HANDBOOK OF FOOD SPOILAGE YEASTS

SECOND EDITION

TIBOR DEÁK

CRC Press
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Contents

List of Figures

List of Tables

Preface

Yeasts are undoubtedly the most important group of microorganisms exploited for commercial purposes. Yeasts that bring about the leavening of bread and the fermentation of wine and beer are essential parts of everyday life. In addition to playing a crucial role in the production of fermented foods and beverages, yeasts are also the source of a wide range of valuable products and useful ingredients made by the various branches of biotechnology. Their benefits to humans, however, are counterbalanced to some degree by the detrimental role they play in the spoilage of processed and stored foods.

Yeasts are capable of growing in a wide range of foods if environmental conditions are favorable. Under such conditions, the growth and metabolic activities of yeasts in foods may result in extensive economic losses to the food industry. To control the spoilage of foods by yeasts, the physicochemical and biological parameters associated with various foods should be adjusted to inactivate or inhibit the growth of yeasts. These parameters are collectively called ecological factors.

Microbial ecology has become a very inspiring concept in food microbiology. Though artificial and man-made, foods can be considered as ecosystems that provide specific niches and habitats for microorganisms whose activities are governed by intrinsic and extrinsic ecological factors. By understanding these factors and the implicit properties of food-borne microorganisms, both the beneficial and harmful activities of bacteria, molds, and yeasts can be controlled. Preservation of foods and beverages can be based on the same ecological principles that are used in the exploitation of yeasts, and govern, in general, the activity, growth, survival, and death of microorganisms. A more thorough understanding of these ecological factors is becoming increasingly important to avoid spoilage risks. In recent years, these risks have increased because of new product formulations, novel, non-thermal preservation methods, and milder processing and preservation introduced to meet consumer demands.

The bulk of this book focuses on the microbial ecology of yeasts. In characterizing food-borne yeasts, the effects of physical, chemical, and biological factors on the physiological properties of yeasts are summarized. The metabolic and enzymic activities of yeasts are directly responsible for unwanted changes in sensory properties and for loss of storage quality of foods. Strategies for food preservation are based on ecological principles, with goals of prohibiting growth or killing spoilage microorganisms. Some gross ecological parameters render certain foods particularly vulnerable to yeast spoilage. An overview of the yeasts present in major types of food and beverages is the second main feature of this book, which has been maintained and further elaborated in the second edition.

In the decade since the first edition, however, there has been a great increase in knowledge about yeasts, which has forced changes in many ways in this book. The biodiversity of yeasts has developed as a new disciplinee, resulting in the description of novel species in various habitats. New insights have been gained into the understanding of the physiological and genetic backgrounds of stress responses of yeasts to ecological factors applied in processing and preservation. The great progress in molecular biology has revolutionized the taxonomy and phylogeny of yeasts. Consequently, many changes in names have come about, confusing many uninitiated in the subject. Considerable progress has been made in the detection and identification of yeasts, resulting in more reliable recognition of spoilage agents. In the professional field, new strategies have emerged for the production of more natural, more convenient products. The industrial production of food and beverages has been

changed by the introduction of novel and improved processing, packaging, and storing technologies, mostly based on a combination of ecological factors. In an effort to follow all these developments, this book is more than an update of the first edition.

This second edition of the *Handbook of Food Spoilage Yeasts* barely contains an unchanged paragraph from the first edition. This is a new book rather than a revised and updated edition. The scope and content of the handbook have been extended and restructured. Chapter 1 gives a concise summary of the morphological and phenotypic characteristics of yeasts, illustrated with photographs. Chapter 2 on classification has been completely revised to follow the continuous development and changes in the taxonomy of yeasts. It is, nevertheless, a restrained treatment, focusing on groups of foodborne yeasts. "Ecology" (Chapter 3) is one of the main lines followed throughout the book; it outlines the most important ecological factors encountered in foods, supplemented by new sections on biofilms and interactions. Chapter 4 gives a brief but concise overview of the metabolic activities of yeasts, also pointing out the regulation of processes. Chapter 5 deals with the growth, life cycle, and death of yeast cells, also touching upon on kinetics and predictive modeling, in addition to stress responses. This edition includes a separate chapter (Chapter 6) on preservative treatments used to inhibit and inactivate yeasts, including both the traditional treatments (heating, freezing, drying, and chemical preservation), and the new and alternative methods such as irradiation, high pressure, pulsed electric fields and others, as well as novel combinations. Chapter 7, which makes up one-third of the total volume of the book, is a comprehensive coverage of the biodiversity and ecology of yeasts in various food types and commodities. The emphasis is on spoilage aspects; however, the beneficial role and application of yeasts are also explored. The thoroughly revised Chapter 8 on methods of enumeration and detection provides new insights into conventional methods and novel rapid and automated techniques. In line with the phylogenetic classification, Chapter 9 on identification focuses on the molecular techniques of identification, pointing also to recent and possible future developments of these methods. The traditional identification procedures based on phenotypic characters are also summarized, and the simplified method developed by the author is found in the Appendices. The number of species discussed has been increased by 20%, and an outline of the most important foodborne yeasts is also provided. Chapter 10 is a new addition, an outlook on the industrial application of yeasts, both on food fermentation and their exploitation in the broader field of biotechnology. The discussion of industrial strain developments inevitably draws on the field of genomics. In addition to the new and revised text, the second edition includes 30 new tables and 40 new figures. A total of more than 2000 references are cited. While most of the reports in the first edition are retained, more than half of the references are from recent literature of the past 10 years.

It is hoped that the book will serve as a practical guide to understanding the ecological factors governing the activities of yeasts in foods and beverages. Knowledge of the underlying ecological principles, the sources, and routes of spoilage can be recognized, quality assurance programs planned, and control measurements implemented. By the same token, food and beverage processing technologies can be improved, and both the preservation and fermentation of products can be executed in a more effective way. The text will be useful for advanced study, and the comprehensive repository of relevant literature included may provide helpful reference for research. Last but not least, this book can be used as a teaching aid in academia.

This book would not have been possible without the foundation of the first edition made jointly by the present author and Professor Larry R. Beuchat. Over the years, our collaboration has remained continuous, and Larry Beuchat's encouragement to compile this new edition is sincerely acknowledged. The author also wishes to express his thanks to Dr. Gábor Péter for critical appraisal of the manuscript and for providing the photographs in Chapter 1. The author is greatly indebted to those who granted permission to use or adapt illustrations from published works.

Tibor Deák

About the Author

Tibor Deák, Ph.D., D.Sc., is Professor Emeritus in the Department of Microbiology and Biotechnology, Faculty of Food Science, of the Corvinus University, Budapest, Hungary.

Dr. Deák received his B.Sc. degree in biology and chemistry from the University of Szeged in 1957, and his M.Sc. degree in microbiology from the Eötvös University of Budapest in 1963. He received his Ph.D. and D.Sc. degrees in biological sciences in 1970 and 1989, respectively, from the Biology Section of the Hungarian Academy of Sciences, Budapest. After gaining experience at the Budapest Canning Co. and the Research Institute for Canning Industry, he was appointed assistant professor in microbiology at the University of Horticulture and Food Science in 1967. He became associate professor in 1970 and full professor in 1980. He was the head of the department from 1970 to 1996, and served two terms as dean of the faculty of food science and technology (1986–1991) and one term as the rector of the university (1993–1996).

Dr. Deák has served as president of the Hungarian Scientific Society for Food Industry. He is a board member of numerous scientific societies including the International Committee for Food Microbiology and Hygiene, the International Committee for Yeast, the International Committee for Food Mycology, and the Hungarian Society of Microbiology, and member of the World Federation of Culture Collection, European Culture Collection Organization, and the American Society of Microbiology.

He has been granted postdoctoral research fellowships by the Hungarian Academy of Sciences, the British Council, FAO-UNO, and has twice been Senior Fulbright Scholar and visiting research professor affiliated with the Department of Food Science and Technology of the University of Georgia. His awards include the Sigmund Award for Food Science and the Manninger Award for Food Microbiology. He has twice received the Magister Optimus Teaching Award. He is a Fellow of the American Academy of Microbiology, a Distinguished Fellow of the Kansas State University, Manhattan, and an appointed External Examiner of the Free State University, Bloemfontein, South Africa.

Dr. Deák is author or co-author of more than 330 research papers and 24 books and chapters, as well as 15 textbooks and manuals. His continuing research interests include the microbial ecology of foods, biodiversity of yeasts in agrofood-ecosystems, yeasts as spoilage agents in foods and beverages, and yeast detection and identification.

List of Abbreviations

Abbreviations for genera of most common foodborne yeasts (used only for binomial species names)

Bret.	*Brettanomyces*
C.	*Candida*
Citerom.	*Citeromyces*
Clsp.	*Clavispora*
Cry.	*Cryptococcus*
Cysto.	*Cystofilobasidium*
Db.	*Debaryomyces*
Dek.	*Dekkera*
F'ella	*Filobasidiella*
Filob.	*Filobasidium*
Gal.	*Galactomyces*
Geo.	*Geotrichum*
Guehom.	*Guehomyces*
Hsp.	*Hanseniaspora*
Hyphop.	*Hyphopichia*
Iss.	*Issatchenkia*
Kazach.	*Kazachstania*
Klc.	*Kloeckera*
Klu.	*Kluyveromyces*
Leucosp.	*Leucosporidium*
Lodd.	*Lodderomyces*
Met.	*Metschnikowia*
P.	*Pichia*
Rho.	*Rhodotorula*
Rhosp.	*Rhodosporidium*
S.	*Saccharomyces*
S'codes	*Saccharomycodes*
S'copsis	*Saccharomycopsis*
Schizo.	*Schizosaccharomyces*
Schwan.	*Schwanniomyces*
Spb.	*Sporobolomyces*
Sporid.	*Sporidiobolus*
Trichomon.	*Trichomonascus*
Trisp.	*Trichosporon*
Tsp.	*Torulaspora*
Ya.	*Yarrowia*
Zygo.	*Zygosaccharomyces*
Zygotsp.	*Zygotorulaspora*

Other Abbreviations

5.8S, 18S, 26S	rDNA in ribosomal subunits
am.	Anamorph
bp	Basepair
cfu	Colony-forming units
ITS	Internal transcribed spacer
kb	Kilobase
LSU	Large subunit ribosome
Mb	Megabase
mtDNA	Mitochondrium DNA
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
SSU	Small subunit ribosome
syn.	Synonym
tel.	Teleomorph

1 Characteristics and Properties of Foodborne Yeasts

Yeasts are traditionally characterized, classified, and identified by morphological and physiological criteria (Kreger-van Rij, 1987; Kurtzman and Fell, 1998). Since the 1970s, a large amount of data on the chemical composition of cell walls, capsular polysaccharides, whole-cell hydrolysates, antigenic determinants, and enzyme patterns have provided valuable information for yeast taxonomy (Phaff, 1984). However, the most important criteria for classification are DNA base composition, nuclear DNA (nDNA) homology, and the sequences of ribosomal DNA molecules, which can be used not only to elucidate the degree of relatedness but also to reveal evolutionary relationships of yeasts (Kurtzman and Phaff, 1987; Wilmotte et al., 1993; Kurtzman and Robnett, 1998). Sophisticated biochemical and molecular methods, however, cannot be easily applied in routine identification procedures. Hence, in both classification and identification of yeasts, the morphological and physiological criteria, among them the characteristics of sexual reproduction, are still important (von Arx and van der Walt, 1987; Yarrow, 1998; Boekhout and Phaff, 2003) (Table 1.1).

The criteria used in the description and classification of yeasts have been described in detail by van der Walt and Yarrow (1984) and Kreger-van Rij (1987). Only the most important morphological and physiological criteria that serve to identify foodborne yeasts are discussed here. Yeast classification is changing, and now based on molecular characteristics. Trends and recent developments in this field will be summarized as well.

TABLE 1.1
Criteria Traditionally Used in the Characterization of Yeasts

Morphological	Physiological	Biochemical, Molecular
Sexual reproduction	Fermentation of sugars	Diazonium Blue B reaction
Mode of conjugation	Assimilation of carbon sources	Urease reaction
Forms of spores and sporangia	Assimilation of nitrogen sources	Type of coenzyme Q
Formation of basidiospores	Vitamin requirements	Cell wall composition
Vegetative reproduction	Temperature of growth	Whole-cell carbohydrates
Budding	Growth at low water activity	Long-chain fatty acids
Fission	Resistance to cycloheximide	Protein electrophoretic pattern
Arthroconidia	Formation of starch	Isoenzymes
Ballistoconidia	Production of acetic acid	
Microscopic growth		Guanine + cytosine mol%
Size and shape of cells		DNA–DNA homology
True hyphae and pseudohyphae		DNA restriction fragments
Chlamydospores		Gene probes
Macroscopic growth		Chromosome karyotyping
Colonies on solid media		rDNA sequences
Liquid cultures		Complete sequences

1.1 MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS

For practical purposes, yeasts may be defined as unicellular fungi, in which the vegetative (asexual) reproduction occurs mainly by budding. According to terminology introduced for fungi (von Arx, 1979), budding is a type of conidiation and the buds are blastoconidia. The large majority of yeasts (e.g., *Saccharomyces*) exhibit multilateral budding, a special type of cell division in which daughter cells (buds) appear over a large area of the cell surface. In contrast, in bipolar budding, the buds are formed only at the poles of the cells (annelloconidiation), resulting in characteristic lemon-shaped cells (e.g., *Hanseniaspora*; Figure 1.1). Otherwise, yeast cells are usually rounded, ovoid, or cylindrical. Buds may also arise on stalks or sterigmata, often forcibly discharged by a turgor mechanism, and these are known as ballistoconidia (e.g., *Bullera*). Cell division in some yeast takes place by the formation of a cross wall, called fission, resulting in the formation of arthroconidia (e.g., *Schizosaccharomyces*; Figure 1.2). Some yeast is capable of both budding and fission (e.g., *Trichosporon*).

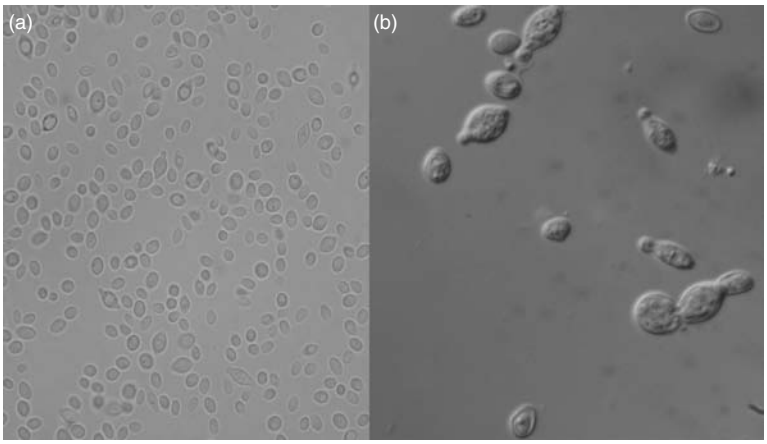

FIGURE 1.1 Bipolar budding, *Kloeckera apiculata* (tel. *Hanseniaspora uvarum*). (a) Bright field microscopy, magnification: ×400 (courtesy of Dr. G. Péter); (b) Nomarski microscopy, magnification: ×2000.

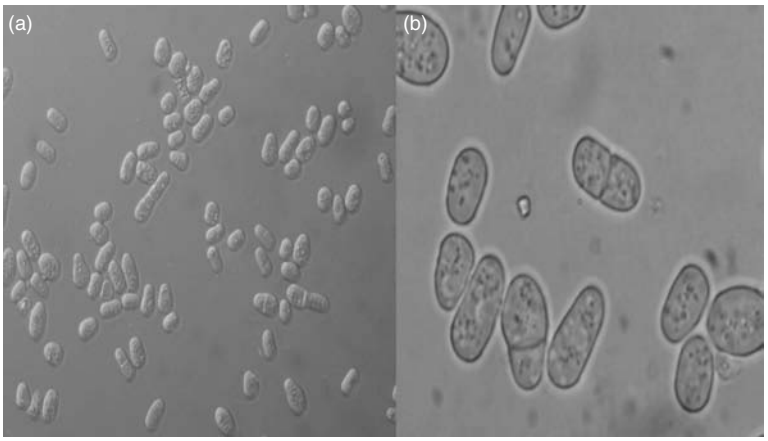

FIGURE 1.2 Splitting cells of *Schizosaccharomyces pombe*. Bright field microscopy, (a) magnification: ×400; (b) magnification: ×2000.

