *Filobasidium*, *Hanseniaspora*, *Kluyveromyces*, *Torulaspora* and *Zygosaccharomyces* (Campbell and Msongo 1991; Campbell 1996; van der Aa Kühle and Jespersen, 1998). The non-*Saccharomyces* wild yeasts cause various types of spoilage, e.g. *P. membranifaniciens* is known to produce film, haze and off-flavours such as phenolic, estery and acidic notes. According to Campbell and Msongo (1991) spoilage caused by wild yeasts belonging to the genera *Pichia*, *Hansenula* and *Debaryomyces* is commonly associated with aerobic conditions even though the yeast species to some extent are capable of anaerobic growth.

2.7 Yeasts in Other Alcoholic Beverages (Cider, Sherry Wine, Tequila)

Cider is an alcoholic beverage commonly consumed in numerous European countries, such as France, Spain, Ireland and Slovenia, where it is still produced by a natural

fermentation process involving the sequential development of indigenous yeast species. Wild microflora performing the alcoholic fermentation of apple must into cider typically originate from the fruits or from the surfaces of the process equipment. A great similarity was demonstrated between yeast populations involved in both wine and cider fermentation processes. Recently Morrissey et al. (2004) by using molecular techniques and differential media isolated, identified and tracked the yeast species involved in the overall process of a traditional Irish cider fermentation, finding as predominant yeast species *M. pulcherrima*, *P. anomala*, *B. anomalus*, *B. bruxellensis*, *D. polymorphus*, *H. uvarum*, *P. fermentas*, *P. guilliermondii*, *S'codes ludwigii* and *S. cerevisiae*. The Irish cider fermentation could be subdivided into three principal phases depending on the prevalent yeast species. *K. apiculata*/*H. uvarum* yeasts are the predominant species in the first phase, representing over 90% of the initial yeast count of 6.0×10^6 cfu/ml. As the alcohol level rises to above 4%, the numbers of *Hanseniaspora*/*Kloeckera* yeasts decrease and *Saccharomyces* yeasts begin to dominate, reaching at the fermentation peak (on day 5) 8.3×10^8 cfu/ml. Then a marked decrease occurs in the population, which drops to 5.0×10^6 cfu/ml by day 18.

Naumov et al. (2001) analysed genetic and molecular data of 21 strains, isolated from cider juice produced in France, and found that 18 out of the 21 cider strains belong to the variety *uvarum* of the species *S. bayanus*. The specific ecologic niche of *S. bayanus* var. *uvarum* in winemaking is at low temperatures and also cider production is controlled at low temperatures. It is relevant from a technological point of view that the production of pectinolytic enzymes has been reported as a specific character of *S. bayanus* var. *uvarum*.

The last phase of Irish cider fermentation, the so-called maturation phase, is dominated by *Dekkera*/*Brettanomyces* species, which begin to be detected after day 12, with their overall numbers increasing from 11% of the total yeast population to over 90% of the population by day 22. At the 25th day the fermented cider is racked and submitted to the maturation phase for up to 18 months. During this phase *Brettanomyces*/*Dekkera* are the only yeast species detected. The presence of these species has also been reported in French cider (Le Quere and Drilleau 1996). Owing to their prevalence, these species likely represent the principal contributors to the overall organoleptic properties of this alcoholic beverage. Regarding the sources of these species in these traditional cider-making process, for *Saccharomyces* species the main sources are the apples themselves, with high numbers (2×10^4–5×10^6 cfu/ml), and the process utensils, which have substantial yeast populations even some months after the last pressing. *Hanseniaspora*/*Kloeckera* species are the prevalent yeasts of the fresh must, so it is possible to conclude that the apple might be the principal source for this species, while *Brettanomyces* yeasts could be traced back to the press house and also to the fruit.

The production of sherry wine starts with an alcoholic fermentation of must by yeasts to produce white wine, followed by long ageing (5–12 years) in oak casks. Sherries comprise three different types of white wines, *fino*, *amontillado* and *oloroso*, depending on the different ageing procedures, giving wines with aroma compounds. *Fino* wines result from biological ageing, using the "solera system", under a velum produced by the so-called flor yeasts growing on the wine surface when the ethanol

content is lower than 15% v/v. The aerobic metabolism developed by these yeasts causes changes in the aroma fraction that endows the wine with its typical flavour. In addition, these yeasts protect against browning, allowing the wine to retain its pale colour for years. *Oloroso* wines are obtained by oxidative ageing, after the addition of ethanol up to a content of about 18% v/v, which prevents the growth of flor yeasts. Under these conditions, *oloroso* wine develops a dark colour as a result of the oxidation of phenolic compounds. *Amontillado* wines are obtained by ageing in a two-step process involving biological ageing under similar conditions to those of *fino* wines, followed by an increase in the ethanol content; after that they are subject to oxidative ageing, as for *oloroso* wine. *Amontillado* wines are thus the oldest and the most valued of the three types, in consequence of the development of a more complex flavour than the other two. Yeasts involved in velum formation during ageing have been included into four races formerly described as *S. beticus*, *S. cheresiensis*, *S. montuliensis* and *S. rouxii*. In a recent revision of the *Saccharomyces* genus (Esteve-Zarzoso et al. 2004) the first three yeasts were included in the species *S. cerevisiae*, whereas *S. rouxii* is now included in *Z. rouxii* species. The yeast ecology in sherry wine is affected by overproduction of acetaldehyde and the rate of film formation. A correlation between high acetaldehyde production and specific *S. cerevisiae* strains was found, such as a relationship between the number of days required to form a yeast film on the wine surface and the ability to dominate the flor yeast population. Although molecular techniques has been applied to study film-forming yeasts and for race characterisation (Martinez et al. 1995), the differentiation of four races of flor yeasts is still based on their ability to ferment different sugars. Analysis of the mtDNA restriction pattern (Martinez et al. 1995, Ibeas and Jimenez 1997) of flor yeasts yielded a high genetic variability, whereas the analysis of chromosomal profiles showed less polymorphism (Esteve-Zarzoso et al. 2004). Since ethanol is a powerful inducer of respiration-deficient mutant, the polymorphism found in mtDNA has been attributed to the mutagenic effects of ethanol upon the mitochondrial genome, followed by the selection of those mtDNA sequences which make the mitochondria metabolically active under these conditions.

Yeasts also play an important role in the production of alcoholic beverages typical of Mexico (tequila, mezcal, sotol, bacanora and raicilla), which are obtained from different agave juices. The first part of this process is the transformation by yeast fermentation of agave must in an alcoholic aromatic product, which then is distilled yielding each typical agave beverage. As regards tequila fermentation, a wide variety of yeasts are present at the beginning of the fermentation (Lachance 1995), such as in wine production. Among the fermenting yeasts, the most frequent isolates belong to the species *S. cerevisiae*, *K. africana*, *C. magnolia* and *C. krusei*.

A recent study (Fiore et al. 2005), conducted on *Saccharomyces* and non-*Saccharomyces* yeasts isolated from grape and agave musts, has revealed a correlation between strain technological aptitude and origin, explained as a specific adaptation to fermentation conditions, which probably determine different physiological and enological properties. Thus, the significant differences in β-glucosidase and β-xylosidase activities between *S. cerevisiae* agave and grape strains could indicate a certain specialisation to metabolise different cellulosic materials from grape juice and agave plant.

2.8 Yeasts in Indigenous Foods, Beverages and Cash Crops

Indigenous fermented foods and beverages play a major role in the diet of many people, especially in Africa, Asia and South America. The fermentations are predominantly conducted spontaneously without the use of starter cultures. For some products back-slopping may be used. The food processing normally takes place at household level or on a small industrial scale and the products are consequently often of varying quality and stability. Cereals, legumes and tuber roots are the major raw materials used for indigenous fermentation but milk is also fermented, e.g. in East Africa. Important cash crops such as cocoa and coffee are also produced by spontaneous fermentation. Especially in developing countries where the lack of appropriate storage facilities is a major problem, fermentation is a very effective way of food processing. Also the socioeconomic and cultural effects of the production of traditionally fermented foods and beverages from local crops should not be neglected. An overview of indigenous fermented foods from all over the world has been published previously (Steinkraus 1996).

A wide range of yeast species are involved in the fermentation of indigenous foods and beverages and as these products to a great extent are made by spontaneous fermentation consequently several different yeast species will be present especially at the initial phases of the fermentation. Also at strain level a pronounced biodiversity is found in these products. The yeast population found will primarily depend on the raw materials and processing conditions and also the occurrence of other microorganisms may influence the composition of the yeast population. *S. cerevisiae* is apparently the commonest yeast in indigenous fermented foods and beverages, where it has been shown to be very important especially in the fermentation of cereals and alcoholic beverages (Jespersen 2003). Even though it has not been described in detail, it may further play a role during fermentation of cocoa (Jespersen et al. 2005).

Examples on alcoholic beverages where *S. cerevisiae* plays a dominant role are *pito*, *dolo*, *burukutu* and *otika*, all different names for indigenous fermented beers made from guinea corn (*Sorghum vulgare*). The beers have a fairly thick consistency owing to a large amount of solids (5–7%) and the alcohol content is rather low (1–3% v/v). The beers are consumed in an actively fermenting state and therefore have quite a short shelf life. Owing to their low alcohol content and the large quantity of suspended solids, many consumers consider these indigenous fermented sorghum beers as much as a food as a beverage. For samples of dried yeasts harvested from previous brews and reused as inocula in the next beer fermentation, 99% of the isolates could be identified as *S. cerevisiae* (van der Aa Kühle et al. 2001). The dominance of *S. cerevisiae* in the fermentation of *pito* and other indigenous fermented sorghum beers seems to be a general observation even though the composition of the yeast population responsible for the fermentation may vary depending on regional area and local deviations in the production.

S. cerevisiae also plays a leading role in the fermentation of maize dough that forms the basis for a variety of different foods in Africa and South America where they in certain areas contribute to a large proportion of the daily food intake. From investigations on fermented maize dough used for production of *kenkey* in Ghana, West Africa, it is known that the yeast population on the raw maize, during steeping

and early phases of fermentation, consists of a mixed flora comprising *Candida* spp., *Saccharomyces* spp., *Trichosporon* spp., *Kluyveromyces* spp. and *Debaryomyces* spp. However, after 24–48 h of fermentation *S. cerevisiae* dominates with counts exceeding 10^6 cfu/g and after 72 h of fermentation *C. krusei* is the dominant yeast species (Jespersen et al. 1994). The microbial succession leading to the dominance of *C. krusei* at the advanced stage of fermentation is likely to be due to an increased tolerance of this yeast species against high levels of organic acids present at the later stages of fermentation (Halm et al. 2004).

A tremendous biodiversity at both intraspecies and interspecies level is observed during fermentation of cocoa and coffee. For cocoa production the seeds and pulp from the fruit pods of the tree *Theobroma cacao* Linné are fermented. The first step in cocoa fermentation is the spontaneous fermentation of the cocoa beans including the surrounding pulp. The cocoa beans are either fermented in heaps, boxes, baskets or trays. The methods of fermentation vary considerably from country to country and even adjacent farms may differ in their processing practices, which might influence the composition of the yeast population (Jespersen et al. 2005). During fermentation, microbial activity leads to the formation of a range of metabolic end-products such as alcohols, acetic acid and other organic acids, which diffuse into the beans and cause their death. This induces biochemical transformations within the beans that lead to formation of precursors of the characteristic aroma, flavour and colour, which are further developed during drying and are finally obtained during roasting and further processing (Schwan and Wheals 2004). A recent study (Jespersen et al. 2005) has shown that *C. krusei* is the dominant species during heap fermentation followed by *P. membranifaciens*, *P. kluyveri*, *H. guilliermondii* and *Trichosporon asahii*, whereas *S. cerevisiae* and *P. membranifaciens* are the dominant species during tray fermentation, followed by low numbers of *C. krusei*, *P. kluyveri*, *H. guilliermondii* and some yeast species of minor importance. Isolates of *C. krusei*, *P. membranifaciens*, *H. guilliermondii*, *T. asahii* and *Rh. glutinis* could be found on the surface of the cocoa pods and in some cases on the production equipment, whereas the origin of *S. cerevisiae* isolates was not indicated by the results obtained. For the predominant yeast species determination of chromosome length polymorphism (CLP) by PFGE showed a pronounced biodiversity involving several different strains within each species. During fermentation of coffee *P. kluyveri*, *P. anomala* and *H. uvarum* have been shown to be the dominant yeast species during "wet" processing, which is the method used for removal of the pulp, mucilage, parchment and silver skin covering *arabica* coffee beans (Masoud et al. 2004).

As mentioned previously, biodiversity at the strain level is often observed in spontaneously fermented products. Especially the biodiversity of *S. cerevisiae* strains has been investigated intensively. For strains isolated from fermented maize dough several different chromosome profiles can be observed as well as differences in their assimilation profiles (Hayford and Jespersen 1999; Naumova et al. 2003). The diversity has further been confirmed by PCR amplification using primers against the 5′ termini of the delta elements flanking the Ty1 retrotransposon. Strains that cannot be separated by the PFGE methods can sometime be separated by the PCR method and vice versa (Hayford and Jespersen 1999). For strains from fermented sorghum beer produced in the northern part of Ghana and Burkina Faso the majority of the isolated

S. cerevisiae strains (52.5%) were only able to assimilate glucose and maltose (van der Aa Kühle et al. 2001) and this is thereby not consistent with the accepted description of the species *S. cerevisiae* by Vaughan-Martini and Martini (1998) but is in accordance with the description given by Barnett et al. (2000). By sequence analysis of the D1/D2 domain of the large subunit (26S) ribosomal DNA a deviation from the type strain of *S. cerevisiae* (CBS 1171) of three nucleotides equivalent to 0.5% of the DNA was found, which according to Kurtzman and Robnett (1998) is typical of conspecific strains. Also several physiological variants of *S. cerevisiae* have been found to be involved in the fermentation of palm wine (Owuama and Saunders 1990), during fermentation of *cachaca*, an alcoholic beverage produced from sugar-cane juice in Brazil (Guerra et al. 2001) and during fermentation of *aguardente*, another alcoholic beverage produced from sugar-cane juice in Brazil (Pataro et al. 2000). Also differences in multiple locus genes such as the *MAL* genes have been found between strains of *S. cerevisiae* isolated from cereal-based indigenous fermented foods and strains of *S. cerevisiae* used for industrial applications (Hayford and Jespersen 1999; van der Aa Kühle et al. 2001). In general, a low number of *MAL* genes are seen for the strains isolated from the indigenous products compared with the industrial strains, which can imply that the selection pressure for maltose utilisation has not been as pronounced for these strains as for industrial strains of *S. cerevisiae*. Also other differences are seen, including the fact that *MAL41* has never been observed for *S. cerevisiae* strains isolated from indigenous fermented foods and beverages, whereas *MAL41* is nearly always present in industrial strains of *S. cerevisiae* and *S. pastorianus* (Jespersen et al. 1999). Also for a significant number of isolates from indigenous fermented foods and beverages a yet undescribed *MAL* locus can be observed (Hayford and Jespersen 1999; van der Aa Kühle et al. 2001).

The functions yeasts might have in indigenous fermented foods, beverages and cash crops are several even though the topic has not been investigated in detail for many of these products. In general, yeasts are involved in the fermentation of carbohydrates and in the production of aroma compounds. However, depending on the raw materials and processing, yeasts might further be involved in stimulation of lactic acid bacteria, inhibition of mycotoxin-producing moulds, improvement of the nutritional value, degradation of cyanogenic glucosides, production of tissue-degrading enzymes and some strains might additionally have probiotic properties (Jespersen 2003).

The most intensively studied function of yeasts in the fermentation of foods and beverages is the conversion of carbohydrates into alcohols and other aroma components such as esters, organic acids and carbonyls. For spontaneously fermented maize dough a total of 76 aroma compounds have been identified by gas chromatography–mass spectrometry. The compounds included 21 carbonyls, 19 alcohols, 17 esters, 12 acids, a furan, five phenolic compounds, an alkene and one unidentified compound. In general, alcohols and esters are produced in much higher amounts than other aroma compounds when yeasts are present in high concentrations (Annan et al. 2003).

In indigenous fermentations yeasts often coexist with other microorganisms. Depending on the type of product a microbial succession involving both yeasts and other microorganisms will normally take place. Yeasts have been reported to stimulate the growth of other microorganisms, including lactic acid bacteria by providing essential metabolites such as pyruvate, amino acids and vitamins, and *S. cerevisiae*

has further been reported to utilise bacterial metabolites as carbon sources (Gadaga et al. 2001; Leroi and Pidoux 1993). However, the topic appears to be poorly investigated and the mechanisms appear not to have been described in detail. *S. cerevisiae* together with *C. krusei* have been reported to have an inhibitory effect on the growth of mycotoxin-producing moulds such as *Penicillium citrinum*, *Aspergillus flavus* and *Aspergillus parasiticus*. The inhibitory effects of the yeasts were mainly shown to be due to substrate competition but also inhibition of spore germination might occur owing to the production of high concentrations of organic acids (Halm and Olsen 1996). *S. cerevisiae* as well as several other yeast species have been reported to have pectinase activity that could be of importance for the substrate availability of other microorganisms and subsequent microbial degradation of complex molecules. The presence of pectinase activity in yeasts is especially of importance in the fermentation of coffee where yeasts such as *P. kluyveri* and *P. anomala* have been shown to be able to degrade the pectin in the mucilage layer surrounding the coffee bean (Masoud et al. 2004).

Especially for indigenous fermented products produced in developing areas, bioavailability of vitamins and other nutrients is very important. Even though it has been poorly investigated, yeasts might influence the nutritional value of the fermented products. For products fermented with *S. cerevisiae* and *Lb. plantarum* the crude protein content and the contents of riboflavin, thiamine, niacin and ascorbic acids were found to increase. The contents of some amino acids were improved, while those of others were reduced. The total contents of polyphenols, tannins and phytate were reduced by the fermentation. Also, increased physiochemical properties such as improved starch stability and improved gelatinisation were obtained by the fermentation (Onilude et al. 1999). The last mentioned properties are important in the production of adult foods but might be a drawback in the production of weaning foods. In clinical trials *Saccharomyces* yeasts have been reported to be effective in the treatment of acute infantile gastroenteritis and diarrhoea following treatment with antibiotics, and have been shown to inhibit infections with *C. albicans*, *Salmonella typhimurium* and *Shigella flexneri* as well as *Clostridium difficile* (Berg et al. 1993; McFarland et al. 1994; Ouwehand and Salminen 1998). Also, *Saccharomyces* yeasts have been shown to protect against cholera toxin probably by adhesion of the toxin to receptors on the yeast surface (Brandâo et al. 1998). Further, *Saccharomyces* yeasts have been shown to modulate the host immune response by stimulating sIgA production and the phagocytic system in mice and mammalian cells (Rodrigues et al. 2000) and strains of *S. cerevisiae* isolated from West African fermented maize dough have been shown to lower the expression of proinflammatory cytokines upon exposure to pathogenic bacteria such as toxin-producing *Escherichia coli* (van der Aa Kühle et al. 2005).

2.9 Collections of Food Yeast Cultures

The results of numerous research groups around the world and in the last few years have yielded the isolation, identification and characterisation of yeasts involved in food processing. These strains are often deposited in culture collections of universities or research centres and these collections exist on a variety of scales and with a variety of purposes, but are especially addressed to provide materials and services to scientists

Table 2.2 Principal food yeast collections of the world

Country	Yeast collections Code	Website
Armenia	RCDM	http://wdcm.nig.ac.jp/catalogue/rcdm/rcdm.html
Belgium	MUCL	http://www.belspo.be/bccm
Brazil	CCT	http://www.cct.org.br/
Canada	LCC	
China	CGMCC	http://www1.im.ac.cn/typecc/junzhong/en.html
	YM	http://www.ynst.net.cn/kyjg/kydw/dw15/index.html
Hungary	NCAIM	http://ncaim.kee.hu
Italy	DBVPG	http://www.agr.unipg.it/dbvpg/
Japan	IFO	http://www.ifo.or.jp/index_e.html
	JCM	http://www.jcm.riken.jp/JCM/catalogue.html
Netherlands	CBS	http://www.cbs.knaw.nl/index.htm
Portugal	PYCC	http://www.igc.gulbenkian.pt/
Russian Federation	VKM	http://www.vkm.ru/
Slovak Republic	CCY	http://www.chem.sk/activities/yeast/ccy/
Slovenia	ZIM	http://www.bf.uni-lj.si/zt/bioteh/chair/CIM.htm
Spain	CECT	http://www.uv.es/cect/
Taiwan	BCRC	http://www.bcrc.firdi.org.tw/bcrc/indexe.htm
UK	NCYC	http://www.ifr.bbsrc.ac.uk/ncyc/
USA	ATCC	http://www.atcc.org/
	NRRL	http://wdcm.nig.ac.jp/wdcm1999/a_kurtzman.html
	Herman J. Phaff	http://www.phaffcollection.org/home.asp

Data from WFCC-MIRCEN World Data Centre for Micro-Organisms and from other sources.

and to promote research around the world. Among the numerous yeast collections, we have selected some in different countries, comprising more than 1,000 yeast strains and characterised by the electronic database online (Table 2.2).

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Chapter 3

Molecular Methods to Identify and Characterize Yeasts in Foods and Beverages

M.T. Fernández-Espinar, P. Martorell, R. de Llanos and Amparo Querol

Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), P.O. Box 73, 46100 Burjassot, Valencia, Spain (e-mail: aquerol@iata.csic.es, Tel.: +34-96-3900022, Fax: +34-96-3636301)

3.1 Introduction

Studies aimed at identifying different yeast species, as well as the strains that belong to one species, have been based on morphological and physiological approaches (Kreger-van Rij 1984; Barnett et al. 1990). As an example, Table 3.1 shows the morphological and physiological characteristics of some of the main yeast species associated with foods and beverages as well as the morphological appearance of the cells of some of them (Fig. 3.1). These characteristics can vary according to growing conditions (Scheda and Yarrow 1966, 1968; Yamamoto et al. 1991) and sometimes the species are defined by a unique physiological characteristic that is controlled by a single gene. Therefore, depending on the physiological state of the yeast, as happens with the fermentation of galactose, which has traditionally enabled oenologists to differentiate the species *S. cerevisiae* and *S. bayanus* (Price et al. 1978; Kurtzman and Phaff 1987). More recently, methods have been developed to differentiate yeasts based on the analysis of total proteins in the cell (Van Vuuren and Van der Meer 1987; Vacanneyt et al. 1991), isoenzymic patterns (Duarte et al. 1999) and fatty acid analysis using gas chromatography (Cottrell et al. 1986; Tredoux et al. 1987; Moreira da Silva et al. 1994). However, the reproducibility of these techniques is somewhat questionable, as in many cases they depend of the physiological state of the yeasts (Golden et al. 1994). By contrast, techniques using molecular biology are seen as an alternative to traditional methods since they analyse the genome independently of the physiological state of the cell. Many techniques have been developed using the tools offered by molecular biology and many of them are useful to identify and characterize yeasts at a molecular level. We will go on to talk about those techniques that have had preferential use in the field of the food yeasts.

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
© Springer-Verlag Berlin Heidelberg 2006

Table 3.1 Morphological and physiological characteristics of some of the main yeast species associated with foods and beverages (according to Kurtzman and Fell 1998)

Morphology	Assimilation								Fermentation						
		Gal	Glc	Lac	Mal	Raph	Suc	Thre	Gal	Glc	Lac	Mal	Raph	Suc	Thre
Candida stellata	Globose to ovoidal	–	+	–	–	+	+	–	–	+	–	–	+/l	+	–
Debaryomyces hansenii	Spheroidal to short ovoidal	+	+	v	+	+	+	+	w/–	w/–	–	w/–	w/–	w/–	w/–
									–	–		–			
Dekkera bruxellensis	Ellipsoidal, oval and elongated	v	+	–	v	v	v	v	v	+	–	v	–	v	v
Hanseniaspora uvarum	Apiculate, lemon	–	+	–	–	–	–	–	–	+	–	–	–	–	–
Metschnikowia pulcherrima	Globose to ellipsoidal	+	+	–	+	–	+	+	w/–	+	–	–	–	–	–
									–						
Saccharomyces cerevisiae	Globose, ovoidal	v	+	–	+	+	+	+	v	+	–	v	+	+	–
Saccharomycodes ludwigii	Lemon, sausage	–	+	–	–	+	+	–	–	+	–	–	+	+	–
Yarrowia lypolitica	Spheroidal, ellipsoidal to elongated	v	+	–	–	–	–	–	–	–	–	–	–	–	–
Zygosaccharomyces bailii	Spheroidal to ellipsoid	v	+	–	–	–	v	+/w	–	+	–	–	–	v	w/–

+ positive, – negative, v variable, w/– weak or negative, +/w positive or weak, +/l positive or latent.

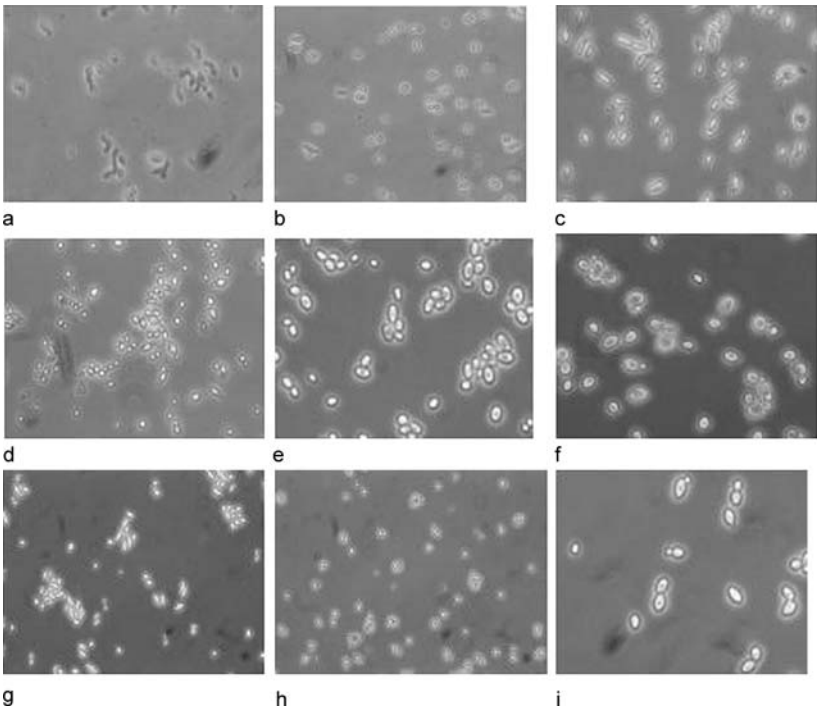

Fig. 3.1. Morphology of the main yeast species in foods and beverages: (**a**) *Candida stellata*, (**b**) *Debaryomyces hansenii*, (**c**) *Dekkera bruxellensis*, (**d**) *Hanseniaspora uvarum*, (**e**) *Metschnikowia pulcherrima*, (**f**) *Saccharomyces cerevisiae*, (**g**) *Saccharomycodes ludwigii*, (**h**) *Yarrowia lypolitica* and (**i**) *Zygosaccharomyces bailii*

3.2 Methods for Species Identification

3.2.1 Methods Based on the Analysis of Ribosomal Regions

The ribosomal genes (5.8S, 18S and 26S) are grouped in tandem forming transcription units that are repeated in the genome between 100 and 200 times (Fig. 3.2). In each transcription unit two other regions exist, the internal transcribed spacers (ITS) and the external ones (ETS), regions that are transcribed but are not processed. In turn, the codifying units are separated by the intergenic spacers, also called NTS. The gene 5S is not included in the previously described transcription unit but is found adjacent in the same repetition unit in tandem in the case of yeasts. The ribosomal genes 5.8S, 18S and 26S, as well as the ITS and NTS, represent powerful tools to establish the phylogenetic relationships and to identify species (Kurtzman and Robnett 1998), owing to the conserved sequences to be found there, as well as their concerted evolution, i.e. the similarity between repeated transcription units is greater within species than between units belonging to different species, owing to mechanisms like the unequal crossing over or genetic conversion (Li 1997).

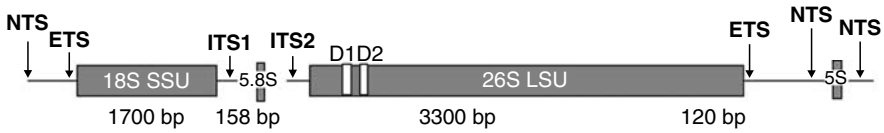

Fig. 3.2. Structure of nuclear ribosomal DNA

Different methods have been developed to identify yeast species using the information contained in these regions, as we will describe in the following sections.

3.2.1.1 Sequencing Ribosomal Regions

One of these methods is based on the determination and comparison of the nucleotide sequences in these regions. The two most commonly used regions are those corresponding to the domains D1 and D2 located at the 5′ end of gene 26S (Kurtzman and Robnett 1998) and the gene 18S (James et al. 1997). The availability of these sequences in databases, especially in the case of the D1/D2 region of gene 26S, makes this technique very useful to assign an unknown yeast to a specific species when the percentage of homology of its sequences is over or similar to 99% (Kurtzman and Robnett 1998). The database comparison is carried out using the program WU-BLAST2 at the internet address http://www.ebi.ac.uk/Blas2/index.html.

Moreover, the development of the DNA PCR, which enables direct sequencing of the regions of interest, together with modern technologies of automatic sequencing make this technology relatively quick to use. In this process, which is outlined in Fig. 3.3, the domain under consideration is amplified by PCR starting off with total DNA. The PCR products are purified using commercial kits to eliminate the primers and the excess of deoxynucleotides that would interfere in the sequencing reaction. In the automatic sequence systems, four fluorescent dyes are used to identify each of the bases (A, G, C and T). The dyes are incorporated by means of PCR amplification using the same primers. The DNA fragments marked in this way are separated in fine capillaries in terms of their size and are simultaneously excited by a laser, producing an emission that is different for each of the dyes. The signals generated are later transformed by software into peaks of colour, each of which corresponds to a nucleotide. The separation is quick and allows approximately 600 nucleotides to be read in 2 or 3 h, according to the sequencer model. Recent applications of the technique are shown in Table 3.2.

3.2.1.2 Restriction Analysis of Ribosomal Regions

With an industrial application in mind, other simpler identification methods were developed in parallel, based on PCR amplification of these regions of the ribosomal DNA and later restriction of the amplified fragment. The basis of the PCR technique is detailed in Sect. 3.3.3. Although it is usual to use DNA as a template in the amplification reaction, in a number of studies a small quantity of an isolated colony

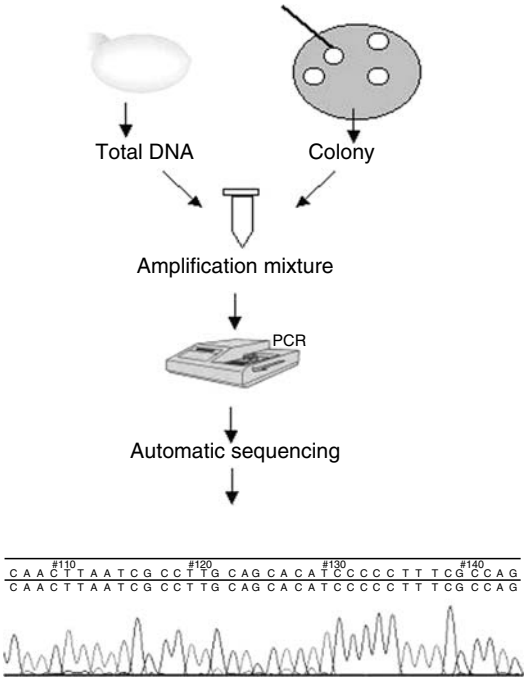

Fig. 3.3. Method for species identification based on PCR amplification and subsequent sequencing of ribosomal regions

has been used as the template. This approach represents a great saving in time and only needs a previous 15-min step at 95°C in the amplification protocol in order to liberate the DNA into the reaction mixture. The amplification products are visualized in agarose gels at 1.4%. The differently sized amplification products correspond to different species; however, when the amplified fragments are the same size they do not always correspond to the same species and it is necessary to resort to the digestion of these fragments to be able to identify them definitively. The digestion of the PCR products is carried out directly without needing a previous purification step and the generated fragments are separated by electrophoresis in agarose gels at 3% and their size is determined by comparison with appropriate markers. This technique, which is schematized in Fig. 3.4, is characterized by its easy execution and its reproducibility. Dlauchy et al. (1999) used this methodology to amplify the ribosomal gene 18S and the intergenic region ITS1 of 128 species mainly associated with foods, wine, beer and soft drinks using the primers ns1 (5′-GTA GTC ATA TGC TTG TCT C-3′) and its2 (5′-GCT GCG TTC TTC ATC GAT GC-3′) and digesting enzymes *Alu*I, *Hae*III, *Msp*I and *Rsa*I. Later, this methodology was used by Redzepovic et al. (2002). Another ribosomal region that is very useful to differentiate at species level is the one that includes the gene 5.8S and the adjacent intergenic regions ITS1 and ITS2, amplified using the primers its1 (5′-TCC GTA GGT GAA

Table 3.2 Molecular techniques most frequently used for identification of yeast species in foods and beverages and their application in the last 5 years

Technique	Target species	Matrix (food or beverage)	References (from 2000 to 2005)
Sequencing	*S. cerevisiae*	Sorghum beer	van der Aa Kuhle et al. (2001)
	S. cerevisiae/*S. uvarum*/non-*Saccharomyces*[a]	Orange juice	Arias et al. (2002)
	S. cerevisiae/*C. humilis*	Sourdoughs	Foschino et al. (2004)
	Non-*Saccharomyces*[b]	Fermentation of coffea arabica	Masoud et al. (2004)
	S. cerevisiae/non-*Saccharomyces*[c]	Fermentation of cocoa beans	Jespersen et al. (2005)
PCR-RFLP of 5.8S ITS	*S. cerevisiae*/non-*Saccharomyces*[d]	Wine	Esteve-Zarzoso et al. (2001), Torija et al. (2001), Beltrán et al. (2002), Rodríguez et al. (2004)
		Poultry	Deak et al. (2000)
	S. cerevisiae/*S. kluyveri*/non-*Saccharomyces*[e]	Wine	Pramateftaki et al. (2000)
	Pichia anomala	Yoghurt	Caggia et al. (2001)
	S. cerevisiae	Sorghum beer	van der Aa Kuhle et al. (2001)
	S. cerevisiae/*S. uvarum*/non-*Saccharomyces*[a]	Orange juice	Arias et al. (2002)
	S. cerevisiae/*S. unisporus*/non-*Saccharomyces*[f]	Orange fruit and juice	Las Heras-Vazquez et al. (2003)
	S. cerevisiae/non-*Saccharomyces*[g]	Wine	Ganga and Martínez (2004)
	S. cerevisiae/non-*Saccharomyces*[h]	Cider	Morrissey et al. (2004)
	S. cerevisiae/*S. exiguous*/non-*Saccharomyces*[i]	Sourdough	Pulvirenti et al. (2004)
	Zygosaccharomyces spp.	Candied fruits and marzipan	Martorell et al. (2005)
PCR-DGGE	*S. cerevisiae*/non-*Saccharomyces*[j]	Cocoa fermentation	Nielsen et al. (2005)
	S. cerevisiae/non-*Saccharomyces*[k]	Coffea fermentation	Masoud et al. (2004)
	Non-*Saccharomyces*[l]	Grapes	Prakitchaiwattana et al. (2004)
	S. cerevisiae/non-*Saccharomyces*[m]	Sourdough	Meroth et al. (2003)
	Saccharomyces/non-*Saccharomyces*[n]	Wine	Mills et al. (2002)
	S. cerevisiae/non-*Saccharomyces*[o]	Wine	Cocolin et al. (2000)

Real-time PCR	*D. bruxellensis*	Wine	Phister and Mills (2003)
	Food spoilage yeasts[p]	Yoghurts, milk, cheese, fruit juice	Bleve et al. (2003)
	Food spoilage yeasts[q]	Fruit juice	Casey and Dobson (2004)
	D. bruxellensis	Wine	Delaherche et al. (2004)

[a]*Candida*, *Clavispora*, *Geotrichum*, *Hanseniaspora*, *Issatchenkia*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Saccharomycopsis* and *Torulaspora* spp.
[b]*Candida*, *Hanseniaspora*, *Issatchenkia*, *Kluyveromyces*, *Pichia* and *Torulaspora* spp.
[c]*Candida*, *Hanseniaspora*, *Pichia*, and *Trichosporon* spp.
[d]*Dekkera anomala*, *Issatchenkia terricola*, *Kloechera apiculata*, *Kluyveromyces thermotolerance* and *M. pulcherrima*; *Candida*, *Hanseniaspora*, *Schizosaccharomyces* and *Zygosaccharomyces* spp.
[e]*C. stellata*, *H. uvarum*, *M. pulcherrima* and *Torulaspora delbruckii*.
[f]*Candida*, *Clavispora*, *Hanseniaspora*, *Pichia*, *Rhodotorula* and *Trichosporon* spp.
[g]*C. krusei* and *Rhodotorula glutinis*; *Zygosaccharomyces* spp.
[h]*Brettanomyces*, *Debaryomyces*, *Hanseniaspora*, *Metschnikowia*, *Pichia* and *Saccharomycodes* spp.
[i]*I. orientalis* and *Candida* spp.
[j]*H. guilliermondi and P. membranifaciens*; *Candida* spp.
[k]*H. uvarum*, *I. orientalis*, *K. marxianus* and *T. delbrueckii*; *Candida* and *Pichia* spp.
[l]*Aureobasidium pullulans*, *Hanseniaspora* and *Metschnikowia* spp.
[m]*C. humilis*, *D. hansenii* and *S. uvarum*.
[n]*Candida*, *Hanseniaspora*, *Kluyveromyces*, *Pichia* and *Metschnikowia* spp.
[o]*C. ethanolica*, *K. apiculata* and *M. pulcherrima*.
[p]*Candida*, *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, *Saccharomyces* and *Zygosaccharomyces* spp.
[q]*Z. bailii*, *Z. rouxii*, *C. krusei*, *R. glutinis* and *S. cerevisiae*.

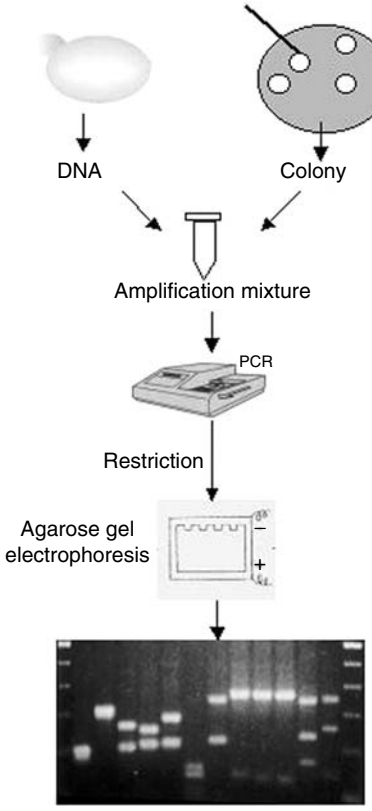

Fig. 3.4. Method for species identification based on PCR amplification and subsequent restriction analysis of ribosomal regions

CCT GCG G-3′) and its4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) as described by White et al. (1990). Guillamón et al. (1998) used this technique to identify wine yeasts quickly and later its use was extended to a total of 191 yeasts (Esteve-Zarzoso et al. 1999; Fernández-Espinar et al. 2000; de Llanos et al. 2004) related to food and drinks. The amplified fragments and restriction profiles of these species with the enzymes *Hae*III, *Hin*fI, *Cfo*I and *Dde*I are currently available online at the address http://yeast-id.com. The utility of the technique has been proved by studying reference strains (Ramos et al. 1998; Fernández-Espinar et al. 2000; Cadez et al. 2002; Esteve-Zarzoso et al. 2003; Naumova et al. 2003) and has been applied by numerous authors for species identification in different foods and beverages. Recent applications of the technique are shown in Table 3.2.

Restriction analysis of other ribosomal regions has also been used to identify yeast species, especially those belonging to the complex *Saccharomyces* "*sensu stricto*." This is the case for the ribosomal regions denominated NTS (Baleiras Couto et al. 1996; Nguyen and Gaillardin 1997; Pulvirenti et al. 2000; Nguyen et al. 2000a, b; Caruso et al. 2002; Romero et al. 2005), the gene 18S with the neighbouring region NTS

(Capece et al. 2003) or ITS (Vasdinyei and Deak 2003), the gene 18S (Tornai-Lehoczki and Dlauchy 2000) and different domains of the gene 26S (Smole-Mozina et al. 1997; Van Keulen et al. 2003). However, the fact that a database is not available means that these techniques using these regions cannot be generalized to the identification of yeasts.

3.2.2 PCR–Denaturing Gradient Gel Electrophoresis

Recently, a genetic fingerprinting technique based in PCR amplification, denaturing-gradient gel electrophoresis (DGGE), was introduced into microbial ecology (Muyzer et al. 1993).

In PCR-DGGE, which is schematized in Fig. 3.5, DNA fragments of the same length but with different sequences can be separated. Separation of DNA amplicons is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in polyacrylamide gels containing a linear gradient of

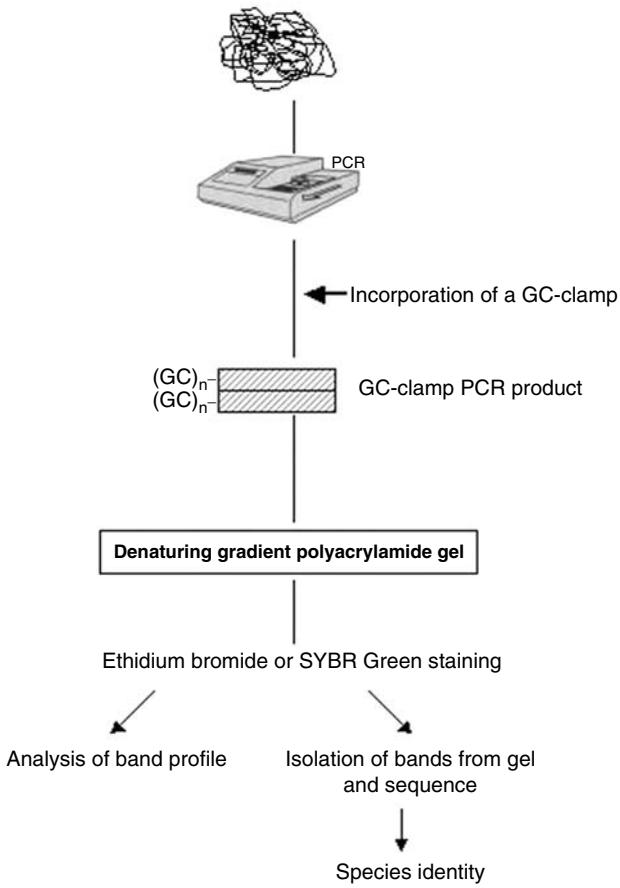

Fig. 3.5. Denaturing gradient gel electrophoresis

DNA denaturants (a mixture of urea and formamide). The mobility of the molecule is retarded at the concentration at which the DNA strands dissociate. Complete strand separation is prevented by the presence of a high melting domain which is artificially created as follows. DNA is specifically amplified by PCR using particular groups of universal primers. A sequence of guanines (G) and cytosines (C) is added to the 5′ end of one of the PCR primers, coamplified and thus introduced into the amplified DNA fragments.

A related technique is temperature-gradient gel electrophoresis (TGGE), which is based on a linear temperature gradient for the separation of DNA molecules. DNA bands in DGGE and TGGE profiles can be visualized using ethidium bromide. Recently, SYBR Green I was introduced as an alternative to ethidium bromide. PCR fragments may be isolated from the gel and used in sequencing reactions for species identification as described in Sect. 3.2.1.

The use of DGGE and TGGE in microbial ecology is still in its infancy, but their future perspectives are promising (Muyzer and Smalla 1998). Their application to yeast identification in food and beverage settings is very recent as is shown in Table 3.2.

3.2.3 Real-time PCR

The real-time PCR technique was developed in 1996 and from then on its use for different applications has increased almost exponentially (Wilhelm and Pingoud 2003). In this technique, the amplification products are observed as the PCR cycles take place. The technique is based on the detection and quantification of a fluorescent donor whose signal increases in direct proportion to the quantity of PCR product in the reaction. The process, which is schematized in Fig. 3.6, is carried out in a thermocycler that has a detection system able to capture and quantify the signal emitted by the donor at the end of each cycle for each sample. The information obtained is represented as an amplification curve that provides the cycle number for which the intensity of donor emission increases compared with the background noise. This cycle number is called the cycle threshold (Ct) and is inversely proportional to the number of copies of the sample; thus, it can be used to evaluate the initial quantity of sample numerically (DNA or cells) with great precision, within a wide range of concentrations.

The fluorescence can be obtained through binding agents or probes. As a binding agent SYBR Green is used, which binds to the double-chained DNA, increasing the

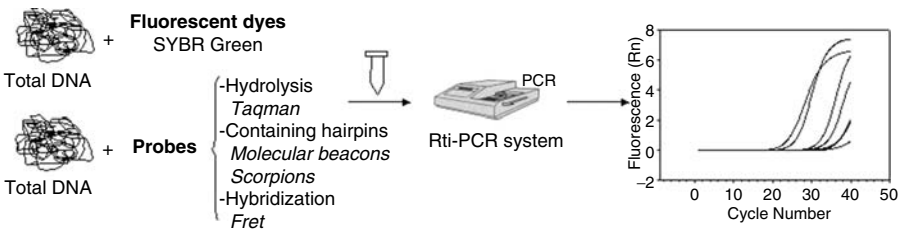

Fig. 3.6. Real-time PCR technique

fluorescence as the quantity of PCR product increases. Regarding the probes, three types can be distinguished: hydrolysis probes, loop-shaped probes and hybridization probes. The most commonly used hydrolysis probe is the so-called Taqman probe and it is characterized by having a donor photochrome binding to an acceptor photochrome. When both photochromes are bound to the probe, the donor does not emit a signal. But, when the probe binds to the sequence of interest during the PCR reaction, the exonuclease activity of the Taq polymerase activates the donor photochrome of the rest of the probe, leading to the emission of a fluorescence signal. The fluorescence signal of the donor is monitored, and it increases in the successive PCR cycles. The loop-shaped probes (Molecular Beacons, Scorpions) have inverted repeated sequences (ITR) at their 5′ and 3′ ends. This design allows a loop shape to be formed owing to the complementarity of the two ITR regions, in the absence of the target sequence. When the probe binds to the target DNA, the separation of the fluorochromes leads to efficient fluorescence. Lastly, the hybridization probes consist of two probes, donor and acceptor, binding to the region to be amplified, each one marked with a fluorophore. Resonance energy transfer only occurs when both probes bind to the target DNA, and they are very close together. All these fluorescence systems have advantages and drawbacks that determine when they are chosen. For example, if one wants a simple, economic and easy-to-use system, SYBR Green is chosen. However, during the PCR reaction this can bind to primer dimers and other non-specific products, leading to an overestimation of the target DNA concentration. If greater specificity is required, one must resort to the system with probes.

Real-time PCR has numerous advantages compared with other identification techniques. It is necessary to stress its high specificity and sensitivity, its ability to quantify and the fact that analysis after PCR is not necessary (electrophoresis). The latter, together with the use of reduced reaction times and cycles, makes it very fast, which is very useful in the event of routine analysis and especially in applications that require correction measures. Given all the advantages of a system of this type, one must bear in mind that the design of the primers and probes is very demanding because the specificity and sensitivity of the method will depend on them. There is software that helps to design primers and probes that are suitable for the conditions of real-time PCR. The design approach usually starts off with data concerning the sequence of genes or regions whose usefulness in establishing the phylogenetic relationships among yeast species has been demonstrated and that also has the advantage of being easily found through the internet. These sequences are those corresponding to the ribosomal region D1/D2 (Kurtzman and Robnett 1998), to the mitochondrial gene COX2 (Belloch et al. 2000; Kurtzman and Robnett 2003) and to the nuclear gene of actin (Daniel and Meyer 2003). Recent applications of real-time PCR for the identification of yeasts in foods and beverages are shown in Table 3.2.

3.2.4 New Technologies

DNA microchips began to be operative between 1993 and 1995 and were consolidated around 2000–2001 as a research technique, thus representing one of the most recent tools that researchers can count on to face the demands of modern winemaking techniques. With this technique, through the hybridization of nucleic acids,

one can find out which yeast species are present. Until now, the microchip technique has not been used to detect yeasts; however, given its high specificity and sensitivity, as well as the quantity of information it provides, this technique is seen as a good alternative in the near future and therefore it is interesting to know its basis.

Microchips, manufactured by specialized companies, are small devices that contain thousands of fragments of biological material (DNA, RNA, proteins) arranged in an orderly and well-known way on a solid support (slides, glass, plastic). The sample of DNA that will come into contact with the microchip must be marked to allow its detection. The most commonly used markers are fluorescent ones, but radioactive markers or chemio-luminescence can also be used. When they come into contact with a sample, only those chains that are complementary to those on the chip bind and form a characteristic pattern of light, which can be read with a scanner and interpreted with a computer.

Nucleic acid sequence based amplification (NASBA) is a promising diagnostic tool for the analysis of viable microorganisms, since it is based on amplification of RNA rather than DNA. NASBA was first described by Compton (1991). Amplification involves the coordinated activities of three enzymes, AMV reverse transcriptase, RNase H and T7 RNA polymerase. Oligonucleotide primers, complementary to sequences in the target RNA, deoxyribonucleotide triphosphate and ribonucleotide triphosphate are incorporated in the reaction. The first primer allows the reverse transcriptase to form a complementary DNA (cDNA) strand. Then, the RNAse digests away the RNA and the second primer binds to the cDNA, allowing the reverse transcriptase to form a double-stranded cDNA copy. The cDNA is used as a template and as result RNA will be produced exponentially. The reaction is performed at a single temperature, normally 41°C. At this temperature, the genomic DNA from the target microorganism remains double-stranded and does not become a substrate for amplification. The NASBA reaction requires fewer "cycles" than conventional PCR to produce a desirable amplification, only four to five cycles are required. There are several approaches for the detection of products. The simplest one is the use of standard agarose gel electrophoresis and ethidium bromide staining. The use of molecular beacons has recently been developed to allow real-time detection of NASBA products. This procedure facilitates the establishment of strategies for quantification (e.g. similar to that used in Rti-PCR assays).

NASBA for detection of microorganisms is at around the same stage as PCR was a decade or so ago, with a few methods being published sporadically in the scientific press (Cook 2003). Hence, considerable further development is required before NASBA can be used for routine use. However, since the technique can equal the rapidity and accuracy of PCR and has additional potential advantages, NASBA is a very promising tool for detection of viable food yeasts.

3.3 Methods to Differentiate at Strain Level

3.3.1 Pulsed Field Electrophoresis of Chromosomes

In this technique, the alternating application of two transverse electrical fields means the chromosomes are forced to change their migration direction continually,

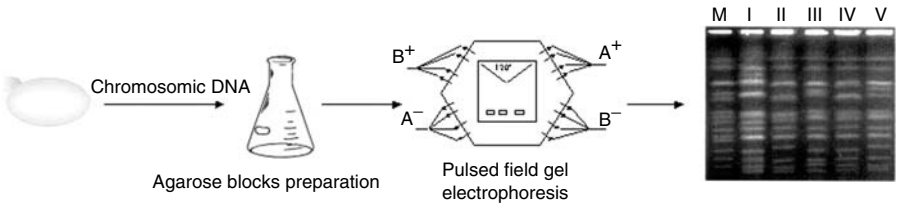

Fig. 3.7. Pulsed field electrophoresis of chromosomes technique

thus avoiding their being retained in the lattice of the agarose gel and enabling large fragments of DNA to be separated (Lai et al. 1989).

The yeasts are grown in liquid medium and then they are combined with melted agarose and placed in small moulds. The absorbed yeast cells undergo lyses in situ and then the free DNA is immobilized in the agarose matrix. The blocks are inserted in agarose gels which are subjected to electrical fields (Fig. 3.7). The parameters that condition the resolution of the bands are the variation intervals in the force of the electrical field, the agarose concentration, the temperature and the angle between the electrical fields.

Karyotype analysis is demonstrated to be a highly efficient technique to differentiate strains of *S. cerevisiae*. The polymorphism revealed by this technique is the result of the addition or elimination of long fragments of DNA in homologous chromosomes during the evolution of the yeast genome (Wolfe and Shields 1997; Casaregola et al. 1998; Keogh et al. 1998).

Numerous authors have applied karyotype analysis to the characterization of reference and commercial yeasts belonging to different species (Blondin and Vezinhet 1988; Degré et al. 1989; Vezinhet et al. 1990; Yamamoto et al. 1991; Querol et al. 1992; Fernández-Espinar et al. 2001; Petersen and Jespersen 2004; Schuller et al. 2004). These works demonstrate that karyotype analysis is an efficient technique to differentiate yeasts at strain level. Recent applications of the technique to *S. cerevisiae* and other yeast species associated with different foods and beverages are shown in Table 3.3.

3.3.2 Restriction Analysis of the mitochondrial DNA

The mitochondrial DNA (mtDNA) of *S. cerevisiae* is a small molecule of between 65 and 80 kb, whose degree of variability can be shown by restriction. The high degree of polymorphism revealed by this technique among strains of *S. cerevisiae* makes it one of the most commonly applied techniques in the characterization of this type of isolate (Table 3.3).

Several methods have been developed to isolate yeast mtDNA (Aigle et al. 1984; Gargouri 1989; Querol and Barrio 1990). However, Querol et al. (1992) have developed a method of mtDNA analysis (Fig. 3.8) that avoids using gradients in ceasium chloride and an ultracentrifuge, factors which restrict use in industry. The simplification of the technique is based on the fact that the mtDNA of yeasts is a molecule

Table 3.3 Molecular techniques most frequently used for yeast strain characterization in foods and beverages and their application in the last 5 years

Technique	Target species	Matrix (food or beverage)	References (from 2000 to 2005)
Karyotyping	*Y. lipolytica/C. zeylanoides*	Poultry	Deak et al. (2000)
	S. bayanus	Wine	Naumov et al. (2000)
	S. cerevisiae/S. cerevisiae "flor"	Wine	Esteve-Zarzoso et al. (2001)
	S. cerevisiae	Cachaca	Guerra et al. (2001)
	S. cerevisiae/S. bayanus	Cider	Naumov et al. (2001)
	S. bayanus	Wine	Naumov et al. (2002)
	S. uvarum	Wine	Demuyter et al. (2004)
	S. cerevisiae	Wine	Martínez et al. (2004)
	S. cerevisiae/non-*Saccharomyces*[a]	Grape and wine	Rodríguez et al. (2004)
	S. cerevisiae	Must	Antunovics et al. (2005)
	S. cerevisiae/non-*Saccharomyces*[b]	Fermentation of cocoa beans	Jespersen et al. (2005)
RFLP mtDNA	*S. cerevisiae*	Grape	Comi et al. (2000)
	S. cerevisiae/non-*Saccharomyces*[c]	Wine	Pramateftaki et al. (2000)
	S. cerevisiae/K. marxianus/K. lactis	Cheese	Suzzi et al. (2000)
	S. cerevisiae/S. cerevisiae "flor"	Wine	Esteve-Zarzoso et al. (2001)
	S. cerevisiae	Must and wine	Fernández-González et al. (2001)
	S. cerevisiae	Wine	Torija et al. (2001)
	S. cerevisiae	Wine	Beltrán et al. (2002)
	S. cerevisiae	Wine	Lopes et al. (2002)
	D. hansenii	Cheese	Petersen et al. (2002)
	S. cerevisiae	Wine	Granchi et al. (2003)
	S. cerevisiae	Wine	Torija et al. (2003)
	S. cerevisiae	Must	Cappello et al. (2004)
	S. cerevisiae "flor"	Wine	Esteve-Zarzoso et al. (2004)
	S. cerevisiae/C. humilis	Sourdoughs	Foschino et al. (2004)
	S. cerevisiae	Wine	Martínez et al. (2004)
	S. cerevisiae/non-*Saccharomyces*[a]	Grape and wine	Rodríguez et al. (2004)
	Z. bailii/Z. rouxii	Candied fruits and marzipan	Martorell et al. (2005)

RAPDs	*Y. lipolytica/C. zeylanoides*	Poultry	Deak et al. (2000)
	Y. lipolytica	Sausages	Gardini et al. (2001)
	S. cerevisiae	Cachaca	Guerra et al. (2001)
	Schizosaccharomyces pombe	Cachaca	Gomes et al. (2002)
	S. cerevisiae	Sourdoughs	Succi et al. (2003)
	Geotrichum candidum/D. hansenii	Dairy products	Vasdinyei and Deak (2003)
	Non-*Saccharomyces*[d]	Cheese	Fadda et al. (2004)
	S. cerevisiae/C. humilis	Sourdoughs	Foschino et al. (2004)
	Z. bailii/Z. rouxii	Candied fruits and marzipan	Martorell et al. (2005)
Micro- and minisatellites	*S. cerevisiae/Kl. apiculata*	Wine	Caruso et al. (2002)
	S. cerevisiae	Wine	Howell et al. (2004)
	Z. bailii/Z. rouxii	Candied fruits and marzipan	Martorell et al. (2005)
δ PCR	*S. cerevisiae*	Wine	Pramateftaki et al. (2000)
			Lopes et al. (2002)
			Ciani et al. (2004)
			Demuyter et al. (2004)
		Must	Cappello et al. (2004)
		Sourdough	Pulvirenti et al. (2004)

[a]*C. guillermondi*, *C. pulcherrima* and *Kl. apiculata*.
[b]*C. krusei*, *H. guilliermondii*, *P. membranefaciens* and *S. kluyveri*.
[c]*C. stellata*, *H. uvarum*, *M. pulcherrima* and *T. delbruckii*.
[d]*C. lambica*, *C. zeylanoides*, *Debaryomyces hansenii*, *G. candidum* and *K. lactis*.

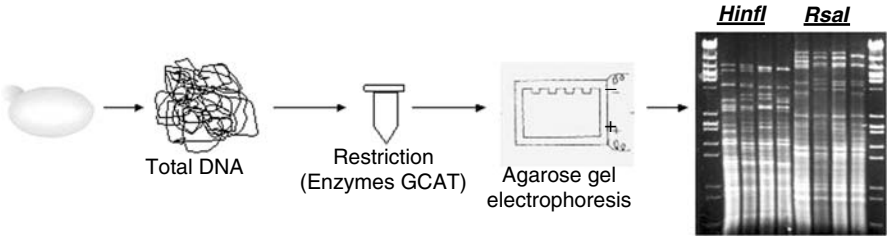

Fig. 3.8. Method based on the restriction analysis of the mitochondrial DNA

with 75% of A and T (Gray 1989) and although it is rich in AT, some 200 regions rich in GC have also been found. Therefore, digestions of total DNA with GCAT-type enzymes do not recognize the sequences rich in either GC or AT. Given the small number of restriction sites in the mtDNA and the high number of cutting sites in the nuclear DNA, the latter breaks into small fragments, which enables one to visualize the bands corresponding to the mtDNA as clearly defined bands, superimposed on the shadow of the nuclear DNA digested. Not all the enzymes reveal the same degree of polymorphism and it depends greatly on the species. In the specific case of *S. cerevisiae* the enzymes that are most suitable to differentiate at strain level are *Hin*fI and *Hae*III (Guillamón et al. 1994).

This rapid technique enables a greater number of strains to be analysed in less time, and is ideal for industry given its speed, safety and economy and because it does not require sophisticated material or very specialized employees. López et al. (2001) simplified the method: the 77 h that was needed to complete the protocol following the original method has been reduced to 25 h with the adapted method.

Numerous authors have applied restriction fragment length polymorphism (RFLP) mtDNA to the characterization of reference and commercial wine yeast strains (Vezinhet et al. 1990; Querol et al. 1992; Guillamón et al. 1996; Mesa et al. 1999; Fernández-Espinar et al. 2001; Esteve-Zarzoso et al. 2004; Schuller et al. 2004) and strains belonging to other species (Romano et al. 1996; Guillamón et al. 1997; Petersen et al. 2001) These works demonstrate that RFLP mtDNA analysis is an efficient technique to differentiate at strain level. Recent applications of the technique to strains from yeast species associated with different foods and beverages are shown in Table 3.3.

3.3.3 Methods Based on the PCR Technique

The quickest molecular techniques are those based on the PCR technique (Saiki et al. 1985, 1988) and they have been used to discriminate between strains of wine yeasts. Some variants of the basic PCR technique have been developed that can be used to detect polymorphisms of DNA fragments without the need to use restriction enzymes. The techniques most frequently used to differentiate yeasts at strain level are randomly amplified polymorphic DNA (RAPD) and microsatellites. Other techniques, such as δ-sequence amplification and amplification of "intron splice sites", have been developed specifically to differentiate strains of the species *S. cerevisiae*.

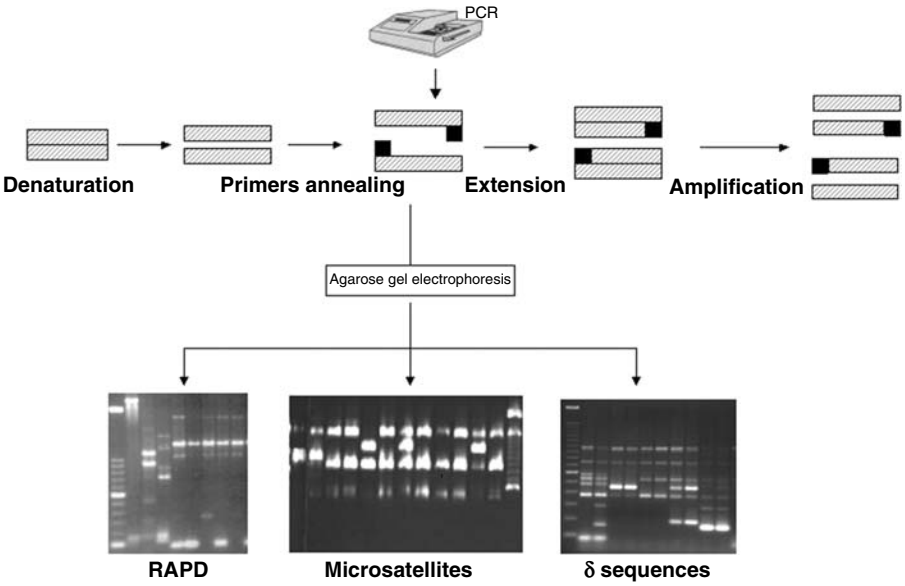

Fig. 3.9. Methods to differentiate at strain level based on PCR amplification

All these techniques use oligonucleotides as primers, which bind to target sequences in each DNA strand of the yeast. The sequence of the primers varies according to the technique, as we will see later. The amplification is carried out with a thermostable polymerase DNA and the amplification protocol always includes a variable number of cycles (generally between 25 and 45) that include denaturation of the DNA followed by hybridization and a period of extension. The result is the amplification of the DNA duplicating the quantity of target DNA in each cycle. The amplification conditions, especially the hybridization temperature, also differ. The amplification profiles are visualized in agarose gels at 1.4% with strain-specific profiles that enable us to identify and differentiate them.

We will go on to talk about each of these techniques in detail and Fig. 3.9 shows examples of the amplification profiles obtained for some of them.

3.3.3.1 Randomly Amplified Polymorphic DNA

The RAPD technique (Williams et al. 1990), is characterized by the fact it uses just one primer, which has the special characteristics of being particularly short (approximately ten nucleotides) and having an arbitrary sequence. The RAPDs-PCR reaction is also characterized by the low hybridization temperature used (37°C). Thus, the pairings between the oligonucleotide and the DNA are determined by the short and arbitrary sequence this has, and favoured by the low temperature used, setting off the amplification of diverse fragments of DNA distributed all the way along the genome. The

result is a pattern of amplified products of different molecular weight that can be characteristic of the species or of the different strains or isolates within the same species (Bruns et al. 1991; Paffetti et al. 1995).

The main advantage of the method is that one does not need previous information about the sequence to design the primer. Moreover, the technique enables one to analyse the variability along the whole genome, thus revealing more polymorphism than other techniques that analyse specific regions. However, owing to the low hybridization temperature used (37°C) the amplification profiles obtained are unstable and difficult to reproduce and it is necessary to carry out several repetitions for each sample, starting off with different DNA extractions. Only the bands present in all the repetitions will be taken into account. This fact together with the need to combine the amplification results with several oligonucleotides to obtain a good resolution power means that the technique is not apt for routine application at an industrial level. Consequently, the technique has not been used much for the characterization of strains.

The efficiency of the technique to differentiate at strain level has been demonstrated by analysing reference strains belonging to different species (Quesada and Cenis 1995; Baleiras Couto et al. 1996; Romano et al. 1996; Tornai-Lehoczki and Dlauchy 2000; Pérez et al. 2001a; Cadez et al. 2002). Recent applications of the technique to *S. cerevisiae* and other yeast species associated with different foods and beverages are shown in Table 3.3.

3.3.3.2 PCR of Repetitive Regions of the Genome (Microsatellites and Minisatellites)

There are repeated regions in the genome that represent potential targets for molecular identification at strain level, as they show great variability. These areas are the microsatellites and the minisatellites that constitute motifs of very varied length, repeated in tandem abundantly and at random along the genome. The microsatellites are usually less than 10 bp in length, while the minisatellites are between 10- and 100-bp long. The variability found in these regions can be shown by means of PCR amplification using specific oligonucleotides, such as $(GTG)_5$, $(GAG)_5$, $(GACA)_4$ or M13. The ability of these oligonucleotides to reveal polymorphism among strains of *S. cerevisiae* was demonstrated by Lieckfeldt et al. (1993) using hybridization techniques. The same authors were the first to use these sequences as primers in a PCR reaction, showing the usefulness of this technique for characterization at strain level. The technique was used by other authors later for the study of reference strains (Baleiras Couto et al. 1996; González Techera et al. 2001; Hennequin et al. 2001; Pérez et al. 2001a, b; Marinangeli et al. 2004) and recent applications are shown in Table 3.3. The amplified products obtained are approximately 700 and 3,500 bp in size; therefore, they can be visualized in agarose gels. Visualization of the amplified products obtained is usually carried out in acrylamide gels, although it can also be done in automatic sequencers. This means that the technique is not very useful for routine application, in spite of its high resolution and its high reproducibility. The resolution power of this technique is comparable to δ elements and restriction analysis of mtDNA.

3.3.3.3 Amplification of δ Sequences

δ sequences are elements measuring 0.3 kb that flank the retrotransposons Ty1 (Cameron et al. 1979). Around 100 δ copies are present in the yeast genome as part of the retrotransposons Ty1 or as isolated elements. However, these δ sequences are concentrated in genomic regions adjacent to the transfer RNA genes (Eigel and Feldmann 1982). The number and the localization of these elements demonstrate certain intraspecific variability that Ness et al. (1993) took advantage of to develop specific primers (δ_1 and δ_2) that are useful to differentiate strains of *S. cerevisiae*. These authors showed that the δ elements are stable enough for this technique to be used as an identification method of *S. cerevisiae* strains at an industrial level, as demonstrated by other authors later (Table 3.3). Some of these studies show the great variability this technique reveals between isolates of the *S. cerevisiae* species compared with other highly resolving techniques, such as restriction analysis of the mtDNA and electrophoresis of chromosomes (Pramateftaki et al. 2000; Fernández-Espinar et al. 2001).

Recently, Legras and Karst (2003) optimized the technique by designing two new primers (δ_{12} and δ_{21}) that are located very near to δ_1 and δ_2. The use of δ_{12} and δ_{21} or of δ_{12} with δ_2 reveals greater polymorphism, which is reflected by the appearance of a greater number of bands. Consequently, the new primers are able to differentiate more strains: 53 commercial strains were differentiated unequivocally (Legras and Karst 2003). Shuller et al. (2004) confirmed it later, showing that the combination of δ_2 and δ_{12} identified twice as many strains as the set of primers designed by Ness et al. (1993).

An important drawback of this technique is the influence that the concentration of DNA can have on the profile obtained, as shown by Fernández-Espinar et al. (2001) and commented on by Shuller et al. (2004). Although this problem is avoided by standardizing the concentration of DNA, the comparison of results between laboratories is complicated. Another problem of this technique is the appearance of "ghost" bands due to the low annealing temperature (42°C) used during the amplification reaction. Recently, Ciani et al. (2004) used an annealing temperature of 55°C to characterize wine strains of *S. cerevisiae*. In this way, the amplification profiles obtained are much stabler, although fewer bands are obtained.

3.3.4 Amplified Fragment Length Polymorphism

Although this technique is fundamentally based on PCR amplification, we will consider it in a different section, owing to its complex methodology, which implies the use of other methodologies, as we will see later.

Amplified fragment length polymorphism (AFLP) is a technique that involves the restriction of genomic DNA followed by the binding of adapters to the fragments obtained and their selective amplification by PCR. The adapter sequence and the restriction sites are used as the primers' target for PCR amplification. The fragments are separated in DNA sequencing gels and visualized by auto-X-ray or in automatic sequencing (Vos et al. 1995). Figure 3.10 outlines this technique.

As in the case of RAPDs, previous information about the sequence is not needed to design the primer, it is easily reproduced and it offers a great deal of information.

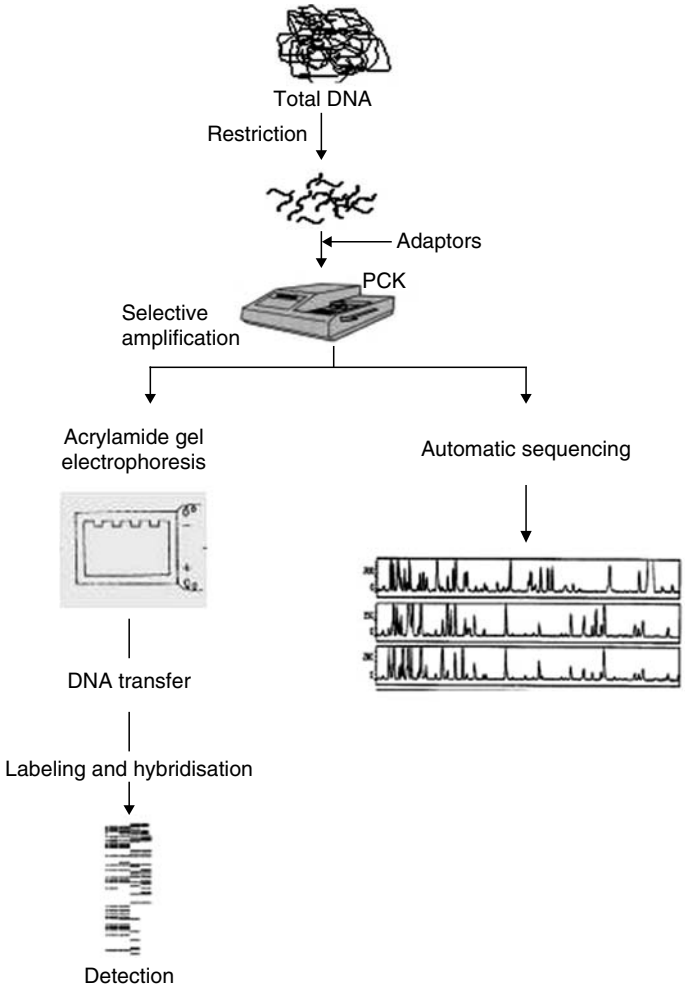

Fig. 3.10. Amplified fragment length polymorphism

AFLP is a useful technique to discriminate between yeasts at strain level, as shown by de Barros Lopes et al. (1999); however, it has the drawback of being a very laborious technique, since it requires automatic sequencers, which are very sophisticated for use in industry, and also the data are difficult to interpret. Although the technique has been very widely used to study bacteria, plants and animals, in the case of yeasts, there are few works in this respect (de Barros Lopes et al. 1999; Azumi and Goto-Yamamoto 2001; Boekhout et al. 2001; Theelen et al. 2001; Borst et al. 2003; Dassanayake and Samaranayake 2003; Trilles et al. 2003).

Acknowledgements

We thank all our colleagues and collaborators who contributed in the different works reviewed and discussed in this chapter. Thanks are also to CICYT grants (ref. AGL2000-1492 and BIO2003-03793-C03) from the Spanish Ministerio de Ciencia y Tecnología and a Generalitat Valencia grant (ref. GRUPOS03/012). P.M. is a recipient of a FPI predoctoral fellowship from the Ministerio de Ciencia y Tecnología and R. de L. is a recipient of a predoctoral I3P fellowship from CSIC.

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Chapter 4

Yeast Ecological Interactions. Yeast–Yeast, Yeast–Bacteria, Yeast–Fungi Interactions and Yeasts as Biocontrol Agents

Bennie C. Viljoen

Department of Microbial, Biochemical and Food Biotechnology, U.F.S., Box 339, Bloemfontein 9300, South Africa
(e-mail: viljoenbc.sci@mail.uovs.ac.za)

4.1 Introduction

When the domains of individual microorganisms overlap, it is likely that interactions will occur (Boddy and Wimpenny 1992). The outcome of these interactions is evaluated on the basis of the effect they have on population size (Odum 1953) regardless of whether the interactions are detrimental, neutral or beneficial. The types of interaction found in mixed populations of microorganisms are classified on the basis of these effects as direct or indirect interactions (Bull and Slater 1982). Indirect interaction refers to competition, commensalism, mutualism, amensalism or antagonism and neutralism (Linton and Drozd 1982), and direct interaction to predation and parasitism (Frederickson 1977; Bull and Slater 1982). However, fermented foods and beverages develop their nutritional and organoleptic qualities as a result of the metabolic activity of a succession of different microorganisms and it is unlikely that the interactions will separate into these discrete groups since more than one type of interaction occurs simultaneously (Verachtert et al. 1990).

Present understanding of the positive, negative or neutral role of interactions between yeasts, bacteria and fungi has its origins the first time fermentation was employed. The fermentation of many products includes interaction both within and between different microbial groups (e.g. yeast–yeast, yeast–bacteria, yeast–moulds), the physiological activity of which brings about desirable changes which decisively determine the character of a product and stabilise the population in a specific ecological niche (Wood and Hodge 1985; Leroi and Pidoux 1993; Geisen et al. 1992; Rossi 1978; Challinor and Rose 1954). However, interaction does not necessarily only imply the positive or negative attributes within fermentation but it also involves the antagonistic activity of yeasts against other microorganisms by means of the production of microcins (Baquero and Moreno 1984; Golubev and Boekhout 1992), secretion of antibacterial and antifungal compounds, co-fermentation, and their role as in biological control.

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
© Springer-Verlag Berlin Heidelberg 2006

4.2 Ecological Interaction Between Microorganisms

4.2.1 The Secretion of Antifungal or Antibacterial Compounds

It is well known that certain fungi (Punja and Utkhede 2003) and members of the bacterial groups (Williams and Vickers 1986) possess the ability to synthesise and secrete secondary metabolites that exhibit antagonistic activities against other microorganisms. However, little attention has been given to yeasts as possible producers of similar substances despite positive indications already published early in the twentieth century (Hayduck 1909; Fernbach 1909).

Hayduck (1909) obtained a volatile thermolabile toxic extract from yeast which was confirmed by Fernbach (1909) to be an amine that inhibited the growth of *Escherichia coli* and staphylococci. Schiller (1924) demonstrated the presence of an inhibitory enzyme active against the staphylococci, while Bachmann and Ogait (1935) argued that the main reason for the inhibitory action of baker's yeast was due to the production of acetaldehyde. Barglowski (1938) found that *Saccharomyces cerevisiae* and *Mycotorula albicans* strains suppressed the growth of *Mycobacterium tuberculosis*, while Cook et al. (1941) prepared an antibiotic from baker's yeast which inhibited the growth of *Aspergillus niger* and *Penicillium glabrum*. Baker's yeast grown in rye decoction is also reported to exhibit strongly bactericidal activities against *Aerobacter aerogenes* owing to thermolabile enzymes (Tikka and Itkonen 1941). Owing to the development of acid, *Torulopsis utilis* showed antibiotic action against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas pyocyaneus* (Carpentier 1945), while Sartory and Meyer (1946) obtained an inhibitor from baker's yeast active against *Escherichia coli* and *Proteus vulgaris*. Florey et al. (1949) noted that unsaturated fatty acids from baker's and brewer's yeasts, *Debaryomyces mucosus* and *T. utilis*, as well as succinic acid from *T. utilis* var. *major* possess antibacterial properties inhibiting a variety of bacterial organisms. Complete inhibition of *Penicillium glaucum* and *Salmonella typhosa* by yeasts was reported by Toda (1950), while similar inhibiting effects were noted by Motzel (1956) due to cyclic peptides. Despite the inhibition of *Bacillus subtilis* and pediococci obtained by substances produced by the yeasts *Brettanomyces bruxellensis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*, no attempts were made to isolate or identify the substances. Parfentjef (1953) isolated a fraction containing protein possessing anti-infectious properties, malucidin, from baker's and brewer's yeast. This protein protected animals against infection by a number of microorganisms, which included the yeast *Candida albicans*, pathogens like *Proteus* and *Shigella endotoxins* and many species of gram-negative and gram-positive bacteria (Parfentjev 1958).

Robinson et al. (1958) in studies on the decrease of the bacterial population in preferments, isolated two antibiotic substances designated as I1 and I2 from yeasts which possessed inhibitory properties for *Micrococcus pyogenes* and *Escherichia coli*. In a survey of the antibiotic powers of yeasts, MacWilliam (1959) examined 150 yeast strains for their antibiotic powers against bacteria and moulds. Strong inhibition against *Fusarium*, *Mucor* and *Penicillium* was achieved with the yeast strain *Candida pulcherrima* producing pulcherriminic acid, a derivative of the red pigment

pulcherrimin. Robinson et al. (1962) continued their research on the two antibiotic substances they had previously isolated from *Saccharomyces cerevisiae* which they identified as polypeptides, capable of surviving baking and showing antibacterial activity against *Staphyllococcus aureus*.

Despite ongoing arguments that the possible role of yeasts as a source for antimicrobial compounds is merely attributed to the natural effect of competition for nutrients; Fatichenti et al. (1983), in a study on the antagonistic activity of *D. hansenii* against bacteria, found that the yeast species produced extracellular and intracellular antimicrobial compounds that inhibited the growth of *Clostridium tyrobutyricum* and *Clostridium butyricum*. Antibacterial activity was also detected in *Kloeckera apiculata* and *Kluyveromyces thermotolerans*, secreting substances that inhibited the growth of beer-spoilage bacteria (Bilinski et al. 1985). The expression of antibacterial activity by these two yeasts against the gram positive bacteria *Bacillus megaterium* and *Lactobacillus plantarum* involves transformation of methylene blue into a pharmacologically active form. Antibacterial activity against *Staphyllococcus aureus* was noted by the production of extracellular glycolipids, called sophorosides, by *T. bombicola* (Cavalero and Cooper 2003). The sophorosides also proved to be active against *Candida albicans*.

Probably the most significant and well-known antagonistic action by yeasts in recent years comprises the production of killer toxins (Young 1987; Rosini and Cantini 1987; Shimizu 1993; Walker et al. 1995; Suzuki et al. 2001; Marquina et al. 2002). These toxins are extracellular proteins or glycoproteins that disrupt cell membrane function in susceptible yeasts. Although these killer toxins were originally considered species-specific, clear evidence indicated that they occur across species in different yeast genera (Palpacelli et al. 1991; Llorente et al. 1997; Suzuki et al. 2001), and they can kill various filamentous fungi (Walker et al. 1995).

4.2.2 Yeast Co-Interrelationships with Other Microorganisms

Other than the antagonism exhibited by yeasts as just described, ecological theory describes a wide variety of interactions between yeasts and other microorganisms. Yeasts are added to foods and feeds as a source of proteins and vitamins, are represented in waste-treatment facilities, and are used for industrial purposes. These processes frequently rely on a variety of microorganisms (Linton and Drozd 1982; Kuenen and Harder 1982; Frederickson 1977; Hesseltine 1965).

The use of mixed cultures resulted in a higher growth rate, better biotransformations and higher yields in products (Verachtert et al. 1990). Although it has been stressed that the main interaction between the different microorganisms relied on microbial competition for the growth-limiting substrate (Bull and Slater 1982; Alexander 1971), various additional interactions occur simultaneously (Meyer et al. 1975; Yoon et al. 1977; Bungay and Bungay 1968). The consequence of other interactions often results in the interrelationship or co-existence of different species growing on a single growth-limiting substrate (Kuenen and Harder 1982). If physiochemical intrinsic and extrinsic conditions are within specified limits and the environment contains sufficient available energy and required nutrient sources for microbial growth, microbial communities will develop (Meers 1973). Interrelationships between and

within the communities develop, and as a result the stability of the environment is altered (Nakamura and Hartman 1961) by one species to stimulate the growth of other species because of changes in pH, growth factors, oxygen depletion, etc. For example, the growth of lactic acid bacteria reduces the pH value of media to encourage yeast growth, the removal of substances (osmophilic yeasts metabolise high sugar concentrations) that would otherwise prevent the growth of a second species (Mossel and Ingram 1955) or the excretion of relevant enzymes for the breakdown of complex carbohydrates (Antuna and Martinez-Anaya 1993). Owing to the change in the abiotic environmental conditions, the nature of the interactions between the populations may also change (Megee et al. 1972).

4.2.2.1 Yeast–Bacteria Interactions

When bacterial strains grow, environmental alterations may inhibit the growth of other species owing to the removal of essential nutrients or by the production of organic and inorganic toxic compounds (Meers 1973). Bacteria, predominantly lactic acid bacteria, commonly excrete organic acids which lead to a lowering in the pH, which either inhibits the growth of undesired pathogens or promotes yeast growth. Therefore, the interrelationship between lactic acid bacteria and yeasts, as applied in many fermented foods and beverages, plays an essential role in product preservation. In these ecosystems, they may compete for the same substrates (Bull and Slater 1982; Fleet 1990) or synergistically promote the growth of each other. Moreover, the antagonistic and synergistic effects exhibited by using the microorganisms in co-culture, may also be applied in converting wastes into feeds and in industrial processes.

Yeasts (*Trichosporon cutaneum*, *Candida krusei*, *C. valida* and *Pichia membranaefaciens*) and lactic acid bacteria (*Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus buchneri* and *Lactobacillus delbrueckii*), grown in co-culture during the fermentation of animal waste and corn were responsible for an increase in the total amino acid content, total nitrogen and protein content of the final product (Hrubant 1985). Moreover, indigenous enteric bacteria, coliforms and faecal streptococci were destroyed and even selected faecal coliforms and *Mycobacterium paratuberculosis* strains added to the media died within 9 th. In addition, the yeast *Saccharomyces boulardii* may be applied as a probioticum in feeds, preventing the development of the toxigenic *Clostridium difficile* (Elmer and McFarland 1987; Castex et al. 1990; Kimmey et al. 1990) and the consequent diarrhoea, leading to an improvement in the performance of steers (Mir and Mir 1994), lactating dairy cows (Swartz et al. 1994), sheep (Jouany et al. 1998) and poultry (Bradley et al. 1994). The pharmacological protective action of yeasts against pathogenic organisms has even been applied in aquaculture. Other than serving as sources of vitamins and proteins, yeasts increase the non-specific local immunity by changing the production and activity of bacterial toxins (Isayev and Nagornaya 1992). The interaction between bacteria and yeasts in aquaculture, however, remains very vague and needs attention.

Megee et al. (1972) described the symbiosis between *Saccharomyces cerevisiae* and *Lactobacillus casei* and indicated that by varying the concentration of the substrate's different types of symbioses like commensalism + competition, competition, and

mutualism and competition were present. When no riboflavin was present in the medium, the bacteria were dependent upon the yeast for supplying the riboflavin, but competed for limited supplies of glucose in the medium when sufficient riboflavin was present. "True commensalism" was reported by Shindala et al. (1965) on the symbiosis between *Saccharomyces cerevisiae* and *Proteus vulgaris* based on an essential niacin-like factor, and between *Saccharomyces cerevisiae* and *Proteus vulgaris* based on riboflavin deficiency both elaborated by the yeasts and required by the bacterium. Challinor and Rose (1954) observed 13 interrelationships between yeasts, mainly *Saccharomyces cerevisiae*, and *Lactobacillus* spp., and in each of them the yeast appears to be the active organism, synthesising the missing substances, like vitamins, amino acids or purines, essential for the growth of *Lactobacillus*. Symbiotic growth in a chemostat between *Acetobacter suboxydans* and *Saccharomyces carlsbergensis* was reported by Chao and Reilly (1972) based on the inability of the yeast to utilise mannitol which was added as the only carbon source, but actively ferments the fructose once it has been oxidised by the bacterium. On the other hand, during alcoholic fermentation of molasses worts, increasing yeast inocula enhanced the lactobacilli growth and contributed to the consumption of monosaccharides liberated during hydrolysis of sucrose by yeasts (Ngang et al. 1992). In a similar way, when *Lactobacillus plantarum* and *Saccharomyces cerevisiae* were grown in co-culture in a glucose–citrate medium under acid conditions, *Saccharomyces* reduced the lactic acid produced by *Lactobacillus* and thereby stabilised the pH, encouraging the fermentation of citrate by the *Lactobacillus* (Kennes et al. 1991a).

4.2.2.2 Yeast–Yeast Interactions

Mixed microbial populations are intentionally applied in industry to improve flavour and yield (Verachtert et al. 1990), to lower pH to inhibit undesired species and to create stability or to obtain desired physiological properties (Harrison 1978). Yeasts are an integral part of these populations and help to secure quality by a range of mechanisms and activities. Detailed yeast–yeast interactions, however, are not studied systematically as observed with bacterial interactions. Other than the most commonly found interrelationship between yeasts, namely the competition for nutrients to survive (Nissen et al. 2004), significant contributions similar to those for bacteria based on symbiosis between yeasts comprise typical mutualism, commensalism, amensalism and predation. These interrelationships have been successfully applied in industry.

Yeast–yeast co-fermentation of glucose and xylose, as obtained after the breakdown of polymers in agricultural waste streams, with immobilised *Pichia stipitis* and *Saccharomyces cerevisiae* resulted in higher ethanol yields from the mixed substrates (Grootjen et al. 1991). The treatment of the effluent of waste starch with *Endomycopsis fibuliger* and *Candida utilis* yielded high concentrations of single-cell protein (Jarl 1969, 1971) when the former hydrolysed the starch to dextrins and low molecular weight sugars, enabling *Candida utilis* to assimilate the soluble products released. The use of mixed yeast cultures for single-cell protein production from *n*-alkanes was used to overcome vitamin requirements. By culturing the biotin-requiring yeasts *Candida novellus*, *Candida tropicalis* or *Pichia sake* with B_1-requiring yeast species, such as *Trichosporon pululans* or *Candida lipolytica*, good growth was

obtained without any added vitamins as the yeasts supply each other's vitamin requirements. Another application of yeasts (*Candida utilis* and a *Mycotorula* sp.) in co-culture, grown on sulphite waste liquor for the production of single-cell protein, contributed to high yields when *Candida utilis* enhanced the growth of the *Mycotorula* sp. The invaluable role of autolysis of yeasts, as a means of indirect interaction between yeasts, should not be overlooked, as the amino acids and vitamins released may encourage the growth of other yeasts (Fleet 2001).

Direct interaction between yeasts mainly relies on the antagonistic interaction involving yeasts capable of producing soluble killer toxins. The secreted proteinaceous killer toxins are lethal to a wide variety of susceptible yeasts and have many potential applications in environmental, medical and industrial biotechnology (Young 1987; Rosini and Cantini 1987; Suzuki et al. 2001; Marquina et al. 2002). Recently, it was observed that zygocin, a protein toxin produced and secreted by the yeast *Zygosaccharomyces bailii* effectively kills pathogenic yeasts like *Candida albicans*, *Candida krusei and Candida glabrata* (Weiler and Schmitt 2003). In the late 1990s, predacious yeasts based on haustorium-mediated predation were also observed (Lachance and Pang 1997) as another means of direct interaction between yeasts. More information on predation between yeasts other than in laboratory situations, however, is needed.

4.2.2.3 Yeast–Filamentous Fungi Interactions

The most prominent interactive relationships between yeasts and filamentous fungi definitely comprise the antagonistic application of yeasts as biocontrol agents against fungi, and the mutualistic relationship with fungi during the processing of predominantly Asian fermented foods. Both topics will be dealt with later in this chapter. Commensalism and mutualism rely on the co-culture of yeasts and filamentous fungi and the latter provide the necessary enzymes to break down complicated substrates like cellulose. A typical example is when *Candida utilis* species contribute to high single-cell protein content when grown in co-culture with the cellulotic *Aspergillus niger* on apple promace (Bhalla and Joshi 1994). The higher yield of protein from the yeast–fungi co-culture relies on the hydrolysis of lignocelluloses by the fungi releasing hexoses and pentoses which the *Candida utilis* can efficiently metabolise.

On the other hand, yeasts exudates may also stimulate hyphal growth like *Rhodotorula mucilaginosa* enhancing the growth of the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora rosea* (Fracchia et al. 2003).

4.3 Yeast Interactions in Foods and Beverages

Microbial communities with their combined physiology, interactions and enzymatic activities are responsible for the major biochemical and nutritional changes that occur in the substrates of fermented foods and beverages (Steinkraus 1982; Hesseltine and Wang 1967; Wood and Hodge 1985; Wood 1981). Antimicrobial effects present in fermented foods and beverages are attributed to organic acids, antibiotic factors, volatile acids, hydrogen peroxide and to a number of substrates

excreted in the products. These antimicrobial effects are the result of the presence of several kinds of microorganisms involved in the fermentation and putrefaction of foods which inevitably lead to beneficial or detrimental interaction among the populations (Noda et al. 1980; Frederickson 1977; Bull and Slater 1982; Slater and Bull 1978).

Microbial interactions involving yeasts, bacteria and/or fungi have been indicated from a number of examinations of food products like bread (Lues et al. 1993), meat, fish, fruit, vegetables, protein foods, dairy products and cereals. The metabolic interactions are governed by the inherent technological characteristics and biochemical activities of yeasts providing essential growth metabolites, such as amino acids, vitamins, removing toxic end products of metabolism, inhibiting the growth of undesired microorganisms by lowering the pH, secreting alcohol, producing CO_2, or encouraging the growth of the starter cultures by increasing the pH owing to the utilisation of organic acids.

These properties have been applied successfully in the processing of foods and beverages as a means of biological control to enhance food safety and shelf life by destroying, retarding or preventing the growth of pathogenic and spoilage microorganisms (Ray and Daeschel 1992; Campbell-Platt 1994). The most successful application of interactions in foods and beverages comprises the presence of yeasts and lactic acid bacteria in a product. The interactions rely on several modes of action; however, despite the many references to the occurrences of yeasts in co-culture with lactic acid bacteria (Wood and Hodge 1985; Steinkraus 1982), only a few researchers have studied the interactions systematically in defined media (Gobbetti et al. 1994a, b; Kennes et al. 1991b). Except for the studies in wine and to an extent in bread making, none of the other fermented foods or beverages have been studied in detail.

Yeast–bacteria associations are by far the most prominent interactions occurring in food and beverage production determining the flavour and other qualities by a range of mechanisms and activities. While lactic acid bacteria, comprising *Pediococcus*, *Leuconostoc*, *Lactobacillus*, *Lactococcus* and *Bacillus*, are the main species associated with fermented products, other species have significant roles. Yeast–yeast associations, on the other hand, are frequently indicated in foods and beverages, but few studies have reported the interactions between the yeasts in detail other than referring to the presence of them and their association with bacteria or moulds. Interactions between the different species occur at various stages throughout production, sometimes as multicultures, unimulticultures or as polycultures. In addition, these interactions may be initiated spontaneously, when the organisms originate from the environment or are inoculated as mixed cultures. These interactions again may appear simultaneously or sequentially to achieve a specific goal. A few typical yeast–bacteria interactions as encountered in foods and beverages are highlighted. As these interactions vary between different products, only the major groups will be discussed.

4.3.1 Microbial Interactions in Fermented Starch-rich Materials

Various fermentations of starch-rich raw materials utilising yeast–lactic acid bacteria associations or mixed cultures including fungi are evident in the literature. The processing involves acid fermentation or alcohol fermentation. Both exhibit distinct

advantages like prevention of spoilage, flavour development, preservation and creation of stability within the products. The fermenting processes relying on alcoholic production will be dealt with later under the heading alcoholic fermentation.

It is important to maintain an equilibrium between the yeasts and the lactic acid bacteria during acid fermentation (Wood 1985; Nout 1991). Excessive acid production by the lactic acid bacteria will result in a decline in the number of surviving yeasts, which consequently leads to a deficiency of growth factors. As a result of such deficiencies, the lactic acid bacteria would produce less acid, and in turn allow an increase in yeast numbers (Nout 1991; Nout et al. 1989). The interactive behaviour between yeasts and lactic acid bacteria creates environmental conditions that protect the products from spoilage by fungi and pathogens owing to the low pH and high compositions of acetic and lactic acids.

4.3.1.1 Cereal Fermentations

Sourdough bread leaven relies on various associative interactions whereby the lactic acid bacteria (*Lactobacillus sanfrancisco*) and yeasts (*Saccharomyces cerevisiae* and *Saccharomyces exiguus*) survive in co-existence (Gobbetti and Corsetii 1997; Gobbetti et al. 1995). The *Lactobacillus* sp. utilises the carbohydrate, maltose, made available owing to amylase action, providing the yeasts with glucose, a stage that may be best described as commensalism, since *Saccharomyces exiguus* strains lack the ability to utilise maltose. However, *Saccharomyces cerevisiae* strains may consume maltose competitively, leading to a decrease in bacterial metabolism (Gobbetti et al. 1994a). Under normal fermentation conditions the yeasts utilise the glucose liberated from the breakdown of maltose and in return produce CO_2 for leavening (Sugihara 1985; Steinkraus 1979). *Lactobacillus sanfrancisco* has a positive influence on yeast leavening and gas production (Gobbetti et al. 1995). A similar positive tendency in yeast fermentation and gas production was observed in the Corleywood baking process by Viljoen and Lues (1993) and Lues et al. (1993). The yeasts secrete compounds like amino acids (Gobbetti et al. 1994b; Spicher and Schroder 1979), peptides (Berg et al. 1981) and vitamins (Spicher and Schroder 1979; Spicher and Nierle 1984) that stimulate the growth of the lactic acid bacteria (Spicher et al. 1981, 1982). Moreover, the yeasts produce phenolic compounds, glycerol (Yong and Wood 1976), etc., which are specific for the aroma (Noda et al. 1980), while the synthesis of antimicrobial compounds by the lactic acid bacteria inhibits spoilage organisms like *Bacillus subtilis*, coliforms and others (Corsetti et al. 1994). The increased protective association in co-culture is expanded in bread by the inclusion of *Propionibacterium freudenreichii* in order to prevent ropy bread induced by *Bacillus subtilis* (Odame-Darkwah and Marshall 1993). Killer activity, however, may cause a serious decrease in the quality of the product if the inoculated yeasts are killed, as indicated in the Turkish baking industry.

This associative interaction between lactic acid bacteria and yeasts, as applied in the processing of sourdough bread (Sugihara et al. 1971; Kline and Sugihara 1971; Wood et al. 1975; Martinez-Anaya et al. 1990; Boraam et al. 1993; Gobbetti et al. 1994a, b, 1995; Oura et al. 1982), is also applied during the production of Pannettone, rye sourdough (Spicher et al. 1981) and soda crackers. For more details, Sugihara (1985) reviewed these processing methods.

Various mixed-culture fermentations are initiated spontaneously in cereal ferments from organisms present in the natural environment, equipment, substrates or through the repeated use of inocula originating from a previous fermentation (Hesseltine 1965, 1983; Verachtert et al. 1990). These mixed inocula may be added simultaneously or sequentially. Unfortunately, most of the cereal fermented foods have been inadequately studied, and contribute little to the modes of interaction between yeasts, bacteria and fungi. It is therefore very difficult to refer to precise interactions as they occur. Most of the literature only refers to the microorganisms present or the biochemical changes, with no indications of interaction.

References to the aspects of the microbiology of ogi preparation are abundant (Akinrele 1970; Odunfa 1999; Banigo and Muller 1972; Odunfa and Adeyele 1987; Banigo et al. 1974). Ogi is a natural fermentation, the microbial flora originate from the maize, sorghum or millet grains (Odunfa 1999; Steinkraus 1982). The grain fungal flora *Aspergillus*, *Penicillium*, *Cephalosporium* and *Fusarium* spp. are eliminated early during the steeping period (Akinrele 1970) and their contribution to the product or other organisms is not clear. The cause for their early elimination is probably due to their inability to compete under the acidity and low oxygen conditions prevailing in the fermenting dough-like mass. *Corynebacterium* hydrolyses the starch and initiates acidification owing to the production of organic acids. *Lactobacillus plantarum* and *Aerobacter cloacae* are also involved in the acidification. The *Lactobacillus* utilises the dextrins from the corn following depletion of the fermentable sugars and contributes most to the acidification by producing lactic acid, while *Aerobacter* increases the niacin and riboflavin content of the mash (Akinrele 1970). The lowering of the pH encourages the yeasts *Saccharomyces cerevisiae* and *Candida mycoderma* to grow, contributing to the flavour and enrichment of vitamins. The lactic acid is a good growth substrate for *Candida*, and the species is therefore considered to play an important role in the preparation of ogi involving the partial destruction of organic acids (Akinrele 1970). Consequently, this will increase the pH and may allow the growth of undesired bacteria. The associative action between the yeasts and the bacteria may therefore be explained as mutualism, since the bacteria create growing conditions for the yeasts by hydrolysing the starch and lowering the pH. The yeasts, in return, provide growth stimulants such as vitamins needed by *Lactobacillus plantarum* (Akinrele 1970) and increase the pH. This was shown earlier with lactic acid bacteria from sourdoughs which required vitamins (Spicher and Schroder 1979) and amino acids to be supplied by yeasts.

Similar associative interactions were observed by Nout (1991) studying the ecology of natural lactic acid fermentation of sorghum-based infant food formulas during repetitive fermentation cycles. During the early fermentation stages, *Leuconostoc* and *Lactococcus* spp. dominated, inhibiting yeast growth owing to excessive production of organic acids. When the nutrients became deficient, *Lactobacillus plantarum* and *Candida* spp. succeeded, which consequently led to an interactive equilibrium. The pH was regulated by the lactic acid bacteria producing organic acids, which allowed adequate yeast growth, and the yeasts supplying the micronutrients enabled the growth of the lactic acid bacteria. Moreover, the fermented mixtures of cereals exhibited a strong antimicrobial effect towards a range of pathogenic bacteria (Nout et al. 1989).

Other cereal fermented foods relying on spontaneous fermentation such as kenkey, koko, banku, panjabi waries, papadams, jalebies, pozol, etc., are prepared in much the same way as described for ogi, although with a different microbial composition. Despite inadequate information on the associative interaction among the microorganisms, the same mutualistic relationships as discussed earlier might be possible. Kenkey fermentation is dominated by *Aspergillus*, *Rhizopus* and *Penicillium* in the initial fermenting stages. The acid-producing *Leuconostoc* spp. soon decrease in numbers during the fermentation, succeeded by *Lactobacillus brevis* and *Acetobacter* spp. in the fermenting dough. Wild types of yeasts, including *Saccharomyces cerevisiae*, are present at all stages of the fermentation, contributing to the flavour by producing esters and ethanol (Muller and Nyarko-Mensah 1972). Koko fermentation comprises the lactic acid bacteria (*Pediococcus cerevisiae*, *Leuconostoc mesenteroides* and *Leuconostoc fermenti*) and yeasts. Panjabi waries and papadams include the yeasts *Saccharomyces cerevisiae* and *Candida* spp., while jalebies are prepared with *Saccharomyces bayanus* (Batra and Millner 1974). Pozol includes fungi (*Geotrichum* and *Mucor*), *Trichosporon* and *Agrobacterium* (Verachtert and Dawoud 1990).

4.3.1.2 Cassava

Cassava is considered a major source of starch-rich food, but with low levels of protein (Steinkraus 1982; Odunfa 1999; Akinrele et al. 1975). The fermentation of gari, the most important fermented cassava product, is anaerobic and follows a two-stage process. In the first stage, *Corynebacterium manihot* and *Bacillus* spp. break down the starch owing to the production of pectinolytic enzymes (Okafor et al. 1984) and release organic acids, which consequently lowers the pH (Collard and Levi 1959; Akinrele 1970). *Bacillus* spp. cause hydrolysis of starch by disintegrating the cell components (Ejiofor and Okafor 1981). According to Okafor (1977), the lactic acid bacteria (*Leuconostoc* and *Lactobacillus*) and *Alcaligenes* are also present during the first stages of fermentation, utilising the free fermentable sugars originating from the tuber and adding to the acidity. Abe and Lindsay (1978) supported by Ngaba and Lee (1979) reported the presence of *Streptococcus faecalis* and claimed that the species is the primary fermentative organism in acidic cassava fermentation. In the second stage, the acid condition stimulates the growth of the fungus *Geotrichum candidum* and presumably also the yeast *Candida*. Collard and Levi (1959) and Akinrele (1970) reported that the fungus added to the acidification, and for the production of aldehydes and esters that are responsible for the taste and aroma. The yeast species quickly proliferates and appeared to be essential as part of the microbiota present during gari fermentation (Okafor 1977). However, no indication of the contributions of the yeasts was reported by any of the authors, despite their growing to numbers as high as 10^6 cfu/g. Moreover, no reference to the associative interaction between the yeasts and the other microorganisms was reported.

During the fermentation of foo-foo, similar microbial populations and interactions were evident, with the exception of *Klebsiella* and the absence of the fungi (Okafor et al. 1984). The *Bacillus*, *Klebsiella* and *Corynebacterium* spp. develop early and contribute to acid formation and the hydrolysis of starch, but are overgrown

by the lactic acid bacteria that further increase the acidic conditions. At the same time, the yeast *Candida* develops in large numbers, and contributes to the lowering of the pH. The prevailing acidified environment permits only these organisms to grow.

4.3.1.3 Fermented Flavouring Products

The production of soy sauce represents a typical sequential inoculation method making use of a two-stage process. The first stage is an aerobic process growing *Aspergillus oryzae* or *Aspergillus sojae* on soybeans and wheat which amylolytically hydrolyses the starch (Yokotsuka 1985; Hesseltine and Wang 1967; Verachtert and Dawoud 1990). As predominant in most mixed-culture fermentations, the fermentation relies on the development of yeasts and lactic acid bacteria. This association is only visible during the second stage. After relying on simple sugars liberated from the first stage, an anaerobic fermentation with *Lactobacillus delbrueckii*, *Pediococcus halophilus* and *Zygosaccharomyces rouxii* takes place. The lactic acid bacteria proceed to grow and produce lactic acid, which decreases the pH, encouraging the growth of *Zygosaccharomyces rouxii*, which results in vigorous alcoholic fermentation (Yong and Wood 1976). Excessive lactic acid fermentation by *Pediococcus halophilus*, however, results in the depression of alcoholic fermentation (Noda et al. 1980). Other osmophilic yeasts such as *Candida etchelsii* and *Candida versatilis* present produce phenolic compounds and furfural, which are desirable flavour enhancers (Morimoto and Matsutani 1969; Yokotsuka 1985; Noda et al. 1980; Wood and Hodge 1985). Similar processes occur in the preparation of miso, except for the use of barley or rice and soybeans, kaffir beer, merissa brewing in Sudan, etc.

4.3.2 Microbial Interactions in Dairy Products

4.3.2.1 Milk-Based Beverages

The commensalistic interaction between *Lactobacillus acidophilus* and the lactose fermenting yeast *Kluyveromyces fragilis* in acidophilus-yeast milk (Subramanian and Shankar 1985) relies on the co-existence of both organisms to secure a good product. Although the lactic acid fermentation originally relied on the fermentation of *Lactobacillus acidophilus* either alone or in mixed cultures with other lactic acid bacteria, the overgrowth of these organisms resulted in fewer viable cells of *Lactobacillus acidophilus*, which consequently reduced the species contribution to gastrointestinal disorders (Lang and Lang 1975). The co-culture of *Lactobacillus acidophilus* with lactose-fermenting yeasts reduces the time of coagulation of the milk owing to acid production by the yeasts, elevates the number of viable lactic acid bacteria cells attributed to stimulating influences of yeasts, and inhibits the growth of *Escherichia coli* and *Bacillus cereus* (Subramanian and Shankar 1985).

Mutualism (synergism) occurs between yeasts and lactic acid bacteria during the fermentation of milky kefir (Rossi 1978) and sugary kefir (Leroi and Pidoux 1993). The predominant species isolated from milky kefir are *Saccharomyces kefir*, *Candida kefyr*, *Lactobacillus caucasicus*, *Lactobacillus casei* and *Leuconostoc* spp. (Oberman 1985; Loretan et al. 2003). The yeasts provide growth factors like amino acids,

vitamins and other compounds for bacterial growth, which consequently lead to elevated acid production, while the bacterial end products are used by the yeasts as an energy source (Challinor and Rose 1954; Wood and Hodge 1985). This phenomenon creates stability in the products. However, a decrease in alcohol production by the yeasts might occur owing to excessive lactic and acetic acid production by osmophilic lactic acid bacteria (Noda et al. 1980; Essia Ngang et al. 1990; Tani et al. 1963), competition for the carbon source or lysis of the yeast cell walls by bacterial enzymes (Lonvaud-Funel et al. 1988; Borregaard and Arneborg 1998).