Although yeasts are primarily unicellular, some may develop hyphae or pseudohyphae. True hyphae are characterized by the lack of a constriction at the cross walls (Figure 1.3), whereas pseudohyphal cells are formed by budding and elongation and show a constriction at the attachment of cells (Figure 1.4). When yeasts form true (septate) hyphae, buds may arise from them, or sometimes the hyphae breaks up into arthroconidia (e.g., *Geotrichum*). These filamentous forms, collectively called yeast-like organisms, show some properties of both hyphal molds and unicellular yeasts. Moreover, many filamentous fungi can be induced to form a yeast phase, and some pathogenic fungi are also dimorphic in that they are capable of forming yeast-like cells in the invaded tissue (Shepherd et al., 1985; de Hoog, 1987).

The fungal world is classified mainly by characteristics of sexual reproduction (sporulation). Accordingly, those yeasts that form spores by sexual reproduction can be classified into the appropriate higher fungal taxa. The so-called true yeasts, which form ascospores, are traditionally grouped as a class, Hemiascomycetes, in the fungal division Ascomycota. However, the endogenous

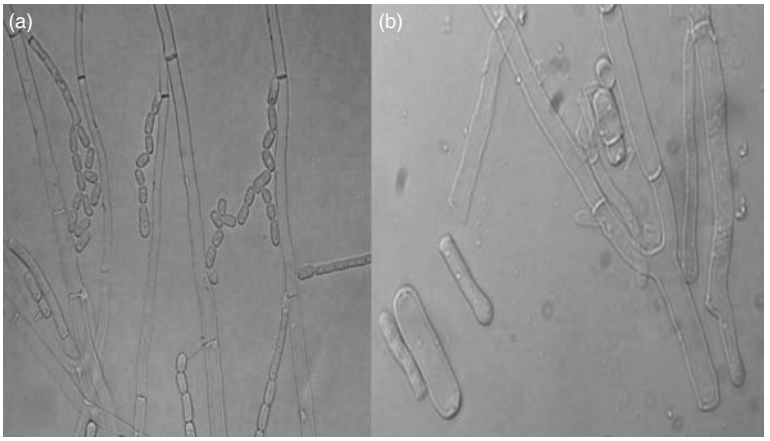

FIGURE 1.3 True hyphae with septa and arthroconidia of *Geotrichum candidum* (tel. *Galactomyces geotrichum*). (a) *In situ* bright field microscopy of slide culture, magnification: ×400 (courtesy of Dr. G. Péter); (b) Nomarski microscopy, magnification: ×2000.

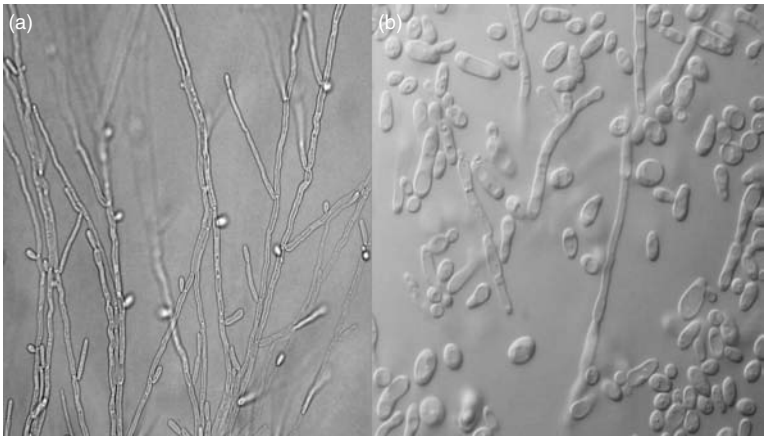

FIGURE 1.4 Pseudohyphae and blastoconidia of *Candida tropicalis.* (a) *In situ* bright field microscopy of slide culture, magnification: ×400 (courtesy of Dr. G. Péter); (b) Nomarski microscopy, magnification: ×2000.

formation of sexual spores in these yeasts differs substantially from the characteristic development of ascospores in that yeasts lack dikaryotic ascogenous hyphae and ascocarps. Hence, this group of yeasts can be considered as a separate division of fungi, for which the name Endomycota has been suggested (Barr, 1983). Sexual spore development in some other yeast shows obvious similarities with certain groups of Basidiomycota. Some develop basidia directly on hyphae, whereas others form thick-walled teliospores from which a basidium originates. This latter group is close to the smuts, and together with them, may also be grouped into a separate division, Ustomycota (Moore, 1972, 1996).

The mode of spore formation varies among yeasts (Moore, 1998). Formation of sexual spores usually occurs through conjugation of opposite mating types. These mating types may be found in the same (homothallic) or separate (heterothallic) vegetative colonies. Complementary mating types must meet in order for sporulation to occur. An isolate of heterothallic species sometimes contains only one haploid mating type, and this is asporogenous. Homothallic yeasts containing both haploid mating types may show some kind of conjugation prior to spore formation. Conjugation can occur between independent cells or between a cell and its bud (mother–daughter conjugation). Cells of *Saccharomyces* species are diploid and transform directly into sporangia (asci) when the spores form (Figure 1.5). In *Zygosaccharomyces*, conjugation between independent haploid cells precedes spore formation (Figure 1.6), whereas conjugation occurs between the mother cell and its bud in *Torulaspora*. Other modes of sporulation also occur among yeasts.

Spores may remain in the sporangium until the time of germination (e.g., *Saccharomyces*), or they may liberate easily from sporangia (e.g., *Kluyveromyces*). This trait is of diagnostic value, as is the shape of spores. Sexual spores vary considerably in shape and surface structure. They may be spheroid, ellipsoidal, kidney or needle-like, or hat or Saturn-shaped. Although morphological features of spores play an important role in yeast classification, they can be properly studied only by electron microscopy. Moreover, spores may not be consistently formed for a variety of reasons. For example, when the yeast strain is haploid and heterothallic, spore formation does not occur in isolated culture. Many homothallic yeasts often require special conditions to induce spore formation. Hence, many have been initially described in asexual form, called anamorphic (or imperfect) state, as contrasted to the teleomorphic (perfect) state, which can reproduce sexually by the formation of spores. Unfortunately, these two states often bear different names. For example, the teleomorph *Hanseniaspora uvarum* has its anamorphic form as *Kloeckera apiculata*. According to the rules of nomenclature, the name of the teleomorph is preferably used, if it exists; however, many species occur

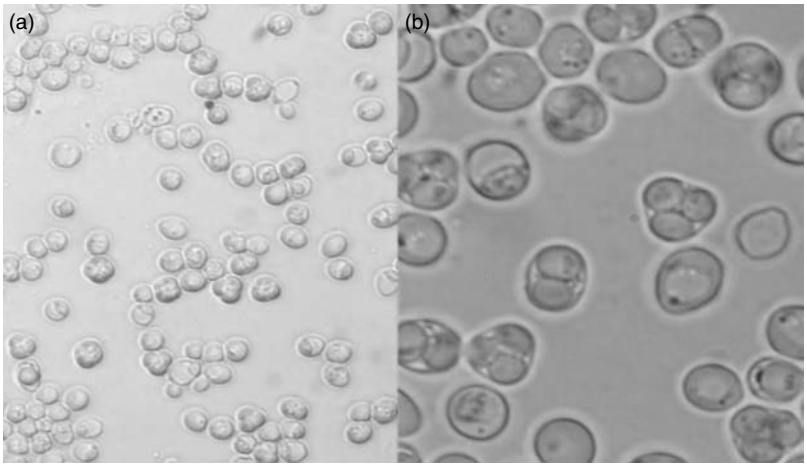

FIGURE 1.5 Cells and asci of *Saccharomyces cerevisiae*. Bright field microscopy, (a) magnification: ×400; (b) magnification: ×2000.

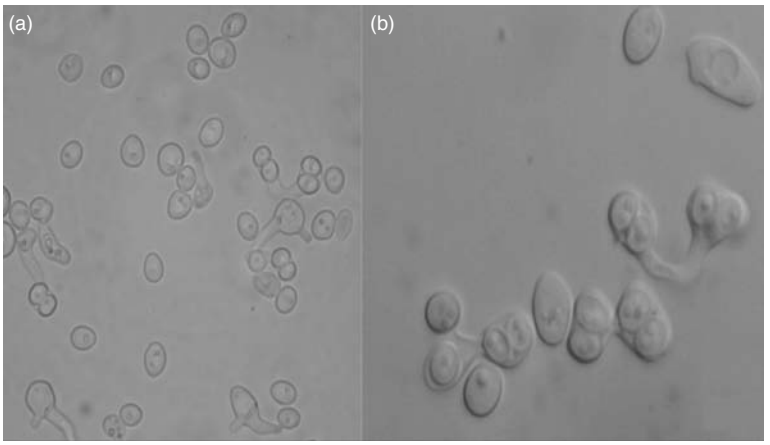

FIGURE 1.6 Conjugating cells and spores of *Zygosaccharomyces bailii*. (a) Bright field microscopy, magnification: ×800; (b) Nomarski microscopy, magnification: ×2000.

primarily in the anamorphic form, which is far more common, and the names too are better known. Moreover, numerous synonyms are created by nomenclatural changes as a consequence of taxonomic rearrangements. Synonymy presents a problem to those not familiar with classification. For example, the older name *Torula utilis* became *Torulopsis utilis*, and both are synonyms of *Candida utilis*, which in turn is the anamorph of *Pichia jadinii*. Table 1.2 lists the names of teleomorph–anamorph pairs of the most important foodborne yeasts, and Table 1.3 lists some frequently used synonyms.

The basidiospore is also a product of a complex sexual cycle in basidiomycetous yeasts. After germination, basidiospores take on a budding yeast form. The spore-bearing structures, basidia, may arise directly from hyphae or from thick-walled teliospores, showing similarities to Heterobasidiomycetes or Ustomycetes. In addition to spore-bearing structures, hyphae may also form clamp connections, another basidiomycetous character. Basidiomycetous yeasts are usually isolated in the anamorphic state, because self-sporulating, homothallic strains are rare. Their haploid anamorphs have been grouped into various imperfect genera such as *Rhodotorula*, *Cryptococcus*, *Sporobolomyces*, and so forth.

Classification of yeasts into higher taxa is usually based on the morphology of sexual states. Physiological tests, in conjunction with microscopic observation of vegetative cells, are necessary for the delineation and identification of species. Recognition of species is primarily based on the fermentation of, and growth on, a variety of carbon and nitrogen compounds. Some identification schemes rely entirely on fermentation and assimilation reactions.

Yeasts vary in their ability to ferment sugars as measured by the production of carbon dioxide. Some species ferment vigorously (e.g., *Saccharomyces*), while others are capable of only weak fermentation or no fermentation at all (e.g., *Rhodotorula*). Sugars and other carbon compounds used in assimilation tests (i.e., growth under aerobic conditions on a given substrate) are numerous. Other tests include growth on nitrate, in the absence of vitamins, tolerance to high sugar concentration, cycloheximide, or preservatives, and growth temperature requirements. A single chemical test that distinguishes between ascomycetous and basidiomycetous yeasts is the diazonium Blue B (DBB) test (Hagler and Ahearn, 1981). The cell wall of basidiomycetous yeasts reacts with the dye, resulting in dark red colonies, in contrast to ascomycetous yeasts, which fail to react with DBB.

1.2 BIOCHEMICAL CHARACTERISTICS

The reliability of morphological and physiological criteria traditionally used to characterize yeasts is often questioned. A single gene can enable the yeast to ferment or assimilate a specific sugar.

TABLE 1.2
Teleomorph–Anamorph Connections of Foodborne Yeast Species

Teleomorph	Anamorph
Bulleromyces albus	*Bullera alba*
Citeromyces matritensis	*Candida globosa*
Clavispora lusitaniae	*Candida lusitaniae*
Cystofilobasidium infirmominiatum	*Cryptococcus infirmominiatus*
Debaryomyces hansenii	*Candida famata*
Dekkera anomala	*Brettanomyces anomalus*
Dekkera bruxellensis	*Brettanomyces bruxellensis*
Dipodascus ingens	*Candida ingens*
Filobasidiella neoformans	*Cryptococcus neoformans*
Filobasidium capsuligenum	*Candida japonica*
Galactomyces geotrichum	*Geotrichum candidum*
Hanseniaspora guilliermondii	*Kloeckera apis*
Hanseniaspora osmophila	*Kloeckera corticis*
Hanseniaspora uvarum	*Kloeckera apiculata*
Hanseniaspora valbyensis	*Kloeckera japonica*
Hyphopichia burtonii	*Candida variabilis*
Issatchenkia orientalis	*Candida krusei*
Kazachstania exiguus	*Candida holmii*
Kluyveromyces lactis	*Candida sphaerica*
Kluyveromyces marxianus	*Candida kefyr*
Kregenvanrija fluxuum	*Candida vini*
Lachancea thermotolerans	*Candida dattila*
Leucosporidium scottii	*Candida scottii*
Metschnikowia pulcherrima	*Candida pulcherrima*
Metschnikowia reukaufii	*Candida reukaufii*
Pichia anomala	*Candida pelliculosa*
Pichia fermentans	*Candida lambica*
Pichia guilliermondii	*Candida guilliermondii*
Pichia jadinii	*Candida utilis*
Pichia membranifaciens	*Candida valida*
Pichia nakasei	*Candida citrea*
Rhodosporidium toruloides	*Rhodotorula glutinis*
Saccharomyces cerevisiae	*Candida robusta*
Sporidiobolus salmonicolor	*Sporobolomyces salmonicolor*
Trichomonascus ciferrii	*Sporothrix catenata*
Torulaspora delbrueckii	*Candida colliculosa*
Wickerhamiella domercqiae	*Candida domercqiae*
Yarrowia lipolytica	*Candida lipolytica*
Zygoascus hellenicus	*Candida steatolytica*

Both the formation of ascospores and ascospore morphology depend on the pairing of complementary mating types. These uncertainties cast some doubt on the reliability of traditional criteria and on the validity of classification based on these criteria. Hence, biochemical tests have been used to characterize yeasts for several decades, and recent advances in molecular biology have led to the development of new criteria for differentiation and characterization of yeast species and strains.