Similar symbiotic relationships based on acid or alcohol fermentation occur when lactic acid bacteria are responsible for lowering of the pH as a result of the secretion of organic acids (Wood 1981) allowing the yeast population to be competitive in the immediate environment, followed by yeast fermentation as in various milk-based fermentations like Leben, Dahi, Koumiss, etc. (Wood 1981; Bankole and Okagbue 1992; Steinkraus 1982). Oberman (1985) and Vedamuthu (1982) reviewed the fermented milks, whereas Narvhus and Gadaga (2003) reviewed the role of interactions in African fermented milks. The combination of conditions (acidic, saturated with CO_2 and alcohol), is inhibitory to many spoilage bacteria and filamentous fungi and thereby substantially increases the shelf life and safety of the products (Wood and Hodge 1985).

4.3.2.2 Cheese

The production of cheeses involves a maturation stage characterised by a complex ecology of yeasts, bacteria and filamentous fungi (Devoyod and Desmazeaud 1971; Fleet 1990; Jakobsen and Narvhus 1996; Viljoen 2001). The microbial interaction between this microbiota determines the quality, safety and acceptability of the final product.

Several yeasts assist the starter cultures in cheeses by proteolytic activity (Besançon et al. 1992), lipolytic activity (Siewert 1986), the formation of aroma components and participation in the maturation (Welthagen and Viljoen 1999). The positive interaction of yeasts with the starter cultures in surface-ripened cheeses has been well reviewed (Fleet 1990; Jakobsen and Narvhus 1996; Corsetti et al. 2001; Addis et al. 2001). The yeasts, by utilising the accumulated lactic acid in the cheeses, increase the pH and secrete growth factors which promote the growth of *Brevibacterium linens*, which is essential for cheese ripening (Marth 1978). Yeasts also assist the development of fungi in blue-veined and Camembert cheeses (Kaminarides and Laskos 1992; Schlesser et al. 1992) by gas production leading to curd openness (Coghill 1979). In contrast, however, strain-specific interactions between *Yarrowia lipolytica* and *Penicillium roqueforti* may result in the inhibition of mycelial growth and sporulation of the mould mainly owing to competition for nutrients (Van den Tempel and Jakobsen 1998).

Similar yeast–lactic acid bacteria associations were detected in harder cheeses like cheddar (Fleet and Mian 1987; Welthagen and Viljoen 1998, 1999), Parmesan (Romano et al. 1989) and Gouda (Welthagen and Viljoen 1999). On the basis of these associations, Guerzoni et al. (1996) proposed the inclusion of *D. hansenii* and *Y. lipolytica* as adjunct starter cultures during the making of cheese to support the

starter cultures during ripening based on proteolytic and lipolytic activity. In addition, the ability of *D. hansenii* to inhibit *Clostridium* species further adds to the justification (Deiana et al. 1984). Ferreira and Viljoen (2003) applied these yeast species as adjunct starter cultures in cheddar cheese and clearly indicated the mutualistic interaction not only between the yeasts and the lactic acid bacteria, but also between the two yeast strains. When the yeast strains were inoculated individually, a much lower survival was evidenced. The exact mutualistic association between the yeast strains, however, was not clarified other than the indication that both strains survived better when co-inoculated and enhanced flavour development was, detected. Addis et al. (2001), however indicated the yeast–yeast interaction between the two strains in blue-veined cheeses, evidenced by an enhancement in the growth of *Y. lipolytica* caused by *D. hansenii*.

4.3.3 Microbial Interactions in Meat Products

The low initial numbers and reduced growth rates at low temperatures of yeasts in meat products are constraints that prevent them from effectively competing with psychrotrophic bacteria (Walker and Ayres 1970; Dillon and Board 1991; Fleet 1990). However, storage and processing conditions that reduce bacterial competition favour the growth of yeasts (Fleet 1990) and they may cause spoilage or add to the flavour. In fermented meat sausages, when *D. hansenii* species is added as an adjunct starter culture, the species adds a yeast flavour and stabilises the reddening reaction (Hammes and Knauf 1994; Geisen et al. 1992). The sulphite-tolerant species (Banks 1983) *D. hansenii* and *Candida* spp. are responsible for encouraging the growth of pseudomonads and members of the *Entobacteriaceae*, which is usually inhibited by sulphite (Banks et al. 1985). Owing to acetaldehyde production and thereby sulphite binding (Dillon and Board 1991) the yeast species reduce the antibacterial activity of the preservative. Similarly, yeasts utilise organic acids playing a preservative role in processing, and thereby increase the pH, favouring the growth of spoilage bacteria (Walker 1977). No specialised studies on the interaction between these microorganisms, however, have been attempted, and therefore data regarding microbial associations remain very vague.

4.3.4 Microbial Interactions During Vegetable Fermentations

The fermentation and the subsequent storage of olives rely on various interactions between a developing yeast flora and bacteria. During the first phase, when active lactic acid fermentation occurs, fermentable sugars are present in the brine under anaerobic conditions. The strong fermentative yeasts predominate when bacteria are inhibited, outcompeting the other yeasts for the available sugars or they may disrupt the lactic acid fermentation under normal conditions causing "stuck" fermentations (Vaughn et al. 1972). When the available sugars are depleted, oxidative yeasts like *Pichia membranaefaciens* and *Candida mycoderma* develop, utilising the desirable organic acids in the brines and thereby increasing the pH, which allows spoilage bacteria to grow (Mrak et al. 1956). The commonest spoilage incurred by fermenting pectolytic or cellulolytic yeasts during this period is gas formation and softening

(Garcia et al. 1992; Vaughn et al. 1972). Similar results were reported for cucumber fermentation (Vaughn 1983). In contrast to the detrimental effects of yeasts in olive brines, Marquina et al. (1992) reported on significant contributions of yeasts which utilise the lipids present in olives or produce lipases, which resulted in the formation of compounds that stimulate the growth of desirable lactic acid bacteria. Moreover, yeasts utilise the bitter oleuropein, an olive component with antibacterial action, which consequently stimulates bacterial growth (Marquina et al. 1992). Halotolerant yeasts contribute to the flavour (Suzuki et al. 1989), and the occurrence of killer activity might be used to avoid the growth of undesired yeast contaminants.

4.3.5 Microbial Interactions During Alcoholic Fermentations

Several types of fermented beverages which include alcoholic production are evident in the literature derived from fruit, sorghum, rice, barley, plants, etc. Many of the beverages rely on mixed-culture fermentations, reviewed by Wood and Hodge (1985), Steinkraus (1982), Verachtert and Dawoud (1990), Wood (1981), and others.

4.3.5.1 Wine

The interaction between the microorganisms associated with wine fermentation relies on a series of inter-relationships: yeasts–fungi, yeasts–yeasts, yeasts–acetic acid bacteria and yeasts–lactic acid bacteria (Fleet 1992). The existing interactions have been reviewed in detail by Fleet (2003). A typical yeast–filamentous fungi interaction occurs when infection is incurred by *Botrytis cinerea* favouring the presence of non-*Saccharomyces* yeasts and causes a slower fermentation and an increase in glycerol and acetic acid production. The extracts of grapes infected with *Botrytis cinerea* will inhibit (Ribéreau-Gayon 1985) or activate (Reed and Nagodawithana 1988) alcoholic fermentation.

Except for killer yeast activity (Young 1987; Shimizu 1993; Guriérrez et al. 2001), the recognition of non-*Saccharomyces* yeasts as important contributors to wine fermentation (Fleet et al. 1984; Heard and Fleet 1987; Martinez-Anaya et al. 1990; Mora et al. 1992; Schutz and Gafner 1993) results in various yeast–yeast associations that can be exploited. Other than the production of ethanol, organic acids, sulphur, etc. (Fleet 1990, 2001; Bisson 1999, Soden et al. 2000; Mills et al. 2002) by some yeasts, inhibitory to the growth of competing yeasts, the medium-chain fatty acids, decanoic and octanoic acids (Lambrechts and Pretorius 2000), their corresponding ethyl esters (Lafon-Lafourcade et al. 1984; Ribéreau-Gayon 1985) and yeast ghosts (Edwards et al. 1990) produced all contribute to yeast–yeast interactions. Inhibitory effects by *Kloeckera apiculata* against *Saccharomyces cerevisiae* (Mortimer 2000) and *Metschnikowia pulcherrima* against a range of other yeasts have been reported (Nguyen and Panon 1998). The interaction between the non-*Saccharomyces* and *Saccharomyces* species based upon competition for carbohydrates, nitrogen, other compounds and dominance during the fermentation remains largely unexplored.

Yeast–bacteria interaction in wine production relies predominantly on the yeast association with the lactic acid bacteria and acetic acid bacteria. Detailed studies on

the combined growth of acetic acid bacteria (*Acetobacter aceti*, *Acetobacter pateurianus* and *Gluconobacter oxydans*) and wine yeasts (*Saccharomyces cerevisiae*, *Kloeckera apiculata* and *Candida* spp.) were performed (Lafon-Lafourcade et al. 1984; Drysdale and Fleet 1988, 1989). Antagonistic effects by *Acetobacter*, due to acetic acid excretion, result in decreased fermentation by *Saccharomyces cerevisiae* and may cause stuck fermentation (Ludovico et al. 2001).

Wine yeasts vary in their interaction with lactic acid bacteria (Fornachon 1968; Thornton 1991; Suzzi et al. 1995) as they may inhibit or stimulate the growth of lactic acid bacteria. The naturally present lactic acid bacteria occur at low numbers, and die during alcoholic fermentation (Fleet 1993, 2003; Fleet and Heard 1993) and exert little or no effect on yeast growth. If the alcoholic fermentation is restricted or retarded, multiple yeast–lactic acid bacteria interrelationships occur that play a substantial role during malolactic fermentation (King and Beelman 1986; Lemaresquier 1987; Markides 1993; Fleet 1990; Martineau and Henick-Kling 1995), which commenced after alcohol fermentation. The antagonism of the yeasts is related to alcohol production (Wibowo et al. 1985), SO_2 (Wibowo et al. 1988), proteins (Dick et al. 1992), fatty acids (Edwards et al. 1990; Edwards and Beelman 1987; Lonvaud-Funnel et al. 1988; Lafon-Lafourcade et al. 1984), antibacterial factors (Fornachon 1968) and the removal of substances important to bacterial growth (King and Beelman 1986). Growth stimulation of the lactic acid bacteria is encouraged by yeast autolysis (Fleet 1992; Charpentier and Feuillat 1993; Crouigneau et al. 2000), removal of inhibitory fatty acids (Edwards and Beelman 1987), yeast ghosts (Lafon-Lafourcade et al. 1984), amino acids (Lonvaud-Funel et al. 1988), vitamins (Lemaresquier 1987), sucrose hydrolysis (Ngang et al. 1992) and ethanol at low concentrations (King and Beelman 1986).

Although amensalism is indicated during wine fermentation, whereby the *Saccharomyces cerevisiae* strains prevent the growth of initially present non-conventional wine yeasts owing to elevated concentrations of ethanol, useful commensal relationships between yeasts occur when wine is allowed to become partially aerobic, leading to the formation of sherry (Amerine and Kunkee 1968; Carr et al. 1969). According to these authors, the interaction between the flor-filming yeasts relies on competitive, amensal and commensal relationships. In addition, neutralism is reported as *Saccharomyces diasticus* strains possess glucoamilase, which enables them to ferment polysaccharides which cannot be metabolised by other yeasts found during beer and wine fermentations.

4.3.5.2 Fruit Juices and Cider

The yeast–bacteria interrelationship between *Saccharomyces cerevisiae* and *Leuconostoc oenos*/*Lactobacillus plantarum* plays an important role in the degradation of glucose, malate and citrate, the major carbon sources in fruits and fruit juices like orange and cider, during fruit fermentations under acidic and anaerobic conditions (Kennes et al. 1991a, b). The microbial ecology follows the principles of wine fermentations. The microflora of the apples includes yeasts (*Saccharomyces cerevisiae*, *Kloeckera apiculata* and *Candida* spp.), lactic acid bacteria (*Lactobacillus brevis*, *Pediococcus* spp., *Leuconostoc mesenteroides* and *Leuconostoc oenos*) and

acetic acid bacteria (*Acetobacter* and *Gluconobacter* spp.). The non-proliferating population of yeasts initiates the fermentation, but is inhibited by a lack of nutritional growth factors and the toxic effect of ethanol owing to competition and amensalism from the ethanol-tolerant species *Saccharomyces cerevisiae*. Similarly, competition for nutrients between lactic acid bacteria and the yeast also exists as well as positive or negative contributions from the indigenous microflora present. The yeast outcompetes the lactic acid bacteria for the utilisation of the sugars, which results in the production of ethanol without changing the pH. The ethanol present in the media favours the subsequent conversion of citric acid (with oranges) or malic acid (with apple juice) to acetic acid by the lactic acid bacteria *Lactobacillus plantarum* and *Leuconostoc oenos*, respectively (Kennes et al. 1991a). *Leuconostoc oenos* usually fulfils a similar role during wine fermentation (Fleet et al. 1984), although it has been proposed that *Schizosaccharomyces pombe* can remove excess organic acids with inferior results. Although the lactic acid bacteria compete for carbohydrates, they also depend on essential stimulants excreted by the yeasts as reported earlier. Similar competitive/commensal interrelationships may occur between *Pediococcus cerevisiae* and the yeasts during beer production when the bacteria form diacetyl, which spoils the taste of the beer, or polysaccharides, which cause ropiness.

4.3.5.3 Beer

Beer is a product derived from malted barley, hydrolysed under controlled conditions by amylases to maltose and glucose to make it available to yeasts which produce ethanol (Rainbow 1981; Priest and Campbell 1996). Under normal conditions, the inoculated brewer's yeast *Saccharomyces cerevisiae* quickly dominates during fermentation and suppresses the growth of spoilage *Enterobacteriaceae*, lactic acid bacteria and other competitive microorganisms by elevated alcohol concentrations, low pH, CO_2 production, SO_2, co-sedimentation and organic acid secretion, while the anaerobic conditions that prevailed prevent the growth of aerobic acetic acid bacteria (Jespersen and Jakobsen 1996). Brewer's yeast inhibition of lactic acid bacteria is also attributed to competition for nutrients (Pfenninger et al. 1979), heat-labile yeast metabolites (Dolezil and Kirshop 1980), alanyl dipeptides and co-sedimentation of certain bacteria with brewing yeasts (White and Kidney 1979, 1981). The hop bitters present also inhibit the growth of lactic acid bacteria. Competition for nutrients, low pH conditions and hops, moreover, inhibits *Pediococcus damnoses* and related *Pediococcus* spp. However, if insufficient cleaning, heating, hops-resistant bacteria (Fernandez and Simpson 1995) and delays in pitching of the wort occur, beer contaminants derived from barley, wort and the equipment may cause spoilage (Flannigan 1996). Filamentous fungi affect the flavour of the beer and mycotoxins have a concentration-dependent effect on yeasts, resulting in reduced CO_2 evolution and ethanol production. Similarly, *Pediococcus* spp. and *Lactobacillus* spp. contribute to the flavour (Priest 1996) and may compete for the available nutrients. The role of "wild yeasts" such as *Pichia membranaefaciens*, *Pichiasubpelliculosa* and species of *Schizosaccharomyces*, *Brettanomyces*, *Kloeckera*, *Debaryomyces*, *Candida* and *Torulaspora* is well reviewed by Rainbow (1981) and Campbell (1996). Although these yeast contaminants are able to grow under anaerobic conditions (Campbell

and Msongo 1991), they do not compete well under the acid and ethanol concentrations of the beer. Access of air stimulates their growth, and competition for the nutrients. Killer strains of *Saccharomyces cerevisiae* may cause the severest competition. The killer strain kills sensitive culture yeast strains, and establish itself as the dominant yeast of the fermentation. Yeast autolysis, which occurs after lengthy secondary fermentation, and nitrogen released by *Saccharomyces carlsbergensis* may also encourage microbial growth in providing essential growth stimulants.

Verachtert et al. (1990) in unravelling the complex mixed-culture process during Lambic and Gueze beer fermentation identified a succession of different microbial species during four fermentation phases. The spontaneous fermentation starts with the development of *Enterobacteria* and low numbers of maltose non-fermenters such as *Kloeckera apiculata*, *Saccharomyces globosus* and *Saccharomyces diarensis*. When these yeasts disappear, a second group of fermenting yeasts, *Saccharomyces cerevisiae* and related *Saccharomyces* spp., are responsible for ethanol fermentation, followed by actidione-resistant yeasts belonging to *Brettanomyces* that metabolise the sugars not assimilated by *Saccharomyces*. A fourth group of oxidative yeasts remain in the yeast layer found on top of the fermenting wort with no significance to the fermentation. After the main ethanol fermentation, lactic acid bacteria, usually *Pediococcus* spp., are responsible for the synthesis of lactic acid, adding to the acid–vinous character of the beer. Despite clear indications of numerous yeast–yeast interactions present during the production of these beers, the data remain vague and need attention.

4.3.5.4 Distilled Beverages

Similar interesting interrelationships may occur in distilled alcoholic beverages, such as whiskey and rum. But, as with beer, the interactions between yeasts, fungi and bacteria have been inadequately studied, despite numerous references to spoilage (Barbour and Priest 1988) and advances in brewing and distilling yeasts. Whiskey fermentation relies on a mixed fermentation of added yeast (*Saccharomyces cerevisiae*) and indigenous bacteria (Barbour and Priest 1988). The fermentation comprises the bacterial species *Lactobacillus*, *Enterobacteriaceae*, *Peddiococcus* and *Leuconostoc*, which originate from the malted barley and equipment. *Enterobacteriaceae* spp. are eliminated in the early stages of the fermentation by the low pH and alcohol concentration, while the lactic acid bacteria proliferate and compete with the yeast, which reduces the ethanol yield. The yeast, however, encourages the growth of lactic acid bacteria by the excretion of glycerol and products due to yeast autolysis (Barbour and Priest 1988).

4.4 Yeast Antagonism Applied as Biocontrol Agents in Preventing Plant-Spoilage Fungi

Numerous yeasts capable of playing a significant role in interactions have been isolated from fruit, fermented products, soil and other natural environments over the last few decades (Fleet 2003). However, our knowledge of the ecological distribution of such yeasts is still very limited. The interest of biological control in the

ecology of representative yeasts species arises from the necessity to control their metabolic activity by factors that can be influenced by technological means. Ideally it should be possible to predict that a yeast which possesses certain characteristics regarding biocontrol occurs most frequently in a certain environment within a defined habitat.

There is little doubt that various yeasts afford some protection to post-harvest spoilage (Chalutz and Wilson 1990; Chalutz and Droby 1998); consequently there is renewed interest in the possibilities of harnessing and accentuating the mechanisms of biological control as awareness of the dangers and disadvantages and public resistance to chemical control by fungicides posing potential oncogenic risks increase (Wilson and Wisniewski 1989). Moreover, the stresses of modern concepts of quality assurance require products of a high standard in quality, and biological control contributes to an improvement in hygienic safety, constant levels of quality and shelf life.

During the last decade a steady flow of reports claimed that particular post-harvest diseases and temperate fruit can be controlled to some extent by the antagonistic interaction between yeasts and mycotoxic fungi (Guinebretiere et al. 2000). Some of these yeasts like *Candida oleophila* and *Pseudozyma flocculosa* have been commercialised, known as Aspire and Sporodex, respectively (Droby et al. 1998; Punja and Utkhede 2003). Yeast antagonistic efficiency is also successfully reproduced in the inhibition of spoilage or toxin-producing fungi in high-moisture wheat stored under airtight conditions (Petersson and Schnurer 1995; Bjornberg and Schnurer 1993).

The high efficiency of yeasts applied as biocontrol agents is related to their indigenous adaptation to the immediate environment, the nutritional conditions prevailing at the wound site (Chalutz and Wilson 1990), their resistance to fungicides, survival at varying temperatures and ability to colonise (Roberts 1990). To evaluate the usage of yeasts as biocontrol agents, an understanding of the antagonistic interaction between the yeasts and fungal pathogens is needed.

Since limited evidence related to the production of antimicrobial compounds is evident, alternative ways of inducing biocontrol activity have been claimed. According to Avis and Belanger (2001) the antifungal metabolites produced by *Pseudozyma flocculosa* are a mixture of fatty acid containing derivatives that affect membrane permeability of the target organisms, thereby inhibiting the growth of powdery mildews. Nutrient competition, however at the wound site is regarded as the principal mode of antagonism (Droby et al. 1989; Punja and Utkhede 2003), although other modes of action like induced resistance, pathogen and antagonist density, age of the cells (Droby et al. 2002), cell wall degradation by β-(1-3)-glucanase enzymes (Wisniewski et al. 1991), killer toxins produced by yeasts (Walker et al. 1995), antifungal toxins like zygocin (Weiler and Schmitt 2003), and attachment of the yeasts by cell-surface proteins or lectin (Wisniewski et al. 1991).

Naturally occurring yeast antagonists like *Metschnikowia pulcherrima*, *D. hansenii*, *Pichia anomala* and *Pichia guilliermondii* are continuously isolated and reapplied to the fruit or silaged grain as effective biocontrol agents. Scientists will continue to collect more species, as our current knowledge regarding yeasts as biocontrol agents has only just begun.

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Chapter 5

Physiological and Molecular Responses of Yeasts to the Environment

Graeme M. Walker[1] and Patrick Van Dijck[2]

[1]*Division of Biotechnology & Forensic Science, School of Contemporary Sciences, University of Abertay Dundee, Bell Street, Dundee DD1 1HG, UK (e-mail: g.walker@abertay.ac.uk)*
[2]*Flanders Interuniversity Institute for Biotechnology, Department of Molecular Microbiology, VIB10, Laboratory of Molecular Cell Biology, K.U. Leuven, Institute of Botany & Microbiology, Kasteelpark Arenberg 31, 3001 Leuven, Belgium (e-mail: patrick.vandijck@bio.kuleuven.be)*

5.1 Introduction

Understanding the ways by which yeasts respond to changes in their physicochemical environment is very important in the food and beverage industries. For example, it is important for the maintenance of yeast viability and vitality in the production and utilisation of yeasts for food and fermentation processes, and it is additionally important for the control of yeasts that act as spoilage agents of foods and beverages. In the former situation, yeasts are confronted with several environmental stresses including insults caused by changes in temperature, pH, osmotic pressure, ethanol concentration and nutrient availability that individually or collectively can deleteriously affect yeast physiology. These changes may result in lowered yeast growth yield and impaired fermentation performance. In the case of food spoilage yeasts, such organisms have adapted to survive stress caused by low temperature and oxygen levels, anhydrobiosis and high salt/sugar concentrations and their effective elimination is often based on measures to counteract the inherent stress tolerance of these yeasts. Chapter 11 covers food spoilage yeasts in more detail.

The present chapter describes both physiological and molecular aspects of stress on yeast cells and will focus on yeasts' responses to changes in their environment which are pertinent in situations where survival of the yeast is both desirable (e.g. industrial fermentations) and undesirable (e.g. foods and beverages spoilage). The stresses of particular relevance for the food industry are thermostress, pH shock, osmostress, nutrient starvation, ethanol toxicity, oxidative stress, prolonged anaerobiosis, and exposure to chemical preservatives. This chapter will not review biologically related stress factors in yeasts such as cellular ageing, genotypic changes and competition from other organisms, the last of these having been dealt with in Chap. 4.

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
© Springer-Verlag Berlin Heidelberg 2006

5.2 Yeast Nutrition and Growth

5.2.1 General Comments About Cell Physiology of Important Food Yeasts

The premier industrial yeast *Saccharomyces cerevisiae* is widely employed in the production of foods and fermented beverages. As such, it is by far the most economically important microorganism known to mankind. The metabolic activities of *S. cerevisiae* have been exploited for millennia in the leavening of bread and in the fermentation of cereal wort and grape must – these activities will continue to be exploited for future millennia. Why has *S. cerevisiae* found such dominance in baking and alcoholic beverage production? The reasons lie both in the ability of numerous "industrial" strains of *S. cerevisiae* to effectively transform sugars into ethanol, carbon dioxide and numerous secondary flavour compounds and it's ability to withstand stress caused primarily by temperature, osmotic pressure, ethanol toxicity and competitive bacteria and wild yeasts. Figure 5.1 summarises major stresses encountered by industrial fermentation (brewing) yeast strains. Of course, most yeasts are similarly able to ferment sugars, but they may not be able to tolerate the rigours of a large-scale industrial fermentation plant. *S. cerevisiae* is clearly able to do so and has found niches well-suited to it's physiological behaviour in wineries and fermentation plants (Martini 1993; Vaughan-Martini and Martini 1995). In short, *S. cerevisiae* is arguably the most resilient industrial yeast that we currently have at our disposal. Nevertheless, new approaches to improve stress-tolerance of *S. cerevisiae* have been developed with potential benefits for food and beverage production processes (Chap. 13).

Stress-tolerance attributes in other yeast species also impact significantly in foods and beverages. Several non-*Saccharomyces* yeasts have also found beneficial production applications, whilst some species are detrimental after production in storage situations, especially with regard to yeast spoilage of high-sugar and high-salt foods. Some examples of stress-tolerant yeasts important in both food production and spoilage are listed in Table 5.1.

5.2.2 Major Nutrients Encountered in Foods/Fermentation Media for Yeast Growth

Foods and beverages designed for human nutrition also represent rich sources of nutrients for yeasts. In the production of alcoholic beverages, substrates such as malt wort or wine must are complete nutrient sources and contain fermentable sugars (mainly maltose in the case of wort, and glucose and fructose in wine must), assimilable nitrogen (amino acids), minerals, vitamins and oxygen (necessary in initial stages of fermentation), together with minor growth factors. Occasionally, the availability of certain key nutrients in fermentation media may be limiting and this can adversely affect efficiency of yeast fermentation processes. For example, if there is insufficient oxygen available in the initial stages of fermentation, *S. cerevisiae* cells will be unable to synthesise ergosterol and unsaturated fatty acids, which are essential plasma membrane components. In turn, this can affect the ability of cells to with-

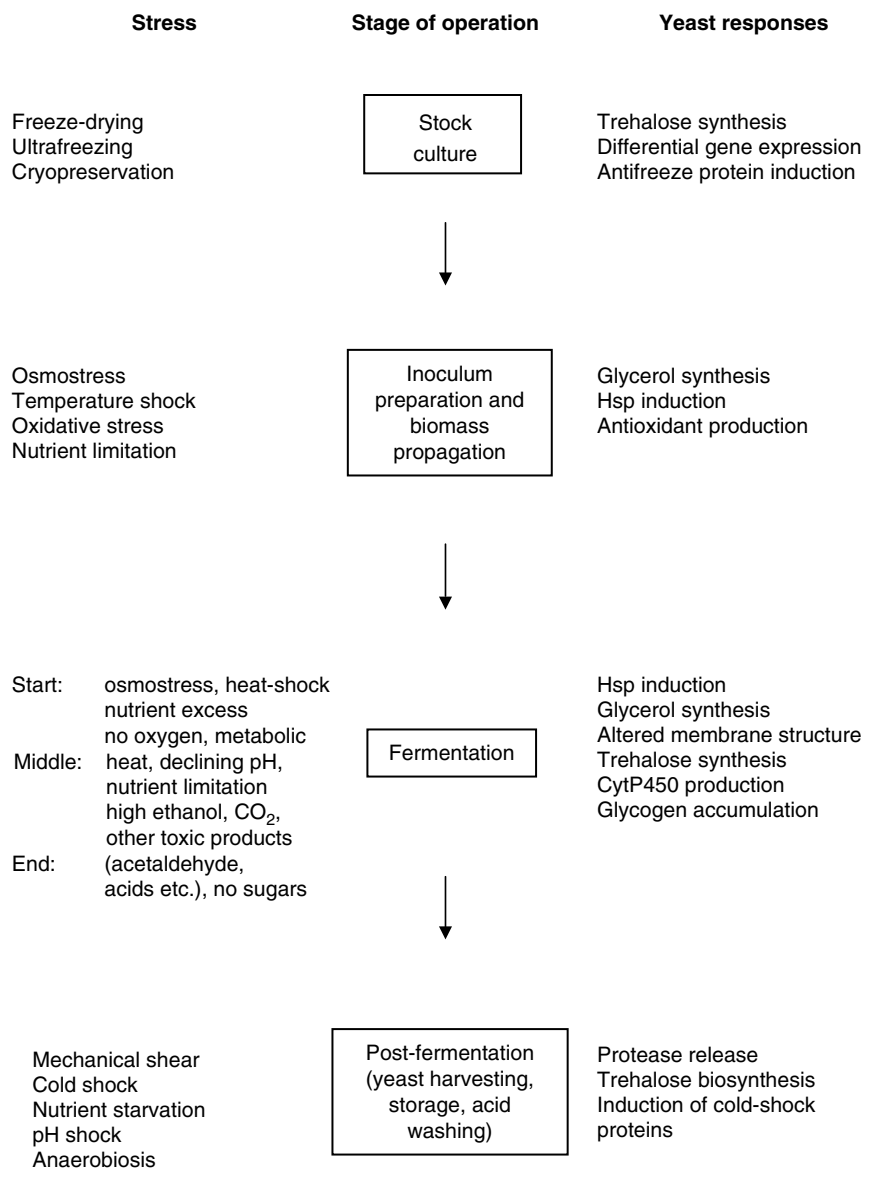

Fig. 5.1. Stresses encountered by industrial fermentation (e.g. brewing) yeasts

Table 5.1 Some important food yeasts

Yeast genus	Importance in foods
Candida spp.	Production of microbial biomass protein, vitamins, citric acid. Some food spoilers (e.g. *C. zeylanoides* in frozen poultry)
Cryptococcus spp.	Some strains are used as biocontrol agents to combat fungal spoilage of post-harvest fruits. *C. laurentii* is a spoilage yeast (poultry)
Debaryomyces spp.	*D. hansenii* is a salt-tolerant food spoiler. It is also used in biocontrol of fungal fruit diseases
Kluyveromyces spp.	Fermentation of lactose in cheese whey for potable alcohol; source of food enzymes (pectinase, microbial rennet, lipase); cocoa fermentations. Some species are spoilage yeasts in dairy
Metschnikowia spp.	*M. pulcherrimia* is used in biocontrol of fungal fruit diseases. Osmotolerant yeasts
Phaffia spp.	*P. rhodozyma* is a source of astaxanthin food colorant
Pichia spp.	Production of microbial biomass protein, riboflavin (*P. pastoris*). *P. membranifaciens* is an important (surface-film) spoiler of wine and beer
Rhodotorula spp.	*R. glutinis* is used as a source of food enzymes (lipases). Some species are food spoilers of dairy products
Saccahromyces spp.	*S. cerevisiae* is used in traditional food and beverage fermentations (baking, brewing, winemaking, etc.); source of savoury food extracts and food enzymes (e.g. invertase). It is also used as a livestock growth factor. *S. bayanus* is used in sparkling wine fermentations. *S. diastaticus* is a wild yeast spoiler of beer. *S. boulardii* is used as a probiotic yeast
Schizosaccharomyces spp.	*S. pombe* is used in traditional African beverages, rum and for wine deacidification. It is regarded as an osmotolerant yeast
Schwanniomyces spp.	Microbial biomass protein (from starch – *S. castellii*)
Yarrowia spp.	*Y. lipolytica* is used in production of microbial biomass protein, citric acid and lipases
Zygosaccharomyces spp.	*Z. rouxii* and *Z. bailii*, being osmotolerant, are important food and beverage spoilage yeasts. *Z. rouxii* is also used in soy sauce production

stand the toxic effects of ethanol produced later in fermentation. Limited availability of metal ions can also influence fermentation performance of yeasts (Birch et al. 2003). For example, in brewing, zinc deficiency may result in slow or incomplete fermentations (Walker 2004). In addition, during fermentation, the concentrations of various nutrients change and yeasts must respond dynamically to such changes. Knowledge of physiological and molecular responses of yeasts to the concentration and availability of specific nutrients is of particular interest for the alcoholic beverage industries because these responses will impact on the progress and efficiency of fermentation. In winemaking, yeast starter culture strain selection focuses on good nutrient utilisation as well as on resilience to unfavourable growth environments (Bauer and Pretorius 2000). These two characteristics are inextricably linked.

For food spoilage yeasts, in addition to nutrient availability, physico-chemical conditions play important roles in dictating the extent of spoilage caused. For example, high osmotic pressure caused by high-sugar- or high-salt-containing foods represents hostile conditions for the survival and growth of most yeasts, but not for osmotolerant strains of the spoilers *Zygosaccharomyces rouxii* and *Debaryomyces hansenii*, respectively. Such yeasts have clearly adapted to such adverse environments by sensing the external stressor(s) and responding accordingly to maintain their survival. Again, knowledge of these responses can assist in control of yeast food spoilage.

5.2.3 Nutrient Sensing and Translocation

Nutrients exert a myriad of effects on living cells as a consequence of their function in both supplying energy and providing substrates for biosynthesis and catabolism. In addition, nutrients exert many regulatory effects that are mediated by nutrient-sensing systems, sometimes largely or even completely independent of their metabolism. Such systems have extensively been studied in yeasts during recent years (see reviews by Boles and André 2004; Holsbeeks et al. 2004). At the level of nutrient sensing, one can distinguish genuine receptor proteins and transporter-like receptor proteins. The first category includes the G-protein-coupled receptor Gpr1 and the second category includes glucose and amino acid transporter-like receptors. Whereas these proteins lost their capacity to transport the ligand and only function as sensors, some active transporters are also able to function as a sensor.

For yeast cells, glucose is the most important nutrient and therefore it is not surprising that yeast cells have developed a number of glucose-sensing pathways. The glucose-repression pathway, the Rgt2-Snf3 glucose-sensing pathway and the glucose-induced cyclic AMP (cAMP)–protein kinase A (PKA) pathway have recently been reviewed (Geladé et al. 2003; Rolland et al. 2002).

The *S. cerevisiae* G-protein-coupled receptor Gpr1 belongs to a small subfamily of the large GPCR family (Graul and Sadée 2001). Sequence database searches have revealed other closely related members of this GPCR subfamily in other fungi like *Schizosaccharomyces pombe*, *Candida albicans*, *Aspergillus* sp., *Neurospora crassa* and other *Saccharomyces* species (Versele et al. 2001 and unpublished results). Whether all the Gpr1 homologues have glucose as their ligand remains to be seen. Recently evidence against glucose as the ligand of the CaGpr1 has been obtained. Moreover, there is evidence that in *C. albicans* this receptor may be a methionine sensor (Maidan et al. 2005). The *S. cerevisiae* G-protein-coupled receptor Gpr1 senses sucrose and glucose in the millimolar range, whereas mannose acts as an antagonist (Lemaire et al. 2004). Binding of glucose or sucrose to the receptor activates the Gα protein Gpa2 (Colombo et al. 1998). Apart from the receptor, Gpa2 also interacts with Gbp1/Krh2 and Gbp2/Krh1, two proteins that have been proposed to act as Gβ-mimicking subunits on the basis of structural resemblance with classical Gβ proteins (Battle et al. 2003; Harashima and Heitman 2002) and also with Plc1 (Ansari et al. 1999). The activity of Gpa2 is controlled by the RGS protein Rgs2 (Versele et al. 1999). Hence, a GPCR system composed of Gpr1, Gpa2 and Rgs2 has been proposed to act as a glucose-sensing system for control of the cAMP pathway (Thevelein and de Winde 1999; Versele et al. 2001). An unusual feature of this GPCR system is that the rapid stimulation of cAMP synthesis requires a low level

of glucose phopshorylation by any one of the three glucose kinases encoded by *GLK1*, *HXK1* and *HXK2*. This means that partial metabolism of the ligand by phosphorylation is required to sustain stimulation of the adenylate cyclase encoded by *CYR1* (Rolland et al. 2002). Activation of Gpa2 stimulates adenylate cyclase and the cAMP that is produced results in the activation of PKA, resulting in a plethora of changes (Thevelein and de Winde 1999).

Important downstream targets of the PKA pathway are the redundant Msn2 and Msn4 transcription factors that interact with the stress response element (STRE) and the Gis1 transcription factor that interacts with the post-diauxic shift (PDS) element. These transcription factors are under negative control of the PKA pathway. Conditions that result in a high PKA phenotype (no stress, cells growing on glucose) result in phosphorylated Msn2 and Msn4 which are localised to the cytoplasm. The cytoplasmic localisation is also regulated by the target of rapamycin (TOR) kinase pathway. Glucose activates TOR kinases that inhibit the dephosphorylation of Msn2 (Beck and Hall 1999). Upon various stress conditions or under nutrient limitation, the PKA activity is low, the transcription factors are dephosphorylated and are translocated into the nucleus, where they regulate gene expression. Inside the nucleus, the Msn2 protein is rapidly degraded despite a constant MSN2 messenger RNA (mRNA) level (Bose et al. 2005; Durchschlag et al. 2004; Lallet et al. 2004). The degradation of Msn2 is dependent on the cyclin-dependent protein kinase Srb10, a member of the transcription machinery. The degradation of Msn2 upon heat shock is mediated via the 26S proteasome (Lallet et al. 2004). Among the targets regulated by the Msn2/4 transcription factors are heat shock protein (Hsp) encoding genes, such as *HSP12* and *HSP104*, which are rapidly repressed upon activation of the PKA pathway (Marchler et al. 1993; Varela et al. 1995). These proteins play important roles in various processes that help yeast cells cope with a broad array of stresses, including heat and ethanol stress (Piper 1995, 1997; Sanchez et al. 1992). Furthermore, high PKA activity also causes repression of the trehalose synthase-encoding genes *TPS1* and *TPS2* (Winderickx et al. 1996). Trehalose plays a prominent role in cellular stress resistance by protecting membranes from desiccation and by preventing protein denaturation (reviewed by Bonini et al. 2004).

A similar phosphorylation-dependent nuclear localisation has recently been shown for the Crz1p/Tcn1p transcription factor. Under normal growth conditions, Crz1p is negatively regulated by PKA phosphorylation and localises to the cytosol (Kafadar and Cyert 2004). Under specific stress conditions, such as high salt, alkaline pH or cell wall damage, this transcription factor is dephosphorylated by calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, translocating it into the nucleus, where it activates the expression of genes whose products promote adaptation to stress (Yoshimoto et al. 2002). In the nucleus, the Czr1 transcription factor binds to the calcineurin-dependent response element (CDRE). All known calci-neurin-dependent transcriptional changes (ion homeostasis, cell wall maintenance, vesicle transport, lipid biosynthesis and small molecule transport) are believed to be mediated through Crz1.

The role of glucose and sucrose signalling through the cAMP–PKA pathway during industrial applications has recently been reviewed (Verstrepen et al. 2004). As mentioned before, the nutrient mixture during the production of wine and beer is very different from the nutrient mixture on which yeast cells are grown in the labora-

tory. The main sugars in grape must are glucose and fructose; beer wort contains glucose, fructose, sucrose, maltose and maltotriose, and the fermentation medium for ethanol production is usually a mixture of any of these sugars in variable concentrations, depending on the origin of the substrate (fermentation feedstock) (Bamforth 2003; Yoon et al. 2003). In most cases, the initial concentration of glucose and/or sucrose in the growth medium is well above the threshold concentrations (20–40 mM) for induction of the sugar signalling cascades (Meijer et al. 1998; Meneses et al. 2002). Hence, both the main glucose-repression pathway and the Ras–cAMP–PKA pathway are triggered at the start of the process. This has three major consequences: the repression of respiration, the arrest of the consumption of other carbohydrates and the loss of cellular stress resistance (Thevelein and de Winde 1999; Van Dijck et al. 1995). While these effects help *S. cerevisiae* survive in its natural habitat, sugar signalling causes several problems in various yeast-based industrial processes.

The low stress resistance during active fermentation of yeasts is disadvantageous for their use in industrial applications (Attfield 1997). A striking example will be discussed later when we describe the process of frozen dough preparation.

In the last few years, genome-wide expression analysis after the addition of glucose or other nutrients to yeast cells has been performed. The results obtained with these arrays have confirmed the strong interconnections between different pathways (Schneper et al. 2004). To determine the function of some of the components in the Ras–cAMP–PKA pathway, various research groups have performed experiments by addition of glucose to mutants in this pathway (Jones et al. 2003, 2004; Lin et al. 2003; Roosen et al. 2005; Wang et al. 2004). These arrays identified novel targets of the cAMP–PKA pathway, including targets that play a role in cell wall biogenesis. The experiments also identified new transcription factors and response elements in regulated promoters. Many of the targets are redundantly activated by a Ras–cAMP–PKA-dependent pathway and by one or more PKA-independent pathways.

Other examples of nutrient-sensing plasma membrane proteins have been discovered in yeasts. Snf3 and Rgt2 are two non-transporting homologues of glucose carriers which are proposed to have a function in glucose sensing (Özcan et al. 1998) and Ssy1 is a non-transporting homologue of amino acid carriers which is proposed to have a function in amino acid sensing (Didion et al. 1998; Iraqui et al. 1999; Klasson et al. 1999).

Snf3 and Rgt2 have a role in the glucose-induced expression of specific glucose transporter genes. The Snf3 protein senses low levels of glucose and is responsible for inducing several *HXT* genes in response to low glucose. The Rgt2 protein senses high levels of glucose and is responsible for inducing *HXT1* in response to high glucose. Both sensor proteins have a long C-terminal cytoplasmic tail that is required for signalling. Recently, the plasma membrane-associated protein kinase casein kinase I was identified as a signal transmitter in Rgt2-mediated glucose signalling. It is now proposed that glucose binding to Rgt2 activates casein kinase I, which then phosphorylates Mth1 and Std1, two proteins that were previously shown to interact with the C-terminal tail of Snf3 and Rgt2. This phosphorylation leads to their degradation and finally to the induction of *HXT* gene expression (Moriya and Johnston 2004; Özcan et al. 1998). Mth1 inhibits the interaction between two different domains of the Rgt1 transcription factor. This intramolecular interaction inhibits DNA binding and proper

expression of the *HXT* genes. The current hypothesis is that Mth1 promotes transcriptional repression by Rgt1 by binding to it and preventing the intramolecular interaction, thereby enabling Rgt1 to bind to DNA, resulting in Rgt1-dependent repression of *HXT* genes in the absence of glucose and Rgt1-dependent activation of *HXT1* gene expression in the presence of high levels of glucose (Polish et al. 2005).

A similar sensing system has been described for amino acid transporter induced expression. The amino acid sensing protein Ssy1 forms a sensor complex with Ptr3 and Ssy5 called the SPS complex. Activation of the SPS complex by amino acids triggers the proteolytic activation of two latent transcription factor precursors, Stp1 and Stp2, that are located in the cytosol, and the active proteins are then targeted to the nucleus, where they induce expression of specific amino acid transporter genes (Andreasson and Ljungdahl 2002). An extensive review on both glucose and amino acid transporter-like sensors has recently been published (Boles and André 2004).

Apart from the non-transporting sensors, there also exist active transporting nutrient permeases that also function as sensors (reviewed by Holsbeeks et al. 2004). These include Mep2 (ammonium permease; Lorenz and Heitman 1998), Gap1 (general amino acid permease; Donaton et al. 2003) and Pho84 (phosphate transporter; Giots et al. 2003). In these proteins, specific mutations have been identified that distinguish transport and sensing capacity. These three permeases are important for activation of PKA targets upon sensing their respective ligands. The downstream targets that are activated by these sensors largely overlap with those of the cAMP–PKA pathway (Roosen et al. 2005). So far, the only component that has been identified in the signal transduction pathway activating PKA is the Sch9 protein kinase which is essential for the nitrogen-induced activation through Gap1.

5.2.4 The Physicochemical Environment and Yeast Growth

Yeasts generally grow well in warm, moist, sugary, acidic and aerobic environments. Those few species which prefer exceptional physical or chemical conditions are, nonetheless, very important in industry, often as spoilage organisms.

With regard to temperature, most laboratory and industrial yeasts are mesophilic and generally grow best between 20 and 30°C, although some species associated with warm-blooded animals will not grow well below 24–30°C, whilst some psychrophilic yeasts grow optimally between 12 and 15°C (Table 5.2). Psychrophilic yeasts that adapt to low temperatures are very important spoilage organisms in frozen foods. Yeasts exhibit characteristic, or "cardinal", minimum, optimum and maximum growth temperatures (T_{min}, T_{opt} and T_{max}, respectively). For *S. cerevisiae*, T_{max} values range from 35 to 43°C, whereas strains of *S. bayanus* and *S. pastorianus* fail to grow above 35°C.

Most yeasts are mildly acidophilic, being able to grow well between pH 4.5 and 6.5, with several species being are able to grow in more acidic or alkaline conditions. During alcoholic beverage fermentations, the pH declines owing to a number of factors including dissolution of CO_2, proton extrusion and organic acid secretion. Brewing and winemaking strains are able to tolerate low fermentation pH values

Table 5.2 Yeast growth temperature limits

Thermal domain	Broad definition	Examples of yeasts
Psychrophile	A yeast capable of growing between 5 and 18°C. Obligate psychrophiles have an upper growth limit at or below 20°C	*Leucospiridium* spp. (e.g *L. frigidum*). *Torulopsis* spp. (e.g. *T. psychrophila*)
Mesophile	Yeasts with growth limits at 0°C and up to 48°C	Vast majority of yeast species
Thermophile	Minimum temperature for growth at or above 20°C	*Candida sloofii*, *Cyniclomyces gluttulatus*, *Saccharomyces telluris*, *Torulopsis bovina*

Information from Watson (1987)

and the former are additionally tolerant of acid-washing, a practice designed to eliminate contaminant bacteria.

With regard to water requirements, yeasts need water in high concentrations for growth and enzymatic activity. As is the case with temperature, yeasts have cardinal water potentials for growth; namely, ψ_{min}, ψ_{opt}, and ψ_{max} (where ψ_w, water potential, is expressed in megapascals). Most yeasts can grow reasonably well at low water potentials, with those able to grow well in conditions of low water potential (i.e. high sugar or salt concentrations) referred to as osmotolerant or xerotolerant. Such yeasts include *Candida mogii*, *D. hansenii*, *Metschnikowia bicuspida*, *Schizosaccharomyces octosporus* and *Z. rouxii* and are very important economically as food spoilage yeasts. The last species is generally unaffected by ψ_w values between −1.0 and −5.6 MPa.

Concerning requirements for oxygen, yeasts need this nutrient not only as a terminal electron acceptor for respiratory metabolism, but also as a growth factor for the biosynthesis of sterols and unsaturated fatty acids (Walker 1998). In fact, few yeasts can tolerate complete lack of oxygen and anaerobiosis is an effective means of preventing food spoilage (e.g. vacuum packing under nitrogen). For *S. cerevisiae*, the propagation of this species for yeast biomass for food and fermentation applications necessitates vigorous oxygenation (typically using molasses and fed-batch processes), whilst the subsequent employment of this yeast in baking or brewing necessitates very low levels of oxygenation (only at the start of fermentation). *S. cerevisiae* is able to tolerate these two fundamentally different situations of oxygen availability by using either respiratory or fermentative metabolism as appropriate to the prevailing growth conditions.

5.2.5 General Survival Mechanisms of Yeasts

During yeast propagation and fermentation processes in food and beverage production, yeast cell survival is paramount to ensure efficient bioconversion of substrate to biomass or metabolite. In food or beverage storage, however, the opposite is true in that yeast survival is undesired. In essence, yeast cell death needs to be minimised during industrial fermentations to maintain culture viabil-

ities at high levels; whilst on the other hand, yeast cell death needs to be maximised to eradicate undesired yeasts in foods and beverages. Yeasts will obviously die if confronted with excessive heat, extreme cold, high-voltage electricity, ionising radiation, reactive oxygen species, and high hydrostatic and osmotic pressures. Several physical treatments can be used to eradicate food and beverage spoilage yeasts. Some food spoilage yeasts are listed in Table 5.1 and include *Cryptococcus laurentii* and *Candida zeylandoides* (frozen poultry), *Z. bailii* and *Z. rouxii* (fruit and vegetables) *Kluyveromyces* spp., *Rhodotorula* spp. and *Candida* spp. (dairy products) and those spoiling fermented beverages include wild strains of *Saccharomyces* (e.g. *S. diastaticus*), and species of *Candida*, *Debaryomyces*, *Torulaspora*, *Pichia*, *Hanseniaspora*, *Metschnikowia*, *Szhizosaccharomyces* and *Zygosaccharomyces*. Such yeasts may be eradicated by heat (e.g. beer is commonly pasteurised at 70°C for 20 s) or by high-pressure–low-temperature treatments that have potential applications as an alternative to heat in the sterilisation of foods. Spoilage yeasts may also be prevented using chemical preservatives such as the weak acids and sulphur dioxide (Sect. 5.4.5).

Yeast cell death results when cellular damage caused by severe physical or chemical stress overcomes yeast cells' protection responses. These responses, which will now be discussed, are multifarious and involve physiological and molecular genetic level adaptations to ensure yeast survival in the face of environmental insults.

5.3 Yeast Responses to Physical Stresses

5.3.1 Temperature Stress

Table 5.3 summarises some of the general adverse influences of high-temperature stress on yeast cell physiology. Yeasts cannot regulate their internal temperature and thermal stress causes cellular damage by disrupting hydrogen bonding and denaturing proteins and nucleic acids, leading to rapid loss in yeast cell viability. Intrinsically thermotolerant yeasts possess T_{opt} values above 40°C and are able to survive sudden heat shock temperatures (e.g. to 50°C), whereas induced thermotolerance occurs when cells are preconditioned by exposure to a mild heat shock (e.g. 30 min at 37°C) prior to a more severe heat shock. pH also influences yeast thermotolerance and Coote et al. (1991) have provided evidence which implicates alterations in intracellular pH as the trigger for acquisition of thermotolerance in *S. cerevisiae*. Cells growing quickly in a glucose-rich medium are more heat-sensitive than stationary-phase cells, perhaps owing to glucose repressing the synthesis of stress-defence proteins.

Yeast cells exhibit a heat shock response when exposed to transient, sublethal temperatures. This involves induction of synthesis of a specific set of proteins, the highly conserved Hsps. Table 5.4 summarises the major Hsps of *S. cerevisiae*. Note that these proteins are also induced when yeasts are exposed to stresses other than heat shock (Piper 1997), meaning that they should more correctly be referred to as stress proteins.

Table 5.3 General effects of high temperature on yeast cell physiology

Physiological function	Comments
Cell viability	At the highest growth temperature of many yeasts, there is also appreciable cell death. At supramaximal growth temperatures, thermal death rate is exponential
General cell morphology	Atypical budding, irregular cell wall growth and increased cell size
Cell division and growth	Growth of non-thermotolerant yeasts are inhibited at temperatures above 40°C. Actively dividing cells in the S phase are more thermosensitive compared with resting cells. Heat shock transiently arrests cells in the G1 phase of the cell cycle
Plasma membrane structure/function	Increased fluidity and reduced permeability to essential nutrients. Ergosterol is known to increase thermotolerance. Decrease in unsaturated membrane fatty acids. Stimulation of ATPase and RAS-adenylate cyclase activity. Decline in intacellular pH
Cytoskeletal integrity	Extensive disruption of filaments and microtubular network
Mitochondrial structure/function	Decrease in respiratory activity and induction of respiratory-deficient petite mutants. Aberrant mitochondrial morphology
Intermediary metabolism	Inhibition of respiration and fermentation above T_{max}. Immediate increase in cell trehalose and MnSOD following heat shock
Protein synthesis	Repression of synthesis of many proteins, but specific induction of certain heat shock proteins. Mitochondrial protein synthesis more thermolabile than cytoplasmic
Chromosomal structure/function	Increased frequency of mutation of mitotic cross-over and gene conversion. Increased mitotic chromosomal non-disjunction. Inefficient repair of heat-damaged DNA

Several Hsps contribute to yeast thermotolerance by acting as molecular "chaperones" to prevent protein aggregation (Morano et al. 1998). They also promote protelolysis of aberrant stress-damaged proteins. The molecular basis of the heat shock response in *S. cerevisiae* fundamentally involves increased transcription of heat shock element (HSE) genes induced by a sublethal heat shock, but not by other stresses (e.g. osmostress, oxidative stress, DNA damage). The activation of the heat shock transcription factor (HSF) in *S. cerevisiae* does not appear to be required to induce tolerance against severe stress, but is required for high-temperature growth. Although Hsps may not play direct roles in conferment of yeast thermotolerance, Hsp104 does appear to ameliorate effects of thermal stress in respiring cultures of *S. cerevisiae* (Lindquist and Kim 1996) but in brewing strains under industrial fermentation conditions the role of Hsp104 in conferring stress resistance has been questioned (Brosnan et al. 2000). Other Hsps and stress proteins appear

Table 5.4 Major heat shock proteins of *S. cerevisiae*

Heat shock protein	Proposed physiological function
Hsp104	Acquisition of stress tolerance. Constitutively expressed in respiring, not fermenting cells and on entry into stationary phase
Hsp83	Chaperone function
Hsp70 family	Interact with denatured, aggregated proteins and assists in solubilising them with simultaneous refolding (i.e. chaperone function). Also involved in post-translational import pathways
Hsp60	Similar to Hsp70. This chaperone family facilitates post-translational assembly of proteins
Small Hsps – Hsp30, Hsp26, Hsp12	Cellular role still elusive, but may be involved in entry into the stationary phase and the induction of sporulation. Hsp30 may regulate plasma membrane ATPase
Others	
Ubiquitin	Responsible for much of the turnover of stress-damaged proteins
Some glycolytic enzymes	Enolase (Hsp48), glyceraldehyde 3-phosphate dehydrogenase (Hsp35) and phosphoglycerate kinase
Catalase	Antioxidant defence
GP400 and P150	Secretory heat shock proteins (unknown function)

Information from Watson (1990), Mager and Ferreira (1993), Parsell and Lindquist (1994), Mager and De Kruijff (1995) and Tsiomemko and Tuymetova (1995)

constitutively expressed in ale-brewing strains of *S. cerevisiae* in production-scale fermentation conditions (Kobi et al. 2004).

Estruch (2000) and Trott and Morano (2003) have reviewed molecular-level responses of yeasts to heat shock and other stresses. The exact sensing mechanism to activate the heat shock response is not clear. There is evidence that cells may sense heat shock via the accumulation of thermally misfolded proteins. Addition of the imino acid analogue azetidine-2-carboxylic acid (AZC) to yeast cells causes reduced protein stability and strongly induces heat shock factor regulated genes. This induction is dependent on the heat shock factor, indicating that this factor is activated by the presence of the misfolded proteins (Trotter et al. 2002). Addition of azidothymidine (AZT) also resulted in a strong reduction of the expression of ribosomal protein genes, which is also a HSF-dependent process. The sensing of the misfolded proteins seems to be specific for the HSF since the STRE-regulated genes are not strongly induced. Apart from the misfolded proteins other pathways, not activated by misfolded proteins, have been suggested as being important for the heat shock induction (Kamada et al. 1995). The best studied is the cell integrity pathway which senses thermal stress through the plasma membrane sensors Hcs77 and Mid2 (Gray et al. 1997; Verna et al. 1997).

Well-characterised components of the heat shock response are the transcription factors that are required to induce or repress heat shock regulated genes. These include transcription factors that are specific for the heat shock response, such as the HSF (Hsf1), or transcription factors that respond to a variety of cellular and environmental stress conditions, such as the Msn2 and Msn4 transcription factors. The Hsf1 activates gene expression through binding of the HSE, nGAAn, in the promoter of regulated genes, whereas the Msn2/Msn4 transcription factors bind the STRE, CCCCT (Bienz and Pelham 1986; Marchler et al. 1993). The binding of HSF to the HSE is cooperative, and some deviations from the canonical NGAAN sequence are tolerated in functional HSEs. The minimum requirement for recognition by HSF seems to be three canonical inverted repeats of this sequence, since it forms a homotrimer. Chromatin immunoprecipitation combined with microarray analysis identified approximately 165 *in vivo* targets for HSF. Thirty percent of these genes are also induced by the diauxic shift (Hahn and Thiele 2004).

Recently new components important for the signal transduction pathway inducing HSE-mediated gene expression have been identified. These include the ubiquitin ligase Rsp5 and Bul1/Bul2, two homologous proteins interacting with Rsp5 (Kaida et al. 2003). Mutagenesis analysis of the interacting part indicated that these three proteins function as a complex and there is also evidence that this complex functions independently from the Hsf1. As the bul1bul2 double mutant is sensitive to various stresses, such as high temperature, high salt and non-fermentable carbon sources, these proteins may be involved in the general stress response. How this Rsp5–Bul1/2 complex exerts its effect at the level of gene expression remains to be determined.

The question remains how partially denaturated proteins or changes at the level of the cell wall can activate the HSF. It has long been known that Hsf1-regulated response is a transient response, despite the continuous presence of stress. This indicates that Hsf1 activity must be controlled in a very precise manner. In contrast to the situation in metazoan HSF activation, the yeast Hsf1 transcription factor is constitutively bound to the promoters of regulated genes. Many of the regulated genes are also expressed under non-stress conditions and that is why *HSF1* is an essential gene (whereas Msn2 and Msn4 are non-essential genes). In contrast to Msn2 and Msn4, of which activity is regulated by nuclear translocation and DNA binding, the Hsf1 transcription factor can only be regulated by derepression or by activation. Under high-temperature conditions, Hsf1 is activated by phosphorylation (Sorger and Pelham 1988; Wiederrecht et al. 1988). In addition heat shock and the superoxide-generating agent menadione induce distinct patterns of HSF phosphorylation (Liu and Thiele 1996), suggesting that different stresses may cause differential phosphorylation.

Apart from heat stress, Hsf1 activity is also regulated by glucose starvation (Amoros and Estruch 2001). Recently it was shown that Hsf1 and Snf1 interact *in vivo* and that Hsf1 is phosphorylated by Snf1 *in vitro* (Hahn and Thiele 2004). In response to glucose starvation, HSF undergoes phosphorylation in an Snf1-dependent manner. This may indicate that different kinds of stress may activate different kinases that then activate HSF. Differential phosphorylation of HSF by stress-specific kinases might allow sophisticated levels of control of HSF activity to induce a subset of targets in response to specific stress signals.

One form of Hsf1 activation by heat stress is through a conformational change. The HSF contains a DNA binding and trimerisation domain, a repression element (CE2), a C-terminal modulator (CTM) and two transcription activation domains (AR1 and AR2), located at the N and C termini, respectively. Under normal conditions these two activation domains are repressed by interaction with the central regulatory domains and upon heat shock, this interaction is broken and the activation domains become functional (Chen and Parker 2002).

A further level of fine-tuning the response to heat shock is at the level of the HSE. The C-terminal modulator domain of the yeast HSF is required for the activation of genes containing atypical HSE but not typical HSE. This CTM domain is responsible for the hyperphosphorylation of HSF upon heat shock. It is required for activation of genes that have atypical HSE elements in their promoter (Hashikawa and Sakurai 2004). CTM is thought to modulate the activator function of ScHsf1 depending on the architecture of the HSE.

Yeast cells also respond to heat shock by accumulating other stress-protective compounds such as trehalose (Ertugay and Hamamci 1997; Neves and François 1992). Trehalose functions as a thermoprotectant and a cryoprotectant by stabilising cell membranes and accumulates markedly in cells exposed to a non-lethal heat shock (reviewed by Bonini et al. 2004). Together with Hsp104, trehalose acts synergistically to confer thermoprotection in *S. cerevisiae*. Recently a new mechanism of interaction between trehalose and Hsf1 was described. The α-helical content (and therefore the transactivating capacity) of the C-terminal activation domain of Hsf1 can be increased by the addition of trehalose but not by the addition of sucrose. During a heat shock both trehalose and Hsf1 are induced and it seems that in addition trehalose modifies the structure of the C-terminal activation domain (Bulman and Nelson 2005).

Heat shock may also enhance oxidative damage caused by oxygen free radicals, resulting in stimulation of the antioxidant enzymes catalase and superoxide dismutase. The polyamines spermine and spermidine also play important roles in thermal protection of *S. cerevisiae* cells by stabilising the structural integrity of yeast membranes during thermostress.

There is functional overlap between thermal, oxidative and ethanol stress responses of yeasts (but not necessarily between thermal and osmotic stress responses). It should also be noted that combined stresses, commonly encountered in industry, may act synergistically to affect yeast survival (Piper 1995). For example, high ethanol concentration together with high temperature may negatively affect yeast growth and metabolism during beverage fermentations. This would be particularly problematic if large-scale fermentation vessels were not properly cooled.

Cold stress in yeasts affects yeast cells in different ways (Table 5.5) and understanding this is important for controlling the psychrophilic yeast spoilage of foods, cryopreserving yeast stock cultures and maintaining viability of stored yeasts. The last of these is important in brewing when yeasts are cold-stored for later pitching into fermenters – a practice that can adversely affect subsequent fermentation performance (Boulton et al. 1989). Brewer's yeast with elevated levels of trehalose is able to maintain cell viability in cold storage conditions (4°C for several days in 5% v/v ethanol). Cryopreservation of yeasts in liquid nitrogen (–196°C) is a suitable

Table 5.5 Influence of low temperature on yeast cell physiology

Low-temperature stress	Yeast cells' response
Low-temperature exposure (e.g. 15°C)	Reduced sterol synthesis and increased polyunsaturated fatty acids in the cell membrane
Cold shock (e.g. 4–10°C)	Cell division arrest. Differential protein biosynthesis
Freezing	Uniform cell shrinkage. Vacuolar membrane damage
Freeze–thaw stress	Resistance of stationary phase cells. Antioxidant responses (due to reactive oxygen species generated during thawing)
Cryo-stress (liquid nitrogen, –196°C)	Significant induction of genes encoding proteins involved in cellular defence, energy transduction and metabolism

method for long-term maintenance of stock cultures but freezing and thawing does constitute a considerable stress and also causes severe mechanical injury to cells. Yeast cells do respond to such stress at a molecular level and it has been shown using differential hybridisation (Kondo and Inouye 1991) and more recently by DNA microarrays (Odani et al. 2003) that specific genes are induced following cold shock and cryopreservation treatments of *S. cerevisiae*, respectively. Concerning the latter, the genes in question encode a variety of "cell rescue" proteins that may be involved in repairing damage to the yeast cell envelope and to cellular organelles.

Similar to an adaptation to high temperatures, yeast cells can also adapt to low temperatures. Exposure of yeast cells to a temperature of 10°C reveals that there are two groups of transcriptional responses. A cold-adaptation-specific early response and a late cold response, which largely overlaps with the general environmental stress response as it is also dependent on Msn2 and Msn4 (Schade et al. 2004). Genes such as *NSR1* (nucleolin-like protein), *TIP1*, *TIR1* and *TIR2* (serine- and alanine-rich cell wall proteins) are all part of the early response. Genes that are expressed in the late response largely overlap with the genes expressed during the near-freezing response. The near-freezing response is controlled by the Msn2/Msn4 transcription factors and is similar to the late cold response (cells are conditioned for an even colder temperature). The long-term cold adaptation enhances survival at lower or even freezing temperatures. This is mainly because of the strong accumulation of trehalose and molecular chaperones such as Hsp104, Hsp42, Hsp12 and Ssa4 (Kandror et al. 2004). The mRNAs for these proteins are also stabilised more at these low temperatures.

Specific freeze-stress protection mechanisms include the production of antifreeze proteins and ice nucleators. Antifreeze proteins (AFPs) cause thermal hysteresis where they lower the freezing point without affecting the melting point. They also inhibit ice recrystallisation, a process that is the main cause of cell damage. Expression of natural fish AFPs as well as a chemically synthesised DNA fragment encoding an artificial antifreeze protein in *E. coli* has been demonstrated to improve both salt and freeze tolerance (Holmberg et al. 1994; Meijer et al. 1996). Ice nucleators (INAs) limit supercooling and induce freezing at high subzero

temperatures by mimicking the structure of an ice crystal surface. They establish protective extracellular freezing instead of lethal intracellular freezing (Zachariassen and Kristiansen 2000).

Frozen dough technology is a typical example of an industrial application where the freeze tolerance of the microorganism used is of paramount importance. When the flour is mixed with the yeast for the preparation of the dough, the yeast rapidly initiates fermentation and at the same time the freeze resistance of the yeast rapidly drops (Nagodawithana and Trivedi 1990; Rose and Vijayalakshmi 1993). To minimise the problem, freeze doughs are prepared by rapid mixing of the dough at low temperature. However, a minimal prefermentation of the dough appears to be necessary for a good quality of the bread (Hsu et al. 1979a, b; Richard-Molard et al. 1979). The rapid, striking transition from high freeze tolerance to high freeze sensitivity during the start-up of yeast fermentation constitutes a major obstacle for optimal use of the frozen dough technology. Attempts to obtain strains better suitable for use in frozen doughs have been based on the few microbial freeze-tolerance mechanisms identified so far, and have stimulated further research to unravel new underlying mechanisms. The rapid drop in freeze tolerance during the start-up of yeast fermentation also provides an unique model system to identify novel mechanisms involved in freeze tolerance because of the rapid and dramatic change in freeze tolerance over a short time period within the same organism. Screens to identify mutants that do not show the fermentation-induced loss of freeze resistance have been developed (Teunissen et al. 2002; Van Dijck et al. 2000). Recent genome-wide gene expression analyses and Northern blot analyses of freeze-tolerant and freeze-sensitive yeast strains have revealed a correlation between freeze tolerance and expression of the aquaporin encoding genes *AQY1* and *AQY2* (Tanghe et al. 2002). This relationship was confirmed by deletion and overexpression of *AQY1* and *AQY2*, clearly reducing and enhancing yeast freeze tolerance, respectively (Tanghe et al. 2002). Recently, similar results were obtained for the *Candida albicans* and *Schizosaccharomyces pombe* aquaporin genes (Tanghe et al. 2005a, b). Whereas there is a clear correlation between the level of aquaporin expression and freeze tolerance, this correlation is only present under rapid freezing conditions (Tanghe et al. 2004). Normal 800-g frozen dough prepared with a yeast strain overexpressing aquaporin genes did not show improved leavening capacity after thawing, whereas this was clearly the case in 0.5-g doughs. By using various fast and slow freezing conditions, Tanghe and coworkers could nicely show that more aquaporins in the membrane are only beneficial during rapid freezing. Most probably under slow freezing conditions, there is enough time for dehydration of the yeast cells in order to prevent intracellular ice crystal formation (Tanghe et al. 2004).

Yeast cells that have a higher intracellular content of glycerol, either by growing them in glycerol medium or by deletion of the Fps1 glycerol transporter, acquire tolerance to freeze stress and retain high leavening ability in dough after frozen-storage (Izawa et al. 2004, 2005).

Freeze tolerance has also been shown to correlate with the accumulation of specific amino acids. A dominant mutation in the *PRO1* gene, encoding γ-glutamyl kinase, which catalyses the first step in L-proline biosynthesis from L-glutamate, results in L-proline accumulation and a mutation in the *CAR1* gene, encoding

arginase, results in arginine and/or glutamate accumulation (Morita et al. 2003; Shima et al. 2003). Strains harbouring these mutations are more freeze-tolerant.

Other yeast species that are more tolerant to freeze stress have been used to study the cellular and biochemical basis of freeze tolerance. All cells of *Torulospora delbrueckii* strain PYCC5323, isolated from traditional corn and rye bread dough, survive long periods of freezing, whereas under similar conditions only 20% of *S. cerevisiae* cells survive (Hernandez-Lopez et al. 2003). The biochemical basis for the higher stress tolerance was its preservation of the plasma membrane integrity by a lower increase in lipid peroxidation and a higher resistance to H_2O_2. Interestingly, the higher freeze stress tolerance was not based on the trehalose level (Alves-Araujo et al. 2004). The high freezing resistance depends on a period of slow freezing during which the cells adapt their metabolism. This is also the reason why de novo protein synthesis is required. This phenotype, together with the fact that the growth and fermentation capacity of *T. delbreuckii* similar to that of *S. cerevisiae*, holds promise that this strain may become the best strain for freeze-tolerant yeasts for the frozen dough market. Previously, other groups had isolated *Torulaspora* strains from nature and showed that they were freeze-tolerant (Hahn and Kawai 1990).

5.3.2 Reduced Water Availability

Severe water stress (i.e. reduced water availability) occurs when yeast cells are dehydrated, for example, using spray and drum or fluidized-bed driers for use in production of dried yeast for the food and fermentation industries. Dehydration causes a rapid efflux of water through the cell membrane, resulting in collapse of the cytoskeleton. To compensate for this, cells adapt by recruiting intracellular water from the vacuole into the cytoplasm and by synthesising compatible solutes such as glycerol. This anhydrobiosis may deleteriously affect yeast cell physiology by altering the structure and function of vacuolar, nuclear and cell membranes. Remarkably, growth arrest resulting from such changes is reversible and the aim of dried yeast manufacturers is to maintain cell viability as high as possible following rehydration. This causes water influx into cells, increasing turgor pressure, and yeasts with high levels of the osmolytes trehaolse (Eleutherio et al. 1995) or glycerol (Hohmann 1997) appear more resilient to dehydration–rehydration stresses. Bauer and Pretorius (2000) have reviewed these stresses with regard to wine yeasts.

Hyperosmotic shock results in a loss of cell turgor pressure and a rapid decrease in cytoplasmic water content and cell volume (i.e. cells shrink). Conversely, hypoosmotically shocked cells increase in volume owing to the high water permeability of the plasma membrane (they also lower intracellular levels of potassium and glycerol).

Mild water stress in yeasts occurs during osmostress caused either by hypersomotic shock by increasing the solute concentration, or hypoosmotic shock by reducing the solute concentration. Note that both ethanol and heat shock may cause water stress in yeasts by reducing intracellular water (Halsworth 1998; Piper 1995). Generally speaking, *S. cerevisiae* cells are moderately resilient to short-term osmostress with regard to cell survival and growth tolerance. Osmotolerant yeasts are able to adapt to conditions of very low water potentials and are found in natural solute-rich habitats (e.g. honey, tree exudates) and as spoilers of salt-rich or sugar-rich foods.

There are various physiological responses of yeasts to osmostress, including efflux of intracellular water, rapid reduction in cell (and vacuolar) volume, transient increase in glycolytic intermediates, and accumulation of osmolytes in the cytosol. Loss of intracellular water induces the synthesis of osmolytes (or compatible solutes) that are able to restore cell volume, stabilise membrane proteins and maintain enzymatic activity. Glycerol is the major compatible solute in yeasts and can effectively counterbalance the loss of water due to osmotic stress. Increased levels of intracellular glycerol can be the result of increased uptake, increased biosynthesis, increased retention and decreased dissimilation, as summarised in Fig. 5.2. Glycerol is synthesised during glycolysis by reduction of dihydroxyacetone phosphate to glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase (GPD), the activity of which is increased under osmotic stress. When cells are osmotically stressed, there is a requirement for cytosolic NADH that is met by decreased reduction of acetaldehyde to ethanol together with increased oxidation to acetate.

Osmotolerant yeasts like *Z. rouxii* are able to retain their cell volume in media of low water potential and in this yeast an active glycerol transport mechanism enables retention of glycerol as part of an osmotic stress response. As shown in Table 5.6, other polyols (arabitol, sorbitol, mannitol and erythritol), ions (e.g. K^+) and the disaccharide trehalose may accumulate in yeast cells in response to water stress caused by specific solutes. Note that stress induced by high salt (NaCl) conditions creates two different phenomena: ion toxicity and osmotic stress (Serrano et al. 1997). Defence responses to salt stress are based on osmotic adjustments by glycerol synthesis and cation transport systems for sodium exclusion.

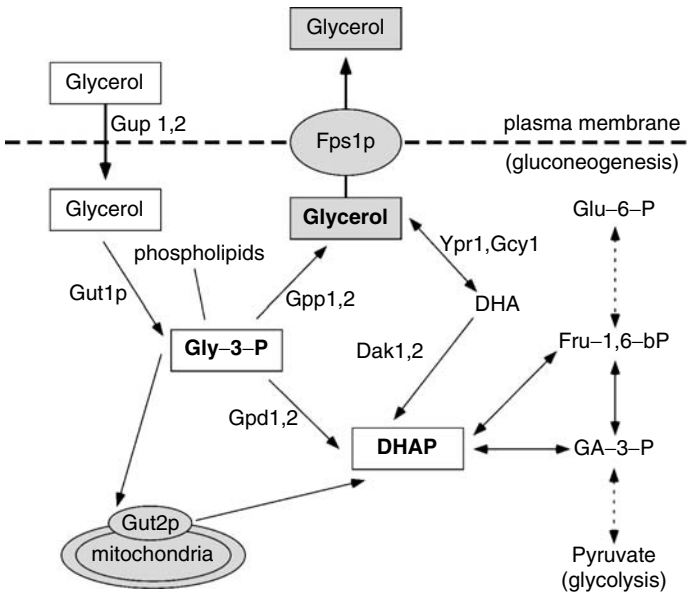

Fig. 5.2. Summary of glycerol uptake and utilisation by yeast

Table 5.6 Responses of yeasts to changes in media osmotic potential

Yeast	Treatment[a]	Intracellular solute
S. cerevisiae	Upshock with NaCl	K^+, Na^+ glycerol and trehalose increased
	Upshock with sorbitol	Glycerol initially, then trehalose increased
Z. rouxii	Upshock with NaCl	Glycerol, arabitol increased
	Upshock with glucose	Arabitol initially, then trehalose increased
D. hansenii	Upshock with various solutes	Glycerol increased
	Downshock by reducing NaCl concentrations	Glycerol decreased

Adapted from Jennings (1995)
[a]Upshock refers to the lowering of osmotic potential in the medium, whereas downshock refers to the raising of osmotic potential due to decreasing solute concentrations

The molecular basis of osmotic stress responses in yeast cells is now well characterised, particularly in *S. cerevisiae*. In this yeast, a variety of stress conditions (heat shock, UV irradiation, osmostress, etc.) induce transcription of genes containing STREs to enable cells to grow under potentially lethal conditions. A signalling system specific to osmostress called the high osmolarity glycerol (HOG) pathway activates a mitogen-activated protein kinase (MAPK) cascade (Fig. 5.3).

The pathway starts with the activation of cell-membrane-bound receptor proteins (Sho1, Msb2 and Sln1) that act to sense the external osmolarity and ends with synthesis of osmoprotectants such as glycerol (Cullen et al. 2004; Hohmann 2002). These osmosensors are encoded by genes (e.g. *SLN1*) which are upstream regulators of the HOG pathway. The Sln1 branch is required to induce the expression of several reporter genes in response to very high solute levels and this indicates that the Sln1 branch operates over a broader range of osmolarities than the Sho1/Msb2 branch. Under osmotic stress, Sln1 leads to phosphorylation of a downstream target protein Ypd1, which continuously transfers a phosphate group to the response regulator protein Ssk1. This pathway activates two partially redundant mitogen-activated protein kinase kinase kinases (MAPKKK) Ssk2 and Ssk22. The Sho1/Msb2 branch requires Cdc42, Ste20 and Ste50 to activate the MAPKKK Ste11. Any of the three MAPKKKs is able to activate Pbs2, which then phosphorylates Hog1. This MAPK regulates the expression of numerous genes by controlling the activity of several transcription factors (activators and repressors) (Saito and Tatebayashi 2004; Tamas and Hohmann 2003; Westfall et al. 2004).

Hog1 dual phosphorylation by the mitogen-activated protein kinase kinase (MAPKK) Pbs2 results in its nuclear localisation and causes regulation of gene expression through several transcription factors, Hot1, Sko1, Smp1, and probably also through Msn1, Mns2 and Msn4 (Gasch et al. 2000; O'Rourke and Herskowitz 2004; Rep et al. 2000). About 580 genes are regulated by Hog1, some of which require the general stress response to be fully activated. Genes such as *CTT1*, *ALD2*

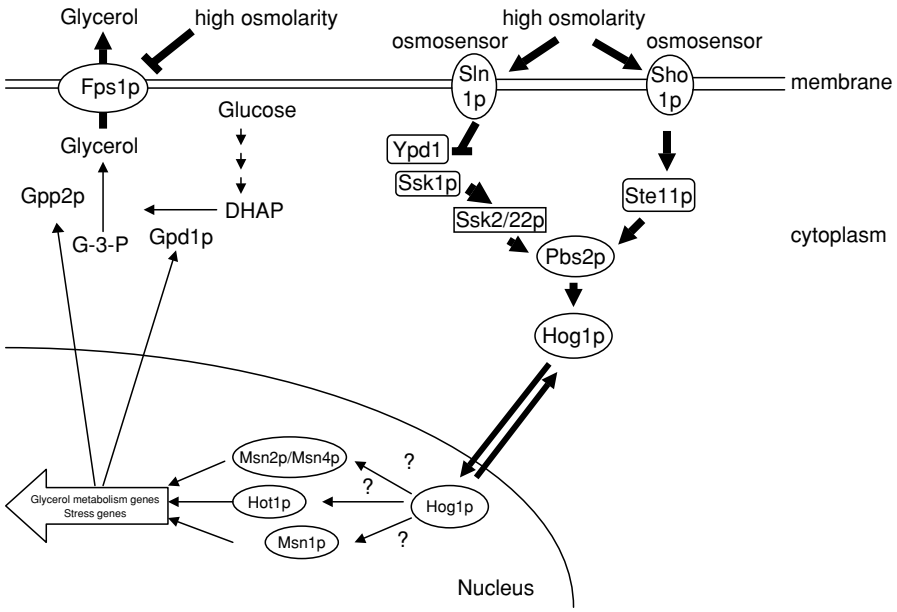

Fig. 5.3. The high osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase cascade pathway

and *HSP26* require both a HOG-dependent process and the general stress-response pathway. This is in contrast to genes such as *STL1* and *GRE2*, which are directly regulated by Hog1 and the two transcription factors Hot1 and Sko1. Activation of Hog1 also results in a transient cell-cycle arrest in the G1 phase of the cell cycle. Escoté and coworkers have recently shown that the Sic1 cyclin-dependent kinase inhibitor is a direct target of Hog1. Hog1 phosphorylates Sic1, which results in a stabilisation of this protein. This together with the downregulation of cyclin expression results in cell-cycle arrest under stress conditions which allows cells to recover before they progress into the S phase (Escoté et al. 2004).

Hog1 regulates gene expression through interaction with the Rpd3–Sin3 histone deacetylase complex by recruiting this complex to the promoters of regulated genes. Histone deacetylation of the promoters allows proper transcription by the RNA polymerase II under stress conditions (de Nadal et al. 2004).

Recently, a new and physiologically more relevant function for Hog1 was put forward by the group of Peter Coote when they characterised the response of *S. cerevisiae* to citric acid. This organic acid occurs naturally in the juice of lemons and other sour fruits, a habitat populated by different species such as *Candida parapsilosis*, *Candida stellata*, *S. cerevisiae*, *Torulaspora delbrueckii*, and *Z. rouxii* (Lawrence et al. 2004). Functional screening of the yeast genome deletion set, combined with whole genome transcript and proteomics analysis have revealed that the HOG pathway plays a key role in the adaptation to citric acid stress. Only 13% of the genes

whose expression was induced upon citric acid addition displayed a citric acid sensitive phenotype when the corresponding gene was deleted. Addition of citric acid rapidly results in phosphorylation and activation of Hog1 and deletion of *HOG1* (and also of other components of the HOG pathway, such as *PBS2* and *SSK1*) makes the strain sensitive to citric acid. Citric acid does not cause osmotic stress but it does induce a general stress response and glycerol biosynthesis (Lawrence et al. 2004).

Recently, methylglyoxal (MG), a toxic glycolytic metabolite, has been suggested as the putative signal initiator of the Sln1 branch of the HOG pathway. Hog1 is rapidly phosphorylated and activated upon treatment of the cells with low concentrations of MG. Further investigation has also shown that MG activates the Msn2 transcription factor and stimulates the uptake of Ca^{2+} in yeast cells, thereby activating the calcineurin/Crz1-mediated pathway (Maeta et al. 2005).

The MAPK cascades transduce signals in yeasts that are triggered not only by osmotic stress but also by other environmental stresses and external stimuli such as nutrient availability and the presence of growth factors or mating pheromones. There is also considerable crosstalk between different MAPK cascades and recently it was shown that different interaction regions on the Sho1 sensor for either Ste11 or Pbs2 may result in the crosstalk between the osmotic stress sensing pathway and the pheromone pathway (Zarrinpar et al. 2004). In the absence of Pbs2 or Hog1, osmotic stress leads to activation of the MAPKK of the pheromone pathway, and induction of genes normally only expressed in response to the mating pheromone (Rep et al. 2000).

In addition to glycerol, the disaccaride trehalose plays an important role in protecting yeasts against water stress and this has relevance in the baking and brewing industries. For example, baker's yeast with elevated trehalose levels (more than 10% of dry weight) is relatively resistant to the drying process in terms of retention of leavening capabilities. Similarly, baker's yeast with a high trehalose content maintains its viability in frozen doughs (but refer to the previous discussion). Baker's yeast manufacturers therefore employ measures to minimise trehalose loss (e.g. through endogenous fermentation) in baking strains of *S. cerevisiae*.

In brewing yeasts, osmotic stress is encountered when cells are pitched into high-gravity malt wort (e.g. 18°P) and this may result in slow or incomplete fermentation. Intracellular trehalose in brewing yeasts may serve an important role as an osmoprotectant (and stress indicator) during very high gravity fermentations by *S. cerevisiae*. Trehalose acts by stabilising membranes against osmotic shock and trehalose accumulation in stationary phase cells is thought to be partly due to their increased osmotolerance compared with that of actively dividing cells. Trehalose is now widely recognised as a general "stress metabolite" in yeasts since it has been shown to act in yeasts not only as an osmoprotectant, but also as an antidessicant, a cyroprotectant, a thermoprotectant and a chemical detoxicant.

5.3.3 pH Stress

pH_2 stress is encountered by brewing yeasts when they are acid-washed at about pH_2 to eliminate spoilage bacteria prior to fermentation. Phosphoric acid is normally used, rather than organic acids (e.g. acetic acid, lactic acid), which are more inhibitory to yeasts owing to undissociated organic acids lowering intracellular pH following cell

membrane translocation. This is the basis of the action of weak acid preservatives in inhibiting food spoilage yeast growth (Sect. 5.4.5).

5.3.4 Miscellaneous Physical Stresses

In large industrial fermentation vessels, yeasts may encounter stress due to both hydrostatic and gaseous pressure. For example, in brewing cylindro-conical fermenters are employed which create stressful environments for yeast cells owing to hydrostatic pressure and pressure due to endogenous CO_2 produced during fermentation. CO_2-induced pressure effects are exacerbated in the presence of ethanol, the other major fermentation metabolite, and may lead to yeast growth inhibition and production of beer off-flavours. *S. cerevisiae* is not particularly barotolerant and fails to grow at high hydrostatic pressures (above 10 MPa) owing to damage to the cytoskeleton and mitotic apparatus. Barotolerance in yeasts may be linked to trehalose accumulation.

For elimination of spoilage yeasts, the use of high pressure–low temperature as an alternative to heat sterilisation has potential in food preservation.

Yeasts suffer from radiation stress when exposed to UV radiation, γ-rays or X-rays, which cause DNA damage (such as dimerisation, nicks and lesions) and cell-cycle arrest in yeast cells. Ionising radiation effects on yeast cells are indirectly mediated by reactive oxygen species (generated by water radiolysis), which damage DNA and cell membrane function. Both *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* have been widely studied as model eukaryotes in fundamental studies of DNA repair following radiation damage. In relation to yeast biotechnology, low doses of γ-radiation may enhanced alcoholic fermentation processes.

Mechanical and gravitational stresses may be encountered by yeast cells during industrial processes such as agitation in stirred-tank bioreactors or high-speed centrifugation. However, owing to the thick cell walls, *S. cerevisiae* is relatively resistant to mild shear stress and quite severe mechanical stress (e.g. glass bead homogenisation or high-pressure extrusion) is needed in order to rupture the cell walls to extract cell components. Similarly, ultrasound, which is capable of rupturing bacterial cells, is generally ineffectual in rupturing *S. cerevisiae*, but may stimulate certain yeast-catalysed transformations (perhaps owing to increased flux of substrates through the cell membrane).

Application of electrical fields affects yeast cell membrane permeability and this is exploited during electroporation or electrofusion to transform yeasts with exogenous DNA. The amplitude, frequency and duration of electrical exposure is crucial for the success of such techniques in yeast recombinant DNA technology. Some experiments have shown stimulatory effects of electrical fields on yeast growth and metabolism, particularly with regard to fermentative activity.

5.4 Yeast Responses to the Chemical Environment

5.4.1 Nutrient Starvation

Yeasts deprived of essential nutrients will be cell division cycle arrested, generally in the unbudded G1 phase. In diploid cells of *S. cerevisiae*, nutrient starvation may

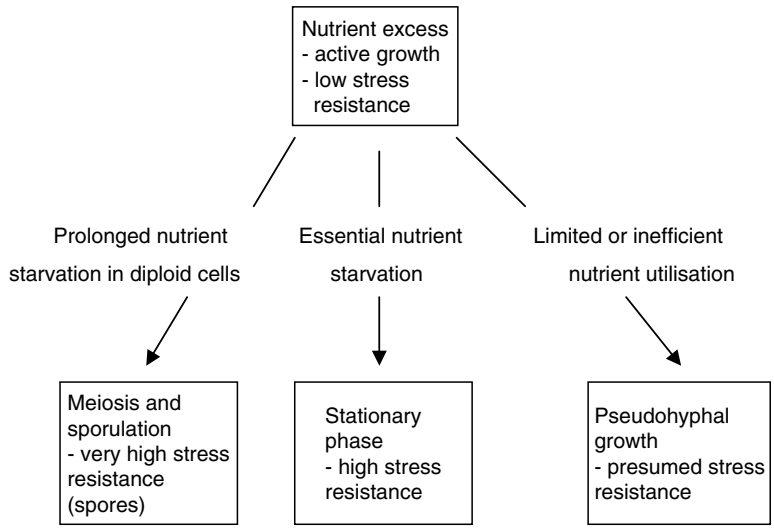

Fig. 5.4. General growth responses of *S. cerevisiae* to availability of nutrients

result in meiosis and sporulation, with formation of an ascus encasing very stress resistant haploid spores (Fig. 5.4). Although some yeasts may be described as oligotrophic and are able to grow in very low nutrient concentrations (Kimura et al. 1998), generally in yeasts nutrient exhaustion represents a stress that requires a cellular response. It is known that when yeast cells enter the stationary phase of growth, following depletion of nutrients, their cell walls thicken, they accumulate reserve carbohydrates (glycogen), they synthesise several stress proteins (Puig and Perez-Ortin 2000) and they generally become more stress-tolerant compared with actively dividing cells (Fuge and Werner-Washburne 1997). Conversely, it means that cells during the exponential phase of growth are less stress-tolerant. This would occur during fed-batch propagation of baker's yeast under conditions of high oxygenation and limited glucose supply (designed to avoid the Crabtree effect (Walker 1998). At the end of the propagation processes of baker's yeast, nutrient (molasses) supply is turned off to induce the biosynthesis of trehalose (Van Dijck et al. 1995) and stress proteins (Sales et al. 2000) that are required to maintain yeast viability and protect cells during subsequent dehydration.

With regard to beverage fermentations, if brewing yeasts suffer from prolonged periods of nutrient starvation in storage tanks, subsequent fermentation performance and flocculation capabilities may be adversely affected. In brewing, nutrient availability is key to efficient yeast metabolism and the best strains are those that adapt quickly to nutrient excess (during fermentation) following periods of nutrient starvation (during storage). Nutrient-related stress responses of wine yeasts have been discussed by Bauer and Pretorius (2000).

In *S. cerevisiae*, starvation of particular amino acids leads to the rapid inhibition of ribosomal RNA (rRNA) synthesis and is signalled when cells sense a rapid reduction in protein biosynthetic rate. In industrial strains of *S. cerevisiae*, if levels of

assimilable amino acids drop below certain threshold levels (e.g. around 150 mg/l), then fermentation may be curtailed prematurely. Also in these strains there is sequential uptake and utilisation of specific amino acids and this may lead to deficiency of certain amino acids at different stages of fermentation, necessitating their de novo biosynthesis. de Winde et al. (1997) have discussed specific molecular-level responses of *S. cerevisiae* to amino acid limitation and starvation.

The molecular response of nutrient limitation has mostly been studied after a switch from a richer to a poorer nutrient source (Winderickx et al. 2003). Yeast cells starved for a single essential nutrient will complete their current cell cycle and arrest in the next G1 phase at "START A". Subsequently, they will progress into an "off-cycle", G0 or stationary phase, in which they can survive nutrient starvation much longer than when arrested elsewhere in the cell cycle. They accumulate elevated levels of the storage carbohydrates glycogen and trehalose and expression of STRE- and PDS-controlled genes is induced. The transcription of ribosomal protein genes is repressed. The same phenotype is observed in cells growing on non-fermentable carbon sources, indicating that these phenotypes are not just a growth-arrest phenotype. Re-addition of nitrogen, sulphate or phosphate to cells starved of these nutrients, respectively, in each case causes within a few minutes a rapid, post-translational activation of the neutral trehalase enzyme (Hirimburegama et al. 1992). Nutrients modulate the critical size threshold, such that cells are large in rich medium and small in poor medium.

Recently, the mechanism by which nutrient availability is sensed and converted into a signal regulating cell cycle progression has been studied in great detail. Nutrient effects are mediated in part by the Ras–PKA pathway and by the TOR pathway. Both pathways seem to regulate the activity and cellular localisation of the transcription factor Sfp1. This transcription factor plays a role in the control of the cell size and regulates ribosomal protein gene expression. Under optimal growth conditions, Sfp1 is localised to the nucleus and regulates ribosomal protein gene expression but upon inhibiting TOR signalling, stress or changes in nutrient availability, Sfp1 is released from the ribosomal protein gene promoters and translocates to the cytoplasm (Jorgensen et al. 2004; Marion et al. 2004). Apart from Sfp1, also the fork-head-like transcription factor Fhl1 and two cofactors Ifh1 (a co-activator) and Crf1 (a co-repressor) are involved in ribosomal protein gene expression under the control of both TOR and PKA. Similar to Sfp1, its activity is regulated by nuclear-cytoplasmatic translocation (Martin et al. 2004). The exact mechanism of Sfp1 regulation and localisation remains to be determined.

The TOR pathway is also involved in other functions. Under nutrient limitation conditions, Tor activity is inhibited and this results in a block in translation initiation through inhibition of eukaryotic initiation factor 4E (eIF4E), in repression of ribosomal genes and rRNA and transfer RNA (tRNA) synthesis and in uptake of amino acids by degradation of high-affinity amino acid permeases. On the other hand, Tor inactivation causes an induction of several genes through nuclear localisation of different transcription factors (Gln3, Rtg1, Rtg3, Msn2 and Msn4) (Schmelzle and Hall 2000).

Apart from a general nutrient limitation response, various other signal transduction pathways exist that sense the nutrient availability. The carbon catabolite

repression pathway is a signalling cascade activated by carbon source depletion. Limitation of sugar availability stimulates the activity of the central protein kinase Snf1. Snf1 will then phosphorylate Mig1 and phosphorylated Mig1 will translocate to the cytoplasm, resulting in the derepression of Mig1-repressed genes in the presence of low levels of glucose. Under amino acid starvation conditions, the sensor kinase Gcn2 detects uncharged tRNAs and phosphorylates and thereby activates the α-subunit of eukaryotic initiation factor 2 (eIF-2) (Kubota et al. 2000). Phosphorylated eIF-2α inhibits general translation but selectively derepresses the synthesis of the transcription factor Gcn4 at the translational level (Grundmann et al. 2001). Gcn4 controls a network of amino acid biosynthetic pathways, genes involved in glycogen homeostasis, genes encoding protein kinases and transcription factors (Natale et al. 1993).

S. cerevisiae prefers inorganic phosphate (Pi) as the phosphorus source. In the presence of sufficient Pi in the medium, all genes required for the utilisation of organic compounds as a source for phosphorus are repressed. Upon Pi depletion, the transcription of some phosphate-starvation response genes such as *PHO5* (encoding a secreted acid phosphatase) is activated by the transcription factor Pho4. In the presence of high concentrations of Pi, Pho4 is negatively regulated through binding of the specific inhibitors Pho80 and Pho85.

An important phenotype observed under nutrient-limitation conditions is the morphogenetic switch from yeast cells to pseudohyphal cells. This occurs during nitrogen limitation on rich medium. Both diploid pseudohyphal development and haploid invasive growth are controlled by the activity of the cAMP–PKA pathway (via Tpk2), the MAPK pathway (via Kss1) and the glucose repression pathway (via Snf1) (Gagiano et al. 2002; Gancedo 2001; Truckses et al. 2004). This morphogenetic switch allows the yeast cells to forage for nutrients.

5.4.2 Ethanol Stress

Ethanol accumulation during fermentation represents a potent chemical stress towards yeast cells. Low ethanol concentrations are inhibitory to yeast growth and cell division, but higher concentrations can be lethal. *S. cerevisiae* (being the predominant fermentative microorganism) is quite ethanol-tolerant, with some strains able to produce over 20% v/v ethanol by glycolytic metabolism. Other yeasts die at around 5% ethanol. Therefore, a fundamental understanding of ethanol-induced toxicity and ethanol tolerance in yeasts is of distinct commercial significance for alcohol producers, especially those involved in bioethanol (fuel alcohol) processes. Ethanol tolerance may be defined as the ability of yeasts to withstand higher levels of ethanol without any deleterious effects on growth and metabolic activities. Table 5.7 summarises some effects of ethanol on yeast physiology.

Although ethanol passively diffuses out of yeast cells, the plasma membrane is the primary target of ethanol toxicity. Whilst ethanol does deleteriously affect many aspects of yeast cell physiology, increased membrane fluidity and disruption of membrane structural integrity represent major consequences of yeast cell exposure to toxic levels of ethanol. Dissipation of cellular pH gradients and inhibition of the proton pumping plasma membrane ATPase are major deleterious effects of ethanol

Table 5.7 Important effects of ethanol on yeast cell physiology

Physiological function	Ethanol influence/cellular response
Cell viability and growth	General inhibition of growth, cell division and cell viability Decrease in cell volume Induction of morphological transitions (e.g. promotion of germ-tube formation in *Candida albicans*) Enhancement of thermal death
Intermediary metabolism and macromolecular biosynthesis	Denaturation of intracellular proteins and glycolytic enzymes Lowered rate of RNA and protein accumulation Reduction of V_{max} of main glycolytic enzymes Enhancement of petite mutation Enhanced mitochondrial superoxide dismutase activity Induction of heat shock-like stress proteins Elevated levels of cellular trehalose Increase in oxygen free radicals Induced synthesis of cytochrome P450
Membrane structure and function	Decrease in membrane saturated fatty acids (e.g. palmitic) Increase in membrane unsaturated fatty acids (e.g. oleic) Acceleration of sterol biosynthesis (squalene, ergosterol) Induced lipolysis of cellular phospholipids Increased phospholipid biosynthesis (e.g. phosphatidyl inositol) Increased ionic permeability Inhibition of nutrient uptake Inhibition of H^+-ATPase and dissipation of proton motive force Uncoupling of electrogenic processes by promoting passive re-entry of protons and consequential lowering of cytoplasmic pH Hyperpolarisation of plasma membrane

on yeasts (Alexandre et al. 1994). Tolerance to ethanol may be induced in yeast cells following a sublethal heat shock and this indicates that the latter confers a degree of cross-protection at the level of membrane stabilisation. Interestingly, both heat shock and ethanol induce the biosynthesis of a set of common stress proteins and is part of the cells' *adaptive stress response*. Numerous physiological adaptations in yeasts confer protection against ethanol and many of these operate at the level of plasma membrane structural maintenance. For example, *S. cerevisiae* responds to ethanol by increasing fatty acyl chain length and the proportion of unsaturated fatty acids and sterols in the plasma membrane. Ethanol-stressed *S. cerevisiae* also accumulates trehalose, which can also stabilise membranes. That ethanol can induce the synthesis of oxygen free radicals is evident by increased activity of the antioxidant enzyme mitochondrial superoxide dismutase.

Several environmental factors, including media composition, play important roles in dictating yeast ethanol tolerance. For example, divalent cations, particularly Mg^{2+} ions, are able to act as stress protectants against both temperature stress and ethanol toxicity, presumably at the level of cell membrane stabilisation (Birch and

Walker 2000; Walker 1999). Lipid supplementation of fermentation media is also known to improve the ability of yeast cells to withstand otherwise toxic levels of ethanol.

The response of yeast cells to ethanol stress is very similar to the response of cells encountering a heat shock (Piper 1995). The main target of ethanol is the plasma membrane. The fluidity alters during ethanol stress, resulting in changes in permeability to ionic species, especially protons. Microarray analysis of yeast cells after treatment with ethanol (7% v/v) confirmed that the cells try to rescue themselves by altering the expression of genes involved in ionic homeostasis and energy metabolism (Alexandre et al. 2001). In addition there is an activation of the plasma membrane $H(^{+})$-ATPase protein (Piper 1995). The microarray analysis resulted in a large number of differentially expressed genes. About 3% of all genes were upregulated and a similar number of genes were downregulated (Alexandre et al. 2001). About half of these genes are regulated by the Msn2 and Msn4 transcription factors and are also regulated by other environmental stresses. Similar to what has been observed during heat stress, so far only for a few genes altered in gene expression, it has been shown that they are required for the tolerance to high concentrations of ethanol. These include Hsp104, the trehalose biosynthesis genes and the mitochondrial superoxide dismutase Sod2 and the alcohol-sensitive ring/plant homeodomain (PHD) finger protein Asr1 (Betz et al. 2004; Costa et al. 1997; Piper 1995). Apart from microarrays, metabolomics (Martini et al. 2004) and proteomics (Zhou et al. 2004) have also been performed after treatment of yeast cells with ethanol.

However, none of these studies resulted in the identification of an ethanol-specific stress response. Recently two important steps in elucidating ethanol-specific signal transduction mechanisms have been described. First, two-hybrid interaction studies using the nucleoporin Nup116 as a bait resulted in the identification of Asr1. The N-terminal region of Asr1 contains two regions with homology to the interesting new gene (Ring) type or PHD-type finger domain (Betz et al. 2004). These authors found that the localisation of Asr1 is specifically and rapidly regulated by the external level of different kinds of alcohols. Treatment of exponentially growing cells with 7.5% ethanol very rapidly (10 min) relocated the Asr1 protein to the nucleus in a reversible way. As mentioned before, Asr1 is required for ethanol tolerance but not for any other stress type.

Second, one of the phenotypes observed after heat or ethanol stress is the selective mRNA export from the nucleus. Whereas the majority of the mRNAs accumulate in the nucleus, Hsp mRNA is exported under such conditions. The mechanism behind this selective export system has recently been determined. In cells treated with 10% ethanol there is a rapid and reversible nuclear localisation of the DEAD box protein Rat8p. This change correlates very well with the blocking of bulk poly(A)(+) mRNA export. The nuclear localisation is caused by a defect in the Crm1p exportin, the same protein that also interacts with Yap1 to keep it in the cytoplasm under non-oxidative stress conditions (see further). Interestingly, the localisation of Rat8p did not change in heat shocked cells, indicating that there is a different response after heat shock than after ethanol stress (Takemura et al. 2004).

Both ethanol-specific responses open the possibility to characterise the specific signal transduction pathway involved. In addition to ethanol, other fermentation

metabolites are toxic to yeasts, including the other major fermentation product, carbon dioxide, together with secondary products like acetaldehyde.

5.4.3 Oxidative Stress

Oxidative stress causes damage to yeast DNA, proteins and lipids owing to reactive oxygen species such as the superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). These species may be formed endogenously by mitochondrial respiration or exogenously by increasing dissolved oxygen tension of yeast growth media. Oxidative stress arises when yeast antioxidant defences are insufficient to maintain the intracellular redox balance. These defences include non-enzymic (e.g. glutathione, metallothioneins) and enzymic (e.g. catalases, superoxide dismutases) mechanisms (Table 5.8). Glutathione is a thiol compound that protects yeasts by scavangeing oxygen radicals and the metallothioniens can detoxify metal ions (e.g. copper) as wells as protect yeast cells against the damaging effects of oxidants. The antioxidant enzymes able to neutralise oxygen free radicals in yeasts include peroxisomal and cytosolic catalases (encoded by *CTA1* and *CTT* genes, respectively); mitochondrial and cytoplasmic superoxide dismutases (Mn Sod and Cu/Zn Sod, respectively encoded by *SOD2* and *SOD1* genes) and peroxidases (e.g. cytochrome c peroxidase). Interestingly, overexpression of catalase and superoxide dismutase genes increases yeasts' thermotolerance, indicating that oxidative stress may form the underlying basis of other environmental insults.

Pretreating yeasts with sublethal levels of oxidants confers protection owing to an adaptive oxidant stress response, in a similar way to conferment of thermotolerance by pre-heat-shocking cells. One of the adaptive mechanisms seems to be a decrease

Table 5.8 Main antioxidant defences of yeasts

Defence system	Function
Enzymes	
CuZn superoxide dismutase	Dismutation of superoxide anion (cytoplasm)
Mn superoxide dismutase	Dismutation of superoxide anion (mitochondria)
Catalase A	Decomposition of hydrogen peroxide (peroxisome)
Catalase T	Decomposition of hydrogen peroxide (cytoplasm)
Cytochrome c peroxidase	Reduction of hydrogen peroxide
Glutathione reductase	Reduction of oxidised glutathione
Chemicals	
Glutathione	Scavenging of oxygen free radicals
Metallothionein radicals	Cu^{2+}-binding, scavenging of superoxide and hydroxyl
Thioredoxin	Reduction of protein disulphides
Glutaredoxin	Similar function to thioredoxins, protection against H_2O_2
Polyamines	Protection of lipids from oxidation

Modified from Moradas Ferreira et al. (1996) and Estruch (2000)

in H_2O_2 plasma membrane permeability during adaptation to H_2O_2 (Branco et al. 2004). Heat shock will also confer protection against oxidative stress, indicating close interrelationships between various physiological stress responses in yeasts. The signal transduction pathways involved in this oxidative stress tolerance have recently been reviewed (Ikner and Shiozaki 2005).

The sensors to activate the pathways that control the oxidative stress adaptive response were first identified in *E. coli* and have resulted in the identification of specific H_2O_2 and O_2^- sensors, OxyR and SoxR, respectively (Zheng and Storz 2000). In contrast to most other sensors that are located at the plasma membrane, these sensor proteins are transcription factors, localised in the cytoplasm. Genome-wide expression analysis upon treatment of yeast cells with sublethal doses of H_2O_2 has resulted in more than 100 induced spots and about 50 repressed spots on two-dimensional maps and more than 900 induced genes and about 600 repressed genes by DNA microarray mRNA profiling (Causton et al. 2001; Godon et al. 1998). Most of these genes seem to be regulated by only three different transcription factors, functioning alone or in combination. These are Yap1, Skn7 and Msn2/4. These three transcription factors are not only activated by oxidative stress but also by various other types of stress.

Yap1 is important for oxidative, cadmium and drug stress responses. The oxidative and chemical stress sensing seems to occur directly at the level of the Yap1 protein and involves protein phosphorylation followed by cellular redistribution. It has recently been shown that Mtl1, an upstream activator of the PKC1–MAPK cell integrity pathway, may be a cell wall sensor for oxidative stress (Vilella et al. 2005). Addition of diamide or H_2O_2 results in actin cytoskeleton depolarisation. Mtl1, Rom2 and Pkc1 functions are all required to restore the correct actin organisation. Pkc1 is also required to overcome the effects of oxidative stress by enhancing the machinery required to repair the altered cell wall and to restore actin cytoskeleton polarity by promoting actin cable formation.

A hypothesis that has been proposed is that the signal transduction pathway that is activated upon oxidative stress may constitute the upper part of the cell wall integrity pathway which then at the level of Pkc1 may branch into the cell integrity MAPK pathway and into a specific oxidative stress induced pathway consisting of the downstream transcription factors Yap1, Skn7 and Msn2/Msn4. Although this may be true for some inducers of oxidative stress, such as diamide, it is not the case with H_2O_2, where no difference in the expression pattern of genes regulated by Yap1, Msn2/Msn4 or Skn7 between wild-type and *mtl1*Δ strains was observed.

Similar to what we have previously mentioned for Msn2 and Msn4, Yap1 cellular localisation is also dependent on the stress situation. Under non-stress conditions, Yap1 is rapidly exported out of the nucleus by interaction with the nuclear export protein Crm1 (Yan et al. 1998). Upon oxidative stress induction, the interaction between Yap1 and Crm1 is lost, probably by Yap1 phosphorylation, and Yap1 is rapidly redistributed to the nucleus, with the help of the nuclear import receptor Pse1 (Isoyama et al. 2001), where it can bind to Yap1 response elements (YRE) in the promoter regions of different genes.

Skn7 as well as Msn2 and Msn4 seem to be stress-response coordinators as they are involved in many types of stress.

5.4.4 Anaerobiosis

Most yeasts are aerobic and few tolerate strictly anaerobic conditions. *S. cerevisiae* has an absolute requirement for oxygen that is necessary for the synthesis of certain fatty acids and sterols This species is auxotrophic for oleic acid and ergosterol under strictly anaerobic conditions. Oxygen is also required as the terminal electron acceptor for yeasts' respiration. Yeasts can be placed in different groups based on their growth responses to oxygen availability (Table 5.9). The molecular basis of anaerobic stress tolerance and the signal transduction pathways involved have not yet been studied at the molecular level.

5.4.5 Yeast Biocides

In addition to the chemical stresses mentioned earlier, yeast cells may also be subjected to chemicals that are purposely designed to control their growth and metabolic activities. This is precisely the case when endeavouring to prevent growth of spoilage yeasts in foods and beverages. Such yeasts may be controlled by temperature, anhydrobiosis or by addition of yeast preservatives such as weak acids. These weak acids include sorbic, benzoic and acetic acids that have wide uses as anti-yeast agents in foods and beverages. These acids are transported into yeasts in their undissociated form and act by dissipating plasma membrane proton gradients and depressing cell pH when they dissociate into ions in the yeast cytoplasm.

To counteract the effects of weak acids, *S. cerevisiae* is endowed with a stress response that acts to reduce the possibility that the weak acid will accumulate within its cells to potentially toxic levels. The high anion accumulation may influence free-radical production, leading to severe oxidative stress. In the presence of oxygen the

Table 5.9 Classification of yeasts based on responses to oxygen

Class	Examples	Comments
Obligately fermentative	*Candida pintolopesii* (*Saccharomyces telluris*)	Naturally occurring respiratory-deficient yeasts. Only ferment, even in presence of oxygen
Facultatively fermentative		
Crabtree-positive	*S. cerevisiae*	Such yeasts predominantly ferment high-sugar-containing media in the presence of oxygen
Crabtree-negative	*Candida utilis*	Such yeasts do not form ethanol under aerobic conditions and cannot grow anaerobically
Non-fermentative	*Rhodotorula rubra*	Such yeasts do not produce ethanol, either in the presence or in the absence of oxygen

Adapted from information by Van Dijken and Scheffers (1986) and Scheffers (1987)

energy crisis is further exacerbated because of the mitochondrial electron transport chain dysfunction. This results in high endogenous levels of superoxide free radicals (Piper 1999).

Different yeast species have developed different strategies to cope with this kind of stress. The spoilage yeast *Z. bailii* seems to limit the diffusion of the acid into the cell. In addition, this yeast can also oxidatively degrade sorbate and benzoate, two of the most commonly used food preservatives. This explains the recent finding that tolerance to weak acid stress in this yeast occurs at no energy cost (Leyva and Peinado 2005).

This is in contrast to the situation in *S. cerevisiae* where weak acid stress results in a strong induction of the expression of the ATP binding cassette (ABC) transporter Pdr12 that is located in the plasma membrane. This transporter extrudes the acid from the cell at a high cost of ATP. In addition, to keep the electrochemical potential difference across the membrane the protons are extruded by the plasma membrane H^+-ATPase (Pma1). The strong upregulation of Pdr12 upon being challenged with 1 mM sorbate at pH 4.5 seems to be very specific as it is not present upon being challenged with other types of stress (Piper et al. 1998). This indicates that there must exist a weak-acid-induced signal transduction pathway that is different from the other stress-induced pathways.

Recently, the combined efforts of functional genomics and microarray analysis resulted in the identification of a transcription factor, War1p, for weak-acid resistance. This factor has been identified in two independent screening assays. In a functional screening assay, strains deleted for putative transcription factors were tested for their capacity to grow in the presence of 1 mM sorbate (Kren et al. 2003). Alternatively, classical mutagenesis in a strain that harbours the *PDR12* promoter–*LacZ* reporter construct was performed and mutants that were not able to induce the *LacZ* reporter gene were identified as carrying loss-of-function alleles of *WAR1* (Bauer et al. 2003). The probable mechanism of action is presented in Fig. 5.5 (Kren et al. 2003). Weak acids can enter the cell by passive diffusion at low pH. The higher intracellular pH dissociates weak acids, generating protons and $RCOO^-$ anions that accumulate intracellularly. Within minutes, the transcription factor War1p, which is constitutively bound to the weak-acid response element (WARE) present in the promoter of *PDR12*, becomes more phosphorylated, resulting in the induction of transcription of the Pdr12 efflux pump. Cells lacking either War1 or Pdr12 are weak-acid-hypersensitive. There is no increased sorbate sensitivity in the *war1*Δ *pdr12*Δ strain, suggesting that Pdr12 is the most important War1 target (Kren et al. 2003). Ectopic expression of *PDR12* from the *GAL1-10* promoter fully restored sorbate resistance in a strain lacking War1p, demonstrating that *PDR12* is the major target of War1p under sorbic acid stress. An important question that remains to be answered is the identification of the upstream parts of this new signalling pathway. What is the sensor? What is the signal transduction pathway upstream of War1? Is War1 activated directly by the organic monocarboxylate anions? To identify novel components of the pathway, genome-wide expression analysis of the response of yeasts to weak-acid stress was performed and this resulted in more than 100 genes that were induced. Three different pathways seem to be responsible for the weak-acid response. These are pathways mediated by War1,

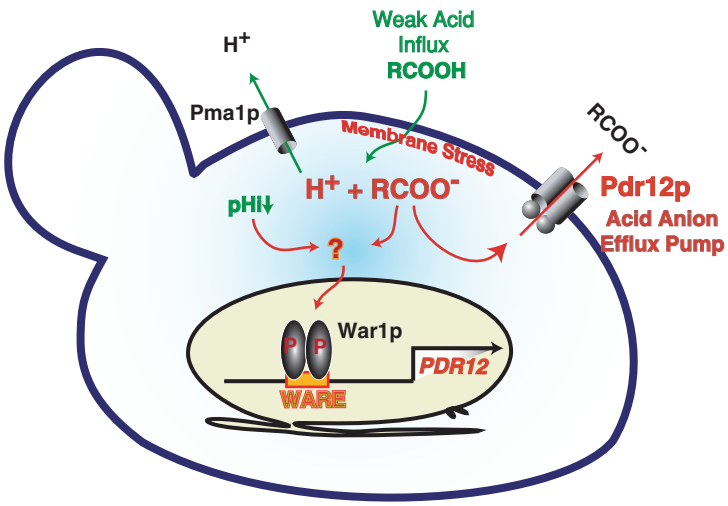

Fig. 5.5. Weak-acid-specific signal transduction pathway in *S. cerevisiae* (Kren et al. 2003)

Msn2/Msn4 and a third pathway. Only one of the targets, *PDR12*, turned out to be both stress inducible and required for weak-acid resistance (Schüller et al. 2004). In an alternative approach, the yeast deletion mutant collection was screened for mutants whose growth is affected in the presence of sorbic acid. Two hundred and thirty-seven mutants were identified as incapable of growing at pH 4.5 in the presence of 2 mM sorbic acid, whereas 34 mutants were more resistant compared with the wild-type strain (Mollapour et al. 2004). The direct role in weak-acid-stress signalling for these various genes awaits further investigation.

Important for the food industry is that similar to heat or cold adaptation, yeast cells can also adapt to weak acids. Challenging *S. cerevisiae* cells at low pH (4.5) with low concentrations of sorbate or benzoate (0.5–2.5 mM) results in a rapid entry into the G0 phase of the cell cycle. After several hours, they resume growth because they are then weak-acid-adapted (Holyoak et al. 1999; Piper et al. 1998). A first clue in the molecular mechanism behind this behaviour came from the *S. cerevisiae* Cmk1 mutant. This mutant did not show the long period of growth arrest. Cmk1 is a Ca^{2+}/calmodulin-dependent protein kinase. These data indicated that the weak-acid-induced cell cycle arrest must be repressed by this kinase (Holyoak et al. 2000). Whether Cmk1 is responsible for the phosphorylation of War1 remains to be investigated.

Sulphur dioxide has long been used as a yeast (and bacterial) preservative in the manufacture of alcoholic beverages, especially wine. It acts by dissociating within the yeast cell to SO_3^{2-} and HSO_3^-, decreasing intracellular pH. Unlike *S. cerevisiae*, *Z. bailii* growth is very sensitive to increasing extracellular Ca^{2+} concentrations, which suggests a simple expedient to limit spoilage by *Z. bailii* (Demidchik et al. 2004).

In clinical mycology, infective yeasts like *Candida albicans* are controlled using agents that act by targeting the plasma membrane by inhibiting the biosynthesis of ergosterol and cell wall components (e.g. glucan, mannoprotein and chitin) and the activity of the plasma membrane H^+-ATPase. Chauhan and Calderone (2004) have recently reviewed adaptive stress responses in human pathogenic yeasts.

5.5 Summary and Conclusions

Table 5.10 summarises some of the physiological responses of yeasts to physical and chemical stresses that impair growth and metabolism.

It is apparent that yeasts, particularly industrial strains of *S. cerevisiae*, are actually quite resilient organisms that are able to respond quite well, and often very rapidly, to sudden changes in their physico-chemical environment. Of course, some yeasts are better than others in adapting to stress and there are many examples of yeast species that can be described as osmotolerant, ethanol-tolerant, etc. In an effort to survive multiple environmental stresses, yeast cells may activate certain common molecular-level responses, such as trehalose accumulation, antioxidant production and stress protein biosynthesis. Stress responses are therefore inter-related and yeasts will succumb to stress and die when protective measures (either general or specific) initiated by cells are overcome. A lot of fundamental physiological and molecular knowledge has been accumulated in recent years concerning the stress responses of *S. cerevisiae*. However, we now need to extend this knowledge to production strains of this yeast growing in industrial environments, and to other important food yeast species. Deeper understanding of stress responses in such yeasts is key to their successful exploitation in food fermentations and to control of food spoilage yeasts.

Table 5.10 Major physiological responses of yeast to environmental stress

Stress	Physiological responses
High temperature	Heat shock protein biosynthesis Decreased membrane lipid unsaturation Altered cell pH Polyamine biosynthesis
Low temperature	Enhanced trehalose accumulation Increased membrane lipid polyunsaturation
Osmotic pressure	Accumulation of compatible solutes (glycerol, trehalose) Increased K^+ uptake/Na^+ efflux
Dehydration	Trehalose accumulation
Oxidants	Enzymic: superoxide dismutase, catalase, cytochrome peroxidase Non-enzymic: glutathione, thioredoxin, metallothionein, polyamines, carotenoids
Toxic chemicals	Ethanol: stress proteins, altered membrane transport, mitochondrial superoxide dismutase Xenobiotics: glutathione Heavy metals: stress proteins, metallothioneins

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Chapter 6

Molecular Mechanisms Involved in the Adaptive Evolution of Industrial Yeasts

Eladio Barrio[1], Sara S. González[1,2], Armando Arias[1], Carmela Belloch[2] and Amparo Querol[2]

[1]*Institut 'Cavanilles' de Biodiversitat i Biologia Evolutiva, Universidad de València, Parc Científic de Paterna, P.O. Box 2085, 46071 Valencia, Spain (e-mail: eladio.barrio@uv.es, Tel.: +34-963-543667, Fax: +34-963-543670; e-mail: aarias@cucba.udg.mx)*
[2]*Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), P.O. Box 73, 46100 Burjassot, Valencia, Spain (e-mail: sargongon@iata.csic.es; e-mail: belloch@iata.csic.es; e-mail: aquerol@iata.csic.es)*

6.1 Introduction

Adaptation is obviously a key concept in modern biology, but its precise meaning has often been controversial (Mayr 1982). At the most basic level, the concept of adaptation is related with function. This way, some trait, or integrated suit of traits, of an organism is adaptive if it performs a function that is, in some way, beneficial to the organism to live in an environment. Adaptations can involve aspects of an organism's behavior, physiology, morphology, etc., or the ability of an individual to alter those properties depending on the environment (phenotypic plasticity). The originality of the theory of natural selection proposed by Charles Darwin lay in the fact that it provided a hypothesis to explain the origin of adaptations. Since then, adaptive traits have been considered the result of adaptive evolution, i.e., an evolutionary process directed by natural selection.

The neo-Darwinian theory of evolution by natural selection, also known as the new synthesis, was based on the idea that most natural populations contain enough genetic variation to respond to any sort of selection. Most of this genetic variation is due to the presence of different alleles generated by mutation and homologous recombination. Adaptation may then be explained by the gradual evolution resulting from small changes in the allele frequencies acted upon by natural selection.

However, with the advent of molecular methods, the potential importance of major, new mutations (novelties) in adaptive evolution has been emphasized (Nei 1987; Li 1997). Molecular studies have shown that mutations include not just the generation of new alleles by nucleotide substitution, but such important processes as the generation of new genes, not only by gene duplication (Long et al. 2003), or radically new alleles by unequal crossing over. The complete sequencing of different

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
© Springer-Verlag Berlin Heidelberg 2006

yeast genomes as well as the study of the molecular basis of the physiological properties of yeasts have provided unique tools to study the molecular mechanisms involved in the adaptive evolution of yeast traits of industrial interest.

In the present chapter, we are not going to deal with the procedures to identify, demonstrate or understand the adaptive significance of the traits and properties of industrial yeasts. Rather, we are going to review the different molecular mechanisms involved in the generation of these major genetic novelties that can explain the adaptive evolution of industrial yeasts.

6.2 The *Saccharomyces sensu stricto* Complex Includes the Most Important Industrial Yeasts

Yeasts are defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth results predominantly from budding or fission, and which do not form their sexual states within or upon a fruiting body (Kurtzman and Fell 1998). Of the more than 700 known yeast species, several dozen are used in industrial processes, mainly in the production of fermented products and metabolites. Among them, the most useful and widely exploited species are those from the *Saccharomyces* genus, especially *S. cerevisiae* and its relatives, included in the *Saccharomyces sensu stricto* complex.

The *Saccharomyces sensu stricto* complex (Vaughan-Martini and Martini 1998) consists of three species associated with industrial fermentation processes, *S. bayanus*, *S. cerevisiae*, and *S. pastorianus*, and four species isolated from natural habitats, *S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, and *S. paradoxus*.

S. cerevisiae has been found associated to very diverse fermentation processes, including baking, brewing, distilling, winemaking, and cider production, and also in different traditional fermented beverages and foods around the world. The origin of *S. cerevisiae* is controversial. Some authors propose that this species is a "natural" organism present in plant fruits (Mortimer and Polsinelli 1999). Others argue that *S. cerevisiae* is a "domesticated" species found only in association with human activities, because attempts to find this species in regions remote from human activities have been unsuccessful (Naumov 1996). Moreover, some authors suggested that this species could originate from its closest relative *S. paradoxus*, a wild species found all around the world (Vaughan-Martini and Martini 1995). This debate is important in postulating the original genome of *S. cerevisiae* and how the strong selective pressure applied since its first unconscious use in controlled fermentation processes has reshaped it.

The cryophilic *S. bayanus* has been found in nature in cold areas of Europe and also appears associated with different fermentation processes: winemaking, cider production, brewing, and as grape must contaminants. The type strain of this species, originally isolated from beer, has recently been described as a hybrid possessing also a nuclear genome from *S. cerevisiae* (Nguyen et al. 2000; de Barros Lopes et al. 2002; Nguyen and Gaillardin 2005), which led to the proposal of the reinstatement of *S. uvarum*, a former taxon included in *S. bayanus*, as a distinct species (Pulvirenti et al. 2000; Nguyen and Gaillardin 2005) or as a different variety within *S. bayanus* (Naumov 2000).

S. pastorianus (synonym *S. carlsbergensis*) is the bottom-fermenting yeast responsible of the production of lager beer, although it has also been found in musts and

wines. Different studies (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001) have demonstrated that strains of this species correspond to natural hybrids between *S. cerevisiae*, and a *S. bayanus* like yeast. Chromosome sets from both parental species are present in strains of *S. pastorianus* (Tamai et al. 1998; Yamagishi and Ogata 1999), while the mitochondrial DNA (mtDNA) was inherited from the non *S. cerevisiae* parent (Piškur et al. 1998).

The wild yeast *S. paradoxus*, the closest relative to *S. cerevisiae*, according to phylogenetic reconstructions (Rokas et al. 2003), is a natural species distributed worldwide with a fortuitous presence in fermentation processes. However, it has recently been described as the predominant yeast in Croatian vineyards (Redzepović et al. 2002).

Finally, the *Saccharomyces sensu stricto* complex also includes three other wild species, *S. cariocanus*, *S. mikatae*, and *S. kudriavzevii*, whose description (Naumov et al. 2000a) was based on a few strains isolated from natural habitats in Brazil, the first one, and Japan, the other two.

Saccharomyces sensu stricto yeasts possess a series of unique characteristics that are not found in other genera (Vaughan-Martini and Martini 1998). One of these unique characteristics is their high capability to ferment sugars vigorously, both in the presence and in the absence of oxygen, to produce ethanol. This ability allows them to colonize sugar-rich substrates (plant saps and fruits) and compete with other yeasts, which are not so tolerant to alcohol. The aparition of angiosperm plants with sugar-rich saps and fruits introduced a new ecological niche with a different selection regime that likely imposed altered physiological demands on the ancestors of *Saccharomyces* yeasts (Wolfe and Shields 1997). Under such circumstances, adaptive evolution took place in this new ecological context favoring the acquisition of such high fermentative capability.

This capability has unconsciously been used by humans to produce fermented foods and beverages, which introduced new selective pressures on these yeasts. Neolithic human populations likely observed that fruit juice spontaneously ferments producing an alcoholic beverage (Mortimer et al. 1994). Since then, the yeast *S. cerevisiae* and related species have become an essential component of many important human activities, including baking, brewing, distilling, and winemaking.

In general, these industrial *Saccharomyces* strains are highly specialized organisms which have evolved to utilize the different environments or ecological niches that have been provided by human activity. This process can be described as "domestication" and is responsible for the peculiar genetic characteristics of the industrial yeasts. During the last few years, intensive research efforts have been focused on elucidating the molecular mechanisms involved in yeast adaptation to the industrial process, and the reshaping of genomic characteristics of the industrial yeast which have been unconsciously selected over billions of generations (Querol et al. 2003).

6.3 Adaptive Evolution by "Genome Renewal"

Although *Saccharomyces sensu stricto* yeasts are becoming ideal model organisms to test population genetics models (Zeyl 2000) and to study speciation mechanisms (Greig et al. 2002a), very little information is available about the genetic variability of natural *Saccharomyces* populations.

The analysis of natural populations of *S. cerevisiae* from spontaneous wine fermentations (Mortimer et al. 1994) showed that, although genetic diversity was high, almost all strains were homozygous for most of the genes analyzed. This observation, together with the high fertility of the strains and their homothallic character, led the authors of the study to propose a mechanism of evolution for natural wine yeasts, termed genome renewal. This hypothesis is based on the ability of homothallic haploid *S. cerevisiae* cells to switch their mating type and conjugate with cells of the same single-spore colony to produce completely homozygous diploids. Strains of *S. cerevisiae* accumulating heterozygous recessive mutations can change to completely homozygous diploids by sporulation and homothallic switching of individual haploid spores. This process would favor the action of selection, removing recessive deleterious genes and fixing recessive beneficial alleles, thereby enabling yeasts to adapt efficiently to changing environmental conditions. However, Puig et al. (2000) demonstrated that homozygosis could also be achieved by mitotic recombination or gene conversion during vegetative growth.

6.4 Molecular Mechanisms Involved in the Generation of Evolutionary Novelties

Decades of genetics research and the development of large-scale genomic approaches led to the complete sequencing of the genome of *S. cerevisiae* (Goffeau et al. 1996), the first eukaryote to have its genome sequenced. The available molecular techniques and the rapidly expanded genome data with recent publication of new genome sequences from yeasts (Cliften et al. 2003; Kellis et al. 2003, 2004; Dietrich et al. 2004; Dujon et al. 2004), including other *Saccharomyces sensu stricto* species, provided a new approach to decipher the molecular mechanisms involved in the generation of evolutionary novelties in yeasts. Also, molecular evolution and molecular population genetics have provided useful analytical tools for the detection of the processes and mechanisms that underlie the origin of these evolutionary novelties.

Recently, Long et al. (2003) reviewed the different molecular mechanisms that are known to be involved in the creation of new gene structures, the details of which are understood to varying degrees. In the next sections, we will provide evidence of the role of several molecular mechanisms in the adaptive evolution of yeasts.

6.4.1 Gene Duplication

Gene duplication as the most important source of new genes was postulated by Haldane (1933). He proposed that redundant gene copies generated by gene duplications (called paralogues, i.e., genes that are homologous by duplication of an ancestral gene, in contrast to orthologues, genes that are homologous by descent) are not constrained to maintain their original function and, hence, they can accumulate divergent mutations, resulting in new gene functions.

Gene duplications can be produced by different mechanisms resulting in the duplication of a single gene or a group of adjacent genes (Koszul et al. 2004), in the duplication of a chromosome, called aneuploidy (Hughes et al. 2000), or in the duplication of the whole genome content, called polyploidy (Wolfe and Shileds 1997).

In some cases, redundant genes could be retained if there is an evolutionary advantage to having extra dose repetitions. In others, one duplicate will be free to accumulate mutations because only one of the duplicates will be under purifying selection owing to the restrictions to maintain the ancestral gene function. The classical model of acquisition of new genes by duplication proposes that both paralogues could be preserved if one of them acquires a mutation with a new, beneficial function and the other retains the original function (a process called neofunctionalization). However, this process was assumed to be extremely rare (Wagner 1998), because most changes neutrally fixed in the unrestricted duplicate will be loss-of-function mutations, and, hence, this copy will become a pseudogene to be finally lost (a process known as nonfunctionalization). Accordingly, the classical model predicted that few duplicates should be retained in the genome over the long term, but the sequencing of complete genomes showed that retention of ancient duplicates is very common (Wagner 1998).

To explain the preservation of paralogous genes, Force et al. (1999) proposed an alternative process, called subfunctionalization, whereby both members of a pair acquire complementary degenerative mutations in independent subfunctions, originally present in the ancestral gene. This way, both duplicates are required to produce the full patterns of activity of the single ancestral gene, and subsequent adaptive evolution will promote their subfunctional specialization.

The *GAL1* and *GAL3* paralogous genes of the *Saccharomyces sensu stricto* species provide an example of subfunctionalization in yeasts (Hughes 1999). The galactose-inducible *GAL1* gene encodes a galactokinase that catalyzes the production of galactose-1-phosphate from galactose and ATP, whereas the galactose-inducible *GAL3* gene encodes a regulatory protein involved in the activation of both *GAL1* and *GAL3* genes in the presence of galactose and ATP. *Kluyveromyces lactis* contains a single *GAL1* gene encoding a protein with both regulatory and structural functions. The phylogenetic analysis of these genes indicates that *K. lactis GAL1* diverged from the *Saccharomyces sensu stricto GAL1-GAL3* genes before the gene duplication event, indicating that each paralogue specialized by subfunctionalization.

6.4.1.1 Polyploidization: Whole Genome Duplication in Yeasts

The importance of whole genome duplication in the evolution of higher eukaryotes was postulated by Ohno (1970). The complete sequencing of diverse eukaryote genomes revealed that whole genome duplications occurred several times during the evolution of certain eukaryotic lineages (some plants, fishes, amphibians, etc.).

One of the most striking results obtained from the sequencing of the *S. cerevisiae* complete genome was the presence of 376 gene pairs within 55 large duplicated regions. This observation led Wolfe and Shields (1997) to propose that a whole-genome duplication event, polyploidization, occurred in an ancestor of *S. cerevisie* after the split from *K. lactis*, some one hundred to two hundred million years ago. Polyploidization followed by extensive gene loss of most paralogues by pseudogenization and the accumulation of chromosomal rearrangement events explains the observed pattern of dispersed, large segmental duplications present in the *S. cerevisiae* genome (Keogh et al. 1998).

The hypothesis that *S. cerevisiae* is a paleopolyploid was initially very controversial. Other authors suggested that the duplicated segments could arise via independent local duplication events (Souciet et al. 2000), but the comparative analysis of gene order (Wong et al. 2002) in the genomes of different yeast species, partially sequenced by the Genolévures consortium (Souciet et al. 2000), corroborated this hypothesis and also allowed the location of the polyploidization event in the phylogeny of the hemiascomycetous yeasts. The complete, or almost complete, sequencing of genomes from yeast species of the *Saccharomyces* complex diverged before the genome duplication event (*Saccharomyces sensu stricto*, Kellis et al. 2003; Cliften et al. 2003; *S. castellii*, Cliften et al. 2003; *Candida glabrata*, Dujon et al. 2004) and after (*S. kluyveri*, Cliften et al. 2003; *Ashbya gossypii*, Dietrich et al. 2004; *K. waltii*, Kellis et al. 2004; *K. lactis*, Dujon et al. 2004) confirmed that the duplication event encompassed the entire genome, and was produced by polyploidization of an ancestor of *S. cerevisiae* and related species. The comparison of pre- and postduplication genomes allowed the conclusion to be drawn that the whole genome duplication event doubled the number of chromosomes in the *Saccharomyces* lineage, but subsequent gene-loss events, 88% of paralogous genes were lost, led to the current *S. cerevisiae* genome, which contains only about 500 more genes than the preduplication species, but distributed among 16 chromosomes instead of eight. The polyploid genome returned to functional normal ploidy, not by meiosis or chromosomal loss, but instead by a large number of deletion events of small size (average size of two genes), balanced between the two duplicated regions.

Polyploidization in yeasts can theoretically occur by different mechanisms: (1) an error during meiosis can lead to the production of diploid spores and subsequent conjugation between diploid cells, (2) an error during mitosis in unicellular organisms, (3) rare mating between two diploid yeasts of the same species that became mating-competent by interchromosomal mitotic recombination at the MAT locus (de Barros Lopes, 2002), (4) interspecific hybridization by conjugation of spores from different species, and subsequent genome duplication by errors during mitosis or meiosis, or (5) rare mating between two mating-competent diploid strains belonging to different species (de Barros Lopes 2002). In the first three cases, the result is an autotetraploid yeast, whose nucleus contains four allelic copies of each chromosome; however, in the last two cases, the result is a fertile allotetraploid (also called amphidiploid) yeast, containing pairs of "homeologous" chromosomes, i.e., homologous chromosomes coming from two different species. Examples of both types of polyploid yeasts have been described (Naumov et al. 2000b).

Andalis et al. (2004) demonstrated that isogenic autopolyploidy is accompanied by defects affecting viability and subsequent survival of the new organisms, and, hence, postulated that the entire genome duplication event that occurred in an ancestor of *S. cervisiae* was likely generated by allopolyploidization.

But the most important consequence of the whole genome duplication event was the sudden acquisition of extra copies of each gene in the genome. Wolfe (2001) suggested that these duplicated genes formed by polyploidy should be called "ohnologues", after Susumu Ohno, to distinguish them from other kinds of paralogues because they are all the same age.

The complete genome sequences of *K. waltii* (Kellis et al. 2004) and *A. gossypii* (Dietrich et al. 2004), species that diverged before the polyploidization event, were used to map and analyze the fate of the ohnologues during the evolution of the *S. cerevisiae* lineage. The different expected outcomes with respect to the fate of duplicated genes, described in Sect. 6.4.1, were observed. This way, nonfunctionalization was the most frequent process: 88% of paralogous genes generated by polyploidization were lost.

Of the approximately 460 surviving ohnologues, 60 pairs showed decelerated evolution and tend to be highly similar, even at the silent codon positions, suggesting that they may be subject to periodic gene conversion. Moreover, in about half of these cases, the two paralogues in *S. cerevisiae* are closer in sequence to each other than either is to its orthologue in *S. bayanus*, showing that gene conversion occurred after the relatively recent divergence of the two *Saccharomyces* species. These cases often involve proteins known to be highly constrained, such as ribosomal proteins, histone proteins, and translation initiation/elongation factors, indicating that they have likely been retained because of the advantage of having extra dosage of the genes.

The remaining ohnologues have diverged in sequence and often also in function. Kellis et al. (2004) found that more than 100 gene pairs show a higher rate of protein evolution relative to *K. waltii*, with one ohnologue accumulating significantly more amino acid replacements than the other. They also argue that, in many of these cases, accelerated evolution was confined to only one of the two paralogues, which strongly supports a process of neofunctionalization, the slowly evolving paralogue has probably retained the ancestral gene function and the rapidly evolving paralogue probably corresponds to the copy relieved of selective constraints, which is free to evolve more rapidly to acquire a derived function after duplication. Most of these ohnologues correspond to protein kinases and regulatory proteins, generally involved in metabolism and cell growth.

The other approximately 300 ohnologue pairs did not show significant differences in their rates of evolution. In some cases, the functional changes may be similar to those just described but subtler. In other cases, gene pairs may have been retained by subfunctionalization. Specialization to different ancestral subfunctions may explain the similar rates of evolution in both ohnologues. Moreover, this subfunctionalization may have occurred by divergence in regulatory sequences.

The polyploidization event suddenly provided new gene functions that have had a profound impact in the evolution of the *Saccharomyces sensu stricto* lineage (Piškur and Langkjær 2004; Wolfe 2004). The partitioned functions of most ohnologues, retained in the *Saccharomyces sensu stricto* lineage, indicate that the genome duplication provided new genes that played a direct role in the adaptation of these species toward a highly efficient fermentation performance under anaerobic conditions. Wolfe and Shields (1997) indicated that many ohnologue pairs are differentially regulated in the presence and absence of oxygen (DeRisi et al. 1997), including genes of proteins of the electron transport chain complexes (e.g., *CYC1*/*CYC7* encoding cytochrome c isoforms, or *COX5A*/*COX5B* encoding cytochrome c oxidase subunit 5 isoforms) and genes encoding enzymes of the glycolysis/gluconeogenesis pathway (e.g., *PYK1*/*PYK2* coding for pyruvate kinases, *ENO1*/*ENO2* for enolases, etc.).

The polyploidization also allowed the development of efficient glucose-sensing and glucose-repression pathways (Kwast et al. 2002). Ohnologues encoding regulatory proteins are involved in the development of the two glucose-sensing pathways of high affinity and low affinity, the Snf1 pathway of glucose-repression of gluconeogenesis and respiration, and in the glucose-responsive protein kinase A pathway (Wolfe 2004).

In conclusion, the polyploidization event provided the basis for the evolution of new gene functions during the competition to colonize sugar-rich substrates supplied by fruit-bearing plants. The competitive advantage of a fermentative metabolism, fast growth and the production of toxic ethanol put the ancestors of the industrial *Saccharomyces* yeasts in the pole position to become, under the selective pressures unconsciously imposed to improve controlled fermentation processes, the highly efficient mono- and oligosaccharide fermenters that exist today.

6.4.1.2 Aneuploidy: Chromosome Duplication

An alternative mechanism to provide potential new genes is by changing chromosome copy numbers, which is known as aneuploidy. However, the most important consequence of aneuploidy is the increase of gene dose.

Aneuploidy arises by nondisjunction, i.e., inaccurate chromosome segregation, during meiosis or mitosis. The increase in copy numbers for some genes results in an imbalance of the gene products and disruption of the regulatory interactions, which could be deleterious or even lethal for many organisms. Although aneuploidy is tolerated in industrial yeasts, it is one of the causes of the poor sporulation exhibited by some strains.

Wine *Saccharomyces* strains are frequently aneuploid, with disomies (two chromosome copies), trisomies and, less frequently, tetrasomies (Bakalinsky and Snow 1990). This aneuploidy, and also autopolyploidy, has been postulated as a mechanism that may confer advantages for adaptation to variable external environments by increasing the number of copies of beneficial genes or by protecting the yeasts against recessive lethal or deleterious mutations (Bakalinsky and Snow 1990; Guijo et al. 1997; Salmon 1997).

Hughes et al. (2000) observed that the deletion of a gene strongly favors the acquisition of a second copy of a whole chromosome or a chromosomal segment containing a paralogue of the deleted gene. About 8% of 300 yeast deletion mutants examined had acquired a detectable aneuploidy, and in six of the cases they examined, the amplified chromosome contained a close paralogue of the deleted gene, implying that characteristic aneuploidies can act as dominant suppressors and under some circumstances lead to increased fitness.

Kellis et al. (2004) correlated these deletion results with the identification of the ancestral and derived functions of paralogues (Sect. 6.4.1.1). Strikingly, deletion of the ancestral paralogue was lethal in 18% of cases, whereas deletion of the derived paralogue was never lethal. The derived paralogue is thus not essential under these conditions, either because it does not function in a rich medium or because the ancestral paralogue can complement its function. Along with possibly gaining a new function, the derived copy has lost some essential aspect of its function, and cannot typically complement deletion of the ancestral gene.

6.4.1.3 Single Gene and Segmental Duplications

Gene duplication can also involve either a single gene or a group of adjacent genes (segmental duplication). Genome sequencing projects have revealed that multigene families, i.e., groups of identical or similar genes generated by successive single gene or segmental duplications, are common components of all genomes. This way, the *S. cerevisiae* genome contains 265 multigene families with three or more paralogues, including a family with 108 members (Llorente et al. 2000), which indicates that successive gene duplications should have occurred.

Genome comparisons (Souciet et al. 2000; Dujon et al. 2004) showed that tandem repeated gene duplication is very common among yeasts and illustrates the importance of ancestral duplications that occurred before divergence of hemiascomycetous yeasts. Sequence divergence between paralogues in different yeast species shows bimodal distributions, with a fraction of multigene families showing high sequence identities, probably reflecting recent duplications and/or sequence homogenization by gene conversion, and an important fraction with low identities, corresponding to ancient duplications that occurred before species divergence.

Single-gene and segmental duplications mainly correspond to intrachromosomal direct tandem-repeat duplications. Although there are some examples of segmental duplications that are dispersed throughout the genome, most gene families are located in subtelomeric regions (adjacent to chromosome telomeres). Classical examples of redundant genes in subtelomeric regions are the *MEL*, *SUC*, *MGL* and *MAL* genes involved in the assimilation of sugars. Yeast strains differ by the presence or absence of particular sets of these genes, which could be attributed to selective pressure induced by human domestication, as it appears that they are largely dispensable in laboratory strains.

Clusters of duplicated genes have also been found internal to chromosomes. A typical example is the large gene cluster on chromosome VIII near *CUP*1. The *CUP*1 gene encoding copper metallothionein, is contained in a 2-kb repeat that also includes an open reading frame (ORF) of unknown function (Fogel and Welch 1982). The repeated region has been estimated to span 30 kb in laboratory strains, which could encompass 15 repeats, but the number of repeats varies among yeast strains.

Different mechanisms have been postulated to explain the origin of single-gene and segmental tandem duplications. The critical step is the origin of the first tandem duplication, which requires the presence of similar nucleotide sequences flanking the duplicated region. These similar sequences may also be provided by transposable elements. Ectopic recombination between homologous chromosomes or unequal sister chromatide exchange, at the similar sequences, will result in the duplication of the genome region. Subsequent duplications can occur by ectopic recombination between paralogous repeats.

The fate of the duplicated genes is discussed in Sect. 6.4.1. However, many tandemly duplicated genes exhibit identical or nearly identical sequences, indicating that these multigene families evolve in a concerted way to preserve gene function, and, hence, increase gene dosage. Ectopic recombination and gene conversion are the mechanisms postulated to explain the concerted evolution observed in the members of multigene families (Li 1997).

Another process, postulated to preserve identical function in the members of a gene family, is the birth-and-death model of multigene family evolution (Nei et al. 1997), in which repeated gene duplications are counterbalanced by gene degeneration or deletion (nonfunctionalization). A systematic analysis of *S. cerevisiae* intergenic regions revealed the presence of many degenerated pseudogenes, called gene relics, homologous to extant *S. cerevisiae* ORFs (Lafontaine et al. 2004). Gene relic distribution is mainly subtelomeric and related to multigene families. Thus, multigene family evolution by a gene birth-and-death mechanism is also compatible with the presence of new paralogues and relics in several yeast strains and the sequence polymorphism within the tandem *DUP240* family, one of the largest *S. cerevisiae* gene families (Leh-Louis et al. 2004a, b).

Many of the tandemly repeated genes, especially the subtelomeric multigene families, are involved in secondary metabolism. These genes are not essential, but they play an important role in the adaptation to new environmental conditions. For example, subtelomeric gene families in *S. cerevisiae* are often related to cell membrane and cell wall components, such as lectine-like proteins (the *FLO* family), sugar transporters (the *HXT* family), genes related to cell–cell fusion (the *PRM* family), and assimilation and utilization of nutrients (*GAL*, *MAL*, *SUC*, and *PHO* families) (Vega-Palas 2000; Harrison et al. 2002). Some dispersed gene families may also be related to adaptation to environmental conditions, such as the *CUP1* gene tandem repeats present in copper-resistant *S. cerevisiae* strains (Fogel and Welch 1982).

Other species, including those that diverged before the whole genome duplication event, also contain subtelomeric gene families that are probably involved in adaptation to changing environments. For example, the genome of *K. waltii* also contains several families of membrane proteins, hexose transporters, and flocculins (Kellis et al. 2004); and multigenic families encoding multidrug resistance proteins and hexose transporters are specifically more expanded in *Debaryomyces hansenii* than in the other yeasts (Dujon et al. 2004).

Many of these subtelomeric repeats were likely advantageous to industrial strains during selection for thousands of years of human biotechnology practices. Rapid changes in the gene composition of these families may increase the chances of acquiring a selective advantage and improving their industrial fitness. In fact there are several examples of spontaneous gene duplications selected as a response to limiting conditions (Brown et al. 1998).

6.4.2 Lateral Gene Transfer: Acquisition of New Genes from Another Species

Another possible way in which a genome can acquire new genes is to obtain them from another species. This process, known as lateral or horizontal gene transfer, has been proven to be very important in prokaryotes, but not so frequent in eukaryotes. In the case of eukaryotes, allopolyploidy and introgression due to interspecific hybridization could be considered as mechanisms of lateral gene transfer, and they will be treated in Sect. 6.4.3.

Genome sequencing has revealed the presence of a few genes occurring in a single yeast species that have close homologues in bacteria. These genes, most of them

encoding metabolic enzymes, are rare in the yeast genomes less than 1%), but do appear.

A recent study (Gojković et al. 2004) demonstrated that lateral gene transfer has played, together with the whole-genome duplication event, a major role in the evolutionary history of the *Saccharomyces* complex yeasts. These authors proposed that horizontal gene transfer promoted evolution of the ability to propagate under anaerobic conditions in *Saccharomyces* yeasts. In strict aerobic yeasts, the "de novo" pyrimidine biosynthesis, more precisely the fourth enzymic activity catalyzed by a mitochondrial dihydroorotate dehydrogenase (DHODase) is dependent on the active respiratory chain. However, the facultative anaerobic *Saccharomyces sensu stricto* yeasts have a cytoplasmic DHODase independent of the respiratory chain, which is phylogenetically related to a bacterial DHODase from *Lactococcus lactis*. Gojković et al. (2004) demonstrated that *S. kluyveri*, which separated from the *S. cerevisiae* lineage more than one hundred million years ago, represents an evolutionary intermediate, having both anaerobic cytoplasmic and aerobic mitochondrial DHODases. From these observations, they suggested that a *Saccharomyces* yeast ancestor, which originally had a eukaryotic-like mitochondrial DHODase, acquired a bacterial DHODase, which subsequently allowed cell growth gradually to become independent of oxygen.

6.4.3 Interspecific Hybridization and Introgression

In the case of *Saccharomyces sensu stricto*, one of the most interesting mechanisms observed in the adaptation of these yeasts to industrial process is the formation of interspecific hybrids. Allopolyploidy and introgression by interspecific hybridization are the main mechanisms of lateral gene transfer in eukaryotes.

Artificial interspecific hybridization experiments indicated that *Saccharomyces* "*sensu stricto*" interspecific hybrids can easily be formed (Naumov 1996), and, although sterile, they are viable and can be maintained by asexual reproduction. *Saccharomyces sensu stricto* species are present in the same ecological niche and could hence be involved in the formation of hybrids because haploid cells or spores of these species are able to mate with each other and form viable, but sterile, hybrids. Hybrids produce spores with extensive imbalance in chromosome number and low frequencies of genetic exchange. The mismatch repair system plays a major antirecombination role in these yeast hybrids. The ways in which yeast hybrids may escape this postzygotic barrier are achieved either by doubling of the chromosome number, which results in an allotetraploid (Naumov et al. 2000b), or by recovering euploidy by homothallic diploidization of spores, which results in a homoploid (Greig et al. 2002a).

The best described example of hybrid yeasts is the lager yeasts, included in the taxon *S. pastorianus* (synonym *S. carlsbergensis*) (Vaughan-Martini and Kurtzman 1985). This yeast is a partial allotetraploid hybrid between two species of the *Saccharomyces sensu stricto* group, *S. cerevisiae*, and a *S. bayanus* related yeast (Hansen and Kielland-Brandt 1994; Nguyen et al. 2000; Casaregola et al. 2001). Chromosome sets from both parental species are present in strains of *S. pastorianus* (Tamai et al. 1998; Yamagishi and Ogata 1999), while the mtDNA was inherited

from the non *S. cerevisiae* parent (Piskur et al. 1998). Extensive and variable aneuploidy is found in different *S. pastorianus* isolates (Casaregola et al. 2001), and many of them are chimerical, with part from each parent indicating recombination sometime in their history (Bond et al. 2004).

Moreover, the type strain of *S. bayanus*, originally isolated from beer, has recently been described as possessing also a nuclear genome from both *S. cerevisiae* and *S. bayanus* (Nguyen et al. 2000; de Barros Lopes et al. 2002; Nguyen and Gaillardin 2005).

New natural hybrids have been found in environments different from brewing. Masneuf et al. (1998) characterized a *S. bayanus* × *S. cerevisae* hybrid strain (S6U) isolated from Italian wine, and a triple hybrid present in a homemade French cider (CID1). This hybrid contained two copies of the nuclear gene *MET2*, one coming from *S. cerevisiae* and the other from *S. bayanus*, and the mitochondrial genome originated from a third species, which Groth et al. (1999) demonstrated corresponded to the type strain of the species *S. kudriavzevii*. This was the first report indicating that a rare *Saccharomyces sensu stricto* species, for which only two strains isolated from tree exudates in Japan were known (Naumov et al. 2000a), was involved in interspecific hybridization.

New hybrids *S. cerevisiae* × *S. kudriavzevii* isolated from both natural habitats and fermentation processes, and natural *S. cerevisiae* × *S. paradoxus* hybrids have also been postulated on the basis of their patterns of hybridization with repetitive elements (Liti et al. 2005). Natural hybrids are not restricted to the *Saccharomyces sensu stricto* complex: James et al. (2005) have recently described hybrids between species of the genus *Zygosaccharomyces*.

In two recent studies, new hybrids resulting from the hybridization between *S. cerevisiae* and *S. kudriavzevii* have been described among wine strains (González et al. 2005a) and among brewing yeasts (González et al. 2005b). These wine hybrid strains were predominant in spontaneous fermentations from eastern Switzerland (Schütz and Gafner 1994), and different brewing hybrids were isolated from three Belgian Trappist beers, and also from English, German and New Zealand beers. These authors also found a *S. bayanus* × *S. cerevisiae* × *S. kudriavzevii* hybrid strain, also isolated in Switzerland in 1951, that shows a different genome structure than the other triple hybrid CID1. The sequencing analysis of gene regions located at different chromosomes and the comparative genome hybridization to *S. cerevisiae* DNA microarrays showed that *S. kudriavzevii* hybrid strains contain aneuploidy differences and chimerical chromosomes resulting from recombination between "homeologous" chromosomes of different parental origin (S.S. González, A. Querol, J. García-Martínez, J.E. Pérez-Ortín, and E. Barrio, unpublished results).

The diversity of *Saccharomyces sensu stricto* hybrids, their distinct origins and their presence in different habitats indicate that, in spite of the homothallic character of most natural *Saccharomyces* strains and the persistence of their asci, interspecific hybridization is not so infrequent. Pulvirenti et al. (2002) proposed that yeast-feeding invertebrates may provide the appropriate conditions promoting intra- and interspecific hybridization, because these animals produce, in their digestive tracts, enzymes that hydrolyze the ascus wall, releasing free spores able to conjugate.

As an alternative to haploid cell conjugation, de Barros Lopes et al. (2002) proposed that rare mating between diploid strains of the *Saccharomyces sensu stricto* complex could be involved in the generation of interspecific hybrids. They demonstrated that rare mating is possible not only between nonhybrid diploid strains, but also between CID1, S6U, and lager hybrids with *S. paradoxus* and *S. cerevisiae* diploids, indicating that this mechanism may be involved as well in the generation of multiparental hybrids also from allopolyploids, such as S6U (Naumov et al. 2000b).

Natural interspecific hybridization in yeasts is more frequent that suspected and has probably been undervalued as an important mechanism in the evolution of yeasts by providing new gene combinations of adaptive value (Masneuf et al. 1998; Greig et al. 2002b), genetic robustness due to redundancy, new or specialized functions from divergence of redundant genes (Wolfe and Shields 1997), and also new species through allopolyploid (Naumov et al. 2000b) or homoploid (Greig et al. 2002b) speciation.

In fact, interspecies hybridization might have been a key event in evolution of the high fermentation capabilities of the species of the *Saccharomyces sensu stricto* complex. As mentioned in Sect. 6.4.1.1, Andalis et al. (2004) proposed that the whole-genome duplication in the ancestor of the *Saccharomyces sensu stricto* complex was probably generated by allopolyploid hybridization.

6.4.4 Recruited Autonomous Mobile Elements as a Source of New Genes

There are different examples in eukaryotic genomes indicating that an autonomous mobile element could be directly recruited by host genes to generate a new gene function (Long et al. 2003). In fact, 4% of new exons of human protein-coding genes correspond to recruited autonomous mobile elements.

In the case of yeasts, Butler et al. (2004) demonstrated that homothallic mating (self-fertility based on a mating type switch mediated by HO endonuclease) in the *Saccharomyces* complex originated through the acquisition of an intein-like sequence. Inteins are selfish DNA elements inserted in-frame and translated together with their host proteins (Gogarten et al. 2002). This precursor protein undergoes an autocatalytic protein splicing reaction resulting in two products: the host protein and the intein peptide, which exhibits endonuclease activity involved in the intein mobility.

The close resemblance between HO endonuclease and the endonuclease encoded by the VMA1 intein suggests that, shortly before the whole duplication event, an intein from an unknown origin invaded the *VMA1* gene of the ancestor of the *Saccharomyces sensu stricto* yeast, which gave rise to the HO endonuclase encoding gene after subsequent duplication (Butler et al. 2004). The *HO* mating type switching gene facilitated the change from a cell cycle with a major haploid phase to a cycle with a major diploid phase, which increased the level of genetic robustness of the yeast genome, at least owing to dominance, and promoted the evolution of a repair system based on efficient homologous recombination (Piškur and Langkjær 2004).

6.4.5 New Genes Generated by Retroposition

Retroposition may create duplicate genes in new genomic positions through the reverse transcription of expressed parental genes (Long et al. 2003). This way, messenger RNAs (mRNAs) can be retrotranscribed to complementary DNAs (cDNAs) by a retrotransposon reverse transcriptase and inserted in a new genome position. These retrotransposed genes differ from their parental genes in the absence of introns and the presence at the 3′ end of an A–T stretch coming from the retrotranscription of the mRNA poly(A) tail. As a retroposed protein-coding gene copy lacks internal promoter sequences, it has to recruit a new regulatory sequence to be functional or it will become a processed pseudogene.

Schacherer et al. (2004) recently described in yeasts experimental evidence for the recovery of a function involving duplication by retroposition. They used a positive selection screen of *S. cerevisiae URA2* mutants to isolate spontaneous revertants containing a duplication of the terminal part of the *URA2* gene.

The molecular characterization of the duplicated *URA2* regions showed that they were generally punctuated by a poly(A) tract and were always located in Ty1 sequences. Schacherer et al. (2004) demonstrated that the duplication mechanism involves the reverse transcription of *URA2* mRNA packed in Ty1 viruslike particles, and the subsequent integration of the cDNA into a Ty1 resident copy. Reverse transcription was initiated in the poly(A) region via the terminal part of the URA2 gene and switch at the level of the 5′ junction observed on a Ty element template, leading to the formation of the chimerical structure observed: a δ long terminal repeat (LTR) TyA segment in frame with the duplicated terminal part of the *URA2* gene. Integration was mediated by a homologous recombination event resulting from gene conversion between preexisting chromosomal Ty elements and the 5′ end of the cDNA. Finally, in order to be transcribed to mRNA, the chimerical gene was likely using the promoter located in the δ-LTR region.

6.4.6 Domain Shuffling: New Chimerical Genes Generated Unequal Crossing Over

The ectopic recombination either between similar short sequences (microhomology) present in nonhomologous genes or between divergent paralogous genes could generate new chimerical genes with a different function. An ectopic recombinational event that combines a gene with a new promoter may be a way to generate a dramatic change in the pattern of expression and, thus, may be important in adaptive evolution.

Experimental evolution with yeasts has shown that natural selection can rapidly favor new gene functions generated by ectopic recombination between paralogous genes and subsequent duplications. Brown et al. (1998) analyzed a population of *S. cerevisiae* yeasts that underwent 450 generations of glucose-limited growth. Relative to the ancestral strain, the evolved strain grew at significantly lower steady-state glucose concentrations and demonstrated enhanced cell yield per mole of glucose, significantly enhanced high-affinity glucose transport, and greater relative fitness in pairwise competition. The analysis of the evolved strain revealed the existence of more

than three tandem duplications of a chimerical gene, derived from unequal crossing over, containing the upstream promoter of *HXT7* and the coding sequence of *HXT6*, two adjacent highly similar genes encoding high-affinity hexose transporters originating from a recent duplication. Selection under low glucose concentrations favored a strain containing these duplicated *HXT7*/*HXT6* chimaeras, which increase the ability of *S. cerevisiae* to scavenge glucose at low substrate concentrations.

Another example comes form the study of *S. cerevisiae* yeasts present in spontaneous wine fermentations. Pérez-Ortín et al. (2002) found in several wine strains a new allele of *SSU1* (SSU1-R), a gene that mediates sulfite efflux and, hence, confers sulfite resistance. This new allele was the product of a reciprocal translocation between chromosomes VIII and XVI owing to unequal crossing over mediated by microhomology between very short sequences on the 5′ upstream regions of the *SSU1* and *ECM34* genes. This ectopic recombination put the coding sequence of *SSU1* under the control of the promoter upstream region of *ECM34*, which resulted in a significant increase of *SSU1* expression. They also showed that this chimerical gene (and the translocation) is only present in wine yeast strains, suggesting that the use for millennia of sulfite as a preservative in wine production could have favored its selection.

6.4.7 Domain Duplication: Gene Elongation Generated by Tandem Duplications

Internal duplications have occurred frequently in eukaryote evolution. This increase in gene size, or gene elongation, is an important mechanism to generate complex genes from simple ones (Li 1997).

In the case of yeasts, the most important source of gene elongation is the presence of codon repeats, i.e., trinucleotide microsatellite expansions in coding regions. The most abundant codon repeats found in yeasts are those coding for the amino acids glutamine, asparagine, aspartic acid, glutamic acid, and serine (Albà et al. 1999; Malpertuy et al. 2003).

In most cases, codon repeats show a significant bias toward long tracts of one of the possible codons, suggesting that "trinucleotide replication slippage" is the most important mechanism generating these reiterations (Albà et al. 1999). Replication slippage occurs when a template strand containing contiguous short repeats, in this case trinucleotide repeats, and its copy shift their relative positions during replication owing to mispairing between neighboring repeats, so that part of the template is either copied twice or missed out (Hancock 1999).

However, these different codon repeats are concentrated in different classes of proteins. Thus, acidic and polar amino acid repeats, particularly glutamine, are significantly associated with transcription factors and protein kinases (Richard and Dujon 1997). Changes in the length of repeats in such cellular components of the cell signaling system could alter their biochemical properties, and, hence, modify their interactions with DNA, with other DNA binding proteins, or with other transcription factors and contribute to their evolutionary diversification (Albà et al. 1999; Malpertuy et al. 2003). This modified protein can then be selected for its new function, allowing the cell to increase diversity among its transcription factors,

to specialize them, to adapt to a new environment, and eventually to speciate (Malpertuy et al. 2003). Such diversification could be relatively rapid on an evolutionary time scale because of the high mutation rates of microsatellites (Hancock 1999), which is congruent with the overrepresentation among these transcription factors containing trinucleotide repeats of hemiascomycete-specific genes, which were shown to diverge more rapidly during evolution (Malpertuy et al. 2000).

6.5 Gross Chromosomal Rearrangements in Yeast Evolution

It has largely been proposed that speciation frequently occurs when a population becomes fixed for one or more chromosomal rearrangements that reduce fitness when they are heterozygous. This way, chromosomal rearrangements induce the formation of multivalents during meiosis, resulting in a loss of gamete viability (50% reduction for each translocation).

In the case of *Saccharomyces sensu stricto* species, chromosomal rearrangements have been suggested to account for their postzygotic reproductive isolation (Ryu et al. 1998). However, Fischer et al. (2000) characterized the translocation differences in the species of the *sensu stricto* complex, and concluded that these rearrangements are not required for speciation, since translocations are present only in three species and are not shared between species, indicating that occurred after species divergence.

Delneri et al. (2003) used a reverse approach to determine the role of translocations in speciation. They engineered the genome of a *S. cerevisiae* strain to make it collinear with that of two different *S. mikatae* strains differing in one and two translocations, respectively, with respect to *S. cerevisiae*. Interspecific crosses between strains with collinear genomes resulted in hybrids showing an increase in spore viability (up to 30%). These results indicate that although chromosome rearrangements are not a prerequisite for yeast speciation, they may likely contribute to the reduction of gene flow by suppressing recombination.

The comparative analysis of genomes (Kellis et al. 2003) showed that paralogous genes, transposons, and transfer RNAs (tRNAs) are located at the rearrangement breakpoints, which indicates that ectopic recombination may have been involved in the origin of these chromosomal rearrangements. Indeed, Ty elements or δ-LTRs are well known to induce chromosomal deletion, duplication, translocation, and inversion events by allelic or ectopic recombination in yeasts (Kupiec and Petes 1988; Rachidi et al. 1999). Ectopic recombination, between similar sequences present in nonhomologous genes, between divergent paralogous genes, or between transposable elements could generate evolutionary novelties such as new chimerical genes with a different function of adaptive value (discussed in Sect. 6.4.6) or changes in gene regulation caused by transposable elements on nearby genes.

The fact that selected industrial yeast strains display differences in fitness and in phenotypic traits of industrial relevance that are associated with chromosomal variation (Codón and Benítez 1995) suggests that gross chromosomal rearrangements may be involved in the adaptive evolution of yeasts and account for the high

capacity of industrial yeasts to rapidly evolve. There are several studies whose conclusions support the role of chromosomal rearrangements in the adaptive evolution of yeasts.

Dunham et al. (2002) analyzed the karyotypic changes in six yeast strains, evolved after 100–500 generations of growth in glucose-limited chemostats. These strains contained different chromosomal rearrangements mediated by Ty and tRNA recombinations. Moreover, evolved strains from three independent cultures shared a similar translocation in a chromosome XIV region immediately adjacent to *CIT1*, which encodes the citrate synthase involved in the regulation of tricarboxylic acid cycle. The fact that the same genomic rearrangements recur in different strains suggests that they may be adaptive and responsible for the increased fitness of these strains. Dunham et al. (2002) also postulated that some of the approximately 300 transposon-related sequences found in the *S. cerevisiae* genome are in positions that may provide a selective advantage by allowing adaptively useful chromosomal rearrangements.

Colson et al. (2004) used *S. cerevisiae* strains with artificial translocations, introduced to make their genomes collinear with those of *S. mikatae* strains (see earlier; Delneri et al. 2003), in competition experiments under different physiological conditions. Their experiments showed that the translocated strains of *S. cerevisiae* consistently outcompeted the reference strain with no translocation, both in batch and chemostat culture, but especially under glucose limitation. These results also suggest that chromosomal translocations in yeasts may have an adaptive significance.

Another example comes from the analysis of natural strains. Pérez-Ortín et al. (2002; Sect. 6.4.6) demonstrated that the translocation between *S. cerevisiae* chromosomes VII and XVI, found very frequently in wine strains, was generated by ectopic recombination between genes *ECM34* and *SSU1*, resulting in a chimerical gene that confers a higher resistance to sulfite, a preservative used during winemaking.

Finally, Infante et al. (2003) used the method of comparative genome hybridization with DNA chips, to analyze the genomes of two variants of *S. cerevisiae* flor yeasts, which are adapted to grow aerobically on the surface of sherry wines by transforming ethanol into acetaldehyde. This analysis showed that both strains differ in 116 rearranged regions that comprise 38% of their genomes. These authors concluded that the presence of genes that confer specific characteristics to the flor yeast within these regions supports the role of chromosomal rearrangements as a major mechanism of adaptive evolution in *S. cerevisiae*.

Acknowledgements

We would like to acknowledge the financial support from the Spanish Ministerio de Ciencia y Tecnología (CICYT grant BIO2003-03793-C03) and from the Valencian Autonomous Government (Generalitat Valenciana grant GRUPOS03/012). C. B. and A. A. acknowledge an I3P postdoctral contract from the Spanish Research Council (CSIC) and a PROMEP fellowship from the Mexican Secretaria de Educación Pública, respectively.

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Chapter 7

Principles and Applications of Genomics and Proteomics in the Analysis of Industrial Yeast Strains

URSULA BOND[1] AND ANDERS BLOMBERG[2]

[1]*Moyne Institute for Preventive Medicine, Microbiology Department, University of Dublin, Trinity College, Dublin 2, Ireland (e-mail: ubond@tcd.ie)*
[2]*Department of Cell and Molecular Biology, Lundberg Laboratory, Göteborg University, 413 90 Göteborg, Sweden (e-mail: anders.blomberg@gmm.gu.se)*

7.1 Introduction

Yeasts are presumably the oldest commercially cultured microorganisms and are widely used in the food and beverage industries. *Saccharomyces cerevisiae* belongs to the phylum *Hemiascomycetes*, and at least 1,000 separate strains of *S. cerevisiae* are currently being used in the baking, brewing, distilling and wine-making industries with at least two million tons of yeasts produced per year in the brewing and baking industries alone. Industrial yeast strains can be obtained from a number of repositories, such as the National Collection of Yeasts Culture (http://www.ncyc.co.uk/), The Culture Collection of Yeasts (http://www.chem.sk/yeast/culture_collection_of_yeasts.htm), The European Culture Collections' Organization (ECCO; http://www.eccosite.org/) and the Collection de Levures d'Interêt Biotechnology (http://www.inra.fr/Internet/), to name but a few. The high fermentative capacity of yeasts, together with their ability to withstand the extreme environmental conditions experienced during industrial fermentations, has led to the selection of strains with unique characteristics. In this chapter we focus on the recent use of genomics and proteomics approaches to gain an understanding of the nature of the genomes of industrial strains of yeasts, the expression of genes within these genomes and their final proteome complement.

7.2 DNA Sequencing of Yeast Genomes

To completely understand the molecular and physiological composition of any organism, it is essential to have the complete DNA sequence of its genome. This systematic approach, at a minimum, allows the researcher to determine the number of genes encoding functional proteins and provides opportunities for the theoretical

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
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and experimental analysis of all these genes. In this section we outline the background to the sequencing of the haploid genome of the yeast *S. cerevisiae* and show how this endeavour acts as a paradigm for the analysis of the more complex genomes of the industrial strains of yeasts.

The establishment of the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org) has been an important hub in the dissemination of information regarding the yeast genome and allows one to access the DNA sequence information of all open reading frames (ORFs). Additionally, the SGD provides links to other web-based databases such as GenBank (DNA) and GenPept (protein) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov), Munich Information Centre for Protein Sequences (http://mips.gsf.de/genre/proj/yeast/), Protein Information Resources (http://pir.georgetown.edu/) and SwissProt (http://us.expasy.org/sprot/), to name but a few. Links to gene expression and other functional genomics databases are also available which provide the possibility for direct comparisons of the gene expression profiles for the entire genome under a variety of environmental conditions and in a variety of genetic backgrounds (see later). Presently, the Yeast Genome Sequencing Project and the subsequent annotation of the sequences into a user-friendly database at the SGD is the prototype for the sequencing and annotation of other genomes.

7.2.1 Sequencing of the Genome of *Saccharomyces cerevisiae*

In order to obtain the entire genome sequence of an organism, it is essential to first create a complete representative genomic library. In the early 1980s, the pioneering work of Burke and Olsen allowed the cloning of large genomic fragments in the order of several 100 kb in length into yeast artificial chromosomes (YACs) (Burke et al. 1987). YAC plasmids contain both centromere and telomere sequences, thus allowing the plasmid and the inserted DNA to replicate like a chromosome. Yeast genomic libraries were also prepared in cosmids which are replicating plasmids containing the cos sites of the bacteriophage λ. Genomic fragments as large as 45 kbp can be cloned into cosmids. By sequencing and examining overlapping sequences, of YAC or cosmid clones, a physical map of the yeast genome was created (Cherry et al. 1997). This map with its linked library of DNA clones provided the starting point for the yeast genome sequencing project.

The DNA sequence of the *S. cerevisiae* genome was completed in 1996 (Goffeau et al. 1996). This was achieved through the cooperation of over 600 scientists from Europe, the USA and Japan using automated robotic machines to sequence DNA from random YAC or cosmid clones. Adding to this was DNA sequence information emerging from a "network" of small to medium-sized yeast laboratories which also led the interpretation and verification of the DNA sequences emanating from the DNA sequencing centres. From this information, it was possible to deduce the organisation of the *Saccharomyces* genome, which was shown to be composed of twelve million base pairs arranged on 16 chromosomes (Mewes et al. 1997). Originally, 6,275 theoretical ORFs containing greater than 100 amino acids were identified in the genome sequence. Following subsequent reanalysis of the data and the inclusion of additional information from the scientific literature, this number

was revised downward as of May 2002 to 6,062, of which 3,966 represented ORFs previously identified by genetic analysis or by the presence of structurally and functionally related orthologues in other species and 2,096 genes of unknown function. The estimate for the number of protein coding genes has more recently been further refined and the current estimate at the SGD (as of January 2005) is 5,798. In addition to ORFs, sequences corresponding to non-protein coding genes such as those coding for ribosomal RNA, transfer RNAs (tRNAs), small nuclear RNAs, small nucleolar RNAs, non-coding RNAs, transposable elements (Ty) and long terminal repeats (LTRs) have been identified in the genome.

7.2.2 Genome Sequencing of Other Yeast Species

Since the initial sequencing of the *S. cerevisiae* genome, the DNA sequences of a number of other members of the *Saccharomyces sensu stricto* group have been completed. A comparative analysis of the genomic sequences of *S. bayanus*, *S. mikatae* and *S. paradoxus*, which are separated from *S. cerevisiae* by approximately five million to twenty million years of evolution, confirms the organisation of ORFs onto 16 chromosomes with an average genome size of approximately 11.5×10^6–12×10^6 bp for this genera (Kellis et al. 2003). The four genomes show a high degree of conservation of synteny with only 1.3% of sites of insertions or deletions falling within protein coding regions. The 32 telomeric and subtelomeric regions of the 16 chromosomes represent the regions of greatest sequence diversity. By using a reading frame conservation (RFC) test, together with manual inspection of dubious ORFs for all initially predicted 6,275 theoretical ORFs from the *S. cerevisiae* genome, Kellis et al. (2003) identified 5,458 ORF orthologs in all four species, thus leading to a re-evaluation of the number of true ORFs in the *S. cerevisiae* genome.The comparative genomic analysis from multiples members of the *Saccharomyces sensu stricto* group, in addition to defining the number of true ORFs in these species, has allowed a comprehensive analysis of the rate of evolution of these genomes and the identification of ORFs unique to each species. Additionally, the genome alignments of all four species has allowed the identification of regulatory elements in the intergenic regions of the genomes. The sequencing of more distantly-related yeasts such as *S. kluyveri* (Cliften et al. 2003; Kellis et al. 2003), *Candida glabrata*, *Kluyveromyces lactis*, *Debaryomyces hansenii*, *Yarrowia lipolytica* (Dujon et al. 2004), *Ashbya gossypii* (Dietrich et al. 2004) and *Kluyveromyces waltii* (Kellis et al. 2004) has added to our understanding of the evolution of the genomes of the *Hemiascomycetes*.

7.3 Whole Genome Approaches to the Characterisation of Industrial Strains of Yeasts

The complete genome sequence is an important resource allowing scientists to examine the physiology and evolution of related organisms. It is also the prerequisite for some of the developed genome-wide techniques that have revolutionised the way biological systems are studied. In this section we outline some of the basics of these techniques aiming at the analyses of the complete complement of transcripts (the transcriptome) and proteins (the proteome) in a cell.

7.3.1 Microarray Technology for Genome and Transcriptome Analysis

Microarray technology grew out of the complete DNA sequencing of the haploid *S. cerevisiae* genome and involves robotic application of DNA, representing each gene in the genome, to glass slides, silicone or nylon membranes. The DNAs are arrayed in an orderly fashion to allow easy identification of genes within the array (Chittur 2004; Epstein and Butow 2000; Gerhold et al. 1999; Hardiman 2004). Two types of DNA microarray chips are currently used. Firstly, DNA sequences (500–5,000-bases long) representing individual ORFs are PCR-amplified using DNA oligos specific to each gene. These DNAs are robotically "spotted" onto the glass slides or nylon membranes. The second method generates arrays of oligonucleotides (20–80-mer oligos) representing each gene. These oligonucleotides can be directly synthesised using photolithographic techniques in situ or by conventional synthesis followed by on-chip immobilisation. A variation on this second approach is to include internal controls on the microarrays in which single nucleotide mismatches of each oligonucleotide are included on the chips to allow quantification of the specificity of hybridisation to a given probe. The prototype oligonucleotide microarrays were developed at Affymetrix, which sells its products under the GeneChip trademark.

The arraying of the whole genome, representing each individual gene, on a single matrix, allows the simultaneous analysis of the complete messenger RNA (mRNA) profile (transcriptome) of an organism in a single experiment. Relative steady-state levels of mRNAs are normally examined temporally or spatially under experimental conditions where perturbations from the normal growing conditions are imposed. These perturbation may include changes in genetic background, environmental changes or pharmacological changes, to name but a few. This is achieved through the principles of nucleic acid hybridisation. Briefly, heat denatured double-stranded DNA or single-stranded DNA on the microarray chips is incubated with a labelled probe. For transcriptome analysis, the probes are prepared by first converting RNA to complementary DNAs (cDNAs) by random priming using the enzyme reverse transcriptase. This cDNA probe represents a "snapshot" of the total pool of mRNAs present in the cell under a specific set of experimental conditions. A similar cDNA pool is prepared from RNA extracted from cells grown under "control" conditions. The cDNA probes are differentially labelled by incorporating the fluorescently tagged nucleotides Cy3 (green; 635 nm; control) and Cy5 (red; 532 nm; experimental), respectively, during the reverse transcription reaction. The differentially labelled cDNAs are then mixed and added to the microarray chip, where they compete for hybridisation to the DNA sequences on the chip. The extent of hybridisation is directly proportional to the amount of cDNA, representing a specific mRNA in the sample, that is complementary to a given DNA sequence on the chip and the degree of sequence homology between the two sequences.

Hybridisations are normally carried out in a solution containing 5X SSC (0.6 M sodium chloride, 0.06 M sodium citrate), 7% sodium dodecyl sulfate (SDS) and 50 mM sodium phosphate, pH 7.0. Blocking reagents such as Denhardt's solution [0.02% poly(vinylpyrrollidone), 0.02% bovine serum albumin, 0.002% Ficoll 400] or commercially available blocking reagents are added to the hybridisation solution to

increase the effective concentration of the probe and to reduce non-specific hybridisation. Hybridisations are normally carried out at 68°C or at a temperature below the melting temperature of the probe. Under these hybridisation conditions the labelled probe will bind to its complementary sequence on the DNA chip. The stringency of hybridisation can be varied by changing the temperature of hybridisation or the composition of the hybridisation solution. Following hybridisation, the filters are washed to remove unhybridised probe. Again the stringency of hybridisation can be adjusted here by varying the washing conditions; high-stringency washing (0.5X SSC, 0.1% SDS; 68°C) will allow only completely identical sequences to hybridise, while low-stringency conditions (2X SSC, 0.1% SDS; 68°C or at a lower temperature) will allow hybridisation between DNAs containing mismatches.

The hybridised DNA can then be measured using a fluorescence scanner. The readout from the fluorescence detector is then analysed using programs such as Genepix Pro or ScanAlyze and is expressed as pixels of green or red fluorescent light per square millimetre for each gene. Following normalisation of the data to correct for different efficiencies of labelling and corrections for size of the spot, subtraction of background and removal of spurious readings, the data can be directly fed into spreadsheet programs. The data are presented as a normalised linear ratio or a normalised $\log_2$ ratio of red-to-green fluorescence. The red-to-green fluorescence ratio gives a direct measurement of the relative proportions of RNA (cDNA) in the starting samples. A $\log_2$ ratio of greater than zero indicates a higher level of RNA (cDNA) or DNA in the experimental sample compared with the control sample, while a $\log_2$ ratio of less than zero indicates the opposite.

The red-to-green fluorescence ratios can then be fed directly into clustering programs such as Cluster (http://rana.lbl.gov/eisensoftware.htm) (Eisen et al. 1998). Clustering programs use hierarchical or *K*-means algorithms as a means of identifying and correlating patterns of gene expression and can be used to group together, into expression classes, genes showing similar gene expression patterns. Clustering programs also allow data from multiple microarray experiments to be analysed simultaneously. The cluster output can be viewed in TreeView (http://rana.lbl.gov/eisensoftware.htm) as a colour-coded graphical representation of expression profiles at a glance (Fig. 7.1).

Microarray technology has been exploited to generate a vast amount of data examining the gene expression patterns of *S. cerevisiae* under a variety of experimental conditions. The majority of these can be accessed through the SGD or directly at the site Yeast Microarray Global viewer (yMGV; www.transcriptome.ens.fr/). The latter site contains data from 1,544 experiments mainly showing gene expression patterns for the haploid yeast *S. cerevisiae*. Comparison of gene expression data generated from different sources is often hampered by differing experimental parameters being examined. However, as mentioned before, programs such as Cluster allow the side-by-side clustering of gene expression patterns from any number of differing sources and will reveal overall similarities and differences in the patterns. The yMGV site provides a graphical representation of gene expression variations for each published genome-wide experiment. Additionally, one can examine the effects of experimental conditions on one or a group of genes and identify groups of genes sharing similar transcription profiles in a defined subset of experiments.

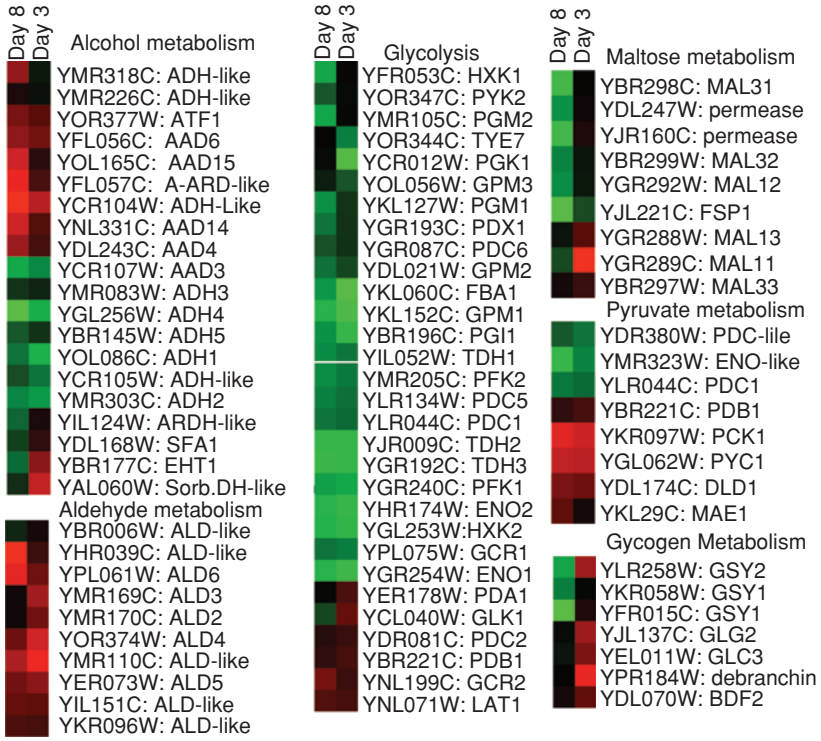

Fig. 7.1. TreeView display showing expression profiles of genes encoding proteins involved in carbohydrate metabolism. Log_2 ratios of transcript levels on days 3 and 8 of a brewing fermentation with a lager strain of a yeast relative to the levels on day 1 were obtained from a microarray analysis (James et al. 2003). Genes were first grouped together by cellular process and then clustered to reveal transcripts showing similar levels of expression (log_2 ratios) using the program Cluster. The range and intensity of colours from *red* to *green* represents a continuum of highest levels of induction (*red*) or repression (*green*)

One of the most useful datasets is that of gene expression patterns of *S. cerevisiae* under a variety of environmental conditions experienced by yeasts, such as heat and cold shock, amino acid starvation, nitrogen depletion, and during the exponential and stationary growth phase (Gasch et al. 2000).

7.3.2 Technologies for Proteome Analysis

The mRNA expression changes estimated by microarray analysis should ideally reflect the change in the amount of protein under the same experimental conditions. However, in many instances this is at its best a good approximation and rather large discrepancies are revealed. Additionally, transcriptome analysis does not reflect the complex myriad of post-translational features, like protein modifications

(e.g. phosphorylation, N-terminal acetylation or ribosylation), protein association with cofactors (e.g. NADH or zinc), protein complex formation (e.g. the ribosome contains almost 100 components), protein localisation (e.g. into mitochondria or the nucleus) and protein degradation (e.g. ubiquitination and breakdown via the proteasome), that eventually define the active proteome component of the cell. Ideally all these levels of complexity should be examined for a full understanding of protein activity in the cell and hence the system under study. This, in short, defines the great challenge in proteomics. In addition, the quantitative range of proteins in the cell is huge; recent estimates indicate that protein abundance in yeasts covers roughly 5 orders of magnitude (Ghaemmaghami et al. 2003), which of course adds to the technological challenge. Proteomics on *S. cerevisiae* currently includes a plethora of techniques, where many of the methodologies rely on the fact that the full genome sequence is available. In the analysis of industrial yeast strains where the full genome has not yet been released, the most fundamental proteomics approach with electrophoretic separation of cell extracts, subsequent image analysis and protein identification has so far been applied. However, the application of some large-scale techniques in proteomics, presently only within reach for the analysis of laboratory *S. cerevisiae* strains, will most likely in the near future also be applied for the analysis of industrial strains (Sect. 7.6).

7.3.2.1 Two-Dimensional Polyacrylamide Gel Electrophoresis

The standard methodology in proteomics has been the combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separation and quantification with mass spectrometry (MS) based identification of resolved proteins. Separating/arraying all proteins in a yeast cell provides a substantial experimental challenge. The technique applied must deal with the great qualitative and quantitative complexity and should be able to do so with a reasonably high throughput of samples. The technique most frequently used to obtain high-resolution separation of proteins is 2D-PAGE, which resolves proteins in two consecutive steps that separate on the basis of independent protein-specific properties, i.e. the isoelectric point (pI) and the molecular weight (M_r). The principle is simple and elegant and when first applied in the mid-1970s it was a major breakthrough in the molecular global analysis of biological samples (Klose 1975; O'Farrell 1975). The major procedure has not changed much over the years; however, substantial refinements in chemistry and hardware have made the currently applied technique vastly superior to the initial setup.

Isoelectric focusing of native proteins is well established and has been extensively used. To increase the resolving power, separation under denaturing conditions by adding high amounts of urea (about 9 M) and 1–4% of a non-ionic detergent was developed. The denaturing capacity is increased even further by inclusion of thiourea in addition to urea as a chaotroph, and the use of zwitterionic amphiphilic compounds (e.g. 3-[3-(Cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS, or SB 3–10) (Rabilloud et al. 1997). This procedure is particularly useful for the resolution of problematic proteins (Rabilloud 1998), but has become the standard procedure in many laboratories.

Separation based on different protein pIs was initially achieved by the inclusion of carrier ampholytes, molecules that in an electric field will generate a pH gradient in which proteins migrate until they reach the pH where their net charge is zero. However, when applying a wide-range ampholyte mixture, such as one with pH 3–10, the separation will not cover this wide a pH range at the end of the run; the final gradient will cover not more than about pH 4–6.5 (O'Farrell et al. 1977). The consequence is, of course, that proteins with pI values outside the produced pH range of the gradient will not focus. One solution to this problem is to pursue isoelectric focusing for shorter times, leading to a gradient with greater pH coverage (O'Farrell et al. 1977). This technique is then a non-equilibrium one, and proteins will, in general, not reach their true pI value. These non-equilibrium pH gradient gel electrophoresis (NEPHGE) gels have been applied to the separation of yeast samples, especially in the past (Bataillé et al. 1988; Boucherie et al. 1995; Brousse et al. 1985; Iida and Yahara 1984; Shin et al. 1987). The main problem with the NEPHGE procedure is reproducibility and standardisation.

The technical solution to at least some of the problems mentioned before for ampholytes came with the invention and application of immobilines (Bjellqvist et al. 1982; Görg et al. 1988, 1999). Immobilines produce a pH gradient that is generated by the covalent anchoring of the immobilines to the polymer matrix. The consequence is that the gradient will be stable and not collapse during prolonged running time. In addition, shallow (zooming in on narrower pH regions) or very broad gradients can be formed, and even the use of sigmoid-shaped non-linear pH 3–10 gradients for optimal protein pattern spread has been applied for the analysis of yeasts (Muller et al. 1999; Norbeck and Blomberg 1997, 2000). In addition, with the development of more alkaline pH gradients (up to pH 12.5) the 2D system can resolve very alkaline proteins like histones and many ribosomal proteins (Wildgruber et al. 2002). The use of shallower gradients, where better physical separation is obtained between proteins with closely matching pI values, can be very useful, and will minimise the shielding effect on low-abundance proteins from abundant neighbours (Wildgruber et al. 2000).

For the 2D separation there is presently no good system for the parallel separation of proteins in the range 1–600 kDa, which is the size range provided by the yeast proteome. Size differences can be dealt with but only if we apply slightly different techniques for different size classes: i.e. altering the concentration of the polyacrylamide matrix will enable better separation of small proteins (high total percentage of acrylamide, %T) or large proteins (low %T) (Garrels 1979). However, choosing either of these extremes will of course compromise the resolution of the opposite size class. In practice this means that more gels have to be run and fewer samples will be analysed. Most groups have thus adopted some intermediate concentration (10–12%T) as the standard, thus accepting minor losses in resolution of extremes in the process.

7.3.2.2 Post-Separation Analyses – Image Analysis

Visualisation of 2D-PAGE-separated proteins can be performed by different means, and when dealing with the industrial-scale visualisation this is achieved by the use of

some sensitive stain. Silver deposits at protein spots can detect low nanogram quantities of protein; however, different silver staining protocols are more or less sensitive and some are more useful for quantitative purposes (Blomberg 2002; Rabilloud 1992). The signal-response curve for silver staining is linear for all protocols over a rather narrow dynamic range, and proper quantification over a wider range can only be achieved by the use of calibration strips. A recent development of great use in protein quantitation is the use of fluorescent dyes. The currently most frequently used dye is Sypro Ruby, which has been documented to generate a linear response over some orders of magnitude. This staining procedure is, however, a bit less sensitive than silver staining (Rabilloud et al. 2001; Blomberg et al., unpublished data). In Fig. 7.2a the 2D pattern of lager yeast strain CMBS33 from Sypro Ruby staining can be observed from cells grown under laboratory conditions in synthetic defined medium containing glucose. Recently dual labelling with covalent linkage of fluorescent dyes prior to electrophoretic separation was also applied, i.e. fluorescence-based multiplexed proteomics (Patton and Beechem 2002). Different samples are labelled with dyes with different spectral properties, mixed and separated by 2D-PAGE. The resulting gel is scanned in different spectral windows and the individual quantities estimated. The main advantage of this methodology is that between-gel variation is cancelled, which makes sample comparison and spot matching more straightforward. However, if more than two or three samples are to be analysed, which is usually the case in proteome analysis if statistics are to be applied (which it should), the problem still persists with gel-pattern matching. Any of these means for

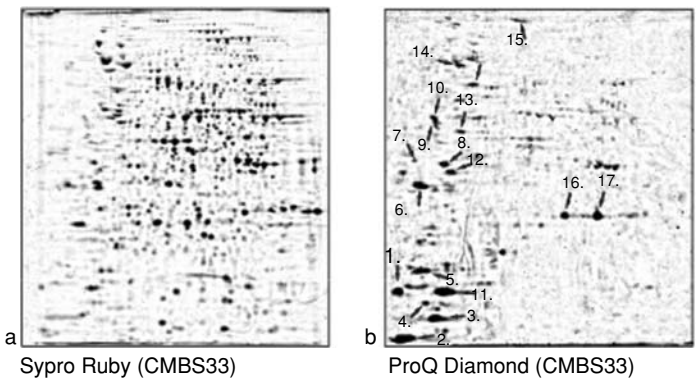

Fig. 7.2. Differential staining with Sypro Ruby and ProQ diamond of 2D-separated proteins from a lager yeast strain. The lager strain CMBS33 was grown in synthetic defined medium with glucose as the carbon and energy source and harvested in the mid-exponential growth phase. (**a**) Total proteins were separated using a wide-range non-linear pH 3–10 gradient in the first dimension and were visualised by the fluorescent dye Sypro Ruby. (**b**) The same gel as in **a** was subsequently stained with the phosphoprotein specific stain ProQ diamond to visualise proteins in the pattern that were modified by phosphorylation (about 25 proteins displayed significant phosphorylation specific staining). The acidic side in the first dimension is to the *left*. (Adopted from R. Caesar, E. Pettersson, J. Gustafsson, A. Hashemi, A. Blomberg, unpublished results)

visualisation can be utilised for protein expression analysis of samples cultivated on an industrial scale. However, the most precise quantitative method is not really applicable to large-scale fermentations – isotopic labelling of proteins during cultivation by addition of a radioactive amino acid, usually ^{35}S-methionine. After separation the gels are dried and exposed to image plates that have a higher sensitivity and a wider linear range of response compared with those of ordinary X-ray film (Blomberg 2002).

Protein stains have also been developed to specifically capture protein features. One good example of this is the recently developed stain ProQ diamond, where gels are fluorescently stained and imaged to reveal phosphorylation levels using this fluorescent phosphosensor dye (Schulenberg et al. 2003). The initial staining step is subsequently followed by staining and imaging to reveal general protein expression levels using a total protein indicator such as Sypro Ruby. ProQ diamond specific proteins can subsequently be identified by peptide mass fingerprinting. Figure 7.2b displays the phosphorylated proteins indicated by ProQ diamond staining in the lager strain CMBS33. Some of the phopsphorylated proteins were identified earlier as phosphoproteins in *S. cerevisiae*, while others are novel phosphorylation targets (R. Caesar, E. Pettersson, J. Gustafsson, A. Hashemi, A. Blomberg, unpublished results).

Generated images can be analysed for quantitative changes in protein spot volume. For this task there are currently a number of different commercial software packages. All of them are based on some procedure for background subtraction, smoothing and subsequent spot identification and quantification (Blomberg 2002). Different software packages apply different strategies in the analysis of spot volumes. Some use mathematical modelling based on spot width and peak height, while others identify the outer borders of the spot and sum all the pixel values; however, the quantitative results are usually more or less similar, at least for well-resolved spots. The final analysis is to compare the quantitative values of individual protein spots between different samples. This matching process is supposed to be automated in many of the software packages, but still much time is spent in manual editing and matching of different images. Currently procedures are being developed that hopefully will lead to more accurate automatic handling of large collections of image data; these are based on image warping prior to matching (Gustafsson et al. 2002; Veeser et al. 2001).

7.3.2.3 Protein Identification: a Historical Survey

The 2D map obtained is of rather limited biological value if resolved protein spots are not identified. This was initially performed by co-migration with purified preparations of enzymes. Applying this procedure, Calvin McLaughlin and colleagues were able to produce the first annotated 2D map of *S. cerevisiae*, which in 1978 contained identity for 17 proteins (Elliott and McLaughlin 1978). The proteins identified were mostly very abundant and involved in glycolysis, like hexokinase, triosephosphate isomerase and enolase.

Another early procedure for the identification of proteins in the 2D patterns was by the use of antibodies. Proteins were separated and subsequently blotted onto

filters where polyclonal antibodies were used for identification. This approach was first attempted for yeasts by the Bordeaux group lead by Helian Boucherie. Antibodies were produced against purified preparations of enolase and glyceraldehyde-3-phosphate dehydrogenase, and these antibodies were used for protein identification on 2D gels (Brousse et al. 1985) and were also applied in the first analysis of some wine yeasts (Brousse et al. 1985). The antibodies detected both Eno1p and Eno2p as well as Tdh2p and Tdh3p (as we know them today). This exemplifies a weak point when using polyclonal antibodies; they are prone to cross-reactivity. The future use of antibodies in protein characterisation would most likely be found in the large scale analysis of diverse protein modifications or in the analysis of low-abundance proteins.

The advent of molecular biology techniques provided novel avenues for protein identification since genes could be cloned and overexpressed by the use of multicopy plasmids. This strategy was explored for the identification of the location of *PGI* and *PGM* encoded polypeptides in the 2D pattern (Bataillé et al. 1987), as greatly enlarged spots. A variation on this theme would be the 2D analysis of a gene knockout, where the gene product in question is scored as a missing 2D spot in the mutant.

A cheap, rapid, yet efficient way of identifying proteins on a laboratory scale is based on the determination of their amino acid ratios (Garrels et al. 1994; Maillet et al. 1996). Specific double-labelling with ^{3}H and ^{14}C or ^{35}S-labelled amino acids, chosen among those that are specifically incorporated into proteins without interconversion, allowed an accurate measurement of different amino acid ratios for roughly 200 proteins. This double-labelling technique is particularly suited for the analysis of proteins that are only transiently expressed; these proteins will be difficult to analyse by other means since in many instances they will be present at very low levels.

The first technique for direct identification of resolved proteins was microsequencing. In principle, microsequencing works extremely well in most cases; however, a number of problems have also been encountered, e.g. proteins frequently carry modifications in their N-terminus, which blocked and stopped the sequencing reaction. Thus, novel procedures for generating material to be analysed in the microsequencer were developed. The most appealing and experimentally straightforward method was in-gel digestion with a specific protease, mostly trypsin, and subsequent elution and fractionation of the peptides generated (Norbeck and Blomberg 1995; Rosenfeld et al. 1992). The in-gel digestion turned out to be a major breakthrough in the analysis of proteins and is still the main method in use today for the generation of peptide cocktails from 2D-resolved proteins. A number of laboratories have successfully adopted this strategy of microsequencing of isolated peptides and used it for the annotation of their yeast map (Garrels et al. 1997; Norbeck and Blomberg 1995, 1997; Sanchez et al. 1996). Microsequencing is a very powerful technique when it comes to stringent identification of proteins, in particular for closely related proteins (Norbeck and Blomberg 1995). However, this technique also has a number of drawbacks: (1) it is rather expensive to run and maintain (e.g. chemicals have to be ultrapure), (2) the analysis time for each amino acid is about 30–45 min, at least for the somewhat older microsequencers, which results in few protein identifications per day, and (3) rather large amounts of protein are required (1–10 pmol). This

excludes the use of microsequencing in any type of really large scale programme when linked to the characterisation of hundreds of proteins resolved by 2D-PAGE.

7.3.2.4 Protein Identification: Mass Spectrometry

Currently the methodology of choice for large-scale protein identification is based on the use of mass spectrometry. Molecules to be analysed by mass spectrometry are ionised in the gas phase and are subsequently resolved in relation to their mass-to-charge ratio. Key in the protein analyses by mass spectrometers was to get large molecules like proteins and peptides into the gas phase as ions. Much effort was spent in the past to develop non-destructive "soft" ionisation methods that are compatible with studies of proteins and peptides. Two different ionisation methods were eventually developed that proved versatile, non-destructive and robust in the ionisation of a wide spectrum of peptides and proteins.

In matrix-assisted laser desorption ionisation (MALDI) a large excess of matrix material is mixed with the analyte molecule and a small volume of the mixture is placed on a metal target where it is allowed to dry and form crystals (Mann et al. 2001). Nanosecond laser pulses from nitrogen lasers are then used to irradiate the crystals under vacuum. The matrix is a small organic molecule like the commonly used compound α-cyano-4-hydroxycinnamic acid, and it is believed that the matrix molecules absorb the laser energy, resulting in desorption, and throw out of a small volume of matrix and embedded analytes. The matrix thus serves the purpose of propelling the non-volatile proteins and peptides into the gas phase. The ionisation process of a peptide competes with ionisation of all other peptides in the mixture, and this suppression phenomenon results in a context-dependent signal intensity for a particular peptide (Larsson et al. 1997). This phenomenon makes MALDI-MS non-quantitative and also explains why some peptides never "fly" in the analysis of complex peptide mixtures.

In the other ionisation method, electrospray ionisation (ESI), a low flow rate of liquid (microlitres per minute or less) is pumped through a very narrow and high electric potential needle. This procedure leads to a dispersion of the liquid into micrometre-sized charged droplets, which is called an electrospray (Mann et al. 2001). When these small droplets evaporate charge is transferred to the analyte molecules. Electrospray is regarded as a very soft ionisation method since it rarely fragments the analyte ions. ESI works well on many types of macromolecules and usually results in multiply charged larger molecules. Since the separation and analysis in the mass spectrometer is based on mass over charge, multiple charges produced by ESI bring even very large proteins into the mass/charge analytical range of most mass spectrometers. This ionisation method can be directly coupled in-line with a high-performance liquid chromatography system. This system design is rather robust since sample cleanup, separation and concentration is performed in a single in-line chromatographic step. Many of the very potent applications of ESI-MS in proteomics are currently based on in-line separation of peptides by 1D or 2D chromatography systems.

Mass spectrometers measure the mass-to-charge ratio of ions. This can be achieved by separation based on time-of-flight (TOF-MS), quadrupole electric fields

generated by metal rods (quadrupole MS), or selective ejection of ions from a 3D trapping field (ion-trap MS) (Mann et al. 2001). When structural information is to be gained, e.g. in peptide sequencing, two different steps of *m*/*z* analysis are performed in tandem MS (MS/MS) with some kind of fragmentation procedure, in a collision-induced dissociation (CID) chamber, placed in between. The MS/MS analysis can in principle be performed by employing the same *m*/*z* separation principle twice (e.g. TOF-TOF) or by combining two different ones (e.g. quadrupole TOF). Both ionisation procedures described, MALDI and ESI, can be coupled to any of the *m*/*z* separation methods; however, some combinations appear to be a better match and are more frequently found in current commercial instruments (e.g. MALDI-TOF; ESI ion trap).

In the TOF-type mass spectrometers ions are accelerated to a fixed amount of kinetic energy by a strong electric field, applied some 100–500 ns after the laser pulse, and the ions travel down a flight tube. This procedure leads to molecules with low *m*/*z* values having higher velocities and getting to the detector before the ones with high *m*/*z* values. The mass accuracy of MALDI-TOF instruments is roughly 10 ppm (0.01 Da for a 1-kDa peptide). The quadrupole is a mass filter that is composed of four metallic rods to which an oscillating electric field is applied. This filter lets through only ions with a certain *m*/*z* value, with the other ones not reaching the detector. The mass-over-charge spectrum is obtained by changing the amplitude of the electric field and recording the ions that reach the detector. Most peptide sequence analyses have been performed on triple quadrupole instruments where the design is divided into three sections. Two sections that provide *m*/*z* filters and one central quadrupole section that contains the ions during fragmentation thus constitute the CID chamber. Quadrupole mass spectrometers are capable of unit mass resolution and mass accuracy of 100–500 ppm. In ion traps the ions are physically trapped in a 3D electric field. Ion traps capture the continuous beam of ions up to the limit of the maximum number of ions that can be introduced into the trap without distorting the electrical field. After capture, the ions are subjected to additional electrical fields that lead to one ion after the other being ejected from the trap. The ejected ions are detected and this produces the *m*/*z* spectrum. In cases where MS/MS analysis is to be performed with the ion trap, all except the desired ion are first ejected. After that the remaining ion is fragmented by collision to a gas and the fragments are analysed by the same procedure as before. This construction allows for multiple rounds of analysis and fragmentations, with the result that a large number of MS/MS analysis can be performed (MS^n). Ion traps are compact, robust and very versatile instruments for which the operation can be highly automated. The mass accuracy of the ion traps is slightly less than for the quadrupole instruments, which is usually not a great problem in the analysis of yeast proteins because of the relatively small genome (only roughly 6,000 proteins).

Algorithms for protein identification based on MS data has been developed and refined (Mann et al. 2001). Originally protein identification was almost exclusively performed using MALDI-TOF-MS data, where the peptide mass fingerprint generated was compared with the theoretically predicted masses of peptides for each entry in the protein database. Trypsin digestion has most frequently been used (cleavage after arginine and lysine residues), but in principle any type of specific protease

could be used in the generation of peptides. The first step in the procedure is that visualised proteins are physically cut out from preparative gels. These gel pieces are destained and washed, dehydrated and then rehydrated with trypsin solution. With the growing number of sequences in the databases, more sophisticated algorithms have been developed that generate lists of protein hits with corresponding probability scores. However, as a rule of thumb for an unambiguous identification, when high mass accuracy in the range 10–50 ppm is achieved, at least five peptide mass MALDI-MS data need to match that should cover at least 15% of the length of the protein. MS/MS data can also be used for database searches. Since sequence information is revealed in the tandem mass spectra, these searches provide higher confidence and greater discrimination. Several alternative algorithms exist, but here it suffices to mention that experimental spectra are matched against calculated fragmentation spectra for all peptides in the database. The power of the MS/MS approach truly comes into play when mixtures of proteins (or whole proteomes) are to be analysed (Washburn et al. 2001), since single peptide fragmentations can be sufficient to identify a particular protein in the sample.

7.3.2.5 Yeast Proteome Databases

Currently the most impressive and complete global analysis of the yeast proteome includes the analysis of 400 proteins corresponding to 279 different genes for the laboratory strain S288c during exponential growth in synthetic defined medium (Perrot et al. 1999). In this analysis, a combination of different techniques was used in the identification of the protein spots; genetic alterations (gene overexpression or deletion) 121 proteins, amino acid composition 114 proteins, and MS 221 proteins. Many of the most abundant proteins in the 2D pattern are involved in energy metabolism, of which proteins encoded by 48 genes have been identified. A large proportion of the proteins identified are involved in biosynthesis of either small molecules (65 different genes) or macromolecules (79 genes). Some of the metabolic pathways are almost completely covered, like purine synthesis, which was also used for the first complete study of all its components (Denis et al. 1998). However, only a small proportion (five proteins) of the subunits in the ribosome were separated and identified. A good proportion of the aminoacyl-tRNA synthetases have been identified, 13 out of the 20 believed to be present in the genome. In addition, a high number of chaperones are present; these are mostly rather dominant and are represented by a large variety of proteins and different families in yeasts. Also identified are components involved in the cellular response to growth perturbations (stress); osmotic adaptation (four proteins) and detoxification (six proteins). This data can be explored at the Bordeaux 2D database (YPM; http://www.ibgc.u-bordeaux2.fr/YPM/).

7.4 Genome Constitution of Industrial Strains of Yeasts

The genomic analysis of industrial strains of yeasts has lagged behind that of the prototype haploid *S. cerevisiae* species. This is partly due to the complex nature of their genomes. Industrial strains of yeasts, in particular those involved in the beer and wine industries, all belong to the *Saccharomyces sensu stricto* group. Currently,

this taxonomic group includes seven yeast species, *S. cerevisiae*, *S. paradoxus*, *S. pastorianus*, *S. cariocanus*, *S. kudriavzevii*, *S. bayanus* and *S. mikatae*.

Yeasts isolated from wine fermentation in which no starting inoculum is used tend to belong to the species *S. cerevisiae* or *S. bayanus*, with the majority being *S. cerevisiae* (Mortimer 2000). A small number of wine yeasts appear to be interspecies hybrids of *S. cerevisiae* and *S. bayanus*, while one particular cider yeast strain (CID1) contains DNA from three separate species (de Barros Lopes et al. 2002; Mortimer 2000). The majority (approximately 70%) of wine yeasts are prototrophic, heterozygous and homothallic and most possess diploid genomes (Mortimer 2000).

Industrial beer fermentations in modern-day breweries use two uniquely different strains of yeasts, the top-fermenting ale strains and the bottom-fermenting lager strains, the names "top" and "bottom" reflecting whether the yeast rises to the top of the fermentation or settles to the bottom at the end of fermentation (Kielland-Brandt et al. 1995; Kodama et al. 2005). Top-fermenting yeasts produce ales and closely resemble *S. cerevisiae*, while the bottom-fermenting lager strains appear to have arisen from a fusion between two yeast species, one closely resembling *S. cerevisiae*, while the other appears similar to *S. bayanus* and/or *S. pastorianus* CBS1503 (*S. monacensis*) (Kodama et al. 2005). The lager yeasts have been grouped with *S. pastorianus* (Vaughan-Martini and Martini 1987) but are commonly referred to as *S. carlsbergensis*.

The lager yeast genome is believed to have undergone a genome duplication following the species fusion, leading to an allotetraploid strain containing varying numbers of *S. cerevisiae* and non-*S. cerevisiae* chromosomes. Generally lager yeast genomes are considered to be aneuploid in nature, possessing unequal numbers of chromosomes, e.g. a strain could have one copy of chromosome III and five copies of chromosome IV. In addition, recombination between the two homeologous sets of chromosomes has occurred, generating mosaic chromosomes (Kodama et al. 2005). These complex genomes appears to confer unique selective characteristics to these strains, e.g. the ability of lager yeasts to grow at low temperatures (7–13°C) and to withstand high osmotic pressure, high hydrostatic pressure, and high ethanol and CO_2 concentrations.

7.4.1 Classical Approaches to the Analysis of Industrial Yeast Genomes

7.4.1.1 Single Chromosome Transfer of the Lager Yeast Genome

The poor sporulation ability of lager yeast strains has hampered classical genetic analysis of these polyploid strains. However, preceding the development of molecular approaches for genome analysis, studies using the technique of single chromosome transfer from lager strains to well-defined laboratory *S. cerevisiae* strains helped to elucidate the polyploid and mosaic nature of lager yeast genomes (Casey 1986; Kielland-Brandt et al. 1995; Nilsson-Tillgren et al. 1981). Using this experimental approach three chromosome types in the lager strains have been defined: (1) *S. cerevisiae* chromosomes capable of recombining with *S. cerevisiae* chromosomes, (2) non-*S. cerevisiae* chromosomes showing no recombination and (3) mosaic chromosomes where regions of the lager yeast chromosome can recombine with an

S. cerevisiae chromosome (Kielland-Brandt et al. 1995). The conclusions reached by these seminal experiments defined the complex makeup of the lager yeast genomes and have been borne out and refined by subsequent analysis using molecular and whole genome approaches (see later).

7.4.1.2 Electrophoretic Analysis of Lager and Wine Strains of Yeasts

The characterisation of the structure and composition of industrial yeast chromosomes has been greatly aided by the development of pulsed-field gel electrophoresis. Classical single (continuous) field gel electrophoresis on agarose gels in tris(hydroymethyl)aminomethane acetate buffers has an upper limit of separation of approximately 30–50 kb. Pulsed-field gel electrophoresis, in which the direction and the duration of the current are varied (Coulson et al. 1988), relieves this upper restriction and allows the separation of DNA fragments in the size range from 10 kb to 10 Mb. The earliest models of pulsed-field gel electrophoresis employed a single field inversion of the electric current: field-inversion gel electrophoresis (FIGE) (Carle et al. 1986). FIGE periodically inverts the polarity of the electrodes subjecting the DNA to a 180° reorientation. The more complex pulsed-field systems such as contour-clamped homogeneous electric field (CHEF) (Chu 1990; Chu et al. 1986) transverse alternating field electrophoresis (TAFE) (Gardiner et al. 1986) and rotating gel electrophoresis (RGE) (Anand and Southern 1990; Gemmill 1991; Southern et al. 1987) all subject the DNA to smaller changes in orientation, generally between 96 and 120°. This results in the DNA moving forward in a zigzag manner allowing for separation over a wider range of sizes.

The first yeast chromosome fingerprint of the haploid strain of *S. cerevisiae*, using FIGE (Carle and Olson 1985), confirmed the presence of 16 chromosome bands. Separation of industrial yeast chromosomes on CHEF or TAFE gels revealed a wide heterogeneity in the chromosome banding pattern; however, in each case chromosomes equivalent in size to the *S. cerevisiae* chromosomes are apparent. (Casey 1996). Strains of lager yeasts vary in the number of chromosome bands present and in subtle electrophoretic mobilities of individual chromosomes. For example, chromosome fingerprints of a number of yeast cultures from the Stroh Brewery Culture Collection distinguished two unique fingerprints of type "Tuborg" and type "Carlsberg" (Casey 1996). These two strain types differ in the presence or absence of the small chromosomes I and X. By combining the techniques of pulsed-field gel electrophoresis and Southern blotting, the identity of individual chromosome bands was deduced. In a seminal paper by Casey (1986) the presence of three types of chromosome X in *S. carlsbergensis* was revealed using a DNA probe specific to chromosome X. Likewise, by varying the stringency of the hybridisation and washing conditions, the *S. cerevisiae* like, the non-*S. cerevisiae* homeologues and mosaic chromosomes, present in the lager strains, can be distinguished.

Electrophoretic karyotyping of wine yeasts from different sources reveals that strains differ in the number, electrophoretic mobilities and band intensities of chromosomes. Up to 21 chromosome bands have been identified in certain wine yeast strains (Castrejon et al. 2004). Hybridisation of chromosomes separated by pulsed-field gel electrophoresis, using chromosome-specific DNA probes, has allowed the

identification of individual chromosomes of wine yeast (Puig et al. 2000). Furthermore, karyotyping has revealed that the genomes of wine yeasts are subject to genetic rearrangements by undergoing recombination between homologous chromosomes and between paralogous or repeated DNA sequences (Codon et al. 1998; Infante et al. 2003). The recombination events, resulting in the generation of mosaic chromosomes in lager and wine yeasts, appear to occur during mitosis rather than being meiotic in nature (Puig et al. 2000). The resulting complex genomes of lager and wine yeasts lead to poor mating ability, poor sporulation and spore viability and thus contributes to the genetic isolation of these strains.

7.4.2 Comparative Genomic Microarray Analysis

7.4.2.1 Comparative Competitive Genomic Hybridisation of Lager Strains of Yeast

While pulsed-field gel electrophoresis allows the identification of the number of individual chromosomes and chromosome heterogeneity between yeast strains, it cannot reveal any information on the actual gene makeup of these industrial yeast strains. The development of whole genome microarray technology has allowed researchers to address this issue and to ask pertinent questions regarding the biological consequences of the hybrid nature of many industrial strains of yeasts. Such questions include what is the nature of the mosaic chromosomes in industrial strains of yeasts, how many copies of *S. cerevisiae* and non-*S. cerevisiae* genes are present in the genome and what effect does the presence of multiple non-identical genes have on the final transcriptome and proteome content of the yeast?

The technique of comparative competitive genomic hybridisation (CCGH) (Watanabe et al. 2004) to microarrayed *S. cerevisiae* genes on DNA chips can be used to determine the relative copy number of *S. cerevisiae* like genes, at single-gene resolution, in industrial strains of yeasts. In this case, the genomic DNA populations from two different yeast strains are differentially labelled by random priming with Cy3- and Cy5-tagged nucleotides by PCR amplification using enzymes such as Taq polymerase and hybridisations are carried out as described in Sect. 7.3.1. In this experiment, the differentially labelled DNA samples compete for hybridisation to their complementary sequence on the DNA chips. As with microarrays using two differentially labelled cDNA pools (Sect. 7.3.1), a ratio of hybridisation, reflected in red-to-green fluorescence ratios for each gene, is obtained. The ratio of hybridisation for the two competing DNA samples will depend on (1) the degree of sequence homology between the labelled DNA probes and the *S. cerevisiae* DNA arrayed on the chip and (2) the relative abundance (copy number) of the DNA sequence in the labelled DNA sample. In the simplest example, in which differentially labelled DNAs from two haploid strains of *S. cerevisiae* are competitively hybridised to *S. cerevisiae* DNA chips, one expects the ratio of red-to-green fluorescence to be 1.0 for all genes except for individual genes which may differ in the two strains. For polyploid and, in particular, aneuploid strains of yeasts, depending on the gene copy number and the homology between the reactants, the ratio of hybridisation (red-to-green fluorescence ratio) will be greater or less

than 1. This deviation from the control mean ratio of hybridisation is indicative of a higher or a lower copy number, respectively. The caveat in using CCGH analysis for industrial strains of yeasts is that only genes with extensive homology to *S. cerevisiae* will be detected.