Several biochemical methods have been applied for determining the relationships between yeast genera, species, and/or strains (Kurtzman and Phaff, 1987). Analysis of the ubiquinone system is

TABLE 1.3
Frequently Used Synonyms for Food Yeast Species

Current name	Synonyms
Brettanomyces anomalus	*Brettanomyces claussenii*
Brettanomyces bruxellensis	*Brettanomyces intermedius*
Candida albicans	*Candida claussenii*, *Candida stellatoidea*
Candida catenulata	*Candida brumptii*, *Candida ravautii*
Candida diddensiae	*Trichosporon diddensiae*
Candida etchellsii	*Brettanomyces sphaericus*
Candida famata	*Torulopsis candida*
Candida kefyr	*Candida macedoniensis*, *Candida pseudotropicalis*
Candida lactiscondensi	*Torulopsis caroliniana*
Candida tropicalis	*Candida vulgaris*, *Candida paratropicalis*
Candida valida	*Candida mycoderma*
Candida versatilis	*Torulopsis anomala*
Candida vini	*Candida mycoderma*, *Mycoderma vini*
Cryptococcus albidus	*Torulopsis aeria*
Cryptococcus curvatus	*Candida curvata*
Cryptococcus humicolus	*Candida humicola*
Debaryomyces hansenii	*Deb. kloeckeri*, *Deb. subglobosus*, *Deb. nicotianae*
Debaryomyces etchellsii	*Pichia etchellsii*
Debaryomyces polymorphus	*Pichia polymorpha*
Dekkera bruxellensis	*Dekkera intermedia*
Geotrichum candidum	*Oidium candidum*, *Oospora lactis*
Hyphopichia burtonii	*Pichia burtonii*, *Trichosporon variable*
Issatchenkia terricola	*Pichia terricola*
Kluyveromyces marxianus	*Klu. bulgaricus*, *Klu. fragilis*, *Saccharomyces fragilis*
Kluyveromyces lactis	*Kluyveromyces drosophilarum*
Kodamaea ohmeri	*Pichia ohmeri*, *Endomycopsis ohmeri*
Lachancea thermotolerans	*Saccharomyces veronae*
Lachancea fermentati	*Zygosaccharomyces fermentati*, *Saccharomyces montanus*
Pichia anomala	*Hansenula anomala*
Pichia angusta	*Hansenula polymorpha*
Pichia jadinii	*Hansenula jadinii*
Pichia subpelliculosa	*Hansenula subpelliculosa*
Rhodotorula mucilaginosa	*Rhodotorula rubra*
Saccharomyces cerevisiae	*S. ellipsoideus*, *S. italicus*, *S. oviformis*, *S. diastaticus*
Saccharomyces bayanus	*S. willianus*, *S. globosus*, *S. inusitatus*
Saccharomyces pastorianus	*S. carlsbergensis*
Saccharomycopsis fibuligera	*Endomycopsis fibuligera*
Schizosaccharomyces pombe	*Schizosaccharomyces malidevorans*
Torulaspora delbrueckii	*Saccharomyces rosei*, *S. fermentati*, *S. vafer*
Torulaspora globosa	*Saccharomyces kloeckerianus*, *Debaryomyces globosus*
Trichosporon cutaneum	*Trichosporon beigelii*
Yarrowia lipolytica	*Saccharomycopsis lipolytica*
Zygotorulaspora florentinus	*Zygosaccharomyces florentinus*, *Saccharomyces eupagycus*
Zygosaccharomyces rouxii	*Saccharomyces rouxii*, *Zygosaccharomyces barkeri*

Many *Candida* species have *Torulopsis* synonyms with the same species epithet, for example, *C. apicola*, *C. cantarellii*, *C. dattila*, *C. domercqiae*, *C. famata*, *C. glabrata*, *C. holmii*, *C. inconspicua*, *C. magnoliae*, *C. stellata*, *C. utilis*, *C. versatilis*.

suitable for the study of relatedness at generic level. The number of isoprene units in the side chain of ubiquinones or coenzyme Q molecules varies from 5 to 10 among yeasts. In many genera, this number is uniform and has been used as an exclusionary value (Yamada et al., 1977, 1987).

Protein electrophoretic fingerprinting has been successfully used for grouping and identifying species in the genus *Candida* (Vancanneyt et al., 1991), and in the basidiomycetous species (Vancanneyt et al., 1992). SDS–PAGE protein profiles provide a valuable tool for identifying yeasts. Comparison of electrophoretic patterns of specific enzymes is valuable for discriminating strains at the species level and for assessing teleomorph–anamorph relationships (Yamazaki and Komagata, 1982; Yamazaki et al., 1998).

Analysis of whole-cell carbohydrate composition (von Arx and Weijman, 1979; Sugiyama et al., 1985; Weijman and Golubev, 1988; Boekhout et al., 1992) and purified cell walls (Prillinger et al., 1993) provides powerful taxonomic information at the generic or higher taxa level, and has proved to be a highly indicative way to discriminate between yeasts of various basidiomycetous affiliation (Weijman and R de Miranda, 1988). Yeast cell wall is mainly composed of a glucomannan–protein complex. Ascomycetous budding yeasts contain only 1.2% chitin, confined mainly to the bud scars. Filamentous forms may contain some more chitin, but significantly less than yeasts of basidiomycetous affinity with up to 10% chitin content (Phaff, 1998).

Analysis of cellular long-chain fatty acids is also valuable in the identification of yeast species, although a considerable variation in fatty acid composition may impose a constraint in applying this technique to classify yeasts (Augustyn et al., 1992; Rozes et al., 1992). Fatty acid profiles of yeasts are also affected by growth conditions (Golden et al., 1994).

1.3 MOLECULAR CHARACTERISTICS

Although biochemical methods have proved to be useful for taxonomic purposes, the exploration of phylogenetic relationships among yeasts became possible only after the introduction of molecular techniques about 40 years ago. Various DNA-based methods have reformed the taxonomy and classification of yeasts, including the recognition and identification of species as well as their infraspecific relationships. The armory of molecular methods extends from determining DNA composition to sequencing of parts and even the whole genome of yeasts (Kurtzman, 2006).

The guanine and cytosine (G + C) content of yeasts ranges from ~28 to 70 mol%. Although some overlapping occurs in the narrow range of 48–52%, the G + C content of ascomycetous yeasts is generally less than 50%, whereas that of basidiomycetous yeasts is generally above 50% (Kurtzman et al., 1983; Kurtzman, 1998). From the base composition, the taxonomic class of imperfect yeasts can usually be inferred. The range in G + C content among species within a genus is often 10% or less, with the exception of some obviously heterogenous genera. On a species level, the use of G + C content is only exclusionary in that a difference of $\geq$2 mol% indicates strains belonging to different species (Price et al., 1978).

Although G + C values are helpful for the separation of species, their use is clearly limited. Considerably greater resolution can be achieved from information on nuclear DNA (nDNA) complementarities as determined from reassociation reactions. The associated methodology has been described in detail by Kurtzman et al. (2003). Results from comparisons are expressed as a percentage of DNA/DNA homology (relatedness), and it is generally accepted that a homology of >70–80% demonstrates conspecificity.

Comparison of mitochondrial DNA (mtDNA) sequences offers a similar degree of relatedness to that afforded by nDNA reassociation. Restriction patterns for mtDNA have been successfully used to correlate teleomorph–anamorph relationships between the genera *Dekkera* and *Brettanomyces* (Hoeben and Clark-Walter, 1986).

DNA coding for ribosomal RNA (rRNA) appears to be highly conserved, and offers a means of assessing relationships among higher taxa. Woese (1987) effectively used the cataloging of sequences

generated from 16S rRNA to assess phylogenetic relationships among the prokaryotes. This approach revolutionized the understanding of prokaryote taxonomy. Because of the much larger number of fragments obtained from 18S or 25S ribosomal subunits of eukaryotes, this method did not appear feasible for fungi for some time. Instead, the much smaller 5S rRNA sequences were studied to infer phylogenetic relatedness among fungi. These studies were extended to include several yeast species and genera (Blanz and Gottschalk, 1984; Walker, 1985). Recently, methods to study the longer rRNA molecules in small and large subunits of ribosomes have been developed, and results from these sequencing studies have been extremely useful in assessing yeast phylogeny (Kurtzman and Blanz, 1998). Hundreds of complete or almost complete rDNA sequences have been determined that enable the assessment of evolutionary relationships among yeasts and higher fungi. The main conclusions drawn from these studies are discussed in the next chapter on yeast classification.

Molecular techniques have been increasingly applied in the identification of yeasts. Electrophoretic karyotyping has proved to be of value in determining the relationships among different yeast taxa. The value of the technique is largely confined to assessing specific relationships and infraspecific variations (Viljoen et al., 1988; Vezinhet et al., 1990; Boekhout et al., 1993; Vaughan-Martini et al., 1993). The sensitivity of the technique can be improved by hybridization probes prepared against specific genes or whole chromosomes (Török et al., 1992). Various gene probes and rapid assays on the basis of the polymerase chain reaction have been developed for the identification of *Saccharomyces* (Seehaus et al., 1985) and *Zygosaccharomyces* species (Pearson and McKee, 1992), as well as the clinically important *Candida* species (del Castillo Agudo et al., 1993; Niesters et al., 1993). Molecular detection and identification methods will be discussed in detail in later chapters as well.

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2 Classification of Yeasts

Since the first scientific description of *Saccharomyces cerevisiae* (Meyen, in 1838; Reess, in 1870), the development of yeast taxonomy has been burdened with controversy and continuous changes in names (Barnett, 2004). Over the years, a number of classifications have been proposed for the ever-increasing number of yeast species described. In 1912, Guilliermond was the first to outline a phylogenetic scheme of yeasts among Ascomycetes. In the years between 1931 and 1942, three systematic monographs were published by the collaborators of yeast collection developed at the Delft Technical University; they founded the "Dutch school" of yeast classification, resulting in several successive editions of taxonomic treatises (Lodder and Kreger-van Rij, 1952; Lodder, 1970; Kreger-van Rij, 1984). The fourth edition of *The Yeasts, a Taxonomic Study* was edited by Kurtzman and Fell (1998), and the fifth edition, edited by Kurtzman, Fell, and Boekhout, is in preparation at the time of this writing. Meanwhile, Barnett and coworkers (Barnett et al., 1983, 1990, 2000) have published another series of monographs on yeast characteristics and identification with taxonomic background. Barnett (2004) has published a historical account on yeast taxomomy that can be consulted for further details. Table 2.1 summarizes the increase in the number of yeast species and genera over the years.

2.1 TRADITIONAL CLASSIFICATION

Yeasts are traditionally considered to be unicellular fungi that reproduce by budding (or fission). In contrast to the majority of fungi having filamentous vegetative cells, yeasts are predominantly unicellular, and the sexual spores are not enclosed in a fruiting body. Their sexual states (teleomorphs) can be classified into two major fungal classes, the Ascomycetes and the Basidiomycetes; however, there is a large number of species whose perfect (sexual) state is not known. Previously, these anamorphic yeasts were grouped in the form-class Deuteromycetes or Fungi imperfecti. Though lacking a sexual state, on the basis of various other characters, the recent trend is to include these forms also in one of the two higher classes.

This definition covers yeasts with ascomycetous and basidiomycetous relationships, as well as teleomorphic and anamorphic forms. Although this morphological concept of yeasts may be extended to fit many stages in the life cycle of hyphal fungi, for traditional reasons not all fungi with a yeast phase have been included among yeasts (Kreger-van Rij, 1984). Van der Walt (1987)

TABLE 2.1
Number of Species and Genera Described in Taxonomic Monographs on Yeasts

Reference	Taxon Species	Genus
Lodder and Kreger-van Rij (1952)	164	26
Lodder (1970)	349	39
Barnett et al. (1983)	469	63
Kreger-van Rij (1984)	500	60
Barnett et al. (1990)	597	83
Kurtzman and Fell (1998)	692	96
Barnett et al. (2000)	678	93
Kurtzman et al. (2007)	~1500	~141

defined yeasts as unicellular, ontogenic stages of the Ascomycetes and Basidiomycetes, and pointed out the artificial delimitation of yeasts among fungi. Budding stages of dimorphic Basidiomycetes and Ustomycetes may well be classified as yeasts, and similarly, the yeast phases of the Taphrinales as well as the so-called black yeasts may also qualify as yeasts. This would expand the dimension of the yeast domain among fungi.

Von Arx and van der Walt (1987) made an effort toward a more natural classification of yeasts. They assigned the ascogenous yeasts to the Hemiascomycetes, in the order Endomycetales, of the Ascomycota. The Endomycetales are restricted to unicellular, budding stages of ascomycetous fungi, in which hyphae may be present. The fission yeast, *Schizosaccharomyces*, though not budding, has traditionally been considered among yeast, and has been placed in a separate family. Among the heterobasidiomycetous filamentous fungi with yeast stages, von Arx and van der Walt (1987) distinguished three groups showing affiliation with Ustilaginales, Filobasidiales, and Tremellales. The differentiation between ascomycetous and basidiomycetous anamorphic yeasts has been made possible by reliable chemotaxonomic markers that provide a rational classification of anamorphs in natural families (Table 2.2). On the basis of conidiation and the carbohydrate composition of whole cells (von Arx and Weijman, 1979), three families have been proposed by van der Walt (1987): Candidiaceae, Cryptococcaceae, and Sporobolomycetaceae, restricted to anamorphic Endomycetes, Basidiomycetes, and Ustomycetes, respectively.

The progress of yeast classification resulted not only in the enlargement of the number of species and genera recognized, but also in an increase in the number of physiological tests involved in the differentiation and the consideration of biochemical features included. Although only six carbon or nitrogen sources were used in the fermentation and assimilation tests in the first edition of the taxonomic monograph (Lodder and Kreger-van Rij, 1952), following Wickerham's proposal, the number of different compounds tested increased, and Kreger-van Rij (1984) based the species description on the utilization of 18 substrates, while Barnett et al. (2000) employed 99 different tests for identification. As a consequence, depending on the tests employed, strains can be divided into

TABLE 2.2
Some Gross Morphological and Chemotaxonomical Differences between Yeasts of Ascomycetous and Basidiomycetous Affinity

Characteristics	Ascomycetous	Basidiomycetous
Morphological		
Mucous colonies	–	+
Carotenoid pigments	–	+
Ballistoconidia	–	v
Cell wall structure	Bilayered	Multilayered
Budding	Holoblastic	Enteroblastic
Septal pores	None or simple	Micro- or dolipore
Biochemical		
Cell wall composition	Glucan, mannan	Glucan, mannan, chitin
DBB reaction	–	+
Urease reaction	–	+
Production of starch	–	v
Type of coenzyme Q	6, 7, 8, (9)	(8), 9, 10
G + C mol%	<50	>50

Note: −: negative; +: positive; and v: variable.
Source: From Deák, T. and Beuchat, L. R. (1996) *Handbook of Food Spoilage Yeasts.* CRC Press, Boca Raton, FL. pp. 12–14.

different species that can be grouped in different ways. The problems associated with traditional classifications based on conventional morphological and physiological tests manifest not only in the frequent changes in grouping and naming, but also in the uncertainties of test results. Some morphological features (e.g., shape of spores, presence of filaments) often vary in strains belonging to the same species. Physiological properties (e.g., production of an enzyme for utilization of a substrate) are often determined by a single gene that can be changed by mutation. Hence, traditional classifications based on phenotypic characters have serious limitations. Despite considerable progress, such systems of classification for yeasts have been far from complete. A reliable classification should consider genetic similarities and differences and has to be based on genotypic properties. Molecular techniques provide tools required to achieve this, and their application already has had great impact on the classification of yeasts. Moreover, the molecular approach allows a phylogenetic arrangement of systematic categories.

2.2 MOLECULAR TAXONOMY AND PHYLOGENY

The use of molecular methods has extended the scope of classification to consider the evolutionary relationships among species and higher taxa. In phylogenetic studies, the similarities and differences in nucleotide sequences of single genes are determined and compared. Similarity is assumed to reflect common ancestry, whereas the degree of sequence divergence marks evolutionary distance (Petersen et al., 2000). In the previous chapter, various molecular methods that can be used for characterization and identification of species were discussed. Their use to infer phylogenetic relationships will be further expanded here.

The genes coding for ribosomal RNA (rRNA) are most frequently studied for phylogenetic purposes. Ribosomes are present in all organisms, and rDNA occurs in multiple copies in both small and large subunits (SSU and LSU). These molecules consist of different domains, some of which are highly conserved, while others are more variable, and can hence be used, respectively, for evolutionary studies of major groups of higher taxa and for assessing phylogenetic relationships among species. In yeasts, the size of rDNA in SSU is 18S, whereas LSU contains 5S, 5.8S, and 26S rDNA molecules. All these are transcribed in one unit (repeated in many copies), and the parts are separated by internal and external spacers (ITS and ETS). Between each unit, there is an intergenic non-transcribed spacer (IGS or NTS), and the 5S rDNA usually occurs in this region and is transcribed separately from the rest of gene cluster (Figure 2.1).

Comparisons of rDNAs have become the basis of assessing both close and distant relationships among yeasts (Kurtzman and Phaff, 1987; Kurtzman, 1994). Before sequencing methods were developed, relatedness between pairs of rDNAs could be estimated with the extent of reassociation, and also by analysis of restriction fragment length polymorphism. Sequences could be determined first for the small 5S rDNA consisting of about 120 nucleotides. Initial sequencing studies targeted 5S rRNA, and revealed relationships among groups of basidiomycetous yeasts in correlation with the ultrastructure of septal pores (Walker and Doolittle, 1983), and also pointed out a large evolutionary distance between *Schizosaccharomyces* and budding yeasts (Walker, 1985). However, the conserved structure of 5S rRNA genes allows us to estimate only broad phylogenetic relationships.

As rapid and automated PCR-based sequencing methods have become available, partial and complete sequences (~1700 nucleotides) of 18S rDNA, the D1/D2 variable domains (~600 nucleotides)

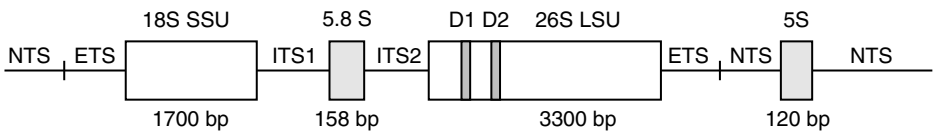

FIGURE 2.1 Structure of nuclear ribosomal DNAs.

of 26S rDNA, and the ITS1–5.8S–ITS2 regions (~600 nucleotides) have been determined from many yeast species building up large databases (Wilmotte et al., 1993; Kurtzman and Robnett, 1998; Sugita et al., 1999; Takashima and Nakase, 1999; Fell et al., 2000). The conserved sequences of SSU rDNA allow broad phylogenetic analyses, whereas the LSU domains and ITS sequences are more variable and permit resolution at the species level. The latter two, also due to their smaller sizes, have become universally used in establishing phylogenetic relatedness, and also for the identification of species. The compilation of large sets of data on sequences of the D1/D2 domains of LSU rDNA shows that conspecific strains, with few exceptions, ordinarily differ only in 0–3 nucleotides (0–1%), whereas intrageneric differences between species may be 3–25% (Kurtzman and Robnett, 1991; Peterson and Kurtzman, 1991; Kurtzman, 1992, 1994). The degree of nucleotide differences correlates with percent nuclear DNA (nDNA) relatedness, which is usually larger than 70% between conspecific strains. However, in the region of 50% or less nDNA relatedness, nucleotide differences may vary, being less than predicted (Kurtzman, 1990). A recent example is some closely related *Pichia* species and *Candida* anamorphs ranging 37–68% in DNA relatedness but differing by only 1–3 nucleotides in D1/D2 domain (Vaughan-Martini et al., 2005). Variations among species in different genera may be due to unequal rates of nucleotide substitutions in different rDNA regions, and the length of time elapsed since the species diverged. The rates of base substitution can also differ in different regions of the rDNA molecule. Although the resolution provided by analyses of D1/D2 and ITS regions is generally similar, sometimes ITS sequences differ between species with no differences in D1/D2 domain, and vice versa (Scorzetti et al., 2002; Kurtzman and Robnett, 2003). Consequently, definition of yeast taxa solely based on the number of nucleotide differences in a single gene is not always reliable.

Recently, more and more data are available for gene sequences other than rDNA. Yeast species have been separated using sequence determination of various protein genes such as cytochrome oxidase II (Belloch et al., 2000), elongation factor 1α and RNA polymerase II (Kurtzman and Robnett, 2003), and actin (Daniel et al., 2001; Daniel and Meyer, 2003). Phylogenic conclusions derived from other genes are usually comparable to those drawn from rDNA sequence analysis. However, multigene analyses can corroborate phylogenetic trees determined from single genes, and strengthen, in particular, support for basal lineages. From combined analysis of eight genes, Kurtzman and Robnett (2003) resolved the phylogenetic relationships of 75 species of the *Saccharomyces* complex. In a study including a large dataset of more than 100 genes, Rokas et al. (2003) demonstrated that almost any selection of at least 20 genes resulted in a comparable phylogenetic tree. Lutzoni et al. (2004), in a broad evaluation of relationships among major fungal divisions using combined data of two, three, and four genes, concluded that four-gene analysis revealed deep relationships within fungi not recognized in other studies.

The eventual understanding of fungal phylogenies would be arrived at by the analysis based on complete genomes. Although there are many ongoing fungal genome sequencing projects, the number of whole genomes would probably never match the large datasets available on single genes. A recent study based on the 21 available fungal genome sequences, including 14 yeast species, demonstrated that hemiascomycetous yeasts form a sister group of filamentous Euascomycetes with a basal position of the fission yeast *Schizosaccharomyces* within Ascomycota (Kuramae et al., 2006).

2.3 CURRENT CLASSIFICATION OF YEASTS

Molecular phylogenetic studies showed that yeasts are derived from two main monophyletic lineages of fungi: Ascomycota and Basidiomycota. It has, however, also become clear that yeast growth form was adapted by numerous unrelated groups of filamentous Ascomycota and Basidiomycota (Swann and Taylor, 1993, 1995; Kurtzman and Robnett, 1995). Many dimorphic fungi take yeast form in certain life stages or live as yeast under certain conditions. Even some fungi belonging to Zygomycota can form yeast cells anaerobically. Hence, yeasts are neither a natural nor a formal taxonomic group, but an assembly of unicellular growth forms shown by a range of unrelated fungi. It is a matter of

convention which fungus is considered yeast, and with the increasing number of recognized species, the boundaries of the group of yeasts become even wider. Treated among yeasts are some species, for example, *Eremothecium* and *Ascoidea*, which are permanently filamentous and do not form budding cells. Others, such as *Dipodascus* and *Geotrichum*, are also filamentous, breaking into unicellular parts by fission. In turn, dimorphic fungi and the so-called black yeasts, although showing typical budding, are excluded from yeasts. Incidentally, some of the latter species (e.g., *Aureobasidium pullulans*) commonly colonize the same habitats (leaves, fruits, etc.) as yeasts.

Phylogenetic analyses of the fungi based on rDNA and other gene sequences indicated that both Ascomycota and Basidiomycota comprise three classes each, and with the exception of Euascomycetes, yeasts can be classed in every one (*Oosporidium*, a peculiar endospore-forming red yeast, appears to be an Euascomycete).

2.3.1 Ascomycetous Yeasts

Phylogenetic analyses of 18S and D1/D2 domain of 26S rDNA concordantly demonstrate that the phylum Ascomycota is composed of three major lineages: the sister classes of Hemiascomycetes and Euascomycetes, as well as an assemblage of basal groups provisionally termed Archiascomycetes (Wilmotte et al., 1993; Kurtzman and Sugiyama, 2001). Hemiascomycetes includes budding yeasts and yeast-like forms without fruiting bodies; Euascomycetes comprises filamentous fungi that produce sexual spores in asci enclosed in fruiting bodies. The basal ascomycetes, for which the name Archiascomycetes was suggested (Nishida and Sugiyama, 1994), do not form a monophyletic line and represent various early forms. Among Euascomycetes no yeast forms can be found (except *Oosporidium*), and that class will not be discussed here.

2.3.1.1 Archiascomycetes

Following earlier proposals supported by 5S rRNA sequence differences, Berbee and Taylor (1995) demonstrated from 18S rDNA analysis that *Schizo. pombe*, *Taphrina deformans*, and *Pneumocystis carinii* form a basal group within Ascomycota, separate from the major lineages Hemiascomycetes and Euascomycetes. Nishida and Sugiyama (1994) expanded the group by including *Saitoella*, *Protomyces*, and further *Taphrina* species, and suggested the name Archiascomycetes to indicate the early evolutionary position of these yeasts and yeast-like forms, to which later an apothecial ascomyceta, *Neolecta*, was also added. Undoubtedly, these species show some traits (cell wall composition, fission or enteroblastic budding, urease reaction, etc.) intermediate between Ascomycetes and Basidiomycetes that may have originated before the divergence of the two major lineages of higher fungi (Sjamsuridzal et al., 1997). Nonetheless, the members of the group differ to a large extent from each other and do not appear to be of monophyletic descent. Hence, recently, the group is no longer considered a uniform class; instead, the lineages are separated at class and order level (Eriksson, 2006; Kurtzman and Fell, 2006) (Table 2.3).

The genus *Schizosaccharomyces* represents a classical group of yeasts, first described in 1893 by Lindner. The name refers to a special way of vegetative propagation—fission. Although *Schizosaccharomyces* species differ sharply from budding yeasts in means of propagation, they ferment vigorously and have always been considered yeast. Other properties (no chitin in cell wall, urease positivity, ubiquinone Q10) and rDNA sequence place them into a phylogenetic lineage far removed from budding yeasts (Sipiczki, 1995).

2.3.1.2 Hemiascomycetes, Saccharomycetes

Previously, all ascomycetous yeasts were placed in the class Hemiascomycetes and the order Endomycetales. However, the identity of the genus type *Endomyces* has become doubtful; hence, it has been replaced by *Saccharomyces*, providing the names for the family, order, and class as well.

TABLE 2.3
Classification of the Basal Ascomycota

Archiascomycetes (Taphrinomycotina)
Neolectomycetes
  Neolectales
    Neolectaceae
      Neolecta
Pneumocystidiomycetes
  Pneumocystidales
    Pneumocystidaceae
      Pneumocystis
Schizosaccharomycetes
  Schizosaccharomycetales
    Schizosaccharomycetaceae
      Schizosaccharomyces
Taphrinomycetes
  Taphrinales
    Protomycetaceae
      Protomyces
      Saitoella (A)[a]
    Taphrinaceae
      Taphrina
      Lalaria (A)[a]

[a] (A) anamorphic genus.

With new descriptions, the number of species belonging to Saccharomycetes is now greater than 1000, but the separation of diverse genera and assignment to families remain largely uncertain. Data from molecular phylogenetic analyses are often in conflict with morphological and physiological traits. Species relationships have been outlined from single gene (D1/D2 26S rDNA) analysis (Kurtzman and Robnett, 1995, 1998), but higher taxa could be revealed by multigene phylogenetic studies that are not yet completed to the whole group (Kurtzman and Robnett, 2003, 2006). Since the classification presented in the fourth edition of *The Yeasts* (Kurtzman and Fell, 1998), the number of genera increased from 55 to at least 71, and some 11–13 families appear to be circumscribed, though the family Candidaceae assembling anamorphic genera will not be maintained. Anamorphic species and genera are intended to be included together with related teleomorphs; however, these relationships, as well as the phylogenetic position of many ascomycetous yeasts, are unclear, and several genera are temporarily listed in Saccharomycetales as *incertae sedis* until their family belongings become cleared. Table 2.4 outlines the classification of Saccharomycetes as it is expected to take shape (Kurtzman and Fell, 2006; Kurtzman and Robnett, 2006).

The extensive efforts made over the years by Kurtzman and coworkers have been influencing and determining the taxonomy and classification of ascomycetous yeasts. It should be noted, however, that some diverging ideas have also been proposed. On the basis of the analysis of cell wall composition and complete 18S rDNA sequences, Prillinger et al. (2002) and Lopandic et al. (2005) divide Hemiascomycetes into four orders: Saccharomycetales, Dipodascales, Lipomycetales, and Stephanoascales, and also recognize a class of basal lineages under the name Protomycetes that may be the common ancestors for both Ascomycetes and Basidiomycetes.

2.3.2 Basidiomycetous Yeasts

Although the existence of some yeast that belong to Basidiomycetes was realized only in the 1960s, the interest in studying these yeasts, which are of economic, agricultural, and medical importance,

TABLE 2.4
Families and Genera in the Class Saccharomycetes, Order Saccharmycetales

Families	Genera	Families	Genera
Ascoideaceae	*Ascoidea*	Saccharomycetaceae	*Saccharomyces*
Cephaloascaceae	*Cephaloascus*		*Kazachstania*
Dipodascaceae	*Dipodascus*		*Kluyveromyces*
	Galactomyces		*Lachancea*
	Geotrichum (A)		*Nakaseomyces*
	Yarrowia (?)		*Naumovia*
Trichomonascaceae	*Trichomonascus*		*Tetrapisispora*
	Sporopachydermia		*Torulaspora*
	Sugiyamaella		*Vanderwaltiozyma*
	Wickerhamiella		*Zygosaccharomyces*
	Zygoascus		*Zygotorulaspora*
	Blastobotrys (A)	Pichiaceae	*Pichia*
	Trigonopsis (A)		*Saturnispora*
Eremotheciaceae	*Eremothecium*		*Komagataella*
Lipomycetaceae	*Lipomyces*		*Kodamaea*
	Dipodascopsis		*Kuraishia*
	Myxozyma (A)		*Ogataea*
	Zygozyma		*Nakazawaea*
Metschnikowiaceae	*Metschnikowia*		*Yamadazyma*
	Clavispora		*Issatchenkia*
	Kodamaea (?)		*Williopsis* (?)
	Aciculoconidium (A,?)		*Dekkera*
Saccharomycodaceae	*Saccharomycodes*		*Brettanomyces* (A)
	Hanseniaspora	Debaryomycetaceae	*Debaryomyces*
	Nadsonia (?)		*Citeromyces*
	Kloeckera (A)		*Lodderomyces*
	Wickerhamia (?)		*Schwanniomyces*
Saccharomycopsidaceae	*Saccharomycopsis*		*Wickerhamia*
	Ambrosiozyma (?)		*Hyphopichia*
	Botryozyma (A)	Candidaceae	*Candida* (A)

Note: (A): anamorph genus; (?): uncertain position.
Sources: Adapted from Kurtzman, C. P. and Robnett, C. J. (1998) A. van Leeuwenhoek 73:331–371. From Kurtzman, C. P. and Sugiyama, J. (2001) *In*: The Mycota. Vol. VII Part A. Systematics and Evolution (eds. McLaughlin, D. J., McLaughlin, E. G., and Lemke, P. A.). Springer Verlag, Berlin. pp. 179–200; From Kurtzman, C. P. (2003) FEMS Yeast Res. 4:233–245; From Kurtzman, C. P. (2006a) *In*: Food Spoilage Microorganisms (ed. Blackburn, C. W.). CRC Woodhead Publ. Cambridge, England. pp. 28–54.

increased in parallel with the growing number of species with recognized basidiomycetous affinity (Fell et al., 2000, 2001; Scorzetti et al., 2002). Currently, about 300 yeast species in more than 60 genera have been described. These belong to all three classes recognized among Basidiomycota fungi: Urediniomycetes, Ustilaginomycetes, and Hymenomycetes (Swann and Taylor, 1995).

Characteristic morphological features for basidiomycetous yeasts such as shape of basidia, probasidia (teliospores), ballistoconidia, dikaryotic hyphae, and clamp connections generally do not correlate with molecular sequence data, whereas cell wall composition and septal ultrastructure corroborate molecular phylogenetic grouping (Table 2.5). Basidiomycetous yeasts are all positive in urease reaction and Diazonium Blue B staining. Buds (blastoconidia) develop enteroblastically and generally on one or both poles of cells, sometimes on stalks. In *Trichosporon*, cells also

TABLE 2.5
Taxonomic Characteristics of Selected Basidiomycetous Yeasts

Species	Septal Pore	Xylose	Ubiquinone Type in Cell Wall	Ballistoconidium	Teliospore	Basidium Type
Urediniomycetes						
Leucosporidium scottii	Simple	−	9, 10	−	+	Phragmo
Rhodosporidium toruloides	Simple	−	9	−	+	Phragmo
Sporidiobolus johnsonii	Simple	−	10	+	+	Phragmo
Kondoa malvinella	Simple	−	10	−	+	Phragmo
Sporobolomyces singularis	Simple	−	10(H_2)	+	−	−
Hymenomycetes						
Cystofilobasidium capitatum	Doli	+	8	−	+	Holo
Mrakia frigida	Doli	+	8	−	+	Holo
Filobasidium floriforme	Doli	+	10	−	−	Holo
Filobasidiella neoformans	Doli	+	10	−	−	Holo
Bullera crocea	Doli	+	10	+	−	Phragmo
Ustilaginomycetes						
Pseudozyma fusiformata	Micro	−	10	−	−	−
Malassezia furfur	Micro	−	9	−	−	−

reproduce by fission (arthroconidiogenesis). Special buds, called ballistoconidia, are discharged actively. In teleomorphic species, the haploid monokaryotic yeast phase alternate with dikaryotic hyphal phase, usually with clamp connections. Life cycle and formation of basidia differ in homothallic and heterothallic species. Basidia often develop from thick-walled teliospores (probasidia).