Using this approach, Bond et al. (2004), Kodama et al. (2005) and Infante et al. (2003) have examined the copy number of *S. cerevisiae* like genes in lager and wine yeasts. A number of interesting findings have emerged from these studies. In one of the lager yeast studies (Bond et al. 2004), competitive hybridisations were carried out for two lager strains of yeasts and the yeasts were compared with a haploid *S. cerevisiae* strain. When the ratio of hybridisation for each individual gene was arranged according to the *S. cerevisiae* gene order, it was observed that the ratios of hybridisation for genes on a given chromosome were remarkably similar. However, in eight of the 16 chromosomes, distinct "jumps" in the ratios of hybridisation occurred at discrete loci along the chromosome. Examples of two of these jumps in the ratios of hybridisations are shown in Fig. 7.3. For chromosome III (Fig. 7.3a), a distinctive jump occurs at the MAT locus, between YCR039C and YCR040C. In lager strain 6701, genes to the left of the MAT locus show a ratio of hybridisation of 0.6, while for strain CMBS, the average ratio of hybridisation is 1.0. The genes to the right of the MAT locus in both strains show an average ratio of hybridisation of 1.7. It is interesting to note that the majority of the jump locations are conserved between the two lager strains of yeasts (they are definitely different isolates) and also between a third lager strain (Bond and James, unpublished data). However, each strain also possessed its own unique jumps; for strain CMBS a unique jump is observed on chromosome VIII after YHR165C and on chromosome XIII after YMR302C, while strain 6701 has a unique jump on chromosome X after YJR009C. The simplest explanation of the ratios of hybridisation data is that the jump locations represent regions where the homeologous chromosomes have undergone inter-and/or intra-chromosomal translocations. In fact, previous studies have used CCGH to identify genomic rearrangements such as translocations, deletions and amplifications in *S. cerevisiae* strains during experimental evolution conditions (Dunham et al. 2002). While CCGH analysis can reveal relative differences in copy number between two yeast species and/or strains, it does not allow an absolute quantitative determination of gene copy number. However, the relationship between the ratios of hybridisation and the gene copy number can be determined by combining CCGH analysis with quantitative real-time PCR. The technique of real-time-PCR allows the determination of the degree of amplification of known DNA quantities. By comparing the rate of PCR amplification of ORFs displaying unique ratios of hybridisation to the rate of PCR amplification of a known single copy gene, Bond et al. (2004) have shown that there is a direct correlation between the ratio of hybridisation and gene copy number for *S. cerevisiae* like genes in lager strains of yeasts.

An analysis of chromosome XVI in two lager strains of yeasts reveals the presence of four distinct regions with unique ratios of hybridisation (Fig. 7.3b). The region immediately following the left telomere up to the ORF YPL242C shows a ratio of 1.8. This is followed by a long stretch up to YPR159W, where a ratio of 1.0–1.2 is observed. Surprisingly, the region from YPR160C to YPR190C, encompassing 30 genes, many of which are essential in the haploid *S. cerevisiae* species,

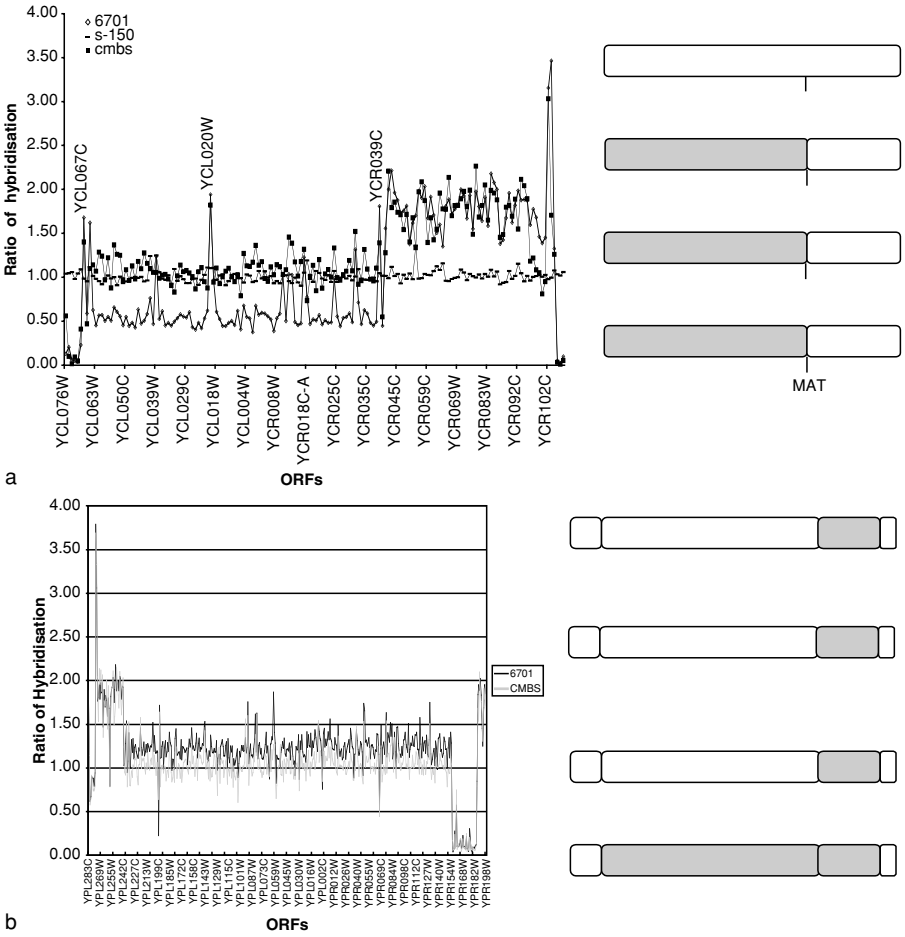

Fig. 7.3. Comparative competitive genomic hybridisation for the determination of the copy number of *S. cerevisiae* like genes in industrial yeast strains. (**a**) Ratio of hybridisation along chromosome III as determined by comparative competitive genomic hybridisation (*CCGH*) for the lager strains 6701 (*diamonds*) and CMBS (*squares*) relative to the haploid strain S-150B (*bars*). Schematic representation of chromosome III in strain 6701 as determined by quantitative-real-time PCR and CCGH. On the basis of these analyses the minimum copy number is one *S. cerevisiae* like chromosome III (*open box*) and three mosaic chromosome IIIs, where the genes to the left of the MAT locus are non *S. cerevisiae* like (*grey*) and those to the right of the MAT locus are *S. cerevisiae* like (*open boxes*). (**b**) Ratio of hybridisations along chromosome XVI for lager strains 6701 (*black line*) and CMBS (*grey line*) relative to the haploid strain S-150. Schematic representation of the minimum copy number and types of chromosome XVI in these strains as determined by CCGH and quantitative real-time PCR. *Open boxes S. cerevsiae* like, *grey boxes* non-*S. cerevisiae* like

shows a ratio of 0.2. From YPR190C to the telomere, the ratio once again rises to 1.8. The low hybridisation signal in the region YPR160C–YPR190C suggests that the *S. cerevisiae* genes in this region are absent in the lager strains or have significantly diverged from the *S. cerevisiae* sequence and most likely the region only contains non-*S. cerevisiae* gene equivalents. Southern blotting analysis of chromosome XVI, separated on CHEF gels, indicates that the latter is the case as a very weak hybridisation signal can be detected with DNA probes prepared from *S. cerevisiae* ORFs in this region (Usher and Bond, unpublished data).

Using the relationship between the ratio of hybridisation and the gene copy number one can deduce that the minimum chromosome XVI complement in these lager strains consists of three *S. cerevisiae* like chromosomes containing the non-*S. cerevisiae* region YPR160C–YPR 190C and one non-*S. cerevisiae* chromosome containing the telomeres and subtelomeric regions of an *S. cerevisiae* like chromosome (Fig. 7.3b). In a CCGH study with a number of lager strains of yeasts, Kodama et al. (2005) showed that the mosaic makeup of chromosome XVI is conserved amongst the lager strains although some variations do exist. These chromosomes most likely arose from recombination events between homeologous chromosomes. It is interesting to note that in the majority of cases, these recombination sites (as defined by the jumps in the ratios of hybridisation) are located at known sites of high genetic recombination (as in the case of the MAT locus on chromosome III) or at sites containing clusters of Ty or tRNA genes. However, the lager-specific jump sites, such as that observed at YHR165W in strain CMBS, do not contain any of these elements. These unique recombination sites may truly represent examples of adaptive evolution conferring specific selective advantages to that particular strain. Using CCGH analysis, Kodama et al. (2005) further showed that *S. pastorianus* (CBS 1538), *S. carlsbergensis* (IFO11023) and *S. monacensis* (CBS1503) lack certain *S. cerevisiae* like chromosomes. Remarkably, the *S. pastorianus* strain CBS1538 appears to lack *S. cerevisiae* like chromosomes II, III, IV, VI, VIII, XII, XV and XVI.

7.4.2.2 CCGH Analysis of Wine Yeast Genomes

Like lager yeasts, wine yeast strains show properties of aneuploidy, polyploidy and hybrid chromosomes. For example, a subgroup of wine yeasts, isolated from the flor vellum of aging sherry wines, have been shown to contain genetic heterogeneity as identified by electrophoretic karyotyping (Infante et al. 2003). Flor vellum is a biofilm that develops on the surface of sherry wines after the fermentative processes is complete. CCGH analysis of two flor yeast strains, *S. cerevisiae* var. *beticus* 11.3 and *S. cerevisiae* var. *montuliensis* 1.28, confirmed the aneuploid and hybrid nature of their chromosomes. By comparing the ratios of hybridisation for the two flor yeast strains, Infante et al. (2003) revealed that certain chromosomes, such as chromosome IV, show identical ratios of hybridisation (and therefore gene copy numbers), while chromosomes I, III, VI, X and XI show ratios of hybridisations 6 times that observed for chromosome IV. The aneuploid nature of the chromosomes is revealed by plotting the log ratios of the hybridisations between the two strains for each ORF as a function of their position on the *S. cerevisiae* chromosomes. As with the lager strains, the points at which the ratios of the hybridisations

change coincide with positions of Ty elements, LTRs and tRNA gene clusters. A number of other breakpoints correspond to positions of known meiosis-induced double-strand breaks (Gerton et al. 2000). Copy number difference between the two flor yeasts involved 38% of *S. cerevisiae* ORFs present in 116 regions of the genome.

Thus, the picture emerging for industrial yeast strains is one of aneuploidy arising from recombination events, mostly between homeologous chromosomes but also including non-homologous interchromosomal recombination events, resulting from the selective pressures experienced by these strains. This hypothesis, put forward by Infante et al. (2003), is supported by results from a number of model systems set up to examine genome evolution. In a study by Delneri et al. (2003), interspecies crosses between two different *S. mikatae* strains and an *S. cerevisiae* strain engineered to contain chromosomes that are collinear with the *S. mikatae* strain resulted in progeny whose genomes displayed widespread aneuploidy. The authors suggest that aneuploidy imposes genetic isolation on the strains, leading to stable genetic properties. In another experiment to examine the effects of selective pressures on the evolutions of genomes, Dunham et al. (2002) grew strains of *S. cerevisiae* for 100–500 generations in glucose-limited chemostats. Again using CCGH analysis, these authors found that these selective pressures resulted in the emergence of aneuploid strains showing gross chromosomal rearrangements such as amplifications, deletions and translocations. Experiments have also shown that spores of *S. cerevisiae*, *S. bayanus* (*S. uvarum*) and *S. pastorianus* are capable of mating to produce diploids (Delneri et al. 2003). However, upon sporulation, the spores produced are sterile and rarely produce meiotic offspring, leading to the genetic isolation of these strains and the maintenance of stable polyploid genomes.

That such chromosomal rearrangements result from selective pressures in nature was elegantly shown by Perez-Ortin et al. (2002) in the analysis of a wine yeast strain T73. In this strain, a reciprocal translocation between chromosomes VIII and XVI was observed. This genome rearrangement confers sulfite resistance to the strain by generating a fusion between the 5′ upstream region of the *SSU1* gene, encoded on chromosome XVI, and the promoter region of the *ECM34* gene from chromosome VIII. The *SSU1* gene mediates sulfite efflux in *S. cerevisiae* (Park and Bakalinsky 2000). Currently no known function has been ascribed to the *ECM34* gene. The resultant chimera (SSUI-R) results in higher expression levels of the *SSU1* gene, thus conferring sulfite resistance on this strain. The translocation event seems to be mediated by a short stretch of sequence homology between the two regions: The SSU1-R allele contains four repeats of a 76-bp sequence which is identical to a single copy of a 77-bp sequence in the *ECM34* gene. It is interesting to note that the site of translocation on chromosome VIII at YHL043W co-localises with a recombination site in the lager yeasts identified by CCGH (Bond et al. 2004), suggesting that this region may represent a particular "hotspot" of recombination common to wine and lager yeasts. However, an analysis of recombination sites identified in flor yeasts and lager yeasts shows few or no sites in common other than sites in subtelomeric regions. This lack of similarity may reflect the different adaptive pressures on these yeast strains owing to the different fermentation conditions encountered in beer making and wine making. It is interesting to note that none of the translocation sites

identified by sequencing in *S. bayanus*, *S. mikatae* and *S. paradoxus* strains (Kellis et al. 2003) have been identified by CCGH in the lager strains, suggesting that none of these species are the true parent of the hybrid lager strains. Alternatively, the recombination sites identified by CCGH may be *S. cerevisiae* specific. It is also possible that the strains have undergone further genomic rearrangements so that the original translocations have been lost or altered.

7.4.3 Comparative Proteomics to Reveal the Hybrid Constitution of Industrial Strains

The molecular details of the mixed constitution of industrial yeast strains can also be analysed by the application of proteomics.

7.4.3.1 Comparative Proteomics of Lager Strains

Boucherie and colleagues (Joubert et al. 2000) used 2D-PAGE in a comparative analysis of proteomes of different lager yeast isolates from various breweries. In agreement with the hypothesis that the genome of lager yeast is a hybrid from at least two different genomes, it was observed that many of the more abundant lager yeast 2D spots seemed to be duplicated (compared with what was found for the laboratory *S. cerevisiae* strain). The authors mentioned in their report that many of these duplicated spots exhibited the same relative abundance while displaying slight differences in their pI and/or M_r. For three of the duplicated non-*S. cerevisiae* spots, their identity as sequence variants of Pdc1p, Eno1/2p and Fba1p was confirmed by the use of microsequencing. Differential labelling of proteins from different strains and subsequent sample mixing followed by 2D-PAGE analysis allowed reliable scoring of the number of co-migrating proteins on a global scale. Independent analysis by two different labelling methods yielded essentially the same result: roughly 85% of the proteins in the lager strain K11 (patent no. FR 2 750 703-A1) co-migrated with proteins from *S. cerevisiae* (based on the analysis of about 300–500 proteins, respectively, in the two independent studies). Comparative proteome analysis via co-migration on 2D gels was also applied earlier to the analysis of strain variants of *S cerevisiae*, where one isolate from fish intestine was related to a laboratory strain (Andlid et al. 1999); for all 984 matched proteins in that study it was apparent that in total 98% of the proteins co-migrated, leaving only 16 strain-specific proteins in the fish isolate. Thus, in the comparison between *S. cerevisiae* and the lager beer strains a much lower similarity was seen compared with what was found between *S. cerevisiae* strains. The overlap to the 2D pattern of *S. cerevisiae* (Joubert et al. 2000) for the lager strain made possible the release of the first 2D map of an industrial yeast encompassing 185 identified proteins. The lager reference 2D pattern was later extended by MALDI-MS analysis to include an additional 30 lager-specific protein spots (Joubert et al. 2001).

The 2D pattern of the lager strain K11 was also compared with that of other type strains/species in an attempt to identify the non-*S. cerevisiae* genetic component of lager strains. It was reported that the proteome of lager brewing yeasts and of the type strains of *S. carlsbergensis*, *S. monacensis* and *S. pastorianus* could be

interpreted as the superimposition of two elementary patterns. One of them originates from proteins encoded by an *S. cerevisiae* like genome and the other apparently from the *S. pastorianus* NRRL Y-1551 strain. Surprisingly it was found that the two different type strains of *S. pastorianus* displayed rather different 2D patterns, despite supposedly being identical isolates. The reason for this discrepancy was not known, and complicates the final interpretation of their result. However, this work constitutes an important example for the use of 2D analysis for the comparative proteomics of yeast strains using spot position (x and y dimensions) as an indicator of protein identity.

More recent analysis of different lager strains adopted the procedure of liquid chromatography (LC) MS/MS analysis of resolved protein spots. In this work (R. Caesar, E. Pettersson, J. Gustafsson, A. Hashemi, M. Rudemo, A. Blomberg, unpublished results) MS/MS analysis of non-*S. cerevisiae* spots in the 2D gel and subsequent comparison to the complete proteomes of the yeasts within the *Saccharomyces sensu stricto* group revealed a phylogenetic tree with great similarity to the one obtained using gene sequence data. In this comparison the non-*S. cerevisiae* proteins in the total protein 2D pattern of the lager strains are clearly most similar to those of *S. bayanus* (Fig. 7.4).

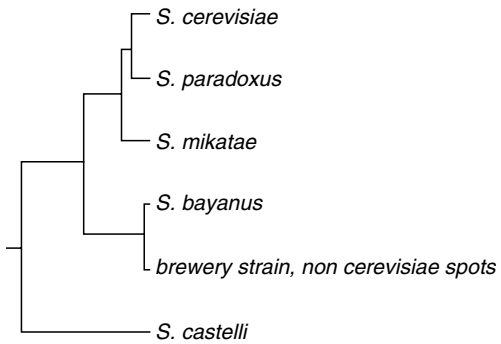

Fig. 7.4. Phylogenetic tree of the non-*Saccharomyces cerevisiae* proteins in the lager strain CMBS33. Seven dominant spots on a CMBS33 2D gel lacking corresponding spots on the gels from the laboratory strain BY4742 were identified as Eft1p, Shm2p, Ilv5p, Pdc1p, Met6p, Pgk1p and Pyk1p using liquid chromatography tandem mass spectrometry (MS/MS). The SEQUEST algorithm was subsequently used to correlate MS/MS spectra from these spots to theoretical mass data derived from a FASTA database containing the amino acid sequences of the identified proteins and their homologues in *S. paradoxus*, *S. mikatae*, *S. bayanus* and *S. castelli*. For each protein the peptide sequences identified from any of the species were compiled to a sequence for the CMBS33 form of the protein. In total, the peptides identified contained 1,237 amino acids covering 32% of the seven proteins. Multiple alignment of the regions covered by the MS/MS analysis were performed between the five *Saccharomyces* species and the brewery strain using ClustalW and a hierarchical tree based on the result was constructed. The brewery strain non *S. cerevisiae* spots differed with one amino acid from *S. bayanus*, with 36 amino acids from *S. cerevisiae*, with 34 amino acids from *S. paradoxus*, with 32 amino acids from *S. mikatae* and with 134 amino acids from *S. castelli*

7.4.3.2 Comparative Proteomics of Ale Strains

The first protein 2D map of an ale-fermenting yeast was recently presented (Kobi et al. 2004). In this analysis 205 spots corresponding to 133 different proteins were identified by MALDI-TOF analysis in the A38 ale strain. Comparison of the proteome of this ale strain with a lager brewing yeast and the *S. cerevisiae* strain S288c confirmed that ale strains are much closer to S288c than lager strains. A zoom-in on a central part of the 2D patterns of three different lager strains, *S. cerevisiae* S288c, and two different ale strains clearly displays these differences and similarities (Fig. 7.5). The A38 ale strain exhibited a very similar pattern to the *S. cerevisiae*

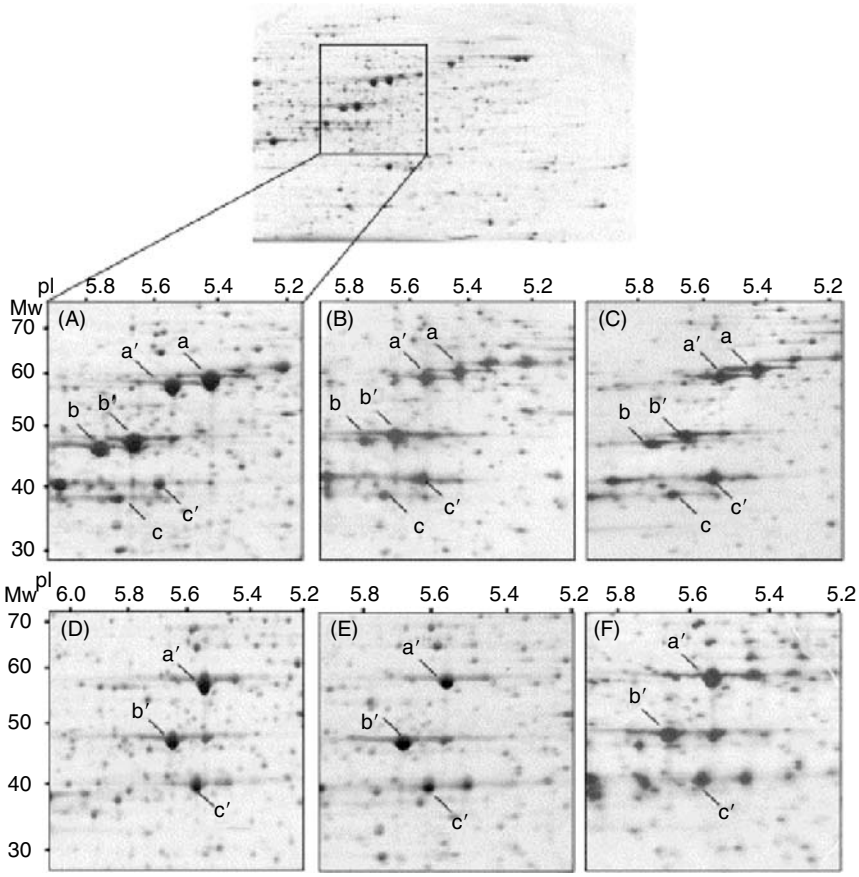

Fig. 7.5. The 2D patterns of ale strains are similar to the one found in laboratory strains. 2D polyacrylamide gel electrophoresis analysis of ale (**e**, **f**), lager (**a–c**) and laboratory (**d**) strains. The protein spots indicated are Pdc1p (*a* and *a*'), Eno2p (*b* and *b*') and Fba1p (*c* and *c*'). 2D images are displayed with the acidic side to the *right*. (Adopted from Kobi et al. 2004 with permission from Elsevier)

strain, and this observation could also be extended to other ale strains. However, this does not mean that the 2D patterns of these ale strains were identical to the laboratory strain S288C. On the contrary, looking at the entire gel for these strains, some discrepancies appear, e.g. the alcohol dehydrogenase isoform Adh4p is present in the ale-brewing strains but not in the laboratory strain under these growth conditions. This is interesting since this zinc-dependent Adh4 protein is reported to be regulated by zinc deficiency (Yuan 2000), which could indicate that the industrial ale strain might exhibit alterations in zinc metabolism. The Adh4p isoform was also found expressed under normal laboratory growth conditions in an industrial baker's yeast strain (Nilsson et al., unpublished results).

7.4.3.3 Comparative Proteomics of Baker's Yeast Strains

Four industrial baker's yeast strains with various fermentative capacities were compared with a laboratory *S. cerevisiae* strain during fed-batch cultivation. After 2D-PAGE analysis the strains were grouped according to differences in protein expression and 2D spot positional differences (Nilsson et al., unpublished results). It was found that the baker's yeast strains were more similar to each other than to the laboratory strain. However, overall the 2D patterns were rather similar among all strains and the most outstanding difference was found for the protein spots corresponding to alcohol dehydrogenase I and II, where all strains examined exhibited large variations. In particular it was noticed that the baker's yeast strains contained several alcohol dehydrogenase spots not present in the laboratory strain. Subsequent MALDI-MS analysis of these alcohol dehydrogenase spots revealed a rather large apparent variability in the alcohol dehydrogenase protein sequence, and some of the sequences seemed to be sequence hybrids between Adh1p and Adh2p.

7.4.4 Genomic Sequencing of Industrial Strains of Yeast

The ultimate characterisation of industrial yeast strains will require the complete genome sequence at a nucleotide level; however, this has been hampered by the polyploid nature of their genomes which requires sequencing and analysis of both the *S. cerevisiae* and the non-*S. cerevisiae* components of the genome. The first description of the sequencing of an industrial lager strains was reported by Suntory, Japan (Nakao et al. 2003). This group sequenced the lager strain Weihenstephan 34/70 (*S. pastorianus*) using a random shotgun approach. Two types of contigs covering 23.2 Mbp (95% coverage) were identified, displaying either 98% identity or 85% identity to the *S. cerevisiae* DNA sequence (Kodama et al. 2005; Nakao et al. 2003). The sequence data confirmed the findings of classical genetics of the presence of three types of chromosomes in the lager strains, *S. cerevisiae* like, non-*S. cerevisiae* like and mosaic chromosomes, arising from recombination events between homeologous chromosomes and identified 37 chromosomal varieties. In general, with the exception of a number of translocations and inversions, the gene synteny is identical to that of *S. cerevisiae*. The non-*S. cerevisiae* like DNA sequences most closely resemble those of *S. bayanus* (*uvarum*); however, the sequences are not identical. This may be due to the difference in strains of *S. bayanus* as only a single *S. bayanus* sequence

is currently available. Interestingly, this study identified 20 ORFs with no significant identities to *S. cerevisiae* ORFs. Furthermore, the recombination sites on the mosaic chromosomes, identified by CCGH analysis (Bond et al. 2004), are confirmed by the sequence analysis, although the Weihenstephan strain contains a number of unique mosaic chromosomes that are not present in the 6701 or CMBS strains characterised by CCGH analysis and vice versa (Fig. 7.6).

7.5 Analyses of the Industrial Process

The novel tools for genome-wide analysis of the yeast transcriptome and proteome are not only instrumental for a better description of industrial strains but also allow a more comprehensive analysis of the industrial-scale fermentation process. The environmental conditions experienced by yeasts in industrial settings are quite unique in their complexity and this complexity is rarely experienced by strains propagated under laboratory growth conditions. For example, lager and ale yeasts during industrial fermentation simultaneously experience a wide array of conditions such as anaerobiosis, high alcohol concentrations, high hydrostatic pressure and high cell density. Likewise, wine yeasts are exposed to a unique set of conditions such as high sugar concentrations, anaerobiosis, low pH and high ethanol concentrations during oenological fermentations. One might consider all of these conditions as imposing severe stress on the yeasts, and therefore it is of particular interest to examine the gene expression profiles in order to understand the stress responses of these yeasts

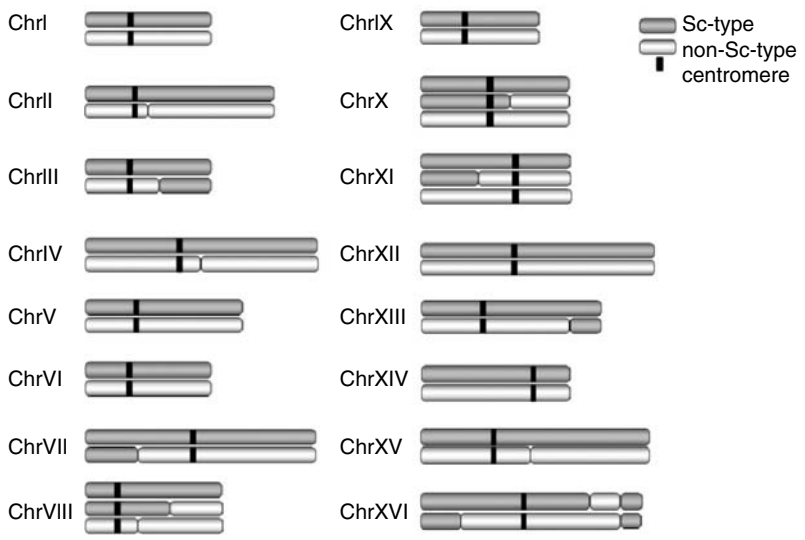

Fig. 7.6. Genome composition of the lager strain Weihenstephan 34/70 (*S. pastorianus*) as deduced by DNA sequencing. (Adopted from Kodama et al. 2005 with permission from Springer-Verlag)

to their environment and to examine the expression patterns of genes involved in metabolic processes, in particular those associated with carbohydrate metabolism.

A number of transcriptome datasets are currently available profiling the gene expression patterns in lager yeasts (Dawes et al. 2002; Higgins et al. 2003a, b; James et al. 2002, 2003; Olesen et al. 2002; Panoutsopoulou et al. 2001; Pugh et al. 2002) and wine yeasts (Backhus et al. 2001; Cavalieri et al. 2000; Erasmus et al. 2003; Rossignol et al. 2003) under fermentation conditions. It should be pointed out that in all transcriptome studies outlined in this section, it is only possible to examine gene expression of *S. cerevisiae* like genes in either lager yeasts or wine yeasts. The contribution of the non-*S. cerevisiae* genes to the overall gene expression patterns remains unexplored at this stage. Likewise the effects of multiple copies of similar but non-identical genes on the overall gene expression pattern are currently unknown, as is whether control of gene expression is conserved between the *S. cerevisiae* and non-*S. cerevisiae* genes. Currently these datasets have not been compiled into a format (website) that allows direct comparison of the different datasets; however, some comparisons have been carried out (James and Bond, unpublished data). While direct comparisons of the data are hampered by the varying experimental conditions used (parameters such as time points sampled during the fermentation, starting cell densities, industrial growth media and temperature used all vary between the datasets), an overall picture of the physiological state of the yeast under fermentation conditions can be deduced.

7.5.1 Beer Production

7.5.1.1 Gene Expression Patterns in Lager Yeasts Under Fermentation Conditions

Among the common gene expression themes that emerge from the analysis of lager yeast fermentations is the co-ordinate upregulation of genes affected by anaerobiosis and those required for ergosterol and fatty acid metabolism. The latter set of genes are required for continued cell membrane biosynthesis, a process requiring the presence of molecular oxygen. Thus, brewers generally actively aerate the wort at the start of the fermentation. This small quantity of initially added O_2 is sufficient to allow continued ergosterol biosynthesis during the fermentation. The co-ordinate upregulation of genes required for fatty acid and sterol metabolism is further verified by the upregulation of a number of genes involved in peroxisomal metabolism such as the PEX genes *PEX*, *5*, *10*, *14*, *21*, *11* and *18*. Likewise, the PAU gene family, initially identified as genes responding to anaerobic growth conditions, but more recently suggested to be sterol carriers (Wilcox et al. 2002), are co-ordinately upregulated as fermentation proceeds (James et al. 2003). High levels of ergosterol may be essential to protect yeast from ethanol stress (Alexandre et al. 1994). Another interesting finding from the transcriptome analysis was the co-ordinate upregulation during large-scale fermentation of genes involved in aldehyde metabolism (Fig. 7.1).

Of the genes that show decreased transcript levels on days 3 and 8, with respect to day 1, of fermentation, the most abundant classes are those required for protein and amino acid biosynthesis. These account for more than 30% of the downregulated genes. The co-ordinate downregulation of these genes most likely reflects the

low level of general cell metabolism occurring during industrial fermentation, where cells may only undergo one to two cell divisions. The co-ordinate downregulation of protein synthesis genes has also been observed in stationary-phase yeast cultures (Gray et al. 2004). The majority of genes involved in glycolysis are similarly downregulated as fermentation proceeds (Fig. 7.1). These genes are generally regulated by catabolite repression (glucose repression) and their downregulation may reflect continued levels of useable carbohydrates even at day 8 of fermentation.

7.5.1.2 Application of Proteomics to Analyse Ale Fermentation

Kobi et al. (2004) followed proteome changes during the fermentation process of the ale strain A38 in a 10-hl pilot device, for the first, second and third generation. The A38 strain was initially grown in aerobic conditions with saccharose as the sole carbon source before pitching in wort under anaerobic conditions for the first fermentation. To mimic the industrial process, the yeasts were harvested at the end of the fermentation and re-inoculated in the fresh wort for a second and then third generation. In particular, proteome analysis and comparison was performed during each generation (at the start and after roughly 200 h). A comparison between the beginning and the end of the first generation showed that 50 of the 85 differentially expressed proteins were repressed, mostly glycolytic enzymes, proteins involved in acetylcoenzyme A formation, proteins of the tricarboxylic acid cycle, and proteins involved in respiration. It is clear that most of the changes reveal an adaptation to anaerobic conditions. In addition, most of the proteins induced at later times in the fermentation process were protein fragments belonging to either proteins in carbon metabolism, or protein or amino acid biosynthesis pathways. It was suggested that intracellular proteolysis influenced the regulation of these proteins during the industrial fermentation process.

Many fewer changes in protein abundance were scored between the beginning and the end of the third generation, and the observed changes exhibited lower levels of response. Among the proteins that displayed at least a twofold change were proteins involved in methionine biosynthesis (e.g. Sah1p, Met6p and Met3p) as well as some involved in carbon metabolism (e.g. Fba1p, Adh1p and Ald6p); all the example proteins indicated displayed induction except Ald6p, which was repressed.

7.5.1.3 Stress Responses During Beer Fermentation

Considering the extreme environmental conditions experienced by yeasts during the fermentation process, one might expect the induction of stress genes in these cells. Stress-responsive genes are generally regulated by transcription factors such as the heat shock factor, Hsf1 and the Msn2/Msn4 proteins, which bind to heat shock elements (HSE) and stress responsive elements (STREs), respectively. Msn2/Msn4p are zinc-finger proteins that are activated by a number of stress conditions encountered by yeasts such as entry into the stationary phase, carbon source starvation and osmotic stress. Surprisingly, transcriptome analysis during fermentation indicates that genes under the control of Hsf1 and Msn2/Msn4 are downregulated as fermentation proceeds (James et al. 2003). These results are consistent with previous

data showing that Hsp104p levels were repressed during fermentation (Gray et al. 2004; Brosnan et al 2000). The lack of expression for stress-responsive genes appears to be unique to lager strains of yeasts. A comparative analysis of genes encoding heat shock proteins (HSP genes) in a haploid laboratory strain of *S. cerevisiae* and the lager strain 6701, grown under identical fermentation conditions, revealed that a subset of the HSP genes (*HSP104*, *HSP30*, *HSP26* and *HSP12*) are in fact highly induced late in fermentation (on day 8) exclusively in the haploid laboratory strain (James et al. 2002; James and Bond, unpublished results), a response not encountered for the lager strain. These results therefore suggest that the transcriptional regulation of HSE-/STRE-regulated HSP genes is significantly different in lager and laboratory strains of yeasts.

The regulation of genes responding to oxidative stress has also been extensively examined in lager strains of yeasts during fermentation (Higgins et al. 2003a; James et al. 2003). These genes are generally under the control of the transcription factor Yap1p. Expression levels of these genes are elevated on days 1 and 3 of fermentation most likely in response to the production of O_2 free radicals. The expression patterns of these genes differ in the various reported datasets (Higgins et al. 2003a; James et al. 2003; Olesen et al. 2002). This most likely reflects the different regimes for oxygenation of the media at the beginning of fermentation. A number of oxidative responsive genes are also induced late in fermentation; these include the genes *LYS7*, *SOD1*, *TSA1* and *MXR1* (James et al. 2002, 2003). The *MXR1* gene encodes the protein methionine sulfoxide reductase, which reverses the oxidation of methionine residues and which has been shown to be required for the reduction of dimethyl sulfoxide to dimethyl sulfide, which is a thioester of major importance for the aroma and flavour of beer (Hansen et al. 2002).

Kobi et al. (2004) reported that a number of stress proteins exhibited changes in ale yeasts during the first-generation fermentation, like Hsp26p and Ssa4p. However, the only stress proteins that also increased during the third generation of fermentation were Kar2p and Ssa1p (both these proteins encode chaperones that are required for protein folding). Yeast strains must cope with various stresses during the fermentation process; however, there is clearly no strong stress response in the ale strain.

7.5.2 Wine Fermentation

7.5.2.1 Gene Expression Patterns in Wine Strains Under Fermentation Conditions

The conditions for wine and lager fermentations differ in a number of important aspects, such as starting cell concentrations, growth media (wort, rich in maltose for beer, and grape juice, rich in fructose and glucose for wine), pH, temperature and length of incubation. However, yeasts in both fermentations share some common environmental conditions, such as anaerobiosis and high ethanol concentrations at the end of the fermentation. In general, carbohydrates do not become limiting during wine fermentations. Instead cells enter the stationary phase owing to limiting nitrogen concentrations and/or attainment of maximal cell density. Surprisingly, the expression patterns are remarkably similar in both lager and wine fermentations;

however, unique expression patterns are also observed under both conditions. As observed in lager strains of yeasts, there is a co-ordinate downregulation of genes involved in protein and amino acid biosynthesis and upregulation of PAU genes and genes encoding proteins involved in aldehyde metabolism. Significant differences between the expression patterns observed in the wine and lager fermentations include the downregulation of genes for ergosterol biosynthesis in wine fermentations and the upregulation of genes required for glycolysis. The former results from the inclusion of ergosterol in the culture medium, while the latter most likely reflects the concentrations of carbohydrates at different times during the wine and lager fermentations. One interesting similarity is the upregulation of genes encoding aryl alcohol dehydrogenases. The contribution of these genes to total alcohol synthesis is presently unknown. It has also been suggested that these genes are induced in response to oxidative stress (Rossignol et al. 2003).

Transciptome analysis during wine fermentation also revealed major changes in gene expression patterns as a result of nitrogen depletion as the fermentation proceeded and confirmed that growth arrest (entry into the stationary phase) is a direct consequence of nitrogen depletion (Rossignol et al. 2003). The major changes in the gene expression pattern associated with nitrogen depletion were the induction of genes required for the metabolism of poor alternative nitrogen sources such as genes required for proline, allantoin and urea utilisation, nitrogen permeases and genes encoding proteins required for the management of glutamate pools. All of these genes are under the control of the TOR pathway.

7.5.2.2 Stress Responses During Wine Yeast Fermentation

One of the major differences in gene expression patterns between wine and lager yeast fermentations is the upregulation of HSP genes in wine yeast (Rossignol et al. 2003). As mentioned before, this group of genes appear to be actively repressed during lager yeast fermentations. The expression patterns of HSP genes in wine yeasts most closely resembles that observed in haploid *S. cerevisiae* strains grown under fermentation conditions (James et al. 2002). The HSP genes, such as *HSP30*, *HSP26* and *HSP104*, which are under the control of STREs are particularly induced during wine fermentations. Such genes have previously been shown to be induced following diauxic shift and upon entry into the stationary phase. Both conditions see a shift from usage of fermentable carbohydrates to non-fermentable carbon sources such as acetate and ethanol. It is possible that conditions at diauxic shift are radically different in wine and lager production. Alternatively, the differential expression of HSP genes in wine and lager yeasts raises the possibilities that chromatin structure and/or the presence of homeologous non-*S. cerevisiae* genes contribute to these gene expression patterns.

In addition to HSP genes, 58% of genes previously defined as being regulated as part of the common environmental response (CER) and the environmental stress response (ESR) (Gasch et al. 2000) are upregulated during wine fermentations (Rossignol et al. 2003). Additionally, genes responsive to ethanol stress are also induced. Many of these genes are involved in cell wall biogenesis, suggesting that cell wall alterations may help yeasts cope with ethanol stress. It is interesting to note

that ten genes involved in cell wall biogenesis are also induced late in lager yeast fermentations (James et al. 2003).

A wild-type *S. cerevisiae* wine strain isolated from the natural must of spontaneous grape fermentation was analysed for proteome changes during semiaerobic growth conditions (Trabalzini et al. 2003). In particular the response of this wine strain to the exhaustion of glucose from the medium was of interest since this is believed to be the main reason for unwanted stuck fermentation during vinification. When glucose was depleted from the medium a large number of changes in protein abundance were apparent. Roughly 50 proteins displayed decreased amounts at the end of fermentation, usually in the range twofold to threefold repression and, interestingly, some of these proteins are currently functionally not characterised (e.g. Ybr025cp and Yir035cp). In addition, a large number of protein spots with low molecular weight increased during the later phases of fermentation. Identification of these spots indicated that they belonged to protein fragments of larger proteins, which indicated proteolytic breakdown as part of the cellular response. However, also well-known stress responsive proteins like Sod1p, Tsa1p and Ctt1p exhibited increased expression when glucose was depleted, indicating a clear stress response in this wine yeast.

7.5.2.3 Application of Proteomics to Understand Factors That Affect Wine Haze

The clarity of white wine is highly important for the winemaker. Bottles showing haziness, likely to be rejected by the consumer, result from the aggregation of grape proteins naturally present in wine. To prevent haze formation, winemakers usually lower the concentration of wine proteins through the use of bentonite. Unfortunately bentonite also removes wine aroma components, hence lowering wine quality. Thus, alternative methods of protein stabilisation are being investigated by the wine industry.

Mannoproteins from yeast cell walls are known to be released into the extracellular medium during yeast growth, in particular during the stationary phase (Dupin et al. 2000). One of the proteins released, invertase, was shown to lower haze formation, probably by competing with grape-derived proteins for some unknown factor(s) in wine that is required to form large highly light scattering protein aggregates that are responsible for the haze. To better characterise the protein components of wine a 2002 vintage Sauvignon Blanc wine was analysed by LC-MS/MS (Kwon 2004). Wine proteins were concentrated by the use of a 5-kDa cellulose membrane centrifugal filter tube, followed by salting-out precipitation in saturated ammonium sulfate aqueous solutions and subsequent centrifugation to isolate the protein pellets. Proteins were separated by SDS-PAGE and Coomassie visualised bands were cut out for trypsination and peptide elution. The LC-MS/MS analyses resulted in the identification of 12 different cell wall or plasma membrane associated proteins from *S. cerevisiae*: e.g. Gas1p, a GPI-anchored 1,3-glucosyltransferase, Pho3p, a periplasmic acid phosphatase, Suc4p, invertase, Bgl2p, *endo*-1,3 glucanase and Yju1p (Cwp1p), reported as a structural component of the cell wall. Many of these released yeast proteins could have an influence on the wine haze formation. It was proposed that MS identification of proteins could be used as a quality indicator of wine.

7.5.3 Industrial Production of High-Quality Baker's Yeast

Two aspects of industrial baker's yeast production have been investigated using proteomics. One important feature of baker's yeast performance is high fermentative activity even after long-term cold storage. To identify molecular effects during this storage regime on a baker's yeast strain, 2D analysis was performed before and after storage for 26 days at 4°C (Nilsson et al. 2001a). It was clear from this analysis that cold storage resulted in large changes in the protein content. However, even after this long period of storage the original state of the culture, e.g. exponential growth in the respiro-fermentative state or in the transition phase to respiration, could still be distinguished by analysis of the 2D pattern. The fermentative activity after storage was also shown to be dependent on the initial state, and thus it was concluded that the initial state at harvest was important for the long-term performance of this industrial yeast strain. The baker's yeast cells experience different types of starvation regimes during industrial production. To investigate the effects on these industrial strains from either nitrogen or carbon limited starvation, a differentially starved baker's yeast strain was analysed for protein changes (Nilsson et al. 2001b). It was found that for certain glycolytic enzymes a significant change in the amount of protein could be observed during these starvation regimes, e.g. Pdc1p decreased during nitrogen starvation, while Adh1p was downregulated during carbon starvation. However, the recorded changes in the protein levels did not correlate with the observed changes in fermentative capacity.

7.6 Future Perspectives

The complete genome sequence of the first industrial yeast strain, the lager yeast Weihenstephan 34/70 (*S. pastorianus*), will be instrumental in our analysis of these hybrid genomes. It will not only potentially indicate the full compliment of genes in these cells, but will also provide the basis for a number of novel technological possibilities in the analysis of industrial yeast strains. The genome sequence will soon be opened up for expression analysis of not only the *S. cerevisiae* part of the genome but will also put the non *S. cerevisae* part within experimental reach; not before long the first microarrays with a full complement of genes from industrial yeast will be available. In addition, it will make possible a more complete and precise proteome analysis by providing a better template for database searches using MS data. It will also make possible non-gel-based proteomics approaches that are based on whole cell trypsination prior to high-resolution LC-MS/MS analysis (Washburn et al. 2001). Hopefully these novel possibilities will lead to the publication of studies where combined transcriptome and proteome analyses are presented, which are missing at present. Of great general interest will be the analysis of differences in the regulatory components of these mixed genomes where industrial strains might have evolved/been selected to respond quite differently to the well-studied laboratory *S. cerevisiae* strains (see previous sections on the stress response). Hopefully the genome sequence and the novel experimental possibilities will also lead to the development of dedicated databases where transciptome and proteome data from industrial yeast strains can be presented, compared and analysed. The interplay between

the hybrid proteomes will be another important avenue for future studies, since the genome sequence puts within experimental resolution a view of the physical protein interaction network, by either two-hybrid analysis or co-immunoprecipitation (e.g. tandem affinity purification tagging), in particular regarding the mixed composition of protein complexes. These studies will also be of fundamental importance in our understanding of the functionality of hybrid protein complexes, where different components compete in the formation of complexes and play different functional roles in their final activity.

Many challenges remain and will require further advancements both in the tools of bioinformatics as well as in analytical techniques. One important aspect is the finite gene number for industrial strains, a number that even for the well-studied laboratory *S. cerevisiae* strain, as mentioned earlier, has changed over the years and most likely will continue to change. In this respect the mixed genomes of some industrial yeast strains will provide an even greater challenge in gene identification. However, maybe the greatest obstacle in our strive for a more detailed functional analysis of individual genes in the genetic background of industrial strains will be the difficulty in generating gene deletions in these mixed genomes of several similar and identical gene copies. This problem will certainly ensure that the evolutionary link to *S. cerevisae* will persist not only in the industrial production line but also in future functional analysis of non-*S. cerevisae* genes in the potent laboratory test-bed *S. cerevisiae*.

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Chapter 8

Carbohydrate Metabolism

J. Richard Dickinson[1] and Arthur L. Kruckeberg[2]

[1]*Cardiff School of Biosciences, Cardiff University, P.O. Box 915, Cardiff CF10 3TL, UK*
(e-mail: dickinson@cardiff.ac.uk)
[2]*Gothia Yeast Solutions AB, Terrassgatan 7, 41133 Göteborg, Sweden*
(e-mail: arle.kruckeberg@gothiayeast.com)

8.1 Introduction

Carbohydrate metabolism by yeasts has been the topic of scientific investigation for well over a century, owing to the central roles that yeasts play in food and beverage biotechnology and to the ease with which yeasts can be studied in the laboratory. The literature on the biochemistry, genetics, and molecular biology of yeast carbohydrate metabolism is too vast to cover thoroughly in this chapter. General reviews of the field (Zimmermann and Entian 1997; Kruckeberg and Dickinson 2004) as well as specialised reviews (vide infra) can be consulted for more detail. Most of the research on carbohydrate metabolism has been with the baker's or brewer's yeast, *Saccharomyces cerevisiae*. However, many other yeast species play important roles in the food sector, as agents of fermentation, as sources of biomass components, or as spoilage organisms. The roles of these other species are often due to unique features of their carbohydrate metabolism, and examples of these are given here.

8.2 Carbon Sources

Yeasts are chemoheterotrophic organisms, obtaining all of their energy and carbon from organic compounds. "Yeast" actually refers to a taxonomically and physiologically disparate group of organisms, including ascomycetes, basidiomycetes, and deuteromycetes (fungi imperfecti). Collectively, yeasts are able to grow on a wide range of carbon sources, including carbohydrates, alcohols, organic acids, amino acids, *n*-alkanes, and lipids. Carbohydrates are the commonest carbon source, and also the most important in terms of the biotechnological applications of yeasts. Yeasts are able to grow on monosaccharides, oligosaccharides, and polysaccharides (Barnett 1976). Among monosaccharides, metabolism of hexoses (glucose, fructose, mannose) is general, and is carried out largely by the glycolytic pathway (Käppeli 1986). Galactose utilisation additionally requires the action of the Leloir pathway (De Robichon-Szulmajster 1958; Leloir 1964; Douglas and Hawthorne

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
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1966). In contrast, pentose utilisation is quite restricted taxonomically (e.g. *Pichia stipitis* and a few other species; Maleszka and Schneider 1982; Verduyn et al. 1985), and requires xylose reductase, xylitol dehydrogenase, and xylulokinase activities and the pentose phosphate pathway.

Utilisation of oligosaccharides and polysaccharides is initiated by hydrolysis to the component monosaccharides, and expression of the appropriate hydrolase can also be taxonomically restricted. For example, not all yeasts are able to metabolise sucrose, presumably owing to the absence of invertase expression (Ahearn et al. 1977; Lachance et al. 2001). *Kluyveromyces* species are unusual amongst yeasts in expressing β-galactosidase activity, which is required for lactose utilisation (but see Sect. 8.10) (Algeri et al. 1978; Dickson and Markin 1980). *S. cerevisiae* var. *diastaticus* is an example of an amylolytic yeast; it expresses and secretes glucoamylase activity that is required for starch utilisation (Yamashita et al. 1985; Pretorius et al. 1986). Species of *Schwanniomyces* are amylolytic as well (Clementi et al. 1980). Pectinolytic (Wimborne and Rickard 1978; Gainvors et al. 1994) and xylanolytic (Kratky and Biely 1980; Lubomir and Peter 1998) yeasts have been isolated and characterised. In contrast, no yeasts with cellulolytic capability have been identified (but see Sect. 8.10). Yeast biomass and yeast extracts are, however, stimulatory to cellulolytic rumen bacteria, and *S. cerevisiae* is often used as a feed additive or probiotic for ruminants (Callaway and Martin 1997; Jouany et al. 1998).

8.3 Modes of Metabolism

A number of regulatory phenomena have been described for the modes of carbohydrate metabolism by yeasts. The *Crabtree effect* refers to the occurrence of alcoholic fermentation (rather than respiration) of glucose under aerobic conditions. This is a characteristic of *S. cerevisiae* and some other facultatively fermenting yeasts, and occurs during aerobic batch cultivation and during aerobic glucose-limited chemostat cultivation at high dilution rates (Fiechter et al. 1981; Postma et al. 1989). Numerous physiological differences distinguish Crabtree-positive species from Crabtree-negative species such as *K. lactis* and *Schw. castellii* (van Urk et al. 1989; Zimmer et al. 1997; Kiers et al. 1998; Zeeman et al. 2000), but the causal factor regulating the onset of Crabtree metabolism has not yet been identified and remains controversial (Diderich et al. 2001; Otterstedt et al. 2004; Thierie 2004).

The *Pasteur effect* refers to inhibition of fermentation by aerobiosis; for example, *P. anomala* displays respiratory metabolism under aerobic conditions even at high glucose concentrations, and switches to fermentative metabolism under hypoxia irrespective of the glucose concentration (Fredlund et al. 2004). In *S. cerevisiae* the Pasteur effect is largely confined to non-growing cells or to cells grown at very low dilution rates in aerobic, glucose-limited chemostats (conditions in which the glucose transport capacity is low; Lagunas 1986).

The *Custers effect* refers to the inhibition of fermentation by anaerobiosis; for example, the wine spoilage yeast *Brettanomyces* ferments glucose to ethanol and acetic acid only under aerobic conditions (Wijsman et al. 1984). The *Kluyver effect* refers to an inability to utilise some disaccharides anaerobically, although (some

of) their constituent monosaccharides can be metabolised under anaerobic conditions. For example, *Candida utilis* is able to ferment glucose but not maltose (4-α-D-glucopyranosyl-D-glucopyranose) anaerobically, and *S. cerevisiae* is able to ferment glucose but not trehalose (α-D-glucopyranosyl-(1,1)-α-D-glucopyranose) anaerobically (Sims and Barnett 1991; Malluta et al. 2000; Fukuhara 2003). In these situations the capacity to transport the disaccharide into the cell may be too low to sustain anaerobic growth.

8.4 Substrate Transport

Carbohydrate uptake is mediated by transport proteins which are integral in the plasma membrane. Transport of monosaccharides as well as disaccharides and oligosaccharides is mediated by proteins in the Sugar Porter family of the Major Facilitator Superfamily of solute transporters (Busch and Saier 2004). In yeasts, these proteins all display discernible sequence homology (Kruckeberg 1996).

Hexose transport is carried out by a facilitated diffusion (i.e. uniport) mechanism in *S. cerevisiae* (Kruckeberg 1996). Most other yeasts also display a hexose-H^+ symport mechanism, e.g. in *Schizosaccharomyces pombe* (Lichtenberg-Frate et al. 1997; Heiland et al. 2000) and *Kluyveromyces* species (Gasnier 1987; Postma and van den Broek 1990). A fructose-specific symporter, Fsy1, occurs in the *Saccharomyces sensu stricto* yeasts *S. pastorianus* and *S. bayanus* (Goncalves et al. 2000; Rodrigues de Sousa et al. 2004). Disaccharide transport is generally mediated by a symport mechanism as well. Examples include the maltose-H^+ and α-glucoside-H^+ symporters of *S. cerevisiae* (Serrano 1977; Cheng and Michels 1991; Stambuk et al. 1999) and the lactose-H^+ symporter of *K. lactis* (Dickson and Barr 1983; Chang and Dickson 1988). The uptake of sugars by proton symport may be adaptively advantageous as it allows for accumulation of the substrate against a concentration gradient; in other words, the nutrient can be taken up by the cell despite low extracellular concentrations. Furthermore, the substrate affinities of the cytosolic enzymes that act on transported sugars (e.g. hexokinase, β-galactosidase) are low enough that a concentrative mode of transport may be required to maintain a sufficient metabolic flux to sustain growth.

Some carbohydrates that are fermented by yeast are not transported intact, but are instead hydrolysed by extracellular hydrolases. Examples include sucrose and raffinose, which are typically cleaved to glucose and fructose (or, for raffinose, fructose and melibiose) in the periplasm by invertase; the monosaccharides are then taken up by the hexose transport system (Moreno et al. 1975; Lazo et al. 1977; Bisson et al. 1987). Strains of *S. cerevisiae* differ in their ability to utilise the melibiose; some express an extracellular melibiase that hydrolyses this disaccharide to glucose and galactose, both of which can then be taken up and metabolised (Buckholz and Adams 1981; Naumov et al. 1990).

S. cerevisiae has a family of 20 genes encoding hexose transporters and related proteins, the so-called *HXT* gene family (Boles and Hollenberg 1997; Kruckeberg 1996). Of these, only *HXT1–HXT7* encode transporters that are important for growth and metabolism of glucose (Reifenberger et al. 1995; Diderich et al. 1999a). The galactose transporter, encoded by *GAL2*, is also a member of the *HXT* gene family (Nehlin et al. 1989; Szkutnicka et al. 1989). Two members of the family,

encoded by *SNF3* and *RGT2*, have lost the ability to transport hexoses; instead, they function as sensors of the extracellular glucose concentration. This glucose signal is involved in regulating the expression of various *HXT* genes (Özcan and Johnston 1999). The remaining members of the family (*HXT8–HXT17*) are phenotypically silent, and may not be expressed under normal physiological conditions (Diderich et al. 1999a).

The affinity of Hxt hexose transporters for glucose differs; for example, Hxt1 and Hxt3 have a low affinity (approximately 100 mM), whereas Hxt2, Hxt6, and Hxt7 have a high affinity (approximately 1 mM; Reifenberger et al. 1997). The low-affinity transporters are expressed at high glucose concentrations (e.g. early in batch cultivation on glucose or in chemostat cultivation with high residual glucose concentrations), whereas the high-affinity transporters are expressed at low glucose concentrations (e.g. as batch cultures approach the diauxic shift or in chemostat cultures with low residual glucose concentrations; Diderich et al. 1999a). Thus, the substrate affinity of cellular glucose transport is appropriate for the glucose concentration in the environment. This regulation is achieved by the combined action of the Snf3/Rgt2 signal transduction system (Özcan and Johnston 1999) and by glucose repression of the high-affinity glucose transporter genes (Bisson 1988; Petit et al. 2000).

Sugar transporters are subject to inactivation when they are no longer required by the cell; this is true for the transporters of maltose (Lucero et al. 1993) and galactose (DeJuan and Lagunas 1986), and for the high-affinity glucose transporters (Busturia and Lagunas 1986). In all cases, the proteins are "tagged" by ubiquitination and removed from the plasma membrane by endocytosis; subsequently they are translocated to the vacuole and degraded by proteolysis (Riballo et al. 1995; Horak and Wolf 1997; Krampe and Boles 2002).

8.5 Glycolysis

Glycolysis oxidises glucose to pyruvate with the concomitant reduction of NAD^+ to NADH. The pathway consists of ten enzymes: hexokinase phosphorylates glucose and fructose at the expense of ATP, and phosphoglucose isomerase interconverts the two hexose-6-phosphates. Phosphofructokinase adds a second phosphate group to fructose-6-phosphate to form fructose-1,6-bisphosphate; this reaction consumes another ATP molecule. Aldolase then cleaves fructose-1,6-bisphosphate to form dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. These can be interconverted by triose phosphate isomerase. The glyceraldehyde-3-phosphate formed by the last two reactions is oxidised to 1,3-bisphosphoglycerate by glyceraldehyde-3-phosphate dehydrogenase, with concomitant reduction of NAD^+; an inorganic phosphate is added in this reaction. The phosphoryl group is then transferred to ADP by phosphoglycerate kinase, to form ATP and 3-phosphoglycerate. The latter compound is rearranged to 2-phosphoglycerate by phosphoglycerate mutase, and further to phosphoenolpyruvate by enolase. A second phosphoryl transfer reaction occurs, catalysed by pyruvate kinase, to form pyruvate and a second ATP molecule. Note that two ATP molecules are consumed in upper glycolysis, and four are produced

in lower glycolysis (two each for the two three-carbon compounds produced by aldolase). Thus glycolysis yields a net of 2 moles of ATP per mole of glucose, via substrate-level phosphorylation (Stryer 1995; Zimmermann and Entian 1997).

Glycolysis is not only an important source of ATP, and of pyruvate for subsequent dissimilatory reactions, but also provides carbon skeletons for amino acid biosynthesis. For example, phosphoenolpyruvate is used for aromatic amino acid biosynthesis, 3-phosphoglycerate is used for serine biosynthesis, and pyruvate is used for biosynthesis of alanine, valine, and leucine.

The glycolytic pathway is ubiquitous amongst yeast species, despite the diversity in substrate range and metabolic modes displayed by yeasts. The biochemical basis for the differences in metabolism has not received much attention; from the limited number of studies done on yeasts other than *S. cerevisiae* it seems that the regulation of glycolytic enzyme gene expression is the primary source of diversity; in addition, differences in the kinetics and allosteric regulation of some enzymes has been noted among yeast species (Weusthuis et al. 1994; Passoth et al. 1996; Flores et al. 2000).

In *S. cerevisiae*, glycolytic enzymes are expressed to very high levels, especially during growth on glucose. Most of the enzymes occur as isoenzymes, and the genes encoding them are differentially expressed. For example, hexokinase activity is encoded by the *HXK1*, *HXK2*, and *GLK1* genes (the *HXK* gene products act on both glucose and fructose, whereas glucokinase, the enzyme encoded by *GLK1*, recognises only glucose). *HXK2* expression is stimulated by glucose, whereas transcription of *HXK1* and *GLK1* is repressed by glucose (Herrero et al. 1995).

8.5.1 Glycerol Biosynthesis

Glycerol biosynthesis is an important side-reaction of the glycolytic pathway. The dihydroxyactone phosphate formed by aldolase can either be metabolised further through glycolysis (after isomerisation by triose phosphate isomerase) or be reduced to glycerol-3-phosphate by glycerol-3-phosphate dehydrogenase, with concomitant oxidation of NADH to NAD^+. Subsequently, glycerol is formed by glycerol-3-phosphatase. Glycerol biosynthesis is important as a means of replenishing the pool of NAD^+ that is consumed by glycolysis.

Glycerol is also important to yeasts because it plays a role in protection of the cell from osmotic stress, since it is an effective compatible solute. In yeasts such as *S. cerevisiae* the enzymes required for glycerol formation are up-regulated in response to high osmolarity conditions in the environment. This response is mediated by the high osmolarity glycerol (HOG) signal transduction pathway (Hohmann 2002; Saito and Tatebayashi 2004). Some yeast species are osmotolerant, and are able to grow on substrates with low water activities such as jam and honey. These yeasts, such as *Debaryomyces hansenii* and *Zygosaccharomyces* species, can cause food spoilage. One physiological characteristic of these species is their high capacity for glycerol production, and their increased ability to retain glycerol within the cell for osmoprotection (Larsson and Gustafsson 1987; Larsson et al. 1990; Vindelov and Arneborg 2002).

8.5.2 Fates of Pyruvate: Ethanolic Fermentation

The pyruvate formed by glycolysis has two catabolic fates: it can be fermented to ethanol or it can be oxidised to CO_2 by the citric acid cycle. In either case, the NADH produced by glycolysis is re-oxidised to NAD^+. In fermentation, pyruvate is cleaved to acetaldehyde and CO_2 by pyruvate decarboxylase. The acetaldehyde so formed is then reduced to ethanol by alcohol dehydrogenase; NADH is oxidised by this reaction. *S. cerevisiae* has 20 genes for alcohol dehydrogenases. The two cytosolic isoenzymes most important for central carbon metabolism are encoded by the *ADH1* and *ADH2* genes (Lutstorf and Megnet 1968). Adh1 is the isoenzyme involved in ethanolic fermentation, and transcription of *ADH1* is up-regulated during growth on glucose (Denis et al. 1983). This regulation is due to the activity of the Rap1 and Gcr1 transcription factors (Santangelo and Tornow 1990). *ADH2* is glucose-repressed, and Adh2 functions primarily during gluconeogenic growth on ethanol; its transcription is activated via the action of the *ADR1* transcriptional activator (Denis and Young 1983; Young et al. 2002).

Acetaldehyde is also used in the production of cytosolic acetyl-coenzyme A (CoA) via the action of the acetaldehyde dehydrogenase and acetyl-CoA synthase enzymes. Cytosolic acetyl-CoA is used for the biosynthesis of lipids and amino acids, and can be translocated into mitochondria by the carnitine–acetyl transferase shuttle (for a review see Pronk et al. 1996).

8.5.3 Fates of Pyruvate: Respiration

The oxidative catabolism of pyruvate begins with its transport into the mitochondria, followed by conversion to acetyl-CoA and CO_2 with the reduction of NAD^+. The reaction is catalysed by pyruvate dehydrogenase, an enzyme complex which is composed of five different subunits which in *S. cerevisiae* are encoded by *PDA1* (E1α), *PDB1* (E1β), *LAT1* (E2), *LPD1* (E3), and *PDX1* (protein X), (reviewed in Steensma 1997). The E3 component (lipoamide dehydrogenase) also forms part of the related multienzyme complexes α-ketoglutarate dehydrogenase, branched-chain ketoacid dehydrogenase, and glycine decarboxylase (Dickinson et al. 1986; Ross et al. 1988; Dickinson and Dawes 1992; Sinclair et al. 1993; Sinclair and Dawes 1995).

It has long been held that in *S. cerevisiae* the flux of pyruvate to the fermentative and oxidative branches is a function mostly of the kinetic properties of pyruvate dehydrogenase and pyruvate decarboxylase. Pyruvate dehydrogenase has a tenfold higher affinity for pyruvate than pyruvate decarboxylase but its activity in the cell is quite low. Hence, low concentrations of pyruvate favour its oxidation via pyruvate dehydrogenase, whereas high concentrations of pyruvate (which occur when the flux through glycolysis is high, such as during growth on high glucose concentrations) result in "overflow" fermentative metabolism via pyruvate decarboxylase and alcohol dehydrogenase with the resultant formation of ethanol. Further control of the distribution of pyruvate between fermentation and respiration is effected at the level of gene expression, since glucose induces expression of pyruvate decarboxylase, and represses the expression of acetyl-CoA synthetase and the Lpd1 subunit of pyruvate dehydrogenase.

8.5.4 Acetate Metabolism

Acetate can be utilised as a carbon source by most yeasts. In *S. cerevisiae* it is converted to acetyl-CoA using ATP by acetyl-CoA synthetase. There are two isoenzymes: that encoded by *ACS1* is repressed by glucose, while that encoded by *ACS2* is constitutively transcribed (Steensma et al. 1993; van den Berg and Steensma 1995; van den Berg et al. 1996). The regulation of *ACS1* is complex. A carbon source responsive element (CSRE) and a binding site for Adr1 combine to mediate about 80% of derepression. The balance between repression by Ume6 and induction by Abf1 is also important (Kratzer and Schüller 1997). In anaerobic conditions Acs2 is used. An *acs2* mutant is unable to grow on glucose but grows on ethanol or acetate. Acs1 is the isoenzyme required for growth in aerobic conditions as an *acs1*Δ mutant can grow on all carbon sources. *acs1 acs2* double mutants are inviable (van den Berg and Steensma 1995). The cytosolic acetyl-CoA produced in this way can be used for lipid and amino acid biosynthesis, and can also be transported into the mitochondria by the carnitine–acetyl transferase shuttle. In *K. lactis* there are also two isoenzymes of acetyl-CoA synthetase encoded by *KlACS1* and *KlACS2* (Zeeman and Steensma 2003). The former is expressed at a low level on glucose or ethanol and induced on acetate or lactate; the latter is preferentially expressed on glucose and ethanol (Lodi et al. 2001; Zeeman and Steensma 2003).

8.5.5 Tricarboxylic Acid Cycle

Acetyl-CoA derived from ethanol or acetate via acetyl-CoA synthetase, from pyruvate via pyruvate dehydrogenase, or from fatty acid oxidation reacts with oxaloacetate to form citrate, the first intermediate in the tricarboxylic acid (TCA) cycle. The reaction is catalysed by citrate synthase. *S. cerevisiae* has three isoenzymes of citrate synthase with different subcellular locations: these are encoded by *CIT1* (mitochondrial matrix), *CIT2* (peroxisome), and *CIT3* (mitochondrial matrix) (McAlister-Henn and Small 1977). *CIT2* is taken as the classic example of a gene that is subject to the retrograde response – the situation in which the expression of nuclear genes is regulated by the mitochondria. (Key players in this phenomenon are the retrograde response genes *RTG1*, *RTG2*, and *RTG3*.) In wild-type cells the expression of *CIT2* is low, but in cells which are mitochondrially compromised the transcription of *CIT2* is greatly increased (Liao and Butow 1993; Chelstowska and Butow 1995). Metabolites produced in the peroxisomes can also be used in the TCA cycle (in the mitochondria). Thus, the retrograde regulation of *CIT2* can regulate the efficiency by which the cells use two-carbon compounds in anaplerotic pathways, especially the glyoxylate cycle. The retrograde response is also valuable in offering metabolic flexibility in a variety of different developmental scenarios (e.g. sporulation, filamentation).

Aconitase catalyses the stereospecific isomerisation of citrate into isocitrate. The enzyme is encoded by *ACO1* and is located in both the cytosol and the mitochondrial matrix. There is no genuine *ACO2* gene but Yjl200c has considerable sequence similarity to Aco1. The expression of aconitase is repressed by glucose and glutamate where its presence would be unnecessary. As one would predict, *aco1* mutants

are unable to grow on non-fermentable carbon sources and are glutamate auxotrophs on glucose because α-ketoglutarate (the precursor to glutamate) is not made (Crocker and Bhattacharjee 1973).

Next is the oxidative decarboxylation of isocitrate to α-ketoglutarate. There are three isoenzymes of isocitrate dehydrogenase. NAD-specific isocitrate dehydrogenase is an allosterically regulated octamer composed of four subunits of Idh1 and four of Idh2 (Lin and McAlister-Henn 2002). It is located in the mitochondrion. Both subunits can bind the substrate: Idh1 for allosteric activation by AMP and Idh2 for catalysis (Lin et al. 2001). There is a strong correlation between the level of isocitrate dehydrogenase activity and the ability to grow on acetate or glycerol (Lin et al. 2001). *S. cerevisiae* has two NADP-dependent isocitrate dehydrogenases: Idp1 (mitochondrial) and Idp2 (cytosolic). A mutation in either *IDH1* or *IDH2* renders it unable to grow on acetate or pyruvate but it can still grow on ethanol. In contrast, *idp1* or *idp2* mutants as well as *idp1 idp2* double mutants can grow on either acetate or ethanol. NAD-specific isocitrate dehydrogenase binds specifically and with high affinity to the 5′ untranslated leader sequences of all mitochondrial messenger RNAs (mRNAs). It is thought that this suppresses inappropriate translation because when *S. cerevisiae* has been disrupted for NAD-specific isocitrate dehydrogenase it displays increased mitochondrial translation (de Jong et al. 2000). However, despite the increased rate of synthesis, subunits 1, 2, and 3 of cytochrome c oxidase, and cytochrome b (all encoded by mitochondrial genes) are all reduced in the absence of NAD-specific isocitrate dehydrogenase owing more rapid degradation (de Jong et al. 2000). Evidently, several lines of evidence point to NAD-specific isocitrate dehydrogenase having a role in regulating the rate of mitochondrial assembly besides its role in the TCA cycle.

α-Ketoglutarate dehydrogenase catalyses the oxidative decarboxylation of α-ketoglutarate via succinyl-CoA to succinate. The multienzyme complex comprises a dehydrogenase (E1) encoded by *KGD1*, succinyl transferase (E2) encoded by *KGD2*, and lipoamide dehydrogenase (E3) encoded by *LPD1*. Mutants of *S. cerevisiae* in E1, E2, or E3 components are able to grow on glucose but not on acetate or glycerol (Dickinson et al. 1986; Repetto and Tzagoloff 1989, 1990). The complex assembles spontaneously in vivo. Succinyl-CoA ligase α and β subunits are encoded by *LSC1* and *LSC2*, respectively. All of the aforementioned are subject to catabolite repression.

Succinate dehydrogenase catalyses the conversion of succinate into fumarate. In *S. cerevisiae* the flavoprotein precursor of this enzyme is encoded by *SDH1*. The mature Sdh1 binds to Sdh2 (the iron–sulphur protein) to form an active dimer. Two hydrophobic proteins (Sdh3 and Sdh4) anchor the active dimer to the mitochondrial inner membrane. The expression of the *SDH* genes is repressed by glucose and derepressed on respiratory carbon sources (Lombardo et al. 1990; Scheffler 1998). The turnover of *SDH2* mRNA is crucial to the control of succinate dehydrogenase activity by the carbon source. Mutants defective in succinate dehydrogenase are unable to grow on all respiratory carbon sources. The situation in *K. lactis* is very different. Despite the fact that the orthologous gene *KlSDH1* is 84% identical to the *S. cerevisiae SDH1*, it is highly expressed under both fermentative and nonfermentative conditions. Furthermore, strains carrying mutations in the *KlSDH1* gene are still able to grow on lactate, but strangely cannot grow on acetate, ethanol,

or glycerol (Saliola et al. 2004). It would appear that in *Kluyveromyces* lactate is metabolised to pyruvate by lactate ferricytochrome c oxidoreductase (just as in *Saccharomyces*) and that the pyruvate is channelled into the TCA and glyoxylate cycles using enzymes which are differently regulated. In the *K. lactis Klsdh1* mutant both of the genes encoding acetyl-CoA synthetase are expressed more highly than in the wild type when grown on lactate as are its genes for the glyoxylate cycle enzymes malate synthase and isocitrate lyase when grown on both lactate and glucose. Since pyruvate decarboxylase is highly expressed (in both the mutant and the wild type) on lactate, the *Klsdh1* mutant can grow on lactate by having high levels of glyoxylate cycling and accumulating some succinate (Saliola et al. 2004). There must be an additional unknown special effect by lactate on gene expression or enzyme activity in *Kluyveromyces* because it is not clear why this mutant cannot grow on pyruvate.

Fumarase, which catalyses the conversion of fumarate to malate, is encoded by a single gene in all yeasts which have been examined. In *S. cerevisiae* it exists as separate cytosolic and mitochondrial forms. Various explanations have been proposed to explain this situation, including different transcription initiation sites to produce two different mRNAs (a longer one encoding the mitochondrial isoenzyme and targeting sequence and a shorter one for the cytosolic form) (Wu and Tzagoloff 1987), or dual translational initiation and selective splicing; the last two having been established for other proteins. However, the localisation and distribution of fumarase appears to be unique because there is only one translation product which is targeted to the mitochondria by an N-terminal presequence which is then removed by the mitochondrial processing peptidase. Some of the fully mature fumarase molecules are then released back into the cytosol. In vivo translocation into the mitochondria only occurs during translation and in vitro translation of the *FUM1* mRNA requires mitochondria (Sass et al. 2001).

Malate dehydrogenase catalyses the oxidation of malate to oxaloacetate. In *S. cerevisiae* the three isoenzymes, encoded by *MDH1* (mitochondrial), *MDH2* (cytoplasmic), and *MDH3* (peroxisomal), are all subject to catabolite repression. The majority (90%) of malate dehydrogenase activity is due to Mdh1, except when acetate or ethanol is the carbon source, in which case Mdh2 constitutes 65% of total malate dehydrogenase (Steffan and McAlister-Henn 1992). Oxaloacetate levels are crucial. If oxaloacetate levels were to become insufficient then further turns of the TCA cycle would not be possible. This could arise owing to the consumption of TCA cycle intermediates e.g. α-ketoglutarate and oxaloacetate in the formation of glutamate and aspartate (respectively), both of which contribute to the synthesis of other amino acids. The metabolic requirement is ensured in two ways: the anaplerotic (filling-up) glyoxylate bypass and Mdh2. Mdh2, like phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase, is critical in gluconeogenesis and is similarly rapidly inactivated and then proteolytically degraded if glucose is supplied to cells which had been growing gluconeogenically.

8.5.6 Mitochondria

It is impossible to describe the TCA cycle without mentioning its location – the mitochondria. Study of the function and biogenesis of these organelles has been

facilitated by the availability of respiratory-deficient *petite* mutants of *S. cerevisiae* which can live anaerobically by fermentation. Subsequently, for a variety of reasons, attempts were made to isolate similar mutants in other yeast species. This soon gave rise to the notion that there were "*petite*-positive" and "*petite*-negative" yeasts: being those from which it was possible or impossible (respectively) to isolate *petite* mutants (Bulder 1964a, b). Notable *petite*-negative species included *K. lactis*, *Sch. pombe*, and *Z. bailii*. For 3 decades researchers pondered why certain yeasts were apparently unable to survive without mitochondria. It was reasoned that these organisms must have certain functions needed for an anaerobic or a fermentative existence which were either contained within the mitochondria or encoded by mitochondrial genes. It now appears that the classification was fallacious and arose simply because in *S. cerevisiae petite* mutants arise at very high frequencies and in other supposed *petite*-negative yeasts the responsible *nuclear* genes had not been identified. However, the authors are still puzzled by the fact that, as far as we know, no-one has ever reported a *petite* mutant of *Z. bailii*. Maybe in this yeast certain vital functions required for anaerobic growth really do reside in its mitochondria.

8.5.7 The Glyoxylate Cycle

The glyoxylate cycle comprises two enzymes: isocitrate lyase and malate synthase. In *S. cerevisiae* isocitrate lyase is encoded by *ICL1* (Fernández et al. 1992). Isocitrate lyase catalyses the conversion of isocitrate into glyoxylate and succinate. The enzyme is a homotetramer. Its synthesis is induced by ethanol and repressed by glucose. Transcription of the gene is controlled by the global regulator Snf1. The enzyme is rapidly inactivated and then proteolytically degraded if glucose is added to gluconeogenically grown cells (Ordiz et al. 1996). As with the other enzymes, mentioned already, whose activity is similarly controlled, phosphorylation by cyclic-AMP-dependent protein kinase is the trigger. Malate synthase catalyses the formation of malate from glyoxylate and acetyl-CoA. There are two isoenzymes. *MLS1* encodes an enzyme with both peroxisomal and cytosolic locations. Mls1 is abundant in the peroxisomes of cells grown on oleic acid but in ethanol-grown cells it is mostly cytosolic (Kunze et al. 2002). *MLS1* is repressed by glucose. *MLS2* (better known as *DAL7*) encodes an enzyme required when allantoin is the sole source of nitrogen; it is subject to repression by NH_4^+ (Hartig et al. 1992). The different regulatory controls over the expression and activity of the two forms ensure that *S. cerevisiae* can metabolise C-2 compounds, allantoin, or both. It has been observed that *S. cerevisiae* which have been phagocytosed have up-regulated both *ICL1* and *MLS1* and that *C. albicans* lacking *CaICL1* have reduced virulence, leading to the suggestion that the glyoxylate cycle is an important component of fungal virulence (Lorenz and Fink 2001).

8.6 The Pentose Phosphate Pathway

The pentose phosphate pathway (hexose monophosphate pathway) is crucial to the operation of many other metabolic pathways. It can be considered to start with the dual glycolytic/gluconeogenic intermediate glucose-6-phosphate, which is converted to 6-phosphogluconolactone by glucose-6-phosphate dehydrogenase

(Zwf1) (Nogae and Johnston 1990; Thomas et al. 1991). It was once believed that this enzyme was the limiting step of the pathway in *S. cerevisiae*, but this is now known not to be the case (Dickinson et al. 1995) Glucose-6-phosphate dehydrogenase is an important source of NADPH, which is required for a variety of reductive biosyntheses. The subsequent conversion of 6-phosphogluconate to ribulose-5-phosphate catalysed by 6-phosphogluconate dehydrogenase (major isoenzyme Gnd1, minor isoenzyme Gnd2; Lobo and Maitra 1982) also yields NADPH. Ribulose-5-phosphate can be epimerised to xylulose-5-phosphate (catalysed by ribulose-5-phosphate 3-epimerase, Rpe1) or isomerised to ribose-5-phosphate by ribose-5-phosphate ketolisomerase, Rki1 (Miosga and Zimmermann 1996). Xylulose-5-phosphate and ribose-5-phosphate can then react together in a reaction catalysed by transketolase to produce glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate. There are two isoenzymes of transketolase: Tkl1 and Tkl2. Tkl1 is the major isoenzyme (Fletcher et al. 1992; Schaaff-Gerstenschläger et al. 1993; Sundström et al. 1993). The glyceraldehyde-3-phosphate can re-enter glycolysis/gluconeogenesis or can undergo further reaction with sedoheptulose-7-phosphate to yield fructose-6-phosphate and erythrose-4-phosphate; the latter reaction being catalysed by transaldolase (Tal1). The fructose-6-phosphate formed in the transaldolase reaction can also re-enter glycolysis/gluconeogenesis or can undergo a transketolase-catalysed reaction with erythrose-4-phosphate to produce glyceraldehyde-3-phosphate and xylulose-5-phosphate.

The pentose phosphate pathway fulfils many metabolic requirements: the provision of NADPH, ribose skeletons (needed for the synthesis of histidine, tryptophan, and purine ribonucleotides and deoxyribonucleotides), and erythrose-4-phosphate for the synthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan.

8.7 Gluconeogenesis

When yeasts are growing on non-fermentable carbon sources they use gluconeogenesis to synthesise glucose-6-phosphate, which is required in various biosynthetic reactions, including the formation of ribose-5-phosphate (via the pentose phosphate pathway, see Sect. 8.6) for the synthesis of histidine and of purine ribonucleotides and deoxyribonucleotides; and erythrose-4-phosphate (also via the hexose monophosphate pathway) for the synthesis of aromatic amino acids. The carbon skeletons to be built into glucose-6-phosphate originate from the TCA and glyoxylate cycles in the form of oxaloacetate which is converted into pyruvate by phoephoenolpyruvate carboxykinase (the first enzyme unique to gluconeogenesis). The pyruvate is then converted to 2-phosphoglycerate and subsequently by "reverse glycolysis" as far as fructose-1,6-bisphosphate. Here the second enzyme unique to gluconeogenesis (fructose-1,6-bisphosphatase) converts the fructose-1,6-bisphosphate to fructose-6-phosphate. The fructose-6-phosphate is subsequently converted to glucose-6-phosphate by phosphoglucose isomerase. Two other enzymes (isocitrate lyase and malate synthase) are required to compensate for the extraction of oxaloacetate from the TCA cycle which would otherwise cease owing to depletion of oxaloacetate. These two activities which comprise the glyoxylate bypass have already been described (Sect. 8.5.7).

It would clearly be metabolically futile for cells which are growing on a non-fermentable carbon source to simultaneously use glycolysis, or to operate gluconeogenesis when glucose is plentiful. *S. cerevisiae* avoids such wasteful futile cycling by controlling the activities of the key enzymes phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase. Phosphoenolpyruvate carboxykinase is encoded by *PCK1* (Valdes-Hevia et al. 1989). This gene's expression is strongly up-regulated in cells growing on gluconeogenic carbon sources (Mercado et al. 1994). *FBP1*, which encodes fructose-1-6-bisphosphatase (Sedivy and Fraenkel 1985; Entian et al. 1988), and *PCK1* are both repressed in cells growing on glucose (Sedivy and Fraenkel 1985; Mercado et al. 1991) This glucose repression is extremely sensitive as it is triggered at glucose concentrations of only 0.005% glucose (Yin et al. 1996) and gluconeogenically grown cells rapidly inhibit and proteolytically degrade phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase if glucose becomes available (Gancedo 1971; Haarasilta and Oura 1975; Müller and Holzer 1981). The artificial expression of *FBP1* and *PCK1* when glucose is present resulted in an increase in generation time of approximately 20% (Navas et al. 1993).

8.8 Trehalose, Glycogen, and Cell Wall Glucans

Trehalose is produced from glucose-6-phosphate in two steps. First, UDP-glucose (or ADP-glucose) and glucose-6-phosphate are converted into trehalose-6-phosphate and UDP (or ADP) by trehalose-6-phosphate synthase (Vuorio et al. 1993). Then, the phosphate group of trehalose-6-phosphate is removed by trehalose-6-phosphate phosphatase (De Viriglio et al. 1993). Both enzymes are present in a complex (Thevelein and Hohmann 1995; Ferreira et al. 1996). Trehalose is important to many organisms (not just yeasts) in resistance against many adverse conditions, including heat, cold, dehydration, osmotic stress, solvents, and free radicals; hence, its synthesis and degradation are highly regulated. In *S. cerevisiae* there are two trehalases which hydrolyse trehalose into two molecules of glucose. The neutral trehalase (Nth1) is cytosolic; the acid trehalase (Ath1) is vacuolar (Kopp et al. 1993; Destruelle et al. 1995).

Glycogen synthesis also uses UDP-glucose but results in a polymer, not simply a disaccharide as with trehalose. An α-1,4 glucosyl "primer" is required initially; this is formed from UDP-glucose (by Glg1 and Glg2 in *S. cerevisiae*; Cheng et al. 1995). Glycogen synthase performs the subsequent elongation. Gsy1 and Gsy2 represent the minor and major isoforms, respectively (Farkas et al. 1991). Gsy3 forms α-1,6 branch points. The breakdown of glycogen into glucose and glucose-1-phosphate is accomplished by glycogen phosphorylase (Gph1) (Hwang et al. 1989). Phosphorylation inactivates glycogen synthase and activates glycogen phosphorylase; dephosphorylation has the opposite effect on both activities (for a complete description see Stark 2004). Dual reciprocal control is obviously efficient as phosphorylation will increase the availability of glucose units by stopping glycogen synthesis and starting glycogen breakdown; whilst dephosphorylation stops glycogen breakdown and simultaneously starts glycogen synthesis.

The cell wall of yeasts is composed of glucans, mannans, chitin, and mannoproteins (as well as other minor components). The biosynthesis of these structural

carbohydrates is complex. As with glycogen and trehalose, the hexose molecules that make them up are initially charged by condensation with UTP (or ATP). In the case of glucose monomers, glucose-6-phosphate is converted to glucose-1-phosphate by phosphoglucomutase (encoded in *S. cerevisiae* by *PGM1* and *PGM2*). Glucose-1-phosphate then combines with UTP to form UDP-glucose in a reaction catalysed by UDP-glucose pyrophosphorylase (encoded by *UGP1* in *S. cerevisiae*; Daran et al. 1995). Synthesis of glucans and other cell wall polysaccharides from UDP-glucose and other constituents is a complex process involving multiple proteins, and its description is beyond the scope of this chapter (for reviews, see Cid et al. 1995; Shahinian and Bussey 2000; Smits et al. 2001). The glucan fraction of the yeast cell wall has received considerable attention recently as a candidate nutraceutical, as it displays immunostimulatory, anti-mutagenic, and anti-oxidant properties (Krizkova et al. 2003; Lee et al. 2001).

8.9 Regulation

8.9.1 Glucose Repression

Glucose is considered the preferred carbon source of *S. cerevisiae* because if this sugar is present at levels above about 0.1% (w/v) then the utilisation of other carbon sources is prevented. *S. cerevisiae* operates its metabolism in this way partly by repressing the transcription of genes required for the utilisation of the alternative carbon sources. Target genes of this glucose repression pathway include the *SUC* genes encoding invertase, *GAL* and *MAL* genes involved in utilisation of galactose and maltose (respectively), and the *FBP1* gene encoding fructose bisphosphatase. Genes required for utilisation of non-fermentable carbon sources (encoding proteins involved in mitochondrial biogenesis, respiration, the TCA cycle, etc.) are also repressed by glucose. Other fermentable carbon sources (e.g. fructose, galactose, and maltose) can exert repressive effects on members of this set of target genes as well, though they are generally less potent than glucose. The metabolism of *K. lactis* is not subject to glucose repression to anything like the extent seen in *S. cerevisiae* (Schaffrath and Breunig 2000), and the oxidative metabolism of *Schw. occidentalis* is also famously not repressed by high sugar concentrations (Poinsot et al. 1987; Zimmer et al. 1997).

Glucose repression in *S. cerevisiae* involves the binding of transcriptional repressors to the promoters of affected genes; these repressors, the Mig1 (Nehlin and Ronne 1990) and Mig2 (Lutfiyya and Johnston 1996) zinc-finger proteins, require the Ssn6 and Tup1 co-repressors for activity (Keleher et al. 1992; Vallier and Carlson 1994). Mig1 is localised in the nucleus in glucose-grown cells, and upon removal of glucose it is rapidly phosphorylated and translocated from the nucleus to the cytoplasm (De Vit et al. 1997). Derepression requires the Snf1 protein kinase complex (Carlson et al. 1981; Carlson 1999). This heterotrimeric complex includes the Snf1 (α) catalytic subunit, which has a catalytic domain and an autoregulatory domain. The other components of the complex are a regulatory (γ) subunit Snf4 involved in responding to glucose, and an oligomerisation factor (β subunit) (Sip1, Sip2, or Gal83) that mediates interactions with the downstream targets and sets the

subcellular localisation of the complex (Schmidt and McCartney 2000). Each β subunit has a unique pattern of localisation (Vincent et al. 2001). During growth on glucose all β subunits are located in the cytoplasm. On shifting the cells to a non-fermentable carbon source Sip1 localises around the vacuole, Sip2 remains cytoplasmic, and Gal83 becomes enriched in the nucleus. It has been shown recently that the cyclic-AMP-dependent protein kinase (protein kinase A) pathway maintains the cytoplasmic localisation of Sip1 in glucose-grown cells. Furthermore, the Snf1 catalytic subunit mislocalises to the vacuolar membrane in cells which lack protein kinase A (Hedbacker et al. 2004). This explains the long-known, but hitherto unexplained genetic interactions between *SNF1* and the cyclic AMP signalling pathway. The Snf1 kinase phosphorylates Mig1 (Treitel et al. 1998; Smith et al. 1999), resulting in its dissociation from promoter DNA and thus permitting transcription of target genes.

The activity of the Snf1 kinase is regulated by glucose; in the presence of the hexose the catalytic domain is inactive owing to intramolecular interaction with the autoregulatory domain. As glucose concentrations decline, the Snf4 protein liberates the Snf1 catalytic domain, which is then able to phosphorylate Mig1. The process is reversed by the Glc7-Reg1 protein phosphatase, which restores the inactive conformation of the Snf1 catalytic and autoregulatory domains at high glucose concentrations.

The signal initiated by glucose is not yet understood. Two models are currently favoured. The first is based on the homology between Snf1 and the AMP-activated protein kinase (AMPK), which regulates energy metabolism in mammalian cells. AMPK is activated by high AMP concentrations and low ATP concentrations, and it is proposed that changes in adenylate concentrations in response to glucose availability could affect Snf1 in a similar way (Hardie and Carling 1997). The second model is based on two types of mutations that result in expression in the presence of glucose of normally glucose repressible genes. Mutations in *HXK2* encoding the predominant hexokinase in glucose-grown cells lead to derepression, as do mutations that restrict cellular glucose transport activity. Hence, the intracellular concentrations of glucose, glucose-6-phosphate, or a related non-glycolytic metabolite are implicated in signalling the extracellular glucose concentration to the repression apparatus. The role of Hxk2 is worthy of special note because it has been suggested to have intrinsic regulatory functions as well as a catalytic function in glycolysis. Mutations in the *HXK2* gene are known which separate catalytic activity from the protein's role in glucose repression (Hohmann et al. 1999; Mayordomo and Sanz 2001a). Also, Hxk2 has been shown to translocate into the nucleus and act directly on the *SUC2* promoter (Herrero et al. 1998; Randez-Gil et al. 1998a, b). These observations are complicated by results which demonstrate that hexokinases from many other organisms are able to replace Hxk2 in exerting glucose repression (Rose 1995; Petit and Gancedo 1999; Mayordomo and Sanz 2001b). This implies that the catalytic activity of the enzyme is sufficient for its regulatory role.

Another line of evidence supporting the second model of Snf1 regulation by glucose is the identification of a trio of protein kinases that act upstream of Snf1, namely Pak1, Tok1, and Elm1 (Nath et al. 2004; Sutherland et al. 2003). The signalling pathway between glucose and these protein kinases remains to be elucidated.

8.9.2 Activation

A number of mechanisms are known in *S. cerevisiae* which bring about transcriptional activation of genes in conditions where glucose is low or absent. Two are described here. The first involves a complex of the Hap2, Hap3, Hap4, and Hap5 proteins, which binds to the promoters of target genes in the absence of glucose and activates their transcription. In *S. cerevisiae* the target genes are mostly involved in respiration and the utilisation of non-fermentable carbon sources. Regulation of the Hap complex takes place at the level of transcription of its components (Pinkham and Guarente 1985; Forsburg and Guarente 1989), although other levels of control probably exist as well. The mechanism results in expression of genes (e.g. *FBP1*, *PCK1*, *ICL1*, *MDH2*, and *JEN1*, which encodes the lactate transporter) required for the utilisation of non-fermentable carbon sources. These genes are under control of Cat8, a zinc-finger DNA-binding protein that activates transcription of target genes (Hedges et al. 1995; Randez-Gil et al. 1997; Bojunga and Entian 1999; Haurie et al. 2001; Roth and Schuller 2001). The *CAT8* gene itself is repressed by the glucose repression pathway (Hedges et al. 1995). Despite possessing *HAP* genes orthologous to those in *S. cerevisiae*, the Hap complex seems to be of lesser importance in *K. lactis* because *hap* mutations have little effect on respiratory metabolism in this organism (Nguyen et al. 1995).

It has long been believed and often written that, in *S. cerevisiae*, glucose repression is always stronger than oxygen induction. In other words, the conventional wisdom has been that when glucose levels are high *all* of the genes required for oxidative metabolism are repressed. However, very recent two-dimensional transcriptome analysis in chemostat cultures has revealed a subset of 35 genes for which induction by oxygen supersedes glucose repression (Tai et al. 2005). The mechanism(s) by which this is accomplished are not understood for every gene but it is noteworthy that some of the genes are known targets of the Hap2, Hap3, Hap4, Hap5 complex and that the expression of *HAP4* (which encodes the regulatory subunit of the Hap2, Hap3, Hap4, Hap5 complex) is identical to the aforementioned 35 genes.

8.9.3 Metabolic Integration

The regulatory pathways discussed earlier reflect some of the complexity of the regulatory networks that act on carbon metabolism in yeasts. Additional layers of regulation stem from the occurrence of forked regulatory pathways and cross talk between pathways. It should always be remembered that no living cell's metabolism distinguishes between, for example, "carbon metabolism" and "nitrogen metabolism": these are merely convenient conceptual divisions for the human mind. In reality, the pathways are integrated to allow optimal growth and reproduction in the individual organism's environmental niche. Consequently, there are "cross-pathway" regulatory phenomena which operate on individual genes and proteins, on individual pathways, and on groups of pathways. For example, some genes encoding enzymes of carbon metabolism (e.g. *PYC1*, *LPD1*) are regulated by the nitrogen source. Conversely, glucose limitation induces *GCN4* (the "global" transcriptional activator controlling depression of amino acid biosynthetic pathways) (Yang et al. 2000).

The integration of carbon catabolism with amino acid biosynthesis (anabolism) is readily observed in the TCA cycle, which, besides being essential for oxidative metabolism, is also required for the provision of intermediates for amino acid biosynthesis. For example, α-ketoglutarate is essential in the biosynthesis of glutamate, glutamine, proline, lysine, and arginine. Additionally, glutamate, which is derived from α-ketoglutarate via NADP-dependent glutamate dehydrogenase, is itself used in the biosynthesis of aspartate, and hence threonine, methionine, and cysteine and also tyrosine, phenylalanine, serine, leucine, isoleucine, and histidine. Hence, as noted before, *aco1* (aconitase-less) mutants of *S. cerevisiae* are glutamate auxotrophs on glucose. Mutants of this yeast defective in the major (mitochondrial) NAD-specific isocitrate dehydrogenase also show glutamate auxotrophy which can be partially compensated by overexpression of the (mitochondrial) NADP-dependent enzyme Idp1. Recent studies of the kinetic properties of the isoenzymes of isocitrate dehydrogenase have led to the conclusion that Idp1 has an ancillary role in glutamate biosynthesis and that the role of Idp2 is equilibration of isocitrate and α-ketoglutarate levels (Contreras-Shannon et al. 2005). Thus, perhaps independent of genetic controls, a high degree of evolutionary fine-tuning has occurred to the kinetic parameters of the different isoenzymes of isocitrate dehydrogenase. Many other examples are evident at this important node of metabolism (Dickinson 2004).

8.9.4 Metabolic Control

Cellular metabolism is subject to *regulation* at the level of gene expression, enzyme half-life, etc. and by the suite of enzymatic activities encoded by the yeast genome. Metabolism is also subject to *control*; in other words, under given conditions of gene expression and substrate availability, the flux through a metabolic activity is set by the activities of the pathway enzymes. Metabolic Control Analysis has pointed out that the control of metabolic flux is in principle distributed amongst all of the steps in a pathway; some steps may have high control, while others have negligible control (Kacser and Burns 1981; Fell 1997). This is in disagreement with the textbook view that single enzymes can be the "rate-limiting step" of a pathway. The most well-known example is that of phosphofructokinase (or in some textbooks hexokinase or pyruvate kinase), which owing to complex allosteric regulation is considered to be the rate-limiting step of glycolysis. Both experimental (Heinisch 1986) and theoretical (Cornish-Bowden 2004) studies have discredited this point of view. Indeed, the preponderance of control over yeast glycolysis lies in the first step, viz. transport of glucose over the plasma membrane, and not in any of the enzymatic steps of the pathway (Ye et al. 1999; Diderich et al. 1999b).

8.10 Metabolic Modelling and Functional Genomics

Carbohydrate metabolism in yeasts has been subject to numerous modelling efforts over the last few decades. The goals and theoretical bases of these efforts have varied widely, and a comprehensive review is not feasible here. Metabolic models can aid in the description and interpretation of experimental data, and they can have a heuristic value in the design of experiments. The input data for metabolic models

can include metabolic fluxes and intracellular metabolite concentrations (e.g. flux balance analysis and metabolic control analysis) or information about enzyme kinetics (substrate and inhibitor affinity, catalytic centre activity, allosteric effector effects, etc., e.g. biochemical systems theory and kinetic parameter modelling). The advent of functional genomics, such as the ability to screen thousands of single-gene knockouts in parallel for their metabolite profile (the "metabolome"), makes metabolic modelling, combined with other types of bioinformatics, crucial for data reduction and identification of interesting trends and phenotypes (Oliver et al. 2002). Simultaneous determination of dozens or hundreds of metabolites in a cell extract is generally performed with NMR or various mass spectroscopic techniques. The large and convoluted data sets generated by these techniques require computational analysis to discriminate the identities and concentrations of individual metabolites. Alternatively, the overall pattern and abundance of metabolites (metabolite fingerprint) can be compared between two or more strains or cultivation conditions in order to identify trends. An example of the latter approach was the comparison of a set of *S. cerevisiae* strains grown under identical conditions: a wild-type strain, and mutants either in fructose-6-phosphate 2-kinase genes or in respiratory functions. The metabolite composition of the cellular extracts of the strains was determined from ^{1}H-NMR spectra. Statistical analysis of the spectra to generate the metabolite fingerprints was performed using principle components analysis (to reduce the large number of variables in the raw spectra to a smaller number of correlated variables) followed by discriminant function analysis (to determine which of the correlated variables discriminate among the strains); however, the identity of individual metabolites was not determined. From the statistical analysis, the strains were easily resolved into the three classes, namely wild type, respiratory mutants, and fructose-6-phosphate 2-kinase mutants (Raamsdonk et al. 2001). The authors propose that this approach could be generally useful in determining the cellular roles of those genes of unknown function identified by genome sequencing projects. It could also be applied to assess the effects of cultivation conditions, stress, or other environmental parameters on yeast physiology.

Applications of metabolic modelling in biotechnology include providing a rational basis for metabolic engineering. For example, the conclusion from metabolic control analysis that in *S. cerevisiae* the glucose transport step exerts high control on glycolytic flux (Sect. 8.9.4) made the transport step a rational target for reducing the flux to pyruvate. It was hypothesised that decreasing the size of the cytosolic pyruvate pool would prevent overflow into the fermentative pathway (Sect. 8.5.4), and that consequently a strain with a sufficiently diminished transport capacity would respire glucose under aerobic conditions. A series of strains of *S. cerevisiae* were constructed with diminished glucose transport capacities, and were screened for respiratory glucose metabolism. One was found that actually displayed Crabtree-negative glucose metabolism, i.e. it respired glucose even at high glucose concentrations. As a result, the strain had a high biomass yield and a negligible ethanol yield during aerobic batch cultivation (Otterstedt et al. 2004).

Metabolic engineering can be defined in general as the use of genetic engineering to modify cellular metabolism. The goals of metabolic engineering can include changing the mode of cellular metabolism (see earlier) or changing the substrate

range or product output of the cell. For example, as noted before, *S. cerevisiae* is not able to metabolise xylose, lactose, or cellulose (Sect. 8.2); however, strains of this species have been engineered to gain these functions. Lactose metabolism was conferred by transformation of the *LAC4* (encoding β-galactosidase) and *LAC12* (encoding lactose-H^+ permease) genes from *K. lactis* into *S. cerevisiae* (Sreekrishna and Dickson 1985; Rubio-Texeira et al. 1998). Cellulose utilisation by *S. cerevisiae* has been engineered by transformation with three fungal genes that encode secreted enzymes capable of degrading cellulose polymers to glucose (endoglucanase and cellobiohydrolase from *Trichoderma reesei* and β-glucosidase from *Aspergillus aculeatus*). The resulting strain ferments cellulose to ethanol with a high yield (Fujita et al. 2004).

Metabolic engineering for xylose utilisation (for production of biofuel ethanol from plant waste) has received considerable attention, with only moderate success. Genes encoding xylose reductase (NADP-dependent) and xylitol dehydrogenase (NAD-dependent) from *P. stipitis* have been transformed into *S. cerevisiae*, and numerous other alterations have been engineered or selected in the resulting strains as well. However, the rates and yields of ethanolic fermentation from xylose are low compared with those from glucose, and a significant portion of the pentose is converted to xylitol. The low efficiency has been attributed to a redox imbalance created by the different cofactor specificities of the heterologous enzymes, to low xylose transport capacity, or to insufficient flux through the pentose phosphate pathway. The challenges of this important undertaking, and the prospects for the future, have been reviewed (Hahn-Hägerdal et al. 2001; Jeffries and Jin 2004).

8.11 Concluding Remarks

Yeasts have been domesticated for food biotechnology for millennia, and have contributed to food spoilage for even longer. The elucidation of the roles of yeasts in the food sector, starting with the discoveries of Pasteur, has highlighted the importance of carbohydrate metabolism – in fermentation, in biomass formation, and in tolerance of extreme environments. In the twenty-first century the economic importance of these organisms in food is likely to grow, and this will be stimulated by continued research into basic and practical aspects of the carbohydrate metabolism of the yeasts.

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Chapter 9

Yeasts as Biocatalysts

PIERRE STREHAIANO, FELIPE RAMON-PORTUGAL AND PATRICIA TAILLANDIER

Laboratoire de Génie Chimique, INP/CNRS, 5 rue Paulin Talabot, BP 1301, 31106 Toulouse Cedex, France
(e-mail: pierre.strehaiano@ensiacet.fr)

9.1 Introduction

When looking at a biological reaction (a reaction due to a microorganism) it appears clearly that it can take place in two different ways:

– A system where the microorganisms are suspended in the medium (here considered as homogeneous): that is the case of the fermentations in winemaking or in brewing, for example.
– A system where the microorganisms are not free in the medium but where they are attached to a support, giving a two-phase system. Such a system is illustrated in nature by biofilms, for example.

In the controlled use of microorganisms in an industrial frame, like alcoholic fermentation, free cells of microorganisms were used first, surely because of tradition, which existed prior to knowledge of the biological nature of the reaction. In oenology, and also in the major part of industrial fermentation processes, this concept of free cells is largely pre-eminent. In some others fields of applications, in contrast, as early as 1960s the purpose was to attach the microorganisms and some industrial applications quickly followed the conceptual developments: electrodes with attached enzymes, bacterial beds in water treatment, adsorbed bacteria for vinegar production, etc.

Thus it appears quite logical to ask:

– Why should the microorganisms be attached?
– How should it be done?
– What interest and what applications are there for the immobilized yeasts in the food industry?

9.1.1 Why Attach the Microorganisms?

In the fermentations in the food industry the main expected interests in the immobilization of microorganisms are first the increase of the reaction rates due to the high cell concentration, second an easier operation in continuous mode and third

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
© Springer-Verlag Berlin Heidelberg 2006

the easy separation of the microbial cells at the end of the fermentation step. Moreover it allows the reuse of the catalyst for some cycles of production. As it becomes possible to increase the reaction rates it is also possible to have better control of the reaction. Indeed the reaction rate is the product of the specific rate of a cell and the number of cells. To increase the reaction rate it is possible to increase the specific rate, the number of cells or both together. Increasing the specific rate requires giving the microorganism the best environmental conditions, but these conditions are rarely those encountered in the practice of industrial fermentations (low pH, high sugar concentration, alcohol) where the inhibition or limitation mechanisms generally dominate. In contrast, the sole apparent limitation to the increase of the number of microbial cells is the saturation of the culture medium. However, it is worth noting that the microorganisms may slightly modify their metabolism when submitted to excessive conditions of cellular concentrations.

Also some works deal with the immobilization process in order to allow good control of different microorganisms working together (co-immobilization). This co-immobilization may also concern microorganisms of the same kind (for example, different yeast genus or species), very different microorganisms (yeasts and bacteria, for example) or a microorganism and an enzyme. This co-immobilization may be realized using different processes: adsorption, entrapment or membrane retention.

9.1.2 How to Immobilize the Microbial Cells?

The immobilization of microbial cells may be carried out by different methods which (for most of them) are based on the methods initially proposed for the immobilization of enzymes by Chibata (1979). These methods may be classified in four categories: adsorption, covalent union, inclusion and retention of microbial cells without a support.

Each of these methods will be briefly presented and their applications to the use of yeasts in the food industry will be developed.

9.1.2.1 Adsorption

The method based on the adsorption phenomena of microbial cells on a support is certainly the oldest method used in the food industry (Linko and Linko 1984). The adsorption is the result of electrostatic attractions between the support and the microbial wall, which is charged negatively (Kolot 1980). The supports used can be of very different natures: wood, bricks, PVC, silica, bentonite, fragments of vegetables. The affinity of a microbial cell to a support varies depending on each microorganism/support pair and is very difficult to forecast. Generally yeast cells have better adsorption characteristics than bacterial cells (Navarro 1980).

Adsorption is a reversible phenomenon that depends on the age of the cells, cellular wall composition, pH and ionic composition of the medium. A desorption phenomenon can take place, leading to a hybrid system where the free cells are associated to immobilized cells. However, this desorption can be compensated with the growth of the microorganism cells on the support, which induces a permanent regeneration of the “biocatalytic system”.

In the recent works dealing with the immobilization of yeast cells the supports most used for the different applications in winemaking, brewing, ethanol production, etc. are cellulose and cellulose derivates (Koutinas et al. 1995; Bardi et al. 1996; Viljava and Lommi 2000), (diethylamino)ethyl (DEAE) cellulose (Lommi and Ahvenainen 1990; Linko and Kronlof 1991; Kronlof and Linko 1996; Andersen et al. 2000), fragments of vegetables (apple cuts, Kourkoutas et al. 2002b; dried raisin berries, Tsakiris et al. 2004; cane stalks, Chen 2001; pieces of figs, Bekatorou et al. 2002), gluten pellets (Smogrovicova et al. 1999; Bardi et al. 1997a), wood chips (Linko et al. 1998; Pajunen et al. 2000; Viljava and Lommi 2000), quince (Kourkoutas et al. 2003), minerals such as aluminium (Loukatos et al. 2000), ultraporous fired bricks (Opara and Mann 1988), kissiris (a glassy volcanic rock; Bakoyianis et al. 1993), ceramics (Zhang et al. 1992; Horitsu 1993; Cheng et al. 2000), porous glass (Kronlof and Linko 1992; Breitenbuecher and Mistler 1994; Yamauchi et al. 1994; Kronlof and Linko 1996) and silicon carbide (Masschelein and Andries 1996; Tata et al. 1999) and a flocculent strain has been absorbed into a sponge (Scott and O'Reilly 1995).

The adsorption is obtained by keeping the microbial suspension in contact with the support. After incubation the free cells are eliminated by several washings of the support as explained by Kourkoutas et al. (2002b) for yeast cells onto apple cuts.

As far as the industrial applications are concerned, the main fields are winemaking, brewing and alcohol production. Nevertheless, even though a lot of work has been done, few of the industrial applications are being used at this time.

9.1.2.2 Immobilization by a Covalent Link

In order to avoid the desorption phenomenon it is possible to establish true covalent binding between the microbial cell and the support. This is done using a union agent and the support is then called an "activated" support. Glutaraldehyde is the most commonly used agent, especially when the supports are made of proteins (Phillips and Poon 1988). In this way, the attachment becomes irreversible and the biocatalyst offers great stability. But the union agents are generally highly toxic against the microbial cells and induce a decrease of their activity. That is why this method of immobilization is no longer used for the immobilization of microbial cells, but it remains interesting for the immobilization of enzymes.

9.1.2.3 Inclusion

In this system the microbial cells are incorporated in the matrix of a more or less rigid polymer. These polymers are synthetic, such as polyacrylamide or cellophane, but they also can be made of proteins (gelatine, collagene) or polysaccharides (cellulose, alginate, agar, carragheenans, etc.). Inclusion is a technique that is easy to manage and it leads to products having good stability but weak mechanical resistance. In some cases this weakness may be a problem (mechanical stirring of the reactors, growth of microbial cells into the matrix leading to the breaking of the matrix) and could be a disadvantage for long-term continuous operation as discussed for brewing by Virkajarvi (2001). Also the polymer may be a limiting factor for the free

diffusion of the solutes or gases required (or produced) by the microorganism (Hannoun and Stephanopoulos 1986). It is considered that diffusion is not the limiting step for compounds having a molecular weight of less than 5,000. It is obvious that for use in the food industry the support has to be safe for the consumer (stable and non-toxic support) and is allowed by the regulation in force for the specific use considered. At this time immobilization by inclusion is the most widespread process and thus it is interesting to present the main supports used.

The main materials for inclusion of microbial cells are:

– κ-Carragheenan: This polysaccharide extracted from marine microalgae is commonly used as a food additive. It jellifies when the temperature reduces (room temperature) after the dissolution obtained by heat treatment (60–80°C). Different works dealt with the characterization of the optimal conditions to immobilize the cells into the gel and it was observed that the mechanical strength increased with increasing carragheenan concentration, corresponding to a decreased cell release. To avoid the cell release, which may be a major disadvantage in some cases, Nunez et al. (1990) proposed to treat the beads with $Al(NO_3)_3$; this treatment was shown to be efficient to induce gel hardening but it was observed that cell viability and diffusion were reduced. Recent examples of the use of carragheenan beads with 4.5×10^9 cells mL^{-1} in brewing were given by Mensour et al. (1996) and Pilkington et al. (1999).
– Agar and agarose: Agar is a polymer issued from some marine macroalgae and agarose is obtained from agar by separation and purification. The gelation is induced in the same way as for carragheenan. Even though the procedure to immobilize cells in this polymer is simple, it is not widely used owing to the low mechanical strength that makes this gel unstable compared with alginate or carragheenan.
– Chitosan gel: This polysaccharide is obtained from chitin extracted from crustacean cells. Its gelation occurs by an ionotropic reaction like alginate. It was used quite early but it appeared that acid-soluble chitosan affected cell viability and so that it is not used for cell entrapment, except in some cases to coat alginate beads to avoid cell release.
– Poly(vinyl alcohol) (PVA): PVA can form a gel when treated with UV radiation but this causes cell death. Also it is possible to make a gel by treatment with boric acid, but this acid may be toxic for some microorganisms. It is also possible to induce gelation by some freeze–thaw cycles, and recently Martynenko et al. (2004) proposed a process for champagnizing involving the use of champagne yeasts immobilized into PVA cryogels (PVACs). At this time this polymer does not appear to be used much for cell entrapment.
– Calcium alginate gel: Alginic acid is a complex heteropolysaccharide extracted from some species of algae. Its gelation is obtained by contact with a calcium solution. Its composition varies a lot depending on the source, and the nature of the alginate must be well defined when making the beads. Indeed the rheological properties of the gel depend on the composition of the alginate (sequences and ratios of L-glucuronic acid and D-mannuronic acid; arrangement of the monomers).

At this time, to our knowledge, this polymer is the one most commonly used for cell entrapment in the food industry: for example, calcium alginate beads

have been retained with a capacity of 1.2×10^9 cells mL^{-1} of gel beads (Linko and Linko 1981; Hsu and Bernstein 1985; Patkova et al. 2002). Indeed, this polymer is already allowed by the regulations for some applications in the food industry, such as a binding or thickening material, and also has good mechanical properties. The method is as follows. A solution containing yeast cells and alginate is driven to a solution of calcium chloride. A mechanical device allows the continuous film to be separated into droplets. As soon as the alginate meets the calcium chloride solution it gels quickly giving small spheres (1.5–2-mm diameter) called beads. In these beads the yeast cells are held prisoner in the frame of the alginate gel. The first works dealing with the use of these kinds of beads rapidly showed that the cells entrapped at the surface of the beads were able to multiply and thus to release free cells into the medium. In order to avoid this phenomenon, it was proposed (1980s) to make an external layer of sterile alginate using two concentric pipes. The internal pipe brings the alginate–yeast cell solution, while the external pipe brings the solution of sterile alginate. The critical points are the regularity of the spheres, the continuity of the external layer and the firmness of the gel (depending on the nature and the concentration of the alginate and the residence time in the calcium chloride solution). It is undoubtedly the method of inclusion which presents the most advanced industrial applications, particularly for some steps in winemaking. Recently a method to obtain dry double-layered beads was developed by Proenol (Portugal) and so it is possible to find on the market an industrial product which is easy to carry, with a long storage life (2 years) and easy to use (no problem of adhesion of beads to the walls of pipes or bottles as was the case with the wet beads).

9.1.2.4 Cell Retention Without External Support

It is also possible to increase the microbial cell concentration by using a natural process such as flocculation or by confining the cells to a part of the reactor by way of a membrane. Flocculation is a natural phenomenon resulting in cell aggregation. It involves the setting up of ionic bonds between sites of cell wall and cations of the medium. In some cases, these ionic bonds are strengthened by the production of filaments at the surface of the yeast cells (Teixeira 1988). But not all the cells are able to flocculate and also natural aggregates are often unstable and sensitive to the shear. Nevertheless, this spontaneous mechanism of flocculation is used in the waste-treatment process (activated sludge).

In the field of fermentations for the food industry the main applications at this time concern alcohol production (Zani-floc process in Brazil), some kinds of beers, and sparkling wine making (second fermentation). In the case of confinement, the free cells of the microorganism are kept in a part of the reactor thanks to a membrane or are retained inside a hollow fibre. This device makes it possible to reach a very high cell concentration (more than 100 g dry weight L^{-1}). For sure the membrane is needed to be freely permeable to solutes and gases. The great interest in this process is that it leads to a sterile medium at the end of the fermentation step. Different bench tests were made for alcoholic beverage production using a device

coupling fermentation and ultrafiltration or using a specific hollow fibre device for sparkling wine making (Jallerat et al. 1993) but to our knowledge none of them are being used at the industrial level.

9.1.3 Impact of Immobilization on Cell Physiology and Fermentation Activity

The possible effects of immobilization on cell activity were studied early on and the different authors reported very different conclusions. In fact, physiological reactions of cells vary depending on the method of immobilization used. For example, it is clear that the entrapment or adsorption of cells results in changes in their micro-environment and thus affects their metabolism. Anyway, in all the cases of cell immobilization, the main factor which likely influences the cell behaviour is the mass transfer limitation (Onaka et al. 1985) as it results in gradients of oxygen, substrates and products. Thus, immobilization may influence the cell physiology and activity via a lot of mechanisms which are still poorly characterized and which may act in opposite ways. For sure the effects depend on the immobilized complex size and the type and the concentration of the polymer or the matrix used for the entrapment or the adsorption.

9.1.3.1 Mass Transfer Limitations and Cell Physiology

Different studies led to different conclusions so it is quite difficult to have a clear view. As far as entrapped yeast cells are concerned, it appeared from different studies quoted by Groboillot et al. (1994) that the size of the beads may act as well as the alginate molecular weight, the ratio between guluronic and mannuronic acids (G/M) and the alginate concentration, and, for example, it was observed that the ethanol productivity of immobilized yeast cells increased when the alginate concentration or G/M decreased. This was explained by the fact that a weak gel probably facilitates the transport of substrate and product, thus enhancing the cell activity. It was also observed that mass transfer limitations may be due to cell concentration (or growth). Nevertheless, it seems well accepted by many authors quoted by Martynenko and Gracheva (2003) that respiratory and fermenting activities are higher in immobilized cells than in free cells.

But the use of an immobilized cell system may also be an easy way to reduce glucose inhibition. In most fermentation processes the diffusional limitations are generally recognized as a major disadvantage since they reduce the glucose uptake, but they may also be beneficial for the uptake of other sugars which are repressed by the glucose level. This was proved by Willaert (1999): studying the brewing by immobilized cells in calcium alginate, he established that the glucose concentration was high in the outer layer of the gel matrix but went to zero in the core of the gel. The cells which were located on the surface of the gel consumed most of the available glucose and the maltose (or maltotriose) uptake was repressed because of the glucose content. In contrast, cells located in the core were not repressed by glucose and thus maltose uptake was not repressed. The author suggested that the same mechanism may act for amino acid uptake.

9.1.3.2 Effect of Immobilization on Cell Morphology

Many changes in the morphology of yeast cells entrapped in calcium alginate were described by different authors. The well-documented review of Martynenko and Gracheva (2003) quotes studies dealing with the physiological activity and morphological changes of immobilized cells in the special case of the Champagne process. It was said that the adaptation of yeast cells to immobilization was accompanied by vacuolization. Also the thickness of the cell wall increased and ribosomes became scarcely visible. It was also shown that the specific rate of cell division was very low: the yeasts proliferated at the periphery of the bead, while the number of cells in the core remained constant.

9.1.3.3 Effect of Immobilization on Cell Physiology

Fumi et al. (1994) established that immobilized cells of *Saccharomyces cerevisiae* in alginate beads showed some alterations during alcoholic fermentation: they observed that the percentage of phosphomonoesters with respect to total phosphorous increased from 1.8% for free cells to 30.8% for immobilized ones and that the polyphosphates contents were, respectively, 56.7 and 22.6%. Grego et al. (1994) noticed that the immobilized cells of *S. cerevisiae* exhibited a slightly increased ethanol stress resistance and explained it by the impact of the ethanol stress on the fatty acid composition being smaller in the immobilized cells than in the free cells. For the enzyme activity Sarishvili and Kardash (1980) noticed that yeast cells immobilized on solid supports exhibited a greater activity of some enzymes (NAD or NADP-dependent glutamate dehydrogenase, alcohol dehydrogenase and malate dehydrogenase) than suspended cells and thus they suggested that a process using immobilized cells could be carried out at lower temperatures and shorter times.

9.1.3.4 Immobilization and Effects on the Product

Concerning the composition of the product after the use of immobilized cells it is generally assumed that there are not great differences with the product obtained with suspended cells (Busova et al. 1994). Jallerat et al. (1993) compared the second in-bottle fermentation using free cells or cells retained in a hollow fibre cartridge: they did not note any difference in the time needed for the fermentation and in the organoleptic qualities of the wines. Recently Tsakiris et al. (2004) assumed that the wines obtained using yeast cells immobilized onto raisin berries had the same aromatic profiles as the wines obtained using free cells even though, according to Balli et al. (2003), the glycerol content was slightly higher in wines obtained with immobilized cells on delignified cellulosic material and gluten pellets than in wines obtained with free cells. Studying the fermentation of white wines using different sorts of immobilized yeast cells, Yajima and Yokotsuka (2001) established that the concentrations of undesirable products (methyl alcohol, ethyl acetate, etc.) were lower in wines made using immobilized cells (in alginate beads) than in wines produced with free cells. But Bardi et al. (1997b) observed that the immobilization of yeast cells on delignified cellulosic material or gluten pellets led to a higher production

of ethyl acetate (compared with free cells). The same behaviour (greater ethyl acetate production) was observed with yeast cells immobilized on a kissiris support (Bakoyianis et al. 1993). So it seems obvious that it is difficult to draw a strong conclusion and that the effects of the immobilization on sensory evaluation of the product depend on the field of application and on the sort immobilization process. As an example of this we can quote the work of Ageeva et al. (1985): they noticed that yeast cells immobilized on different clay materials did not behave the same in regard to the volatile product synthesis.

9.2 Immobilized Yeast Cells and Winemaking

In winemaking different possibilities for the use of immobilized yeast cells have been described. Surely this area of application is the one where most work has been done. To analyse these data we can classify them according to the step in winemaking where the immobilized cells act: demalication of must (or wine), alcoholic fermentation and treatment of sluggish or stuck fermentations, in-bottle fermentation in sparkling wine making.

9.2.1 Demalication of Musts or Wines

L-Malic acid is one of the two main acids in musts and its concentration depends on grape variety and climatic conditions. Winemakers often rely on malolactic fermentation (MLF) to deacidify the must and thus to achieve the biological stability of the wine as well as to ensure good organoleptic qualities. MLF is performed by lactic acid bacteria (*Oenococcus oeni*) and many factors such as low pH and sulphur dioxide level could affect these bacteria and, in some cases, this MLF becomes impossible. The yeast *Schizosaccharomyces pombe* has been proposed as an alternative to MLF but it was quickly proved that a too important development of these yeasts leads to some off-flavours. So, some wineries have set up a two-step process: first the must is inoculated with *Schiz. pombe* for the consumption of L-malic acid and in a second step the must is inoculated with a selected strain of *Saccharomyces* in order to achieve the alcoholic fermentation. However, it appeared that the complete elimination of free cells of *Schiz. pombe* was not possible and that the risk of obtaining some off-flavours was always present. So, a process based on the use of immobilized cells of *Schiz. pombe* has been proposed. The first works of Magyar et al. (1987) established clearly the feasibility of the process using cells of *Schiz. pombe* entrapped in alginate beads. Later, Taillandier et al. (1991) and Ciani (1995) analysed a continuous process using immobilized cells of *Schiz. pombe*. But all these experiments were made at a laboratory or pilot scale owing to the impossibility to obtain and to store great quantities of entrapped cells of *Schiz. pombe*. More recently Silva et al. (2002b, 2003) described experiments using cells entrapped in dried double-layered alginate beads (see Sect. 9.1) on a laboratory scale as well on a winery scale. The beads were placed into nylon bags and these bags were poured in the fermentation tank and shaken daily to agitate the cells and improve the diffusion of solutes and the release of carbon dioxide. To stop the deacidification reaction at the desired level it was enough to remove the bags from the tank and to add to the tank the suitable

strain of *S. cerevisiae* to achieve the alcoholic fermentation. It was shown that the process was efficient and led to wines of good quality. Also the reuse of these beads was studied and it was shown that they maintained good activity for at least five cycles. The process developed by the Portuguese company Proenol to produce these beads of entrapped cells of yeasts makes it possible to store them for more than 2 years without any loss of activity. Thus, it can be concluded that this process is now well established and ready to be used in wineries. Some experiments were done using these entrapped cells of *Schiz. pombe* on wines (red or white) after alcoholic fermentation (unpublished data) and the first results were promising.

9.2.2 Alcoholic Fermentation

Many papers deal with the use of immobilized cells of yeasts (generally *S. cerevisiae*) to achieve the alcoholic fermentation of musts (red or white). The main purpose is always to ensure better control of this important step in winemaking: low-temperature fermentations, improvement of organoleptic characteristics, increase of reaction rates, good achievement of sugar consumption, etc.

Gorff (1988) patented a process using yeast cells immobilized on derivatized cellulose and later Divies et al. (1990) patented a process to entrap the yeast cells in calcium alginate beads. The same year Sarishvili et al. (1990) described a "technology for manufacture of dry red wines with immobilized yeast": the cells were immobilized on beech, oak or polyethylene and the authors observed that the quality of wines was improved. Malik et al. (1991) then tried ten different strains immobilized in alginate and noticed a reduction in their acidification potential compared with that of unbound cells. But most of the studies on this subject are due to the Department of the Chemical University of Patras (Greece). As early as 1992 Bakoyianis et al. (1992) published a paper dealing with the use of a psychrophilic and alcohol-resistant yeast strain immobilized on kissiris in a continuous process for making wine at low temperature. Later they showed (Argiriou et al. 1996) that this yeast strain was more efficient if some preservation treatments at 0°C were made. Bardi and Koutinas (1994) described experiments where different supports were tested as well as different conditions of fermentation: immobilization of cells on delignified cellulose and use of them in 55 repeated batch cultures at low (10°C) or room (30°C) temperature: the main result was that the fermentation rates are increased (threefold) compared with those for free cells. Also the stability of the biocatalyst was proven. Bakoyianis et al. (1998) using cells of *S. cerevisiae* immobilized on different supports (alumina, kissiris and alginate) compared the volatile by-products obtained at different temperatures in a continuous process. It was observed that the levels of 1-propanol, isobutyl alcohol and amyl alcohols were less than those synthesized by free cells for all supports and temperatures studied. But the most original studies from this group dealt with the immobilization of yeast cells onto supports such as apple pieces or raisin skins. Kourkoutas et al. (2001, 2002b) proposed using a psychrophilic and alcohol-resistant yeast strain immobilized on apple cuts for speeding up the fermentation. They noticed excellent taste and aroma of the wines produced and concluded that this process could be accepted by the industry for scaling up the winemaking process. Tsakiris et al. (2004) immobilized yeast cells

on dried raisin berries and obtained good stability of the device. The wines were not different from those obtained using free cells.

All these data show clearly that the alcoholic fermentation in winemaking may be realized without any damage by using immobilized cells of the suitable yeast strain, and a batch process as in the continuous process. But the main problem in developing these processes to an industrial scale is linked to the legislation for winemaking. At this time the sole support clearly allowed (for some applications) is calcium alginate gel. Also the continuous process is not allowed for all types of wine, but that is another problem.

9.2.3 Treatment of Stuck and Sluggish Fermentations

Sluggish and stuck fermentations are some of the most challenging problems that can occur during the winemaking process. The causes can be attributed to nutritional deficiencies of the must, the presence of high levels of inhibitory products, inadequate temperature, and residual toxic products. Although the causes are numerous, the main result is the decrease of cell growth, fermentative activity and viability of the yeast population. To try to reinitialize the fermentation an inoculation of activated yeast cells into the fermentation tank is usually carried out, but this procedure is not always efficient. Silva et al. (2002a) suggested the use of yeast cells immobilized into double-layered alginate beads. The results obtained on a laboratory scale as well as at the winery level showed very good efficiency of the device to treat the stuck fermentations. The great success of these immobilized cells can be explained by an adaptation of the cells to high concentrations of alcohol during the immobilization step.

9.2.4 Special Applications

9.2.4.1 Sweet Wine Making

Sweet wines are wines where the fermentation is stopped before the complete utilization of sugars. At this time to stop the fermentation activity of yeast cells in this kind of winemaking strong quantities of sulphur dioxide are employed, but this product has some disadvantages for consumer safety and the aim of the legislation is to reduce its content in wine. The use of immobilized cells was investigated by some researchers in order to find another solution for making sweet wines as it made it possible to stop the reaction by removing the particles (containing the cells) from the medium. Okuda et al. (2001) described an original process: the fermentation was carried out by immobilized yeast cells which were removed from the medium at the desired level of alcohol (or remaining sugar). In order to ensure the microbial stability of the wine they added an antimicrobial substance isolated from paprika seeds. In this way, they obtained a very stable sweet wine with no viable cells. Kourkoutas et al. (2004) suggested producing semi-sweet wines by using cells of *Kluyveromyces marxianus* immobilized on delignified cellulosic material, quince or apple pieces. The fermentation was run at high temperature and 3–4% of alcohol was synthesized. The final alcohol level was obtained by the addition of potable

alcohol to the fermented must. In these conditions it was said that the semi-sweet wine obtained showed good flavour and aroma and may be blended with other products to improve their quality. This idea to use immobilized yeast cells other than *Saccharomyces* was already developed by Crapisi et al. (1996). It was expected that the bad alcohol production activity of this kind of yeast and also its ability to produce aroma could lead to a partially fermented and aromatic beverage. It was established that it was effectively possible to carry out the fermentation by immobilized apiculate yeast species and that the wine bouquet was not affected by the use of these yeast species. Silva et al. (2002c) described a process to obtain sweet wines by using cells of *S. cerevisiae* entrapped in double-layered alginate beads and showed that it was a great way to make such wines. The process was tested in wineries. For sure the must had to be prepared in order to have a very small population of indigenous yeast cells. The beads containing the cells of the selected yeast strain were placed in nylon bags so that they were easy to remove from the tank after they had worked. When the desired level of remaining sugar was reached, the bags were taken out of the tank and the wine was stabilized to prevent the further development of free cells. As a conclusion, it appears clearly that the use of immobilized cells associated with a treatment of stabilization can be an efficient and easy way for making sweet wines with reduced sulphur dioxide contents.

9.2.4.2 Sparkling Wine Making

In sparkling wines according to the traditional method, which is the method used for the Champagne process, the problem is to eliminate yeast cells without taking the wine out of the bottle at the end of the in-bottle fermentation. Traditionally it is made by the so-called operation of *remuage*, which requires special know-how.

Thus, for more than 20 years, other solutions have been sought and tested and there is certainly the field of winemaking, where the possible use of immobilized cells has been investigated the most. For sure the simplest method would be to filter the wine from one bottle to another, but if this is done, the wine is not allowed to be called Champagne.

For the last 20 years, entrapped cells have been tested. All the first experiments were done using homogeneous alginate and cell beads. Obviously, the results were quite bad, for it was always observed that yeast cells were able to escape the bead and to grow in the medium, which resulted in a troubled wine. But as early as the 1990s double-layered alginate beads were tested (Zamorani et al. 1989; Crapisi et al. 1990; Godia et al. 1991). All the studies made clear that the use of entrapped cells in double-layered alginate beads led to a perfectly clear wine and that there was no difference with a wine obtained following the traditional method. Nevertheless it must be noted that these applications remained at a laboratory or a pilot scale, except for a quite large-scale application by Moët et Chandon (France). This was due to the difficulty to produce regular and easy-to-use alginate beads at an industrial level. As an example, using a laboratory apparatus we (in our group in 1992) were only able to make 500 g of wet beads per day. Moreover wet beads were difficult to place in the bottle as they stuck to the walls of pipes. Also a special machine was needed to put a constant and fixed quantity of the beads in the bottles. From this point of

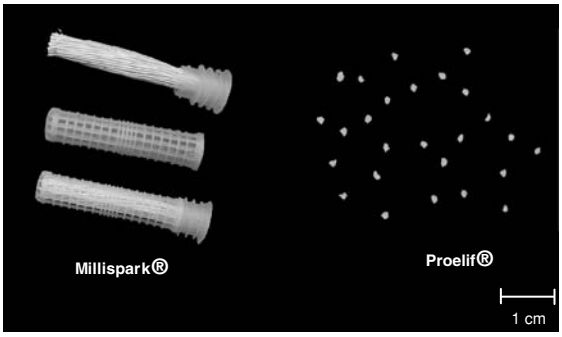

Fig. 9.1. The Millispark device (*left*) and dried double-layered beads (Proelif) (*right*)

view, great progress was made recently (1997) by Proenol (Portugal) and our laboratory working together. So, at this time we are able to produce more than 35 kg of dry beads per hour. These beads are dried so that they can be stored before use for more than 2 years without any loss of activity and also they are easy to put in the bottle thanks to a machine which was developed at the same time.

Another attempt was to place yeast cells in a sort of little cartridge with a membrane or some hollow fibres which separates the cells from the medium: the concept is like a tea bag. This device (called Millispark) developed by Millipore in 1993 (Jallerat et al. 1993) has proven to be very efficient but its development at the industrial level was not possible owing to a too high price.

Figure 9.1 illustrates the Millispark device and dried beads (Proelif) produced by Proenol.

9.3 Ethanol Production

More than 2×10^{10} L of pure alcohol is produced in the world each year and half of that is made in Brazil from sugar cane. It is without doubt the most important fermentation process, and as such the most studied. This alcohol, obtained from the distillation of different kinds of wines, is for the most part used for industrial purposes, such as additives for fuel, solvents for pharmaceutics or food ingredients. The wines to be distilled come from different substrates: sugar (sugar cane or beet root), hydrolysates of starch (maize, wheat or rice), sugar from industrial waste such as lactose from whey or sugars from biomass such as xylose, cellulose or hemicellulose. For sure the main challenge of the fermentation process is in this case to reach the best yield (alcohol produced/sugar used) as well as the highest reaction rates (in order to maximize the use of the fermentation tanks). In contrast to what we observed for the production of drinks, no attention is paid to the organoleptic quality of the wine. The most important thing is to avoid the synthesis of secondary products which can affect the yields and also the distillation process. For these different reasons it is obvious that a lot of studies have been made for 20 years in order to increase the yields, the reaction rates and to minimize the operational costs.

Among these studies a lot deal with the use of immobilized cells. Different processes using different kind of substrates, different kinds of yeasts and also different kinds of apparatus have been described and we will focus here on the most familiar ones or on those developed at the industrial level.

9.3.1 Alcohol from Sugar (Sucrose)

Saccharose (or sucrose) is the main component of sugar cane or sugar beet root. It is extracted from the plant by grinding and water diffusion and the medium obtained contains 120–140 g L^{-1} of sugar. It appears well established that the immobilized cells are more efficient than the free ones: Sree et al. (2000) using a repeated batch fermentation system (*S. cerevisiae* immobilized in alginate beads) noticed that more ethanol was produced by immobilized cells compared with free cells. The maximum amount of ethanol produced by immobilized VS3 cells using 150, 200 and 250 g L^{-1} glucose was 72.5, 93 and 87 g L^{-1} ethanol at 30°C. Using immobilized yeast cells some authors compared the method of immobilization as well as the efficiency of the reaction according to the process used. For example, we can quote the work of Goksungur and Zorlu (2001): they compared the continuous production of ethanol from beet molasses by calcium alginate immobilized *S. cerevisiae* in a packed-bed bioreactor to that obtained in a continuous stirred reactor. They showed that (with a temperature of 30°C and a dilution rate of 0.22 h^{-1}) maximum ethanol (4.62% v/v), yield (0.43 g g^{-1}) and volumetric productivity (10.16 g L^{-1} h^{-1}) were obtained from the beet molasses medium containing 10.90% (w/v) total sugar with 2.0–2.4-mm diameter beads prepared from 2% (w/v) sodium alginate solution. At higher substrate concentrations, substrate was recirculated through the packed-bed bioreactor to increase yields and to decrease residual sugar content. The bioreactor system was operated at a constant dilution rate of 0.22 h^{-1} for 25 days without loss of capacity. In the continuous stirred bioreactor (compared with the packed-bed bioreactor) lower ethanol concentration (3.94% v/v), yield (0.36 g g^{-1}) and productivity (8.67 g L^{-1} h^{-1}) were obtained. Dealing with the continuous fermentation of sugar cane syrup using immobilized yeast cells (*Saccharomyces* sp.) onto chrysotile (fibrous magnesium silicate) in a packed-bed reactor, Wendhausen et al. (2001) showed that the activity of the cells was higher when immobilized, mainly for fermentation of 30–50% w/v glucose solutions. In medium containing 30% w/v glucose, the initial fermentation rate increased 1.2–2.5 times. The yields were in the range 0.41–0.49 g g^{-1} for the immobilized cells and 0.37–0.43 g g^{-1} for the free cells. An average productivity of 20–25 g L^{-1} h^{-1} was obtained in the first 20 days and an average of 16 g L^{-1} h^{-1} was obtained after 50 days of operation. In order to increase the efficiency of immobilized cells, Nagashima et al. (1983) suggested adding some ergosterol and oleic acid to the alginate matrix. In this way they were able to increase the ethanol content of the medium to 57 g L^{-1} instead of 47 g L^{-1} in the same operating conditions but without sterol addition. An example of the use of immobilized cells for ethanol production from molasses on an industrial scale was given by Shi et al. (1995): yeast cells, suspended in the low concentration sodium alginate solution, were immobilized on the fluffy chemical fibre matrix to initiate the associated immobilization. Under factory conditions (four fermentors of 6.5 m^3), the ethanol

production was carried out continuously for 99 days by flowing diluted molasses (16.5–18% w/v sugar), resulting in an ethanol productivity of 6.21 g L^{-1} h^{-1} and an average ethanol concentration in the fermented mash of 9.44% (v/v). It was also proved by Murakami and Kakemoto (2000) that sodium alginate was a better support than κ-carragheenan gel because of its better mechanical strength.

But a natural phenomenon such as flocculation was also used at the industrial level in order to increase the efficiency of the process: Xie et al. (1999) described an industrial plant composed of four air-lift suspended-bed bioreactors in parallel with a total volume of 400 m^3 using cells able to self-flocculate. The process ran for more than 6 months in continuous operation: the effluent contained 70–80 g L^{-1} of ethanol and less than 5 g L^{-1} of residual sugar and an ethanol productivity of 7–8 g L^{-1} h^{-1} was achieved (to be compared with 2–4 g L^{-1} h^{-1} usually observed in classical process).

Studies were also made to appreciate the possible use of yeasts other than *Saccharomyces* and also different immobilization matrices: for example, Gough et al. (1998) analysed the production of ethanol from molasses (140 g L^{-1} sugar) at 45°C using a *Kluyveromyces marxianus* strain immobilized in calcium alginate gels and PVAC. The immobilized cells were used as a biocatalyst in fed-batch reactor systems for prolonged periods. When each system was operated on a fed-batch basis for a prolonged period of time, the average ethanol concentrations produced in the alginate- and the PVAC-immobilized systems were 21 and 45 g L^{-1}, respectively, while the yields remained high (0.41–0.45 g g^{-1}). The results suggested that the PVAC-based immobilization system might provide a more practical alternative to alginate for the production of ethanol by *K. marxianus* IMB3 in continuous or semi-continuous fermentation systems. Love et al. (1998) also tried a mixed matrix made of alginate and kissiris with the same yeast strain and reported good efficiency of the system.

If alginate seems nowadays the most used matrix for immobilization of yeast cells for alcoholic fermentation it must be noted that some different matrices were reported as more efficient. Zhang et al. (1996) suggested that the properties of the ceramic supports compared with those of a calcium alginate gel indicated that the ceramics were the better of the two types of material and had potential for industrial application. Harris and Ghandimathi (1998) immobilized yeast cells of *S. cerevisiae* in a natural rubber coagulum and used them for repeated batch fermentation of molasses. The authors pointed out the fair stability and efficiency of the process and that rubber was inert compared with alginate.

However, the use of a reactor with a very high level of immobilized cells may also have some negative effects and, for example, Yadav et al. (1996) observed that the productivity and the efficiency of a column reactor packed with gel beads might be affected by problems due to gas hold-up and mass transfer effects.

9.3.2 Alcohol Production from Lactose

Lactose is a sugar which may cause environmental damage as it is a major component of whey (waste from the dairy industry). Thus, its use as a substrate for alcohol production was studied early. As *Saccharomyces* sp. are not able to use this substrate the main yeast species used belongs to the genus *Kluyveromyces*. Marwaha

and Kennedy (1985) described a process for the continuous alcohol production from whey permeate using immobilized cell reactor systems. In this process a bioreactor packed with alginate-entrapped *K. marxianus* NCYC179 was used for continuous fermentation of whey permeate to ethanol. A maximum ethanol productivity of 28 g L^{-1} h^{-1} was attained at a dilution rate of 0.42 h^{-1} and 75% lactose consumption (substrate feed rate in the inflowing medium was 200 g L^{-1} lactose). The immobilized cell bioreactor system was operated continuously at a dilution rate of 0.15 h^{-1} for 562 h without any significant change in the efficiency and viability of the entrapped yeast cells (84–81%). More recently, El-Batal et al. (2000) made experiments on whey fermentation by *Kluyveromces* immobilized cells in copolymer carriers produced by radiation polymerization. In this study, yeast cells were immobilized in hydrogel copolymer carriers composed of PVA with various hydrophilic monomers, using a radiation copolymerization technique. Yeast cells were immobilized through adhesion and multiplication of yeast cells themselves by using batch fermentation; the ethanol production was 32.9 g L^{-1}, which was about 4 times higher than that of cells in the free system. Hydrogel copolymer carriers were used in a packed-bed column reactor for the continuous production of ethanol from lactose at different concentrations (50, 100, 150 g L^{-1}). For all lactose feed concentrations, an increase in dilution rates from 0.1 to 0.3 h^{-1} lowered the ethanol concentration in fermented broth, but the volumetric ethanol productivity and the volumetric lactose uptake rate were improved. The fermentation efficiency was lowered with the increase in dilution rate and also at higher lactose concentration in the feed medium, and a maximum of 70.2% was obtained at the lowest lactose concentration, 50 g L^{-1}. More recently, an industrial-scale pilot plant (11,000 L) using kefir yeast immobilized on delignified cellulosic material was described by Athanasiadis et al. (2003): the system showed good operational stability, exhibiting relatively high ethanol yield and ethanol productivity.

9.3.3 Ethanol Production from Starch

Starch is a very abundant substrate but its direct assimilation by yeasts is generally unlikely and thus some pretreatment is often necessary. To avoid this step it is possible to use a specific yeast such as *S. diastaticus* and a process using such a yeast immobilized on wood chip particles was recently described by Razmovski (2000). But it is also possible to use immobilized cells of a good fermenting yeast (*S. cerevisiae*) and immobilized enzymes (glucoamylase) as done by Chithra and Baradarajan (1992) and Giordano et al. (2000): cells and enzyme may be immobilized in separate particles or together in the same particles. However, it appeared that the productivities of these processes were smaller than those for fermentation of glucose-containing solutions.

9.3.4 Ethanol from Other Substrates

Among the substrates having some interest for the production of alcohol, great interest has been devoted to cellulose, hemicellulose and pentoses (xylose). The yeast *Pichia stipitis* appeared as a good species to ferment xylose solutions or hemicellulose

hydrolysates, (Sanroman et al 1994; Liu et al. 2001). *Candida sheateae* was also investigated (Hinfray et al. 1995) and also *Pachysolen tannophilus* (Amin et al. 1988). Chen and Weyman (1989) described a system able to use cellulose directly. In this process, baker's yeast cells were entrapped on glass fibre disks by means of alginate, and the enzymes cellulase and β-glucosidase were precipitated on the yeast cells by tannin. The disks carrying the yeast–enzyme co-immobilizate were installed in a continuous dynamic immobilized bioreactor. Cellulose was added continuously to the bioreactor. In the first few days, the efficiency of the system was good but decreased over the next 5 days to 40%, likely owing to the negative effect of tannin. On this subject of the direct use of cellulose a comprehensive review was made by Chandrakant and Bisaria (1998).

The alcoholic fermentation at a pilot scale from dried sweet potato was investigated by Yu et al. (1994), while Roukas (1994) was interested in the use of carob pod extract as a substrate for the alcoholic fermentation by immobilized cells of *S. cerevisiae* in alginate beads. An interesting substrate may be the Jerusalem artichoke. In this plant the reserves are made of inulin, a polymer of fructose. This polymer may be directly hydrolysed and fermented by *K. marxianus*, and a process using cells immobilized in calcium alginate beads was proposed by Bajpai and Margaritis (1986): the bioreactor was continuously operated with good results (volumetric ethanol productivity of 118 g L^{-1} h^{-1} at a dilution rate of 2.8 h^{-1} and 87% substrate conversion) and its half life was 105 days.

9.3.5 Immobilized Cells and Processes

Because of its economic importance, ethanol production has initiated a lot of studies dealing with the development of specific processes based on the use of immobilized cells.

In order to improve the efficiency of immobilized cell systems, different processes for the fermentation have been analysed for many years. Feng et al. (1989) analysed a continuous fermentation process using *Schiz. pombe* yeast flocs: a suspended-bed bioreactor utilizing air was employed in which the total yeast particles were retained and was allowed to operate over 3 months without interruption. The yeast cell concentration was held at 40 g L^{-1} (dry weight) and a high productivity of 20–24 g L^{-1} h^{-1} was obtained. These *Schizosaccharomyces* yeast flocs may also be used in an immobilized cell reactor separator (ICRS) as described by Dale et al. (1994): an ICRS with gas-phase ethanol product stripping was operated with both sucrose and molasses feeds continuously over 90 days. The feed concentration range was 300–600 g L^{-1}. Using *Saccharomyces* cells, Del Borghi et al. (1985) described a process called rotating biological surface (RBS): a spongy material was employed to trap yeast cells on the disks. In this way, an ethanol productivity of 7.1 g L^{-1} h^{-1} was achieved in the RBS-1CR at a dilution rate of 0.3 h^{-1}.

Many authors developed some apparatus making possible simultaneous bioreaction and separation by a so-called immobilized yeast membrane reactor. Vasudevan et al. (1987) designed a fermentor in which the microbial cells were sandwiched between an ultrafiltration membrane and a reverse osmosis membrane. The ultrafiltration membrane provided free passage for all nutrients which were supplied under

pressure, eliminating diffusional resistance. The reverse osmosis membrane preferentially allowed passage of the product, improving purity and concentration. Ethanol fermentations with *S. cerevisiae* were carried out for 160 h using this reactor with good performances. However, Woehrer (1989) analysed the continuous ethanol production in a three-stage horizontal tank bioreactor (HTR) by yeast cells entrapped in calcium alginate and concluded that "Compared to other continuous ethanol production processes using entrapped yeast cells, the HTR is among the best", and in the same year Shukla et al. (1989) described a novel microporous hollow fibre membrane-based immobilization technique for whole cells making it possible to reach a productivity of 41 g L^{-1} h^{-1} with an initial glucose concentration of 100 g L^{-1} and a yield of 0.45 g g^{-1}. But it is also possible to associate the immobilized yeast cells and a device allowing the elimination of the alcohol (which is a possible inhibitor for the yeast activity). That was done by Shabtai et al. (1991): they developed a system comprising an immobilized yeast reactor producing ethanol, with a membrane pervaporation module for continuously removing and concentrating the ethanol produced. The combined system consisted of two integrated circulation loops: in one, the sugar-containing medium was fed and circulated through a segmented immobilized yeast reactor (the bead matrix was a cross-linked polyacrylamide hydrazide gel coated with calcium alginate), in the other, ethanol-containing medium was circulated through the membrane pervaporation module. Long-term continuous operation (over 40 days) was achieved with a productivity of 20–30 g L^{-1} h^{-1}. As in some cases a possible limitation or inhibition due to a lack of diffusion or to limited escape of carbon dioxide inside the bed of immobilized cells may affect the efficiency of the process, the use of fluidized-bed bioreactors (Busche et al. 1992) or trickled-bed reactors (Jamuna and Ramakrishna 1992) was studied. Ogbonna et al. (2001) presented a study dealing with the scale-up of fuel ethanol production from sugar beet juice using a loofa sponge immobilized bioreactor. They concluded that "by using external loop bioreactor to immobilize the cells (here a flocculent strain of *S. cerevisiae*) uniformly on the loofa sponge beds, efficient large scale ethanol production systems can be constructed". But to our knowledge none of these systems are running on an industrial scale.

Another interesting approach to overcome some technical problems was to use immobilized systems made of a strain of microorganism and something else, such as another strain or species or an enzyme. A study by Andreoni et al. (1983) suggested the utilization of immobilized β-glucosidase enzyme and immobilized growing yeast cells in the ethanol production from municipal solid wastes, and Amin et al. (1983) conducted experiments on the co-immobilization of *S. bayanus* and *Zymomonas mobilis*. A new immobilized biocatalyst called Maxaferm was described later by Noordam et al. (1995) for the continuous production of ethanol from dextrins: the Maxaferm system has been developed for the co-immobilization of enzymes and microorganisms (in this case amyloglucosidase and *S. cerevisiae*).

Recently, Amutha and Gunasekaran (2001) studied the production of ethanol from liquefied cassava starch using co-immobilized cells of *Z. mobilis* and *S. diastaticus*. They noted that the concentration of ethanol produced by immobilized cells was higher than that by free cells of *S. diastaticus* and *Z. mobilis* in mixed-culture fermentation and that in repeated-batch fermentation using co-immobilized cells, the ethanol

concentration increased to 53.5 g L^{-1}. Also, the co-immobilized gel beads were stable up to seven successive batches. Continuous fermentation using co-immobilized cells in a packed-bed column reactor exhibited an ethanol productivity of 8.9 g L^{-1} h^{-1}. An original use of immobilized yeast cells and free cells of a fungus was recently studied by Farid et al. (2002): in this paper the authors analysed the alcohol production from starch by mixed cultures of *Aspergillus awamori* and immobilized *S. cerevisiae*. They concluded that repeated batch by this co-culture were successfully used 12 times without a significant loss in alcohol production.

But the co-immobilization of different species of yeasts did not always lead to a better efficiency as shown by Lebeau et al. (1996) when investigating the continuous alcoholic fermentation of a mixture of glucose (35 g L^{-1}) and xylose (15 g L^{-1}) by *S. cerevisiae* and *C. shehatae* co-immobilized in a double-chambered bioreactor.

9.4 Brewing

In the brewing process, fermentation is made up of two steps: the first step or main fermentation consists in the conversion of most of the fermentable sugars into ethanol by a growing and abundant yeast population; in the second step or maturation, the main objective is to decrease the concentration of diacetyl below the taste threshold for organoleptic reasons, diacetyl being a secondary product of main fermentation. A continuous process would be the most interesting from the economic point of view because of the shortened fermentation time, but the major difficulty lies in keeping a biological system aseptic for a relatively long period. However continuous fermentation of beer has long been attractive since as early as the end of the nineteenth century (Virkajarvi and Linko 1999). The emergence of immobilization technology in the 1970s boosted research on the continuous processes. Huge numbers of papers and patents on this subject have been published in the last 30 years. Among them very few led to industrial applications except for beer maturation or alcohol-free beer production. Nevertheless the feasibility of many proposed processes has been demonstrated at the laboratory or pilot scale (Virkajarvi 2001). The main supports used in the brewing process were discussed in Sect. 9.1.2.

9.4.1 Examples of Proposed System Configurations

Because of the different characteristics of primary and secondary fermentations most of the processes either concerned only one of the two fermentations or have several serial reactors (multistage processes). The most difficult fermentation to manage is the primary one, which is more vigorous, and many critical points have to been solved technically: removal of excess biomass and CO_2, sustaining yeast viability, optimization of oxygen feeding, prevention of clogging the reactor, high residence time and prevention of microbial contamination in a continuous run.

9.4.1.1 Main Fermentation

For the main fermentation high amounts of fermentative yeasts are needed. Very few studies have dealt with batch fermentation. Hsu and Bernstein (1985) modified a

conventional fermenting vessel with two screens that hold alginate beads containing fermenting yeast in the vessel. The whole process lasted 7 days but the organoleptic characteristics of the beer were slightly different. Other authors proposed recycling the same biocatalyst 20 (Ju et al. 1986) to 42 times (Pardonova et al. 1982) or for 3 months (Nedovic et al. 1993), shortening the fermentation time to 12–16 h.

In order to get high productivity a continuous process is much more interesting at the industrial level. As early as 1966 some attempts were made at continuous main fermentation by mixing diatomaceous earth and yeasts and passing malt wort through a kieselghur filter with a residence time of 2.5 h, but the bioreactor had a lifetime of only 7 days before clogging. This system was then improved by Baker and Kirsop in 1973 (Virkajarvi 2001) by operating in a tubular reactor containing the mixture, resulting in an increased lifetime, but it still remained insufficient. Moreover, to achieve satisfactory flavour formation and organoleptic qualities a plug flow reactor, packed or fluidized bed, seemed to be more suitable (Yamauchi et al. 1995). An alternative should be a series of continuous stirred-tank reactors (Linko et al. 1998). In these conditions, immobilization by entrapment always led to swelling of the carrier, preventing long-term operation, and immobilization by adsorption was preferred.

Studies on packed-bed reactors seem to be more extensive with different kinds of carriers: ceramics, glass beads, calcium alginate beads, DEAE-cellulose or wood chips. Shindo et al. (1994) experimented with chitosan beads in a fluidized-bed reactor and by optimizing the recycling flow rate in the reactor obtained a life time of 900 h at a flow rate of 40 mL h^{-1}. Later (Pajunen et al. 2000) this kind of system was extrapolated at the pilot scale with a flow rate of 28 L day^{-1} for 50 days and a bed volume of 1,000 L. A similar process was investigated with alginate beads (Wang et al. 1989): 40% (v/v) beads were used and the ratio of recirculation was 5, giving a fermentation time of 14 h.

Another possible technology would be a loop bioreactor containing a tubular matrix carrier made of silicon carbide (Meura-Delta process, see later; (Virkarjarvi and Linko 1999) or one layer of metal fibres which had been sintered. Alternatively the carrier may be a sintered silicon carbide carrier in a multichannel loop reactor design or may comprise several tubes placed concentrically around each other (Arnaut et al. 2001). Complete attenuation was then achieved in a continuous two-stage bioreactor with a hold-up vessel arranged in series. The total residence time including the time in the hold-up vessel was 2.5 days (van de Winkel et al. 1993).

To achieve the high yeast concentration and activity needed in the first fermentation optimal oxygen feeding is necessary. This can be realized using an air-lift or bubble column bioreactor. This was suggested in several works using various carriers such as alginate (Leskosek-Cukalovic and Nedovic 2002) κ-carragheenan (Mensour et al. 1996; Pilkington et al. 1999) or calcium pectinate beads (Yamauchi et al. 1995; Smogrovicova et al. 1998), spent malt grains (Branyik et al. 2004) and DEAE-cellulose (Branyik et al. 2001). As an example, Labbat breweries in Canada produced beer in a 50-L gas-lift reactor containing yeasts entrapped in carragheenan beads with air in the proportion of 2–5% and with a residence time of 20 h (Mensour et al. 1996). With yeasts immobilized in pectate beads at a ratio of 25% (v/v) in the air-lift the residence time was 13 h.

Another strategy consists in using a multistep system, only one step being an aerated reactor: continuous stirred tank or air-lift. Several configurations have been tested: a fluidized-bed reactor and air-lift with a residence time of 12 h (Smogrovicova et al. 1997); a two-stage packed-bed system (Kronlof et al. 1996), in this case the first stage was aerated and considered as a prefermentor, giving a global production of 60–130 L day^{-1} with a residence time of 20–40 h; an aerated continuous stirred-tank reactor followed by a fluidized-bed reactor (Yamauchi and Kashihara 1996).

9.4.1.2 Secondary Fermentation (Maturation)

The use of immobilized yeasts allows the duration of this step to be shortened, compensating the low reaction rates by high catalyst concentrations. In the case where the main fermentation is continuous (with free or immobilized yeasts) the secondary fermentation is run in one or two reactors in series. Several processes have been run at the industrial level with 7–50-m^3 reactors allowing the duration to be reduced from several weeks in the traditional process to 2–3 h. Two kinds of carrier were used for adsorption. The first one, since 1990, was DEAE-cellulose (Pajunen 1996) in an installation with an annual capacity of 1×10^8 L. The continuous production cycles varied between 2 and 8 months before regeneration. The second one was porous glass beads in two-stage column reactors (Virkajarvi and Linko 1999).

9.4.1.3 Examples of Integrated Processes at Laboratory, Pilot or Industrial Scale

A very attractive design for the brewing process would be an integrated one making it possible to carry out both fermentations in a multistage continuous system. At the laboratory level a three-stage packed-bed reactor containing yeast adsorbed on glass beads had been proposed (Yamauchi et al. 1994). A two-stage system has been investigated (Smogrovicova et al. 1999): primary wort fermentation was conducted in a gas-lift reactor using calcium alginate (residence time 12 h), secondary fermentation was conduced in two parallel packed-bed systems using calcium alginate (residence time 57 h) or gluten (residence time 61 h) for yeast immobilization. Another two-step process could consist of a short aerobic prefermentation of the wort in a continuous stirred-tank reactor followed by a packed reactor filled with calcium alginate beads containing yeast cells. In such a configuration no further maturation is needed (Nakanishi et al. 1985).

Finally in spite of numerous studies and trials, beer production using immobilized yeasts has kept the brewing world waiting for a breakthrough, mainly owing to difficulties in controlling the hydrodynamic and temperature stability of the reactor for a long time and only three processes have reached industrial development.

The first one is the Kirin process in Japan developed since the mid-1980s and exploited for about 10 years producing 185,000 L per year (Virkajarvi 2001). It consists in a three-stage system (four reactors) with yeast adsorbed on porous glass beads (Fig. 9.2): the first stage is an aerated stirred-tank reactor for free yeast growth; the second step is made of two packed beds in series with immobilized yeast for the main fermentation; the last step after heat-treatment is also a packed bed for green beer maturation.

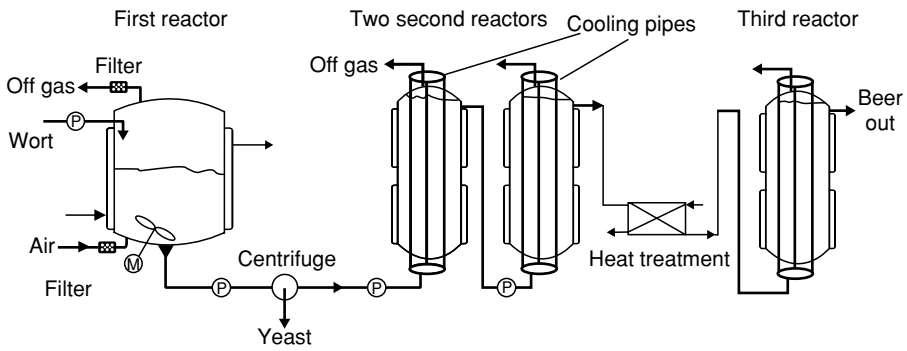

Fig. 9.2. The Kirin process. (Redrawn from Yamauchi et al. 1994)

The second potential industrial process was developed by the VTT technical research centre of Finland (Virkajarvi 2001). It consists of a slightly aerated prefermentor, a buffer tank and a main fermentor (packed-bed reactor containing glass beads); after heat-treatment the green beer enters a second packed-bed reactor for the maturation. The system at a pilot scale was run for 14 months but a problem for longer-term instability and efficiency appeared caused by the decline of yeast viability and plugging. The author proposed solving this problem by substituting glass beads by wood chips. The feasibility at an industrial scale was demonstrated (4×10^6 L per year).

The third semi-industrial process, the Meura-Delta, consists in two loop reactors in series with a matrix of silicon carbide inside for adsorption of yeast (Sect. 9.4.1.1). The first bioreactor is operated at an attenuation of 40% and final attenuation is reached in the second bioreactor. The aeration is arranged by diffusion through plastic tubing in the circulation loop. At least one brewery uses it in Canada with a productivity of 0.5×10^6 –3×10^6 L a year according to the final attenuation.

9.4.2 Alcohol-Free Beer

Traditionally, alcohol-free beers are produced by arrested batch fermentation, vacuum distillation, reverse osmosis, or dialysis. Dealcoholized beers generally lack body and have poor aroma profiles. In order to circumvent these disadvantages, several immobilized yeast cell reactors were developed to produce beer with a final alcohol content below 0.5%. For example, immobilization of yeasts by passive colonization of multichannel silicon carbide membrane carriers with a void volume of 30% and pore sizes ranging from 8 to 100 μm was studied with a view to achieving optimal flavour development (van de Winkel et al. 1991).

Others carriers have been described in the literature. Continuous fermentations with yeasts immobilized on wide-pore sintered glass (Siran) in a fluidized-bed fermentor were carried out at both a laboratory and a pilot plant scale (reactor volumes of 2 and 60 L, respectively) (Aivasidis et al. 1991). Following a colonization phase

at 25–30°C requiring less than 2 weeks, the temperature was lowered to just above 0°C, where the beer fermentation took place. A product containing 0.1–0.3% alcohol (carbohydrate conversion rate of approximately 7%) was obtained after about 2–6 h as compared with 3–4 days with conventional cold/contact fermentation. A system stability of 1 year was observed, with no colonization of wild-type yeast strains from contaminated worts or other sources. Blockages were not observed and yeast or carrier make-ups were not required. The organoleptic properties of the final product corresponded to those of conventionally manufactured low-alcohol beers.

Others processes resort to immobilization on DEAE-cellulose and limited fermentation optimally performed in a packed-bed reactor. This highly controllable system combines short contact times between yeast and wort with the reduction of off-flavours. In some cases this was due to higher activities of hexokinase and pyruvate decarboxylase of immobilized cells compared with those of free cells grown in batch culture (Van Iersel et al. 2000). Using a similar system millions of hectolitres of alcohol-free beer have already been produced. In the reactor a limited fermentation was carried out under strictly anaerobic conditions, very low temperature, relatively high pressure and a short contact time. With the combination of these factors, only a small amount of glucose was metabolized, resulting in a low-alcohol product (less than 0.1% ethanol). In addition, the limited growth under anaerobic conditions stimulated the yeast to restore its redox balance by the reduction of carbonyl compounds (Van Dieren and Bavaria 1996) and simultaneously an increase of ester formation (ethyl acetate and isoamyl acetate) was noted (Van Iersel et al. 1999; Navratil et al. 2002). Nevertheless, in this case, introduction of regular aerobic periods to stimulate yeast growth was recommended to achieve an optimal and constant flavour profile of the alcohol-free beer. A patented method (Lommi et al. 1997) also recommended reactivating the yeast at 2–15°C for 10–30 h. If necessary, the reactor could be regenerated.

During a 12-month pilot-scale project both at Guinness Brewing Worldwide Research Center and at Grolsche Bierbrouwerij Nederland (van de Winkel et al. 1996), a silicon carbide immobilizing carrier system was investigated for the continuous production of alcohol-free beer with 0.05, 0.1 and 0.5% alcohol by volume with a decreased worty flavour and a full beery aroma. The scale-up capability of the immobilized bioreactor system was studied and the operating parameters (dilution rate, fermentation temperature, wort oxygenation, number of bioreactor stages, colonization procedure, cleaning and sterilization procedures) were optimized. From the results it was demonstrated that scaling-up was reproducible and predictable with a single bioreactor stage operating at 10°C with oxygen levels below 1 mg L^{-1} for the production of an acceptable alcohol-free beer.

9.4.3 Miscellaneous

Immobilization of yeasts has been studied for purposes other than conventional fermentation. An oxygen scavenger for beverages has been patented (Edens et al. 1989). It consists of dry yeast immobilized in or on a solid material, e.g. paraffin wax, which allows only very slow penetration by water. Yeast cells were mixed with a slurry of molten paraffin at 95°C. A glass slide was coated with a 0.1-mm layer of

this slurry. The slide was heated at 65°C for 10 min and submerged in air-saturated water. The oxygen concentration of the water was reduced from 7.5 mg L^{-1} to undetectable in 14 days.

For production of diet beer several similar methods have been developed using co-immobilized enzyme (amylase) and yeast (*S. cerevisiae*). This is a way to produce beers with higher attenuation containing less residual sugar. A glucoamylase was bound to living yeast cells, resulting in particle size of approximately 10 μm. The apparent K_m value, the pH and the temperature dependence of the activity and stability of the bound enzyme were significantly different from the characteristics of the free glucoamylase. Compared with the native yeast, the co-immobilizate led to a considerably increased final degree of attenuation when applied for beer wort fermentation or for maturation of beer (Hartmeier and Muecke 1982).

In two others works, brewer's yeast was immobilized in calcium alginate gels. In the first one (Godtfredsen et al. 1981), the immobilized cells were packed in a simple reactor allowing continuous operation. A suitable dextran-coupled amyloglucosidase co-immobilized with brewer's yeast was also applied for production of low-calorie beer in a simple reactor system. In the second work (Juchem et al. 2000), the ability of the beads to reduce the carbohydrate content of the beer was investigated in a repeated batch system and a continuous system using a multistage fluidized-bed bioreactor. In both cases the new technology made possible a significant intensification of the fermentation achieved by a high yeast density coupled with the enzymatic activity.

9.4.4 Influence of Immobilization on the Organoleptic Qualities

Most works report modifications in the minor by-product concentrations for the beer produced by immobilized yeasts. These modifications vary a lot according to the kind of process, batch or continuous (Bardi et al. 1997a), kind of reactor (Yamauchi et al. 1995), fermentation temperature (Bardi et al. 1997a; Bekatourou et al. 2002), dissolved oxygen (Nakanishi et al. 1985; Virkajarvi and Kronlof 1998) and even type of carrier (Smogrovicova and Domeny 1999). Generally succinic acid production was increased (Yamauchi et al. 1995; Shindo et al. 1992, 1993) in connection with an enhanced consumption of isoleucine and acetic acid production was decreased. Most of the time higher alcohols were less concentrated in the final product (Smogrovicova et al. 1998; Smogrovicova and Domeny 1999; Tata et al. 1999) but their concentration could increase for very low temperature (0–7°C) in the batch process (Bardi et al. 1997a); the total nitrogen content was higher (Virkajarvi and Kronlof 1998; Smogrovicova and Domeny 1999), except for immobilization on gluten pellets (Smogrovicova et al. 1999). For production of esters and diacetyl the results are very different according to the studies. It seems that for these metabolites the key parameters, which are the level of yeast activity linked with the sugar flux, the biomass density in the reactor (Smogrovicova et al. 1998) and the redox state of the medium, are strongly dependent on the operating conditions. In fact the organoleptic quality of the beer produced by immobilized yeasts can be controlled by:

– The relative importance of the different stages of a multistage process. For example, yeast metabolism was successfully subdivided into a growth and a

restricted phase through a combination of a continuous stirred-tank reactor and an immobilized yeast packed-bed reactor (Yamauchi et al. 1995). The process control strategy based on the relative intensity of attenuation (proportion of sugar consumption) in the two reactors was optimized with a ratio of 1:2, higher alcohols being mainly produced in the stirred reactor and esters in the packed-bed reactor.
- The level of aeration if the process contains a preaeration step (Virkajarvi and Kronlof 1998).
- The ratio of the biocatalyst volume to the reactor volume (Smogrovicova et al. 1998).

Several authors reported final products with no significant differences from the beer produced in the conventional way (Pardonova et al. 1982; Smogrovicova and Domeny 1999; Umemoto and Mitani 1999; Bekatorou et al. 2002).

9.5 Fruit Wines

As is the case for grape wine, the most frequent technology employed for fruit wine making by immobilized yeasts is entrapment in alginate gel. This method has been studied for:
- The development of a new type of Umeshu (a liqueur made from Japanese apricot fruit, shochu and sugar) (Takatsuji et al. 1992). It was fermented in a reactor containing an immobilized growing yeast strain isolated from Japanese apricot juice. The initial Umeshu juice diluted 2 times with water was circulated through the reactor at 25°C with a dilution rate of 0.13 h^{-1}. Fermented Umeshu with good flavour was manufactured stably by this process for 30 days.
- Fermentation of watermelon juice by a wine yeast (Nakada 1990). The juice with addition of glucose up to 21.1% and adjusted to pH 4 with citric acid flowed through the bioreactor at 0.03 L h^{-1} at 25°C. The alcohol productivity of the bioreactor was 10 g ethanol (L gel)$^{-1}$ h^{-1}. The watermelon wine obtained after 4 days of operation contained 9.3% ethanol and the ratio of isoamyl acetate to isoamyl alcohol (4.47) was high, but formation of ethyl caproate was low compared with the that for wine made by the conventional fermentation method.
- Fermentation of fresh sugar cane juice and fruit juices by mixed yeast strains in a three-stage rhomboid bioreactor. The ethanol concentration in the wine made from cane juice was 9.5% and a mixture of wine yeasts produced a wine containing 10–13% ethanol from mandarin and orange juices at residence times of 6–20 hours for 7 months (Fukushima and Hatakeyama 1983).
- The production of alcoholic beverages from different fruit juices (mango, peach, plum, cherry) containing 8–18% sugar giving 11–12% ethanol (60–84% of the theoretical yield) in batch fermentation by entrapped *S. cerevisiae* (Qureshi and Tamhane 1985).
- Bottle-fermented kiwifruit sparkling wines production by a combination of fermentation using *S. cerevisiae* immobilized in double-layered calcium alginate beads and termination of the fermentation using an antimicrobial substance from paprika seed (PSAS) having strong antimicrobial activity against wine yeasts (Yokotsuka et al. 2004). Secondary alcoholic fermentation in bottles

could be terminated several days after the addition of PSAS to give a concentration of 0.1 g L^{-1} to the fermenting base wine. Beads were easily inserted into the bottles and removed as ice plugs by the conventional disgorging method. The sparkling wines produced had a strong fruity smell and a good balance of sweet and sour flavours.

Entrapment in carragheenan gel has also been proposed for fermentation of ripe Cavendish banana fruit pulp, which contained approximately 126 g L^{-1} total sugars (del Rosario and Pamatong 1985). The volumetric productivity and fermentation efficiency were about 15 g L^{-1} h^{-1} and 94%, respectively. The concentrations of alcohol and residual sugar in the product were 54 and 12.8–14.5 g L^{-1}, respectively.

Adsorption on dicarboxycellulose (Sado et al. 1992) or derivatized cellulose (del Rosario and Pamatong 1985) was tested.

As in grape wine making, the deacidifying yeast *Schiz. pombe* can be useful for malic acid elimination from fruit wines. It has been tested immobilized on oak shavings for apple must amended with red currant must: elimination of 100, 80.7, and 79.8% of initial malic acid, at flow rates of 0.033, 0.079, and 0.092 mL h^{-1}, respectively, was obtained (Czyzycki et al. 1991). Other authors deacidified plum juice by *Schiz. pombe* entrapped in calcium alginate beads (Tachibana et al. 1989) prior to alcoholic fermentation: plum (*Prunus salicina*) juice initially contained 1.2–1.4% malic acid and was batch-treated with 5% of immobilized cells of *Schiz. pombe* (2.1×10^9 cells L^{-1} juice) at 20°C for 3 days to remove 70% of the malic acid. When continuous decomposition of malic acid was carried out using a reactor (2 L) containing 1 L of immobilized cells at 20°C, with a flow rate of 1.5 L day^{-1}, the retention time was 16 h and the concentration of malic acid in the treated juice remained at 27–35 mg L^{-1} for 16 days.

9.6 Cider

For cider production two strategies are possible: the use of yeast alone or the use of co-immobilized yeast and lactic acid bacteria.

For fermentation of pure cultures of yeasts the different methods of immobilization are mainly based on adsorption methods:

- Adsorption on multiple parallel porous ceramic plates. The bioreactors prevented clogging associated with insoluble substances in the sample and gas channelling. Thus, low-fermentation apple juice was possible (Aso et al. 1993).
- Adsorption on an ion-exchange sponge that can have a tailored surface charge was used in high original gravity (1.106) cider fermentation. Continuous circulation of the medium through columns containing weakly basic sponge decreased the batch fermentation time, and increased the final ethanol concentration, possibly aided by sponge-enhanced CO_2 removal from solution (O'Reilly and Scott 1993).
- Adsorption on polyethylene. The use of immobilized yeast at 500×10^6 –600×10^6 cells mL^{-1} for fermentation at 15–20°C improved the quality of fruit wines and decreased the period of fermentation by 3–5 times (Sarishvili et al. 1992).
- Adsorption on foam glass put in a column where the apple juice was circulated with a residence time of 5–6 days. The fermentation was carried out for more

than 3 months at 22°C with no changes in the sensory quality of the product (Bonin and Wzorek 2000).

But entrapment in calcium alginate gel was also carried out for continuous fermentation of apple juice. The average values characterizing the process were as follows: fermentation efficiency, 84.7% of the maximal theoretical yield; ethanol concentration in the mash, 38.9 g L^{-1}; and volumetric productivity, 6.3 g L^{-1} h^{-1} (Dallmann et al. 1987). Alginate gel was shown to be better than pectate gel from an organoleptic point of view (Krasny et al. 1993).

A method for controlling the alcohol and sugar content of cider produced by alginate-entrapped yeast by varying the CO_2 pressure has been patented (Divies and Deschamps 1988). At 0.2 bar of CO_2, the ethanol concentration was 4.6% and the sugar concentration 14.9 g L^{-1} and at 3–5 bar of CO_2, the values were 4.8 and 16.2, respectively. It was possible to produce in the same fermentor a "hard" cider with 3% alcohol and 48 g sugar L^{-1} as well as a "soft" cider with 2% alcohol and 66 g sugar L^{-1}.

For co-immobilization calcium alginate has been proposed for *S. bayanus* and *Leuconostoc oenos* (*Oenococcus oeni*) in a continuous packed-bed bioreactor (Nedovic et al. 2000). The continuous process permitted much faster fermentation compared with the traditional batch process. The flavour formation was also better controlled. By adjusting the flow rate of the feeding substrate through the bioreactor, i.e. its residence time, it was possible to obtain either "soft" or "dry" cider. However, the profile of the volatile compounds in the final product was modified compared with that of the batch process especially for higher alcohols, isoamyl acetate and diacetyl. This modification was due to different physiological states of the yeast in the two processes. Nevertheless, the taste of the cider was acceptable.

A sponge-like material was also used to immobilize both *S. cerevisiae* and *Lactobacillus plantarum* (Scott and O'Reilly 1996). The sponge's open porous network promoted extensive and rapid surface attachment of microorganisms throughout the depth of the material. The matrix surface can also be chemically modified, and basic characteristics enhanced both the initial rate of uptake and also that of final loading (in excess of 10^9 yeast cells g^{-1} sponge and 10^{10} bacterial cells g^{-1} sponge). The flavour of the product was satisfactory.

9.7 Vinegar

For vinegar manufacturing several processes using immobilized yeasts have been proposed using different substrates. Some of them are based on a two-step fermentation, the first one by yeasts and the second one by acetic acid bacteria. For example, continuous production of kiwifruit and persimmon wines in a bioreactor with calcium alginate entrapped yeast cells was studied (Yamashita 2002). When the juice was in the reactor for residence times of 12 and 6 h, the ethanol concentration and the productivity were 11 and 10%, and 7.5 and 13.4 g L^{-1} h^{-1}, respectively. Fermentations of both fruit juices were continued for 50 days without microbial contamination. Continuous production of fruit vinegar using a bioreactor with fixed *Acetobacter aceti* cells on cotton fabrics was then developed. The fabrics were packed into a column and inoculated with *A. aceti*. When the kiwifruit and

persimmon wines fed the column the production rates of acetic acid were 7.4 and 5.2 g L^{-1} h^{-1}, respectively, on the basis of the total column volume with 45 g L^{-1} of the acetic acid in the vinegar. The surface culture using growing cells fixed on the woven cotton fabrics was superior to the submerged culture involving aeration, from the viewpoint of the higher productivity and energy efficiency. Yeast entrapment in calcium alginate beads was also used in view of vinegar production from saccharified rice (Nakajima and Sugiura 1990) or rice flour by repeated batch fermentations (Tamai et al. 1990). When rice flour was used the aim was to reach a high concentration of ethanol (approximately 120 g L^{-1} broth) without residual glucose. This was achieved for a 0.14 L gel L^{-1} packing ratio. The process could be repeated for more than 25 batches with a stable ethanol yield [0.38 g ethanol (g rice flour)$^{-1}$] and cell viability in the gel beads (96%). The ethanol productivity of this system was 3.9 g L^{-1} h^{-1}, which is much higher than that of batch fermentation in vinegar breweries.

A method for manufacturing vinegar from potato comprising (1) liquefaction, (2) saccharification, (3) alcoholic fermentation and (4) acetic acid fermentation was patented (Nagao and Yamamoto 1991). Steps 2 and 3 were combined to reduce microbial contamination in a bioreactor comprising a fermentation chamber packed with beads containing immobilized saccharifying enzymes and yeasts for concomitant reactions.

A process for continuous alcoholic and acetic acid fermentation of onion juice was developed by using yeast and acetic acid bacteria, respectively, immobilized on porous ceramic granules and rings (Takahashi et al. 1993). In continuous vinegar production in multibioreactors, the activities of the immobilized yeast and the acetic acid bacterial cells were not lost during more than 6 months of operation. For acetic acid bacterial cells the method was better than for cells immobilized on calcium alginate, for which a decrease of 50% of initial activity was observed for 3 months of operation. By developing a new cyclic operation, acetic acid yield increased by 16% compared with the yield from steady-state operation. The 4% onion vinegar produced had satisfactory organoleptic properties.

Mixed cultures of immobilized yeasts and another microorganism can be another possible strategy. For this purpose some authors proposed co-immobilizing *Monascus* and *Saccharomyces* in alginate carrier (Wang 1998). Vinegar was prepared by mixed fermentation of glucose mother liquor as the main material. The product ratio of vinegar was 4.5 kg kg^{-1} glucose liquor, and the appearance and flavour were good. Others authors suggested entrapping separately growing yeast cells and *A. aceti* cells in calcium alginate gel as pellets (Sumonpun and Kummun 1989). The immobilized yeast cells converted glucose to ethanol and the immobilized *A. aceti* simultaneously converted ethanol to acetic acid. Preliminary studies showed that a 1:4 ratio of immobilized yeast and immobilized *A. aceti* gave the maximum yield of acetic acid, approximately 3.4% after 22–24 days of cultivation in coconut water medium containing 2% glucose in shake flask culture at 250 rpm.

Finally, immobilized yeasts can also be used for refining fruit vinegar. A method has been patented for apple or apple/Japanese pear (10–30%) vinegar (Panasyuk et al. 1988) which is fed to a packed-bed column containing 300–800 $\times 10^6$ immobilized cells mL^{-1} at a flow rate of 0.02–0.08 h^{-1} for 12–48 h.

9.8 Dairy Products

As far as dairy products are concerned the different utilizations of immobilized cells can be divided into the treatment of whey for its valorization (except for the production of ethanol presented in Sect. 9.3), the treatment of milk and the production of kefir for manufacturing fermented beverages from milk.

The use of salted whey (a liquid by-product from the dairy industry) was investigated (Mostafa 2001) as a substrate for either acetic acid or glycerol production using two yeast strains (*K. fragilis* and another one isolated from waste whey); pH 8.5 and 32°C were the optimum operating conditions for maximal acetic acid production (25.8 g L^{-1}) and supplementation with peptone and pH 7 for glycerol batch production (13.2 g L^{-1}). The experiments in a membrane cell recycle bioreactor gave better results than those obtained for the immobilized cell batch reactors (18.7 g L^{-1} for glycerol production).

The potential of three lactose metabolizing yeasts, *C. pseudotropicalis*, *S. fragilis* and *K. marxianus*, for the removal of biological oxygen demand and chemical oxygen demand from dairy industry wastewater under unsterilized conditions was evaluated (Marwasha et al. 1988). The most efficient was *C. pseudotropicalis*. Using entrapment procedures in an alginate matrix, 40 g (dry weight) cells L^{-1} was the optimum operational cell density. Nitrogen source supplements further improved the ability of immobilized yeast cells to carry out the treatment. For milk or whey treatment two original and similar methods have been investigated.

An immobilized preparation of whole cell-based catalase was obtained by cross-linking the yeast cells permeabilized with toluene in hen egg white using glutaraldehyde for 2 hours at 4°C. Immobilized cells could be reused for the removal of H_2O_2 from milk (Kubal and D'Souza 2004).

Others authors have developed and characterized a new low-cost enzymatic preparation for milk whey saccharification (Gonzalez Siso and Suarez Doval 1994) consisting of β-galactosidase-rich whole cells of the yeast *K. lactis*, previously cultured on milk whey and immobilized by covalent linkage to corn grits (an inexpensive material). Permeabilization of immobilized cells with ethanol increased the intracellular β-galactosidase activity up to 240-fold, and the cells did not further metabolize the glucose and galactose produced. More than 90% milk whey lactose hydrolysis was achieved in a packed-bed bioreactor at 37°C. In another work (Decleire et al. 1985) whey hydrolysis was compared in column reactors containing whole yeast cells immobilized in calcium alginate or in hen egg white in relation to cell β-galactosidase activity, flow rates, temperature and time. With cells having an activity of 1.3 U mg^{-1} (dry weight) immobilized in calcium alginate, 80% hydrolysis was obtained at 4 and 20°C with flow rates of 0.50 and 1.65 bed vol h^{-1}, respectively; the values were 0.2 and 0.4 bed vol h^{-1} with cells entrapped in hen egg white. When the flow rate was expressed as millilitres per hour per gram of wet yeast, no significant difference was observed between either matrix, and 80% hydrolysis was reached with flow rates of 1.7 and 5 mL h^{-1} (g wet yeast)$^{-1}$, respectively, according to the temperature. The best performance was achieved by the yeast egg white reactor. At 4°C, hydrolysis decreased by 10% after 13 days and by 20% after 17 days. Many more applications of purified immobilized β-galactosidase have been studied.

In the field of dairy products many works deal with kefir. In fact, kefir is made from gelatinous white or yellow particles or granules called "grains" formed on cultured milk. These grains contain the lactic acid bacteria/yeast mixture clumped together with casein and a branched polysaccharide composed of glucose and galactose forming an insoluble matrix. They range from the size of a grain of wheat to that of a hazelnut. The grains, then removed after milk fermentation, as well as the fermented beverage are called kefir.

To remove lactose from milk the use of kefir granules obtained by fermentation, containing both bacteria and yeasts, has also been proposed and patented. They were sterilized at a low temperature, inactivated and coated with a semipermeable film-forming material, yielding an immobilized lactase (Snow Brand Milk Products Co. 1982).

A mixture of wheat flour and sour milk was treated according to the method of the traditional Greek fermented food *trahanas*, and was used as a model cereal-based support (starch–gluten–milk matrix) for co-immobilization of lactic bacteria and yeasts for potential use in food production (Plessas et al. 2005). Cell immobilization was proved by microscopy and by the efficiency of the immobilized biocatalyst for alcoholic and lactic repeated fermentations at various temperatures (5–30°C). The stability of the system was always good, revealing suitability for industrial applications. Finally, respectable amounts of lactic acid and volatile by-products were produced, revealing potential application of the immobilized biocatalyst in fermented food production or use as a food additive, to improve nutritional value, flavour formation or preservation time.

The main application of immobilized kefir microorganisms is the production of fermented beverages from milk or whey. For this purpose the same Greek research group has developed different methods (Athanasiadis et al. 2004) They reported a novel whey-based beverage with acceptable organoleptic properties where various treatments were studied. Kefir yeast immobilized on delignified cellulosic materials (DCM) or gluten pellets were proved to accelerate whey fermentation significantly, with the latter support not being so preferable. Kefir granules seemed to achieve similar fermentation times as DCM. The optimal final pH of the product, indicating the amount of fermented lactose, was suggested to be 4.1 since the profile of the volatile by-products was higher than for other final pH values. The addition of fructose seemed to be beneficial for the volatile content of the product, although its acceptability as determined by a preference panel was similar to that of the control.

This delignified cellulosic-supported biocatalyst was also found to be suitable for batch or continuous modified whey fermentation containing 1% raisin extract and molasses (Kourkoutas et al. 2002a). Batch fermentations were carried out at various pH values, and the effect of temperature on the kinetic parameters, in the range 5–30°C, was examined. At pH 4.7 the shortest fermentation time was obtained. The formation of volatiles indicated that the concentration of amyl alcohols (total content of 2-methyl-1-butanol and 3-methyl-1-butanol) was reduced as the temperature became lower. 1-Propanol and isobutyl alcohol formation also dropped significantly below 15°C. The percentage of ethyl acetate increased as the temperature was reduced. At 5°C the content of total volatiles in the product was only 38% of the volatiles formed during fermentation at 30°C.

For the continuous process, ethanol productivities ranged from 3.6 to 8.3 g L^{-1} day^{-1} (Kourkoutas et al. 2002c). The continuous fermentation bioreactor was operated for 39 days, stored for 18 days at 4°C, and operated again for another 15 days without any diminution of the ethanol productivity. The concentrations of higher alcohols (1-propanol, isobutyl alcohol and amyl alcohols) were still low. The main volatile by-products formed in the continuous process were similar to those observed in alcoholic beverages, particularly ethyl acetate, and the fermented whey had a good aroma. The possibility of using such a process for the production of potable alcohol or a novel, low-alcohol content drink was proposed.

Several lactic acid bacteria (*Lactobacillus kefiranofaciens*, *Lactobacillus kefir*, *Lactococcus lactis* subsp. *lactis*), *Enterococcus durans* and yeasts (*S. italicus*, *S. unisporus*), all isolated from kefir-grains from Turkey and Yugoslavia, were immobilized in calcium alginate (Gobbetti and Rossi 1993). A continuous process for the production of a new kefir-like cultured milk was performed at 28°C for 30 days, with dilution rates of 0.03 and 0.06 h^{-1}, respectively, for free and immobilized cells. The pH values were 4.41 and 4.55 and the CO_2 and ethanol concentrations were 0.54 and 0.66 g L^{-1} and 4.50 and 4.58 g.L^{-1}, respectively. The synthesis of aroma compounds (diacetyl and acetoin) was reduced under the conditions of the continuous process, but it increased during storage at 4°C when these compounds reached about the same concentrations as in the traditional kefir. Despite lower values of viability the new cultured milk approached the characteristics of traditional kefir.

9.9 Aroma

In the field of aroma production or aroma enhancement very few applications of immobilized yeasts can be found. They are of two types.

The first one is the production of natural aroma compounds through biocatalysts. 2-Phenylethanol, which has a rose-like odour, can be produced from L-phenylalanine by *S. cerevisiae*. Unfortunately this product inhibits growth even at low concentration. This problem of the inhibitory effect of the product on the yeasts can be tackled by an in situ product removal technique: addition of an ester as a second water-immiscible phase in which partitioning of 2-phenylethanol is very favourable. Thus, the yeast is immobilized in chitosan alginate beads to protect them from the toxic extractant and the production of 2-phenylethanol can be increased compared with production using the conventional method with free cells (Stark et al. 2000).

Another process is based on a simplified double reaction for production of esters which can be added to foods or cosmetics. The first reaction is the production of ethanol by calcium alginate gel immobilized yeast cells and the second reaction is the synthesis of ethyl oleate in presence of oleic acid, in a lipase-catalysed reaction. In this two fluid-phase system the presence of lipase enzyme does not influence the cell growth, the glucose consumption and the concentration of ethanol in the water phase. However, 1 U mL^{-1} lipase in the water phase of the fermentation broth increased threefold the concentration of ethyl oleate in the oleic acid phase (Kiss et al. 1998).

An alternative approach to microbial production of bioflavours, eliminating the need for lengthy product purification, was presented by Kogan and Freeman (1994). It was

based on co-immobilization of precursors for bioflavour generation and microbial cells, traditionally employed for food and beverage processing, within beads made of a food-grade gel matrix. Following incubation under controlled conditions, the bioflavour – or bioflavour mixture – was generated and accumulated within the beads. The flavour-retaining beads might then be employed as a food additive. A feasibility study demonstrated this approach with ethanol production by baker's yeast co-immobilized with glucose medium. Complex bioflavour generation was also demonstrated by baker's yeast co-immobilized with apple juice, generating cider flavours. Beads providing beer taste were also readily made via co-immobilization of brewing yeast with malt.

To efficiently produce a fermented flavoured liquor for improving the taste of bread, a repeated batch fermentation was studied in a bioreactor with the use of immobilized yeast cells (Tamai et al. 1997). It contained about 5% (wt/v) ethanol and ethyl acetate, isoamyl alcohol, isobutyl alcohol as major flavouring component and organic acids with varying concentrations.

The second kind of application is related to the modification of aroma or taste of the product using immobilized yeasts. Several works deal with soy sauce. It can be produced with *Zygosaccharomyces rouxii* and *C. versatilis* immobilized on 4–6-mm porous aluminosilicate glass beads in a two-stage bioreactor (Horitsu et al. 1991). The fermentation time was 6 days. Experiments were also made encapsulating these yeasts in calcium alginate beads, but the fermentation ability was inadequate. A method to produce a soy-sauce-like condiment from a hydrolysed soy sauce raw material by a mixture of immobilized yeasts and immobilized lactic acid bacteria has been patented. *Pediococcus halophilus* and *S. rouxii* were immobilized separately in carragheenan beads (Yamasa Shoyu Co. 1985). The same volume of each kind of bead (60 mL) was then filled in a column (200 mL) and the soy sauce was passed through it in the ascending direction at 28°C for 48 h while flushing with nitrogen gas. A similar process has been tested in order to improve nutritive and organoleptic properties of Worcestershire sauce using immobilized *S. cerevisiae* to increase isoamyl alcohol, ethanol and ethylphenol content, the major aroma components of fermented Worcestershire sauce (Fujimoto et al. 1993).

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Chapter 10

Production of Antioxidants, Aromas, Colours, Flavours, and Vitamins by Yeasts

CHARLES A. ABBAS

ADM Research, JRRRC, 1001 Brush College Road, Decatur, IL 62521, USA (e-mail: abbas@admworld.com, Tel.: 217451-4220, Fax: 217-451-4230)

10.1 Introduction

The application of yeast and yeast-derived products by the food industry continues to be a rapidly growing area. Recent focus on improving human health through capturing greater value from yeast products has led to a rise in the recognition of the nutraceutical potential of many of the current products and has renewed emphasis on research that demonstrates the efficacy of new and existing products. Historically, yeast products have consisted of whole yeasts that are provided as yeast slurry or yeast paste, dried active yeasts, yeast autolysates, yeast extracts, separated components, such as protein isolates and amino acids, cell wall glucans and mannoproteins, vitamins, sterols, carotenoids, other lipids, enzymes, nucleic acids, polysaccharides, and chemically, physically, or enzymically modified components. Some examples of derivatives of yeast cell components are the chemically modified proteins (acylated, phosphorylated, encapsulated enzymes, immobilized enzymes, etc.), physically modified proteins that are partly denatured or texturized, enzymatically modified proteins that are re-partially digested by acid or enzymatic treatment or enzymatically modified proteins with covalently attached amino acids, nucleotides, and nucleosides, flavouring products and flavour substances, salt replacers, and substances or immobilized enzymes that are encapsulated inside the yeast for use as flavours and pharmaceuticals (Abbas 2001, 2003, 2004; Benítez et al. 1996; Halasz and Lasztity 1991; Peppler 1967, 1979; Reed 1981).

Yeast and yeast-derived products contribute to food flavour and aroma in a number of ways as added ingredients or as biocatalysts that carry out fermentation or biotransformation of food components thereby yielding a variety of products with desirable features. The use of yeast and yeast-derived products as bioflavouring agents and biocatalysts for edible meats, breads, other bakery products, cheeses, margarine flavours, yogurts, kefirs, other fermented dairy products, animal feeds, alcoholic beverages, fragrances, fruity flavours, soya-derived products, fermented cocoa beans, fermented tea, fermented vanilla beans, fermented syrups, pickles, ciders, vinegars, and a great variety of other fermented foods and beverages is well

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
© Springer-Verlag Berlin Heidelberg 2006

documented. Yeasts have also been tapped as sources of colorants, vitamins, antioxidants and as supplements for their nutraceutical or health-promoting attributes.

A number of yeast genera have found uses in the previously mentioned applications. These range from the widely used species and strains belonging to the genus *Saccharomyces*, to other genera such as *Candida*, *Debaryomyces*, *Geotrichum*, *Hansenula*, *Kloeckera*, *Kluyveromyces*, *Pichia*, *Schizosaccharomyces*, *Sporobolomyces*, *Yarrowia* and *Zygosaccharomyces*, to name only a few.

The primary goal of this chapter is to provide a background summary and an update on recent developments in this area with emphasis on the production of aromas, vitamins, antioxidants, colours, and flavours. It should be noted that these products are synthesized by metabolic pathways that parallel the biogenetic relationships of other naturally derived materials from plants and animals as illustrated in Fig. 10.1 (Sanderson 1978).

10.2 Background and Definitions

No serious overview of the topics selected can proceed without delineating its scope by providing the reader with concise definitions that can help assemble the products whenever possible into best-fitting groups. This task is made difficult as there is considerable confusion and overlap in the published literature, particularly when it comes to aromas and flavours produced by yeasts. For example, esters produced by yeasts can contribute to both aroma and flavour in the case of alcoholic and nonalcoholic fermentations. Therefore, in many cases of aromas and favours made by yeasts there is no clear or consistent distinction found in the literature. To a food scientist flavour primarily consists of two elements: (1) taste, which is perceived in the mouth and is mainly due to nonvolatile constituents present in food, and (2) aroma, which is perceived in the nose and is mainly attributed to volatile components

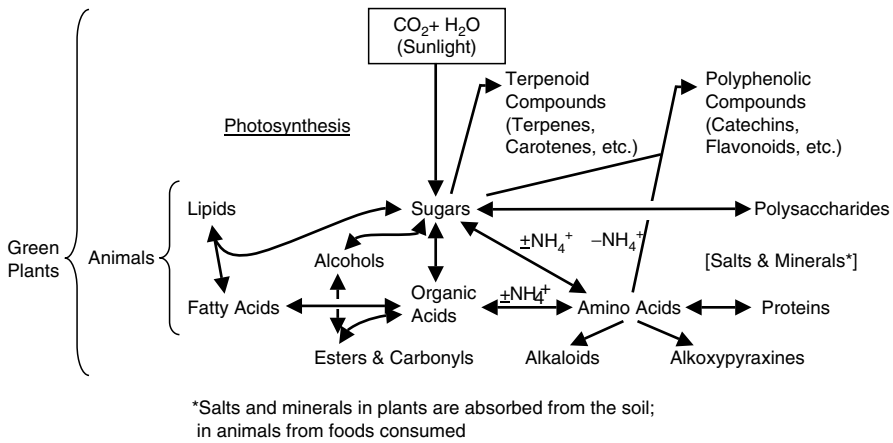

Fig. 10.1. Biogenetic relationship of compounds that comprise living organisms including those that serve as food materials. (Adopted from Sanderson 1978)

(Sanderson 1978). In sensory evaluation, descriptive aroma procedures have been developed which objectively and precisely define aroma as part of flavour (Noble 1978). While the biogeneses of the volatile and nonvolatile components of food are highly interrelated, it is well established that minor variations in a volatile component can lead to a major impact on flavour (Sanderson 1978). For the purpose of this chapter, aromas will be defined as esters, other fusel oil components, and other volatile products that are readily detected by smelling, while flavours will be defined as yeast and yeast-derived products that contribute to organoleptic properties or taste such as yeast extracts, nucleotides, organic acids, and polysaccharides and simple chemical compounds which include polyols such as inositol and glycerol. These products contribute as a whole or in part to the five primary recognized elements of food flavour: sweetness, saltiness, sourness, bitterness, and astringence (Sanderson 1978).

Providing working definitions for yeast-produced colours, vitamins, and antioxidants is a somewhat easier task as long as it is understood that some of the common yeast-derived or yeast-produced pigments may provide in addition to colour enhancement, antioxidant activity as is the case of astaxanthin, a salmon fish feed additive. A similar situation exists for the vitamin B_2, riboflavin, which can be listed as a vitamin as well as a food colorant and as an antioxidant. Keeping the aforementioned in mind, what follows is a summary of the topics under consideration.

10.2.1 Antioxidants

Yeasts and yeast extracts have been recognized as a source of antioxidant compounds for some time (Forbes et al. 1958). In the process outlined by Forbes et al., a 20% suspension of yeast is subjected to a series of organic extractions to yield a white crystalline preparation that can be added to prevent oxidation of food products such as fats, baby foods, whole milk, or milk products. More recently, the increased interest in natural antioxidants has given rise to the screening of microbial sources for compounds to replace the synthetic compounds currently in use as food antioxidants. Natural antioxidants can also be used in nutraceutical applications as supplements (Gazi et al. 2001; Nishino and Ishikawa 1988). Natural oxidants are presumed to be safer for human beings (Gazi et al. 2001). The function of antioxidants and the exact mechanism of their mode of action are still open to conjecture, but it is generally agreed that they act by donating hydrogen protons to substrates, thereby rendering them nonreactive to oxygen-derived free radicals that are referred to as reactive oxygen species or ROS (del Rio et al. 2003; Heath 1981). It is hypothesized that yeast peroxisomes play a similar role to plant peroxisomes. Therefore the response in yeasts to oxygen-derived radicals would involve several enzymes, including catalases, superoxide dismutases (SOD), glutathione (L-γ-glutamyl-L-cysteinylglycine), as well as several NADP-dependent dehydrogenases (del Rio et al. 2003). It is well established that antioxidants are inactivated in the process and that their activity is proportional to the quantity used based on their permitted usage level (Heath 1981).

Yeasts synthesize a number of bioactive compounds which can serve as antioxidants. These have found numerous uses in foods to retard oxidative degeneration of

fatty substances and in nutraceutical supplements to improve health and well-being (Bastin et al. 2002; Doll 2002; Gazi et al. 2001; Ok et al. 2003). They consist of the oxygenated carotenoid torulahodin, both the organic acid and the salt forms of citric acid, coenzyme Q or ubiquinone, glutathione, hydroxymethyl and hydroxylethyl furanone (2*H*), tocotrienol, α-tocopherols (α-TOHs) and other forms of tocopherols, riboflavin (vitamin B_2) and the flavins derived from it, FMN and FAD, and 2,4-hydroxyphenyl ethanol (Cremer et al. 1999; Do et al. 1996; Imai et al. 1989; Kawamukai 2002; Padilla et al. 2004; Penninckx 2002; Sugawara 2001; Suzuki et al. 2003). Other factors that are produced by yeasts and/or that are present in yeast-fermented products or in yeast cell biomass at the end of fermentation that have antioxidant activity or free-radical scavenger activity include several other oxygenated carotenoids, selenium-enriched yeast cells, the wine component resveratrol, octacosanol, yeast-derived cell wall β-glucans, uncharacterized soluble proteins that are produced in yeasts under oxidative stress, sulphur-containing amino acids, cytochrome c, the yeast enzyme Cu,Zn-SOD, and products of SOD genes, CUP1 and SOD1 (An 1996; Archibald 2003; Becker et al. 2003; Combs et al. 2002; Farid and Azar 2001; Forbes et al. 1958; Forman et al. 1983; Imai et al.1989; Lee and Park 1998a, b; Marova et al. 2004; Mast-Gerlach and Stahl 1997; Park 2003; Sakaki et al. 2001, 2002; Shitazawa et al. 2002; Visser et al. 2003).

Glutathione plays an important role as an antioxidant. The depletion of glutathione leads to the accumulation of ROS following the treatment of *Saccharomyces cerevisiae* with the pungent sesquiterpenoid unsaturated dialdehyde, polygodial (Machida et al. 1999). Polygodial exhibited a strong yeasticidal activity against cells of *S. cerevisiae*, in which production of ROS at a significant level could be detected with a fluorescent probe. The production of ROS in polygodial-treated cells was further confirmed by its elimination and the accompanying protection against yeasticidal effects in the presence of antioxidants such as L-ascorbate and α-TOH. Polygodial could accelerate ROS production only in cells of the wild-type strain but not in those of a respiratory-deficient petite mutant (rho0), indicating the role of the mitochondrial electron transport chain in the production of ROS. Unlike antimycin A, which accelerates ROS production by directly targeting the mitochondrial electron flow, polygodial caused depletion of cytoplasmic and mitochondrial glutathione, which functions in eliminating ROS that is generated during aerobic growth. It was hypothesized that the polygodial-mediated depletion of intracellular glutathione was possibly dependent on a direct interaction between its enal moiety and the sulphydryl group of the cysteine in glutathione by a Michael-type reaction. The breeding of high-glutathione-producing strains and their production by fermentation has been described recently (Liu et al. 2003; Sakato 1992; Shimizu et al. 1991; Udeh and Archremowicz 1997; Wei et al. 2003).

Increasing the level of Cu,Zn-SOD in beer was attempted through the genetic engineering of a beer brewing strain of *S. cerevisiae* yeast by the cloning of the Cu,Zn-SOD gene coupled to an α-factor leader (Cremer et al. 1999). The protein product was secreted by the transformed strain (RHS1) and the transformants were active in catalysing the reaction with superoxide. The enzymic activity of SOD was assayed directly and by determining antioxidant activity. The quantity of SOD secreted was insufficient for fermentation processes. Additional expression of the

protein may be achieved by further optimization of the transformation process (e.g. change of the promoter region) and by the use of various recipient cultures.

The antioxidative role of coenzyme Q or ubiquinone in yeasts is well established. Ubiquinone is an essential component of the electron transfer system in both prokaryotes and eukaryotes and is synthesized from chorismate and polyprenyl diphosphate in eight steps. The enzyme *p*-hydroxybenzoate (PHB) polyprenyl diphosphate transferase catalyses the condensation of PHB and polyprenyl diphosphate in ubiquinone biosynthesis. The gene for PHB polyprenyl diphosphate transferase (designated ppt1) was isolated and the gene was disrupted in a strain of the fission yeast *Schizosaccharomyces pombe* (Uchida et al. 2000). This strain could not grow on minimal medium supplemented with glucose. The expression of COQ2 from *S. cerevisiae* in the defective *S. pombe* strain restored growth and enabled the cells to produce ubiquinone-10, indicating that COQ2 and ppt1 are functional homologues. The ppt1-deficient strain required supplementation with antioxidants, such as cysteine, glutathione, and α-TOH to grow on minimal medium. This supports the role of ubiquinone as an antioxidant and the observation that the ppt1-deficient strain is sensitive to H_2O_2 and Cu^{2+}. The ppt1-deficient strain produced a significant amount of H_2S. Thereby the oxidation of sulphide by ubiquinone may be an important pathway for sulphur metabolism in *S. pombe*. Ppt1-green fluorescent protein fusion proteins localized to the mitochondria, indicating that ubiquinone biosynthesis occurs in the mitochondria in *S. pombe*. Thus, analysis of the phenotypes of *S. pombe* strains deficient in ubiquinone production clearly demonstrated that ubiquinone has multiple functions in the cell apart from being an integral component of the electron transfer system.

The formation of several antioxidants can be induced in yeasts grown under stressful conditions or in response to fermentation medium ingredients such as phenolics or additives that are known to be toxic to cells grown aerobically (Cruz et al. 1999; Fung et al. 1985; Larsson et al. 2000; 2001; Millati et al. 2002; Wang et al. 2001). In another variation, synthetic antioxidants are added to fermentation media during the production of fodder yeast to stimulate aerobic growth and to increase cell biomass (Pobedimskii et al. 1998; Larsson et al. 2000). The screening of yeasts for free-radical-scavenging activity is an active area of research (Gazi et al. 2001). The recent publication by Gazi et al. (2001) describes the screening of 25 yeast strains that were cultivated in yeast peptone dextrose broth (YPD) and in yeast malt extract broth (YMB) media under both shaking and stationary conditions. This was followed by measuring the decrease of absorbance at 517 nm of a solution of 1,1-diphenyl-2-picrylhydrazyl after mixing using the supernatant of each cell culture. It was found that all strains tested are capable of producing the activity in at least one condition. Among the tested strains, *Hansenula anomala* (134 units/mL) produced the highest activity during YPD shaking culture. On the other hand, *Rhodotorula glutinis* (199 units/mL) produced the highest activity during YMB stationary culture.

In a recent patent, Nishino and Ishikawa (1998) describe antioxidants that can be used in pharmaceutical, cosmetic, and food applications. These antioxidants comprise whole yeast cells, their cultured products, or their extracts and are selected from a number of genera and yeasts that consist of *Candida gropengiesseri*, *C. parapsilosis*, *C. maltosa*, *C. stellata*, *C. tropicalis*, *Hansenula holstii*, *Hyphopichia*

burtonii, *Pichia membranaefaciens*, *R. glutinis*, *R. minuta*, *R. rubra*, *S. bayanus*, *S. elegans*, *S. unisporus*, *Torulopsis magnoliae*, and *Zygosaccharomyces bisporus*. In this patent, the antioxidant activities of microorganisms were evaluated by adding microorganisms to both Rose Bengal (I) containing medium and in a dye-free medium, culturing yeast under light irradiation, and measuring the viable count or the turbidity of each medium. The antioxidants disclosed inhibit oxidation of low-density-level lipids and are useful as antiarteriosclerotics. A lotion was prepared by combining a 15 mL EtOH extract of *C. parapsilosis* ATCC 6295, 2 g poly(oxyethylene) hydrogenated castor oil, 3 mL 1,3-butylene glycol, 0.2 g perfume, 0.2 g antiseptic, and H_2O to 100 mL.

Another recent publication, by Kakizono et al. (2003), outlines an efficient method for screening antioxidant high-production yeast cells comprising performing a mutation treatment upon a group of cells, reacting the cells with a redox fluorescent indicator capable of generating fluorescence upon being oxidized by ROS, detecting the fluorescence generated from the intracellular fluorescent indicator with an optical detector upon irradiating with excitation light, and isolating the cells possessing the relatively low fluorescence with a fractionation mechanism (Kakizono et al. 2003). This method enables the selection of high-antioxidant-producing cells which appear normally with an extremely low frequency.

A recent patent application describes the isolation and purification of a natural antioxidant compound from natural sources including yeasts [*S. carlsbergenesis*, *S. cerevisiae*, or from a commercially available yeast extract, and saltbush (*Atriplex halimus*)] that can be synthesized chemically, by processes that improve the potency of the product (Mirsky et al. 2001). These antioxidants when used with/without chromium can be formulated for use in animals and humans.

Bio-Catalyzer α-ρ no. 11 (Bio-Normalizer) or BN is a complex natural health food product prepared by yeast fermentation of medicinal plants or unripe papaya fruits which has been reported to possess antioxidant properties (Afanas'ev et al. 2000; Haramaki et al. 1995). The effects of BN have been compared in vivo and in vitro with those of some classical antioxidants to determine their protective properties against free-radical-mediated damage of erythrocytes of thalassemic patients as well as damage to liver, and to peritoneal macrophages of iron-overloaded rats. The principal difference between the protective mechanisms of BN and rutin was observed only in thalassemic cells. Rutin was able neither to remove iron from cells nor to affect haemoglobin oxidation. Thus, rutin's antioxidant effect seems to depend exclusively on its oxygen radical scavenging activity. To better understand the effects of orally administered BN on oxidative damage in the rat heart, the BN-supplemented animals were (1) exposed to ischemia-reperfusion using the Langendorff technique or (2) homogenized and exposed to peroxyl radicals generated from 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). During reperfusion following 40 min of ischemia, leakage of lactate dehydrogenase from hearts isolated from BN-supplemented rats was significantly lower than from hearts of control animals. Furthermore, lower levels of AMVN-induced accumulation of thiobarbituric acid reactive substances and of protein carbonyl derivatives were detected in homogenates prepared from hearts isolated from BN-supplemented rats than in samples from control animals. The findings confirm an antioxidant action of BN

and show that it protects the heart against ischemia-reperfusion-induced damage. From these results, it is clear that yeasts generally have radical scavenging activity and are a good source of potent natural antioxidants.

10.2.2 Aromas

During fermentation yeasts synthesize a vast number of aroma and flavour compounds (Fig. 10.2; Berry 1995; Suomalainen and Lehtonen 1979). The numerically and quantitatively largest groups of aroma compounds synthesized by yeasts consist of fusel alcohols, fatty acids, and their esters (Suomalainen and Lehtonen 1978, 1979). These are generally compounds with a molecular weight of less than 300 (Noble 1978). It has been shown that these are primarily due to yeast metabolism since significant differences in their production have been demonstrated by the use of different yeast genera, species, and strains. In addition to the choice of yeast, several factors contribute to aroma production. These include changes in fermentation conditions such as temperature, pH, aeration, agitation, and the nature and concentration of the substrate utilized (Suomalainen and Lehtonen 1979). Yeast-derived aromas or odours are primarily products of the application of yeasts for the production of alcoholic and nonalcoholic beverages such as beer, wine, sherry, sake, brandy, spirits such as rum and whisky, and other fermented beverages. Modern aroma research has revealed that the complex aroma of alcoholic beverages involves over 400 different chemical compounds which include acids, esters, carbonyl compounds,

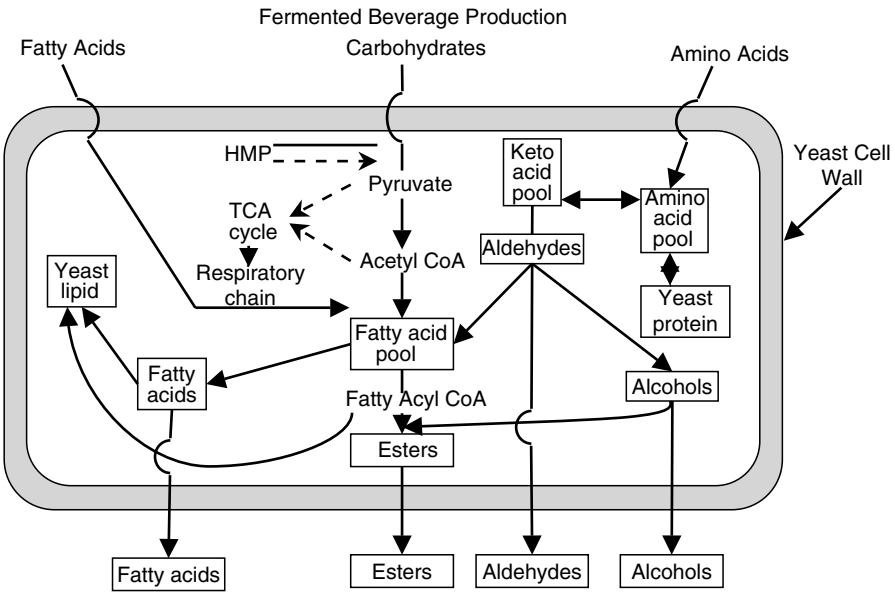

Fig. 10.2. Basic routes by which yeasts form the major flavour groups during fermentation. (Adopted from Ramsay 1982)

acetals, phenols, hydrocarbons, nitrogen compounds, sulphur compounds, lactones, sugars, and a variety of other unclassified compounds that are listed in Table 10.1 in addition to alcohols (Berry 1989, 1995; Dickinson 2003; Garafolo 1992; Suomalainen and Lehtonen 1978, 1979). The unique aromas of the many alcoholic beverages produced by yeasts with some exceptions are frequently the result of a pattern or specific ratios of the previously listed components rather than caused by the presence or absence or a specific concentration of one or a few components (Noble 1978). Yeasts also contribute significantly to the aroma of fermented foods such as breads, cheeses, other dairy products, fermented meat products, vanilla, cocoa, and fermented soy-derived foods. Since many of the aroma compounds that are associated with alcoholic beverages are also present in other fermented foodstuffs, I have chosen to combine all of these into six groups.