Extensive rDNA databases of ITS and D1/D2 sequences contributed greatly to exploring the taxonomic relationships among basidiomycetous yeast species and to establishing connections between anamorphic and teleomorphic forms. Large genera, such as *Rhodotorula*, *Cryptococcus*, and *Sporobolomyces*, comprising anamorphs of similar phenotypic characters, turned out to be phylogenetically diverse; species may belong to 3–4 different higher taxa. In Urediniomycetes, notable for plant pathogenic rust fungi, yeasts belong to four orders. Yeasts associated with Ustilaginomycetes belong to two lineages, both related to the smuts, which are also serious plant pathogens. Among Hymenomycetes, yeasts are found in four orders related to the heterobasidiomycete jelly fungi; the homobasidiomycete lineage does not contain yeast forms. Table 2.6 lists genera and orders comprising yeast forms in classes of Basidiomycota.

2.4 OVERVIEW OF SELECTED YEAST TAXA

In the following, only the families, genera, and species of relevance to the food industry will be discussed in more detail. They will be treated from the taxonomic point of view; however, of the large number of species, a few of the most common ones are mentioned with brief reference to their importance in foods. More references are found in the sections and tables of food types in Chapter 7.

2.4.1 SACCHAROMYCETACEAE

It is proper to start a detailed discussion with yeasts belonging to *Saccharomyces* and related genera, members of which play an important role in human life in the production of bread, beer, wine, and other fermented foods, as well as causing spoilage in various food products.

The history of yeast science, zymology, starts with the discovery of *S. cerevisiae* and other species of the genus in the mid-nineteenth century (Barnett, 2004). Since that time, the adventurous

TABLE 2.6
Classification of Basidiomycetous Yeasts

Urediniomycetes	Ustilaginomycetes	Hymenomycetes
Agaricostilbales	Ustilaginales	Cystofilobasidiales
Kondoa	*Pseudozyma* (A)	*Xanthophyllomyces*
Kurtzmanomyces (A)	*Rhodotorula* (A)	*Phaffia* (A)
Sporobolomyces (A)	Microstromatales	*Cryptococcus* (A)
Sterignatomyces (A)	*Rhodotorula* (A)	Filobasidiales
Microbotryales	*Sympodiomycopsis* (A)	*Filobasidium*
Leucosporidium	Malasseziales	Trichosporales
Rhodosporidium	*Malassezia* (A)	*Trichosporon* (A)
Rhodotorula (A)		*Cryptococcus* (A)
Sporobolomyces (A)		Tremellales
Erythrobasidiales		*Bulleromyces*
Erythrobasidium		*Bullera* (A)
Rhodotorula (A)		*Cryptococcus* (A)
Sporobolomyces (A)		*Fellomyces* (A)
Sporidiobolales		*Dioszegia* (A)
Rhodosporidium		*Filobasidiella*
Rhodotorula (A)		
Sporidiobolus		
Sporobolomyces (A)		

Note: (A): anamorphic genus.

classification of the species and genera has resulted in numerous rearrangements and nomenclatural changes that probably come to an end with establishing phylogenetic grounds (Barnett, 1992; Vaughan-Martini and Martini, 1995; Deák and Beuchat, 1996; Raineri et al., 2003) (Figure 2.2). Reviews on *Saccharomyces* and related genera have been published by Oda and Ouchi (2000), Viljoen and Heard (2000), Fleet (2006), and Kurtzman (2006a).

Drastic changes in the treatment of *Saccharomyces* and related genera can be followed in the successive editions of the taxonomic monographs (Lodder, 1970; Kreger-van Rij, 1984) (Table 2.7). On the other hand, several *Saccharomyces* species recognized earlier were amalgamated into *S. cerevisiae* by Yarrow (1984) (Table 2.8). Further on, *Saccharomyces* species have become divided into two groups in strict and broad sense (*sensu stricto* and *sensu lato*). The former group comprises *S. cerevisiae* and species most closely related to it, that is, *S. paradoxus*, *S. pastorianus*, and *S. bayanus*, to which, more recently, newly described species have been added (*S. cariocanus*, *S. kudriavzevii*, and *S. mikatae*). The *Saccharomyces sensu lato* group has also increased in size over the years, and included at least a dozen species (Table 2.9). It was suggested that two of these, *S. telluris* and *S. transvaalensis*, be placed in separate genera (*Arxiozyma* and *Pachytichospora*, respectively), whereas the newly described species were added to the group (*S. kunashirensis*, *S. martiniae*, *S. naganishii*, *S. humaticus*, *S. yakushimaensis*, and *S. bulderi*). *S. kluyveri* also appeared as an outlier of the genus.

Genetic and molecular evidence has accumulated, suggesting that both *sensu stricto* and *sensu lato* groups overstrain its frame. On the one hand, former species such as *S. uvarum* and *S. carlsbergensis* reduced to synonymy with *S. cerevisiae* have been shown to be proper and distinct species, whereas *S. pastorianus*, *S. bayanus*, and others proved to be heterogenous hybrids (Raineri et al., 1999; Naumov et al., 2000; Pulvirenti et al., 2000; de Barros Lopes et al., 2002; Antunovics et al., 2005; Nguyen and Gaillardin, 2005) (Figure 2.3). However, *S. boulardii*, a type of yeast used as a biotherapeutic agent and claimed to be a separate species, was shown to belong to *S. cerevisiae* (van der Aa Kühle and Jespersen, 2003).

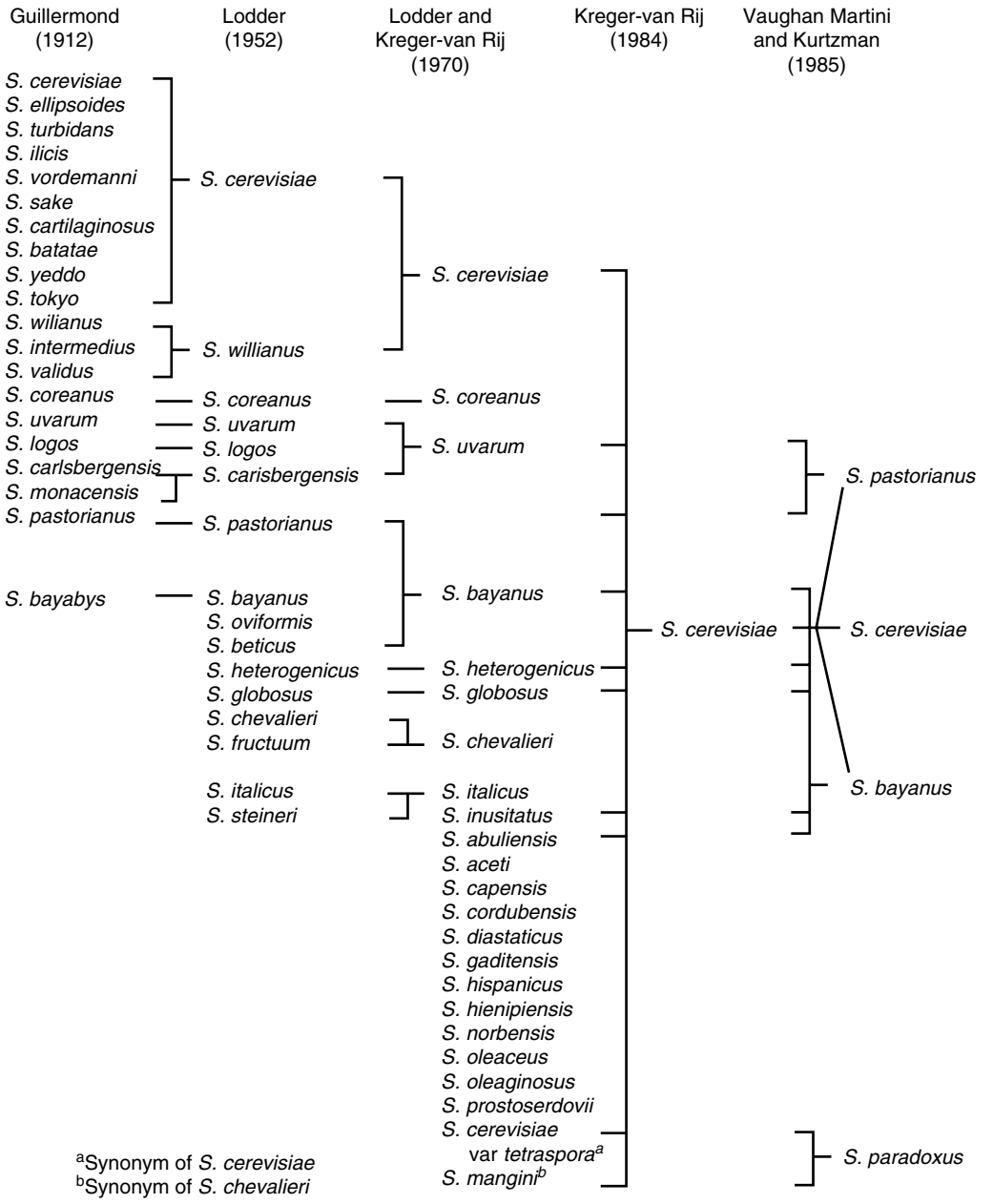

FIGURE 2.2 Taxonomic and nomenclatural adventures of *Saccharomyces sensu stricto*. (From Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL. pp. 12–14.) Three new species added to *Saccharomyces sensu stricto*: *S. cariocanus*, *S. kudriavzevii*, and *S. mikatae* (Naumov et al., 2000).

Species of *Saccharomyces sensu lato*, on the other hand, were clearly intermixed with members of various other genera, which appeared heterogenous themselves. This was the case, in particular, with the genera *Zygosaccharomyces* and *Kluyveromyces* (Belloch et al., 1998, 2000, 2002; James and Stratford, 2003; Kurtzman and James, 2006). Within these genera, as well as in *Torulaspora*, many changes have been made at the species level (Table 2.10). Former species have been reduced to synonymy as in *Klu. marxianus* and *Tsp. delbrueckii*; others have been split and reestablished

TABLE 2.7
Saccharomyces* Species Transferred to the Genera *Zygosaccharomyces* and *Torulaspora

Species by Lodder (1970)	Species by Kreger-van Rij (1984)
S. amurcae	*Zygo. cidri*
S. cidri	*Zygo. cidri*
S. bisporus	*Zygo. bisporus*
S. eupagycus	*Zygo. florentinus*
S. florentinus	*Zygo. florentinus*
S. microellipsodes	*Zygo. microellipsoides*
S. montanus	*Zygo. fermentati*
S. mrakii	*Zygo. mrakii*
S. rouxii	*Zygo. rouxii*
S. delbrueckii	*Tsp. delbrueckii*
S. inconspicuus	*Tsp. delbrueckii*
S. saitoanus	*Tsp. delbrueckii*
S. rosei	*Tsp. delbrueckii*
S. vafer	*Tsp. delbrueckii*
S. kloeckerianus	*Tsp. globosa*
S. pretoriensis	*Tsp. pretoriensis*

TABLE 2.8
Former *Saccharomyces* Species and Synonyms Amalgamated into *S. cerevisiae*

Former *Saccharomyces* Species	
S. aceti	*S. globosus*
S. bayanus (beticus, cheresiensis, oviformis, pastorianus)	*S. heterogenicus*
S. capensis	*S. hienipiensis*
S. cerevisiae (ellipsoideus, intermedius, vini, willianus)	*S. hispalensis*
S. chevalieri (lindneri, italicus)	*S. inusitatus*
S. cordubensis	*S. norbensis*
S. coreanus	*S. oleaceus*
S. diastaticus	*S. oleaginosus*
S. gaditensis	*S. prostoserdovii*
	S. steineri
	S. uvarum (carlsbergensis, logos)

Source: From Yarrow, D. (1984) *In*: The Yeast, a Taxonomic Study, 3rd ed. (ed. Kreger-van Rij, N. J. W.). Elsevier, Amsterdam. pp. 379–395.

as independent species (e.g., *Klu. lactis*, *Tsp. franciscae*, *Zygo. mellis*). New species have also been described (e.g., *Zygo. lentus*, *Zygo. kombuchaensis*, *Klu. nonfermentans*, *Klu. piceae*), and a new genus has been established (*Tetrapisispora*) (Ueda-Nishimura and Mikata, 1999). Molecular phylogenetic studies with 18S, ITS, and 26S rDNA sequences provided evidence that the genera traditionally defined on phenotypic characters do not correspond to phylogenetic lineages. For example, the outlying *S. kluyveri* showed relatedness over generic boundaries to both *Klu. thermotolerans* and *Zygo. fermentati.* The situation has clearly ripened for decision.

Based on multigene sequence analyses, Kurtzman (2003) and Kurtzman and Robnett (2003) reassigned several species among the currently accepted genera and proposed five new genera. The

TABLE 2.9
Reassignment of *Saccharomyces* Species Proposed by Kurtzman (2003)

Genus *Saccharomyces*
- *S. cereviaiae*
- *S. paradoxus*
- *S. bayanus*
- *S. pastorianus*
- *S. mikatae*
- *S. cariocanus*
- *S. kudriavzevii*

Genus *Naumovia*
- *S. castellii*
- *S. dairiensis*

Genus *Lachancea*
- *S. kluyveri*

Genus *Kazachstania*
- *S. exiguus*
- *S. barnettii*
- *S. kunashirensis*
- *S. martiniae*
- *S. rosinii*
- *S. servazzii*
- *S. spencerorum*
- *S. transvaalensis*
- *S. turicensis*
- *S. unisporus*

Source: Proposed by Kurtzman, C. P. (2003) FEMS Yeast Res. 4:233–245.

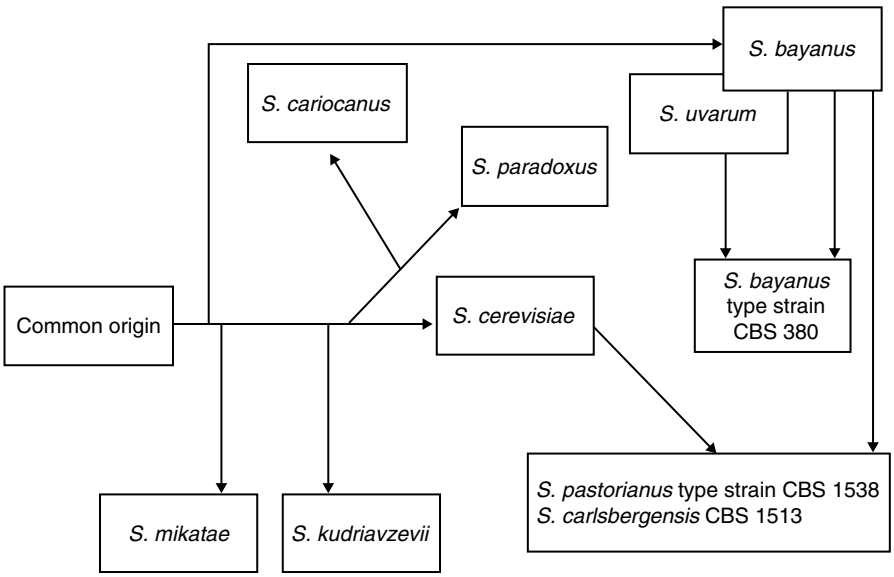

FIGURE 2.3 Origin of *Saccharomyces senus stricto* species and hybrids. (From Raineri, S., Zambonelli, C., and Kaneko, Y. (2003) J. Biosci. Bioeng. 96:1–9. With permission from Elsevier.) Recently, natural hybrids between *S. cerevisiae* × *S. bayanus*, *S. cerevisiae* × *S. kudriavzevii*, and *S. cerevisiae* × *S. bayanus* × *S. kudriavzevii* have been identified by molecular characterization from fermenting wine (González et al., 2006).

phylogenetic structure of the re-evaluated family of Saccharomycetaceae, as shown in Figure 2.4, comprises 11 genera. In this current concept, the genus *Saccharomyces* comprises only the former *sensu stricto* species. Most of *sensu lato* species, together with some former *Kluyveromyces*, are now assigned to a separate genus, called *Kazachstania*, for rules of nomenclatural priority. New genera *Naumovia* and *Nakaseomyces* also include some former *Saccharomyces* and *Kluyveromyces* species, as well as a few *Candida* anamorphs. *Tetrapisispora* has been enlarged by including former members of *Kluyveromyces*; it is closely related to two original *Kluyveromyces* species, now assigned to a

TABLE 2.10
Reassignment of *Zygosaccharomyces* and *Kluyveromyces* Species

Former *Zygosaccharomyces*	Former *Kluyveromyces*
Genus *Zygosaccharomyces*	**Genus *Kluyveromyces***
Zygo. bailii	*Klu. aestuarii*
Zygo. bisporus	*Klu. dobzhanskii*
Zygo. kombuchaensis	*Klu. marxianus*
Zygo. lentus	*Klu. nonfermentans*
Zygo. mellis	*Klu. lactis*
Zygo. rouxii	*Klu. wickerhamii*
Genus *Zygotorulaspora*	Genus *Kazachstania*
Zygo. florentinus	*Klu. africanus*
Zygo. mrakii	*Klu. lodderae*
Genus *Lachancea*	*Klu. piceae*
Zygo. cidri	Genus *Nakaseomyces*
Zygo. fermentati	*Klu. bacillisporus*
Genus *Torulaspora*	*Klu. delphensis*
Zygo. microellipsoides	Genus *Tetrapisispora*
	Klu. blattae
	Klu. phaffii
	Genus *Vanderwaltozyma*
	Klu. polysporus
	Klu. yarrowii

Source: Proposed by Kurtzman, C. P. (2003) FEMS Yeast Res. 4:233–245.

new genus, *Vanderwaltiozyma*. The present genus *Kluyveromyces* is centered on *Klu. marxianus* and *Klu. lactis* with four more species. The former genus *Zygosaccharomyces* is retained for species *Zygo. bailii*, *Zygo. rouxii*, and four more species; two others have been placed in a new genus under the name *Zygotorulaspora*, representing a transition to the closely related genus *Torulaspora* that also includes the former *Zygo. microellipsoides*. A new genus, *Lachancea*, now comprises outlier species of former *Saccharomyces*, *Kluyveromyces*, and *Zygosaccharomyces*.