10.2.2.1 Fusel Alcohols

Fusel alcohols comprise the largest group of aroma compounds in alcoholic beverages. Their concentration varies considerably in spirits, with rums containing an average of 0.6 g/L, whiskeys about 1.0 g/L and brandies about 1.5 g/L (Dickinson 2003; Suomalainen and Lehtonen 1978, 1979). The main fusel oil synthesized by yeasts is isoamyl alcohol, with *n*-propyl alcohol, isobutyl alcohol, phenethyl alcohol, tryptohol, and optically active amyl alcohol as the other long-chain and complex alcohols (Dickinson 2003; Etschmann et al. 2003; Fabre et al. 1997, 1998; Kunkee et al. 1983; Mo et al. 2003; Pan and Kuo 1993; Suomalainen and Lehtonen 1978, 1979; Ter Schure et al. 1998). Several theories have been proposed to explain the formation of fusel alcohols by yeasts, the oldest of which is Ehrlich's so-called catabolic derivation from exogenous amino acids such as leucine, isoleucine, valine, and threonine (Dickinson 2003; Suomalainen and Lehtonen 1979, 1978; Ter Schure et al. 1998). This theory is

Table 10.1 The number of aroma compounds identified in alcoholic beverages

Compound	Number
Alcohols	38
Acids	80
Esters	118
Carbonyl compounds	41
Acetals	17
Phenols	41
Hydrocarbons	11
Nitrogen compounds	18
Sulphur compounds	11
Lactones	17
Sugars	4
Unclassified compounds	11
Total	407

Adopted from Kahn (1969)

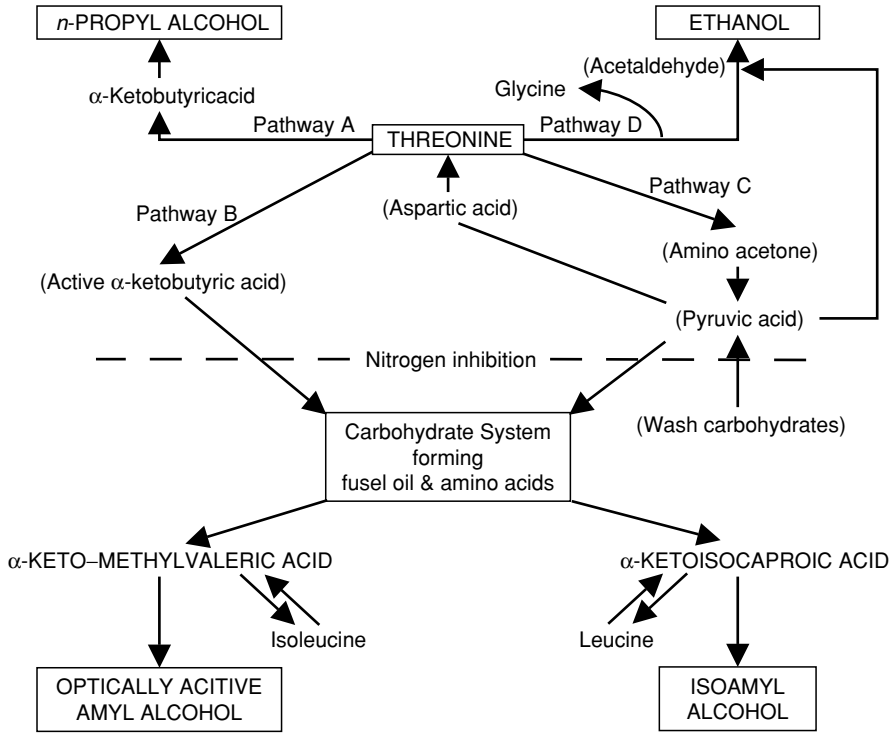

Fig. 10.3. Biochemical relationships of threonine and isoleucine in the formation of fusel alcohols. (Adopted from Reazin et al. 1973)

consistent with more recent work which provides evidence that *S. cerevisiae* grown on isoleucine forms primarily optically active amyl alcohol with significant additional production of *n*-propyl alcohol and isoamyl alcohol when cells are grown on threonine (Suomalainen and Lehtonen 1979). The biochemical pathways describing the production of these alcohols from amino acids is provided in Fig. 10.3 (Suomalainen and Lehtonen 1979). The constitutive expression of the BAP gene which codes for the permease involved in branched amino acid transport has been demonstrated to increase the production of isoamyl alcohol derived from leucine but no comparable increase in isobutyl alcohol derived from valine or amyl alcohol derived from isoleucine was observed (Kodama et al. 2001). The genes for two aminotransferases (ECA39 and ECA40) that actively participate in the transamination of branched amino acids in the mitochondria and cytoplasm have been deleted in order to determine their role in the formation of higher alcohols (Eden et al. 2001). Deletion of the ECA39 and ECA40 genes had little impact on the production of propanol, while the deletion of ECA40 had a drastic impact on the production of isobutyl alcohol and partial impact on the production of active amyl and isoamyl alcohol (Eden et al. 2001). The production of isoamyl acetate in *S. cerevisiae* is regulated at the co-transcriptional level of two genes involved in cytosolic branched-chain amino acid aminotransferase

and L-leucine biosynthesis (Yoshimoto et al. 2002). These findings provide added support to the findings that the catabolic pathway from amino acids fails to explain the formation of fusel alcohols by yeasts grown on carbohydrates with inorganic nitrogen sources or under nitrogen limitation. Therefore, the anabolic formation route from sugars first proposed by Ayräpää provides a plausible alternate explanation (Suomalainen and Lehtonen 1979). Thus, the formation of fusel alcohols in yeasts involves both degradative and synthetic pathways as illustrated in Fig. 10.4 (Suomalainen and Lehtonen 1979). Evidence indicating a high level of specificity of the enzymes involved in the catabolic and anabolic pathways points to the complex operation and regulation of yeast genes involved in the formation of fusel oils as summarized in a recent review by Dickinson (2003). Gaps in the current knowledge of the genetics and biochemical basis for fusel alcohol formation in yeasts will continue to provide the impetus for more research since its delineation will have many practical applications in the production of alcoholic beverages.

10.2.2.2 Fatty Acids

Fatty acids with a carbon chain length ranging from C3 to C16 represent another major group of aroma compounds that are synthesized by yeasts during alcoholic fermentations (Suomalainen and Lehtonen 1979). An important component of alcoholic beverage aroma is provided by the shorter-chain volatile fatty acids such as propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, caproic

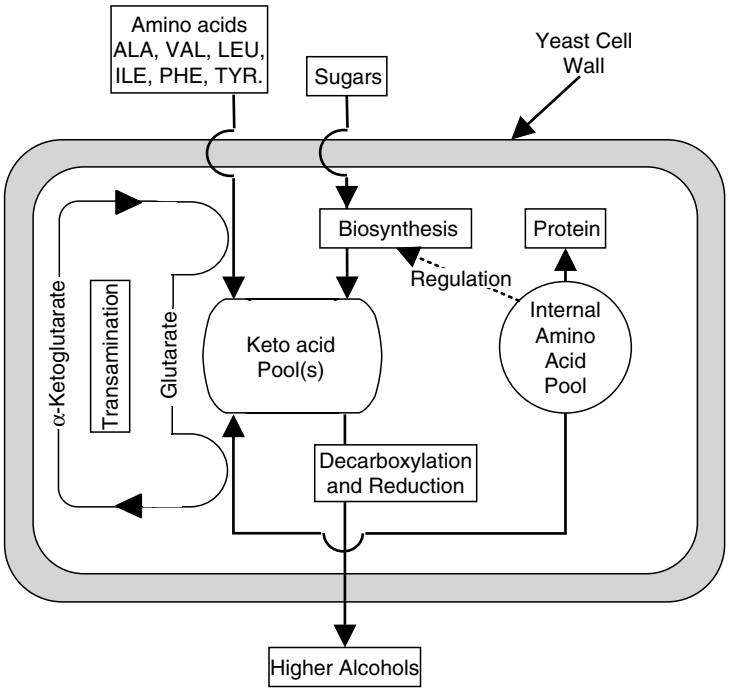

Fig. 10.4. The formation of fusel alcohols. (Adopted from Äyräpää 1973)

or hexanoic acid, caprylic or octanoic acid, and capric acid. These fatty acids are synthesized by yeasts irrespective of the raw material used but with noticeable clear differences in the relative proportions of these acids in the various alcoholic beverages (Edwards et al. 1990; Suomalainen and Lehtonen 1978, 1979). The differences in the relative amounts in the main volatile fatty acids in three spirits are provided in Table 10.2 (Suomalainen and Lehtonen 1979). The acid content of alcoholic beverages varies considerably, with low levels reported for Scotch whisky and cognac, which contain levels in the range 100–200 mg/L, when compared with heavy rum or wines, which tend to have significantly higher levels of volatile acids reported in the range 500–1,000 mg/L (Suomalainen and Lehtonen 1979).

In yeasts, the synthesis of fatty acids begins with acetyl coenzyme A (CoA) formed from the oxidative decarboxylation of pyruvate obtained from glycolysis and involves a multienzyme complex which binds all of the intermediates until the fatty acids are formed (Suomalainen and Lehtonen 1979). This synthesis is illustrated in Fig. 10.5 (Suomalainen and Lehtonen 1979), which provides a scheme for the formation and elongation of fatty acids that leads to even- or odd-numbered chains depending on whether acetyl-CoA or propionyl-CoA is the starting material. The way in which yeasts influence the fatty acid composition of alcoholic beverages is not known. The yeast strain used, the composition of the medium, the temperature, and the aeration employed influence to a great extent the final levels of fatty acids, carbon chain length, and level of saturation (Suomalainen and Lehtonen 1979). At lower temperatures, yeasts synthesize a larger amount of fatty acids when compared with the amounts synthesized at higher temperatures, with more of the unsaturated fatty acids being synthesized under aerobic and semiaerobic conditions. For example, the amount of the fatty acids caproic and caprylic secreted into the fermentation medium at 10°C by *S. cerevisiae* is higher than that secreted at 30°C, whereas

Table 10.2 Relative amounts of the main volatile fatty acids, excluding acetic acid, in the acid fractions of Martinique rum, Scotch whisky and cognac

Acid	Martinique rum (%)	Scotch whisky (%)	Cognac (%)
Propionic	15.7	1.5	2.7
Isobutyric	3.6	4.9	3.6
Butyric	15.3	1.5	3.6
Isovaleric	4.7	5.9	3.3
Valeric	6.5	0.1	Traces
Caproic	5.4	4.2	8.2
Caprylic	14.5	26.7	35.0
Capric	17.5	31.6	30.4
Lauric	6.5	16.2	8.6
Myristic	1.1	2.2	1.6
Palmitic	4.0	1.7	1.1
Palmitoleic	1.0	2.0	0.5
Others	4.2	1.5	1.4

Adopted from Nykanen et al. (1968)

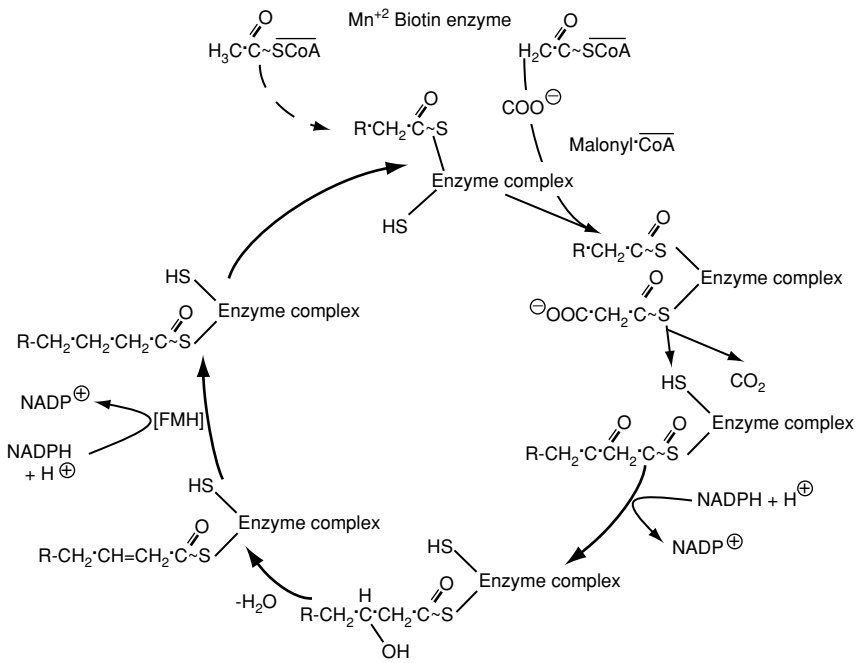

Fig. 10.5. The formation of fatty acids. (Adopted from Lynen 1967)

that of capric and lauric acids is independent of the fermentation temperature (Suomalainen and Lehtonen 1979). When C6–C10 fatty acids are present in beer in levels that exceed 1 mg/L, they contribute to what is known as a "caprylic flavour" characterized by a musty, rancid, or soapy odour (Tressl et al. 1980).

10.2.2.3 Fatty Acid Esters

Fatty acid esters are by far the most prevalent group of aroma compounds produced by yeast fermentation (Buzzini et al. 2003; Cristiani and Monnet 2001; Suomalainen and Lehtonen 1978, 1979; Verstrepen et al. 2003). The ester content of alcoholic beverages is reflected in the perception of their aromas as demonstrated by comparing light rums which have an ester content of 50 mg/L with heavier variety of rums which contain greater than 600 mg/L (Suomalainen and Lehtonen 1979). Fatty acid esters of alcoholic beverages are classified into three major fractions: light, middle, and heavy on the basis of their boiling-point ranges (Suomalainen and Lehtonen 1979). The light fraction consists of fruit esters with pleasant aromas which include all esters that have a boiling point lower than that of isoamyl alcohol such as ethyl, isobutyl and isoamyl esters of short-chain fatty acids (Suomalainen and Lehtonen 1979). The middle fraction comprises ethyl esters that elute during distillation between ethyl caproate and phenethyl alcohol. These include primarily ethyl esters

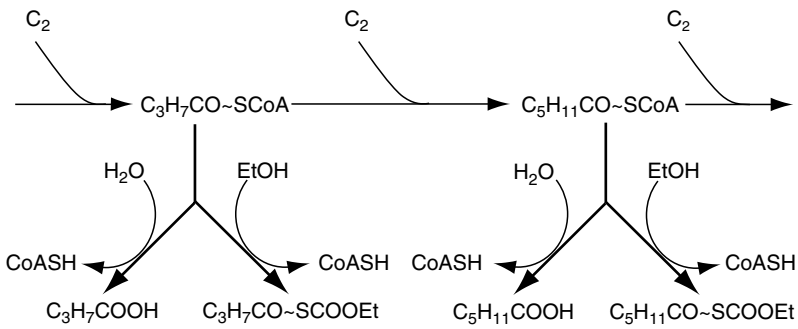

Fig. 10.6. Simplified scheme for the formation of esters and higher fatty acids (Adopted from Nordström 1964)

of caprylic and capric acids. The main components of the heavy distillate fraction are all other esters that elute after phenethyl alcohol (Suomalainen and Lehtonen 1979). The biosynthesis of esters is similar to that of fatty acids and is illustrated in Fig. 10.6 (Suomalainen and Lehtonen 1979). An increase in ethyl esters of caprylic, capric, and palmitoleic acids is noted if the yeast is present during distillation (Suomalainen and Lehtonen 1979; Verstrepen et al. 2003).

During fermentation in yeasts, the formation of esters is carried out by intracellular enzyme-catalysed reactions. The expression level of two genes coding for the yeast alcohol acetyltransferases, ATF1 and ATF2, was shown to influence the production of ethyl acetate and isoamyl acetate (Verstrepen et al. 2003, 2004). Double deletion of the former two genes resulted in partial reduction in the two previously mentioned esters, suggesting the existence of as-yet-unknown other ester synthases (Verstrepen et al. 2003). Ester production by yeasts is dependent to a great extent on the composition of the carbon source and the type of assimiliable nitrogen in the medium used, the fermentation parameters selected, such as pitching rate and top pressure, dissolved oxygen, as well as the genus, species, and strain employed (Verstrepen et al. 2003).

Several fatty acid esters produced by yeasts during alcoholic fermentations have found other applications and an example of this is the use of the short-chain acetate esters of ethyl acetate as a solvent, of isoamyl acetate as a banana food flavour, and of phenyl ethyl as a flowery or rose aroma enhancer in cosmetics and foods (Armstrong 1986; Ashida et al. 1987; Fabre et al.1998; Fukuda et al. 1990a, 1998a, b; Fujii et al. 1996; Furukawa et al. 2003a; Janssens et al. 1987; Quilter et al. 2003; Verstrepen et al. 2003). Screening a selected yeast for 2-phenylethanol production in a molasses-containing medium supplemented with phenylalanine has shown that *Kluyveromyces marxianus* CBS 600 and *K. marxianus* CBS 397 can produce up to 3 g/L at 35°C (Etschmann et al. 2003; Fabre et al. 1997, 1998). The production of the important food and cosmetic aroma compound ethyl oleate can also occur through enzymic catalysis by using immbolized cells of *S. bayanus* to produce ethanol in the presence of oleic acid in a fermentation medium (Kiss et al. 1998). The increased synthesis of ethyl caproate by *S. cerevisiae*, which is an important aroma and flavour compound in sake, has been attributed to inositol limitation

(Arikawa et al. 2000; Furukawa et al. 2003b). Fatty acid ethyl ester synthesis by *S. cerevisiae* is also important in the development of Scotch whisky aroma and flavour (Goss et al. 1999). The release of fatty acids and the production of medium-chain fatty acids and their ethyl esters in the absence of exogenous lipids by yeast strains isolated from musts and wines in the absence of aeration has recently been described (Bardi et al. 1999; Ravaglia and Delfini 1993). Medium-chain fatty acids and their esters are responsible for stuck alcoholic fermentation and their formation is strongly dependent on the yeast strain used and the fermentation medium (Ravaglia and Delfini 1993).

10.2.2.4 Carbonyl, Sulphur, and Phenolic Compounds

Carbonyl, sulphur, and phenolic compounds are other types of yeast fermentation by-products that impact the aroma of alcoholic beverages (Suomalainen and Lehtonen 1979). Owing to their lower sensory thresholds, these compounds are of particular interest as they can contribute to undesirable odours in alcoholic beverages such as beer (Arai 1980; Russell et al. 1983; Suomalainen and Lehtonen 1979; Tressl et al. 1980). The carbonyl compounds of great interest are the aldehydes that are intermediates in the formation of fusel alcohols. Diacetyl and 2,3-pentanedione are formed during fermentation from the decarboxylation of the two α-keto acids, α-acetolactic and α-aceto-α-hydroxybutyric acid, respectively (Suomalainen and Lehtonen 1979). These compounds, illustrated in Fig. 10.7, contribute to a buttery aroma in beer (Tressl et al. 1980). Sulphur compounds derived from the degradation of the amino acids cysteine and methionine lead to the formation of off-flavours in beer, such as the offensive smelling hydrogen sulphide, diethyl sulphide, and dimethyl sulphide (Suomalainen and Lehtonen 1979; Tressl et al. 1980; Van Haecht and Dufour 1995). Sulphites produced by yeasts during alcoholic fermentation can also have a positive effect as they act as antioxidants and flavour stabilizers (Hansen and

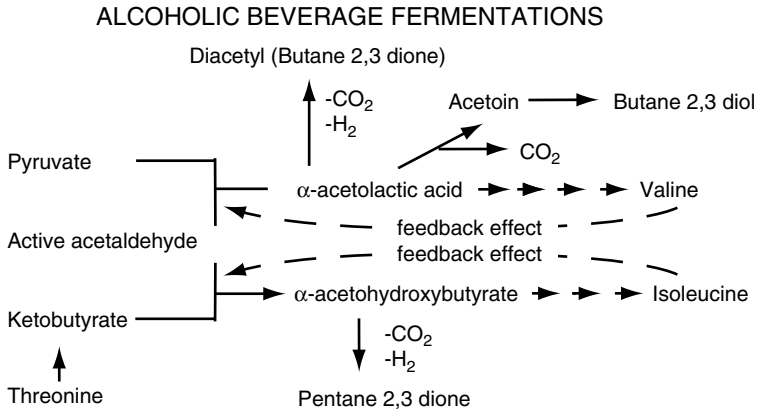

Fig. 10.7. Reduction of the level of diacetyl in wort by reduction of α-acetolactic acid through a feedback mechanism. (Adopted from Berry 1995)

Kielland-Brandt 1996). Inactivation of the MET2 gene which encodes the enzyme homoserine *O*-acetyltransferase increases the level of sulphite in beer (Hansen and Kielland-Brandt 1996). The constitutive expression of MET25 which codes for the enzyme *O*-acetylserine sulphydrylase leads to a reduction in the off-flavour compound hydrogen sulphide (Omura et al. 1995). Phenolic compounds produced during alcoholic yeast fermentations are derived from the catalysis of *p*-coumaric acid, ferulic acid, and vanillin, which are typically of plant origin (Ettayebi et al. 2003; Meaden 1998; Suomalainen and Lehtonen 1979). The products formed by yeast action consist of 4-ethylphenol, 4-ethylguaiacol, and 4-methyl guaiacol as shown in Fig. 10.8 (Dias et al. 2003; Meaden 1998; Suomalainen and Lehtonen 1979). These compounds, while desirable in low levels, can lead to phenolic off-flavours when present in levels in excess of 100 ppb in beer (Tressl et al. 1980). The production of phenolic off-flavours by wine yeast strains of *S. cerevisiae* and other yeast genera such as *Rhodotorula*, *Candida*, *Cryptococcus*, *Pichia*, *Hansenula*, *Dekkera*, and *Brettanomyces* that contribute to phenolic off-flavours in wine products (Coghe et al. 2004; Edlin et al. 1995; Shinohara et al. 2000). Mousy off-flavour production in grape juice and in red and white wines attributed to the yeast *Dekkera* (*D. bruxellensis* and *D. anomala*) and *Brettanomyces* yeasts has been recently described by Grbin and Henschke (2000) and by Dias et al. (2003).

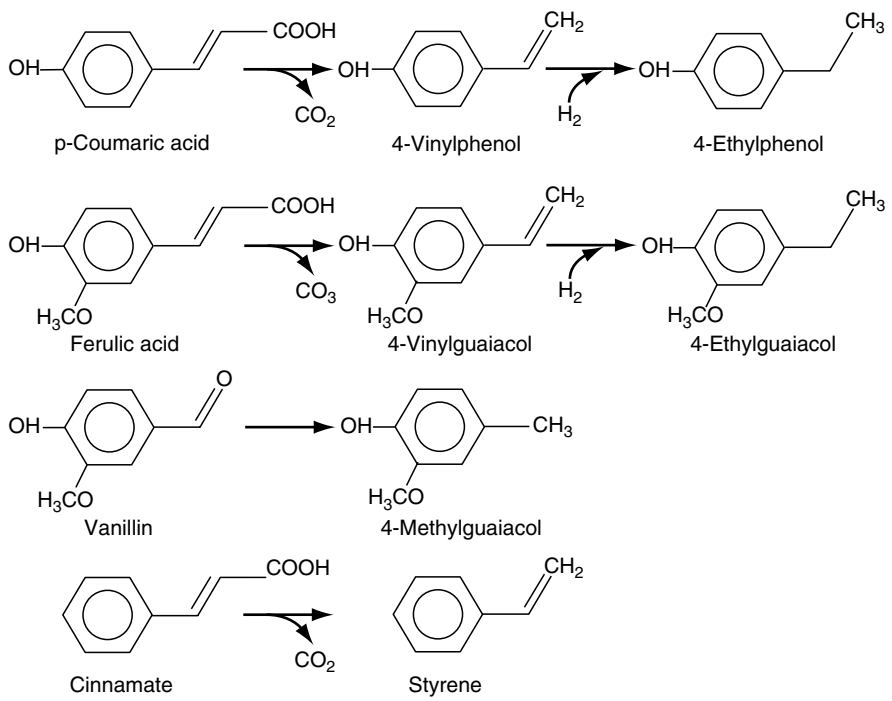

Fig. 10.8. The decarboxlyation of cinnamic acids by *Saccharomyces cerevisiae*

10.2.2.5 Lactones

Lactones are an important group of compounds that are of interest to the food industry as they impart highly pleasant ripened fruit aromas characteristic of peach, apricot, or coconut when added to over 120 foodstuffs (Dufosse et al. 2000, 2002; Endrizzi et al. 1996; Feron 1997; Gatfield et al.1993; Groguenin et al. 2004; Heath 1981; Vandamme 2003; Vanderhaegen et al. 2003; Van Der Schaft et al. 1992; Wache et al. 2003). Lactones are made up of a carbon ring with an oxygen atom and are formed by intramolecular esterification between the hydroxyl and carboxylic group of a hydroxy fatty acid as illustrated in Fig. 10.9. The recognition that the pigmented yeast *Sporobolomyces odorus* can synthesize the lactones responsible for the peach-like odour, γ-decalactone (GC10) and dodecenolactone, has given rise to interest in the de novo microbial production of these compounds from the readily available, natural ricinoleic hydroxy fatty acid (Endrizzi et al. 1996). This hydroxyacid represents over 90% of castor bean oil and thereby provides an economical source as a feedstock which can compete with the chemical synthetic route (Endrizzi et al. 1996). While several yeasts can carry out the aforementioned biotransformation most recent focus has been on using three yeasts *Yarrowia lipolytica*, *Sporidiobolus salmonicolor* (*Sporidiobolus odorus*), and *Sporidiobolus ruinenii*, with reported production of GC10 in the range 0.4–1.2 g/L (Groguenin et al. 2004; Dufosse et al. 2000, 2002; Feron et al. 1997). These levels are below the 5.5 g/L reported earlier for *Y. lipolytica* (Farbood and Willis 1985). A schematic representation for the pathway for the key acyl oxidase enzymes involved in the production of GC10 from ricinoleic acid in *Y. lipolytica* is provided in Fig. 10.10 (Groguenin et al. 2004). The key challenges to the further improvement of industrial processes with the three yeasts are the toxicity, yield, and final concentration and composition of the lactones produced. Several strategies can be employed to overcome these challenges, ranging

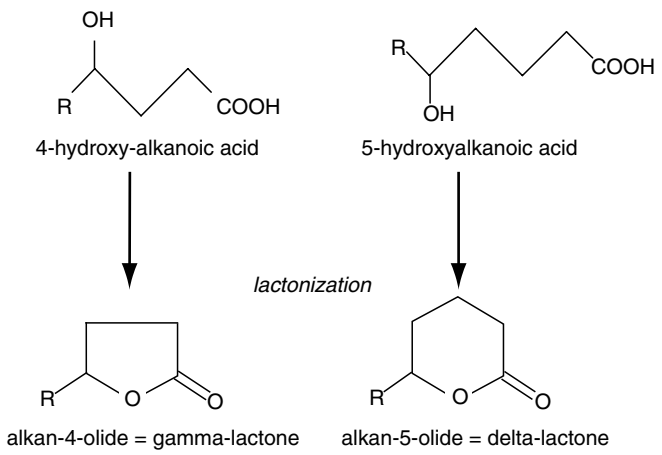

Fig. 10.9. Structure of γ- and δ-lactones and of corresponding hydroxy acids. (Adopted from Groguenin et al. 2004)

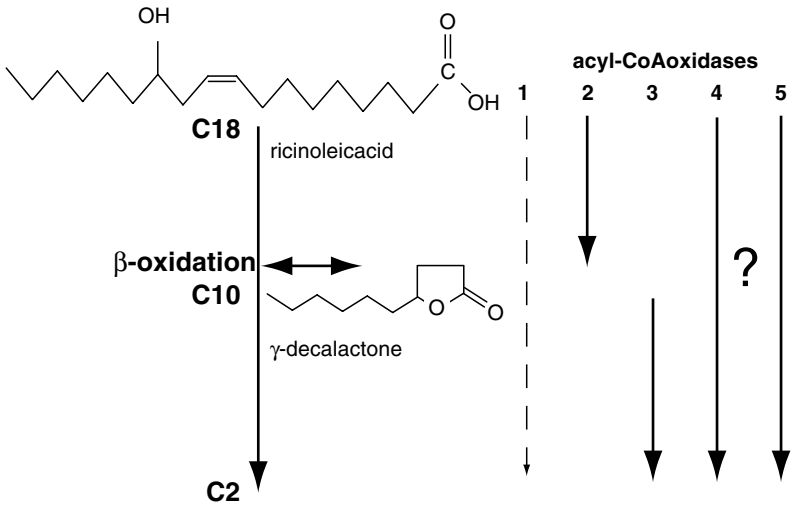

Fig. 10.10. Schematic representation of the pathway from ricinoleic acid to γ-decalactone and activities of the acylcofactor A (*acyl-CoA*) oxidases of *Yarrowia lipolytica*. (Adopted from Endrizzi et al. 1996)

from genetic engineering and/or modification of yeast strains, use of batch vs. fed-batch fermentation, to the continuous extraction of the GC10 decanolide with the use of a membrane based solvent-extraction technique (Akita and Obata 1991).

10.2.2.6 Other Chemical Compounds

Other chemical compounds that contribute to the aroma of yeast-fermented products include breakdown products from sugars such as the furanones, furfural and hydroxymethyl furfuryl, and their alcohol derivatives, production of chiral alcohols from prochiral ketones, volatile organic acids such as acetic acid, which imparts a sour or vinegar smell, pyrazines which are formed from reactions of sugar aldehydes with amino acids which undergo a Strecker degradation reaction followed by self-condensation and oxidation to produce these compounds with burnt or roasted aroma notes, and monoterpene-derived alcohols which are formed from monoterpene hydrocarbons, which are important components of essential oils and convey a fresh fragrance to foodstuffs (Heath 1981; Hecquet et al. 1996; King and Dickinson 2000; Kometani et al. 1995; Maga 1982, 1992; Vandamme 2003).

10.2.3 Colours

Concerns about the use of chemically produced dyes in foodstuffs have resulted in the increased use of naturally produced colorants that are derived from animal, plant, and microbial sources. As a part of this trend, yeasts that are commonly found as part of the food flora and thereby generally recognized as safe (GRAS)

have been tapped as sources of a number of carotenoids for use as food colorants such as astaxanthin, β-carotene and γ-carotene, lycopene, lutein, torulene, torulahordin, and zeaxanthin (An et al. 2001; Bekers et al. 1972; Bhosale and Gadre 2001a–c; Bogdanovskaya 1973; Bogdanovskaya and Zalashko 1982; Bon et al. 1997; Buzzini 2000; Calo et al. 1995; Cang et al. 2002; Cannizzaro et al. 2003; Chen and Liu 1999; Farid and Azar 2001; Fontana et al. 1996a, b, 1997; Frengova et al. 2003, 2004; Guirnovich et al. 1966; Guirnovich and Koroleva 1971; Jacob 1991; Johnson and Schroeder 1995; Johnson 2003; Kilian et al. 1996; Koroleva et al. 1982; Kusdiyantini et al. 1998; Lewis et al. 1990; Liang and Zhang 2003; Martin et al. 1993; Matelli et al. 1990; Misawa 1997, 1999; Misawa et al. 1998; Misawa and Shimada 1998; Miura et al. 1998; Nikolaev et al. 1966; Parajó et al. 1997, 1998a, b; Pshevorskaya and Zabrodskii 1972; Ramirez et al. 2000; Reynders et al. 1996; Sakaki et al.1999, 2001, 2002; Shih and Hang 1996; Shimada et al. 1998; Shitazawa et al. 2002; Simova et al. 2003; Slyusarenko et al. 1976; Squina et al. 2002; Vazquez et al. 1998; Vazquez and Martin 1998; Yang et al. 1993; Zalaskho et al. 1973; Zeile 1972). A number of yeasts are known to produce carotenoids that have been evaluated as food colorants and these include several species of *Rhodotorula* (*R. glutinis*, *R. lactis*, *R. gracilis*, *R. rubra*), *Rhodosporidium*, *Phaffia rhodozyma*, and *Sporobolomyces pararoseus*. Improving the commercial potential for yeast production of the previously mentioned carotenoids has been pursued with improved yeast strains from the aforementioned yeasts and from selected and genetically engineered strains of *S. cerevisiae* (Misawa and Shimada 1998), *C. utilis* (Misawa et al. 1998; Shimada et al. 1998), so-called red yeast, (Fang and Chiou 1996; Yang et al. 1993), and other *Candida* species that have been developed through classical selection and genetic engineering methods (Bekers et al. 1972; Bhosale and Gadre 2001a–c; Bogdanovskaya and Zalashko 1982; Buzzini 2000; Cang et al. 2002; Chen and Liu 1999; Cheng et al. 2004; Frengova et al. 2003, 2004; Girard et al. 1994; Jacob 1991; Koroleva et al. 1982; Martin et al. 1993; Misawa et al. 1998, 1999; Misawa and Shimada 1998; Nikolaev et al. 1966; Pshevorskaya and Zabrodskii 1972; Sakaki et al. 1999; Shih and Hang 1996; Shimada et al.1998; Simova et al. 2003; Slyusarenko et al. 1976; Squina et al. 2002; Zalaskho et al. 1973; Zeile 1972; Yang et al. 1993). The commercial exploitation of one of these yeasts, *Phaffia rhodozyma* or *Xanthophyllomyces dendrorhous*, has resulted in industrial-scale production of the carotenoid astaxanthin (Abbas 2001, 2003, 2004; Jacobson et al. 2000; Palagyi et al. 2001; Kim et al. 2003). Astaxanthin-containing yeasts and astaxanthin extracted from these yeasts has in addition to aquafeed uses as a colorant, other uses as an immunostimulant in poultry, fish, mammalian feed, and food when combined with other antioxidants derived from plant sources such as vitamin E. Other colorants of foods that are produced by yeast fermentation are the vitamin B_2 or riboflavin and caramel colours produced from processing of yeast extracts (Halasz and Lasztity 1991).

10.2.4 Flavours

Flavours can be classified in multiple ways depending on their mode of formation either naturally by biogenetic pathways from known precursors or by processing in which biological, chemical, physical, or a combination of these approaches is

employed (Heath 1981). Natural flavours are mostly metabolites produced in living cells and thereby their formation is strongly dependent on the genetic traits and the environmental conditions under which they are produced (Heath 1981). Flavours produced by processing are generally breakdown products or complex products derived from the interaction of components within a food matrix under the conditions selected (Heath 1981; Chao and Ridgway 1979). Products produced by brewer's yeast that contribute to both aroma and flavour include higher alcohols and their derived esters, carbonyl compounds, and sulphur-containing compounds. Many of these products are derived from amino acid metabolism in yeasts, while others are derived from carbohydrate and lipid metabolism. Yeast-produced flavours can be classified into three very general categories: yeast metabolic products, and this includes products synthesized or derived through yeast biocatalysis, yeast cell mass derived products, which include products prepared through yeast autolysis, and complex products resulting from the interaction of yeast-derived products with other food matrix ingredients. Products representing each category are provided in Table 10.3 and it should be noted that in some cases many of these products have in addition to flavour attributes, aroma, antioxidant, colorant, vitamin, and nutraceutical properties.

10.2.4.1 Yeast Metabolic Products

The metabolic products of yeasts that are used as flavours include a variety of short or long branched or unbranched alcohols, and aromatic alcohols which include in

Table 10.3 Yeast products

Yeast metabolic products	Yeast-derived products	Yeast beverage and food flavour products
Alcohols	Yeast extracts	Baking or bread flavours
Aldehydes	Ribonucleotides	Beer or malt flavours
Amino acids	Cell wall mannoproteins	Cheese and other dairy product flavours
Carbohydrates and other glycosides	Cell wall glucans	Cocoa flavours
Carotenoids	Edible proteins	Distilled spirits flavours
Fatty acids	Choline, glycerol, and inositol	Edible meats flavours
Esters(fragrances)	Mineral-enriched yeast	Roasted flavours
Yeast metabolic products	Yeast-derived products	Yeast beverage and food flavour products
Lactones	Sterols	Soy-derived flavour products
Phenolic compounds flavours	Vitamins	Maple or syrup or caramel
Polyols	Whole cell or single-cell proteins or single-cell oils	Wine, cherry, brandy, and sake flavours
Organic acids	Yeast enzymes	
Terpenes		
Vitamins		

addition to ethyl alcohol, phenyl ethyl alcohol, propyl alcohol, butyl alcohol, octyl alcohol, isoamyl alcohol; amino acids and their breakdown derivatives that are used for their sweet or bitter taste; carbohydrates, glycosides, or polyols such as glycerol, xylitol, and inositol that are used as thickeners, sweeteners, emulsifiers, or for their organoleptic properties; fatty acids and fatty acid esters, and organic acids such as isoamyl acetate, ethyl acetate, ethyl caproate, acetic, formic acid, citric acid, caprylic acid, and lactic acid, which impart a mouth taste of pleasant, sour, or salty notes; and esters, lactones, and aldehydes such as acetaldehyde and GC10 that contribute to a fragrarent, astringent, and sweet taste (Asano et al. 2000; Hecquet et al. 1996; Vandamme 2003; Van den Bremt et al. 2001).

10.2.4.2 Yeast-Derived Products

10.2.4.2.1 Yeast Extracts and Yeast-Hydrolysed Proteins

Yeasts extracts are flavour products that are primarily derived from the soluble fraction of yeasts enriched in amino acids such as glutamic acid in the free acid form or as monosodium glutamate provide a bouillon-like, brothy taste to food (Nagodawithana 1992). These products have found uses by the food industry as lower-cost-flavour protein sources that are competitive with hydrolysed vegetable proteins (HVPs) from soy, wheat, and from other vegetable origins (Cooper and Peppler 1959; Nagodawithana 1992). Yeast extracts are usually derived from yeasts specifically grown under optimized conditions for food application (Chae et al. 2001; Cooper and Peppler 1959; Nagodawithana 1992). In some cases yeast extracts can be derived from spent brewer's yeast, which is readily available from alcohol production facilities in great abundance and thereby represents an inexpensive readily available feedstock (Chae et al. 2001; Nagodawithana 1992). Yeast extracts are provided as powders or pastes and are used at 0.1–0.5% finished dry product basis (Nagodawithana 1992). The manufacturing of yeast extracts employs one of three distinct processes that use autolysis, plasmolysis, or hydrolysis (Chae et al. 2001; Halasz and Lasztity 1991; Lieske and Konrad 1994; Nagodawithana 1992; Peppler 1967, 1970; Reed 1981). The yeast extracts prepared from these processes have different cost structures and product and flavour attributes, and consequently have some tradeoffs. These tradeoffs consist of the cost of using commercial enzymes to autolyse the yeast vs. yeast self-autolysis or the use of high salt, mechanical disruption, or an acid hydrolysis step followed by soluble product recovery (Chae et al. 2001; Lieske and Konrad 1994). Modifying the flavour and colour profile of yeast extract can be accomplished with selected fermentation processes and media as well as by subjecting yeast slurry prior to extraction to O_2 at 60°C for up to 30 min as described in a recent patent (Lieske and Konrad 1994). In this process pasteurized yeast (15% cells by weight) was treated with pure O_2 in a closed reactor at an O_2 partial pressure of 1 atm and pH 6.1, with stirring at 500 rpm. After 15 min, the yeast flavour was modified from very yeasty to mild and the pink colour present initially reduced significantly (Lieske and Konrad 1994). Another mild process for the extraction of yeast was developed that consists of preparing a slurry of a low-moisture active-spray-dried yeast in water at a ratio of 1:5 at 39°C followed by stirring for 30 min and centrifuging the treated material at 2,300 rpm to remove the

soluble fraction which contains trehalose (Cooper and Peppler 1959). The residue after centrifugation was diluted to provide a cream of 16% solids that can be drum-dried. The product derived by this process had a light colour, reduced flavour, and was low in ash but high in protein and nucleic acids when compared with the original composition of the viable yeast. The extract prepared by this process has found many uses in various types of food and feed products. Another yeast extraction process was developed that uses brewer's yeast from a beer factory by combined enzymatic treatments using the enzymes endoprotease, exoprotease, 5′-phosphodiesterase, and adenosine monophosphate (AMP) deaminase (Chae et al. 2001). The effects of enzyme combination, enzyme dosages, and treatment sequence on the recovery of solid and protein, flavour, and compositional characteristics were all investigated (Chae et al. 2001). It was determined that exoprotease dosage strongly affected the recovery of protein and the degree of hydrolysis and sensory characteristics. When the yeast cells were treated using optimal combination of endoprotease and exoprotease (0.6% Protamex and 0.6% Flavourzyme), high solid recovery (48.3–53.1%) and the best flavour profile were obtained. Among various treatment sequences using multiple enzymes, treatment with protease followed by nuclease resulted in the highest 5′-guanosine monophosphate (5′-GMP) content. The optimal concentrations of both 5′-phosphodiesterase and AMP deaminase were found to be 0.03%. After treatments using the optimal combination of enzyme, enzyme dosages, and treatment sequence for four enzymes, a high solid yield of 55.1% and a 5′-nucleotides content of 3.67% was obtained (Chae et al. 2001). A cruder form of yeast extract that is made from both soluble and insoluble fractions by autolysis is employed when a cost-effective product with increased water-binding capability and milder yeast flavour is desired as is the case for meat analogues (Nagodawithana 1992).

Yeast extracts are very powerful savoury flavour ingredients. The sensory quality of yeast extracts is highly influenced by the specific molecular cell composition of the yeast cell. Hydrolysed edible proteins and peptides from yeasts are a good source of the amino acid L-lysine and therefore can be used in food products that need supplementation with this amino acid. The sulphur-containing amino acids cystine, cysteine, and methionine are the limiting amino acids in yeast extracts and therefore attempts have been made to enrich yeast extracts with these amino acids (Reed 1981). Cysteine is a source of sulphur in the generation of meat-like flavours by Maillard chemistry (Hurrell 1982; Stam et al. 2000). Recently, an enriched yeast extract in the amino acid cysteine was produced through the application of genetic engineering to overexpress and overproduce the yeast cysteine-rich protein metallothionein (Stam et al. 2000). This approach can result in the enhancement of the savoury value of yeast extracts prepared by this approach for meat, baked goods, and cheese food applications. Another example of widespread use for yeast proteins and peptides in foodstuffs is as cocoa replacement flavouring components as these can be produced by roasting of yeast extracts in combination with chemical processing (Liggett 1978).

10.2.4.2.2 Nucleotides

The RNA compositions of baker's yeast and of *C. utilis* yeast used to produce single-cell protein have been reported in the range 8–11 and 10–15%, respectively

(Nagodawithana 1992). The ease and cost effectiveness of the methods of production of these two yeasts has resulted in their use to prepare yeast extracts with a high concentration of RNA. The ribonucleotides 5′-inosine monophosphate (5′-IMP) and 5′-GMP are important flavour components of yeast extracts. Ribonucleotide-enriched fractions of yeast extracts are prepared with the nucleotides 5′-GMP and 5′-IMP by enzymic degradation of yeast cellular RNA in hydrolysed yeast extract cell preparations (Chae et al. 2001; Halasz and Lasztity 1991; Nagodawithana 1992; Noordam and Kortes 2004; Patane 2004). A yeast–malt sprout extract was produced by the partial hydrolysis of yeast extract (derived form *S. fragilis* or *C. utilis*) using the sprout portion of malt barley as the source of enzymes, and may be used under the Federal Food, Drug, and Cosmetic Act as a flavour enhancer in food. It contains a maximum of 6% 5′-nucleotides (USA Food and Drug Administration 1973). An improved method for the production of these flavouring agents using liposomes containing 5′-phosphodiesterase that is fused with spheroplasts or protoplast prepared from yeast cells is described in a recent patent application (Patane 2004). The enzymes used are fungal or plant 5′-phosphodiesterases which can release the four free RNA 5′-nucleotides as the final products. Further treatment of the 5′-nucleotide-enriched yeast extract preparations with the enzyme adenyl deaminase from *Aspergillus melleus* converts the 5′-AMP to the flavour-enhancing nucleotide 5′-IMP (Kondo et al. 2001; Nagodawithana 1992; Steensma et al. 2004). The results of the safety evaluation and toxicological data of phosphodiesterases derived from *Penicillium citrinum* and from *Leptographium procerum* are summarized in recent publications (Kondo et al. 2001; Steensma et al. 2004). When used in conjunction with monosodium glutamate or glutamic acid containing peptides in yeast extracts, the aforementioned nucleotides enhance mouthfeel in soups, sauces, marinades, soft drinks, cheese spreads, and seasonings by contributing a meaty flavour with sour, sweet, salty, and/or bitter notes (Noordam and Kortes 2004).

10.2.4.2.3 Yeast Polysaccharides

The major yeast cell wall polymers glucans, mannans, phosphomannans, and mannoproteins have recently been tapped for their potential flavour application as food fibre additives, for their emulsifying properties, and for their nutraceutical potential (Abbas 2003; Halasz and Lasztity 1991; Peppler 1970; Reed 1981; Sucher et al. 1974). These cell wall polymers have been characterized from a number of yeasts which include *S. cerevisiae*, *Pichia holstii*, *Hansensula* sp., a number of *Candida* species, and several *Rhodotorula* sp. Other yeast-produced exopolysaccharides that have gum-like properties that have received recent attention are those produced by *Cryptococcus laurentii* and the yeast-like fungi *Tremella mesenterica* and *T. fuciformis* (De Baets and Vandamme 1999).

Sucher et al. (1974) have described in some detail capturing the greatest value from yeast cell mass from the processing and fractionation of cells to prepare yeast extract proteins, ribonucleotides, and cell wall glucans. The approaches detailed by Sucher et al. in 1974 are still characteristic of current practices employed in the commercial production of yeast-derived products. The process described involved the homogenization of washed yeast cells, followed by alkaline treatment, and centrifugation to remove cell wall constituents. The glucan thus separated was further

washed and dried under vacuum to yield a mild-tasting product which formed a viscous solution upon rehydration. The alkali extract was then acidified to pH 4.5 to produce an isolated yeast protein with a considerable RNA content. The RNA content could be reduced by nuclease treatment and the resultant product used as an amino acid supplement for human consumption. The original RNA contents of 9–14% were typically reduced to less than 3%. After precipitation of the enzyme-treated isolated yeast protein, a yeast extract with a fried-meat flavour is produced with a glutamic acid content of 27–40% of the corresponding remaining protein (Sucher et al. 1974).

10.2.4.2.4 Mineral-Enriched Yeast

Yeasts grown in a medium containing high levels of metallic cations such as chromium, selenium, germanium, or zinc tend to accumulate these metals intracellulary (Halasz and Lasztity 1991; Reed 1981). Owing to their important biological functions as cofactors in enzyme reactions and in other intracellular proteins such as in the case of the chromium-containing glucose tolerance factor (GTF), the use of yeasts cells fortified in these metals as food supplements has gained ground (Halasz and Lasztity 1991; Reed 1981).

10.2.4.2.5 Membrane Lipids and Extracts

Yeast-derived lecithin, inositol, choline, glycerol, and glycolipids can be recovered from the cell membrane lipid extracts or from fermentation broth (Bednarski et al. 2004; Halasz and Lasztity 1991; Harrison 1970). Owing to their physical attributes the use of these components has long been established as flavours in foodstuffs as emulsifiers and surfactants. Owing to their additional health benefits, these components can also be used to fortify foods as food supplements. A commercial process to produce glycerol by fermentation using a strain of *C. glycerinogenes* has been developed and genetically engineered strains of *S. cerevisiae* that can overproduce glycerol or inositol have been reported (Omori et al. 1995; Zhuge et al. 2001).

10.2.4.2.6 Vitamin-Enriched Yeast

Yeast cells are good sources of a number of the B vitamins such as thiamine, pantothenic acid, riboflavin, vitamin B_6, and vitamin B_{12} (Halasz and Lasztity 1991; Harrison 1970; Peppler 1970; Reed 1981). Yeast cells are also good sources of biotin, folic acid, and of ergosterol, which can be converted by UV radiation to vitamin D_2 (Reed 1981). In addition to vitamins, yeasts are also a good source of coenzyme Q.

10.2.4.2.7 Single-Cell Proteins or Oils

A number of other whole-cell yeast products in use consist of use as a single-cell protein or single-cell oil primarily in animal feed applications where yeasts are grown on food processing waste streams from grain, oilseed, candy, brewery, dairy, and wood processing and vegetable oil refinery by-products (Farid and Azar 2001; Ghazal and Azzazy 1996; Halasz and Lasztity 1991; Harrison 1970; Papanikolaou et al. 2002; Peppler 1970, 1979; Reed 1981). Owing to regulatory issues and finished product quality consistency issues, these products have had a limited direct application in foodstuffs.

10.2.4.2.8 Yeast Enzymes

Intracellular yeast enzymes can be prepared from whole yeast cell mass by mechanical disruption and other means. These enzymes have found several food uses (Harrison 1970; Halasz and Lasztity 1991; Mosiashvili et al. 1971; Peppler 1979; Reed 1981). Examples of these are the use of yeast invertase obtained from *S. cerevisiae* and other sucrolytic food yeasts in the confectionary industry to break down sucrose to manufacture liquid-centre candies (Halasz and Lasztity 1991; Reed 1981). Yeast lactases obtained from the GRAS milk sugar fermenting yeast genus of *Kluyveromyces* are used to hydrolyse milk sugar or cheese whey to prepare sweeter sugar slurry. Baker's yeast ribonuclease for is used for RNA digestion during the manufacture of yeast nucleotides. Most recently yeast cytosolic oxidoreductases of brewer's yeast have been used to block Maillard reaction of dicarbonyl intermediates, thereby preventing their decomposition to off-flavour final products (Halasz and Lasztity 1991; Reed 1981; Sanchez et al. 2003).

10.2.4.3 Yeast Beverage and Food Flavour Products

Yeast and yeast metabolic products contribute to the formation of unique distinctive flavours through the formation of complex chemical substances and through other physical interactions with food and beverage matrices in several fermented foods. These reactions involve numerous components that are not fully characterized and contribute to a yeasty character that is frequently used to identify yeast-fermented and/or yeast-containing product.

10.2.4.3.1 Bread and Baked Products

Among these many products are crusty baked breads that are characterized by intense roasted odorant flavours. These flavours are attributed in great part to bread dough composition and preparation methods as nonfermented doughs lack the desired bread taste (Schieberle 1990). It is currently recognized that bread flavour is affected by a great number of compounds produced during fermentation and during baking which include alcohols, diacetyls, esters, organic acids, and carbonyl and other compounds (Annan et al. 2003; Imura et al. 2003; Watanabe et al. 1990). A recent review with 58 references covered many attributes of bread production and included commercial formulations of bakers' yeast, yeast production practices, yeast metabolism and nutrition, application of yeast to bread making, leavening activity, and taste and flavour of yeast-leavened bakery products (El-Dash 1969; Randez-Gil et al. 1999; Van Dam 1986). In this review it is indicated that at least 211 different compounds have been identified in baked breads (Van Dam 1986). Baker's yeast contributes significantly to the formation of these flavour compounds in doughs and breads and these include the alcohols ethanol, propanol, butanol, butan-2-ol, pentanol, pentan-2-ol; the organic acids acetic and lactic; the carbonyl compounds acetaldehyde, propanal, butanal, pentanal, and furfural; and browning reaction products such as melanins and caramels that are concentrated in the bread crust (Van Dam 1986). Several detailed studies have indicated that bakers' yeast is an important source of Maillard-type bread-flavour compounds (Schieberle 1990). The most important odorants present in nonheated

yeast/sucrose and heated homogenates were determined and these consisted in nonheated homogenates, of the odorants 2- and 3-methylbutanoic acid, furaneol, butanoic acid, 2-methylpropanoic acid, 4-vinyl-2-methoxyphenol, and phenylacetaldehyde. After heating the homogenate, 2-acetyl-1-pyrroline (ACPY), methional, GC10, and γ-dodecalactone, followed by 2-acetyltetrahydropyridine (ACTPY), 3-methylbutanol, and 2,5-dimethyl-3-ethylpyrazine became the predominant odorants. A comparison with the primary odorants of wheat bread crust revealed that the yeast is a potent source of the important crust odorants ACPY, methional, ACTPY, and furaneol. Model experiments carried out further to determine the source of these crust odorants showed that ACPY and ACTPY are formed in yeast from the reaction of proline with 2-oxopropanal. The importance of sulphur-containing compounds in enhancing the roasty notes of bakery products is confirmed by the use of the beef broth flavour compound 2-acetyl-2-thiazoline and other sulphur-containing compounds (Bel Rhlid et al. 1999).

The role played by higher alcohols in bread flavour has been demonstrated through the selection of isobutyl and isoamyl alcohols overproducing mutants resistant to 4-aza-DL-leucine from a bakery yeast strain of *S. cerevisiae* (Watanabe et al. 1990). Many mutants that produced more isobutyl alcohol or isoamyl alcohol than the parent strains were obtained. In the evaluation of these mutants, bread containing more isobutyl alcohol was evaluated as giving a favourable characteristic flavour, but bread with more isoamyl alcohol was unfavourable. These mutants were able to ferment dough at similar rates to commercial bakers' yeasts. The mutants overproducing isobutyl alcohol or isoamyl alcohol were released from inhibition of the key enzymes acetohydroxy acid synthase and α-isopropylmalate synthase, respectively, in the pathway of branched-chain amino acid synthesis (Fukuda et al. 1990b; Watanabe et al. 1990).

Baker's yeast has also been demonstrated to contain chemoenzymatic synthetic capability through the synthesis of the aroma active 5,6-dihydro- and tetrahydropyrazines from the aliphatic acyloins that it produces (Kurniadi et al. 2003). The published work described the generation of 25 acyloins by biotransformation of aliphatic aldehydes and 2-ketocarboxylic acids using whole cells of baker's yeast as a catalyst. Six of these acyloins were synthesized and tentatively characterized for the first time. Subsequent chemical reaction with 1,2-propanediamine under mild conditions resulted in the formation of 13 5,6-dihydropyrazines and six tetrahydropyrazines. Their odour qualities were evaluated, and their odour thresholds were established. Among these pyrazine derivatives, 2-ethyl-3,5-dimethyl-5,6-dihydropyrazine (roasted, nutty, 0.002 ng/L air), 2,3-diethyl-5-methyl-5,6-dihydropyrazine (roasted, 0.004 ng/L air), and 2-ethyl-3,5-dimethyltetrahydropyrazine (bread crust-like, 1.9 ng/L air) were the most intensive-smelling aroma-active compounds (Kurniadi et al. 2003). While bread making is one of the oldest food-manufacturing processes, it is only in the past few years that recombinant-DNA technology has led to dramatic changes in formulation, ingredients, or processing conditions. New strains of baker's yeast that produce CO_2 more rapidly, are more resistant to stress, or produce proteins or metabolites that can modify bread flavour, dough rheology or shelf life are now available (Randez-Gil et al. 1999).

10.2.4.3.2 Beer Flavouring

The liquid substances recovered following ethanol removal from beers produced with malts and other grains have found many uses in foods and alcoholic beverages where natural flavours or bioflavours are sought (Vanderhaegen et al. 2003; Ziegler 1972). Recent growth in the use of bioflavouring agents has been fuelled by the increased demand of consumers for natural products that do not pose health or environmental disposal risks (Vanderhaegen et al. 2003). These bioflavouring substances can be produced from beer refermentation using nonconventional yeasts or genetically engineered yeasts that impart unique or additional flavours. In another variation, spent yeast cells from stillage from the production of spirits are reslurried and combined in an aqueous malty fermentation medium that has been treated with enzymes to saccharify the sugars (Vanderhaegen et al. 2003). Where a colourless flavoured malt beverage is desired, enzymes are added first to malt water slurry to convert the nonfermentable sugars in malt (Word et al. 1994). The mash is heated and the liquid extracted and combined with a fermentable carbohydrate to yield a mixture which is boiled and inoculated with yeast. The resulting yeast-fermented product is decolorized to produce a clear and colourless base, which is combined afterwards with a sweetener, tartaric acid, a buffer, and a flavouring agent, followed by carbonation, until the product contains CO_2 in the range 0.48–0.57% by weight. The final product is clear and colourless, has a finished alcohol-to-extract weight percent ratio of 1:0.4–1.5, and is relatively low caloric (8–15 cal/oz). The selected parameters serve to minimize consumer sensations of fullness and excess tartness, sweetness, and astringency, while producing desirable taste characteristics and an attractive appearance.

10.2.4.3.3 Cheeses

When freshly made, most cheeses have little flavour and are often bland (Heath 1981; Moskowitz 1980). It is during ripening that distinctive flavours develop in cheese from the degradation of carbohydrates, proteins, and fats (Heath 1981). These reactions proceed until equilibrium is reached under the conditions of aging employed (Heath 1981). Cheese flavour compounds include a number of hydrocarbons, alcohols, carbonyls, acids, esters, lactones, sulphur-containing compounds, amines, and other miscellaneous flavour components. Yeast flora of dairy products, yeasts included in starter cultures with bacteria and/or moulds, and yeast-derived extracts all contribute to the flavour of a variety and types of cheeses and other fermented dairy products. The yeast flora of dairy products is diverse and includes *Debaryomyces hansenii*, *Geotrichum candidum*, *Y. lipolytica*, *K. lactis*, *C. zeylanoides*, *C. lipolytica*, *C. mycoderma*, *D. kloeckeri*, and *C. lambica* (Anderson and Day 1966; Arfi et al. 2003; Chang et al. 1972; Fadda et al. 2004; Hosono and Tokita 1970; Kalle and Deshpande 1977; Martin et al. 2001; Petersen et al. 2002). The yeast floras of artisanal Fiore Sardo cheese, of a cheese curd, a processed cheese, cheeses produced primarily with yeast, of Limburger cheese, and blue and Roquefort cheese provide examples of the contribution of yeasts to cheese flavour development during ripening (Anderson and Day 1966; Chang et al. 1972; Fadda et al. 2004; Hosono and Tokita 1970; Kalle and Deshpande 1977; Martin et al. 2001). *K. lactis* produced large amounts of alcohols, aldehydes, esters, and terpenes when cultured alone or in association with the mould *G. candidum* and especially *G. candidum* strain G3, and

generated the largest amount of sulphides when cultured alone or in association in cheese curd (Martin et al. 2001). Some of the yeasts tested were shown to metabolize the milk sugar lactose, are tolerant to 3–7% NaCl, grow on lactic acid, a product of bacteria used in starter cultures, and are frequently proteolytic and/or lipolytic (Anderson and Day 1966; Chang et al. 1972; Fadda et al. 2004; Hosono and Tokita 1970; Kalle and Deshpande 1977; Martin et al. 2001). Cheese ripened mainly with *K. fragilis* contained upon analysis four aldehydes, four methyl ketones, seven alcohols, five esters and dimethyl sulphide after 3 weeks (Chang et al. 1972). The amounts of carbonyl compounds were rather small when compared with those for various cheeses of other types but the production of methyl ketones, alcohols, and esters was enhanced by the use of this yeast. The concentrations of ethanol, isoamyl and active amyl alcohol, and ethyl acetate were especially high and these compounds were considered to be responsible for the characteristic flavour of yeast-ripened cheese. *C. mycoderma* and *D. kloeckeri* grew in the presence of 3 and 7% NaCl, respectively, in Limburger cheese and produced relatively high proteolytic activities (Hosono and Tokita 1970). H_2S, volatile fatty acids, and volatile carbonyl compounds were produced by both *C. mycoderma* and *D. kloeckeri*, although the amounts of these volatiles differed. In comparison with the volatiles produced by *Brevibacterium linens*, the kinds and amounts of the volatiles produced by these yeasts were generally limited. These yeasts appear to play an important role in the development of flavour in Limburger cheese (Hosono and Tokita 1970). Yeasts associated with Blue cheese are capable of reducing methyl ketones to secondary alcohols and may play a role in flavour development by producing ethanol, other alcohols, and certain esters. Methyl and ethyl esters and 2-phenylethanol produced by yeasts are important in Blue cheese flavour. Quantitative data on the C3, C5, C7, C9, and C11 methyl ketones in Blue and Roquefort cheese showed considerable variation among samples, but no consistent differences between Blue and Roquefort cheese. This is in contrast to the selective conversion of the C8:0 fatty acids and, to a lesser extent, the 6:0 and 10:0 fatty acids to methyl ketones during cheese curing. The C5, C7, and C9 secondary alcohols were measured in Blue cheese by gas chromatography using the methyl ketones as internal standards. The alcohols were present in lower concentrations than the ketones. Ribonucleotides have been shown to contribute to the taste and/or the aroma and/or the mouthfeel of a low fat spread cheese that resembles the taste and/or the aroma and/or the mouthfeel of the full fat spread cheese (Noordam and Kortes 2004).

10.2.4.3.4 Cocoa Flavours

The yeast flora associated with the fermentation of cocoa bean pods in the fields of the countries of origin contributes to the early development of cocoa flavours (Schwan and Wheals 2004). The first stage of chocolate production consists of a natural, 7-day microbial fermentation of the pectinaceous pulp surrounding beans of the tree *Theobroma cacao*. There is a microbial succession of a wide range of yeasts, lactic acid bacteria, and acetic acid bacteria that carry out a fermentation during which high temperatures of up to 50°C are reached. The physiological roles of the predominant microorganisms are now reasonably well understood and the crucial importance of a well-ordered microbial succession in cocoa aroma has been

established (Schwan and Wheals 2004). During the fermentation process in which the pulp is broken down to release the cocoa beans, the microbial flora produces a number of enzymes which include oxidases, a number of glycohydrolyases, peptidases and proteases, lipases and other esterases, and compounds such as alcohols, the volatile organic acid acetic acid, lactic acid, ketones, amino acids, fatty acids and fatty acid esters, and hydrocarbons that alter the colour of the beans to brownish red and contribute to the elimination of the bitter taste associated with the tannins (Heath 1981). Most of these compounds are removed in the drying step which follows which inactivates all microbial and cocoa bean enzymes and removes all gumminess contributed by polysaccharides, thereby resulting in a stable, dry, brittle product which is traded as a commodity worldwide (Heath 1981). At the processing facilities the cocoa beans are heated to produce the roasted aroma characteristic of cocoa powders and chocolate that is attributed to the aldehyde products from oxidative deamination of amino acids and from the formation of pyrazines, which are the primary Maillard reaction products (Heath 1981; Rasmussen and Bach 1996; Maga 1982, 1992). The roasting step is followed by dehulling and removing of the cocoa bean shell (Heath 1981; Rasmussen and Bach 1996). The nibs recovered are ground and subjected to heating in alkali to form a rich liquor high in cocoa butter. In the following step, the cocoa butter is removed by high-pressure presses and the pressed cakes are recovered, and ground to form cocoa powders that are dried and blended to customer-desired colour and taste specifications.

Ingredient cost consideration and limited availability of cocoa beans has generated interest in cocoa powder extenders and replacers. Blends of spray-dried malted barley and roasted barley or other roasted grains are used in some cases at a rate of 35–50% as a replacement for cocoa powders (Heath 1981). In other cases, the stillage and yeast recovered from malt spirits is recovered following distillation of ethanol and other fusel oils and concentrated by evaporation followed by spray-drying to produce cocoa powder replacements. Cocoa-substitute compounds can also be produced from a number of yeast species such as *S. cerevisiae*, *S. carlsbergensis*, *C. utilis*, *C. tropicalis*, or *Brettanomyces* genera that are preferably propagated on hop-containing or non-hop-containing media and combined with a reducing sugar in an aqueous slurry and heated to high temperature under pressure. Alternative processes that use low-moisture yeast cell preparations that are obtained following spray-drying are heated to elevated temperatures with dry heat to over 250°C in an oven to produce a desired product with a roasted flavour (Liggett 1978). More recently, genetic engineering approaches have been used to clone and produce cocoa-flavour precursor peptides in yeasts or bacteria that when mixed with amino acids and saccharides in the fermentation production medium and heated from 100 to 200 ‘C for 1–60 min can result in the production of cocoa flavours that can be used in food, pharmaceutical, or cosmetic products (Rasmussen and Bach 1996). The lipid extracts from oleaginous yeast have been analysed and evaluated for use as cocoa butter equivalent. The commercial use of oleaginous yeast to produce cocoa butter equivalent while attractive remains untapped (Ratledge 1997).

10.2.4.3.5 Fermented Edible Meats and Edible Meat Flavours

The production of meat flavours from products prepared from yeast extracts has been covered extensively (Van Pottelsberghe de la Potterie 1972) in patents and in a

number of publications (Halasz and Lasztity 1991; Huynh-Ba et al. 2003; Nagodawithana 1992; Peppler 1979; Reed 1981). The use of yeasts in the starter cultures to prepare fermented yeast products, yeast extracts for their flavours, or to support growth of microbial flora used to ferment meats such as sausages or products derived thereof, or other whole-cell or cell wall yeast has been described (Bolumar et al. 2003; Durá et al. 2002; Encinas et al. 2000; Samelis et al. 1994). Meaty flavour or notes can be achieved in non-meat-derived products by processing of aqueous yeast or yeast hydrolysed or non-hydrolysed extracts by treatments that utilize heat in the presence of sugars – monosaccharide or oligosaccharide (preferably xylose or lactose) – with/without other amino acids such as methionine, cysteine, or cystine. The process described produces an improved, durable flavour (Van Pottelsberghe de la Potterie 1972). The sources of carbohydrate can be varied and consist of coffee wastes or other ground plants, oats, rye, or barley. The aqueous medium may also contain a carboxylic acid (lactic, malic, succinic, palmitic, stearic, oleic, or their mixture). After the reaction the medium is evaporated to a paste or converted into a powder. The final formulation consists of hydrolysed plant protein free of cysteine, sodium guanylate, malic acid (or a mixture of lactic, palmitic, and succinic acids), xylose, and water with pH set at 2.5–3.0. This mixture is refluxed for approximately 100 h and concentrated to a paste or to a powder under vacuum. The products possess excellent roast beef aroma, and are suitable for use as food additives.

A number of yeasts contribute significantly to the flavour of fermented meat products and meat-flavoured products and these frequently consist of strains of *D. hansenii* (teleomorph of *C. famata*), *D. kloeckeri*, *Y. lipolytica* (perfect form of *C. lipolytica*), *Citeromyces matritensis* (teleomorph of *C. globosa*), *Trichosporon ovoides* (formerly *T. beigelii*) and several other species of *Candida* (*C. intermedia* or *C. curvata*, *C. parapilosis*, *C. zeylanoides*), *Pichia*, *Cryptococcus*, and *Rhodotorula* (Bolumar et al. 2003; Encinas et al. 2000; Ingram and Simonsen 1980). These yeasts are known to secrete lipases and/or proteases which contribute to flavour by offsetting and modifying the acidic pH produced by mixed bacterial starter culture activities through the degradation of lipids to produce free fatty acids and glyerol and the breakdown of nitrogenous compounds to amino acids with release of ammonia. Several recently published reports describe the isolation and characterization of several such enzymes from *D. hansenii* (Bolumar et al. 2003; Durá et al. 2002). In one of these reports, Bolumar et al. (2003) described the first isolation and characterization of a yeast prolyl and of an arginyl aminopeptidase from *D. hansenii*. In a second report, Durá et al. (2002) described the production of a glutaminase by the same yeast. This yeast is typically dominant in meat fermented products at all stages of sausage manufacturing and is known for its high salt tolerance and its production in addition to proteases, lipases and peroxidase activity. The isolation, characterization, and overproduction of the previously mentoned two groups of enzymes have also been reported for *Y. lipolytica* (Nicaud et al. 2002). Both of these yeasts also contribute to significant flavour development of fermented dairy products such as cheeses.

10.2.4.3.6 Soy-Derived Flavour Products

Soybean products are characterized by a mealy and fatty flavour that is bland (Heath 1981; Kinsella and Damodaran 1980). By comparison yeast-fermented soy

products such as miso prepared from soybean paste or other fermented miso products from rice or barley, soy sauce (shoyu), and other fermented soybean protein hydrolysates have complex flavour and aroma profiles (Hamada et al. 1991; Komai et al. 1987; Kumari and Singh 1990; Sarkar et al. 1994; Sasaki 1996). These flavour and aroma profiles vary with the methods used to prepare the raw materials and other ingredients included, microbial flora used, and the ripening or aging process employed. In the production of soy sauce the high concentration of sodium chloride (more than 12% w/v) used favours the growth of salt-tolerant yeast, such as *Z. rouxii*, *Torulopsis etchellsii*, and *C. versatilis* or *Torulopsis versatilis* (Chien 1974; Dahlen et al. 2001; Halasz and Lasztity 1991; Oro 2001; Sugawara 2001). Other less tolerant yeast genera can be used provided that the initial NaCl level is reduced (below 5% w/v) and these include *S. cerevisiae*, *Hansenula* sp., and *Pichia* sp. (Kayahara et al. 1980; Suezawa et al. 2003; Taiyoji and Watanabe 2002). Industrial yeast strains with improved soy sauce aroma and flavour have been developed and used commercially (Kusumegi et al. 1992; Lee and Kim 1993). The salt-tolerant yeasts used in soy sauce production elaborate a number of flavour compounds which includ: 4-hydroxy-2 (or 5)-ethyl-5 (or 2)-methyl-3 (2*H*)-furanone (HEMF), 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), 3-methyl-1-butanol, and volatile alkyl phenolics such as 2-phenylethanol and 4-ethyl guaiacol (4-EG). HEMF has a strong sweet cake-like aroma with a threshold value of less than 0.04 ppb (Sugawara 2001). HEMF is a strong antioxidant that has been shown to exert an anticarcinogenic effect on benzo[α]pyrene-induced mouse forestomach neoplasia (Sugawara 2001). It is also effective in preventing radiation hazards and has an important physiological function as well as being an aroma component (Sugawara 2001). HEMF was formed during the cultivation of yeast by using a precursor of HEMF which may have been produced by the amino-carbonyl reaction of pentose with amino acids during heating (Sugawara 2001). DMHF is found in many fruits such as strawberries and pineapple (Dahlen et al. 2001). While dilute solutions of DMHF exhibit a strawberry or pineapple-like flavour, in the concentrated form DMHF solutions have a caramel-like aroma (Dahlen et al. 2001). DMHF is a secondary metabolite that is produced by the soy-sauce-fermenting yeast *Z. rouxii* after the addition of D-fructose-1, 6-diphosphate to YPD nutrient media (Dahlen et al. 2001). DMHF concentrations in the range 5–10 g/L have been shown to partially and completely inhibit the growth of *Z. rouxii* cells (Dahlen et al. 2001). While 4-EG is an important aroma in soy sauce, when present in excess over several milligrams per litre it gives an off-odour (Oro 2001). A process for continuous production of 4-EG by the salt-tolerant *C. versatilis* in an airlift reactor has been reported (Hamada et al. 1990, 1991). In this process, large amounts of 4-EG(more than 20 ppm) were produced by immobilized yeast cells for up to 40 days with 1–3 ppm of 4-EG, which is the optimal level in conventional soy sauce, produced within 0.5 h (Hamada et al. 1990). Good soy sauce flavour profiles were reported recently with the salt-tolerant yeasts *C. versatilis* and *Z. rouxii* immobilized on a poly(ethylene oxide) resin in a continuous stirred reactor (Van der Sluis et al. 2001). The production of a soy sauce with quality comparable to that produced by the conventional method was demonstrated in a controlled fermentation process using a genetically modified strain of the red yeast *C. versatilis* in which an inducible ferulic acid

decarboxylase was deleted and replaced with a gene coding for a constitutive enzyme (Suezawa et al. 2003).

The selection and use of improved strains of *Z. rouxii* for production of miso from barley, soybean, wheat, or non-salted rice has been reported (Kasumegi et al.1997, 2001; Kayahara et al. 1980; Matsuda and Yamamoto 1999; Taiyoji and Watanabe 2002; Yoshikawa et al. 1990). Mutants of *Z. rouxii* produced more than twofold aromas of higher alcohols such as isoamyl alcohol, propyl alcohol, isobutyl alcohol, butyl alcohol, and/or β-phenethyl alcohol (Kasumegi et al. 1997, 2001, Kayahara et al. 1980; Matsuda and Yamamoto 1999). The use of wine yeast strains that produce ethanol in the presence of high levels of sodium chloride, yielded miso flavour profiles that are characteristic of the wine yeast used (Kawamura and Kawano 1999). The effect of ethanol addition on the formation of fatty acid esters from the degradation of glycerides present in raw materials that are usually formed during miso fermentation was investigated (Ohnishi 1983). It was found that the ratio of ethyl fatty acid esters formed was determined by the level of ethanol present. This suggests that the production of ethanol and other higher alcohols by yeasts is not only responsible for the production of aroma substances but also for the rate at which lipids are metabolized and for flavour development during miso fermentation (Ohnishi 1983). At reduced salt concentration during the production of non-salted rice miso by *Z. rouxii*, ethanol addition (4%) at 45°C leads to increased sugar and protein metabolism, resulting in bitter flavour and light colour development (Taiyoji and Watanabe 2002).

10.2.4.3.7 Other Fermented Foods and Beverages

Yeasts are also responsible for the complex flavours of many other raw materials, foods, and beverages which include a great number of distilled spirits, other fermented fruit and cereal drinks such as wines, ciders, and sake (Fukuda et al. 1998a, b; Furukawa et al. 2003a, b; Lambrechts and Pretorius 2000; Nykanen 1986; Romano et al. 1999), fermented syrups, and caramel or almond flavours such as salicyl flavour aldehyde produced by the methylotrophic yeast *C. methanolovescens* (Van den Bremt et al. 2000, 2001), fermented dairy products such as kefir and yogurt, and other foods characterized by roasted and fragrant flavours which include tea, vanilla beans, and coffee. Some of the yeasts used in some of these applications are listed with the food type in Table 10.4.

10.2.5 Vitamins

Owing to their ability to incorporate ingredients present in fermentation media, yeast cells are an important source of proteins, vitamins, and minerals (Halasz and Lasztity 1991; Mosiashvili et al. 1971). Among the vitamins and other enzyme cofactors that are accumulated and/or synthesized by yeast are thiamine (vitamin B_1), nicotinic acid containing enzyme cofactors NAD and NADP and their reduced forms, pyridoxine (vitamin B_6), pantothenic acid, or CoA precursor (Peppler 1967), cyanocobalamin or vitamin B_{12}, biotin, folic acid (pteroylglutamic acid) or folacin, and riboflavin or vitamin B_2 (Drewek and Czarnocka-Roczniakowa 1986; Halasz and Lasztity 1991; Oura and Suimalainen 1982). Examples of these are the yeast *Kloeckera apiculatas*, which

Table 10.4 Yeast used in main fermented foods

Fermented food	Yeast employed in process
Alcoholic beverages	*Saccharomyces cerevisiae* and other species; *Schizosaccharomyces* (rum)
Breads and cakes	*S. cerevisiae* and *S. exigus*; *Candida krusei* and *C. tropicalis*; *Pichia* and *Hansenula anomala*
Beers	*S. cerevisiae* and *S. uvarum* (ex *S. carlsbergensis*)
Ciders	*S. cerevisiae* and *S. uvarum*; *Hansenisapora valbyensis*; *Metschnikowia pulcherrima*
Coffee	*S. cerevisiae*
Cocoa	*C. krusei*, *C. famata* and *C. holmii*;
Fermented food	Yeast employed in process *P. membranaefaciens*, *S. chevalieri*
Fermented milks	*S. cerevisiae* and *C. kefir* (for kefir)
Fresh, semihard, pressed, or mould cheeses	*Kluyveromyces* sp., *Debaryomyces hansenii*, *S. unisporus*, *Candida* sp., *Pichia* sp., *Yarrowia lipolytica*, *Clavispora lusitaniae*, *Trichosporoninkin*, *Torulospora delbrueckii*
Fermented meat products	*D. hansenii*, *Candida* sp., *Rhodotorula rubra*
Wines	*S. cerevisiae*

Modified from Cristiani and Monnet (2001)

is known to accumulate thiamine up to a tenth of its dry weight, whereas some strains of the yeasts *S. cerevisiae* and *S. uvarum* (*carlsbergensis*) during ethanol production can release thiamine following treatment with UV light (Halasz and Lasztity 1991). *S. cerevisiae* is also the principal source of CoA and the first important commercial preparations of this coenzyme were made from this yeast (Peppler 1967). Some strains of *S. carlsbergenesis* have also been shown to contain vitamin D_2 and vitamin D_3 as well as 25 hydroxy forms of these two vitamins (Halasz and Lasztity 1991). Ergosterol, a precursor to the aforementioned forms of vitamin D, is an important constituent of yeast cell membrane lipids (Fazekas and Sebok 1959; Tanaka et al. 1971; Xue et al. 2002). Increasing yield of ergosterol in yeast cultures was shown to be influenced by the addition of vitamin B_1 and thioglycolic acid to enhance cell mass in aerated cultures (Fazekas and Sebok 1959). It was concluded that the combined addition of vitamin B_1 and thioglycolic acid enhances sterol production in the cells, most likely by the conversion of pyruvic acid to sterols (Fazekas and Sebok 1959). The cultural conditions for ergosterol production by yeasts were also investigated by using an *n*-alkane mixture as the sole carbon and energy source (Tanaka et al. 1971). Among the yeasts tested, several strains of *C. tropicalis* were shown to produce a relatively large amount of ergosterol (Tanaka et al. 1971). *n*-Alkanes with carbon numbers that range from C10 to C14 and from C17 to C18 were good substrates for ergosterol production by this yeast. The addition of a nonionic detergent (0.02%) and a natural nutrient (0.1%) was also effective. Aeration and the pH of the medium also affected the production of ergosterol. Under the optimal conditions employed, a strain of *C. tropicalis* designated as pK 233 produced ergosterol in a yield of approximately 71 mg/L of broth or

5.8 mg/g of dry cells after 5-day cultivation in a medium containing a mixture of *n*-decane to *n*-tridecane as the sole carbon sources. More recently, a method for transforming ergosterol into vitamin D_2 in yeast was described by Xue et al. (2002). In this method UV irradiation of ergosterol containing yeast grown under optimal conditions with molasses as the carbon source resulted in cells that contain 15,000 IU/g (Xue et al. 2002). Clinical observations showed that the yeast cells generated have good preventive and curative effects for rickets (Xue et al. 2002). Other forms of lipid-soluble vitamins and their precursors that are present in yeast are the vitamin E tocopherols, and vitamin A and its precursor, β-carotene.

In addition to the B vitamins consisting of B_1, B_6, and B_{12}, yeasts are also important sources of flavins derived from riboflavin or vitamin B_2, the water-soluble vitamin C or ascorbic acid (Hancock et al. 2001), and multiple forms of coenzyme Q. The production of yeast cell mass with a predetermined level of vitamin B_1 potency was demonstrated by the incorporation and assimilation of vitamin B_1 from a water-clear vegetable extract with a sugar source provided as the carbon source and used as a growth medium (Gorcia and Levine 1942). Improvement of the biological synthesis of the vitamin B_1 in yeast was shown through the incorporation of vitamin B_1 precursors into the fermentation medium (Harrison 1942). The addition of a suitable thiazole derivative, such as 4-methyl-5-β-hydroxyethylthiazole, and a pyrimidine derivative containing a cyano radical, such as 2-methyl-5-cyano-6-aminopyrimidine, in equimolar quantities to the fermenting mash, preferably toward the end of the process under vigorous aeration at 28–30°C at a pH of 5.5–6.0, resulted in the formation of a methylene linkage between the pyrimidine ring and the nitrogen of the thiazole. Other ethylthiazole derivatives can be used in the aforementioned reaction that yielded vitamin B_1 as a product (Harrison 1942). The production of vitamin B_1 for use in beverages and in pharmaceutical applications was described by Silhankova (1978). In this patent, a vitamin B_1 producing strain of *S. cerevisiae* designated DBM 159 was shown to produce normal levels of ethanol while producing an elevated level of vitamin B_1 (approximately 10 mg/L) in a molasses-containing medium (Silhankova 1978). The formation of vitamin B_6 by several genera of yeasts was examined in hydrocarbon-containing media with vigorous aeration by Tanaka and Fukui (1967). Among the genera studied of *Candida*, *Rhodotorula*, and *Hansenula*, a strain of *C. albicans* exhibited the excellent vitamin B_6 producing ability (300–400 μg/L) in a synthetic medium containing hexadecane as the sole carbon source. The addition of corn steep liquor and an appropriate nonionic detergent, such as Tween 85 or Span 60, stimulated yeast growth and vitamin B_6 production (Tanaka and Fukui 1967). The cell and vitamin yields increased with the rate of aeration. In a medium containing glucose as the carbon source, the vitamin B_6 formed was secreted into the fermentation broth prior to achieving maximum cell density. In cells grown with hydrocarbons as the sole source of carbon, vitamin B_6, mainly in the form of pyridoxal phosphate, accumulated inside the cells (Tanaka and Fukui 1967).

The formation of coenzyme Q, cytochrome c, and flavins by yeasts grown on mixed hydrocarbons was studied using several strains of *Candida* and *Pichia* by Teranishi et al. (1971). These strains included a strain of *C tropicalis* designated as pK 233. The time-course changes in the coenzyme Q, cytochrome c, and flavine contents in the yeast cells were studied during growth on hydrocarbons. The effects of medium

constituents, additional purines, and detergents on flavine production and the type of flavines produced were also investigated. *C. tropicalis* pK 233 was shown to grow well on C9–C14 alkanes under the conditions employed. Undecane was most suitable for cell growth and flavine production. A hydrocarbon mixture of four alkanes which was enriched in *n*-undecane was also suitable as the sole carbon source. Several nitrogen sources, namely NH_4NO_3, $(NH_4)_2SO_4$, and $NH_4H_2PO_4$ were all shown to be effective for flavine production by this yeast. The amount of flavines produced by *C. tropicalis* pK 233 was about 5 mg/L of broth under the conditions employed. The time-course study suggested an interesting correlation between the intracellular contents of coenzyme Q, cytochrome c, and flavines during growth (Teranishi et al. 1971).

The production of protein–vitamin concentrates with yeasts in media containing lipids as the carbon source was described by Szechenyi et al. (1973). Media containing lipids such as grease, lard, rancid and waste fats, or tallow, that are emulsifiable at 30–40°C, were fermented with yeasts (*C. guilliermondii*, *C. utilis*, *S. fragilis*, etc.) in the presence of NH_4^+ salts and (or) urea at pH 4.5–5.0. The cell mass recovered from the fermentation broth had high protein (more than 50%) and vitamin content, which included vitamins B_2, B_6, and B_{12}, nicotinic acid, and pantothenic acid. The cell mass was useful as a food or feed additive without considerable purification. Thus, a medium containing animal-derived lipids or their by-products was shown to yield a 75% dry yeast (based on the lipid consumed) that contained greater than 50% protein, 7% lipids, and 6% ash, as well as vitamin B_1 greater than or equal to 3 mg/100 g, vitamin B_2 greater than or equal to 5 mg/100 g, pantothenic acid greater than or equal to 20 mg/100 g, vitamin B_6 greater than or equal to 1.5 mg/100 g, and ergosterol at a concentration greater than or equal to 3 mg/100 g.

The increased demand for vitamins and enzyme cofactors as food additives and supplements has stimulated research to increase the content of these important components in yeasts intracellularly and in some cases as secreted extracellular products. The development of yeast strains with increased vitamin and enzyme cofactors using classical strain selection and/or genetic engineering has resulted in the development of commercial processes for the production of vitamin B_2, riboflavin (Dmytruk et al. 2004; Lim et al. 2001; Protchenki et al 2000; Stahmann et al. 2000; Voronovsky et al. 2002, 2004). The *C. famata* (*D. hansenii*) strains described are among the most flavinogenic microorganisms developed so far and unlike strains of *Pichia* (*Candida*) *guilliermondii* which concentrate vitamin B_2 intracellularly secrete riboflavin into the growth medium.

The genetic engineering of strains of *S. cerevisiae* that can synthesize the fat-soluble vitamins E and A and its precursor, β-carotene, has been described in great detail in a recent patent application (Millis et al. 2000). The cited patent describes the production of the previously mentioned vitamins by fermentative biosynthesis of intermediates using genetically engineered microorganisms followed by chemical synthesis (Millis et al. 2000). The invention provides methods of producing vitamin E (α-TOH and γ-tocopheryl esters), vitamin A (retinol), or β-carotene. The methods comprise using a biological system with enhanced synthesis of farnesol or geranylgeraniol intermediates to shift microbial metabolism away from sterol biosynthesis via genetic inactivation of the squalene synthase ERG9 gene or by inactivation of squalene synthase by zaragozic acid in a strain with a functional ERG9 gene.

Geranylgeraniol biosynthesis is further enhanced in strains overexpressing any of four different cloned geranylgeranyl pyrophosphate synthase genes from several microbial and plant sources, and these include (1) BTS1 gene from *S. cerevisiae*, (2) crtE gene from *Erwinia uredovora*, (3) a1-3 gene from *Neurospora crassa*, and (4) ggs gene from *Gibberella fujikuroi*. The overexpression of the hydroxymethyl-CoA reductase and/or the ERG20 gene which encodes farnesyl pyrophosphate synthase in *S. cerevisiae* also leads to enhanced biosynthesis of fermentative intermediates. The overexpression of multiple isoprenoid pathway genes or an alternative pathway (Rohmer pathway) was investigated in strains that have an erg9 mutation and elevated levels of hydroxymethylglutaryl-CoA reductase. Further chemical conversion of the fermentation products, farnesol or geranylgeraniol, into α-TOH, γ-tocopheryl ester, vitamin A, or β-carotene was described in some detail (Millis et al. 2000).

The genetic engineering of yeast strains that can synthesize the water-soluble vitamin C or ascorbic acid is described in another recent patent application (Berry et al. 1999). The synthesis of vitamins D_2 and D_3 from ergosterol by *S. cerevisiae* and *C. tropicalis* has long been described in some detail in a number of publications (Fazekas and Sebok 1959; Rao and Raghuntha 1942; Subbotin et al. 1974; Tanaka et al. 1971; Xue et al. 2002). The accumulation and secretion of thiamine or vitamin B_1 in beer wort following treatment of *S. cerevisiae* and *S. carlsbergenesis* has been confirmed at the laboratory and pilot-plant scale and the production of thiamine from its precursors by these two yeasts has been recognized for some time (Bakhchevanska et al. 1984; Popova et al. 1982; Silhankova 1978, 1985). The potential use of several yeasts for production of the vitamin B_{12} has been assessed and *Z. bailii* and fodder yeast were recognized as promising yeasts for its production on industrial media and agricultural residues (Bykhovskii et al. 1972; Mosiashvilli et al. 1971; Popova et al. 1971; Trofimenko and Cheban 1970). The production of vitamin B_6 or pyridoxine has been demonstrated on hydrocarbon-containing media with several yeasts, which include several *Candida*, *Rhodotorula*, and *Hansensula* (Tanaka and Fukui 1967).

10.3 Concluding Remarks and Future Outlook

The economic importance of yeasts to the food and beverage industries continues to outweigh all other commercial uses of yeasts. The revived interest in naturally derived products and the increased concerns about products from animal sources has generated renewed interest in food and nutraceutical uses of yeasts. Among the many advantages of yeasts are their ease of production and for several yeast genera the favourable regulatory environment. New strains of yeasts enhanced through genetic breeding to improve production of food ingredients and for use as supplements provide added promise to biotechnologists and consumers alike (Maraz 2002). It is without doubt that the full potential of the commercial value of yeasts has not been fully realized. With new nutraceutical applications under development the 2–4 % projected annual growth in sales for all yeast products will result in markets that exceed US $20 billion by the end of this decade. The continued expansion in the use of *Saccharomyces* and other genera of so-called nonconventional yeasts represents the rise of yeast biorefineries that aim to capture the full potential of these important biocatalysts.

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Chapter 11

Food and Beverage Spoilage Yeasts

MALCOLM STRATFORD

Mologic, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK
(e-mail: malcolm.stratford@nottingham.ac.uk)

This chapter is dedicated to all those who work in food QA, Quality Assurance laboratories, at the sharp end of yeast spoilage.

11.1 Introduction

Most people have very little knowledge or experience of yeasts; what experience they have is largely confined to the use of yeasts in bread-making or in alcoholic beverages, perhaps through home-brewing. It would surprise many people to realize that they also consume yeast cells daily, by the thousand, particularly in fresh fruit and vegetables. These yeasts are not likely to be the brewing or baking yeast, *Saccharomyces cerevisiae*, but are distributed widely across the 800 known yeast species (Barnett et al. 2000). Consumption of yeast cells in such numbers is not injurious to human health but part of a normal, healthy human diet. But if foods are not consumed but left to incubate at warm temperature, some of these yeasts will grow over time and cause a perceptible alteration in the food. Yeasts of such species may be termed food and beverage spoilage yeasts.

Many excellent reviews of yeast spoilage have previously been published, including those of Ingram (1958), Walker and Ayres (1970), Davenport (1981), Fleet (1992), Tudor and Board (1993), Thomas (1993), Deak and Beuchat (1996) and Pitt and Hocking (1997). In these reviews, the literature of yeast spoilage is examined food by food, listing the yeasts found in and causing spoilage in each individual foodstuff, for example yeast spoilage in cereals; fruit juices and meat. There would therefore seem little need to recapitulate the information already published, in a further review. However, a number of recent technical innovations and social trends in food purchase and consumption made it timely to re-evaluate yeast spoilage in food.

Firstly, DNA sequencing methods applied to yeast identification are revolutionizing the taxonomy and phylogeny of spoilage yeasts (James et al. 1996; Kurtzman and Robnett 1998, 2003). This has meant that very few of the species names associated with spoilage 50 years ago are currently recognized. Sequencing has also enabled a major reassessment of the relationships and phylogeny of many spoilage strains. This has resulted in amalgamation of certain genera and re-naming of others (Kurtzman

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
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2003). This will doubtless cause much confusion at first, and difficulties in matching the old names in the literature with current nomenclature. It is hoped that the proposed new sequence-based structure of yeast taxonomy will not be subject to further changes and that the relationships between different spoilage yeasts shown by the phylogenetic tree will enable prediction of spoilage behaviour in related yeast species.

A second consequence of DNA sequence-based yeast identification has been the revelation as to the extent of misidentification of strains by older taxonomic methods. Many yeast strains, even in recognized culture collections, have been found to have been misidentified. Furthermore, many new species of spoilage yeasts have been found, previously unrecognized. Such species may not be distinguishable by traditional methods of identification and tended to be force-fitted into other genera. The genus *Candida* in particular had become a ragbag assortment of white non-spore-forming yeasts, with no unifying, clearly defined character. It could be speculated that in the older literature of yeast spoilage, where traditional taxonomic tests were used by inexpert researchers, the level of misidentification may exceed 50%.

The third change influencing yeast spoilage is the increased degree of processing and storage of foods. In human prehistory, foods were obtained fresh and consumed immediately. More recently, a proportion of foods were preserved using traditional methods, such as drying, smoking or salting. In modern times throughout the developed world, many foods are sold as pre-prepared meals, minimizing the time spent by consumers in preparing food. Foods are therefore subject to a far greater degree of factory processing than previously. Such foods are also packaged in a very different way. Traditionally, foods were sold with no packaging, allowing easy access to yeast infection in the domestic environment. Processed ready-meals, soft drinks and even fresh fruit/vegetables are now sold packaged in plastic, minimizing microbial contamination, but altering the concentrations of gases within foods. In the future it is likely that much greater attention will be paid to the opportunities available for spoilage yeasts in factory-prepared processed foods.

In the present review, yeast spoilage will be examined largely from the point of view of the "preservation systems" applied to different foods. Knowledge of which yeast species are resistant to which preservation systems will enable prediction of patterns of yeast spoilage in any new foods preserved using these systems.

11.2 Definitions

What is yeast spoilage in foods or beverages? This is a question to which many people can immediately envisage the answer but would find it much more difficult to define precisely. Extreme examples of yeast spoilage include "blown cans" of soft drinks (Fig. 11.1), cloudy re-fermented wine, pink or red slime dripping from refrigerated meat, white yeast colonies on food, and tainted fruit juices. A spoilage yeast species is one with the ability to cause spoilage. As such, yeasts merely isolated from foods are not necessarily spoilage yeasts. Taking a leaf from the medical textbooks of infective agents, a spoilage yeast is one that can be isolated from a spoiled product, and if re-inoculated back into sterile food of the same variety will grow and cause identical symptoms of spoilage. Yeasts are often isolated from spoiled or symptomless foods and should not be termed spoilage yeasts without further tests.

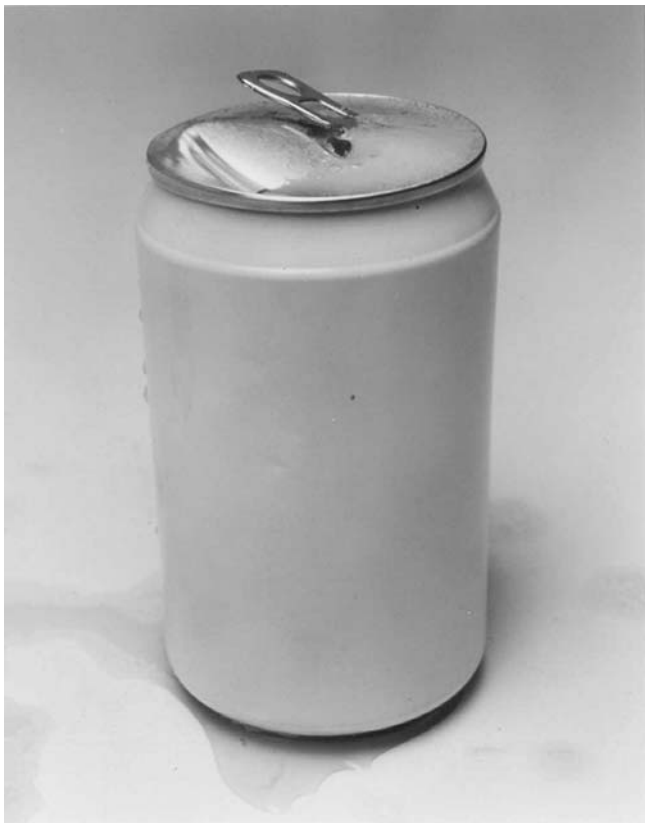

Fig. 11.1. "Blown" packages of food or beverages represent the most obvious sign of yeast spoilage. This soft drink was inoculated with *Zygosaccharomyces bailii* before capping. Similar cans of beverage distended, ruptured or exploded between 1 month and 2 years

However, does the mere presence of a few yeast cells in a food constitute spoilage? and if not, how much can a yeast grow before the food is defined as spoiled? The answers to these more difficult questions will vary with the food type and the individual yeast species involved. Foods frequently contain yeasts, even spoilage yeasts, at low level without perceptible spoilage. An inoculum of 100 cells/ml is only attained with reasonable care in fruit juices (Lloyd 1975). The metabolic activity of yeasts at a cell concentration up to 10,000/ml is insufficient to make any appreciable difference to the food. Since detectable spoilage requires greater numbers of yeasts than this, approximately 1×10^5–1×10^6/ml (Ingram 1949), and very high concentrations of yeasts are not likely to be found through the initial inoculation, it therefore follows that yeast spoilage requires growth of the yeast population.

To our good fortune, yeasts are almost entirely non-pathogenic. To a human in a reasonable state of health, consumption of moderate numbers of viable yeasts of most of the 800 recognized yeast species (Barnett et al. 2000) is not likely to cause

harm (Fleet 1992). Minor gastrointestinal disorders have been attributed to consumption of beverages spoiled by *Saccharomyces* and *Zygosaccharomyces* yeasts but these have not been confirmed (Todd 1983; Muzikar 1984). It has been suggested that such effects may be due to yeast metabolites rather than to live yeasts per se (Thomas 1993). Similarly, yeasts do not form toxins injurious to human health (unlike mycotoxin-forming moulds). Yeasts of many species do form toxins, "killer toxins" (Philliskirk and Young 1975; Young 1987), but these appear targeted primarily at other strains and species of yeasts. The use of killer strains in breweries has even been proposed, to prevent contamination of brewing strains by wild yeasts (Young 1983; Hammond and Ekersley 1984).

The results of yeast spoilage of foods are diverse, but all are the result of large populations of yeast growing in the food and consequent metabolic activity. In spite of this, yeast growth in foods is unlikely to cause significant deterioration in the nutritional value of the food. Yeast growth is likely to remove only a small proportion of the sugars, for example in a fruit juice. Yeast growth may even increase the nutritional value of foods through addition of yeast protein and vitamins.

However, despite not causing a safety hazard or loss of nutritional quality, the results of yeast spoilage are noticeable by any customer. A consumer is unlikely to realize that it is yeast spoilage; a consumer will simply realize that something is wrong or different about the food, *and will reject it*. A common theme of yeast spoilage is therefore that all spoilage symptoms are noticeable and objectionable to the customer and all will cause complaint by any customer purchasing the food.

Since (1) the presence of yeasts in foods is not a safety issue, (2) yeasts do not form toxins adverse to humans or (3) yeasts do not cause significant nutritional loss, food spoilage by yeasts is dependent entirely on what the customer/consumer notices or objects to. Yeast spoilage of foods or beverages can therefore be defined as "Growth of yeast in a food, sufficient to cause an alteration in that food, perceptible to a consumer, and liable to cause dissatisfaction, complaint, or rejection of that food by the customer".

11.3 Which Foods are Prone to Yeast Spoilage?

Yeast and human biochemistry and metabolism are essentially similar. This being so, it is obvious that yeasts and humans are also likely to have a high level of similarity in their nutrition. In a simplified form (Table 11.1), human nutrition is based around carbohydrates (carbon source), proteins (nitrogen source), fats, vitamins and mineral salts. Yeast nutrition also requires carbon and nitrogen sources, vitamins and minerals. It is therefore not surprising that many human foods can fulfil a role as excellent yeast growth media.

However, human and yeast nutrition vary in two important aspects. Firstly, in a yeast growth medium, all of the nutrients must be present simultaneously, whereas in human nutrition, different parts may be found in different foods, for example vitamins in one food and proteins in another. Human foods not containing a complete balanced yeast diet are less likely to be substrates for spoilage yeasts. Secondly, yeast nutrition is strongly oriented towards small molecules rather than giant polymers. Complex polymers of carbohydrate or protein cannot be transported easily through

Table 11.1 The similarity between human and yeast nutrition lends many human foods prone to spoilage by yeasts. Complex polymers predominate in human nutrition, while yeasts almost exclusively utilize monomers. Yeast nutrition is fully discussed in Jennings (1995)

	Human nutrition	Yeast nutrition
Carbon/energy sources	Carbohydrates, sugars, fats	Simple sugars (fats by lipolytic yeasts)
Nitrogen source	Proteins	Amino acids, ammonium ions, (nitrate and nitrite)
Minerals	Iron, calcium, magnesium, phosphate, sodium, potassium	Magnesium, potassium, phosphate
Trace elements	Iodine, sulphur, copper, cobalt, manganese	Iron, sulphur, copper, cobalt, manganese, zinc, (molybdenum)
Vitamins	Vitamins A–K, including B-group vitamins	B-group vitamins by some species

the yeast cell wall (de Nobel and Barnett 1991) and require extracellular degradation by secreted enzymes. Extracellular degradation is common in the filamentous fungi (moulds) but is rare in single celled fungi (yeasts). Preferred carbon sources for yeast assimilation are simple sugars, and to a lesser extent sugar alcohols, or organic acids. Relatively few yeasts can utilize complex carbohydrates such as starch. Where yeast species have the ability to assimilate starch, growth is usually slow or delayed (Barnett et al. 2000). Some yeasts show lipolytic activity, for example *Yarrowia lipolytica*, and are able to utilize fats (Barth and Gaillardin 1996), but again this is slow compared with growth on glucose. Similarly most yeasts have virtually no proteolytic activity (Ingram 1958) and are therefore unable to grow on proteins as a nitrogen source. An exception to this may be the degradation of casein in milk by red yeasts leading to spoilage of milk products (Ingram 1958). Preferred yeast nitrogen sources are again small molecules, notably amino acids, ammonium ions, and nitrate or nitrite for a few yeast species. Nitrogen compounds are generally much more important to bacteria, especially to those that use them as energy sources, leading to the observation that bacterial spoilage is favoured in foods with a high N/C content (Tilbury 1980a). The practical upshot of this is that bacteria form the predominant spoilage flora in high-protein meat-type foods, while fungi assume greater importance in spoilage of foods of vegetable or fruit origin.

The importance of simple sugars to yeast spoilage cannot be overemphasized (including lactose in milk). This is reflected in the naming of the first yeast genus *Saccharomyces*, which means sugar fungus (Mayen 1837 ex Reed and Peppler 1973); one that contains several important spoilage species. As an illustration of this, the writer was once involved in production of a herbal soft drink, prepared with and without sugar. A variety of 150 yeast and mould species were individually inoculated, and in due course spoilage was observed caused by a selection of the yeast and mould species. Unexpectedly, while every spoilage mould species grew equally well

with and without sugar, every single yeast species failed to grow in the formulation lacking sugar. Ingram (1958) stated "with general experience ... it is sugary substrates, above all, which are likely to be spoiled by yeasts".

Foods containing sugars, amino acids, minerals and vitamins are thus likely to be prime media for yeast growth, but such foods may also be colonized by bacteria or filamentous moulds. Fresh foods are heavily contaminated with microorganisms, yeasts, moulds and bacteria. Over time, if the food is not consumed, many of these microbes will grow and spoil the food or cause illness in consumers (food poisoning). Yeasts, moulds or bacteria can cause spoilage, but in foods liable to growth of pathogenic bacteria such as *Clostridium botulinum*, the food is commonly sterilized by heat to kill bacterial spores ("the Botulism Cook"). Such measures will also completely eliminate any fungal contamination in the food. Yeast and mould spoilage of foods is therefore largely restricted to foods that will not support the growth of or toxin production by bacterial pathogens.

Bacterial growth is eliminated to a very large extent by acidity, bacterial spores will not germinate in foods with a pH less than 4.5 and vegetative cells of pathogenic bacteria will not grow at a pH less than 4.0 (Smelt et al. 1982), low temperature or high osmotic strength in foods. Such foods are not heavily heat-treated. Spoilage by yeasts is therefore largely restricted to those foods with low pH, low water activity a_w (caused either by high salt or high sugar), low temperatures or that contain antibacterial agents such as sulphite.

11.4 Symptoms of Yeast Spoilage

As previously defined, yeast spoilage of food is primarily that which the customer or consumer notices and finds objectionable. The symptoms of yeast spoilage are many and varied; not all may be obvious to all consumers and the food may be eaten. Other minor symptoms may be noticed but may go unreported. Fleet (1992) highlighted the problem of estimating the true significance of yeast spoilage, unless very obvious physical effects accompanied yeast growth.

11.4.1 Gas Production

What then are the obvious signs of yeast spoilage? The most visible sign of yeast spoilage is the production of excess gas (Fig. 11.1), leading to swollen containers or Tetra Paks, "blown" cans, or in extreme examples, exploding glass bottles, leading to physical injury (Grinbaum et al. 1994). It is likely that the great majority of spoilage incidents involving exploding containers will result in a consumer complaint, leading to a distortion of spoilage data in favour of yeasts producing excessive gas pressure.

It is not commonly appreciated by the public that not all yeast species produce gas. Excess gas production by yeasts is a result of fermentation of sugars, normally detected in the laboratory by the appearance of gas in an inverted Durham tube, submerged in broth media. Many yeast species do not ferment, and are termed respiring species. Of the yeasts that do ferment, the range of sugar substrates fermented varies considerably (Barnett et al. 2000). Furthermore, the volume of gas produced, and hence the pressure formed, varies between yeast species. The few

yeast species capable of forming sufficient gas pressure by fermentation to explode bottles include *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae*, *Dekkera bruxellensis* and *Saccharomycodes ludwigii*. Tests carried out in the laboratory by the author have demonstrated fermentation pressures formed by these species between 6 and 10 atm overpressure (Fig. 11.2), far in excess of that formed in the manufacture of bottle-fermented wines, such as Champagne. A quick glance at the literature will reveal that these species include the most famous (or notorious) spoilage species, not because they are widespread or are a particular problem, but simply because they have drawn themselves to the attention of the public in such a spectacular way.

Behind these highly fermentative species are a number of other spoilage species that ferment, but to a much lesser extent. These include *Candida parapsilosis* and *Candida pseudointermedia*, which can generate up to 2 atm overpressure (Fig. 11.2), which is insufficient to explode containers but may lead to consumer complaints such as "sparkling" or "tastes like poor Champagne". The lower severity of symptoms in this instance almost certainly has led to a reduced complaint level and underreporting.

11.4.2 Visible Symptoms of Yeast Spoilage

Perhaps the second most obvious sign of yeast spoilage is the visible appearance of yeast cells themselves. In solid foods this can be shown through the appearance of yeast colonies on the food surface, surface discolouration, films or mucous slimes, particularly in yeasts forming extracellular polysaccharides. How obvious this is to

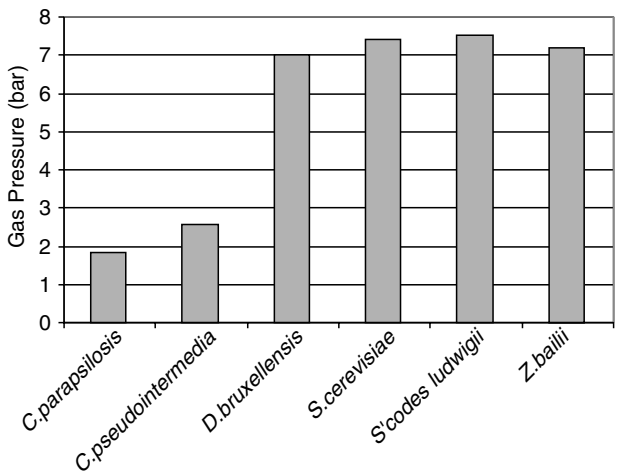

Fig. 11.2. Gas formation by fermentative spoilage yeasts, measured after 2 weeks of growth in a soft drink containing 1 M glucose. The yeast strains were *Z. bailii* NCYC°1766, *Saccharomyces cerevisiae* NCYC°366, *Dekkera bruxellensis* NCYC°823, *Saccharomycodes ludwigii* NCYC°732, *Candida parapsilosis* CMCC°3397 and *C. pseudointermedia* NCYC°2610. All had been identified by D1/D2 26S ribosomal DNA (*rDNA*) sequencing

a customer depends very much on the yeast species and the appearance of the food itself. White yeast colonies are immediately obvious on the surface of tomato soup (Fig. 11.3), but the same yeast colonies might pass unnoticed on the surface of white yoghurt. On most solid foods, pink or red yeasts are more likely to be reported than white or cream yeast colonies, perhaps leading to the regard for *Sporobolomyces* or *Rhodotorula* spp. in yeast spoilage by Pitt and Hocking (1997).