Curiously, in the phylogenetic tree, basal to all these genera related to *Saccharomyces* and forming the family Saccharomycetaceae is the genus *Eremothecium*, with plant pathogenic species, all filamentous yeasts with true hyphae but most of them forming no buds. They are considered to form a separate family, Eremotheciaceae (Kurtzman and Fell, 1998; Kurtzman and Robnett, 1998), but Prillinger et al. (1997), overruling morphological traits by molecular sequence evidence, placed them within the family Saccharomycetaceae. Also basal to genera of Saccharomycetaceae are the genera *Hanseniaspora*, *Kloeckera*, and *Saccharomycodes*, all characterized by species budding bipolarly, forming a well-supported phylogenetic lineage that is generally treated as a separate family, Saccharomycodaceae.

2.4.2 Saccharomycodaceae

Five genera of yeasts, *Nadsonia*, *Wickerhamia*, *Hanseniaspora*, *Kloeckera*, and *Saccharomycodes*, are characterized by a special mode of bipolar budding, lending the cells a peculiar apiculate shape. Phylogenetic analyses showed, however, that this morphological feature does not denote common evolutionary origin. On the contrary, *Nadsonia* is related to Dipodascaceae and *Wickerhamia* to Debaryomycetaceae, whereas *Hanseniaspora–Kloeckera–Saccharomycodes* are associated with Saccharomycetaceae (Kurtzman and Robnett, 1998).

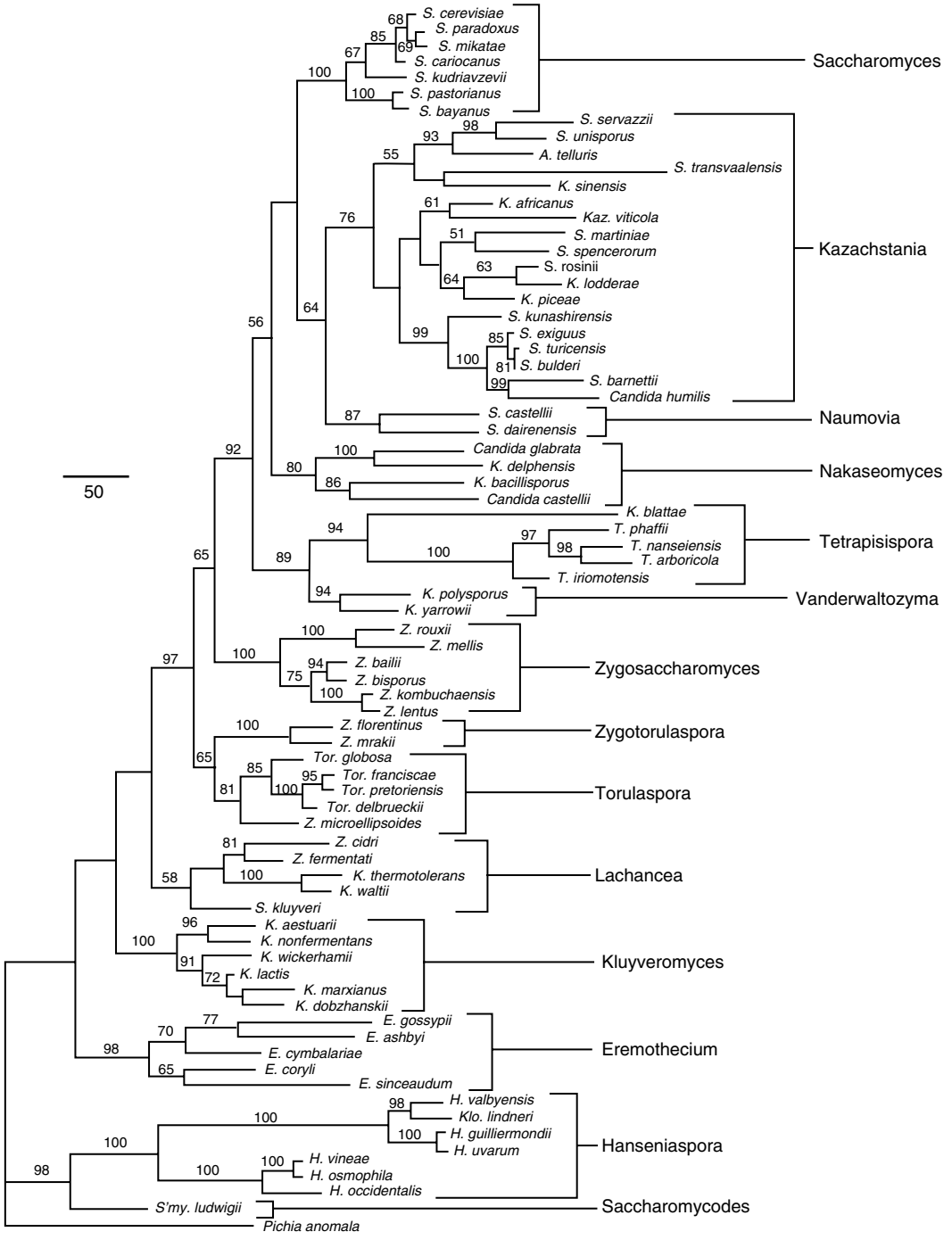

FIGURE 2.4 Phylogenetic structure of Saccharomycetaceae. (From Kurtzman, C. P. and Robnett, C. J. (2003) FEMS Yeast Res. 3:417–432. With permission of Blackwell Publ. Ltd.)

Apiculate yeasts were among the first yeast species discovered in fermenting juice by 1870. Their taxonomy has been the subject of many studies. Molecular studies eventually cleared their relationships, and established teleomorph–anamorph connections between *Hanseniaspora* and *Kloeckera* (Smith, 1984). Although four new species were recently added to *Hanseniaspora* (Cadez et al., 2003),

TABLE 2.11
Currently Described *Hanseniaspora* Species and Their *Kloeckera* Anamorphs

Hanseniaspora	*Kloeckera*
Hsp. guilliermondii	*Klc. apis*
Hsp. occidentalis	*Klc. javanica*
Hsp. osmophila	*Klc. corticis*
Hsp. uvarum	*Klc. apiculata*
Hsp. valbyensis	*Klc. japonica*
Hsp. vineae	*Klc. africana*
Hsp. meyeri	*Klc. lindneri*
Hsp. clermontiae	
Hsp. lachancei	
Hsp. opuntiae	

Kloeckera lindneri remained without teleomorphic counterpart (Table 2.11). Among *Hanseniaspora* and *Kloeckera*, two groups could be outlined; one (comprising *Hsp. occidentalis*, *Hsp. osmophila*, and *Hsp. vineae*) was proposed to be placed in a separate genus *Kloeckeraspora* (Yamada et al., 1992). However, molecular phylogenetic data are not supporting this division (Boekhout et al., 1994; Esteve-Zarzoso et al., 2001), and even reveal four clusters (Cadez et al., 2002). *Saccharomycodes ludwigii* represents a separate basal line to *Hanseniaspora–Kloeckera* (Kurtzman and Robnett, 2003).

Hanseniaspora (*Kloeckera*) species are commonly found in soil, leaves, and fruits; they start alcoholic fermentation of fruit extracts and can also spoil beverages. *S'codes ludwigii* is mainly confined to tree exudates and saps, also occurring in fermenting cider.

2.4.3 Pichiaceae

Pichia is the second largest yeast genus, including more than 100 species. Both phylogenetically and phenotypically diverse, including species with round and hat-shaped spores alike, fermenting strongly or not at all, assimilating nitrate or not, containing types 7, 8, or 9 ubiquinone. Kurtzman (1984) showed 68–75% DNA homology between species of *Hansenula* and *Pichia*, and unified the two genera in *Pichia* considering nitrate assimilation or spore shape poor differentiating traits. Nonetheless, some species of smooth, spherical spores have been reassigned to *Debaryomyces*, others of rough and spherical spores and Q7 to *Issatchenkia*, still others with Saturn-shaped spores to *Williopsis*, *Saturnispora*, and recently to *Kregervanrija* (Kurtzman, 2006b).

Meanwhile, Yamada et al. (1994a), based on partial sequencing of 18S and 26S rRNAs, divided the nitrate-assimilating species with hat-shaped spores into seven groups, reinstated the former genus *Hansenula*, and formed three new genera, *Ogataea* (with *P. minuta*), *Kuraishia* (with *P. capsulata*), and *Nakazawaea* (with *P. holstii*); to these were later added *Komagataea* (*Williopsis pratensis*) and *Komagataella* (*P. pastoris*), and also the genus *Yamadazyma* (e.g., *P. guilliermondii*). Other *Pichia* species have been reassigned to new genera, namely, *Phaffomyces* (e.g., *P. opuntiae*) and *Starmera* (e.g., *P. amethionina*) (Yamada et al., 1997). With the exception of the latter two genera, Kurtzman (1998) did not accept these proposals, even while the genus *Pichia* was clearly polyphyletic.

From the analysis of partial sequences in 26S rDNA, Kurtzman and Robnett (1998) provided evidence that *Pichia* and related yeast genera, including *Dekkera*, and such monotypic genera as *Pachysolen*, *Citeromyces*, and *Lodderomyces*, form at least five or six distinct phylogenetic clades; however, formal taxonomic proposals are yet to be made. The family Pichiaceae may be restricted to genera *Pichia pro parte*, including the type species *P. membranifaciens* and the distantly related

P. (*Komagataella*) *pastoris*, as well as species of *Saturnispora*, *Issatchenkia*, *Dekkera*, and its anamorphs *Brettanomyces* (Kurtzman and Fell, 2006). Several *Candida* species (e.g., *C. inconspicua*, *C. diversa*, *C. vini*) are clearly associated with this clade; *C. vini* is the anamorph of *Kregenvanrija fluxuum*, another new clade of the former *Pichia* complex (Kurtzman, 2006b).

As established by Kurtzman and Robnett (1998), the clade of *P. anomala* (former type species of the genus *Hansenula*) comprises several nitrate-positive *Pichia* and *Candida* species (e.g., *C. norvegica*), as well as some species of the genus *Williopsis*, including *W*.(*Komagataea*) *pratensis*, which was shown to be phylogenetically heterogenous (James et al., 1998). Another lineage appears around *Pichia* (*Ogataea*) species, also including several *Candida* (e.g., *C. boidinii*), and distantly related to *P*. (*Kuraishia*) *capsulata*, *P*. (*Nakazawaea*) *holstii*, *Citeromyces matritensis*, *Pachysolen tannophilus*, and *Ambrosiozyma platypodis*. ITS-RFLP gives identical profiles between teleomorphic and anamorphic states of several *Pichia* and *Candida* species pairs (Villa-Carvajal et al., 2006).

The genus *Dekkera* (anamorph *Brettanomyces*), although evidently related to *Pichia*, represents species of unique metabolic properties regulated by negative Pasteur effect (Custers effect), resulting in the production of acetic acid. The two teleomorphic species (*Dek. anomala*, *Dek. bruxellensis* with respective anamorphs) and three anamorphic *Brettanomyces* without known teleomorphs (*Bret. naardenensis*, *Bret. custersianus*, and *Bret.* [*Eeniella*] *nana*) are important spoilage yeasts; reviewed recently by Loureiro and Malfeito-Ferreira (2006).

2.4.4 *Debaryomyces* and Related Genera

The genus *Debaryomyces* has been created around the species with some intermediate phenotypic properties between *Torulaspora* and *Pichia*. Type species is *Db. hansenii*, one of the most frequent yeasts occurring in foods and causing spoilage though only weekly or not fermenting. Over the years, more species have been added, enlarging the genus, which now includes some 20 species.

Analysis of complete 18S rRNA and D1/D2 rDNA domain of 26S rDNA sequences (Cai et al., 1996; Kurtzman and Robnett, 1997, 1998) showed that species of *Debaryomyces* formed a distinct phylogenetic lineage tightly associated with *Lodderomyces elongisporus*, the group of *C. parapsilosis*, *C. tropicalis*, and *C. albicans*, as well as several other *Candida* species, former *Schwanniomyces* and *Pichia* species transferred to *Debaryomyces* (*Db. occidentalis*, *Db. carsonii*, and *Db. etchellsii*), and other *Pichia* assigned to *Yamadazyma*, such as *P. guilliermondii*. Basal to these clades are *Wickerhamia fluorescens* and *P*. (*Hyphopichia*) *burtonii.*

The close association of these species suggests that a monophyletic higher taxon can be formed around the core genus *Debaryomyces*. *Db. hansenii* is one of the most common foodborne yeast. Although it ferments weakly or not at all, its psychrotolerance and halotolerance render it a serious spoilage organism in various foods (Casas et al., 2004; Prista et al., 2005). On the other hand, it is a potential adjunct for the ripening of cheeses and dry sausages (Petersen et al., 2002; Flores et al., 2004). *Db. carsonii* occurs in wines. Various molecular techniques have been developed for discrimination of the *Debaryomyces* species (Ramos et al., 1998; Petersen and Fespersen, 2004; Martorell et al., 2005; Quirós et al., 2006).

2.4.5 Metschnikowiaceae

Metschnikowia species are characterized by the formation of long needle-shaped spores, in some species originating from large spherical chlamydospores. The genus is not related phylogenetically to *Nematospora* with similar spindle-shaped spores (Yamada et al., 1994b). With the new description, the number of species in the genus almost doubled. According to partial rDNA sequences, the species form two clusters that correspond to their aquatic and terrestrial habitats (Mendonca-Hagler et al., 1993). The latter species live in association with plants, from where they spread to fruits and various foods.

The phylogenetic lineage also comprises a number of *Candida* species, some of which are strongly xerophilic (*C. apicola*, *C. etchellsii*, and *C. lactis-condensi*). Also related are *Pichia* (*Kodamaea*) *ohmeri* and *Clavispora lusitaniae*, as well as *Yarrowia lipolytica*.

2.4.6 Trichomonascaceae

This family has been recently described (Kurtzman and Robnett, 2006) around phylogenetically divergent, morphologically varied teleomorphic (*Stephanoascus*, *Zygosacus*, *Wickerhamiella*) and anamorphic genera (*Blastobotrys*, *Trigonopsis*, *Arxula*). Among members of this group, there are species forming hyphae, pseudohyphae, arthroconidia, and budding cells; if ascospores produced, asci formed by various particular mode on hyphae. *Stephanoascus* proved to be polyphyletic and reassigned to genera *Trichomonascus* and *Sugiyamaella*. *Wickerhamiella* has the smallest cells among yeasts (1.5–3.5 μm), sporulating after conjugation. Several *Candida* species, such as *C. versatilis*, are closely related to *Wickerhamiella*. Other *Candida* species (e.g., *C. cantarellii* and *C. vinaria*) have been put in the genus *Trigonopsis* on the basis of phylogenetic similarity. They also share fermenting must as common habitat, although differ from the unique shape of *Trigonopsis*.