In beverages, liquid yeast media, spoilage yeasts can be seen as hazes, clouds, particulates, surface films or colonies and sediments in undisturbed bottles. In a clear liquid, yeasts are visible as a faint haze at 10^5 cells/ml, but can grow to dense clouds at 10^7–10^8 cells/ml, particularly if bottles are opened and oxygen is permitted to enter. *Dekkera* and *Brettanomyces* spp. can form particularly dense clouds in soft drinks.

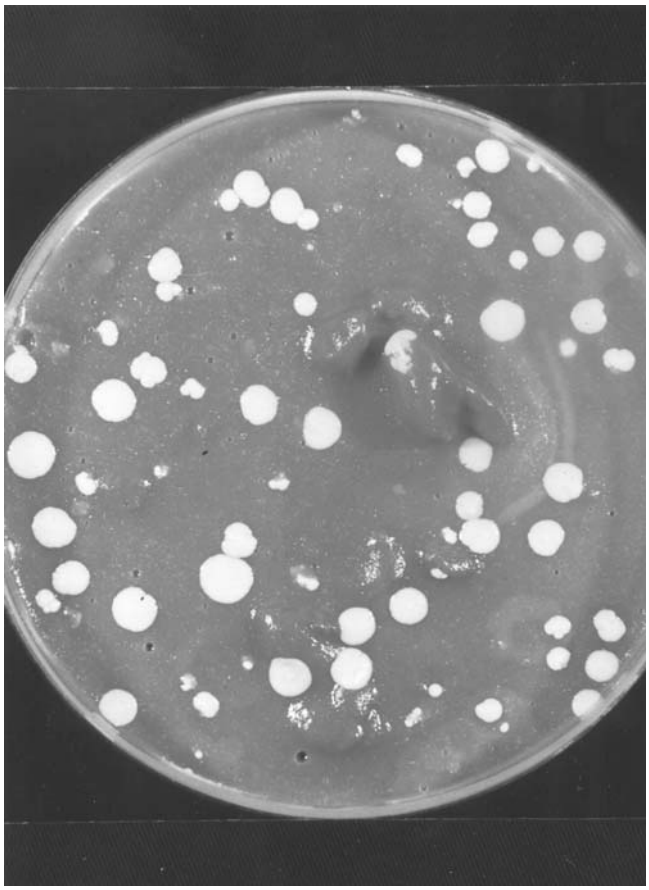

Fig. 11.3. Visible signs of yeast spoilage include white yeast colonies on a sugar-containing tomato soup. Such colonies might pass unnoticed on a white food, such as cheese or yoghurt

Amongst spoilage microorganisms, yeast cells are unusually dense, owing to their thick cell wall, and will sediment rapidly in liquid media. In water, yeast cells fall at 4–5 mm/h (Stratford 1992). As most beverage containers are only 300–400 mm in height, the majority of yeast cells will have formed a sediment within a few days and subsequent yeast growth forms a thick sediment, or a few visible colonies if the inoculum is small. A yeast sediment may be easily visible to the consumer, but the total volume of yeast if dispersed through the beverage would not be.

Yeasts may also aggregate to form more consumer-obvious structures, such as floccs, particulates or surface films. Flocculation has been reported in spoilage yeasts such as *Saccharomyces cerevisiae* and *Z. bailii* (Stratford 1992; Suzzi et al. 1992), but flocculation is most often characteristic of spoilage by *Saccharomycodes ludwigii* (Ribereau-Gayon et al. 1975; Beech and Carr 1977), forming "snowflake" particles in bottled cider.

Surface film formation is characteristic of a number of the less well-known spoilage yeast species, usually in three genera. Film formation is most frequently caused by strains of *Candida boidinii*, *Candida intermedia*, *Candida pseudointermedia*, *Candida pseudolambica* and *Candida tropicalis*, *Issatchenkia occidentalis* and *I. orientalis*, *Pichia anomala*, *P. fluxuum*, *P. fermentati*, *P. galeiformis* and *P. membranifaciens*, but can include spoilage yeasts such as *Dekkera* and *Brettanomyces* spp. (Vollekova et al. 1996). Interestingly, while *P. membranifaciens* is a frequently cited film-forming spoilage in the literature, *P. galeiformis* is a far more frequent cause of spoilage (M. Stratford and H. Steels, unpublished data), suggesting a high level of misidentification in the past.

Surface film formation is characteristic of *all* spoilage yeasts in high-sugar syrups. In sugar syrups with a sugar concentration greater than 35% w/w, the buoyant density of the sugar syrup is sufficiently great to cause yeast cells to float, rather than sediment. In spoilage of high-sugar syrups, it is normal to perceive yeast spoilage as a slick of yeast cells floating on the surface. On the industrial scale, sugar syrups or syrups of fruit juices are frequently spoiled by osmotolerant yeasts floating undetected as surface films on syrups stored in metal tanks. This may be aided by changes in temperature causing condensation on the metal above the headspace, diluting the surface layer and aiding faster yeast growth. Such yeast spoilage cannot be detected in the bulk of the syrup, and only emerges as a heavily contaminated layer when the tank is drained.

11.4.3 Off-Flavours and Off-Odours

To the public, off-tastes and smells in food are probably the least noticeable consequence of yeast spoilage, depending of the perspicacity of the tasters and their familiarity with what the food should normally taste like. One suspects that in a great number of instances, consumers do not notice the altered taste, or if they do, they think that perhaps the food should taste like that. Spoilage from altered taste is probably one of the least reported cases, and the true extent of spoilage from yeasts altering taste but not forming visible changes or gas can only be speculated upon. As an illustration of this fact, there is a story dating from many years ago concerning production of a fruit juice in cardboard packets to be drunk through a plastic straw.

There were many complaints by the public about the plastic straws, because they kept closing or blocking up. After much investigation into the plastic straws, the problem was traced to a mould spoilage problem in the fruit juice. Lumps of mould were blocking the plastic straws. Owing to the opaque cardboard packaging, the public could not see the mould, and not one complaint was ever received concerning the mouldy off-flavours in the spoiled fruit juice.

In theory, yeast growth and metabolism can influence the flavour and smell of food in one of two ways: either by removal of flavoursome food components; or by production of off-flavours or off-odours. In practice, yeast spoilage is nearly always accompanied by the latter, production of small but organoleptically powerful molecules through primary or secondary metabolism. Such molecules are usually volatile and hence give off-odours as well as off-tastes.

The off-taste produced in the greatest quantity is of course that of ethanol. Fermentative spoilage yeasts will generate ethanol in equimolar quantities with carbon dioxide. Ethanol has an unusual slightly sweet taste, and a spoiled fruit juice has a distinct sweet flat note. Comments from consumers reflect their recollections of fermented beverages, "like bad beer" or "like bad wine flavour". Other volatile off-flavours formed by yeasts include acetaldehyde – pungent apples; acetic acid – vinegar; ethyl acetate – sweet pineapple note; diacetal and acetoin – sweet butter.

Spoilage due to certain yeast species may be characterized by distinct off-flavours. *Brettanomyces intermedius* (*Dekkera bruxellensis*) may have a distinct mouse flavour (Beech and Carr 1977; Lafon-Lafourcade 1983), described as leathery, sweaty, farmyard or "Brett" in red wine (Parker 1988). *Pichia* species, notably *P. membranifaciens*, have a yeasty aldehyde off-flavour, forming high levels of acetic acid, acetaldehyde (Rankine 1966; Lafon-Lafourcade 1986) and esters, including ethyl acetate (Lanciotti et al. 1998). *Saccharomycodes ludwigii* forms high levels of acetoin and acetaldehyde (Ribereau-Gayon et al. 1975; Bravo-Abad and Redondo-Cuenca 1985; Romano et al. 1999), while *Kloeckera apiculata* off-flavours in cider comprise high levels of esters and volatile acids (Reed and Peppler 1973; Beech and Carr 1977). The growth of lipolytic yeasts such as *Y. lipolytica*, on fat-rich substrates such as cheese or meat, may result in "free fatty acid rancidity" (Ingram 1958). In an unexpected twist, addition of preservatives to foods may make them more susceptible to spoilage by off-flavours, if the yeasts in question are resistant, and proceed to degrade the preservative. Sorbic acid can be degraded by *Z. rouxii* and *Debaryomyces hansenii* to 1,3-pentadiene, giving a petroleum-like off-odour (Casas et al. 1999).

11.5 Economic Effects of Yeast Spoilage

Any discussion on the economics of food spoilage should first address the problem of estimating the worldwide scale of losses due to yeasts. The following questions require answers. How much spoiled food is eaten, not noticed by the consumer? How much yeast-spoiled food is thrown away without publicity? Either industrially or domestically? To what degree is yeast spoilage complained about?

Frankly, it is impossible to make any true estimation of the levels of yeast spoilage, but the costs must run into millions, possibly billions of euros per year. While any fermentative incidents may be largely reported, visible yeast spoilage is likely to be only par-

tially reported, and spoilage due to off-flavours is likely to be grossly underreported. In addition, there are probably a multitude of instances of minor growth of yeasts causing slight or no customer-perceptible effects that are never reported. By the definition of yeast spoilage used here – being perceptible to the customer– these may not even be classified as food spoilage. Furthermore, as yeast spoilage does not concern public safety, food production companies are not required to publicize any spoilage incidents, and relatively few are ever reported, in order to protect the company brands involved. All that can be said with certainty is that the true scale of yeast spoilage is orders of magnitude greater than the published data, particularly amongst the less obtrusive spoilage species.

Foods in general are "open" or "closed". Open foods can be the non-packaged traditional foods, or packaged foods that have been opened by the consumer. Either way, open foods are freely accessible to infection by any yeast species present. Open foods can be regarded as having an "open shelf life", usually of short duration. Instructions to the customer may be, for example, "refrigerate and consume within 3 days of opening". Such instructions are designed to be fail-safe, allowing a big yeast infection and allowing considerable leeway in temperature. Yeast spoilage of open foods, either domestic or industrial, is very often a result of foods kept for too long or at a very abused temperature.

"Closed shelf life" indicates the stability of a food packaged in a factory and not accessible to yeast infection. Any yeast cells within the food have therefore been derived from the raw materials or from the site of production. Such packages are usually given an antimicrobial treatment to kill or prevent growth of any microbes in the package. Ideally, the antimicrobial measures in place should be sufficient to prevent all microbial growth and completely eliminate food spoilage. In practice, this is often not possible for a variety of reasons; these include legal issues, taste and food quality, public perception and cost. For example, soft drinks may be made immune to spoilage by addition of high levels of sorbic acid, 1,000 ppm at pH 3.4. However, sorbic acid has a legal limit in Europe of 300 ppm (Anon 1989), a taste threshold of approximately 150 ppm and a distinct adverse taste above 300 ppm. Furthermore, the European public is averse to preservatives such as sorbic acid, regarding them as chemical additives. Even when pasteurization is employed, the heat treatment given is always insufficient to kill all microbes every time, occasionally one will survive. As a result, most systems are designed to prevent the growth of most, but not all microbes, and to minimize, but not prevent spoilage. A modicum of good factory hygiene and good manufacturing practice (GMP) is therefore required to fill any gaps left in the preservation system.

When spoilage in foods is reported, the company involved may take one of several courses of action. Major incidents, particularly involving highly fermentative yeasts, may require a public recall of all products involved. This may even extend to television advertising and requests to customers to return products to the shop. Such incidents are rare, but highly damaging to the brand image of the companies concerned. Minor incidents may be on a smaller scale, or may only involve spoilage to a lesser degree. Minor incidents often require a silent recall of all products on shop shelves and in the supply chain. The cost of an incident of food spoilage can be considerable. For example, a soft drinks factory may become infected with a spoilage yeast. Soft drinks are infected, but it may take a month for the yeast to grow and spoilage to become visible. Soft drinks lines run at 30,000 bottles per hour, for perhaps

16 h a day in summer (yeast spoilage is always more prevalent in late summer). Recalling bottles from the infected line could run to more than ten million items. Direct costs of spoilage include the cost of the products, costs of recall and costs of disposal. The indirect cost, the damage done to the company name and the brand image, can be much severer, even to the extent of complete closure and withdrawal of the brand.

11.6 Which Yeasts Cause Spoilage and What are the Properties of the Successful Spoilage Yeasts?

Imagine for a moment a nutritious food, prepared as a hot-water extract of germinated wheat or barley grains, prepared in shallow dishes and exposed to the environment. It is likely that almost all of the 800 yeast species described by Barnett et al. (2000) would grow on this food, and almost all could thus be described as spoilage yeasts (of malt extract). In practice, however, relatively few yeast species are responsible for the majority of instances of food spoilage by yeasts. Pitt and Hocking (1997) noted that while over 110 species from 30 genera were associated with foods, in their experience only about 10–12 species were responsible for spoilage of foods which had been processed and packaged according to normal standards of GMP (Table 11.2). A near identical list of yeast species was presented by Tudor and Board (1993) as the most commonly encountered spoilage yeasts, and Stratford et al. (2000), while only considering spoilage of fruit juices and soft drinks, listed eleven of the most significant spoilage yeasts (Table 11.2). Davenport (1996, 1997, 1998), in

Table 11.2 The most significant and commonly reported food spoilage yeast species

Pitt and Hocking (1997)	Tudor and Board (1993)	Davenport group 1 in Stratford et al. (2000)
Brettanomyces bruxellensis	*B. intermedius*	*B. anomalus*
Candida krusei	*Candida holmii*	*B. bruxellensis*
Candida parapsilosis	*Candida krusei*	*B. naardenensis*
Debaryomyces hansenii	*Debaryomyces hansenii*	*Hanseniaspora uvarum*
Kloeckera apiculata	*Kloeckera apiculata*	*Saccharomyces bayanus*
Pichia membranifaciens	*P. membranifaciens*	*Saccharomyces cerevisiae*
Rhodotorula mucilaginosa	*Rhodotorula* spp.	*Schizosaccharomyces pombe*
Saccharomyces cerevisiae	*Saccharomyces cerevisiae*	*Saccharomyces exiguus*
Schizosaccharomyces pombe	*Schizosaccharomyces pombe*	*Torulaspora delbrueckii*
Torulopsis holmii	*Zygosaccharomyces bailii*	*Z. bailii*
Z. bailii	*Z. bisporus*	*Z. bisporus*
Z. bisporus	*Z. rouxii*	*Z. microellipsoides*
Z. rouxii	–	*Z. rouxii*

Note recent changes in nomenclature (Barnett et al. 2000; Kurtzman 2003); *B. intermedius* = *B. bruxellensis* = *Dekkera bruxellensis*; *B. anomalus* = *Dekkera anomala; Candida krusei* = *Issatchenkia orientalis; Candida holmii* = *Torulopsis holmii* = *Saccharomyces exiguus* = *Kazachstania exigua*; *Kloeckera apiculata* = *H. uvarum*; *Z. microellipsoides* = *Torulaspora microellipsoides*

his "forensic approach " to yeast spoilage considered that it was the properties of the spoilage yeasts that were most significant, not merely the taxonomic names. He considered that the most dangerous spoilage yeasts (group 1) included *Z. bailii*, and abnormal strains of *Saccharomyces cerevisiae* all shared a number of physiological properties (Davenport 1996). Clearly there is general consensus as to which yeasts constitute the greatest threat to foods.

However, there are, in addition, a far larger number of yeast species that also cause food spoilage on a lesser scale. Tudor and Board (1993) referred to these as the "second division yeasts" and listed 19 species. Davenport (1996) also referred to lesser spoilage yeasts, calling them group 2 (spoilage and hygiene yeasts), opportunist species that would only cause spoilage following an error in manufacturing. These were distinct from group 3 (hygiene yeasts) that would not cause spoilage even if inoculated onto a food. Group 3 yeasts could be used as indicator species of factory hygiene.

Having established that the majority of yeast species grow well on a medium like malt extract, why is it that so few yeasts are considered dangerous spoilage species? What extraordinary properties do these species possess? Are these the commonest yeast species? Or do they proliferate more quickly? Data presented by Deak and Beuchat (1996) showed that 99 yeasts species were isolated from all foods, and of these *Z. bailii* only comprised 3.05%, *Z. rouxii* 2.06%, *Saccharomyces exiguus* 1.76% and *B. bruxellensis* 0.14%. The most commonly isolated yeast species from foods were *Candida* and *Pichia* spp. Clearly frequency of isolation does not account for the fame of a few yeasts as dangerous spoilage species. Similarly the growth rate of these yeast species is not exceptional, indeed growth of *Zygosaccharomyces* spp. is often slower than for the majority of yeast species. *Z. bailii* doubles in approximately 2.5 h, *Schizosaccharomyces pombe* doubles in approximately 4 h, while the generation time of *Z. lentus* is near 12 h (Stratford et al. 2000; Steels et al. 1998).

Observations made by Davenport as to the physiological properties of group 1 spoilage yeasts appear relevant here. Group 1 yeasts, comprising the most dangerous spoilage yeasts, were observed to be preservative-resistant, osmotolerant, highly-fermentative and vitamin-requiring (Davenport 1996, 1997). Is it not possible that the highly fermentative property may cause "blown" packages and draw the attention of consumers to spoilage? Similarly the properties of preservative resistance and osmotolerance may enable the spoilage species to proliferate in food environments inimical to the great majority of yeast species. Indeed, does not the list of the most dangerous spoilage species indicate a list of extremeophiles, the only yeast species able to tolerate the high osmotic pressures or elevated concentrations of acidic preservatives used in foods? The individual factors involved in food preservation will now be examined in isolation, and the yeast species most resistant to that preservation factor identified.

11.7 Factors Comprising Preservation Systems

The microbial threat to any foodstuff will be resisted by the "preservation system" used with that food. A preservation system can be regarded as a composite of factors intrinsic to the food, physical and chemical preservation, hygiene and packaging measures. In the food itself this may encompass low pH, low water activity, lack of

nutrients or presence of essential oils. Physical preservative measures include heat and pasteurization, chilled storage, carbonation or modified atmospheres, and chemical preservatives include sorbic, acetic and benzoic acids. Very few foods are protected by a single antimicrobial factor, for example a can of pasteurized soft drink is protected by heat, the acidity of the soft drink, and the packaging restricting oxygen ingress.

11.7.1 Heat

Heat is one of the commonest and most effective methods of preserving foods and beverages. Foods can be very effectively sterilized by retorting sufficiently to kill bacterial spores, or alternatively given a milder heat treatment, pasteurization, that is designed to eliminate vegetative cells. Heat may be applied to the complete package, of food and container, as in tunnel pasteurization, or liquids may be pasteurized "in line" by passage through a plate pasteurizer. The liquid food/beverage can then be hot-filled at 90°C, thus sterilizing the container, or aseptically cold-filled into sterile packaging.

Yeasts are, without exception, relatively sensitive to heat. Temperatures between 45 and 53°C, depending on the species, rapidly kill almost all yeasts. The *D* value indicates the time required to kill 1log (90%) of the inoculum. Yeast *D* values rarely exceed 1 min at 55°C (Corry 1973; Splittstoesser 1996). In comparison, vegetative cells of bacteria such as *E. coli* can also be killed at 52°C but with a *D* value of 18 min (Splittstoesser et al. 1995). The yeast species most resistant to heat include *Saccharomyces cerevisiae*, *P. membranifaciens*, *P. anomala* and *P. galeiformis* (Put et al. 1976; Beuchat 1981; Tchango Tchango et al. 1997) and also *Candida albicans*, *Clavispora lusitania*, *I. orientalis* and *Schizosaccharomyces pombe*. Yeast species showing unusual sensitivity to heat include *Debaryomyces hansenii*, *Z. rouxii* (Beuchat 1981; Samson et al. 1981) and *Cryptococcus magnus* (M. Stratford and H. Steels, unpublished data), which are killed at temperatures as low as 40°C.

Very little yeast spoilage is therefore expected in properly pasteurized foods, except where the yeast cell inoculum is very large. A few survivors might be expected from a yeast population of 10^5 cells/ml heated for 20 min at 60°C. The heat resistance of yeasts may be increased marginally by the presence of high concentrations of sugars or similar solutes (Gibson 1973; Corry 1976), or by cells being in the stationary phase of growth (Iwahashi et al. 1995). The presence of yeast ascospores may also increase heat resistance to a greater degree (Put et al. 1976; Tilbury 1980a), but not to the level achieved by mould genera such as *Byssochlamys* (Tournas 1994) that can survive 25 min at 90°C. The heat resistance of yeast ascospores is also species-dependent, with *Z. bailii* ascospores being less heat resistant than those of *Saccharomyces cerevisiae* (Thomas and Davenport 1985; Raso et al. 1998). As a general rule, yeast ascospores are 10°C more resistant than are their vegetative cell counterparts (Lund 1951; Davenport 1980a).

11.7.2 Acidity and Acetic Acid

In general terms, the pH range of foods and beverages extends from pH 2.5 to pH 7.5. Acidic foods are particularly associated with yeast spoilage, ranging from the

low-pH cola-type beverages, approximately pH 2.5, cider, wine and soft drinks, approximately pH 2.8–3.4, to fruit juices ranging from pH 3.0 in unripe lemon or grapefruit to pH 4.0-4.5 in ripe apple or tomato juices (Hicks 1990). Acidic taste is usually balanced by addition of sugar, with foods of lower pH and higher concentrations of acids, for example citric acid, requiring greater levels of sweetening. This, of course, greatly increases the risk of yeast spoilage in such foods.

The pH range of yeasts, however, extends from below pH 1.0 to pH 10.0 (Aono 1990; Stratford et al. 2000; Steels et al. 2002b). Figure 11.4 shows that the majority of spoilage yeast species are able to grow at pH 2.0 (Pitt 1974) and up to pH 8.5. Furthermore, examination of the growth of yeasts shows that only when the pH falls below 3.0 are yeasts low-pH-stressed, as indicated by slower growth or reduced yield. Acidity in foods per se is therefore of no hindrance to spoilage yeasts, and low pH alone does not contribute directly to protection of foods from yeasts or moulds. However, low pH does have a dramatic effect on food preservation in the presence of weak-acid preservatives, such as acetic acid.

Acetic acid, in small quantities, is a natural component of oils of citrus fruits (Burdock 1995) and strawberries (Willhalm et al. 1966). It can be formed by fermentation in yeasts, notably *Dekkera* and *Brettanomyces* spp. (Geros et al. 2000), but is usually associated with acetic acid bacteria (*Acetobacter* spp.) metabolizing the ethanol in yeast-fermented beverages. Acetic acid is recognized in

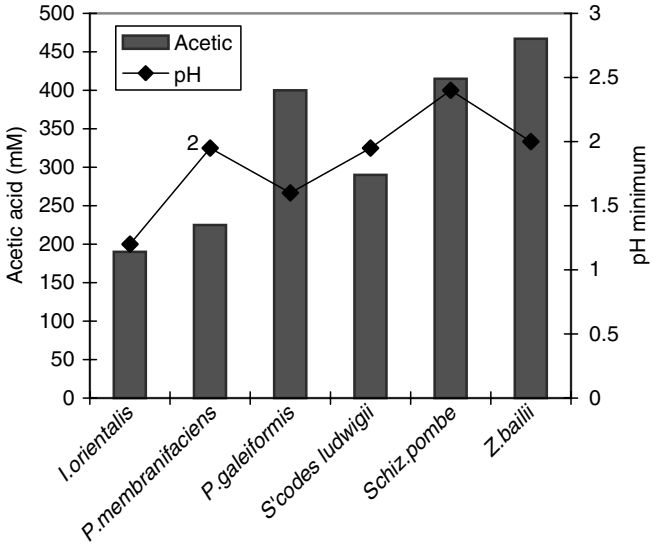

Fig. 11.4. Extreme acetic acid resistance at pH 4.0 shown by certain spoilage yeast species does not correlate with resistance to low pH. The yeast strains were *Z. bailii* NCYC°1766, *Saccharomycodes ludwigii* NCYC°732, *Pichia membranifaciens* 210, *P. galeiformis* 917aH, *Issatchenkia orientalis* CMCC°2528 and *Schizosaccharomyces pombe* NCYC°2722. All had been identified by D1/D2 26S rDNA sequencing

Europe as an acidulant, a food additive that may be used *quantum satis* (Anon 1989). Foods containing acetic acid are easily distinguished by their pungent vinegary taste and smell. These include tomato ketchup, pickles, mayonnaises, salad dressings and kombucha (acetic-fermented tea).

The antimicrobial action of acetic acid is strongly influenced by pH. Acetic acid is a weak acid, forming a dynamic equilibrium in aqueous solution between undissociated acetic acid molecules and acetate anions. The undissociated acid predominates at low pH and appears solely responsible for the antimicrobial activity (Maesen and Lako 1952; Ingram et al. 1956). Undissociated acetic acid is a small, uncharged molecule that is able to dissolve in the hydrophobic lipid plasma membranes of microbes, and thus rapidly pass by diffusion into the cytoplasm (Conway and Downey 1950a, b; Suomalainen and Oura 1955; Walter and Gutknecht 1984; Casal et al. 1998). Once in the cytoplasm, acetic acid dissociates rapidly into acetate ions and protons, causing a severe drop in the pH of the cytoplasm (Neal et al. 1965; Carmelo et al. 1997; Guldfeldt and Arneborg 1998), and inhibiting or killing the microbe.

Yeast species vary widely in their resistance to acetic acid (Fig. 11.4), a fact utilized as a taxonomic test (growth in 1% acetic acid; Barnett et al. 2000). Yeast species most resistant to acetic acid include *I. orientalis* and *I. occidentalis*, *P. galeiformis* and *P. membranifaciens*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*, *Z. bailii*, *Z. bisporus*, *Z. lentus* and *Z. kombuchaensis* (Pitt 1974; Warth 1989a; Kalathenos et al. 1995; Malfeito Ferreira et al. 1997; Saeki 1989; Steels et al. 2002a). *Z. rouxii* strains are unexpectedly sensitive to acetic acid (Giudici 1990). The yeast spoilage flora of any food preserved with acetic acid is therefore predominated by resistant species such as *Z. bailii* and *P. membranifaciens* (Dennis and Buhagiar 1980). Given the only moderate acetic acid resistance of *P. membranifaciens* and the strong acetic resistance of *P. galeiformis* (Barnett et al. 2000) it is likely that many acetic acid resistant *P. galeiformis* strains have been misidentified in the past as *P. membranifaciens*.

11.7.3 High Sugar and Osmotolerance

Yeasts able to grow in high sugar concentrations are often referred to as osmotolerant or osmophilic; however, these names are often also applied to salt-tolerant yeasts, high sugar and high salt being regarded as synonymous in the bacterial field and termed low water activity or a_w. However, research has shown that for yeasts, salt and sugar act inhibit by very different mechanisms (Watanabe et al. 1995) and will therefore be treated separately in this chapter.

High-sugar foods include honey, jams and marmalade, nougat, toffee and caramel, sugar syrups and molasses, marzipan, confectionary and crystallized fruit, fruit cordials and juice syrups. Sugar at a concentration ofmore than 30% w/w has been described as having an antimicrobial effect (Tarkow et al. 1942), yet most yeast species grow well at up to 45% w/w. However high-sugar foods can contain more than 67% sugar w/w and are prone to spoilage only by osmophilic yeasts, amongst which are some of the most xerotolerant organisms known (Tilbury 1980a, b).

Osmotolerant yeasts have been defined as those able to grow at 50% w/w sugar, 0.88 a_w (Sand 1973) while osmophilic species are those able to grow at 60% sugar w/w (Davenport 1975). In addition to causing spoilage, growth of osmophilic yeasts in sugar and syrups can represent a major source of infection of spoilage yeasts, in foods prepared using the sugar as a raw material (Ingram 1949; Scharf 1960).

Detection or enumeration of yeasts from high-sugar foods can be difficult. Plating out yeasts from sugar syrups on normal low osmotic strength media will cause an osmotic down-shock, and prevent yeast growth for several days. It is recommended that samples from high-sugar foods be diluted in 40% sugar to avoid this problem (Beuchat 1998) and grown on high-sugar media containing half the sugar level of the original food.

The antimicrobial action of sugar appears to be primarily via osmosis (Martinez de Maranon et al. 1996, 1997) with the water content of the cytoplasm rapidly removed from cells placed in concentrated sugar. Resistance to high sugar is largely based around accumulation of high concentrations of compatible solutes in the cytoplasm, such as glycerol or arabitol (Tokuoka 1993). Surviving cells grow slowly in high sugar concentrations and are consequentially much reduced in volume.

Yeast species able to grow in high sugar concentrations include *Debaryomyces hansenii*, *Schizosaccharomyces pombe*, *P. ohmeri*, *Z. bailii*, *Z. bisporus*, *Z. lentus* and most notably *Z. rouxii* and *Z. mellis* (von Schelhorn 1950; Scarr 1951, 1968; Sand 1973; Tilbury 1980a, b; Lafon-Lafourcade 1983; Jermini and Schmitt-Lorenz 1987; Tokuoka 1993; Giudici 1990). Spoilage of high-sugar foods is, however, dominated by *Z. bailii*, *Z. bisporus* and *Z. rouxii* (Tokuola 1993).

In addition, there are a family of closely related osmophilic yeast species all associated with spoilage of sugar foods and with insects, such as bees and wasps (Steels et al. 2002b; Stratford and James 2003). These species generally do not form sexual spores and are so termed *Candida*, with the exception of one species that has been sporulated and was given the generic name *Starmerella* (Rosa and Lachance 1998). These are relatively unusual spoilage yeasts and it has been proposed that this group of bee-/wasp-associated yeasts are able to cause spoilage, only following infection from bees/wasps attracted to sugary foods. This group of yeasts includes *Candida bombi*, *Candida apicola*, *Candida etchellsii*, *Starmerella bombicola*, *Candida lactiscondensii*, *Candida davenportii*, *Candida stellata* and *Candida magnoliae*.

11.7.4 High Salt and Halotolerance

Salt toxicity in yeasts is recognized to be primarily due to the toxicity of the sodium ion, Na^+, rather than to the osmotic effects imposed by high-salt concentrations. Resistance to salt is conferred through active sodium efflux pumps or sequestration of sodium ions (Watanabe et al. 1995; Ferrando et al. 1995; Nass et al. 1997; Alepuz et al. 1997; Rios et al. 1997). Salt shock can cause expression of heat shock proteins (Lewis et al. 1995) and accumulation of glycerol (Onishi 1963; Ohshiro and Yagi 1996; Lages et al. 1999). The pH window for optimal yeast growth has been observed to be far narrower in the presence of high salt concentrations (Onishi 1963). Foods containing substantial concentrations of salt include salted meats such as sausage

and bacon, brines and pickled vegetables, and salted fermented foods such as sauerkraut, soy sauce, shoyu and miso.

The majority of yeast species are salt-tolerant microbes and are able to grow in media containing up to 1.5–2 M sodium chloride (8.5–11.5% w/v). The most salt-resistant yeast species able to grow in 20% w/v salt include *Debaryomyces hansenii* and *Debaryomyces tamarii*, *Z. rouxii*, and to a lesser extent, *Z. mellis* (Kurtzman 1998), *Candida etchellsii*, *Candida sorbosivorans* and *Candida parapsilosis* (Onishi 1963; Corry 1973; Tokuoka 1993; Prista et al. 1997; Thome-Ortiz et al. 1998; Betts et al. 1999; Lages et al. 1999). Other yeasts moderately resistant to salt include *P. guilliermondii*, *P. membranifaciens* and *P. subpelliculosa*, *Torulaspora delbrueckii* and *I. orientalis*. Lages et al. (1999) reported *Z. bailii* to be salt-sensitive, on the basis of tests on the atypical type strain: most strains of *Z. bailii* are in fact moderately resistant to salt. Spoilage of foods containing high concentrations of salt is dominated by strains of *Debaryomyces hansenii*, sometimes called *Candida famata*, and *Z. rouxii* (Kato 1981; Fleet 1992; Tokuoka 1993).

11.7.5 Ethanol and Alcoholic Beverages

Ethanol is present in alcoholic beverages fermented by the brewing and winemaking yeast *Saccharomyces cerevisiae* at concentrations up to 20.3% v/v (Peres and Laluce 1998). Ethanol has also been considered as a means of preservation in packaged foods, such as bakery products (Kalathenos and Russell 2003). The degree of protection from spoilage conferred by ethanol depends upon the concentration of ethanol and the species of yeast involved, but overall spoilage is minimized by ethanol in excess of 15% v/v and is eliminated at concentrations greater than 22% v/v. *Z. bisporus* has been isolated from sherries containing 22% v/v alcohol (Hammond 1976; quoted by Thomas 1993). In general terms, fortified wines (17.5% v/v) are spoiled very infrequently, wines (10–15% v/v) are spoiled occasionally, and beers, ciders and alcoholic soft drinks (3–8% v/v) are sufficiently unstable as to require a short shelf life or protection by pasteurization or use of preservatives.

The mechanism of action of ethanol against yeasts is centred upon the integrity of the plasma membrane, ethanol causing leakage and dissipation of the proton motive force, and affecting transport processes (Sa-Correia and van Uden 1986; Cartwright et al. 1986; Jirku et al. 1991; Stevens and Servaas Hofmeyer 1993). Ethanol also affects mitochondria and induces "petites" (Ibeas and Jimenez 1997).

The yeast species most resistant to ethanol are not surprisingly those fermentative species that produce ethanol at the highest concentrations. These include *Dekkera anomala* and *Dekkera bruxellensis*, *I. orientalis*, *P. anomala* and *P. galeiformis*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, *Saccharomycodes ludwigii* and *Z. bailii*. Spoilage yeasts of high-alcohol beverages such as wine are also predominated by *Saccharomyces* spp., *Zygosaccharomyes bailii*, *Saccharomycodes ludwigii* and *Dekkera*/*Brettanomyces* spp. (Peynaud and Domercq 1959; Kunkee and Goswell 1977; Thomas and Davenport 1985; Thomas 1993; Baleiras Couto and Huis in't Veld 1995; Kalathenos et al. 1995; du Toit and Pretorius 2000).

11.7.6 Sulphur Dioxide (Sulphite)

Sulphites are traditional preservatives that have been used in the production of wine, cider and beer for centuries. Flowers of sulphur were burned in wooden barrels, which were then filled, ensuring sterilization of the barrel and thepresence of sulphites, as dissolved SO_2 in the beverage (de Keersmaecker 1996). Sulphites are currently permitted preservatives in Europe (Anon 1989) for use in wine and cider.

Like acetic acid, SO_2 is a weak acid, existing in solution as a pH-dependent equilibrium between molecular SO_2 at low pH and bisulphite and sulphite ions at higher pH (King et al. 1981). Only molecular SO_2 appears to exert an antimicrobial action. Molecular SO_2 passes into cells rapidly, by diffusion through the plasma membrane (Stratford and Rose 1986), dissociates to bisulphite ions and causes a catastrophic fall in cytoplasmic pH (Pilkington and Rose 1988). Membrane transport processes are consequentially inhibited (Freese et al. 1973) and ATP levels fall rapidly (Schimz and Holzer 1979). Sulphites are highly reactive molecules and may also denature a variety of other cytoplasmic targets (Gould et al. 1983).

Resistance of yeasts to sulphites is not always found in all strains of an individual species. However, highly resistant strains (Fig. 11.5) are most likely to be found within the following species: *Saccharomycodes ludwigii*, *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces exiguus; Z. bailii* and *Z. lentus* (Jakubowska 1963; Hammond and Carr 1976; Minarik and Navara 1977; Goto 1980; Usseglio Tomasset et al. 1981; Stratford et al. 1987; Rose 1987; Thomas 1993). Consequentially spoilage in beverages where sulphites are commonly used is

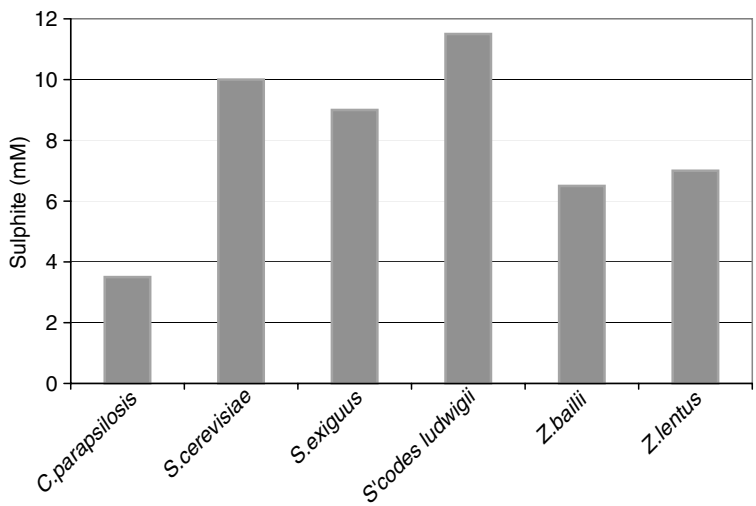

Fig. 11.5. Extreme SO_2, sulphite, resistance at pH 4.0 is shown by certain strains of spoilage yeasts. The yeast strains were *Z. bailii* NCYC°1766, *Saccharomycodes ludwigii* NCYC°730, *Saccharomyces cerevisiae* 292, *Saccharomyces exiguus* 152, *C. parapsilosis* CMCC°3397 and *Z. lentus* NCYC°1601. All had been identified by D1/D2 26S rDNA sequencing

dominated by *Saccharomyces cerevisiae*, *Saccharomycodes ludwigii* and *Z. bailii* (Peynaud and Domercq 1959; Beech 1961; Amerine and Kunkee 1968; Sandu-Ville 1977; Thomas 1993).

11.7.7 Preservatives (Sorbic Acid and Benzoic Acid)

Sorbic acid and benzoic acid are permitted preservatives in Europe, but may be only added up to specified levels in specified foods (Anon 1989). In the USA, however, sorbic acid and benzoic acid have GRAS status and may be found at higher concentrations. The use of these preservatives is widespread in foods as diverse as spreads, fruit syrups, confectionary, bakery products, wine and soft drinks, spent malt grains and almond paste (Sand 1973; Rankine and Pilone 1974; Faid et al. 1995; Jager 1997; Stratford and Eklund 2003). It is also permitted, and fairly common, to encounter mixtures of sorbic acid and benzoic acid in foods, both at reduced concentrations (Taylor 1998). It has been suggested that sorbic acid is more inhibitory to yeasts than benzoic acid (Pitt 1974) but also that benzoic acid is better than sorbic acid at controlling spoilage at pH 3 (Sand and Kolfschoten 1969). As a rule of thumb, sorbic acid and benzoic acid are roughly equal and additive in their anti-yeast effects on a parts per million, milligrams per litre basis.

Sorbic acid is a six-carbon fatty acid, unsaturated in positions 2 and 4, while benzoic acid comprises a carboxylic acid substituted into a benzene ring. Both acids have the great advantage of having relatively little taste and a proven record of safe human consumption and lack of genotoxicity (Ferrand et al. 2000). Benzoic acid is a natural constituent of cranberries and cloves (Swartz and Medrek 1968; Chichester and Tanner 1972). The free acids of both are difficult to dissolve and these preservatives are normally added to foods as soluble salts, sodium benzoate and potassium sorbate.

Both sorbic acid and benzoic acid are weak acids in aqueous solution, and as can be predicted inhibit microbes only in the undissociated acid form that predominates at low pH (Ingram et al. 1956; Azukas et al. 1961; York and Vaughn 1964). Uptake of the undissociated acid molecules is extremely rapid, probably by simple diffusion of these lipophilic acids through the plasma membrane (Macris 1975; Reinhard and Radler 1981b; Walter and Gutknecht 1984; Warth 1989b). Within the cytoplasm, weak acid molecules dissociate and may lower the cytoplasmic pH (Krebs et al. 1983; Holyoak et al. 1996), as has been demonstrated for acetic acid and sulphite.

However, it has been pointed out that the concentrations of protons released by inhibitory concentrations of sorbic acid are insufficient to cause significant pH change (Stratford and Anslow 1998). Alternative sites of action for sorbic and benzoic acids include the membrane (Reinhard and Radler 1981a; Stratford and Anslow 1998), respiration pathways (Palleroni and de Pritz 1960) and glycolysis metabolism (Azukas et al. 1961; Burlini et al. 1993). The effect of subinhibitory concentrations of preservatives is to cause slower growth of yeasts, smaller cell size, and much reduced cell yields (Cole and Keenan 1987; Neves et al. 1994; Stratford and Anslow 1996).

Resistance of certain yeast species to preservatives is a significant threat to the stability of preserved foods. This threat is further exacerbated by the ability of yeasts to adapt to preservatives, enabling growth and spoilage at much higher preservative

concentrations (Ingram 1960; Warth 1977, 1988; Malfeito Ferreira et al. 1997). It is envisaged that foods or beverages containing preservatives may be spilled in production facilities, diluted as they are washed into the drains, and that spoilage yeasts can grow on dilute preservatives, adapting to their presence and thus gain the ability to spoil the preserved foods.

The yeast species most notorious for resistance to preservatives (Fig. 11.6) is *Z. bailii*, together with its close relatives *Z. bisporus* and *Z. lentus* (Ingram 1960; Jermini and Schmidt-Lorenz 1987; Warth 1989a, c; Neves et al. 1994; Steels et al. 1999). These species can grow in soft drinks containing sorbic or benzoic acids at well in excess of the legal limit in Europe. A strain of *Z. bailii* has even been reported that *requires* benzoic acid for growth (Anon 1998). Other less resistant species include *Y. lipolytica* (Rodrigues and Pais 2000) *Candida lambica*, *Candida pseudolambica*, *I. orientalis* and *I. occidentalis*, and abnormal strains of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (Davenport 1996; M. Stratford and H. Steels, unpublished data). Several yeast species only show resistance to single preservatives, such as *Brettanomyces*/*Dekkera* spp., which are sorbic acid resistant but benzoic acid sensitive, or *P. membranifaciens*, *P. galeiformis* or *Schizosaccharomyces pombe*, which are sorbic acid sensitive and benzoic acid resistant (Fig. 11.6).

Spoilage in sorbic acid or benzoic acid preserved foods and beverages is consequentially dominated by these same species, *Z. bailii*, *Z. bisporus*, *I. orientalis* (as *Candida krusei*) and abnormal strains of *Saccharomyces cerevisiae* (Ingram 1960; Lloyd 1975; Thomas and Davenport 1985; Davenport 1996, 1997, 1998)

Much research has been carried out into the mechanisms of yeast resistance to sorbic acid and benzoic acid. Degradation of preservatives by metabolism was

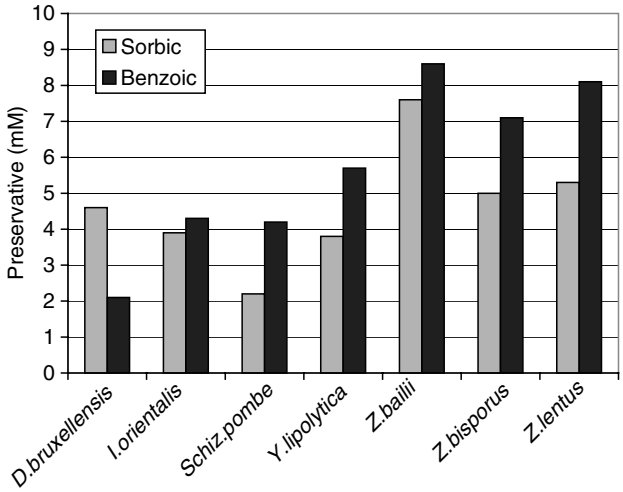

Fig. 11.6. Resistance to sorbic and benzoic acids at pH 4.0 is limited to the spoilage yeasts *Z. bailii* NCYC°1766, *Z. bisporus* NCYC°1555, *Z. lentus* NCYC°1601, *I. orientalis* CMCC°2528, *Yarrowia lipolytica* 474, *D. bruxellensis* 148 and *Schizosaccharomyces pombe* NCYC°2722. All had been identified by D1/D2 26S rDNA sequencing

shown by Warth (1977) not to be a significant factor in resistance. A weak-acid preservative efflux pump was proposed (Warth 1977, 1989b; Henriques et al. 1977) where preservatives were pumped out from the cytoplasm at the expense of ATP. The gene *PDR12* with homology to mammalian multiple-resistance drug pumps was shown to be important in preservative resistance in *Saccharomyces cerevisiae* (Piper et al. 1998). It was proposed that *PDR12* mediated the efflux of preservative anions from the cytosol (Holyoak et al. 1999). However, the work of Cole and Keenan (1987) demonstrated such rapid pH-dependent movements of weak-acid molecules by simple diffusion through the membrane, which must call into question any role for *PDR12* as an anion pump.

11.7.8 Carbonation (CO_2)

Carbonation is the process of dissolving carbon dioxide into beverages under pressure, forming the characteristic taste of sparkling beverages. These include colas, sparkling fruit drinks, mixers such as tonic or ginger ale, cream sodas, lemonades, and sparkling wines such as Cava and Champagne. Carbonation is measured in units of volumes bunsen, the CO_2 volume, at 0°C and atmospheric pressure, dissolved per volume of liquid, or in grams CO_2 dissolved per litre (1.96g/l = 1 vol; Mitchell 1990). Carbonation in soft drinks is typically around 3 vol, ranging from 1.5 vol in sparkling fruit juices to 5 vol in soda water or bottle-fermented wines (Sand 1976b; Mitchell 1990).

It is not often appreciated that carbonation has a considerable antimicrobial effect (Schmidt 1995; Monch et al. 1995), particularly at the higher concentrations permitted by increased pressure. The antimicrobial nature of highly carbonated low-pH soft drinks has enabled successful production of beverages such as "Codds Wallop" in relatively unhygienic conditions since 1870 (Taylor 1998; de Thouars 1999). Inhibition is not caused by pressure per se; yeasts are inhibited by pressurized CO_2, not by nitrogen (Lumsden et al. 1987). The degree of carbonation required to inhibit the growth of most yeasts species is of the order of 2 bar, although yeasts can be killed at 30 bar (Schmitthenner 1949; Amerine 1958; Kunkee and Ough 1966; Eyton-Jones 1987; van der Aar et al. 1993).

CO_2 is also a weak acid in aqueous solution, with a pK_a of 6.3 (Dixon and Kell 1989). Bicarbonate ions predominate at a pH above 6.3, while at more acidic pH, molecular CO_2 is dissolved in solution. The physiological effects of CO_2 at sublethal concentrations may include inhibition of cell division (Lumsden et al. 1987), inhibition of amino acid uptake (Knatchbull and Slaughter 1987), perturbation of cytoplasmic buffering (Sigler et al. 1981), induction of sporulation (Ohkuni et al. 1998) and membrane disruption (Dixon and Kell 1989). An action by CO_2 in lowering cytoplasmic pH is also possible. CO_2 is known to cross membranes so fast by diffusion that concentrations rarely differ across a membrane (Thomas 1995). With a pK_a of 6.3 and a near neutral cytoplasmic pH, CO_2 would certainly dissociate into bicarbonate and protons, forcing the cytoplasmic pH down. Recent work on ultrahigh pressure has shown a substantially greater microbial kill if pressure is applied in the presence of CO_2, for example supercritical CO_2 at 200 bar (Spilimbergo et al. 2002). It has been suggested that the lethality of CO_2 at high

pressure was probably a double effect due to a decline in cytoplasmic pH and cytoplasmic membrane modification.

The yeasts most resistant to carbonation are *Dekkera anomala*, *Dekkera naardenensis* and *Dekkera bruxellensis*, *Saccharomycodes ludwigii*, *Saccharomyces cerevisiae*, *Saccharomyces bayanus*, *Saccharomyces pastorianus* and *Saccharomyces exiguus* (Kunkee and Ough 1966; Reed and Peppler 1973; Ison and Gutteridge 1987; Dixon and Kell 1989; M. Stratford and H. Steels, unpublished data). These yeasts can grow in beverages containing up to 5–6 vol carbonation. Other moderately carbonation-resistant yeasts include *I. orientalis*, *P. fluxuum*, *Candida boidinii*, *Schizosaccharomyces pombe*, *T. delbrueckii*, *Z. bailii*, *Z. cidri*, *Z. microellipsoides* and *Z. fermentati* (Goswell 1986; Ison and Gutteridge 1987; M. Stratford and H. Steels, unpublished data).

Yeasts largely responsible for spoilage of carbonated beverages (some lightly carbonated) include *Dekkera anomala* and *Dekkera bruxellensis*, *Z. bailii*, *T. delbrueckii* and *Saccharomyces cerevisiae* (Pitt and Richardson 1973; Smith and van Grinsven 1984).

11.7.9 Low Oxygen Concentration

It is not often appreciated that low oxygen in a food or beverage is a major factor in preventing microbial spoilage (Sand 1976a, b). Low oxygen concentration is frequently assumed to be responsible for the antimicrobial effects of CO_2 described previously. In reality, these are two separate effects. If CO_2 is dissolved in a beverage, it does not displace the oxygen already there. This will only occur if CO_2 is bubbled through a beverage for some time; CO_2 being a heavy gas will form a layer above the beverage surface. Since oxygen in solution is in equilibrium with the gas in the headspace, there will, in this instance, be a progressive loss of oxygen from the beverage.

The concentration of oxygen in foods depends largely on the diffusion of oxygen from the atmosphere, the food surface being plentifully supplied with oxygen, while deeper into the food, the levels drops sharply. It is no coincidence that the majority of yeasts on fresh fruit and vegetables are found on the surface. Even in an open bottle of liquid, such as a soft drink, the level of oxygen is sufficiently reduced even under a few centimetres of liquid, to greatly inhibit the growth of respiring yeasts. Many processed foods also contain antioxidants, such as vitamin C, to maintain the freshness of the food, which have the effect of lowering the oxygen concentration within the food. Furthermore, most processed foods are contained in a package or bottle. Some packaging is completely oxygen impermeable, such as glass bottles or metal cans. This results in heat-processed bottled or canned foods being under greatly reduced oxygen tension, and only susceptible to spoilage by a limited number of microorganisms (Beuchat and Rice 1979). Different plastics vary considerably in their permeability by oxygen (Rodriguez et al. 1992), which can have a dramatic effect on the microbial species capable of spoiling within the package. It is well known that moulds or *Gluconobacter* spp. in fruit juices are greatly inhibited by oxygen-impermeable packaging (Follstad 1966; Sand 1971a, 1976a; Wyatt et al. 1995).

The benefits of oxygen to yeast growth are generally known, but not completely understood. Primarily, oxygen is required for respiration, as a terminal electron acceptor of the mitochondrial electron transport chain. For respiration, oxygen is required

in large quantities, and in a respiring yeast, respiration may be directly proportional to the oxygen concentration (Johnson 1967). Any yeast totally dependent on respiration will be greatly inhibited in a low-oxygen environment. This includes many very common yeast species in the genera *Rhodotorula*, *Sporidiobolus*, *Aureobasidium* or *Cryptococcus*.

Yeast species capable of fermentation are therefore immediately at an advantage in a low-oxygen environment. However, even fermenting yeasts may require oxygen for purposes other than respiration. Oxygen may be required in some yeast species for active sugar uptake, the Kluyver effect (Barnett and Sims 1982). It has been shown that fermentative brewing yeasts benefit from aeration (Grutzmacher 1991). The explanation for this is that *Saccharomyces cerevisiae* requires low concentrations of oxygen in order to synthesize unsaturated fatty acids and sterols as membrane components. In the complete absence of oxygen, growth of *S. cerevisiae* ceases unless exogenous unsaturated fatty acids and sterols are added (Andreasen and Stier 1953, 1954).

The micro-oxygen requirements of other yeast species have been little researched, but the knowledge available shows that they differ from those of *Saccharomyces cerevisiae*. *Z. bailii* and *Z. bisporus*, despite being fermentative species, are known to require oxygen (van Esch 1987) and would not grow anaerobically in synthetic media, unsupplemented by an unidentified factor present in yeast extract–peptone–dextrose (YEPD) (Rodrigues et al. 2001). Other fermentative spoilage species, *Debaryomyces hansenii* and *Z. microellipsoides*, also grow poorly without oxygen (Tilbury 1976; van Esch 1987).

It appears that spoilage in anaerobic or low-oxygen foods, even by fermentative species, may depend on the presence of as yet unidentified micronutrients.

11.7.10 Lack of Nutrients

Some years ago, a synthetic soft drink was successfully produced and marketed. The manufacturers then improved this by the addition of real fruit juice (Pitt and Hocking 1997). Following juice addition, the levels of spoilage increased to such an extent that the soft drink had to be removed from the market. Fruit juices were adding key nutrients that encouraged proliferation of spoilage yeasts. This illustrates the principle that microbial spoilage may be limited in certain instances by lack of nutrients in a food or beverage.

The nutrients required for growth of microorganisms have been investigated since the early work of Pasteur on fermentation of yeasts. By 1930 numerous studies had been carried out on the nutritional requirements of bacteria and yeasts with the very limited methods available. As a result of these studies, it became apparent that most of the microbial growth-promoting substances were from sources recognized as rich in water-soluble vitamin B, and attention was then centred upon this vitamin. Yeast species differ from each other in their requirements for vitamins. Growth with or without various vitamins has been used as characteristic diagnostic tests in the identification of yeasts (Wickerham 1951; Barnett et al. 2000). B-group vitamins are essential for yeast metabolism but some yeasts can synthesize some or all of their requirements, for example *P. anomala* can synthesize all vitamins, other yeasts can

only synthesize some; hence the remainder must be supplied by the medium (Davenport 1998).

In his "forensic" method of yeast classification, Davenport (1996) divided yeasts into three types: group 1 – spoilage yeasts; group 2 – potential spoilage and hygiene yeasts; group 3 – hygiene yeasts that will not cause spoilage. Davenport (1998) stated that as a defining characteristic, group 1 spoilage yeasts would not grow in the absence of one or more of the B-group vitamins. Spoilage yeasts known to require vitamins include *Z. bailii*, *Z. bisporus* and *Z. mellis* (Barnett et al. 2000), *Dekkera bruxellensis*, *B. naardenensis*, *Saccharomyces exiguus* and *Saccharomycodes ludwigii*. This may form the explanation for the observations of van Esch (1987) that *Zygosaccharomyces* spp. yeasts only occurred in fruit-juice-containing beverages.

In addition to vitamins, yeasts require several metallic ions for growth, including magnesium, potassium, zinc, iron, copper and manganese. In media or foods lacking these metal ions, yeast growth will be prevented. Soft drinks prepared with distilled water were observed to support very little yeast growth (Turtura and Samaja 1975). Many foods also contain acidulants such as citric acid or malic acid, which effectively remove metal ions by chelation. Certain foods may also contain the chelating agent EDTA in the USA, where EDTA has GRAS status. Metal-ion/acid-chelator complexes can be very stable, particularly where acids with multiple carboxyl groups, such as citric acid or EDTA, are complexed with multiply charged transition metal ions (Stratford 1999). Removal of metals by ion-exchange resins has been proposed for prevention of yeast spoilage in grape juice (Feng et al. 1997). Yeasts most resistant to the presence of chelating agents include *Dekkera bruxellensis*, *Dekkera anomala*, *Candida parapsilosis* and *I. orientalis* (M. Stratford and H. Steels, unpublished results). It is possible that resistance to citric acid, together with the ability to utilize nitrate, may enhance the ability of *Dekkera*/*Brettanomyces* spp. to spoil low-nutrient soft drinks (Smith and van Grimsven 1984; van Esch 1987).

11.7.11 Low Temperature

Low temperature in this context means spoilage at chill, at 5°C or less, and includes spoilage of frozen foods below 0°C. The usual definitions of temperature preference as applied to yeasts are psychrophilic, upper temperature limit 20°C, mesophilic, 0–48°C, and thermophilic, 20–50°C (Watson 1987). These definitions do not assist in defining low-temperature-spoilage yeasts as they could be either psychrophiles or mesophiles. A much more practical definition was proposed by Davenport (1980b) – "cold-tolerant yeasts", those capable of growth at 5°C or lower.

Cold temperatures form a hostile environment for the majority of typical spoilage yeasts and foods stored at low temperatures are also not typical substrates for yeast spoilage at moderate temperature. These include frozen vegetables such as peas, chilled and frozen fish and meat. The usual degradation of these foods by bacteria is arrested by the temperature, and there is an opportunity for spoilage by the slower-growing yeasts.

A number of general observations of cold-tolerant yeasts were made by Davenport (1980b). These were (1) basidiomycete yeasts predominated at low temperature;

(2) most were non-fermentative species; (3) an unusually high proportion, 50%, were nitrate positive. Very few of the typical spoilage yeasts are cold-tolerant. Yeasts that will not grow at 5°C or lower include *Z. bailii*, *Z. bisporus*, *Z. mellis* and *Z. rouxii*, *Dekkera anomala* and *Dekkera bruxellensis*, *Candida parapsilosis* and *Candida tropicalis*, *I. orientalis* and *I. occidentalis* (M. Stratford and H. Steels, unpublished results).

Spoilage at low temperature is therefore rarely accompanied by gas production, and spoilage is usually apparent as a result of surface growth. Yeasts reported at low temperatures are often in the basidiomycete genera *Rhodotorula* and *Cryptococcus* (Margesin et al. 2002) and include *Cryptococcus albidus* and *Cryptococcus magnus*, *Rhodotorula graminis*, *R. mucilaginosa* and *R. nothofagi* (M. Stratford and H. Steels, unpublished results), together with *Y. lipolytica*, *T. delbrueckii* and *Debaryomyces hansenii*. The minimum growth temperatures quoted by Davenport (1980b) are –12.5°C for *Debaryomyces hansenii*, –12°C for *Cryptococcus albidus* and –2°C for *R. glutinis*.

A recent development in low-temperature spoilage concerns the recent discovery of two new species in the genus *Zygosaccharomyces*, *Z. lentus* (Steels et al. 1998, 1999) and *Z. kombuchaensis* (Kurtzman et al. 2001). These species are closely related to *Z. bailii* and *Z. bisporus*, but appear to be a low-temperature branch of the family (Steels et al. 2002a). The new species are intolerant of high temperatures but grow well at 4°C. *Z. lentus* has been isolated from a variety of spoiled foods and is preservative-resistant (Steels et al. 1999). It would appear that *Z. lentus* and *Z. kombuchaensis* are capable of fermentative spoilage of refrigerated foods, immune to spoilage by *Z. bailii* and *Z. bisporus*.

11.8 Spoilage Yeast Ecology

The ecology of the great majority of yeast species so far discovered has been relatively little studied, and is consequently poorly understood. Even for a species as well studied as the brewing/baking yeast *Saccharomyces cerevisiae*, the ecology and natural life cycle are still being researched (Naumov et al. 1998; Sniegowski et al. 2002). For most yeast species our knowledge of their ecology is limited to a series of observations as to the locations of isolation of each species; the most notable exceptions to this being the recent revelations as to the interrelationships between yeast and insect ecology (Lachance et al. 1995).

In the yeast spoilage field, there exist extensive lists of yeasts isolated from different foods, which may or may not have been correctly identified and may or may not have been the cause of spoilage in that food. Very little has been written as to where such yeasts originate, and the ecology of these yeasts in the natural environment, in the food production factory or in the home or domestic environment.

11.8.1 The Global Yeast Community

All of the major spoilage yeasts listed by Pitt and Hocking (1997), Tudor and Board (1993) and Stratford (2000), and the group 1 spoilage yeasts listed by Davenport (1996, 1997), are found throughout the world, with the possible exception of Polar or Antarctic regions. Containers of food or beverages may suffer spoilage by *Z. bailii* and related

yeast species at any location from the Orient to the USA, from Russia to South Africa. The lesser-known spoilage yeasts, the "second division yeasts" (Tudor and Board 1993) such as *Candida boidinii*, *Candida parapsilosis* or *Y. lipolytica*, also appear to be universally distributed, and form part of a global community of spoilage yeasts. A possible exception to the universal distribution of spoilage yeast species may be *Z. lentus* and *Z. kombuchaenisis*. These recently discovered species share many of the characteristics of *Z. bailii*, but are notable for their intolerance of heat (Steels et al. 2002). These species fail to grow at temperatures above 30°C and are killed by temperatures above 40°C. It is therefore unlikely that these species are commonly found in tropical countries.

Many of the lesser-known yeasts have indications of geographic location in their specific names. Such distinctions have little or no true validity. The writer of this chapter has isolated *I. occidentalis* in the Orient (Moscow and Thailand) and *I. orientalis* in western Europe. *Candida wyomingensis* has been found in central Russia, *Candida natalensis* in South America, *Cryptococcus uzbekistanensis* in Brazil and *Pseudozyma antarctica* in Thailand. While it is possible that many of the recently discovered non-spoilage yeast species may have unique habitats, such as within beetle intestines and in specific geographic locations, the great majority of well-known spoilage yeasts appear to be universally distributed.

11.8.2 Sources of Infection

Most of the major food spoilage yeasts (Tudor and Board 1993; Davenport 1996; 1997; Pitt and Hocking 1997; Stratford 2000) could be termed extremophiles. This is, of course, a natural consequence of their ability to thrive in foods designed or treated to kill microbes. *Z. rouxii* is an extreme osmophilic yeast; *Debaryomyces hansenii* is a halotolerant species, *Dekkera bruxellensis* and *Saccharomyces cerevisiae* are CO_2- and ethanol-tolerant, *Saccharomycodes ludwigii* is sulphite tolerant, while *Z. bailii* shows extreme resistance to a variety of weak-acid preservatives (Table 11.3). It therefore follows that for a food to be spoiled, it must be infected with yeasts of the right species. Are these extremophilic species common? Particularly in factories where foods are processed and packaged, or do they require a specific mechanism of infection?

The most likely sources of infection of spoilage yeasts are from fruits (and vegetables), sugar and syrups, water and air, insects and dirty, contaminated equipment (Ingram 1958). Fruit, at all stages of development, represent a major natural habitat for many species of yeasts (Do Carmo-Sousa 1969). Dried, shrivelled and mummified fruit form an excellent environment for osmophilic yeasts, including *Z. bailii* and *Z. rouxii* (Beech and Davenport 1970; Davenport 1975; Tilbury 1980a, b). Sugar, either granulated or in the form of high Brix syrup, is also a potential source of infection of osmophilic yeasts (Ingram 1949; Scharf 1960; Schmidt 1995). GMP can eliminate the risks of yeast infection from sugar to a large extent, sugars should be purchased from reputable suppliers, stored carefully, and preferably sterilized by heat treatment. Water can be an ingredient in foods, such as soft drinks, or used as a processing aid, for example in washing or cleaning machinery or raw materials. Contaminated cooling water or washing water used in rinsing bottles are frequent sources of infection (Scharf 1960; Pitt and Richardson 1973; Sand and Kolfschoten 1971; Schmidt 1995). Air can be a source of yeast infection, but less frequently than

Table 11.3 Extremophilic spoilage yeast species. Most are normally rare yeast species and found in foods only when selected for by an extreme environment. Almost all are highly fermentative. Excess gas production is indicated by *an asterisk*. When occurring in environments not containing the specific selective agents, these yeasts become "second division" species. Inclusion of *Debaryomyces hansenii* is debateable, as it is less extreme in proportion to other species, is relatively common and is not highly fermentative

Yeast species	Extreme environmental resistance
(*Debaryomyces hansenii*)	Salt Low temperature
*Dekkera anomala** and *Dekkera bruxellensis**	Carbonated beverages Ethanol Sorbic acid
*Saccharomyces cerevisiae** and *Saccharomyces bayanus**	Carbonated beverages Ethanol Sulphite
*Saccharomycodes ludwigii**	Carbonated beverages Ethanol Sulphite Acetic acid
*Schizosaccharomyces pombe**	Benzoic acid Acetic acid
*Z. bailii**	Sugar Ethanol Sorbic acid and benzoic acid Acetic acid
*Z. bisporus**	Sugar Sorbic acid and benzoic acid Acetic acid
*Z. lentus**	Low temperature Sorbic acid and benzoic acid Acetic acid
*Z. rouxii**	Sugar Salt

might be expected (Ingram 1949), yeast cells being larger and denser than bacteria. Amongst airborne yeasts, basidiomycetes have been reported to predominate (Ingram 1958), the airborne flora being largely composed of black and red yeasts (*Aureobasidium pullulans*, *Rhodotorula* and *Sporidiobolomyces* spp.) together with *Cryptococcus albidus*, *Cryptococcus laurentii* and *Cryptococcus diffluens*. These species are essentially those described by Davenport (1996) as group 3, hygiene-indicator species, not spoilage yeasts. Insects can be regarded as potent carriers of spoilage yeasts, especially fruit flies (*Drosophila* spp.) and bees and wasps. Fruit fly infestation in fruit processing factories is notorious (Ingram 1958) and fruit flies have been shown to carry noted spoilage yeasts such as *Debaryomyces hansenii*, *Candida parapsilosis*, *P. membranifaciens*, *Saccharomyces cerevisiae*, *I. orientalis* and

Saccharomycodes ludwigii (Recca and Mrak 1952; Cooper 1960; Lachance et al. 1995). Bees and wasps may also carry osmophilic spoilage yeasts (Ingram 1958; Tilbury 1976) including *Z. rouxii*. Recently, it has been noted that most members of the *Starmerella*/*Candida* clade have been implicated in spoilage of high-sugar foods (Steels et al. 2002a; Stratford and James 2003). These include *Candida apicola*, *Candida etchelsii*, *Starmerella bombicola*, *Candida lactis condensii*, *Candida stellata*, *Candida davenportii* and *Candida magnoliae*. These yeasts are osmotolerant and most species in this group have been found associated with wasps, bees, bumblebees and leafcutter bees. It has been suggested that bees and wasps attracted to sugary foods form the principle source of infection of these relatively uncommon spoilage yeasts.

Contact with unhygienic, dirty, contaminated equipment is probably the commonest cause of spoilage yeast infection in food-processing factories (Ingram 1958; Tilbury 1976). It has been estimated that 95% of soft drinks spoilage was due to poor factory hygiene (van Esch 1987). The list of possible contaminated equipment includes returned bottles and packaging, filling and capping machines, conveyors and their motors, soap lubricating systems, meters and proportioning pumps and valve seals (Scharf 1960; Sand 1971a, b; Sand and Kolfschoten 1971; Pitt and Richardson 1973; Schmidt 1995; Davenport 1997). This is in addition to the yeasts living within the general factory environment, being particularly concentrated in areas where sugary products are spilled, or washed away and diluted, into the soakaways and drains (Davenport 1996). What are the species that inhibit the general factory environment, the commonest source of spoilage yeast infection?

11.8.3 Species Frequency in Factory Environments

How commonly do different species of spoilage yeasts occur? In a far-sighted attempt to answer this question, Deak and Beuchat (1996) assembled a table of "calculated frequencies (%) of yeasts in foods". Ninety-nine yeast species were considered to be found in foods. The frequency of occurrence was calculated from the literature reports of (1) the number of types of food in which a given species was found, (2) the number of times the species was detected and (3) the number of strains of the species isolated from foods. These frequencies are therefore of reports of species in foods in the literature, and it is not surprising that spoilage yeasts are well represented, particularly the major gas-forming species. The order of frequency in all foods (Deak and Beuchat 1996) is as follows: *Saccharomyces cerevisiae* 7.04%, *Debaryomyces hansenii* 6.72%, *P. anomala* 4.56%, *P. membranifaciens* 4.32%, *R. mucilaginosa* 3.78%, *T. delbrueckii* 3.64%, *Kluyveromyces marxianus* 3.36%, *I. orientalis* 3.20% and *Z. bailii* 3.05%. However, this will include a number of spoiled foods and is understandably biased towards spoilage species. This may not reflect the frequency of occurrence of yeast species, in a food production environment prior to infection.

A series of investigations carried out some 30 years ago by Sand and colleagues may come closer to answering this question. Surveys to determine the yeast flora were carried out in soft drinks factories in Europe, the Middle East and Scandinavia, and on factory machinery (Sand and van Grinsven 1976a, b; Sand et al. 1976a). Yeasts found typically included *Candida stellata*, *Candida sake*, *Candida guilliermondii* and *Z. fermentati*. Unexpectedly, recognized spoilage yeasts were rarely or not detected (Sand et al. 1976a).

Recent surveys of the yeast flora of soft drinks factories support the findings of Sand and colleagues. Typical factory floras of a European and an Asian soft drinks factory are shown on Table 11.4. While several of the second division or group 2 spoilage yeasts are present (Tudor and Board 1993; Davenport 1996), the absence of the *Zygosaccharomyces* and *Saccharomyces sensu stricto* spp. spoilage yeasts was remarkable.

11.8.4 Yeast Frequency by Numbers of Isolates

A species list like that shown in Table 11.4 is misleading in terms of frequency of occurrence. The table lists 14 species and implicitly the reader assumes all to be present in equal proportions. Reality is very different, if the numbers of isolates of each species is taken into account. Red and black yeasts usually predominate in numbers, approximately 50% of isolates; *Rhodotorula*, *Sporidiobolus* and *Sporobolomyces* comprising the red genera and *Aureobasidium* being the black genus. Also common in numbers are *Cryptococcus* spp. and certain *Candida* spp. such as *Candida pseudointermedia*. Interestingly, this factory flora frequency very closely resembles the aerial flora frequency described by Ingram (1958). If the factory flora is plotted against the resistance to an antimicrobial agent, such as sorbic acid, it becomes obvious that the great majority of the factory flora is easily inhibited by very small quantities of preservative (Fig. 11.7). Isolates of resistant species are found in very low numbers, and at the European legal limit for sorbic acid, 300 ppm (just under 3 mM), no isolates were found able to grow. This means that in this factory, no spoilage would be expected in sorbic acid preserved soft drinks, if any of the factory flora of yeast species were to gain access to the product. Similar results and profiles

Table 11.4 The yeast flora of a European soft drinks factory and an Asian soft drinks factory. All species were identified by D1/D2 26S ribosomal DNA sequencing. The species are dominated by red, *Rhodotorula* spp., and black, *Aureobasidium* sp., yeasts. Extremeophilic spoilage species, such as *Z. bailii*, are notable by their absence

European soft drinks factory	Asian soft drinks factory
Aureobasidium pullulans	*A. pullulans*
Bulleromyces albus	*Candida boidinii*
Candida boidinii	*Candida diddensiae*
Candida oleophila	*Candida oleophila*
Candida parapsilosis	*Candida pseudolambica*
Candida pseudointermedia	*Candida pseudointermedia*
Cryptococcus albidus	*Candida silvae*
Debaryomyces hansenii	*Candida sojae*
H. meyerii	*Cryptococcus diffluens*
P. anomala	*P. anomala*
R. mucilaginosa	*P. jadinii*
R. nothofagi	*R. graminis*
Saccharomyces exiguus	*R. dairenensis*
Yarrowia lipolytica	*R. nothofagi*

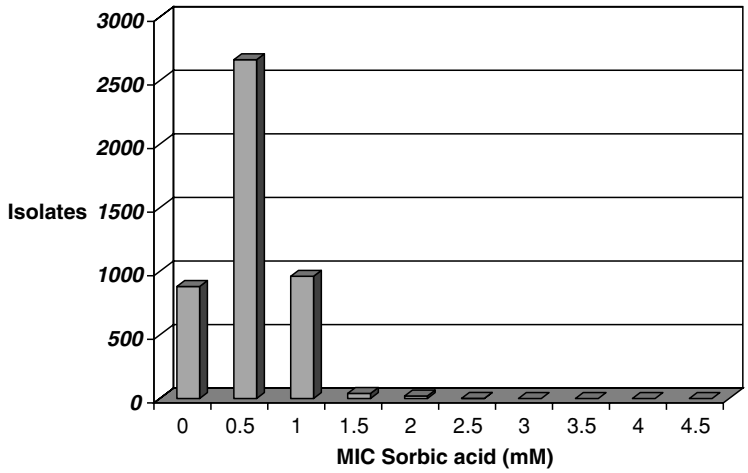

Fig. 11.7. Profile of the resistance of yeast isolates from a European soft drinks factory (Table 11.4) to sorbic acid in a soft drink, pH 3.4. The legal limit for sorbic acid in a soft drink is 300 ppm, 2.7 mM, in Europe (Anon 1989)

of resistance can be obtained with other preservatives systems/factors, for example heat and acetic acid.

The conclusions that can be drawn from this are:

1. The infamous extremophile spoilage yeasts, *Z. bailii*, *Z. rouxii*, *Saccharomyces sensu stricto*, *Saccharomycodes ludwigii*, *Dekkera bruxellensis* (Table 11.3), are rare in factories, either absent or present in very, very low numbers.
2. The second division (Table 11.5), group 2 yeasts (Tudor and Board 1993; Davenport 1996) are commonly present in factories, albeit at moderate/low frequency of isolation.
3. These second division, group 2 spoilage/hygiene species can be controlled by the food preservation system (Fig. 11.7). Group 2 yeasts will not normally cause spoilage unless there is a mistake in manufacturing (Davenport 1996), such as omission of preservation or poor hygiene.
4. Poor factory hygiene has been suggested to account for 95% of yeast infections (van Esch 1987). Improvements in factory hygiene can dramatically reduce spoilage (Beech and Davenport 1970; Sand 1971a; Rankine and Pilone 1974; Windisch and Neumann-Duscha 1974).
5. Poor factory hygiene typically increases the microbial loading of the food, i.e. increases the yeast inoculum, to such an extent that the preservation system/factors are overwhelmed, rather than altering the factory yeast flora to a great extent.

It therefore follows that the great majority of cases of yeast spoilage of foods are caused by the second division, group 2 species. Such food spoilage tends to be

Table 11.5 Commonly encountered second division, group 2 spoilage/hygiene yeast species. These are normally frequently isolated yeasts and can be regarded as opportunistic species, causing spoilage following any mistake in manufacturing or storage. Gas production is indicated by *one asterisk*, excess gas by *two asterisks*. It is likely that the majority of yeast spoilage is caused by these lesser-known species

*Candida albicans**	*H. uvarum**
*Candida boidinii**	*I. occidentalis***
*Candida glabrata**	*I. orientalis*** (*Candida krusei*)
*Candida intermedia**	*P. anomala***
*Candida lambica**	*P. galeiformis*
*Candida parapsilosis**	*P. guilliermondii**
*Candida pseudointermedia**	*P. membranifaciens*
*Candida pseudolambica**	*Saccharomyces exiguus*** = *Kazachstania exigua*
*Candida sake**	*Torulaspora delbrueckii***
*Candida sojae**	*Y. lipolytica*
*Candida tropicalis**	*Z. fermentati*** = *Lachancea fermentati*
Candida zeylandoides	*Z. florentinus*** = *Zygotorulaspora florentinis*
*Clavispora lusitaniae**	*Z. microellipsoides*** = *Torulaspora microellipsoides*

unspectacular and consequently tends to be underreported. The group 1 extremophile species cause spoilage much more rarely, but these are much more likely to be reported, owing to the spectacular nature of their high gas spoilage. To use an analogy, this resembles an iceberg (Fig. 11.8) where the visible 5% of the iceberg is the gas-forming extremophilic species (Table 11.3). However, 95% of the iceberg is beneath the surface, a great underreported mass of yeast spoilage caused by the second division group 2 spoilage yeasts. These common yeasts are opportunists and will cause spoilage following mistakes or poor hygiene, either in the factory or in the domestic environment.

11.9 Future Trends in Yeast Spoilage

11.9.1 Spoilage Yeast Identification and Nomenclature

Future trends in yeast spoilage of foods can potentially originate with the food, or with the yeasts. Any changes in the yeasts themselves are unlikely over a reasonable timescale. However, there will undoubtedly be changes in the nomenclature of spoilage yeast species, through changes in yeast phylogeny and taxonomy, and through more accurate identification of spoilage yeast species using molecular techniques. Fortunately identification of the first division, group 1 yeasts as the cause of the most-obvious spoilage is likely to remain almost unchanged. Yeasts such as *Zygosaccharomces bailii*, *Schizosaccharomyces pombe* or *Saccharomycodes ludwigii* are rarely misidentified owing to their distinctive morphology and physiology. There are, however, likely to be a number of unexpected names appearing in the second division, group 2 spoilage/hygiene yeasts, following better identification. Small, round, budding-yeasts, with smooth white colonies are often very difficult to correctly identify.

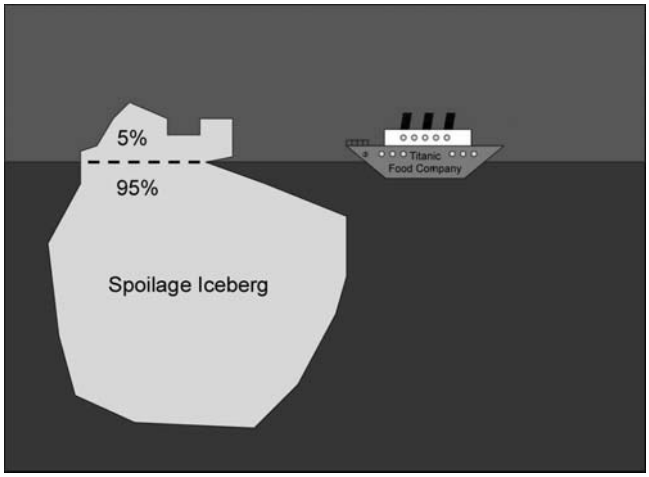

Fig. 11.8. Metaphorically, yeast spoilage resembles an iceberg. The 5% easily visible spoilage is caused by the extremophilic, highly fermentative species (Table 11.3). Ninety-five percent of spoilage is under the surface; caused by poor hygiene allowing the opportunistic "second division" spoilage yeasts (Table 11.5) to proliferate. Spoilage by the second division yeasts is much less obvious, is greatly underreported, but is still sufficient to sink a food company

11.9.2. Packaged and Processed Foods

Any future trends in yeast spoilage of foods and beverages are therefore likely to depend almost entirely on changes in food, food processing and food preservation. The current trend in foods and beverages is for more foods to be purchased ready prepared and packaged. This inevitably means a higher degree of food processing by factories. Over the past 20 years owing to customer demand, a high proportion of processed foods have been prepared containing more sugar and salt than in non-processed foods. It is therefore probable that any increase in food processing will result in more potential targets for yeast spoilage. Recent moves, particularly in the USA, to limit human obesity by lowering sugar concentrations are not likely to affect yeast spoilage. A soft drink, for example, containing 5% sugar is equally likely to suffer yeast spoilage as one containing 10% sugar.

11.9.3 New Preservation Techniques

New mechanisms of food preservation are often discussed in the scientific literature, but are not likely to be widely applied over the next few years. Any new methods require stringent safety testing, must also be acceptable to the public, and must be compatible with current legislation where applicable. Possible new food preservation techniques include use of ultrahigh pressure, irradiation, high-intensity light, pulsed electric fields and use of antimicrobial essential oils. Any new technique is likely to select for extremophilic yeast species able to survive the treatment; these may not be

the species currently recognized as spoilage species. For example, limited studies on irradiation suggest that *Z. bailii* is irradiation-sensitive, but *R. glutinis* is resistant (Youssef et al. 2002).

11.9.4 Less Preserved, More "Natural" Foods

Following the discovery of traces of pesticide and herbicide residues in foods, there is now a strong adverse public reaction to any perceived adulteration to food or additives in food. This has also extended in certain cases to an opposition to physical treatments of food such as pasteurization. Foods are required to be more "natural", with all of the goodness left in and not overprocessed or containing chemical additives, including preservatives. This desire by the public can be counterproductive, as in the USA in natural apple juices. In a celebrated outbreak of verotoxic *E. coli* 0157:H7 food poisoning in California, bacteria in untreated, unpreserved, unpasteurized apple juices survived up to 30 days, and caused 49 cases of illness and one death (McLellan and Splittstoesser 1996).

While food additives may not be completely eliminated from foods, there has been a general trend over the past 20 years for the concentrations of additives in foods to be lowered, often accompanied by changes in legislation. The overall effect of lowering preservative concentrations in foods is that a higher proportion of foods will be subject to yeast spoilage. The extremophilic yeasts will remain a threat, but more of the second division spoilage species will be able to survive in lowered preservative concentrations. It is therefore probable that any increase in yeast spoilage brought about by removal of preservatives will be primarily due to an increase in spoilage by the second division species.

11.9.5 Increased Role of Factory Hygiene

Any shortfall in the preservation systems used to prevent yeast spoilage in foods must result in an unacceptable increase in spoilage, unless the gap is filled by an increase in factory hygiene. There is always an uneasy balance between preservation and factory hygiene. A food containing excessive preservative, for example 1,000 ppm benzoic acid, is almost immune to spoilage and could be produced in very unhygienic conditions. At the other end of the scale, a food with 0-ppm preservatives will require excessive hygiene to prevent *any* microbial infection; an expensive process known as aseptic filling, carried out within clean rooms containing filtered air.

Really good hygiene in factories requires competent and well-educated personnel. Human error or poor training ultimately causes many, perhaps the majority of hygiene breakdowns in factories. As food production becomes more technically complex, it is likely that the human factor will become the weakest link in the successful production of foods and the greatest cause of yeast spoilage in foods and beverages.

QED – the human factor: While working in a British beverage factory some years ago, the writer found that one of the workers in the factory repeatedly did not apply the necessary disinfectant to clean the pipe work. When asked why this was, the worker replied "I knew it was not important, because if it was, you would have come down and done it yourself".

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Chapter 12

The Public Health and Probiotic Significance of Yeasts in Foods and Beverages

Graham H. Fleet[1] and Roostita Balia[2]

[1]*Food Science and Technology, School of Chemical Engineering and Industrial Chemistry, The University of New South Wales, Sydney, NSW 2052, Australia (e-mail: g.fleet@unsw.edu.au, Tel.: +61-2-93855664, Fax: +61-2-93136635)*
[2]*Faculty of Animal Husbandry, University of Padjadjaran, Jatinangor (Km 21), Sumedang 45363, Indonesia*

12.1 Introduction

The microbiological safety of foods is generally discussed in relation to the occurrence and significance of pathogenic bacteria, infectious viruses, mycotoxigenic fungi, parasitic protozoans and toxigenic algae. Mention of yeasts in the context of food safety is conspicuously absent (Lund et al. 2000; Doyle et al. 2001; Hocking 2003). Compared with other microbial groups, yeasts are not seen as aggressive pathogens, but they are capable of causing human disease in opportunistic circumstances (Hurley et al. 1987; Rippon 1988; Segal and Baum 1994; Georgiev 2003; Hazen and Howell 2003). *Candida albicans* (Hurley 1980; Calderone 2002) and *Cryptococcus neoformans* (Campbell and Mackenzie 1980; Casadevall and Perfect 1998) are well known in this regard, and are responsible for causing a range of mucocutaneous, cutaneous, respiratory, central nervous and systemic infections. However, an increasing number of other yeast species are now associated with these disorders and have been added to the list of opportunistic pathogens (Hazen 1995; Georgiev 2003). Although consumption of food contaminated with yeasts may not have a direct role in causing these infections, there is increasing concern that foods could be an underestimated environmental source of these yeast pathogens.

The development of allergic and other adverse responses in humans as a consequence of food consumption is well documented, and food allergy has now become a significant branch of food safety (Metcalfe et al. 2003). The role of yeasts in eliciting these types of responses is relatively minor compared with that of other causative agents, but there is increasing and justifiable interest in this topic.

Public health considerations in relation to microorganisms in foods are not always negative, and the concept of probiotic microorganisms has developed into a major area of scientific and commercial interest (Klaenhammer 2001). For many years, viable and nonviable yeasts have been used as supplements in stock feed to enhance the growth of domesticated animals and poultry (Lyons et al. 1993; Lyons

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
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2002; Dawson 2002). Recently, the yeast *Saccharomyces boulardii* has emerged as a probiotic species for consumption by humans (van der Aa Kuhle et al. 2005), paving the way for a broader consideration of yeasts as probiotic organisms in foods.

Although yeasts are well known for producing fermented foods and beverages, as sources of food ingredients and as spoilage yeasts, their public health significance in foods has largely been overlooked. This chapter addresses this gap in knowledge and brings together a range of topics that broadly cover the relationship between yeasts, foods and public health.

12.2 Yeasts and Foodborne Gastroenteritis

As part of normal, daily food consumption, humans are unknowingly and inadvertently ingesting large populations of viable yeast cells without adverse impact on their health. Table 12.1 lists a range of food and beverage commodities that are likely to harbour significant populations of viable yeasts at the time of consumption. These products contain a diversity of ascomycetous and basidiomycetous yeast species, often at populations as high as 10^6–10^8 cfu/g or 10^6–10^8 cfu/ml. Despite this exposure, outbreaks or cases of foodborne gastrointestinal infections or intoxications attributable to yeasts are rarely encountered. Epidemiological statistics on foodborne microbial disease from various countries over many years are notable for their absence of any data on yeasts (Bean and Griffin 1990; Mead et al. 1999; Lee et al. 2001; Sewell and Farber 2001). Similarly, searches of this topic in computer data bases yield no information. Nevertheless, there are occasional reports of yeast-associated gastroenteritis that are worthy of discussion.

Table 12.1 Diversity of food and beverage commodities with significant populations of viable yeast cells at the time of consumption

Commodity group	Prevalent yeast species	Reference
Fresh fruits	*Aureobasidium, Rhodotorula, Cryptococcus, Candida, Metschnikowia, Hanseniaspora*	Deak and Beuchat (1996), Fleet (2003)
Fruit juices, salads	*Saccharomyces, Zygosaccharomyces, Candida, Hanseniaspora*	Deak and Beuchat (1996), Fleet (2003)
Cheeses, other fermented dairy products	*Debaryomyces, Yarrowia, Candida, Kluyveromyces, Saccharomyces, Galactomyces* (*Geotrichum*)	Fleet (1990), Jakobsen and Narvhus (1996), Frohlich-Wyder (2003)
Delicatessen and fermented meat products	*Debaryomyces, Candida, Yarrowia, Cryptococcus*	Dillon and Board (1991), Samelis and Sofos (2003)
Alcoholic beverages	*Saccharomyces, Hanseniaspora, Pichia, Candida, Brettanomyces, Metschnikowia*	Fleet (1998)
Traditional fermented foods	*Saccharomyces, Candida, Pichia*	Steinkraus (1996), Beuchat (2001)

Jensen and Smith (1976) noted malaise, fever and nausea in a patient who regularly consumed tablets of brewer's yeast as a health-food supplement. These symptoms disappeared when the patient stopped consuming tablets of the yeast. In a review of foodborne disease statistics for Canada over the period 1973–1977, Todd (1983) referred to 39 cases that were attributed to consumption of foods (punctured canned foods, bottled drinks, baked products and infant food) contaminated with yeasts and mould. He noted the need for further research to determine the significance of these observations. *Hanseniaspora uvarum*, (*Kloeckera apiculata*), possibly associated with seafood, was suspected of causing a case of gastroenteritis in Spain (Garcia-Martos et al. 1999).

Under some circumstances, *Candida* species can colonise areas of the intestinal tract, leading to diarrhea and other gastroenteritis symptoms, along with excretion of high yeast populations (more than 10^6 cfu/g) in the faeces (Gupta and Ehrinpreis 1988). Over the years, there have been numerous reports of such cases (reviewed in Talwar et al. 1990; Danna et al. 1991; Levine and Dykoski 1995), where *C. albicans* was the main species involved, but many other *Candida* species were also implicated (e.g. *C. tropicalis*, *C. kefyr*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae*, *C. guilliermondii*). Generally, these cases are associated with individuals who are receiving antibacterial therapy, are immunocompromised, or who have some other underlying disorder. Consumption of yeast-contaminated food was not the initiating factor. Nevertheless, such findings establish the fact that, given appropriate circumstances, yeasts can colonise the intestinal tract, and contribute to gastroenteritis and other infections (Cole et al. 1996; Bernhardt and Knoke 1997).

It may be concluded from the overall epidemiological evidence that, as causative agents of foodborne gastrointestinal infections and intoxications, yeasts present very little risk to consumers. This, of course, correlates with their widespread acceptance as safe agents in the production of fermented foods and beverages. However, some caution and vigilance is required to consider changing demographics, and the increasing numbers of individuals with compromised immune systems.

12.3 Yeasts as Opportunistic Pathogens

As mentioned already, various species of yeasts are considered as opportunistic pathogens. *C. albicans* and *Cr. neoformans* head this list, but many other yeasts are now considered in this category (Hazen 1995; Georgiev 2003). Infection of the blood (fungaemia) is the main pathological effect caused by yeasts. From this source, however, yeast cells are disseminated throughout the human body and can infect almost any organ (e.g. heart, lungs, kidney, brain), often with fatal consequences. They can colonise the skin and membraneous areas, and infections of the oral cavity, vagina, anal region and respiratory system are not uncommon (Hurley et al. 1987; Rippon 1988; Ahearn 1998; Hazen and Howell 2003; Georgiev 2003). Antifungal agents such as amphotericin, fluconazole, itraconazole and ketoconazole are used to treat yeast infections, but the development of resistance to these antibiotics is an on-going issue.

Table 12.2 lists the *Candida* and *Cryptococcus* species that have been reported to cause yeast infections in humans. Only their anamorphic or nonteleomorphic names

Table 12.2 Species of *Candida* and *Cryptococcus* causing opportunistic infections in humans

Candida albicans	*Candida famata*
Candida parapsilosis	*Candida pulcherrima*
Candida tropicalis	*Candida zeylanoides*
Candida krusei	*Candida rugosa*
Candida glabrata	*Candida utilis*
Candida lusitaniae	*Candida lipolytica*
Candida guilliermondii	*Candida dubliniensis*
Candida stellatoidea	*Cryptococcus neoformans*
Candida norvegensis	*Cryptococcus albidus, Cryptococcus laurentii*

Hazen (1995), Hazen and Howell (2003), Georgiev (2003)

are listed, as this is how they are most frequently described in the medical literature. Infections caused by *Candida* species are, by far, the most frequently reported cases, and there is an extensive literature on this topic (reviewed by Hazen 1995; Krcmery and Barnes 2002; Hobson 2003). Although infections with *C. albicans* have been most prevalent and significant in the past, infections by other *Candida* species are increasing in frequency and importance. The species of main concern, here, are *C. parapsilosis*, *C. krusei*, *C. tropicalis* and *C. glabrata* (Pfaller 1996; Krcmery and Barnes 2002). Epidemiological statistics correlate this trend with their increased resistance to antibiotics used to treat infections with *C. albicans*, but other factors are also operative, and include the virulence properties of the yeast and particulars of the host.

Cryptococcus infections generally begin by inhalation of the yeast into the lungs, after which it enters the blood system and is spread to other parts of the body (Hurley et al. 1987). *Cr. neoformans* is the main species involved, but on rare occasions infections by other species such as *Cr. albidus* and *Cr. laurentii* have been reported (Hajjeh et al. 1995; Georgiev 2003).

Table 12.3 lists yeast species other than those of *Candida* and *Cryptococcus* that have been reported to cause infections (primarily fungaemia) in humans. Most notable are species of *Rhodotorula*, *Pichia anomala* (formerly *Hansenula anomala*), *Issatchenkia orientalis* (anamorph *C. krusei*) and *Kluyveromyces marxianus* (formerly *K. fragilis*) (Kremery et al. 1999). The industrialised yeast, *S. cerevisiae*, widely used in the production of foods and beverages, has attracted significant attention as an opportunistic pathogen. It has been linked to various cases of fungaemia, vaginitis and organ infections over the past 50 years (Eschete and West 1980; Aucott et al. 1990; McCullough et al. 1998a; Murphy and Kavanagh 1999; Xu et al. 1999; Cherifi et al. 2004; Llanos et al. 2004). Murphy and Kavanagh (1999) have reviewed the pathogenic significance of *S. cerevisiae* and its implications in the biotechnological applications of this yeast. There is convincing evidence that this yeast is an opportunistic pathogen and that industrialised strains of baker's yeast have been linked to cases of vaginitis (Nyirjesy et al. 1995; McCullough et al. 1998a; Llanos et al. 2004), and brewer's yeast to other infections (Jensen and Smith 1976). Various molecular methods have been used to differentiate

Table 12.3 Yeast other than *Candida* and *Cryptococcus* species causing opportunistic infections in humans

Species	References
Saccharomyces cerevisiae	Eschete and West (1980), Aucott et al. (1990), Bassetti et al. (1998), McCullough et al. (1998a), Murphy and Kavanagh (1999), Wheeler et al. (2003), Llanos et al. (2004)
Saccharomyces cerevisiae var. *boulardii*	McCullough et al. (1998b), Piarroux et al. (1999), Lherm et al. (2002), Cassone et al. (2003)
Rhodotorula spp.	Papadogeorgakis et al. (1999), Petrocheilou-Paschou et al. (2001), Braun and Kaufmann (1999), Diekema et al. (2005)
Pichia anomala	Murphy et al. (1986), Haron et al. (1988), Klein et al. (1988), Yamada et al. (1995), Garcia-Martos et al. (1996), Cermeno-Vivas et al. (1999), Georgiev (2003)
Pichia farinosa	Garcia-Martos et al. (1996)
Pichia membranifaciens	Garcia-Martos et al. (1996)
Issatchenkia orientalis	Merz et al. (1986), Goldman et al. (1993), Abbas et al. (2000), Georgiev (2003)
Kluyveromyces marxianus	Lutwick et al. (1980), Nielsen et al. (1990), Garcia-Martos et al. (1996)
Hanseniaspora uvarum	Garcia-Martos et al. (1999)
Yarrowia lipolytica	Shin et al. (2000), Georgiev (2003)

See also Rippon (1988), Hazen and Howell (2003), Georgiev (2003)

clinical, nonclinical and industrial strains of *S. cerevisiae*, but more research is needed to define the genotypic and phenotypic properties of pathogenic and non-pathogenic strains, as well as the conditions of the host that are predisposing to colonisation and infection by the yeast. Both immunocompromised and immunocompetent hosts have been infected by *S. cerevisiae* (McCullough et al. 1998a; Xu et al. 1999; Llanos et al. 2004). Pathogenic strains of *S. cerevisiae* exhibit the ability to grow at 42°C, produce proteinase and are capable of pseudohyphal growth (Murphy and Kavanagh 1999). However, the composition and the structure of the cell wall are also properties that may confer virulence on strains of *S. cerevisiae* (Wheeler et al. 2003). *S. boulardii* is a yeast with biotherapeutic and probiotic functions and these activities are discussed in a later section. Taxonomically, it is considered to be a variety of *S. cerevisiae*, although it can be differentiated from other strains of *S. cerevisiae* by various molecular criteria (van der Aa Kuhle and Jespersen 2003). Unfortunately, its use as a biotherapeutic or probiotic agent has caused numerous cases of fungaemia in recent years (Piarroux et al. 1999; Lherm et al. 2002; reviewed by Cassone et al. 2003), prompting some authors to suggest that its application for these purposes should be prohibited.

The factors that contribute to infections with opportunistic yeasts are well recognised and are listed in Table 12.4. Usually, healthy, immunocompetent individuals are not at risk of such infections. Generally, individuals with weakened health and immune function are at greatest risk, and include cancer and AIDS

Table 12.4 Factors contributing to human infections with opportunistic yeast pathogens

Weak health; hospitalisation
Cancer, AIDS patients
Weak immune system; treatment with immunosuppressive drugs; chemotherapy
Treatment with broad spectrum bacterial antibiotics
Insertion of catheters
Recent surgery (especially gastrointestinal tract)
Total parenteral nutrition
Hazen (1995), Annaissie et al. (1998), Hobson (2003)

patients, hospitalised patients, and those with catheter insertions (Hart et al. 1969; Hazen 1995; Anaissie et al. 1998; Hobson 2003). Yeasts, especially *Candida* species, are normal inhabitants of the human gastrointestinal tract, and there is sound experimental evidence to demonstrate that they translocate from this source to the blood system (Krause et al. 1969; Cole et al. 1996). This mode of transmission is facilitated by conditions which increase the populations of yeasts in the gastrointestinal tract (e.g. diet, treatment with bacterial antibiotics) and which damage the intestinal mucosa (e.g. immunosuppressive and chemotherapeutic agents, diarrhea episodes). The presence of an indwelling catheter serves as a focus for yeast contamination and growth as a biofilm, that is more resistant to elimination by the host's defence mechanisms, and antifungal agents (Douglas 2003; Kojic and Daroviche 2004). Generally, any factor that increases the exposure of susceptible individuals to yeasts will increase their risk of acquiring an opportunistic infection. Three studies have suggested that foods with substantial yeast loads (e.g. processed meats, soft cheeses) pose a risk to susceptible hospital patients. These patients should not be offered such foods for consumption and, furthermore, these foods introduce yeasts into the general hospital environment, from where they could contaminate catheters and patients (Staib et al. 1980; Radosavljevic et al. 1999; Bouakline et al. 2000). Staib et al. (1980) noted the ability of pathogenic yeasts (*C. parapsilosis*, *C. tropicalis* and *Cr. neoformans*) to grow in some meat products.

Many of the yeast species listed in Tables 12.2 and 12.3 are commonly found in foods at the time of consumption (Fleet 1992) [see Table 12.1 and also the extensive tables of yeasts and foods given in Deak (1991), Tudor and Board (1993) and Deak and Beuchat (1996)]. To establish a stronger linkage between the role of foods in contributing to opportunistic yeast infections, more research is needed to understand (1) the survival and growth of foodborne yeasts throughout the gastrointestinal system, (2) the potential for such yeasts to translocate from the gastrointestinal tract to the blood stream, and (3) the general occurrence and ecology of these yeasts in hospital and health-care environments.

12.4 Allergic and Other Adverse Responses to Yeasts

The ability of foods and food contaminants to elicit allergic and other adverse reactions in humans is attracting increasing scientific and consumer interest, although

there is little reference to foodborne yeasts in this regard (Emerton 1992; Metcalfe et al. 2003; Sampson 2004). Nevertheless, there is a significant body of "lay" and "alternative" literature that connects yeasts to a broad range of allergic and hypersensitive reactions in humans. These include a variety of gastrointestinal, respiratory, skin, migraine and even psychiatric disorders (Truss 1981; Crook 1986; Eaton 2004). Chronic fatigue syndrome, dysfunctional gut syndrome, irritable bowel syndrome and gut dysbiosis are prominent among these disorders. It is thought that overgrowth of yeasts in the gastrointestinal tract leads to the development of these conditions. *C. albicans* is reported to be the main species of concern, but other species are likely to be involved, and this highlights the need for a better understanding of the yeast ecology of the human gut (Bernhardt and Knoke 1997). Metabolites (e.g. acetaldehyde) produced by yeast growth pass into the circulatory system and are believed to trigger the various adverse responses; however, the underlying mechanisms are probably more complex as there is increasing evidence demonstrating the immunogenic or immunomodulating effects of yeast cell-wall components such as the 1,3-β-glucans and mannans (Lindberg et al. 1992; Kim et al. 2002; Instanos et al. 2004). The linkage between yeasts and these human disorders is largely based on dietary observations. If foods suspected to contain yeasts are removed from the diet, symptoms of the disorder generally disappear, but return when these foods are reintroduced into the diet (Grant 1979; Wuthrich and Hofer 1986; Eaton and Howard 1998). Such foods include yeast extract, leavened bread, alcoholic beverages and mould-ripened cheeses (Eaton and Howard 1998).

Biogenic amines (e.g. histamine, tyramine, phenylethylamine, putrescine, cadaverine) cause a diversity of adverse responses in humans, including headaches, hypotension, migraines and digestive disturbances (Shelaby 1996; Silla Santos 1996). Yeasts produce an array of biogenic amines through the decarboxylation of amino acids and contribute to the amine levels found in alcoholic beverages (Izquierdo-Pulido et al. 1995; Torrea-Goni and Ancin-Azpilicueta 2001; Torrea and Ancin 2002; Caruso et al. 2002) and other fermented products such as cheeses (Wyder et al. 1999). The concentrations produced vary with the yeast species (Caruso et al. 2002; Wyder et al. 1999). More research on amine production by yeasts is required, but the evidence to date suggests they do not produce sufficient amounts to be of concern to public health.

Sulphur dioxide has a certain degree of toxicity to some humans, causing respiratory, hypotension, flushing and tingling responses. Its production by strains of *S. cerevisiae* is well documented, where most strains produce less than 10 mg/l of SO_2; however, some strains can produce up to 100 mg/l, which could have public health implications (Romano and Suzzi 1993; Rauhut 1993). The production of SO_2 by other species of yeasts is not well known and requires investigation.

Ethyl carbamate (urethane) is a potential carcinogen, and its production in fermented foods and beverages needs to be considered (Ough 1976). It is formed by the reaction of urea with ethanol under acidic conditions. Yeasts produce urea as a consequence of nitrogen (e.g. arginine) metabolism, with some strains generating more urea than others. Ethyl carbamate formation during wine production is of particular concern where management strategies are used to minimise the presence and production of urea (Henschke and Jiranek 1993).

12.5 Yeasts as Probiotics

Probiotics are viable microorganisms that are beneficial to the host when consumed in appropriate quantities. Benefits include reduction in the incidences of diarrhea, constipation and bowel cancer, stimulation of the immune system, reduction in serum cholesterol levels, and enhanced nutrient uptake (Klaenhammer 2001; Holzapfel and Schillinger 2002; Marteau and Boutron-Ruault 2002). Particular species and strains of lactic acid bacteria (e.g. *Lactobacillus acidophilus*, *Bifidobacterium* spp.) are well known in this context and have received widespread application in the production of yogurts. Generally, greater than 10^6–10^7 viable cells of the probiotic organism need to be consumed on a regular basis for the health benefits to be realised. The probiotic microorganisms survive the ingestion process, and then colonise areas of the intestinal tract to assert their beneficial influence and impact. There is a significant and increasing body of scientific evidence that demonstrates their beneficial functions, but the underlying physiological and molecular mechanisms remain uncertain and require on-going research for their clarification (Mombelli and Gismondo 2000; Sullivan and Nord 2002).

The concept of using yeasts as human probiotics is at an early stage of development. However, there is significant experience in using viable yeasts, principally baker's, brewer's and distiller's yeasts (*S. cerevisiae*), as supplements to feeds for cattle, pigs and poultry, where improvements in growth and health of animals and birds are observed (Lyons et al. 1993; Dawson 2002). There is a substantial literature in this field (Aros-Garcia et al. 2000) and an expanding interest in using yeasts as probiotics in the aquaculture industry (Gatesoupe 1999; Tovar et al. 2002).

S. boulardii has been listed in recent literature as a potential human probiotic (Klaenhammer 2001). It was described in 1984 as an isolate from tropical fruit, and subsequently reported as an effective biotherapeutic agent for the clinical treatment of a range of diarrheal disorders (Surawicz et al. 1989; McFarland and Bernasconi 1993). The yeast is available commercially as lyophilised cultures that are resuspended in sterile saline and administered orally to patients who usually have been hospitalised as a consequence of severe diarrhea. The yeast colonises the intestinal tract, but is eliminated once administration is stopped, or the patient is given fungal antibiotics. The yeast has been reported to be effective in treating antibiotic-associated diarrhea, traveller's diarrhea, Crohn's disease and other inflammatory bowel disorders, acute gastroenteritis in adults and children, chronic diarrhea in HIV infected patients and diarrhea caused by *Clostridium difficile*, *Vibrio cholerae* and various *Enterobacteriaceae* (Czerucka and Rampal 2002; Sullivan and Nord 2002). Although treatment with the yeast has been considered to be safe, an increasing number of outbreaks of *S. boulardii* fungemia are being reported, causing some authors to question its safety status (Table 12.3) (Piarroux et al. 1999; Cassone et al. 2003). Also, there has been significant controversy over the correct nomenclature and taxonomic status for this yeast (McFarland 1996; McCullough et al. 1998b; Mitterdorfer et al. 2002). On the basis of phenotypic criteria, it is difficult to differentiate *S. boulardii* from *S. cerevisiae*. However, a range of molecular methods (sequencing, pulsed-field gel electrophoresis, restriction fragment length polymorphism, randomly amplified polymorphic DNA) clearly distinguish *S. boulardii*

strains from other strains of *S. cerevisiae*, but they fall within the overall cluster or clade for *S. cerevisiae* (Mitterdorfer et al. 2002; van der Aa Kuhle and Jespersen 2003; Posteraro et al. 2005). It is now generally accepted that the original species description of *S. boulardii* is taxonomically invalid, and that the yeast is correctly assigned as *S. cerevisiae* var. *boulardii*.

The mechanisms by which *S. cerevisiae* var. *boulardii* functions as a biotherapeutic or probiotic agent and prevents a range of diarrhea disorders are not fully understood, but multiple activities are probably operating (Czerucka and Rampal 2002). In the case of *Cl. difficile* induced diarrhea, it appears that *S. cerevisiae* var. *boulardii* produces a serene protease which degrades specific diarrhea-causing toxins produced by this bacterium, as well as the receptor sites for these toxins on the colonic mucosa. The yeast may stimulate particular enzymatic activities of the intestinal mucosa, as well as stimulate the host's intestinal mucosal immune response. Also, the cell wall of the yeast could adsorb and bind toxic products of diarrhea-causing bacteria. Among other strains of *S. cerevisiae*, van der Aa Kuhle et al. (2005) screened probiotic *S. cerevisiae* var. *boulardii* for tolerance of low pH (2.5) and bile salts, adhesion to epithelial cells, and effects on proinflammatory cytokine levels. While all strains were acid- and bile-tolerant, only some showed adhesion to epithelical cells and one (only one tested) decreased the expression of cytokine IL-1α. Attachment to intestinal mucosa, therefore, might not be a requirement for probiotic function.