2.4.7 Saccharomycopsidaceae

A group of hyphal yeasts also producing budding cells and sometimes arthroconidia has been divided into several genera on the basis of various modes of spore formation. The frequent changes of different names have ended when it was shown from rDNA analysis that *Arthroascus*, *Endomycopsella*, *Guilliermondella*, and *Botryoascus* are all synonyms of *Saccharomycopsis* (Kurtzman and Robnett, 1995). Some species (e.g., *S'copsis fibuligera*) have strong amylolytic activity and are used in the production of fermented foods in the Orient; by the same token, they cause also spoilage.

2.4.8 Dipodascaceae

A family of yeast-like organisms with true hyphae breaking into arthroconidia, budding cells, and fermentation are usually absent. The two genera, *Dipodascus* and *Galactomyces*, though separate into two clades on the sequence divergence of D1/D2 domain of 26S rDNA, probably belong to a single genus (Kurtzman and Robnett, 1995). Both have *Geotrichum* anamorphs, of which *Geo. candidum* is a common spoilage species, with potential application in biotechnology.

Ascoidea, also a hyphal, yeast-like genus, is closely related, but is usually considered to be a separate family, Ascoideaceae. Interestingly, *Nadsonia*, a genus of bipolarly budding yeasts, shows relatedness to this family.

2.4.9 Lipomycetaceae

Divided into several teleomorphic and anamorphic genera, the family Lipomycetaceae appears to be a monophyletic natural group sharing both molecular and phenotypic unifying characters (van der Walt, 1992). Notable are the production of long-chain fatty acids and extracellular amyloid polysaccharides, lending a mucous appearance to colonies. Many are soil organisms, and may occur as adventitious contaminants on foods.

2.4.10 Candidaceae

The family has been used as a depository for all anamorphic ascomycetous yeasts (Kurtzman and Fell, 1998) and comprises various heterogenous genera, such as *Brettanomyces*, *Candida*, *Geotrichum*, *Kloeckera*, and others. Many anamorphic species have teleomorphic counterparts, and the current taxonomic trend is that classification should be made according to the phylogenetic relationships

TABLE 2.12
Anamorph *Canadida* Species Belonging to Various Teleomorphs

Anamorphs	Teleomorphs
C. colliculosa	*Torulaspora delbrueckii*
C. domercqiae	*Wickerhamiella domercqiae*
C. edax	*Sugiyamaella smithiae*
C. famata	*Debaryomyces hansenii*
C. globosa	*Citeromyces matritensis*
C. guilliermondii	*Pichia guilliermondii*
C. hellenica	*Zygoascus hellenicus*
C. holmii	*Kazachstania exiguus*
C. kefyr	*Kluyveromyces marxianus*
C. krusei	*Issatchenkia orientalis*
C. lambica	*Pichia fermentans*
C. lipolytica	*Yarrowia lipolytica*
C. lusitaniae	*Clavispora lusitaniae*
C. pelliculosa	*Pichia anomala*
C. pulcherrima	*Metschnikowia pulcherrima*
C. sorbosa	*Issatchenkia occidentalis*
C. spherica	*Kluyveromyces lactis*
C. utilis	*Pichia jadinii*
C. valida	*Pichia membranifaciens*
C. variabilis	*Hyphopichia burtonii*

among teleomorphic forms. Accordingly, many genera listed previously in Candidaceae have been removed from it and joined to the respective teleomorphic genera. In this section, several examples have previously been given. Eventually, the genus *Candida* only remained, including about 200 species, several of which with known teleomorphic forms (Table 2.12), still many more without them. Even some of these can be classified into other genera, thanks to the recent progress of phylogenetic analysis applying rDNA and certain protein gene sequences. The genus has recently been reviewed by Daniel (2003) and Deák (2006).

The most thorough phylogenetic reclassification of *Candida* species without known teleomorphs has been made by the analysis of the D1/D2 domain of 26S rDNA (Kurtzman and Robnett, 1997, 1998). These studies revealed the polyphyletic nature of *Candida*, several species of which could be assigned to different main phylogenetic lineages as discussed above under various families. More recently, on the basis of multigene sequence analysis, some *Candida* species have been allocated in *Saccharomyces* and related genera too (Kurtzman and Robnett, 2003). Daniel et al. (2001), using partial sequences of the actin gene, distinguished four groups among some 30 *Candida* and related teleomorphs, largely in conformance to phylogenic lineages generated from rDNA sequences. An exception is the position of the *Metschnikowia*/*Clavispora* clade, which appears related to the *Debaryomyces* lineage rather than separated more distantly.

Another approach for the phylogenetic grouping of *Candida* has been made by Suzuki and Nakase (1998, 1999, 2002) and Suzuki et al. (1999). The Japanese authors examined the cell wall composition, ubiquinone type, and 18S rDNA sequences for almost all species. They arrived at conclusions largely similar to those made by Kurtzman and Robnett (1998). Four phylogenetically distinct clusters were established according to the presence or absence of galactose in cell wall (I: galactose absent; II and III: galactose predominant; and IV: galactose present); and within these clusters, subgroups were formed based on the types of ubiquinone (Table 2.13). Some *Candida* species are related to teleomorphic counterparts and other ascomycetous genera. The four

TABLE 2.13
Grouping of *Canadida* Species and Some Other Genera, Based on 18s rDNA Sequences, Cell Wall Sugars, and Ubiquinone Types

Group I—No Galactose	Group II—With Galactose	Group III—With Galactose
Q6 *C. holmii*	Q8 *C. magnoliae*	Q9 *C. ciferrii*
C. glabrata	*C. etchellsii*	*C. tepae*
C. colliculosa	*C. lactis-condensi*	*C. steatolytica*
Q7 *C. utilis*	*C. stellata**	
C. norvegica	Q9 *C. versatilis*	
C. pelliculosa	*C. azyma*	**Group IV—with galactose**
C. valida	Q9 *C. ingens*	
C. krusei	*C. insectalens**	Q9 *C. cantarellii*
Q8 *C. lusitaniae*	Q8 *C. incommunis*	*C. vinaria*
C. globosa	*Dipodascus*	*Dipodascopsis*
Q9 *C. albicans*	*Galactomyces*	*Lipomyces*
C. tropicalis	*Yarrowia*	*Myxozyma*

* Without galactose.

Sources: Adapted from Suzuki, M. and Nakase, T. (1998) Microbiol. Cult. Coll. 14:49–62. From Suzuki, M. and Nakas, T. (1999) J. Gen. Appl. Microbiol. 45:239–246; From Suzuki, M. and Nakase, T. (2002) J. Gen. Appl. Microbiol. 48:55–65; From Suzuki, M., Suh, S.-O., Sugita, T., and Nakase, T. (1999) J. Gen. Appl. Microbiol. 45:229–238.

clusters are tentatively marked with names of order rank (I: *Saccharomycetales*; II: *Dipodascales*; III: *Stephanoascales*; IV: *Lipomycetales*); however, no formal proposal has been made (M. Suzuki, personal communication).

2.4.11 Basidiomycetous Yeasts

The classification of basidiomycetous yeasts divided into three classes is outlined in Section 2.3.2. From Table 2.6, it can also be seen that the majority of basidiomycetous yeasts exist in anamorphic forms; the most common genera (e.g., *Rhodotorula*, *Cryptococcus*) may belong to different teleomorphic forms. Moreover, based on rDNA sequence data, many anamorphic genera are polyphyletic and include species belonging to different phylogenetic clades. Hence, the previous formal families of anamorphic yeasts (Sporobolomycetaceae and Cryptococcaceae) (Boekhout et al., 1998) have no taxonomic standing. However, it is convenient to discuss basidiomycetous yeasts frequently occurring in foods according to these groups.

The family Sporobolomycetaceae includes anamorphic forms of both Urediniomycetes and Ustilaginomycetes. The two most important genera are *Sporobolomyces*, comprising species that form ballistoconidia, and *Rhodotorula*, most species of which are red pigmented. Several former *Candida* species proved to be of basidiomycetous nature and were reassigned to *Rhodotorula*; thus, the genus now includes cream- and yellow-colored and pseudohyphal species as well (e.g., *Rho. fragaria*, *Rho. bacarum*). The most frequent species in various foods are *Rho. mucilaginosa*, *Rho. glutinis*, and *Rho. minuta*, as well as *Spb. roseus* and *Spori. salmonicolor*. Some hyphal yeast-like anamorphs of Ustilaginomycetes have been reclassified from *Candida* and other genera in *Pseudozyma* (Boekhout, 1995). Many of these species are psychrotrophic and may occur in chilled vegetables.

The form-family Cryptococcaceae comprises anamorphic yeast states belonging to Hymenomycetes. *Cryptococcus* is the largest genus with species of divergent phylogenetic relationships and various teleomorphic forms. Some species have been reassigned from *Candida* and *Rhodotorula*; these produce pseudohyphae (*Cry. curvatus*, *Cry. humicolus*) or red-pigmented colonies (*Cry. macerans*),

respectively. Also red-colored is *Cry. hungaricus*, which has been placed in a separate genus, *Dioszegia*, together with some former *Bullera*-producing ballistoconidia (Takashima et al., 2001). Among cryptococci in foods, the most common are *Cry. laurentii* and *Cry. albidus.* A distinct phylogenetic clade, including *Cry. curvatus* and *Cry. humicolus*, is represented by the genus *Trichosporon*, characterized by true hyphae breaking up into arthroconidia, resembling the ascomycetous genus *Geotrichum.* Species living in soil and water may contaminate foods (e.g., *Trisp. moniliforme*). The most common is *Trisp. pullulans*, which also forms budding cells; because it is phylogenetically distinct, it has been separated and placed in a new genus as *Guehomyces pullulans* (Fell and Scorzetti, 2004).

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3 Ecology

Ecological relations as manifested among higher organisms in the plant and animal world can only be partly related to microorganisms because of their small dimensions, enormous numbers, the greatly diversified environmental factors, and the interactions affecting them. The concept of microbial ecology can even be less adapted to food microbiology since foods are generally not considered biological ecosystems as they represent not naturally existing but artificially created environments. Nevertheless, foods do provide real habitats for microorganisms that colonize and grow on and within them. Considering the huge amount of food produced, they represent an environment of great size in the global dimension.

The concept of microbial ecology, initiated some 50 years ago, has resulted in a basic change of views in food microbiology, bearing on both the preservation and safety of foods, as well as the field of quality control. The ecological principles of food microbiology, as elaborated by Mossel and Ingram (1955) and updated by Mossel (1983), are now widely accepted (ICMSF, 1980). It is being increasingly recognized that foods form an ecosystem, regardless of how artificial and man-made they are (Boddy and Wimpenny, 1992). The borderline between natural and artificial ecosystems is blurred. Agroecosystems are based on natural habitats that have become partially or largely artificial by man through the introduction of crop plants and through livestock replacing natural flora and fauna (Figure 3.1). The artificiality of these systems varies enormously, depending on the intensity of human intervention, from agricultural produces to fully processed foods (Deák, 1998). Every food that has not been packed and heat treated contains a characteristic microbiota. Foods become contaminated from the environment by microorganisms, among them yeasts. Only a part of this primary microbiota will survive under the selective pressures exerted by the intrinsic and extrinsic ecological factors of foods. Microorganisms possessing the appropriate physiological attributes (implicit factors) will survive and develop into a specific spoilage association (Figure 3.2). However, this ecological succession in microbiota can be interrupted if the intrinsic and extrinsic properties of foods are changed by processing. Accordingly, food preservation can be defined as the application of ecological determinants leading to the prevention of colonization and growth or leading to the death of microorganisms (Deák, 1991; Table 3.1). On the basis of these ecological principles, strategies for food preservation can be developed (Gould, 1992).

The ecological principles of food microbiology have been applied to foodborne yeasts (Deák, 1997; Fleet, 1998; Viljoen, 1999) and will be further elaborated in the rest of this chapter.

3.1 BIODIVERSITY OF YEASTS IN NATURAL HABITATS

Biodiversity refers to the variety of living organisms in an ecosystem, whether terrestrial, marine, or other aquatic; from global to minute scales; and including taxonomic, ecological, and genetic levels (Figure 3.3). It is the population level that brings the three approaches characterizing biodiversity to a common denominator (Heywood and Watson, 1995). Biodiversity information on microorganisms has become accessible only recently using molecular techniques, whereas taxonomic (species) and ecological (habitat) approaches are made according to the precedents of macroorganisms.

The pioneering work on yeast biodiversity was done by Phaff and his students (Lachance, 2003). Phaff and Starmer (1987) gave a broad overview of yeasts associated with soils, water, plants, and animals, and the further advancements achieved have been summarized by Spencer and

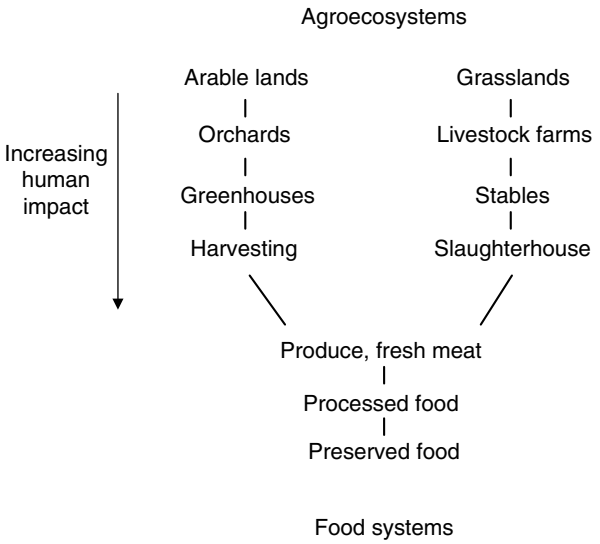

FIGURE 3.1 Agroecosystems and foods with increasing human impact. *Source:* Adapted from Deák, T. (1998) J. Food Technol. Biotechnol. 36:279–283 (Zagreb). With permission.

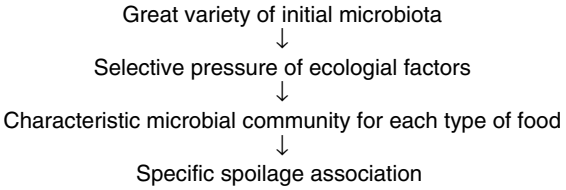

FIGURE 3.2 Principles of microbial ecology of foods.

Spencer (1997), Lachance and Starmer (1998), and most recently in a handbook edited by Rosa and Péter (2006).

Yeasts are widely distributed in nature. They thrive on plant leaves, flowers, and especially fruits. They occur on the skin, hide, feathers, and also in the alimentary tract of herbivorous animals. Some types of yeast are commonly associated with insects. Soil is an important reservoir in which yeasts can survive during unfavorable periods. These natural habitats are important vehicles for carrying yeasts into food processing facilities and hence disseminating to foods.