For *S. cerevisiae* var. *boulardii* to be useful as a probiotic in foods or beverages, it needs to satisfy important technological criteria. Apart from conveying a health benefit to the consumer, the yeast should not have detrimental effects on the shelf-life and sensory properties of the product, and it should remain viable at functional probiotic populations (usually greater than 10^6 cfu/g) until the food/beverage is consumed. To date, there has been little investigation of these requirements. Lourens-Hattingh and Viljoen (2001) demonstrated that populations (10^7–10^8 cfu/g) of *S. boulardii* did not decrease after inoculation into plain yogurts and UHT milk, and remained relatively stable during storage for 28 days at 5°C. However, the yeast exhibited significant growth on the sugars in fruit-based yogurts, producing gas and ethanol that spoiled the product. Heenan et al. (2004) examined the survival and sensory impact of *S. boulardii* inoculated into a frozen, soymilk-based dessert. There was a tenfold decrease in viability of the yeast during the first 10 weeks of storage and, moreover, the yeast gave an unacceptable off-flavour to the product. On the basis of these criteria, more research would be needed to develop *S. boulardii* as an acceptable probiotic in this product. Betoret et al. (2003) have examined the feasibility of incorporating a probiotic mixture of *S. cerevisiae* and *L. casei* into dried apples. Sindhu and Khetarpaul (2001, 2003) described the nutritional enhancement of an indigenous fermented food produced within a mixture of *L. casei* and *S. boulardii*, and its ability to decrease the serum cholesterol level in mice. Psomas et al. (2003) have reported the ability of *S. boulardii*, *S. cerevisiae* and other potential, probiotic yeasts to assimilate cholesterol.

There is a developing interest in using yeast species other than *S. cerevisiae* var. *boulardii* or *S. cerevisiae* as probiotic organisms. Such species include *Debaryomyces*

hansenii, *K. marxianus*, *Yarrowia lipolytica* and *I. orientalis* that are frequently associated with yogurts and cheeses and have been isolated from human faeces (Lourens-Hattingh and Viljoen 2002; Psomas et al. 2001). Other possibilities include *P. farinosa*, *P. anomala* and *Galactomyces geotrichum*, also isolated from human faeces (Mo et al. 2004). The fermented milk products kefir and koumis are frequently noted for their health-promoting, probiotic properties and, in addition to lactic acid bacteria, contain significant populations of yeasts, including *K. marxianus*, *C. kefyr*, *S. cerevisiae* and other *Saccharomyces* species, and *Zygosaccharomyces* species (Oberman and Libudzisz 1998; Beshkova et al. 2002; Frohlich-Wyder 2003; Witthuhn et al. 2005). *D. hansenii* has been studied as a potential probiotic in fish aquaculture where its surface properties and polyamine production appear to be relevant criteria (Gatesoupe 1999; Tovar et al. 2002).

12.6 Other Health and Nutritional Benefits

For many years, now, yeasts and yeast products (principally from *S. cerevisiae*) have been used as ingredients and additives to enhance the sensory and nutritional qualities of foods. Discussion of this topic is beyond the scope of this chapter, but the reader is referred to Dziezak (1987), Halasz and Lasztity (1991), Reed and Nagodawithana (1992), Dawson (2002) and Abbas (Chap. 10) for further information. Recent interests include the use of yeasts to enrich foods and diet with vitamins (e.g. folic acid), antioxidants (e.g. glutathione) and metal ions such as selenium and chromium (Chap. 10; Dawson 2002). The β-(1→3)- and β-(1→6)-glucans of the yeast cell wall exhibit some very attractive functional properties (Nguyen et al. 1998). They can stimulate the immune system (Williams et al. 1992; Jamas et al. 1996; Sutherland 1998), lower serum cholesterol (Robbins and Seeley 1977; Bell et al. 1999) and exhibit antitumour activity (Bohn and Be Miller 1995). In addition, yeast cell-wall polysaccharides also adsorb mycotoxins (Dawson 2002; Yiannikouris et al. 2004; Bejaoui et al. 2004).

12.7 Conclusion

Along with bacteria, viruses and filamentous fungi, yeasts are part of the microflora of many foods and beverages. However, they are rarely (if ever) associated with outbreaks or cases of foodborne illness. In this context, they have an excellent public health track record. Nevertheless, they are opportunistic pathogens, and the role of foods and beverages as a source of the infecting yeast should not be underestimated. Numerous species other than *C. albicans* and *Cr. neoformans* are now considered in the category of opportunistic pathogens, and many of these species frequently occur as contaminants of foods and beverages. More information is required about the ecology of yeasts in the human gastrointestinal tract and how this is impacted by the yeast ecology of foods and beverages. The probiotic and beneficial functions of yeasts in the human diet require further development. While *S. cerevisiae* var. *boulardii* has received most attention in this regard, other species should not be overlooked as having the potential to contribute a diversity of health benefits.

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Chapter 13

The Development of Superior Yeast Strains for the Food and Beverage Industries: Challenges, Opportunities and Potential Benefits

Kevin J. Verstrepen[1,2], Paul J. Chambers[3] and Isak S. Pretorius[3]

[1]*Bauer Center for Genomics Research, Harvard University, Divinity Avenue, Cambridge, MA 02138, USA*
[2]*Centre for Malting and Brewing Science, Department of Microbial and Molecular Systems, Katholieke Universiteit Leuven, Kasteelpark Arenberg 22, 3001 Leuven (Heverlee), Belgium*
[3]*The Australian Wine Research Institute, P.O. Box 197, Glen Osmond, Adelaide, SA 5064, Australia*
(e-mail: sakkie.pretorius@awri.com.au, Tel.: +61-8-83036835, Fax: +61-8-83036601)

13.1 Introduction: Who is Moulding Whom?

When one thinks about domesticated organisms that have been exploited and shaped by humans over the millennia one probably thinks of such things as crop plants, dogs, cats, and livestock. Interestingly many evolutionary biologists and anthropologists who study the relationships between such organisms and humans describe this in the context of co-evolution. They argue that we have been shaped by domesticated species as much as they have been shaped by us. It is argued, for example, that they have 'manipulated' us away from a nomadic existence into the sedentary lifestyle required for growing crops and raising livestock, thereby ensuring their own survival and reproductive success. How would industrial yeasts fare if considered in this light? We have exploited these fungi for millennia in the making of bread, wine and beer, and over this time they have been moulded (forgive the pun) by artificial selection to perform for us in a range of different settings (Fig. 13.1). So central are yeasts to human cultures that we are probably as dependent on them as we are on many of our agricultural domesticated species. From a co-evolutionary perspective one might argue that industrial yeasts such as *Saccharomyces cerevisiae* have used this dependency to exploit us over the millennia; they get us to facilitate their reproduction and dispersal in very large numbers. Because of us, *S. cerevisiae* enjoys phenomenal reproductive success with, for example, an estimated 600,000 t of baker's yeast being produced every year (Pretorius et al. 2003).

This review looks at the next stage in the ongoing relationship between industrial yeasts and humans. We will look at how humans are continuing to make improvements in the performance of such yeasts and how new technologies, particularly recombinant DNA techniques, are being utilised to this end. Or, perhaps we might

The Yeast Handbook
Amparo Querol, Graham H. Fleet (Eds.):
Yeasts in Food and Beverages
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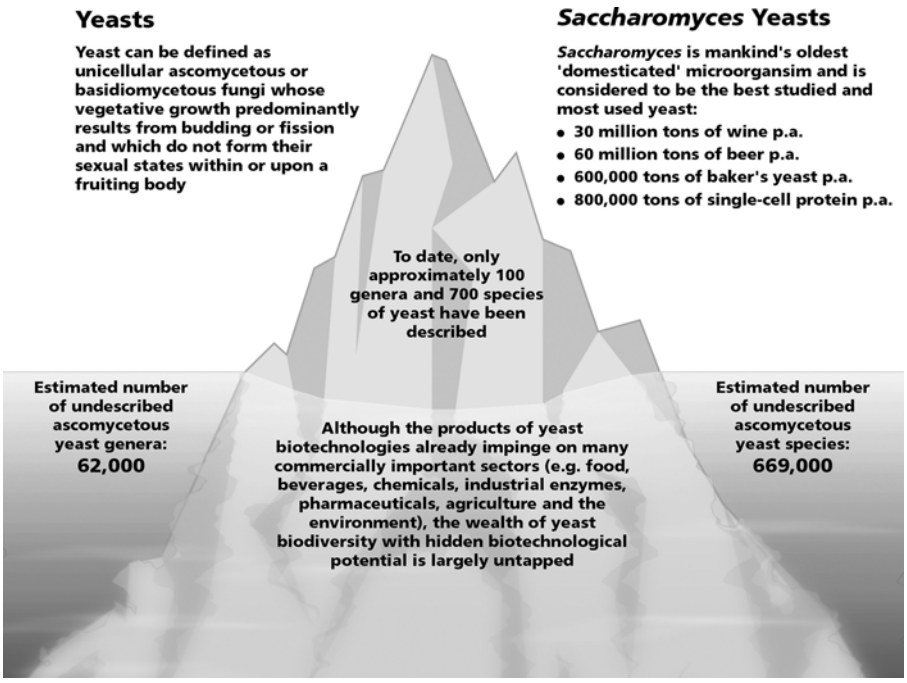

Fig. 13.1. Yeasts comprise a huge group of unicellular ascomycete and basidiomycete fungi. *Saccharomyces* spp., the yeasts that we are most familiar with, represent a tiny tip of this very large iceberg of genera and species, most of which are yet to be described. Who knows what untapped resources lie hidden in the depths of this biodiversity?

just as legitimately say that this review looks at what humans are being 'manipulated' to do by yeasts to find ever more successful ways of using them, thereby increasing their reproductive success.

Our relationship with yeasts probably started at least 7,000 years ago. References to winemaking date back to 5,000 BC, when yeasts were unwittingly used in spontaneous fermentations in Egypt and Phoenicia, and historians believe wine production probably occurred much earlier than this (Robinson 1994). Archeological evidence of a 'brewery' dating back to about 1,500 BC was uncovered beneath the Sun Temple of Queen Nefertiti, suggesting that beer was produced on an industrial scale in ancient Egypt. Selection of yeasts with desirable properties was presumably under way from these very early times but it was not until 1881 that Emil Hansen isolated the first pure yeast culture, a prerequisite for the systematic selection and improvement of strains. Not long after this, Hermann Müller-Thurgau introduced the idea of inoculating fermentations with pure yeast starter cultures and this was rapidly adopted by many wineries of the day. Yeast starter cultures are, of course, now used widely in large-scale wine production, where rapid and reliable fermentations are essential to obtain wines of consistent quality (Henschke 1997).

As far as domesticated organisms go, *S. cerevisiae* is exceptional in what it has to offer us (Fig. 13.2). Not only is it very efficient at fermentation, it also has a long

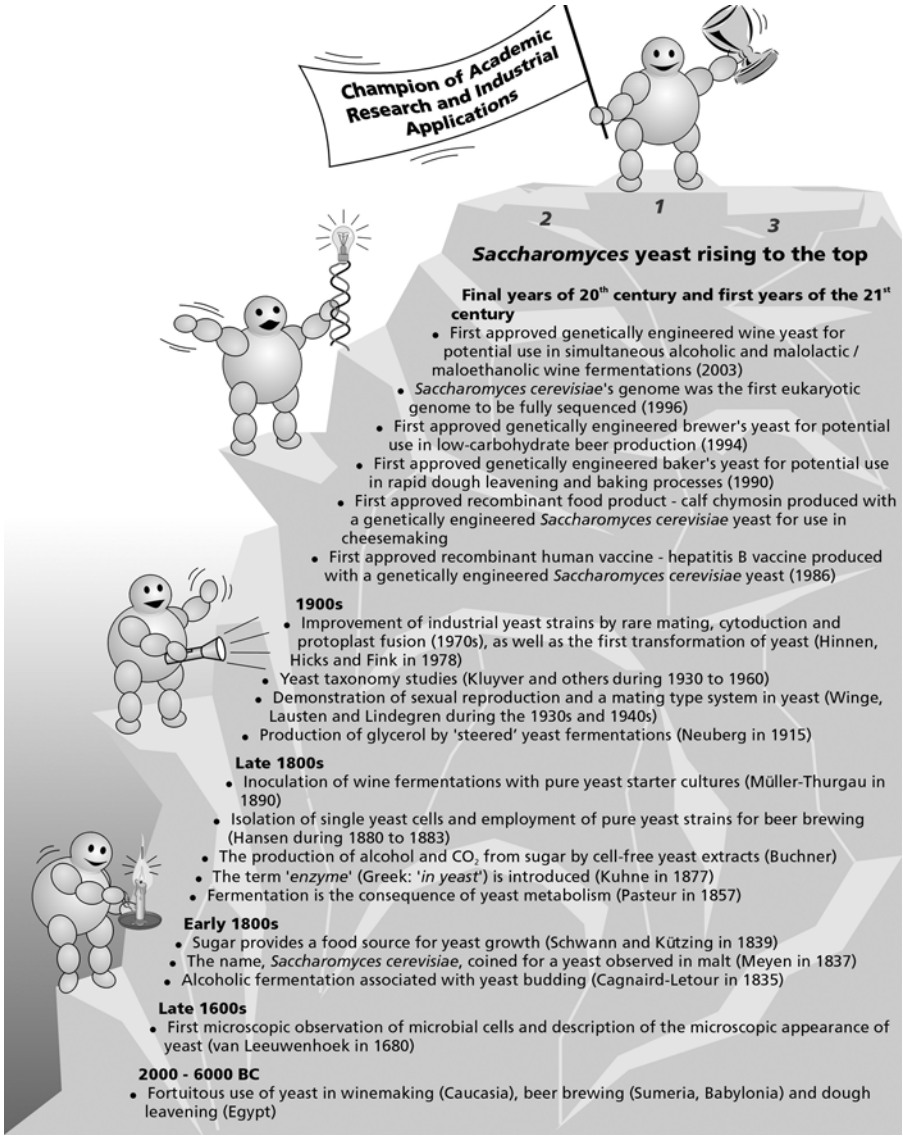

Fig. 13.2. *Saccharomyces* spp. have a very long history of domestication, dating back at least as far as ancient times; however, the past 150 years has seen the greatest growth of knowledge of these yeasts and their systematic application to a range of industrial processes and scientific research programmes. This long history of safe and fruitful association with humans has given *Saccharomyces* spp. a privileged place among domesticated organisms and explains why *S. cerevisiae* has 'generally recognised as safe' (*GRAS*) status and why it was the first genetically modified organism to be approved for applications in a range of industries

history of use in food and beverage production with a proven safety record for human consumption, and this has ensured it of 'generally recognised as safe' (GRAS) status by the US Food and Drug Administration (FDA). It was therefore inevitable that *S. cerevisiae* would be one of the first organisms to be genetically modified (GM) to produce food additives. Indeed, the first GM-based food additive to be approved for use in human food, calf chymosin (which is used to make cheese), was produced using transgenic *S. cerevisiae* cells (Walker 1998). Furthermore, GM *S. cerevisiae* was the first GM organism (GMO), as distinct from a GM product, to be cleared for use in human food production (Gopal and Hammond 1992; Hammond 1995; Walker 1998).

The application of yeasts has now been extended beyond the food world to the bio-ethanol and pharmaceutical industries. In some countries (e.g. Brazil) there is considerable dependence on yeasts for bio-ethanol production, and in a world of depleting fossil fuels and accumulating greenhouse gases it is likely that we will see considerable growth in the production of this renewable fuel. This will, of course, lead to ongoing development of new strains of yeasts that are able to produce greater amounts of ethanol and from cheaper sources of carbon, such as lignocellulose waste (Lynd et al. 2002). The *S. cerevisiae* that we currently have at our disposal is unable to use such carbohydrates, but we can be quite confident that it is already 'working on us' to engineer its genome so that it will be able to access such resources, thus increasing its range even further than is already the case.

Perhaps not surprisingly, given its GRAS status, *S. cerevisiae* was the host of choice for production of the first recombinant human vaccine, against hepatitis B (McAleer et al. 1984). Thus, *S. cerevisiae* continues to make itself more and more indispensable to us as our dependence on it for fuel and medicines grows.

A major issue faced by the fermentation and baking industries is the cost of purchasing or preparing yeast biomass. While beer makers usually repitch (i.e. recycle) their yeast several times before resorting to a fresh starter culture, and some winemakers rely on spontaneous fermentations, most wine and bread producers utilise fresh yeast for each fermentation. Interestingly, some winemakers claim that the unique contributions of different yeast species living on grapes or in the winery confer a complexity upon wine not seen in inoculated, controlled fermentations (Fugelsang 1997), but the risks associated with using such an approach can be considerable. This is why, in beer and bread production and for most wine fermentations, starter cultures of single, known yeast strains are used; they minimise the risk of failed fermentations and spoilage, and ensure a predictable product for the consumer. Thus, despite associated costs, there will be an ongoing need to continue producing large quantities of 'single culture' yeasts. Anything that yeast scientists can deliver with regard to reducing yeast production costs would therefore be welcomed by industry and consumers, who would benefit from savings, passed down the line.

Today's consumers have an increasing interest in luxury, individualised foods and beverages. Thus, there is an ongoing drive to make novel products, but in fermentation industries this is limited when one is restricted to using single strains of yeasts. Thus, scientists associated with the beer and wine industries have an interest in making yeasts capable of delivering more 'interesting' and varied sensory properties to

beverages, without having to resort to mixed ferments. Genetic modification offers numerous possibilities in this regard, enabling the development of products with novel sensory properties and improved quality (for recent reviews, see Pretorius and Van der Westhuizen 1991; Pretorius 2000, 2003, 2004, 2005; Verstrepen et al. 2001a; Akada 2002; Pretorius and Bauer 2002; Pretorius et al. 2003, 2005; de Barros Lopes et al. 2005; Pretorius and Høj 2005). Of course, research and development to produce superior yeast strains for industrial use is very costly, making it accessible only to large multinational companies or highly co-ordinated industry bodies.

Interestingly, when trying to improve yeast-based industrial processes, attention has generally focused on various highly technological solutions ranging from the refinement of bioreactors to the optimisation of various production parameters. It is only recently that there has been a shift to modify yeasts to improve the processes they are applied to, and this is, in no small part, due to the development of modern microbiology and molecular biology (Fig. 13.3). Once the public are more accepting of using GMOs we will be able to construct yeasts in ever more defined and refined ways to meet the demands of industry. The potential of this is already apparent from experimental work that will be described later in this review.

One of the challenges facing scientists working on industrial yeasts is the fact that the strains used in bakeries, breweries and wineries are usually polyploid or aneuploid. This makes crossing and sporulating, two techniques commonly used in 'classic' yeast breeding programmes, difficult (Akada 2002; Estruch and Prieto 2003). In 1978, however, genetic engineering offered an alternative approach when, independently, Hinnen et al. (1978) and Beggs (1978) described a method to genetically transform *S. cerevisiae*, thereby negating the need for sexual reproduction to recombine genes from different genetic backgrounds. But even with this in place industrial strains are still less straightforward to work with than laboratory strains because it is usually undesirable to have auxotrophic markers in strains that are to be used in an industrial setting; auxotrophy can impact on the performance of the yeast and the quality of the product that the yeast is employed to make. This has meant that new dominant genetic markers and new strategies for genetic engineering have had to be developed for selection of transformants in industrial yeasts (Webster and Dickson 1983; Akada 2002; Estruch and Prieto 2003; Verstrepen and Thevelein 2004).

Although this chapter is about industrial yeasts there is only one group of yeasts that we have truly domesticated and co-evolved with, at least in a cultural (again, forgive the pun) sense. That group is the *Saccharomyces sensu stricto* complex, which includes the most thoroughly researched and scientifically described of all domesticated organisms, *S. cerevisiae*. This chapter focuses on industrial strains of *S. cerevisiae*, describing examples of how it has been engineered to improve its performance in the food and alcoholic beverage industries (Figs. 13.4–13.6), future possibilities offered by exciting gene technologies, and some of the hurdles we have to get over to exploit it more fully. Consideration is also given to some of the legal and political issues associated with the application of GM yeasts to the food and beverage industries. Philosophically, however, one might argue that this chapter is really about how this yeast has exploited humans to make it more and more indispensable to us. In this respect we are a means to an end for its reproductive success.

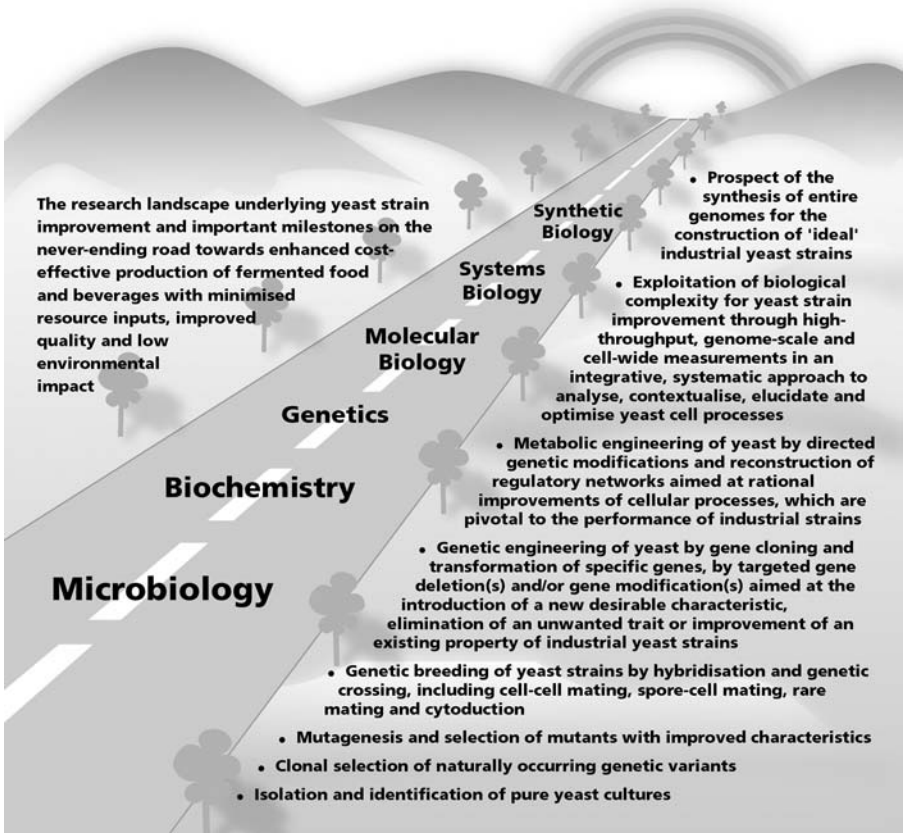

Fig. 13.3. The road of discovery and application in yeast science started in microbiology laboratories. Moving through the twentieth century, the application of biochemistry and genetics to research on yeasts increased our knowledge and understanding of cell physiology enormously, and the advent of molecular biology revolutionised the field. We are now moving into systems biology, and we can only guess what this era will deliver. One thing we can be sure about, however, is that it holds enormous potential for fermentation industries in that it will enable us to design yeasts that are more robust and deliver an increasing array of tailor-made products to meet consumer demand

13.2 Genetically Engineering Yeasts for Improved Performance and Product Quality

This section presents an overview of some of the types of recombinant DNA-based modifications that have been introduced into *S. cerevisiae* to improve fermentation performance or quality of end products (Fig. 13.7). In the current political climate it is unlikely that these yeasts will be used to produce food or beverages, but they give some indication of what will be possible in the future when GMOs are viewed with less suspicion by the general community.

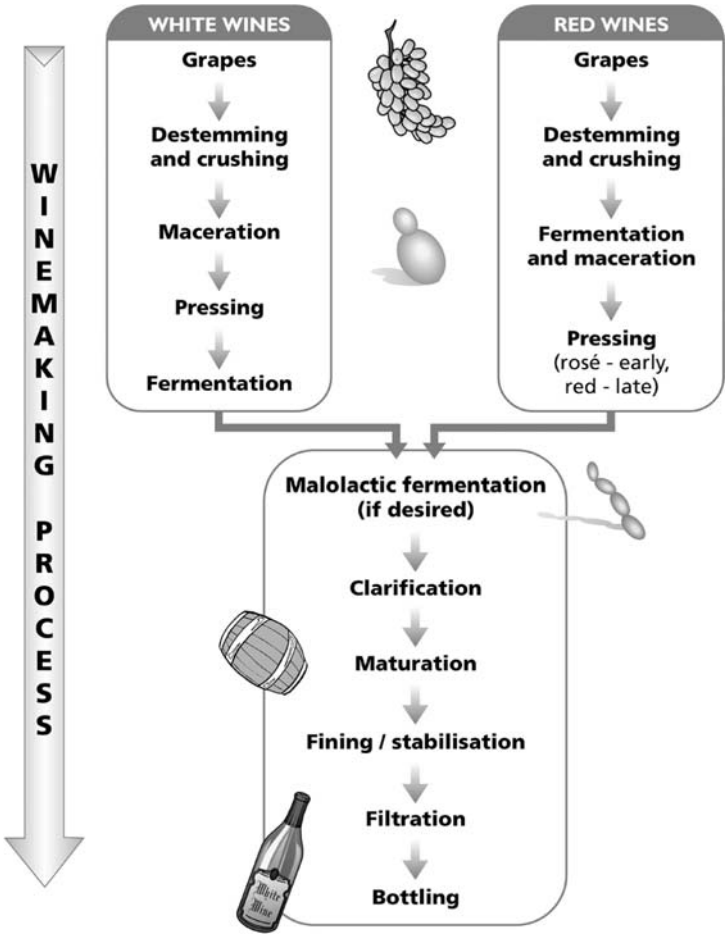

Fig. 13.4. Winemaking is probably the oldest application of yeast fermentation. Illustration of the main steps in this process

13.2.1 Improving Fermentation Performance

Different strains of *S. cerevisiae* vary considerably in their efficiency and reliability in fermentations. This tells us that there is considerable genetic variation that we can tap into when we select or manipulate this yeast for improved performance, and when this variation proves to be too limiting we can use genetic engineering techniques to borrow genes from elsewhere. The following section will consider what are now known as self-cloned (i.e. GM but not transgenic) and transgenic variants of *S. cerevisiae*, that have been created to this end.

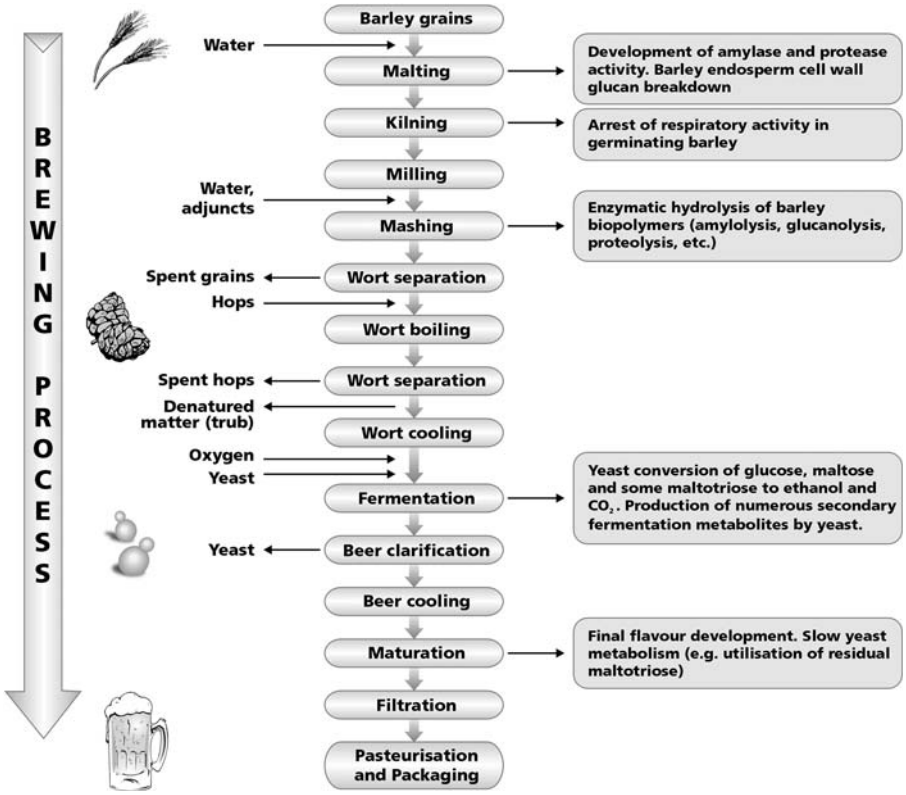

Fig. 13.5. Brewing has been with us for thousands of years and, while there are many variations in the steps involved, the key stages that are central to most brewing processes are illustrated

13.2.1.1 Making Improvements by Manipulating Fermentation Rate, Glycolytic Flux and Sugar Uptake

Fermentation-based production processes (Figs. 13.4–13.6) are time-consuming; beer fermentation takes 4–7 days and wine fermentation often takes more than 20 days. The time needed to complete a fermentation cycle is a major determinant of productivity and cost efficiency. A faster fermentation rate results in lower running costs, greater flexibility, and the total fermenter volume needed to obtain a targeted production volume is dramatically reduced.

Temperature control in the fermentation vessel is the traditional way to manage fermentation rate. The optimal growth temperature of *S. cerevisiae* is 28–30°C and, in general, temperatures close to this result in relatively rapid fermentations. Cooler temperatures slow overall yeast metabolism (Sablayrolles and Barre 1993; Speers et al. 2003) and higher temperatures compromise product quality. For example, many important volatile aroma compounds are lost at higher than optimal temperatures

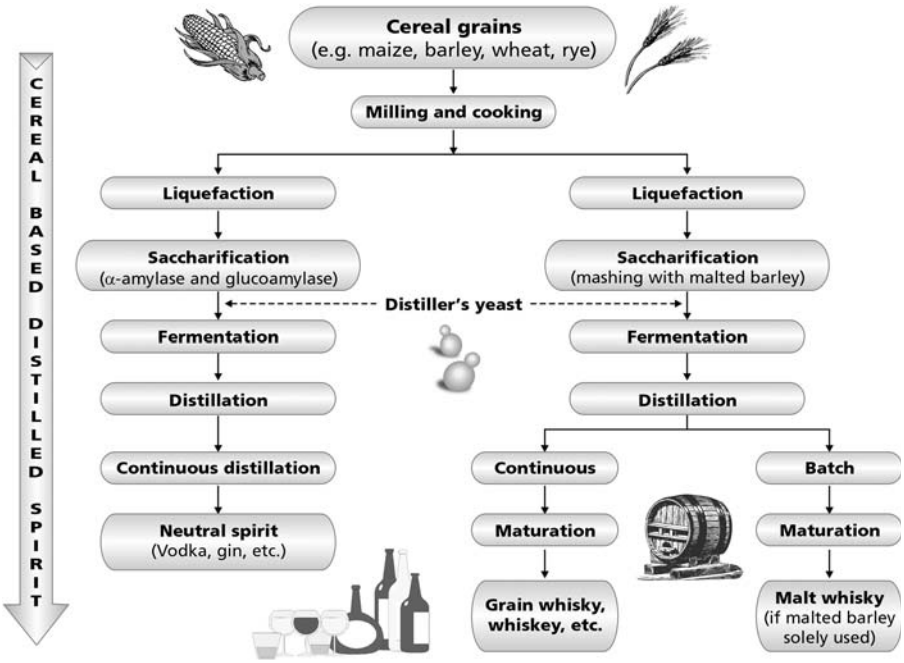

Fig. 13.6. Making alcoholic spirits utilises distiller's yeasts, which are strains of *S. cerevisiae* that produce, amongst other things, high levels of ethanol and have a high level of tolerance to this alcohol. Illustration of the main stages in a fermentation to generate spirits

because of increased evaporation and by the entrainment action of carbon dioxide (Verstrepen et al. 2003a). In addition, production of flavour-active secondary metabolites such as fusel alcohols and esters is disproportionately increased as temperatures increase, causing an unbalanced aroma profile (Sablayrolles and Ball 1995; Verstrepen et al. 2003a). Use of increased fermentation temperatures is therefore limited, particularly in lager beer and white wine production, where temperatures are generally kept below 15°C to preserve the typical aromas associated with these beverages (Nykänen and Suomalainen 1983; Nykänen 1986; Robinson 1994; Dominé 2000; Verstrepen et al. 2003a).

Attempts have been made to change fermentation rates using GM yeast strains engineered to produce higher levels of glycolytic enzymes. In one remarkable study genes encoding key enzymes of the glycolytic pathway were overexpressed in order to increase glycolytic flux (Schaaff et al. 1989). The overexpressed genes encoded hexokinase (HXK), glucokinase (GLK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), aldolase (FBA), triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (TDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase (ENO), pyruvate kinase (PYK), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH), each being overexpressed

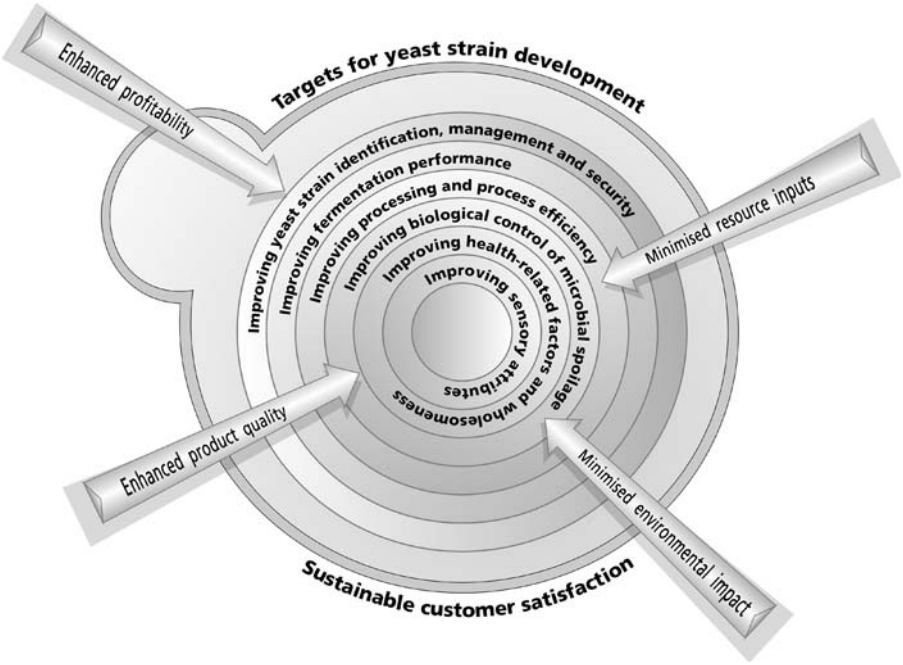

Fig. 13.7. There are many improvements that can be made to yeast performance and product quality in industrial applications. Genetic engineering is a very powerful means to achieving such ends, by enabling the development of new strains of yeasts with novel phenotypes that can lead to, amongst other things, improvements in sensory properties of products and efficiency of processing

in a different strain. However, overexpression of these enzymes had no significant effect on the rate of ethanol formation. Simultaneous overexpression of a group of seven enzymes in the lower glycolytic pathway resulted in a very limited increase of fermentative capacity (Smits et al. 2000). Together, these results suggest glycolytic flux is not regulated at the level of glycolytic gene expression. Perhaps an increase in glycolytic flux may require overactivation of all glycolytic enzymes simultaneously. However, it is likely that the import of sugars into the cell and/or sugar hydrolysis are the main rate-limiting steps for fermentation (Boulton et al. 1996). Over recent years much research has focused on carbohydrate transport and the genes involved.

In most commercial fermentations the medium contains a mixture of carbohydrates. The main sugars in bread dough are sucrose, glucose, fructose and maltose; grape must contains mainly glucose and fructose; beer and whiskey wort contains glucose, fructose, sucrose, maltose and maltotriose; and the fermentation medium for ethanol production usually contains a mixture of any of these sugars in variable concentrations, depending on the origin of the molasses (Bamforth 2003; Yoon et al. 2003). Because carbohydrates are too hydrophilic to cross the cell membrane by free diffusion, specialised carriers are required for their transport into the cell. Yeast cells

have more than 15 general hexose transporters (Stambuk and de Araujo 2001) as well as a set of more specialised transporters, including the five maltose transporters encoded by the *MALx1* genes (where *x* is the number of the *MAL* gene family) (Vanoni et al. 1989; Han et al. 1995), and Fsy1 (Goncalves et al. 2000; de Sousa et al. 2004), a specialised fructose transporter identified in *S. pastorianus* and *S. bayanus*.

Carbohydrate import is a rate-limiting step in fermentation, at least in part, because glucose and sucrose repress the expression of transporters necessary for the import of other sugars such as maltose, maltotriose and galactose (for reviews, see Winderickx et al. 2003; Verstrepen et al. 2004a). Glucose also slows down the uptake of fructose because both sugars are imported by the same HXT carriers, which have a greater affinity for glucose than fructose. Apart from this competitive inhibition of fructose uptake, recent research demonstrates that glucose may also repress the expression of specific fructose transporters such as Fsy1 (Goncalves et al. 2000; Berthels et al. 2004; de Sousa et al. 2004). The minimal concentration at which glucose and sucrose induce signals differs for different signalling pathways and targets but, in general, concentrations higher than 20–40 mM are sufficient to elicit a strong response (Meijer-Michelle et al. 1998; Meneses et al. 2002).

In beer wort, grape must, molasses and bread dough, the initial glucose and/or sucrose concentration greatly exceeds the aforementioned threshold for catabolite repression; therefore, the transport of other sugars is delayed until glucose and sucrose levels drop. In some cases, yeast cells fail to attain a proper de-repressed state, leading to sluggish or 'hanging' fermentations, in which not all of the available sugars are fermented (Oda and Ouchi 1989; Meneses et al. 2002; Meneses and Jiranek 2002; Verstrepen et al. 2004a). Also, towards the end of fermentation, transport and consumption of sugars often slows down. This has been attributed to the various stresses yeast cells experience at the end of industrial fermentations, such as high ethanol and carbon dioxide levels and low levels of carbon and nitrogen compounds (Ivorra et al. 1999; Bauer and Pretorius 2000; Brosnan et al. 2000; Puig and Pérez-Ortin 2000; Blateyron and Sablayrolles 2001; Carrasco et al. 2001). However, the precise mechanisms responsible for this late-fermentation drop in sugar consumption are not yet understood.

On the basis of current knowledge of the regulatory mechanisms that control sugar transport, several attempts have been made to use genetic modification to increase fermentation rates. For example, it has been shown that constitutive expression of the *MAL61* gene, encoding a maltose transporter protein, results in a clearly greater fermentation rate of high-gravity beer worts (Kodama et al. 1995). Similarly, overexpression of maltose permease and maltase resulted in a yeast with improved dough-leavening capacities (Osinga et al. 1988). Increasing sugar uptake through the general hexose transporters, however, is more complicated. This is because some of these have specific roles during fermentation; in fact overexpression of a single *HXT* gene cannot fully restore the fermentation capacity of an *HXT* null strain (Luyten et al. 2002). In order to obtain increased general hexose uptake, it may be necessary to co-overexpress several members of the *HXT* gene family. Other strategies, such as constitutive expression of the heterologus fructose transporter-encoding gene *FSY1*, which may improve the fermentation performance of wine yeasts, remain to be explored.

13.2.1.2 Making Improvements in Oligosaccharide (Dextrin) Utilisation

S. cerevisiae ferments only some mono-, di- and trihexoses, while more complex or branched oligosaccharides are not metabolised. Molasses, a commonly used medium for the production of bio-ethanol, biopharmaceuticals and baker's yeast biomass, contains up to 8% raffinose. This trisaccharide (fructose–glucose–galactose) is hydrolysed by baker's yeast invertase to fructose and the non-fermentable disaccharide melibiose. Expression of the α-galactosidase-encoding *MEL1* gene from *S. bayanus* in baker's yeast resulted in an 8% increase in biomass yield, yet other desirable characteristics were retained (Gasentramirez et al. 1995).

Beer wort contains about 30% non-fermentable higher dextrins. While some residual sugars in fermented beverages contribute to the 'wholesomeness' and the 'mouthfeel' (Ragot et al. 1989), non-fermentable sugars represent a considerable economic loss for brewers. A second disadvantage associated with these carbohydrates is that the popular light beers end up carrying a considerable number of kilojoules. Frequently, glucoamylases are added to break down the higher dextrins in wort into less complex sugars that can be fermented by brewer's yeast. The dextrins are hydrolysed and subsequently fermented, resulting in a relatively high ethanol product. After dilution to an appropriate alcohol level, the resulting beer contains fewer sugars and therefore less energy. By enabling yeast to ferment these dextrins, light beers could be produced without the addition of expensive purified enzymes. In fact, the fermentation of a greater proportion of wort sugars would make the production of 'light' and 'low-carbohydrate' beers cheaper than that of standard beers (Hammond 1995). A commercial lager yeast strain was transformed with a multicopy plasmid in which the *S. diastaticus* glucoamylase-encoding *STA2* gene was placed under the control of the constitutive yeast *PGK1* promoter. Transformants were superattenuating, producing about 1% (v/v) more ethanol than the wild-type parental strain (Perry and Meaden 1988), but they were not very stable. This problem was easily addressed, however, by integrating the *PGKP::STA2* construct into the *S. cerevisiae* genome (Vakeria and Hincliffe 1989). Similar results were obtained with expression of the *S. diastaticus STA1* gene (Sakai et al. 1989) in brewing yeast.

13.2.1.3 Achieving Fermentation of Branched Oligosaccharides and Polysaccharides

Fermentation of media containing a mixture of various complex sugars that are low in cost is important for many ethanol-producing companies, but particularly the bio-ethanol industry; accessing cheaper forms of substrate would make bio-ethanol a more economically viable alternative to fossil fuel (Wheals et al. 1999; Zaldivar et al. 2001). Cellulose and starch are the most abundant and widespread polysaccharides on the planet but *S. cerevisiae* does not have the cellulase or amylase enzymes necessary to access these polymers. The potential of these carbohydrates to be used as cheap sources of carbon in fermentation processes has therefore fuelled research aimed at creating transgenic yeasts carrying the requisite enzymes. Ethanol production using other types of microorganisms that encode their own cellulases and/or amylases is probably impossible because of constraints such as poor ethanol tolerance, poor growth rates and lack of GRAS status (Eksteen et al. 2003).

Starch (and dextrins) are composed of linear α-1,4-linked chains of glucose with various degrees of α-1,6 branching. At least two different classes of enzymes are needed to efficiently digest these molecules into fermentable maltose and glucose: α-amylase (endoamylase) and glucoamylase (exoamylase). Specific debranching enzymes that digest the α-1,6 linkages, such as pullulanase and isoamylase, enable complete digestion of these polymers. Thus to make transgenic amylolytic yeasts requires engineering genes for at least some of the aforementioned enzymes into *Saccharomyces* spp.

Amylolytic yeasts are, of course, also of interest to the whiskey and beer industries because of their potential to ferment all available sugars without the need for extensive malting. In the beer world, digestion of branched carbohydrate molecules has been achieved using enzymes derived from organisms such as *Aspergillus niger* (Gopal and Hammond 1992) and *Aspergillus amawori* (Cole et al. 1988), both of which have enzymes with α-1,6 and α-1,4 hydrolytic activities. An expression cassette of the *A. niger* glucoamylase gene has been inserted into the *S. cerevisiae HO* gene and the resultant strain was tested at a semi-industrial scale in fermentations of up to 10,000 l. The trials were successful, producing beer that was both superattenuated and of good quality (Gopal and Hammond 1992; Hammond 1995). The GM yeast strain was the first (and so far only) GM brewer's yeast to be cleared for the production of human food. Large-scale commercialisation of the beer was not attempted, however, because of the low consumer acceptance of GMOs in food production (see later) (Hammond 1995; Roller and Harlander 1998).

Increased dextrin degradation has similarly been obtained by overexpression of the amyloglucosidase of *Schwanniomyces occidentalis* in a brewing yeast (Lancashire et al. 1989). Like the *A. niger* and *A. amawori* amyloglucosidase activities, the *S. occidentalis* enzyme combines α-1,6 and α-1,4 hydrolytic activities. However, this enzyme has the additional advantage of being heat-labile, and therefore can be inactivated during beer pasteurisation. This reduces the risk of beer sweetening due to further breakdown of unfermented starch during storage (Lancashire et al. 1989).

Research on whiskey yeasts has shown that it is possible to create industrial strains of *S. cerevisiae* that can ferment starch. Amylase genes *LKA1* and *LKA2*, from the yeast *Lipomyces kononenkoae*, were transformed into four different whiskey strains and a wine yeast, and the transformants (unlike the original parent strains) were able to utilise starch for ethanol production (La Grange-Nel et al. 2004). This again highlights the potential of genetic engineering for creating novel yeast phenotypes that are of value to industry.

13.2.2 Breakdown of Haze-Causing Polysaccharides, Phenols and Proteins

Haze is a precipitate that appears in some beers and wines as they mature. The resultant cloudy product can cause filtration problems during processing and is usually regarded as undesirable by consumers. Polysaccharides derived from raw materials and including β-glucans, pectins and xylans are partially responsible for such problems. β-Glucans are found in barley cell walls and are cleaved by a specific *endo*-β-glucanase; however, this cleavage is often only partial because enzymatic activity

is lost owing to the elevated temperatures of kilning, thus leaving residual insoluble carbohydrate. Grapes contain pectins, cellulose (a source of glucans) and hemicellulose (mainly xylans).

Another cause of haze is the precipitation of proteins. In wine this type of haze is caused by grape-derived, pathogenesis-related proteins such as thaumatin-like proteins and chitinases, which aggregate and then precipitate over time, and particularly on heating.

Haze-causing molecules are normally removed by sedimentation, filtration and centrifugation, or by the addition of enzymes (usually bacterial) that break them down. In the case of wine, fining agents such as casein, isinglass, albumin, gelatin, bentonite or polyamide materials are commonly used to bind or filter phenols and colloidal particles that can contribute to haze formation (Boulton et al. 1996). However, filtration, centrifugation, enzymatic treatment and fining are costly and laborious processes. Moreover, as much as 20% of the product can be lost during these clarification and stabilisation steps (Canal-Llauberes 1993). Thus, it would be of great benefit to the alcoholic beverage industries if yeast strains that have an inherent ability to hydrolyse haze-causing molecules could be developed, and there have been several exciting advances in this regard (van Rensburg and Pretorius 2000).

Several yeast strains that can hydrolyse one or more of the known haze-causing agents have been produced. For brewer's yeast, most attention has focused on the development of β-glucan degrading strains. For example a *Bacillus subtilis* β-glucanase gene was fused to the *S. cerevisiae* α-factor secretion signal ($MF\alpha1_S$) and expressed in *S. cerevisiae* (Lancashire and Wilde 1987), leading to reductions in β-glucan content and haze reduction in the final product. More efficient glucan hydrolyis in beer fermentation was obtained by expressing *Trichoderma reesei* β-glucanase, which has a lower optimal pH (4–5 compared with 6.7 for the *B. subtilis* enzyme) (Pentillä et al. 1987a; LaGrange et al. 1996). Industrial brewer's yeast carrying the *T. reesei EG1* gene driven by the *S. cerevisiae ADH1* promoter and devoid of any bacterial sequences has been tested in pilot beer fermentations. The strain hydrolysed virtually all β-glucans, resulting in a significant reduction of beer viscosity, while all other brewing characteristics of the parent strain remained unchanged (Pentillä et al. 1987b; Suihko et al. 1991). Similarly, several glucan-hydrolysing wine strains were constructed that expressed the *Butyrivibrio fibrisolvens* endo-β-1,4 glucanase gene *END1*, the *B. subtilis* endo-β-1,3-1,4-glucanase gene *BEG1*, the *Ruminococcus flavefaciens* cellodextrinase gene *CEL1*, the *Phanerochaete chrysosporium* cellobiohydrolase gene *CBH1* or the *Saccharomycopsis fibuligera* cellobiase gene *BGL1* (van Rensburg et al. 1994, 1995, 1996, 1997, 1998, 2005). However, these strains have not yet been tested in large-scale fermentation trials.

A pectolytic wine yeast was developed by co-expressing the *Erwinia chrysanthemi* pectate lyase gene *pelE* and the *Erwinia carotovora* polygalacturonase gene *peh1* (Laing and Pretorius 1992, 1993a, b), both of which were fused to *S. cerevisiae* $MF\alpha1_S$ secretory signals. Xylan-fermenting yeasts were constructed by expressing the endo-β-xylanase genes from *A. kawachii* (*XYN1*) and *A. niger* (*XYN4* and *XYN5*), *T. reesei* (*XYN2*) as well as the *B. pumilis xylosidase XLO1*, the *A. niger* α-L-*arabinofuranosidase ABF2* and the *A. nidulans* genes *xlnA* and *xlnB* (Pérez-Gonzalez et al. 1993, 1996; Crous et al. 1995, 1996; LaGrange et al. 1996, 1997;

Luttig et al. 1997). While these constructs show great promise in laboratory-scale work, further tests are needed to evaluate the exciting possibilities they offer in large-scale fermentation.

The possibility of creating wine yeasts capable of hydrolysing haze-active proteins has also been investigated. Overexpression of *S. cerevisiae* vacuolar protease A, encoded by the *PEP4* gene, was chosen because of its activity at the low pH of wine. The *PEP4* gene was cloned and expressed in wine yeast using several promoter, leader and terminator sequences. When *PEP4* was driven by the *ADH1* promoter and preceded by the *MFα1* pheromone secretion signal, biologically active protease A was secreted into the medium. However, this was not sufficient to replace bentonite fining because many of the haze-causing proteins are particularly resistant to proteolysis (Pretorius 2000).

A novel method to prevent haze in white wine is the use of haze protective factors (hpfs); specific mannoproteins from *S. cerevisiae* that visually reduce protein haze (Waters et al. 1993, 1994). While the exact mode of action of these proteoglycans is unknown it is possible that overexpression of genes for hpfs in wine yeast during fermentation will lead to a reduction in the levels of haze-causing proteins in wine.

13.2.3 Improving Flocculation and Sherry Flor Formation

At the end of a primary beer, wine or whiskey fermentation, yeast cells have to be separated from the product. The natural tendency of yeast cells to flocculate can make this biomass separation relatively easy. Flocculation is a reversible, asexual and calcium-dependent process in which yeast cells adhere to form flocs consisting of thousands of cells (Verstrepen et al. 2003b). Upon formation, the flocs rapidly separate from the bulk medium by sedimentation or, having entrapped gas bubbles, by rising to the surface. Flocculation is important to the fermentation industry because it provides an environmentally friendly, cost-free and effective way to separate yeast cells from the fermentation product. However, the timing of flocculation is critical: cells should not flocculate before fermentation is complete because this can lead to slow or stuck fermentations, while late or poor flocculation increases the need for extra filtration and centrifugation. The ideal industrial yeast should exhibit strong flocculation towards the end of fermentation. Many of the yeast strains presently used in industrial fermentations have a less than optimal flocculation profile (for a review, see Verstrepen et al. 2003b). Furthermore, the flocculation behaviour of a specific yeast strain is often variable and difficult to predict (Jibiki et al. 2001; Sato et al. 2001; Verstrepen et al. 2003b, 2004b). Improvement and control of flocculation is therefore a constant concern for the alcoholic beverage industries.

Flocculation of *S. cerevisiae* involves lectin-like adhesins, called flocculins, that stick out of the cell wall (Stratford and Carter 1993). Following activation by calcium ions (Stratford 1989; Watari et al. 1994; Teunissen and Steensma 1995; Bony et al. 1997b; Kobayashi et al. 1998), the N-termini of flocculins selectively bind mannose residues of adjacent cells, thereby creating a group or floc of cells. More than 30 genes are known to be involved in this process (Teunissen and Steensma 1995; Teunissen et al. 1995b; Kobayashi et al. 1999; Verstrepen et al. 2003b) but only a few of them encode flocculins; the others are regulatory genes, which are often not

specific for flocculation. The best-known flocculation gene is *FLO1*, which is relatively large, encoding a cell wall flocculin of 1,537 amino acids (Stratford and Carter 1993; Teunissen et al. 1993a, b; Watari et al. 1994; Bony et al. 1997b; Kobayashi et al. 1998). Other dominant genes encoding flocculins include *FLO5*, *FLO9* and *FLO10* (Teunissen and Steensma 1995; Teunissen et al. 1995a; Guo et al. 2000; Batlle et al. 2003). Lager-*FLO1* (Lg-*FLO1*) is a *FLO1*-like gene found in lager yeast (often referred to as *S. pastorianus* or *S. carlsbergensis*). This gene is believed to encode a slightly different flocculin that binds glucose and maltose as well as mannose. Hence, flocculation caused by the Lg-*FLO1* gene product is competitively inhibited by free glucose and mannose in the medium (Kobayashi et al. 1998; Sato et al. 2002).

The *FLO11* gene (also referred to as *MUC1*) encodes a special adhesin that enables cells to adhere to various surfaces, including agar and plastic (Lambrechts et al. 1996; Lo and Dranginis 1996, 1998; Guo et al. 2000; Reynolds and Fink 2001; Verstrepen et al. 2004b). In addition, expression of *FLO11* is required for filamentous growth (Lambrechts et al. 1996; Guo et al. 2000). Filamentous growth occurs when mother and daughter cells do not detach, forming long chains of slightly elongated cells. These chains can penetrate the semi-solid growth medium of agar plates. In liquid medium, filamentous growth is believed to be responsible for flor formation (a layer of floating yeast chains found during the oxidative fermentation stages in sherry production) (Ishigami et al. 2004).

Several attempts have been made to introduce flocculence into non-flocculating *S. cerevisiae* strains. The first strategies were based on the electrofusion or mating of a non-flocculent industrial strain with a flocculating strain (Urano et al. 1993a, b). Later approaches involved the use of genetic engineering to overexpress dominant flocculation genes such as *FLO1* (Barney et al. 1980; Watari et al. 1993, 1994). Self-cloning of *FLO1* into a non-flocculent industrial strain was first reported in 1998 (Ishida-Fujii et al. 1998); self-cloning strains contain no non-*Saccharomyces* DNA sequences and therefore are more likely to be exempt from the strict legislation regulating the use of GMOs in food production (Sect. 13.3.2.1).

Since expression of *FLO* genes results in flocculation, the controlled expression of these genes may lead to controllable flocculation; the main problem with most of the strains produced so far is that they constitutively flocculate, making them unsuitable for industrial use. Recently, a strain was constructed in which *FLO1* was brought under the control of an *HSP30* promoter (Verstrepen et al. 2001b) (Fig. 13.8). This promoter is activated only towards the end of fermentation, when ethanol levels increase and carbohydrates are depleted (Piper et al. 1997; Riou et al. 1997; Donalies and Stahl 2001; Hahn and Thiele 2004). This self-cloning strain showed an appropriate fermentation and flocculation behaviour in small-scale fermentation trials (Verstrepen et al. 2001b); however, further tests are needed to evaluate the performance of this construct in large-scale commercial fermentations. The controlled expression of Lg-*FLO1*, which will potentially prevent early flocculation because Lg-*FLO1* binding is inhibited by residual unfermented glucose and mannose, is also awaiting exploration.

Flor formation is one of the defining features of sherry production. A ‘flor’ is a dense mat of yeast that grows on the surface of maturing sherry after ethanolic

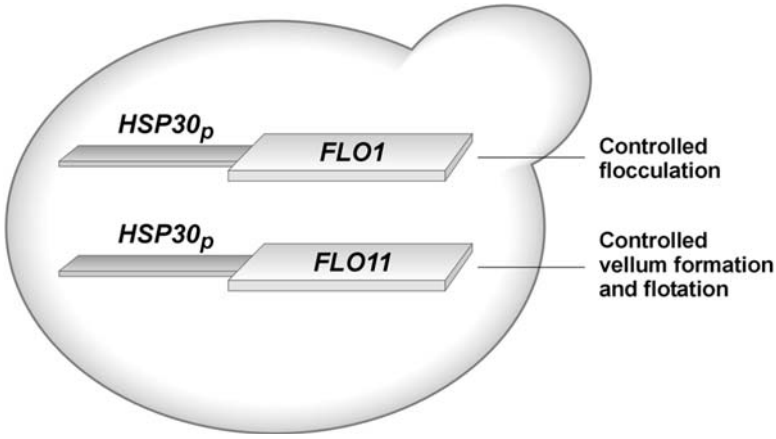

Fig. 13.8. Flocculation of yeast cells involves adhesin molecules called flocculins, one of which is encoded by *FLO1*. Transforming the yeast with this gene behind an *HSP30* promoter ensures that it will be expressed at the end of fermentation, when flocculation is required by the brewer or winemaker. Another flocculin, *FLO11*, is involved in filamentous growth and is important for flor formation during sherry production. If this gene were to be cloned behind the *HSP30* promoter presumably this would also lead to enhanced flor formation at the end of fermentation, when the flor is required

fermentation; failure of the mat to form or its breakdown leads to spoilage. Thus, improving flor formation and stability is of interest to sherry producers. In this context, overexpression of *FLO11* (Fig. 13.8) should help to create strains better able to form stable flors, and with improved adherence to carrier materials sometimes used in fermentations. A first step towards this was recently taken with the investigation of the role of *FLO11* in an industrial sherry yeast (Ishigami et al. 2004).

13.2.4 Engineering Antimicrobial Properties into Industrial Yeast

In contrast to controlled laboratory experiments, commercial-scale industrial fermentations are usually contaminated with other microbes. Industrial equipment is not easy to sterilise or even clean, many raw materials are contaminated with microorganisms and traditional fermenters may have airflow from the industrial plant. Thus there is a risk that 'wild' yeasts and/or bacteria may contaminate the fermentation. This is usually not a problem, but care must be taken to ensure that unwanted microorganisms do not become a predominant species in the fermenter or the packaged product. This can be achieved by arming industrial yeasts with mechanisms that allow them to fight and contain the growth of other microorganisms.

Some feral yeast strains secrete toxins known as zymocins, which kill non-resistant yeasts; in fact contamination with zymocin-producing wild yeasts has been identified as one of the causes of sluggish and stuck fermentations (van Vuuren and Wingfield 1986). Five types of killer toxins have been identified in *S. cerevisiae*: K_1, K_2, K_3, K_{28} and K_{3GR1}, with K_3 and K_{3GR1} probably being variants of K_2. Production and

resistance to killer toxins are conferred by a cytosolic double-stranded RNA fragment; thus, introduction of this RNA into an industrial yeast will confer resistance to zymocin produced by contaminating yeasts and also allows the host cell to fight off non-resistant contaminants (Hammond and Eckersley 1984; Sasaki et al. 1984; Boone et al. 1990). However, even when different killer factors are combined in one yeast strain, there is only limited protection. Application of zymocins produced by non-*Saccharomyces* yeast species, such as *Pichia* and *Hanseniaspora*, will perhaps be more effective and is now being given attention.

The antibacterial capacity of yeasts expressing bacteriocin-encoding genes, such as the *pedA* pediocin gene of *Pediococcus acidilactici* and the *lcaB* leucocin gene of *Leuconostoc carnosum*, has been investigated (Schoeman et al. 1999; du Toit and Pretorius 2000). In laboratory tests, transformants carrying these genes inhibited the growth of sensitive bacterial strains, indicating that it is possible to create bacteriocidal yeasts. Also under exploration is the expression of hydrolytic enzymes that disrupt the microbial cell wall. Lysozyme, for example, is officially approved as an additive to control malolactic fermentation and to enhance wine stability. Lysozyme is an *N*-acetylhexosaminidase capable of lysing the β-1,4 glucosidic linkages of the peptidoglycan layer in some Gram-positive bacteria. The lysozyme-encoding gene from chicken egg white was successfully expressed in *S. cerevisiae*, but the activity was hampered by hyperglycosylation, and the protective effect of this recombinant strain in industrial fermentations has yet to be demonstrated (Nakamura et al. 1993).

Environmental protection is an important issue associated with the development and use of GM zymocidal and bactericidal yeasts. Most industrial fermentations do not guarantee containment of the yeasts used and the liberation of GM yeasts with an obvious selective advantage may be risky, even if their chance of survival and associated disturbance of other ecosystems is very small. Further research is needed to assess and limit these risks before these yeasts can be used in commercial applications (Sect. 13.3.2.2).

13.2.5 Engineering Improved Stress Tolerance in Industrial Yeasts

Industrial yeasts encounter numerous stresses, ranging from mechanical shear stress during handling to complex biochemical stresses during fermentation. Yeasts unable to cope with such stresses exhibit reduced fermentation performance and compromise product quality (Attfield 1997; Bisson 1999; Ivorra et al. 1999; Bauer and Pretorius 2000; Verstrepen et al. 2004a). Attempts have therefore been made to improve stress tolerance in these organisms.

A high level of tolerance to freeze-thawing in yeasts is desirable for the increasingly popular use of frozen dough for bread production; reduced yeast vitality after freezing and thawing makes it necessary to use a larger amount of yeast (Park et al. 1997; Teunissen et al. 2002). UV mutants of an industrial baker's yeast with increased tolerance to this stress have been described, but the mutations that are responsible for the favourable phenotype are not known. In another approach, the yeast aquaporin genes *AQY1* and *AQY2*, that encode proteins involved in transport of water and solutes across cellular membranes, were overexpressed in an industrial yeast strain. The strain showed a remarkable increased resistance to freezing and

thawing, presumably because aquaporins allowed water efflux out of the cells during freezing of the extracellular matrix, thereby preventing cell damage by intracellular ice formation (Tanghe et al. 2002).

Tolerance to other stressors, such as ethanol, is more complex. Attempts have been made to increase the resilience of yeasts by overexpressing genes known to be associated with stress tolerance, but these experiments have met with varying, usually limited, levels of success (Chen and Piper 1995; Kajiwara et al. 2000). This is probably due to the enormous complexity of stress tolerance. For example, it is has been estimated that more than 250 genes are involved in ethanol tolerance (Boulton et al. 1996), and manipulation of one or just a few of these genes may not be sufficient to cause a significant improvement. One area that might be explored is simultaneous overproduction of multiple factors thought to be involved in stress protection, such as trehalose (Majara et al. 1996; Wiemken 1990; Boulton 2000) and some of the heat shock proteins (Sanchez et al. 1992; Varela et al. 1995; Piper et al. 1997). Another approach might be manipulation of the expression of *PMA1* and *PMA2*, which encode cellular ATPases and are known to be important in maintaining a proper intracellular pH and membrane potential under stressful conditions, although again the impacts of the products of these genes appear to be different for different stresses (Monteiro et al. 1994; Attfield 1997; Fernandes et al. 1998). Sterol biosynthesis is also a possible target because sterols are known to be important to maintain membrane integrity during stress (Swan and Watson 1998; Beney and Gervais 2001). But perhaps the most promising strategy is to manipulate genes involved in the various regulatory cascades that control stress resistance, e.g. the Ras/cyclic AMP (cAMP)/protein kinase A (PKA) pathway, the fermentable growth medium (FGM) induced pathway and the target of rapamycin (TOR) pathway. This strategy enables alterations in the expression levels of a large group of genes, and mutations in the Ras/cAMP/PKA pathway have already been shown to increase the general stress resistance (Van Dijck et al. 2000; Teunissen et al. 2002; Versele et al. 2004; Verstrepen et al. 2004a). A recent genome-wide monitoring of the transcriptome during wine fermentation also showed that the TOR pathway plays an important role in the regulation of gene expression during fermentation (Rossignol et al. 2003). Further tests are needed to evaluate whether or not this strategy allows the creation of stress-tolerant industrial strains without affecting the important industrial properties of the respective yeast.

Because of the complexity of stress tolerance, the large number of genes and metabolic processes that are likely to be involved, and our poor understanding of what is required, perhaps approaches other than those targeting small numbers of genes or specific pathways might be more successful for raising stress-tolerant mutants. Adaptive evolution, driven by natural/artificial selection, can lead to the generation of yeasts that are more resilient than the parent from which they were derived (Stephanopoulos 1994; Ferea et al. 1999; Lassner and McElroy 2002; Petri and Schmidt-Dannert 2004; Cakar et al. 2005; Giudici et al. 2005). The approach used involves exposing a parental strain of yeast to ongoing selection in the form of stresses the yeast will encounter during fermentation. After many (typically hundreds of) generations, mutants are raised with increased stress tolerance. The beauty of this method is that no prior knowledge of the genetics or molecular biology of

the system is required, and the strains generated are non-GM and therefore no special approval is needed for their use in food and beverage production. However, stress-tolerant strains generated in this way in the laboratory have not yet been applied in a production-scale industrial setting.

13.2.6 Genetically Engineering Yeasts to Impart Improved Sensory Qualities on Fermentation Products

Yeasts play a significant role in determining the sensory properties and wholesomeness of fermentation products (Swiegers and Pretorius 2005). This section gives an overview of some of the ways *S. cerevisiae* has been genetically engineered to improve these aspects of its performance.

13.2.6.1 Reducing Diacetyl Levels in Alcoholic Beverages

During primary fermentation, yeast cells produce a vast array of secondary metabolites, and despite their low concentrations, some of these contribute enormously to aromas and flavours in fermented beverages. One of the most important flavour-active metabolites is diacetyl, a by-product of yeast valine synthesis. While moderate concentrations of diacetyl impart the typical buttery character of some white wines and Scotch ale beer, diacetyl is unwanted in most other beverages. In fact the prime reason for beer lagering (a 7–20-day maturing period after the primary fermentation) is to reduce diacetyl levels to a concentration below the flavour threshold of about 0.15 mg L^{-1}. During lagering, yeast cells take up the diacetyl produced during primary fermentation and convert it into acetoin and 2,3-pentanediol (Bamforth and Kanauchi 2004).

A variety of strategies have been employed to minimise diacetyl levels in beer, one of which is the introduction of a heterologous gene encoding α-acetolactate decarboxylase (ALDC). This enzyme catalyses the conversion of the diacetyl precursor, acetolactate, to acetoin, which has a far higher organoleptic threshold value than diacetyl (Meilgaard 1975a). ALDC is found in several bacteria, including *Lactococcus lactis* and *Acetobacter* species, which are currently used for food production (Goelling and Stahl 1988). The ALDC gene derived from *Acetobacter aceti* ssp. *xylinum* has been cloned behind the yeast constitutive *PGK1* promoter and introduced into the *S. cerevisiae* genome. The resultant recombinant strain has been used in pilot-scale brewing trials and consistently produced beers of high quality that were comparable to beers produced by the wild-type parent except for drastically reduced diacetyl levels (Yamano et al. 1994a, b, c, 1995). Similarly, the *Enterobacter aerogenes* ALDC, cloned behind the *S. cerevisiae ADH1* promoter and introduced in brewer's yeast, resulted in significantly reduced levels of diacetyl in small-scale fermentation trials (Sone et al. 1988; Fujii et al. 1990).

Another strategy to reduce diacetyl levels is to lower the activity of the *ILV2*-encoded α-acetolactate synthase, which catalyses the synthesis of α-acetolactate. This has been accomplished by screening yeast populations for resistance to the herbicide sulfometuron methyl (SMM), which inhibits the action of α-acetolactate synthase (Casey et al. 1988). Mutants with SMM resistance produce less α-acetolactate and

therefore less diacetyl. However, the reduced activity of this gene product, which is crucial for valine biosynthesis, may affect overall yeast performance. Perhaps the most elegant way to reduce diacetyl formation is to increase flux through the valine biosynthesis pathway. In this way, accumulation of intermediary products such as α-acetolactate is avoided, while valine biosynthesis is not compromised. A high flux was accomplished by overexpressing the *ILV3* and *ILV5* genes and this led to a big reduction in diacetyl production, especially when only *ILV5* was overexpressed (Goossens et al. 1987; Villa et al. 1995).

13.2.6.2 Engineering Yeasts for Improved Flavour Profiles of Alcoholic Beverages

Like diacetyl, volatile esters are only trace compounds in fermented beverages, but they are extremely important for the flavour profile of these drinks (Drawert and Tressl 1972; Engan 1972, 1974; Meilgaard 1975a, b, 1991, Suomalainen 1981; Nykänen and Suomalainen 1983; Nykänen 1986; Kruger 1998a, b; Debourg 2000; Lambrechts and Pretorius 2000; Cristiani and Monnet 2001; Pisarnitskii 2001). The most important flavour-active esters in beer are the three acetate esters: ethyl acetate (solvent-like aroma), isoamyl acetate (fruity, banana aroma) and phenyl ethyl acetate (flowery, roses, honey aroma); and the medium-chain fatty acid ethyl esters ethyl caproate and ethyl caprylate (sour apple). Since most esters are present in concentrations around their flavour threshold values, minor changes in concentration may have dramatic effects on beer flavour (Hammond 1993). This can be problematic in high-gravity brewing because the use of high specific gravity worts results in a severe overproduction of acetate esters. The concentration of acetate esters after dilution of beers produced through ultra-high-gravity fermentations (20°P) can be up to 75% higher than in beers produced with standard 12°P wort (Anderson and Kirsop 1974; Hammond 1993; Meilgaard 2001). In contrast to this, fermentations performed in tall, cylindroconical 'Apollo' fermenters result in decreased formation of esters, so that the beers produced lack desirable fruity tones (Meilgaard 2001; Verstrepen et al. 2003a). Other novel fermentation systems, such as continuous-flow fermenters or fermenters with immobilised yeast also give rise to unbalanced ester profiles (Moonjai et al. 2002; Verstrepen et al. 2003a; Shen et al. 2004).

Much research has focused on the enzymes responsible for the formation of volatile esters. So far, two related alcohol acetyltransferases have been described: Atf1 and Atf2 (Fujii et al. 1996b; Nagasawa et al. 1998). Whereas the physiological role of these ester synthases remains unknown, it is generally believed that volatile esters may be side products of other, physiologically relevant reactions, such as certain steps in the cellular lipid metabolism (Verstrepen et al. 2003e).

The deletion and overexpression of the alcohol acetyltransferase genes (*ATF1* and *ATF2*) have been reported for both brewing and wine strains (Fujii et al. 1994, 1996a, b). From this work it was found that deletion of *ATF1* reduces isoamyl acetate production by 80% and ethyl acetate production by 30%. *ATF2* deletion has similar but smaller effects on ester production (Nagasawa et al. 1998; Verstrepen et al. 2003d). Accordingly, overexpression of these genes in sake yeast led to a tenfold increase in ethyl acetate production and a 30-fold increase in isoamyl acetate

formation. Similarly, it was shown that overexpression of *ATF1* in a commercial wine yeast resulted in wine and distillates with substantially greater ester concentrations (Lilly et al. 2000). In similar experiments on beer fermentations, *ATF1* and *ATF2* were overexpressed in a commercial brewer's strain (Verstrepen et al. 2003c, d). The pilot-scale beers produced with an *ATF1*-overexpressing strain contained 5 times more acetate esters than the beers produced with the wild strain. Overexpression of *ATF2* led to smaller increases in isoamyl acetate formation and no significant change in ethyl acetate levels (Verstrepen et al. 2003c). More detailed analysis using gas chromatography coupled with mass spectrometry revealed that *ATF1* and *ATF2* are capable of esterifying a broad range of different alcohol substrates (Verstrepen et al. 2003d). While most of the esters produced have not been studied intensively, it is possible that these lesser known esters also contribute to the aroma of various fermented beverages.

The work just described indicates that it is possible to use genetic modification to create new yeast strains with desirable characteristics for ester production. In addition, the highly elevated ester levels obtained using these strains clearly indicate that ester synthesis during brewery fermentations is not strictly limited by substrate availability. Indeed, it can be concluded that it is not the substrate concentration, but rather the expression level of the *ATF* genes that is one of the main controlling factors affecting ester synthesis during wort fermentations, as first suggested by Yoshioka and Hashimoto (1981, 1984) and later elaborated by Malcorps et al. (1991).

Genetic engineering can also be used to generate yeast strains with enhanced capacity to liberate volatile flavour compounds from non-volatile precursor molecules that are present in wort or must. A classic example of such precursors is the monoterpenyl glucosides, which release terpenols (e.g. geraniol and nerol) when the glucose residue is cleaved off by glucosidases. However, the glucosidases present in yeasts and grapes are repressed by glucose and are unstable at the pH of wort and must. Expression of other, more active and/or more stable glucosidases, such as the recently described β-glucosidase from *Aspergillus oryzae* (Riou et al. 1998) or glucosidases found in *Candida*, *Hanseniaspora* and *Pichia* species, may increase the varietal aroma (Canal-Llauberes 1993). Another possibility is the use of GM yeasts with alcohol oxidase activity, which can convert fusel alcohols into aldehydes with specific aroma properties (Vanderhaegen et al. 2003).

Another potential application of genetic engineering to construct wine yeasts with increased capacity to release flavour-active molecules from grape juice comes from research showing that carbon–sulfur lyase enzymes release the volatile thiol 4-mercapto-4-methylpentan-2-one (4MMP) from its non-volatile precursors (Tominaga et al. 1995). In subsequent work using deletion strains of *S. cerevisiae* with carbon–sulfur lyase genes knocked out, four genes encoding enzymes capable of releasing 4-MMP in wine fermentations were identified (Howell et al. 2005). In related work it was also shown that Atf1p releases another flavour-active volatile thiol, 3-mercaptohexan-1-ol, from its precursor (Swiegers et al. 2005). With this knowledge it should now be possible to construct yeasts that express carbon–sulfur lyase genes and *ATF1* in a highly regulated manner, thus controlling the amount of flavour-active volatile thiols present in wine. Such yeasts would be of enormous interest to winemakers, brewers and whiskey producers.

13.2.7 Engineering Yeasts with Altered Levels of Ethanol and Glycerol Production

Glycerol, an abundant by-product of fermentations performed by *S. cerevisiae*, is implicated in the mouthfeel, viscosity, perceived sweetness and 'roundness' of wine and beer (Klopper et al. 1986; Scanes et al. 1998). Several attempts have therefore been made to increase glycerol production. Another reason for re-routing glycolysis to glycerol is the expected accompanying decrease in alcohol production, which would be beneficial in the production of low-alcohol beers and wines. Such an approach would be an alternative to current methods of alcohol reduction, which include dialysis, dilution and evaporation, all of which are detrimental to sensory quality. Conversely, decreasing glycerol formation and increasing ethanol production may be useful for producing high-alcohol beverages, such as whiskey, brandy and gin, as well as for the production of bio-ethanol. However, since glycerol serves as an osmoregulator and also has a role in maintaining redox balance (Hohmann 1997; Taherzadeh et al. 2002; White et al. 2003), tampering with glycerol production will probably have its limitations.

Glycerol is an important by-product of glycolysis. About 4–10% of all fermented carbon sources are converted into glycerol, resulting in levels that are about 7–10% those of ethanol (Scanes et al. 1998). Glycerol is produced by the conversion of the glycolytic intermediate dihydroxyacetone phosphate into glycerol 3-phosphate by glycerol 3-phosphate dehydrogenase, of which there are two isozymes, *Gpd1* and *Gpd2*. This step is followed by glycerol 3-phosphatase-driven dephosphorylation. Once produced, glycerol can exit the cell by passive diffusion or facilitated transport via Fps1 protein channels. The rate-limiting steps in glycerol production are the conversion of dihydroxyacetone phosphate into glycerol 3-phosphate and the export of glycerol into the medium (Remize et al. 2001).

Overexpression of *GPD1*, *GPD2* or *FPS1* (Fig. 13.9) can result in as much as a 2% (v/v) reduction in ethanol and a 1.5–4-fold increase in glycerol production, depending on the genetic background of the yeast strains used (Michnick et al. 1997; Remize et al. 2001; de Barros Lopes et al. 2000; Eglinton et al. 2002). However, as a consequence of glycerol overproduction and the resultant changes in redox balance, the formation of other secondary metabolites changed significantly, with increased formation of pyruvate, acetate, butanediol and succinate (Remize et al. 1999; de Barros Lopes et al. 2000; Eglinton et al. 2002). The most negative of these consequences, the increased formation of acetate, can be circumvented, however, by deletion of *ALD6*, which encodes cytosolic $NADP^+$-dependent acetaldehyde dehydrogenase (Eglinton et al. 2002) (Fig. 13.9).

These approaches for diverting carbons away from ethanol production are self-cloning strategies (as long as construction of the *ALD6* deletion does not leave any foreign DNA behind), and this will probably be advantageous for consumer acceptance. However, there is a limit to how much carbon can be diverted to glycerol production without having detrimental effects on cell physiology, which would inevitably lead to problems in industrial applications. An additional strategy which holds great promise for reducing ethanol levels in fermentation products involves constructing transgenic yeasts that carry the glucose oxidase gene, *GOX1*, from

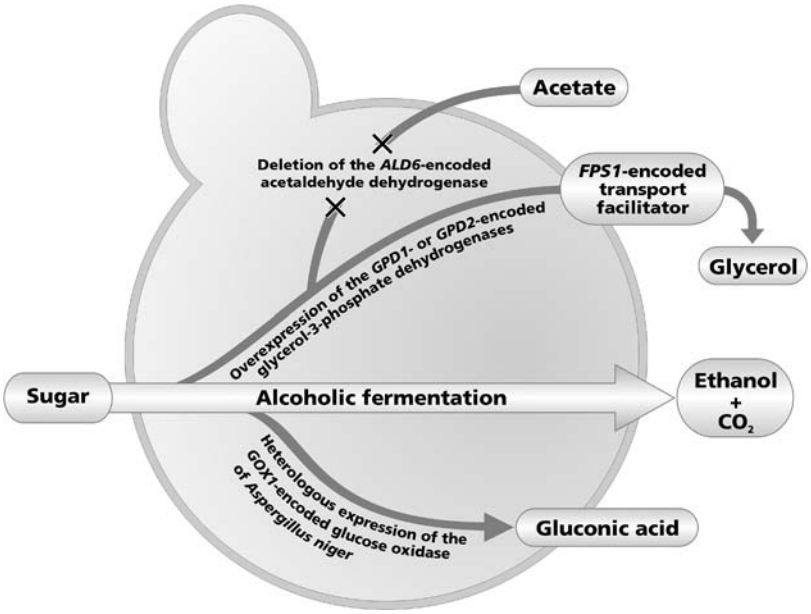

Fig. 13.9. Diverting metabolic processes in yeasts can be used to change the chemical composition of alcoholic beverages. Illustration of how carbons can be diverted from ethanol production to the synthesis of glycerol or gluconic acid. This is achieved by overexpressing native yeast glycerol synthesis genes, *GPD1* or *GPD2*, or introducing a heterologous glucose oxidase (*GOX1*) gene. Overexpressing *FPS1*, the yeast gene for transport of glycerol across the plasma membrane, also helps divert carbons away from ethanol production by facilitating removal of glycerol from the cell. One of the problems of increasing glycerol synthesis is that acetate levels are also increased. This can be ameliorated, however, by deleting the *S. cerevisiae* aldehyde dehydrogenase gene *ALD6*

A. niger. This enzyme converts glucose to gluconic acid, thus diverting carbons away from ethanol production, and it has been demonstrated to reduce ethanol levels by up to 2% in a microvinification trial using a *GOX1*-transformed *S. cerevisiae* strain (Malherbe et al. 2003). While this strain was transgenic, the *GOX1* gene has GRAS status and should therefore not be of great concern to the general community once there is greater acceptance of GMOs in foods and beverages.

13.2.8 Engineering Yeasts to Control Acid Levels in Wine

Acid levels are sometimes too high or too low in wines immediately after fermentation of grape juice. Deacidification processes, performed largely by lactic acid bacteria, are therefore necessary to make some wines more palatable, whereas in others, acids have to be added. In the following sections we will consider how yeasts might be engineered to deal with these problems.

13.2.8.1 Constructing Yeasts that Perform Malolactic Fermentation

Wine producers rely on a secondary fermentation by lactic acid bacteria such as *Lactobacillus* spp., *Pediococcus* spp., and, most importantly, *Oenococcus oeni* (formerly *Leuconostoc*) to convert the very sour and tart malate, found in wine at the end of the primary fermentation, into lactate. This malolactic fermentation is needed for the deacidification and stabilisation of many wines. However, it is difficult to control and delayed or stuck fermentations lead to economic losses and logistic problems. To circumvent the need for secondary malolactic fermentation, *S. cerevisiae* strains that convert malate into lactic acid and carbon dioxide have been constructed (Fig. 13.10). This was achieved by expressing the malolactic enzymes of *Lactococcus lactis* (Ansanay et al. 1993, 1996; Denayrolles et al. 1994, 1995), *Lactobacillus delbrueckii* (Williams et al. 1984) and *Oenococcus oeni* (Labarre et al. 1996) in yeasts. Since *S. cerevisiae* does not have an efficient system for malate uptake, the malate permease of *Schizosaccharomyces pombe* has also been coexpressed with the bacterial malolactic enzyme (Bony et al. 1997a; Volschenk et al. 1997) (Fig. 13.10). This led to the conversion of up to 7 g of malate per litre in 4 days, without significantly affecting the ongoing primary fermentation (Bony et al. 1997a). However, a further

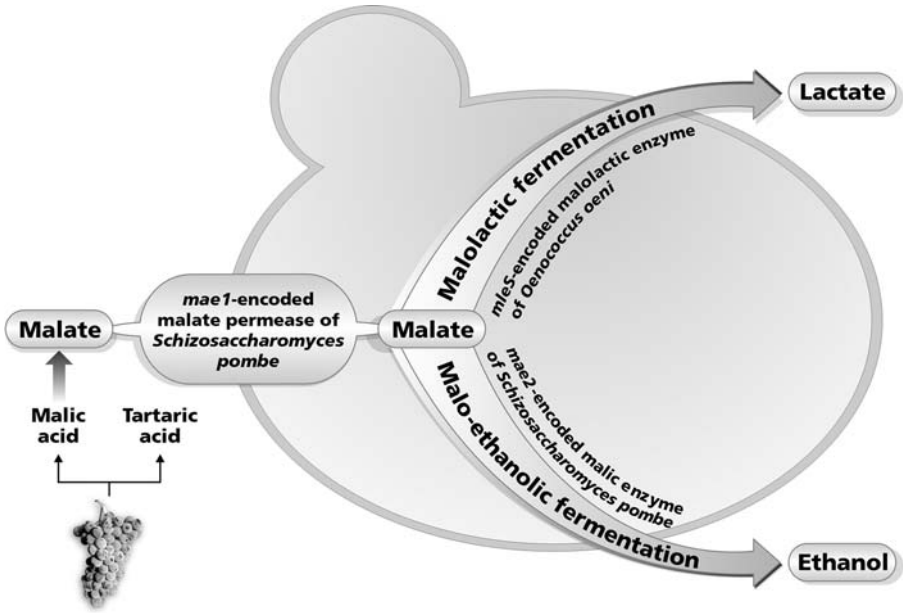

Fig. 13.10. Grape juice often has high levels of malate and this makes wines very acidic and sour to taste. Wine yeasts are unable to ferment this organic acid and winemakers therefore have to rely on bacteria that can convert malate to the less acidic lactate. A strain of *S. cerevisiae* has been constructed that carries a gene for a malate transport protein and genes for the enzymes necessary for malolactate fermentation, thus negating the need for bacterial inputs. The genes that drive this process in the genetically modified *S. cerevisiae* are from a very distant relative of wine yeasts, namely *Schizosaccharomyces pombe* and from the lactic acid bactarium *Oenococcus oeni*

large-scale evaluation is needed to determine the effectiveness of this yeast under industrial conditions and to test the sensory properties of the resultant wine.

13.2.8.2 Constructing Yeasts that Produce Increased Levels of Lactic Acid

A good balance of sweetness and acidity is crucial for wine. In hot climates, grape must often contains low levels of acids and, to correct this, other acids (e.g. tartaric acid) can be added. However, artificial acidification is an added cost to the winemaker and is not always allowed. Attempts have therefore been made to create yeast strains that produce increased levels of lactate from sugar metabolism. This has been achieved by expression of the *Lactobacillus casei* lactate dehydrogenase encoding gene *LDH* (Dequin and Barre 1994; Dequin et al. 1999). The transformants performed a dual fermentation: the normal ethanol-producing, as well as lactic acid-producing fermentations. The strains produced around 5 g L^{-1} lactate, which corresponds to a decrease in pH of around 0.25 units (Dequin et al. 1999). Since part of the alcoholic fermentation is diverted to lactate fermentation, alcohol levels in the wines are about 0.25% (v/v) lower. This is generally not a problem however, because many of the wines produced in hot climates contain high levels of ethanol. Technically, it should be possible to develop a set of yeast strains that produce different amounts of lactic acid, depending on the acid level of the must. In addition, these acid-producing yeasts may be useful in the production of sourdough bread (Dequin 2001).

13.2.8.3 Constructing Yeasts that Produce Low Levels of Volatile Acidity

Acetic acid contributes to volatile acidity in wines, leading to harsh off-flavours and should be kept to below 0.8 g L^{-1}. *S. cerevisiae* forms acetic acid during alcoholic fermentation. While the mechanisms involved in regulating its production remain unknown, it has been shown that the levels of this acid can be controlled by changing the activity of the acetaldehyde dehydrogenase gene *ALD6* (Remize et al. 2000) (Fig. 13.9). Inactivation of both copies of this gene in a diploid wine yeast led to a significant decrease in acetate production during wine fermentation.

In work on sake yeast, a spontaneous mutant with low NADH dehydrogenase activity was selected (Kurita et al. 2003). This mutant showed a 15-fold increase in acetate formation, resulting in a more favourable balance between acetate and lactic acid in the sake wines produced. However, the mutant strains produced altered levels of other secondary metabolites, such as glycerol, which was not desirable. An approach based on the genetic engineering of a target solely involved in acetate formation may help to avoid these undesirable effects.

13.2.9 Engineering Yeasts to Produce Decreased Levels of Hydrogen Sulfide

Hydrogen sulfide is a by-product of sulfur metabolism in yeasts, and with its characteristic ‘rotten eggs’ aroma, it is highly undesirable in fermented beverages. It is formed by the reduction of sulfate during methionine synthesis. Most of the hydrogen sulfide

secreted during primary fermentation is removed from wine by yeast cells during a maturation phase. However, the need for this time-consuming process could be eliminated or the time required greatly reduced by using yeast strains that have been genetically altered to release less of this compound. This can be achieved by increasing the rate of reactions that consume hydrogen sulfide, including the final steps in methionine, cysteine and homocysteine production. Increased expression of the cystathione β-synthase-encoding gene, *NHS5*, in brewer's yeast has been shown to decrease hydrogen sulfide accumulation without affecting other fermentation parameters (Tezuka et al. 1992). Similarly, overexpression of *MET25*, which encodes *O*-acetyl homoserine sulfhydrolase/*O*-acetyl serine sulfhydrolase, resulted in a tenfold decrease in hydrogen sulfide formation in pilot-scale beer fermentation (Omura et al. 1995).

Another method to decrease hydrogen sulfide production is to reduce activity of the methionine, cysteine and homocysteine synthesis pathway, thus reducing the formation of hydrogen sulfide. Elimination of *MET10*, for example, leads to a significant reduction in hydrogen sulfide formation, and an increase in the formation of sulfite, a desirable antioxidant (Hansen and Kielland-Brandt 1996b). However, as the maximum concentration of sulfite in wine is legally limited, care needs to be taken not to exceed this level.

13.2.10 Engineering Yeasts to Make Alcoholic Beverages with Increased Storage (Antioxidative) Potential

As mentioned previously, sulfite is an important natural antioxidant in fermented beverages but it also acts as a flavour-stabiliser because it binds to flavour-active aldehydes, forming stable products with less intense flavours and higher flavour thresholds. Limited amounts of sulfite are sometimes added to white wines to stabilise and protect them from oxidation. Genetic modification can be used to produce yeasts with modified sulfite production, eliminating the need for addition of this preservative. Overexpression of the *MET3* and *MET14* genes, which are involved in the biosynthesis of sulfur-containing amino acids, leads to increased sulfite production in a brewer's yeast (Korch et al. 1991). Alternatively, reduction of the copy number of *MET2*, encoding serine acetyltransferase, also increases sulfite production (Hansen and Kielland-Brandt 1996a). Another strategy that has been tested is the inactivation of *MET10*, which not only led to increased sulfite levels, but also reduced the production of hydrogen sulfite (Hansen and Kielland-Brandt 1996b).

In contrast, some industrial yeast strains produce concentrations of sulfite that are too high, leading to off-flavours and posing possible health concerns for consumers (Rauhut 1993). Presumably decreased sulfite levels could be achieved by using the inverse of the strategies just described.

13.2.11 Engineering Yeasts for Decreased Formation of Ethyl Carbamate

Ethyl carbamate is a suspected carcinogen found in most fermented beverages (Ough 1976) and is thought to be produced from urea and ethanol during storage. Levels of ethyl carbamate increase with elevated storage temperatures and with higher ethanol levels, and can therefore be particularly problematic in fortified wines

and distilled beverages. In *S. cerevisiae*, urea is formed by the *CAR1*-encoded arginase, which is responsible for the conversion of L-arginine into L-ornithine, ammonia and carbon dioxide. Urea is an intermediate in this conversion, and is partially secreted into the medium. Commercial enzyme preparations of acidic urease can be used to hydrolyse this (Ough and Trioli 1988), but having access to yeast strains that produce less urea or strains that produce urease would be highly desirable.

Disruption of the *CAR1* gene in a sake yeast proved to be successful in reducing urea and ethyl carbamate production (Kitamoto et al. 1991), but this strain could not metabolise arginine, resulting in a growth defect that would limit its potential for industrial applications. Expression of the *Lactobacillus fermentum* urease operon was trialled in yeast and, while expression levels of the urease subunits was sufficient it was not able to break down urease, presumably because essential auxiliary proteins were missing (Visser et al. 1997). It therefore seems accessory genes of *Lactobacillus fermentum* would have to be co-expressed with the urease operon in order to create a functional, secreted urease complex.

13.3 GM Industrial Yeasts of the Future

From Sect. 13.2 it is clear that there is enormous potential for the application of genetic engineering to construct yeasts with improved performance, and with the capacity to deliver fermentation products that have improved qualities. The following section looks at what we are moving into with respect to the application of DNA technologies for improving industrial yeasts, and challenges that lie ahead with respect to legislation and public perception of GMOs in food and beverage production.

13.3.1 The Latest Generation of GM Yeasts

The development of several dominant selectable markers (Goldstein and McCusker 1999), as well as gene knockout cassettes that can be removed following gene disruption (Güldener et al. 1996), has enabled researchers to sequentially introduce multiple genetic alterations into a single yeast genome. Perhaps the best example of this has been the development of yeast strains capable of efficient starch and cellulose fermentation, which requires the simultaneous expression of several heterologous genes (for reviews, see Moraes et al. 1995; Petersen et al. 1998; van Rensburg et al. 1998; Zaldivar et al. 2001; Altinas et al. 2002; Lynd et al. 2002; Eksteen et al. 2003). Another remarkable example is the development of a yeast strain capable of producing the steroid hydrocortisone from a simple carbon source (Szczebara et al. 2003). An artificial and fully self-sufficient biosynthetic pathway involving as many as 13 engineered genes was assembled and expressed in a single yeast strain, and endogenous sterol biosynthesis was re-routed to produce compatible sterols to serve as substrates for the heterologous pathway. Biosynthesis involved the expression of eight mammalian genes and the disruption of unwanted side reactions associated with the *ATF2*, *GCY1*, and *YPR1* gene products.

The trend to target complex phenotypes regulated by several genes does not always require genetic alterations in multiple loci. Instead of engineering a multitude of individual structural genes, researchers can instead manipulate genes encoding

proteins involved in cell signalling pathways or regulation of transcription. Indeed, the improved knowledge of biological pathways and systems as a whole (systems biology) makes it possible to target upstream regulators instead of the multitude of downstream effectors. The advantage of such approaches is that multiple targets involved in common processes can be affected by targeting one or a few genes involved in regulatory networks. This technique also has the potential to lead to more balanced changes in a network of protein activities involved in shaping the same aspect of a phenotype, even if all details of this network are not yet known. An example of this approach is found in the work of Roca et al. (2004). In an already GM, xylose-fermenting *S. cerevisiae* strain, these scientists deleted the *MIG1* and *MIG2* genes, which encode repressors of genes involved in the metabolism of carbon sources such as galactose and maltose. Wild-type *S. cerevisiae* is unable to utilise xylose, but this strain had been engineered to carry and express *Pichia stipitis* XYL1 and XYL2 genes, which encode enzymes for xylose catabolism. The original XYL1/XYL2 strain showed unsatisfactory fermentation of xylose but, after deletion of MIG1 and MIG2, and thereby removal of repression of the metabolism of many sugars, the fermentation rate was increased by 25%.

Modification of cell signalling pathways or transcription regulation will, however, only work for pathways or transcription factors that regulate single or a limited number of cell processes. Downstream branches of regulatory pathways often target several physiological processes, and modification of such a pathway would have pleiotropic, probably undesirable, effects. A good example can be seen in the metabolic engineering of the GAL regulatory pathway (a downstream branch of the main glucose repression pathway). Østergaard et al (2000) eliminated three known negative regulators of this pathway (Gal6, Gal80 and Mig1), and a 41% increase in galactose consumption was obtained. However this increased galactose flux did not lead to the predicted increase in biomass formation, but instead caused excessive fermentative metabolism and increased ethanol production (Østergaard et al. 2000).

A third approach by which complex phenotypes can be improved combines old-fashioned breeding and selection with modern techniques such as genome shuffling, stimulation of mutation and/or random insertion or deletion of genetic elements, e.g. the random insertion of strong, constitutive promoter elements into the yeast genome, which can be accomplished using the Ty yeast transposons (Lassner and McElroy 2002; Patnaik et al. 2002; Petri and Schmidt-Dannert 2004; Giudici et al. 2005). These methods have the advantage that no knowledge about underlying cellular biology is needed to improve traits. A large pool of yeast cells is transformed, and the few transformants that show beneficial alterations are selected from the pool. The desired phenotypic trait can be enhanced by repeated rounds of mutation and selection. A great disadvantage of these 'random' methods, however, is the need for an efficient selection method, which may not be trivial. A second drawback is the 'black-box' character of the method; indeed, nothing is known about the alterations that make the mutants better than their progenitors.

'Functional foods', i.e. foods offering a potential health benefit, is an area of increasing interest in the food and beverage industries, and industrial yeast-driven processes are potential sources of several such products, including resveratrol. Resveratrol is a stilbene produced in grapes during infection, radiation or wounding.

This compound is mainly formed in the grape skins, and acts as a protectant. It attracted scientific attention when it appeared to provide an explanation for the 'French Paradox', i.e. the low incidence of heart disease among French people who combine a relatively high-fat diet with the regular consumption of wine; it is thought to have cardioprotectant and anticancer properties (Gao et al. 2002). In order to create a yeast capable of resveratrol synthesis, the co-enzyme A (CoA) ligase encoding gene (*4CL216*) from a hybrid poplar and the grapevine resveratrol synthase gene (*vst1*) were co-expressed in a laboratory yeast (Becker et al. 2003). Expression of *4CL216* enabled the yeast cells to synthesise *p*-coumaroyl-CoA. This compound and 3-malonyl-CoA produced by the yeast were then able to be used by resveratrol synthase to produce resveratrol. While the concentration of resveratrol produced by this recombinant yeast was low compared with levels found in standard red and even white wines, the study proved it was possible to genetically engineer yeasts to produce this important health-promoting substance.

Another example of engineering yeasts to obtain a healthier product for consumers is the construction of a sucrose-fermenting baker's yeast that is incapable of fermenting fructooligosaccharides. Fructooligosaccharides are regarded as functional foods because they stimulate the growth of favourable intestinal bifidobacteria. This carbohydrate would therefore be a useful additive to foods such as bread, but such an approach would be pointless because it is hydrolysed by invertase, secreted by baker's yeast during leavening. However, yeast strains lacking the *SUC* genes, which encode invertase, and overexpressing *MAL* genes, which encode proteins involved in maltose utilisation, still ferment sucrose, but are unable to hydrolyse fructooligosaccharides, so this functional food could be added and retained in bread if recombinant yeast is used (Oda and Ouchi 1991).

13.3.2 Future Challenges: Legislation, Marketing and Consumer Perception

The first GM yeast strains approved for use in food production were a baker's yeast that constitutively expressed maltose permease and maltase genes, resulting in an increased leavening capacity, and a brewer's strain that expressed a glucoamylase gene allowing the partial fermentation of dextrins (Hammond 1995; Walker 1998). However, it is interesting to note that although GM yeasts have been used to create enzymes and additives used in food production, and that *S. cerevisiae* had been granted GRAS status, no GM yeast strain is currently used for food production. This can be attributed to two factors: the complex regulations for the use of GMOs in food production and, more importantly, today's negative consumer perception of genetic modification.

13.3.2.1 Legislation and Its Impacts on the Use of GM Yeasts

Before the early 1990s, there were virtually no regulations on the use of GMOs in food production. Since that time things have changed dramatically. Research scientists and biotechnology companies began to seriously explore the possibilities offered by the newly developed genetic tools and this quickly prompted countries to

develop new guidelines and laws regulating the use of GMOs in food production. But these laws vary greatly from country to country.

In the USA, the FDA issued its 'statement of policy regarding foods derived from novel plant varieties', which also comprises plants that have undergone genetic modification. This statement provides the basis for the FDA's assessment of all GMOs used in food production. The FDA's basic policy with respect to bioengineered foods is that they are not inherently different from other foods: each product is examined on a case-by-case basis for safety and quality (Foramanek 2001). Tests for conventional food and food produced with, or containing, GMOs are similar and include the investigation of the nutritional value and allergenic properties. Moreover, the FDA does not require producers to indicate if their products contain or have been produced using GMOs or GM products. However, in response to pressure from consumer organisations, the FDA has issued guidelines for voluntary labelling that indicates whether foods have or have not been developed using bioengineering. Apart from the FDA, which controls food safety, GMOs such as plants that are released into the environment also need to comply with standards set by the US Department of Agriculture (USDA) and the Environmental Protection Agency (EPA). This ensures the protection of the environment from GMOs that could potentially harm the ecosystem.

In Europe, things are more restrictive. As in the USA, all novel foods are subjected to stringent tests to give assurance of their safety. This 'Novel Food Regulation' or '258/97/EC' is comparable to the FDA's policy in the USA. It is important to note that these regulations apply to all novel foods, not only foods produced with, or containing, GMOs. In addition, the European Union has issued several guidelines, directives and regulations that specifically regulate the use of GMOs in food production. The directive 90/219/EC and its amendment, 98/81/EC, provide the basis for the contained use of GM microorganisms in food production, while 90/220/EC and 2001/18/EC regulate the deliberate release of GMOs into the environment. These regulations basically define precautions, procedures and tests required to permit the use of a GMO for industrial production. Regulation 1829/2003 also stipulates that all food containing, or derived from, GMOs should be tested in order to guarantee its safety for the consumer. Other directives ensure that the GMOs are not harmful for the ecosystem. Finally, 1139/98/EC, 49/2000/EC, 1829/2003/EC, 1830/2003/EC and 641/2004/EC oblige the producer to indicate on the label if a product contains or is derived from a GMO. This is a very important directive, as many companies are afraid that a GM-label may scare consumers, which partially explains why at present hardly any GM-derived food products can be found on the shelves of European supermarkets. Directive 1830/2003/EC also states that producers working with GMOs should assure the traceability of their products, meaning they should keep track of who buys and sells them.

In general, regulations in other parts of the world lie between those of the USA and the European Union. Canada's policies resemble those in the USA. Australia, New Zealand, Japan and China require labelling of GM foods, but the regulations on the use of GMOs are less restrictive than the European guidelines. However, laws concerning the use of GMOs are informed by new scientific findings, public opinion and lobby groups, and therefore are always open to review. It is difficult to predict how the GMO legislation will evolve.

Despite the differences in regulations between different countries there are general guidelines for approval of GMO-derived products. Approval usually requires a few obvious guarantees. For example, the inserted or altered DNA sequence should be fully defined, the GMO should not have any selective advantage that would favour its survival and/or proliferation in nature, and the GMO should not pose any risks to consumers or the environment. A common way for producers to satisfy these demands is to prove that the GMO and/or the derived product is 'substantially equivalent' to analogous conventional organisms or products (Cockburn 2002). When substantial equivalence is shown, few other tests are necessary to comply with safety regulations. If the GMO or the product is not substantially equivalent, further examination is required, including rigorous chemical analyses and animal tests. In the specific case of a yeast used in a contained industrial environment and modified to better suit the producer's and consumer's needs, the risks are very low. In many cases, obtaining a licence for these GM yeasts may be merely a matter of applying and providing the necessary data.

Special, more flexible regulations have been developed for GMOs that contain only 'self-DNA', i.e. DNA sequences that also occur in the respective wild strain. In Japan and Europe 'self-cloning' strains are exempt from the strict regulations governing the use of GMOs, including the important labelling requirement. Many of the possible applications of genetic engineering for the improvement of industrial yeasts are realisable without the introduction of heterologous DNA, especially when some of the recent technologies are used (Ishida-Fujii et al. 1998; Verstrepen et al. 2001b; Akada 2002; Aritomi et al. 2004).

13.3.2.2 Public Perception

Despite complex and strict legislation, the real limiting factor for the introduction of GMO-derived foods and beverages lies with the consumer. A significant proportion of today's consumers are extremely suspicious of the application of gene technology in food production. Many consumers believe GM-derived food may cause long-term health problems, such as cancer. From a scientific point of view, these reservations are not justified (Pretorius 2000; Miles and Frewer 2001; Finucane 2002). There is no reason to suspect that consumption of a GM-derived food could affect the consumer any differently from consumption of a conventional food. Other fears, such as the concern that GMOs could disturb the ecosystem or affect biodiversity, are more realistic (Miles and Frewer 2001; Freckleton et al. 2003). It is precisely for this reason that rigorous GMO legislation was set up. In many cases, there is no reason to expect a problem in this area because the GMOs and their products are subjected to elaborate, extensive testing. In the specific case of industrial yeasts, it has to be highlighted that most of these organisms have been selected for use in an artificial environment, often with copious amounts of nutrients and optimal growth temperatures. Many of these industrial strains therefore cannot compete with feral strains, limiting the threat they pose on the ecosystem. Moreover, most of the traits that are introduced to make these strains better performers for their industrial tasks do not offer any competitive advantage in the 'real world'. In addition, it should be highlighted that relying on traditional

approaches rather than utilising GM yeasts has its consequences; in many cases traditional methods, such as the use of additives and enzymes, can lead to increased costs, additional waste products and higher energy consumption during production process.

What factors need to be addressed so that GMOs can fully realise their great promise? It is clear consumers need to be better informed about the genetic techniques used to create GMOs and the scientific foundation on which gene technology relies. This would arm the general public against the often misleading, biased and in some cases blatantly incorrect information provided by some consumer organisations and pressure groups. GMO legislation should be based on rigorous scientific data and aim solely to protect consumers and the environment, not the interests of pressure groups. Producers, on the other hand, must not take risks, must comply with regulations and should always provide complete and honest information on their products. GM foods and beverages should be designed to deliver benefits for consumers and/or the environment by offering improved products for similar prices, by reducing the price of equivalent products, and/or introducing products with reduced negative impact on the environment. In summary, consumers need to be provided with good information, allowing them to assess correctly the risks and benefits of the use of GMOs (Pretorius and Bauer 2002; Rowe 2004), and GM products must deliver benefits compared with traditional foods and beverages.

13.4 General Conclusion

It is clear that the industrial application of GM yeasts has many potential benefits with exciting possibilities in the bio-ethanol and pharmaceutical industries, as well as providing some potentially huge leaps forward for the traditional food and beverage industries. Biotechnologists in these latter industries would do well to investigate the potential for modifications that can lead to health benefits while ensuring environmental risks are nullified, and without transgressing current laws and regulations.

Community concern over the use of GM products is apparently a great deal less intense for applications in the pharmaceutical and bio-ethanol industries than it is for food and beverage production; ironically we already use GM yeast to express pharmaceutical agents such as vaccines that are injected into us. Consumer information and education about the use of GM yeasts in pharmaceutical and bio-ethanol industries may therefore provide a stepping stone for the introduction of GM yeasts in the food and beverage worlds. The use of self-cloning yeasts may further reduce concerns of consumers, and it may be that such constructs will be the first to find their way into foods and beverage production.

In conclusion then, we can now access and modify the yeast genome in amazingly precise and innovative ways and we are able to tailor this microbe for an increasing array of applications, making it more and more indispensable to us. Thus, our good friend the yeast continues to offer new possibilities for us to increase its usefulness and consequently our dependence on it. Which brings us back to where we started this chapter: who, exactly, is moulding whom?

Acknowledgements

We thank Elizabeth Waters (The Australian Wine Research Institute) for advice on haze formation in alcoholic beverages, and Jeff Eglinton (AWRI) for advice on regulating ethanol and glycerol production. Yeast research at the AWRI is financially supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation, with matching funding from the Australian Government. K.J.V. is a post-doctoral fellow of the Fund for Scientific Research Flanders (FWO Vlaanderen) and a D. Collen Fellow of the Belgian American Educational Foundation (BAEF). Original research at the Centre for Malting and Brewing Science is supported by grants from the Fund for Scientific Research – Flanders (FWO project G.0082.03) and K.U. Leuven Research Fund (grant OT/03/40).

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