3.1.1 Soil

Yeasts commonly occur in both arable land and uncultivated soil of various types and geographic areas from the tropics to the arctic zones. Their population and species diversity are lower than those of bacteria, filamentous molds, and protists. Several yeast genera and species are typical inhabitants (autochthonous members) of soil, whereas others originate from plants and animals, and for these allochthonous yeasts soil serve more as a temporary reservoir than as a specific habitat (Botha, 2006). The diversity of basidiomycetous yeasts usually far surpasses that of ascomycetous species (Table 3.2). Members belonging to the genera *Cryptococcus*, *Cystofilobasidium*, *Sporobolomyces*, *Rhodotorula*, and *Trichosporon* can be regularly isolated from soil. Among the ascomycetous species, *C. maltosa*, *Db. occidentalis*, *Met. pulcherrima*, and *Williopsis saturnus* are found frequently (De Azeredo et al., 1998; Slávikova and Vadkertiová, 2000, 2003). More yeast thrive in the rhizosphere,

TABLE 3.1
Main Ecological Factors Prevailing in Foods

1. Sources of contamination (colonization)
   a. Ubiquitous habitats
      Soil, surface water, air, dust
   b. Specific sources
      Raw materials, equipments, utensils
   c. Vectors
      Insect, rodents, workers, handlers
2. Properties of foods (intrinsic factors)
   a. Physical
      Water activity, acidity and pH, redox potential
   b. Chemical
      Nutrients, antimicrobial compounds
   c. Biological
      Structure, defense mechanism
3. Properties of the environment (extrinsic factors)
   a. Temperature
   b. Humidity
   c. Gaseous atmosphere
4. Properties of microorganisms (implicit factors)
   a. Growth requirements, metabolism, growth rate
   b. Resistance
   c. Microbial interactions
      Synergism, antagonism
5. Processing and preservation
   a. Unit operations, preservation treatments, packaging, storage
   b. Hygienic measures
      Cleaning, disinfection

Taxonomic diversity	Genetic diversity	Ecological diversity
Regnum[1]		Biome
Divisio[2]		Bioregion
Order		Landscape
Family		Ecosystem
Genus		Habitat
Species		Niche
Population	Population	Population
Individuals[3]	Cells[3]	
	Chromosomes	
	Genes	
	Nucleotides	

[1]or kingdom; [2]or phylum; [3]in case of microorganisms: cultures or strains

FIGURE 3.3 The components and levels of biodiversity. *Source:* Adapted from Heywood, V. H. and Watson, R. T. (eds.) (1995) Global Biodiversity Assessment, UNEP, Cambridge Univ. Press, Cambridge, p. 1140.

TABLE 3.2
Most Frequent Species of Yeasts Occurring in Soils

Species	Type of Soil
Candida maltosa	Cultivated
Cryptococcus albidus	Cultivated, forest, grass, tundra
Cryptococcus laurentii	Forest, tundra
Cryptococcus podzolicus	Various
Cryptococcus terreus	Grass, various
Cystofilobasidium capitatum	Forest
Debaryomyces occidentalis	Various
Lipomyces starkeyi	Various
Metschnikowia pulcherrima	Cultivated
Rhodotorula glutinis	Forest, various
Sporobolomyces roseus	Tundra
Sporobolomyces salmonicolor	Cultivated
Guehomyces pullulans	Grass, forest, various
Williopsis saturnus	Cultivated

Source: Data from Sláviková, E. and Vadkertiová, R. (2003) J. Basic Microbiol. 43: 430–436; Wuczkowski, M. and Prillinger, H. (2004) Microbiol. Res. 159: 263–275; and Botha, A. (2006) *In*: Biodiversity and Ecophysiology of Yeasts (eds. Rosa, C. A. and Péter, G.). Springer, Berlin. pp. 221–240.

which is rich in nutrients, than farther from the plant roots, though many yeasts are able to grow under low nutrient (oligotrophic) conditions (Kimura et al., 1998).

Our current knowledge on soil yeasts is mainly based on isolates recovered by cultivation methods. As is the case in other natural samples, the biodiversity of noncultivable microorganisms may be several orders of magnitude larger. The recent application of molecular techniques reveals several unidentified sequence types, while confirming by and large the diversity of soil yeasts recognized by cultivation methods (Wuczkowski and Prillinger, 2004). The ecological role of yeasts in soil is diverse. Exopolysaccharide capsules of *Cryptococcu*s and *Lipomyces* species not only protect the cells from desiccation but also contribute to the aggregation of soil particles. Yeasts enter into various interactions with other soil organisms, and often serve as nutrients for bacteria and predating protozoa. However, soil yeasts are of particular interest from the food ecological point of view as sources of contaminants.

3.1.2 Water

Yeasts are common inhabitants of freshwater and seawater, including rivers, lakes, and ponds, as well as estuaries, oceans, and deep sea (Hagler and Ahearn, 1987; Nagahama, 2006). The number and density of species occurring depend on the type and purity of water. Low populations of yeasts, about 10 cells L^{-1}, are typical for open ocean water, whereas clean lakes usually contain below 100 cells L^{-1}. Pollution increases yeast population, for example, water used for recreational purposes often contains 500–1000 cells L^{-1} (Sláviková et al., 1992), and river discharge dramatically increases both the density and diversity of yeasts (de Almeida, 2005). Seasonal variability appears in rivers and lakes but not in the deep sea (Sláviková and Vadkertiová, 1997; Boguslawska-Was and Dabrowski, 2001). Common species in freshwater are *Aureobasidium pullulans*, *Cry. albidus*, *Cry. laurentii*, *Db. hansenii*, *Iss. orientalis*, and *Rho. mucilaginosa*; those often isolated from seawater are *Met. bicuspidata*, *Klu. nonfermentans*, and red yeasts (Nagahama, 2006). Marine isolates are frequently psychrophilic and are associated with animals.

3.1.3 Air

Yeast cells may become airborne due to wind and human activity, whereas ballistoconidia are actively dispersed from some phylloplane species. Red pigmented yeasts are commonly airborne along with cryptococci and conidia from yeast-like molds (black yeasts) such as *Aureobasidium pullulans* and *Cladosporium herbarum*.

Indoor air carrying house dust may contain more than 10^4 CFU g^{-1} yeast cells with the dominant species of *Cry. albidus*, *Cry. diffluens*, *Rho. mucilaginosa*, and *Db. hansenii* (Glushakova et al., 2004). Yeasts in house dust may act as mycogenic allergens (Hagler, 2006).

3.1.4 Plants

Yeasts commonly occur on the surfaces of aerial parts of plants, from grass to trees, on leaves, bark, flowers, and fruits alike. Leaves provide the largest surface area, referred to as phyllosphere (or phylloplane), providing a natural habitat for microorganisms. Bacteria are most numerous, followed by molds and yeasts (Lindow and Brandl, 2003; Fonseca and Inácio, 2006). In-depth investigations of certain plant habitats were among the pioneering studies on yeast ecology, such as tree exudates and slime fluxes (Phaff and Starmer, 1987), necrotic tissues of cacti (Lachance et al., 1986), and nectar of flowers (Spencer et al., 1970).

It is mostly basidiomycetous yeasts that colonize leaves and other aerial plant surfaces, with *Cry. albidus*, *Cry. laurentii*, *Rho. minuta*, *Rho. glutinis*, and *Spb. roseus* being ubiquitous, beside the yeast-like mold *Aureobasidium pullulans*. Their populations change with the season, in particular during the maturation of fruits when, at the ripened stage, ascomycetous yeasts (*Hanseniaspora* and *Metschnikowia* species) prevail in the basidiomycete community (Prakitchaiwattana et al., 2004). Fruits are important habitats for a variety of yeasts, and the succession in yeast communities is involved in fruit spoilage. This subject will be treated in more detail in Chapter 7.

3.1.5 Animals

Few types of yeast are known to be parasites or pathogens in warm-blooded animals; a much larger diversity of yeasts are associated with invertebrates still waiting to be studied or at least discovered. Considering that arthropods represent about 60% of living beings (more than one million species described; Hammond (1995)) and if only every hundredth would be associated with a yeast species, they would outnumber ten times the species described so far. Indeed, dozens of new yeast species have been described recently from insects in the genera *Pichia, Metschnikowia*, *Candida*, and a new genus, *Starmerella*. Suh et al. (2005) isolated over 600 yeast species over a 3-year period from the gut of beetles, and of these at least 200 were undescribed taxa on the basis of LSU rDNA sequences.

Ganter (2006) has recently published an excellent review of the association between yeasts and invertebrates, among them beetles, termites, ants, bees, flies, and drosophilas, many of which serve as vectors carrying yeast contaminating foods. In most cases, yeasts themselves are the food sources for the animal; however, their associations extend from symbiotic to parasitic relationships. Many arthropods develop special cells or organs (mycetocytes, mycangia) in which they maintain and pass the fungal symbiont to the offspring. Others, for example, ambrosia beetles and termites, cultivate fungal gardens to feed their larvae. At the other end, yeasts infect invertebrates, fishes, birds, and mammals (see Sections 3.2.4.4 and 7.9.4). In 1884, I. Metchnikoff described a yeast species known as *Monospora bicuspidata* (now *Met. bicuspidata*) and noted the role of its needle-shaped spores in the parasitic invasion of the water flea, *Daphnia magna*. *Drosophila*, fleas, and bees, intimately associated with yeasts, play significant roles as vectors for the transmission of yeast contamination to foods.

3.1.6 Biofilms

In most natural habitats, microorganisms do not exist as individual, free-living cells, but instead are associated with one another or attached to surfaces. Cell-to-cell adhesion between yeasts results in the formation of flocs that sediment from suspension (flocculation), whereas cells embedded in a slimy matrix form biofilms on a surface. Biofilms are mostly formed by a mixture of bacterial and yeast cells and fungal filaments.

A complex structure of biofilms occurs on soil particles, leaf surfaces, living and inanimate underwater surfaces, and these biofilms are indeed common life forms of microorganisms (Costerson et al., 1995; Morris et al., 1997; Watnick and Kolter, 2000). Biofilms formed on mucosal membranes covering body cavities are major sources of infections. Biofilms are also commonly formed on food processing units and premises, and serve as origins of food contamination (Borucki et al., 2003; Douglas, 2003; El-Azizi et al., 2004).

Biofilms are composed of exopolysaccharides of bacterial and yeast capsular materials and provide a protecting layer of microbes to resist desiccation and treatment of antimicrobial and sanitizing agents. Within biofilms, special cell-to-cell relations and interspecies cooperation develop, as do mutualistic metabolic interactions, exchange of genetic materials, competition for adhesion sites, and antagonistic action of metabolites (e.g., bacteriocins and zymocins) (Watnick and Kolter, 2000; El-Azizi et al., 2004).

In yeasts, it has been shown recently that the mechanisms of adhesion, biofilm formation, and flocculation have much in common (Verstrepen and Klis, 2006). Each phenomenon is conferred by specific cell surface proteins (adhesins or flocculins), which share a common basic structure but differ between species and strains of the same species. Adhesins are induced by various environmental triggers and regulated by several signaling pathways (see Section 4.6). In all, adhesion and flocculation are responses of cells to stress factors and allow yeasts to adapt to the changing environment.

In the food processing context, biofilms offer greater resistance to cells, are not easily removed from surfaces by the normal cleaning and sanitizing procedures, and could hence be a continuous source of contamination (Joseph et al., 2001). It has been shown, however, that potentially spoiling populations occurred only in 4% of the samples of biofilms formed in breweries (Timke et al., 2005).

3.2 ECOLOGICAL FACTORS

The conditions prevailing in the natural and artificial habitats of yeasts determine their metabolic activity, growth, and survival. A variety of abiotic and biotic factors influence yeasts (Table 3.1) and exert stress conditions that the cells must withstand, or otherwise die. In food microbiology, a basic knowledge of these ecological factors is important in order to control food spoilage microorganisms or to exploit useful microbial activity.

Several comprehensive reviews have dealt with this subject (Phaff and Starmer, 1987; Rose, 1987; Watson, 1987; Deák, 1991, 2004, 2006; Boddy and Wimpenny, 1992; Fleet, 1992).

In the following, first, the physical, chemical, and biological factors are surveyed individually, and then their interactions are discussed. Special attention will be paid to the responses of yeasts to stress.

3.2.1 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.

3.2.1.1 Temperature

The temperature relations of yeasts have been reviewed by Watson (1987). The range of growth temperature of microorganisms can be characterized by cardinal (minimum, optimum, and maximum) temperatures. However, the temperature limits and range for growth of yeasts vary with species. Although in general the temperature range of yeast growth extends from several degrees below 0°C to a few degrees below 50°C, the temperature ranges of individual species or strains do not normally span more than 40°C, and are often much narrower (van Uden, 1984).

In terms of maximum temperature (T_{max}) microorganisms can be subdivided into three groups. The term thermophilic is applied to microorganisms whose T_{max} is well above 50°C; those capable of growth at T_{max} between ~25°C and 50°C are referred to as mesophilic; and psychrophiles are those classified as having a T_{max} below 25°C (van Uden, 1984). Considered in these terms, nearly all known yeasts are mesophilic, and grow best between 20°C and 30°C. In a study covering nearly 600 strains of more than 100 species, including the genera *Saccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Pichia*, *Candida*, and others (Vidal-Leira et al., 1979), the upper limit of growth for 98% of yeasts fell between 24°C and 48°C, a few were below 24°C, but none was above 50°C (Table 3.3). Yeasts such as *Leucosporidium scottii*, *Mrakia frigida*, and a few others can be considered as psychrophilic, having a minimum growth temperature as low as −1°C to 4°C and a maximum of about 20°C. At 37°C, only a limited number of species can grow, mostly those associated, at least temporarily, with warm-blooded animals, such as *C. albicans* and a number of other opportunistic pathogenic yeast. Most strains of *S. cerevisiae* occurring widely in industrial fermentation can grow at 37°C, whereas growth in a similar environment of *S. bayanus* is limited up to 30–35°C.

The majority of yeasts isolated from chilled foods (dairy and meat products) also proved to be of mesophilic character, being recovered better by an isolation regime of 25°C for 7 days than at 5°C for 14 days (Banks and Board, 1987). Many types of yeast possess an optimum growth temperature below 20°C and are capable of growth at or a few degrees below 0°C. These are often called psychrotrophs. Kobatake et al. (1992) isolated 50 strains of yeasts belonging to 21 species from fresh seafood, whose growth temperature range fell between −1°C and 44°C, but only a few of them proved to be truly psychrophile. Guerzoni et al. (1993) found an unexpectedly uniform yeast population in various chilled foods, consisting principally of *Ya. lipolytica*, *Db. hansenii*, and *P. membranifaciens*.

TABLE 3.3
Maximum Growth Temperatures of Some Yeast Species

Species	T_{max} (°C)
Kluyveromyces marxianus	44–47
Candida glabrata	43–46
Candida albicans	42–46
Issatchenkia orientalis	42–45
Pichia guilliermondii	38–43
Metschnikowia pulcherrima	31–39
Pichia anomala	35–37
Yarrowia lipolytica	33–37
Debaryomyces hansenii	32–37
Candida zeylanoides	32–34
Candida vini	27–31
Leucosporidium scotti	22–24

Source: Adapted from Vidal-Leira, M., Buckley, H., and van Uden, N. (1979) Mycologia 71:493–501.

The range of yeasts able to grow above 40°C is limited. Anderson et al. (1988) isolated thermotolerant yeasts from sugarcane mills capable of growing at temperatures above 40°C. Many belonged to *Klu. marxianus*, but other thermotolerant strains were identified with *P. polymorpha*, *Geo. capitatum*, *S. cerevisiae*, and *Candida* and *Debaryomyces* species. *Kluyveromyces* strains are noteworthy for their relatively high T_{max} values. An isolate of *Klu. marxianus* from fermented molasses grew up to 48°C (Hughes et al., 1984), and a few strains of exceptional thermotolerance were found being able to grow and ferment at 52°C (Banat and Marchant, 1995). However, temperatures above 50°C are usually lethal for yeast cells.

The temperature of growth is influenced by other environmental factors. In general, in the presence of antimicrobial compounds, such as ethanol (van Uden, 1984) or bicarbonate (Curran and Montville, 1989), the minimum temperature of growth increases. In turn, ethanol tolerance is decreased at temperatures below or above the optimum temperature range of growth (D'Amore and Stewart, 1987; Gao and Fleet, 1988). The optimum growth temperature of yeasts increases in solutions with high sugar or salt concentrations. Jermini and Schmidt-Lorenz (1987b) observed that an increase in solute concentration (up to 60% w/w glucose) raised the T_{max} by 4–6°C up to 42°C for *Zygo. rouxii*. The minimum temperatures for growth of xerotolerant yeasts also increase with decreasing water activities.

3.2.1.2 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 communities from depths of 2000 to 6500 m. Red yeasts (*Rhodotorula* and *Sporobolomyces*) were most common among the isolates; some of them represented new species such as *Klu. nonfermentans* (Nagahama et al., 1999, 2001).

Further data on the baroresistance of yeast cells come from studies on the possible application of high hydrostatic pressure in food preservation (Smelt, 1998). High pressure exerts a destroying 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 may hint at the function of a general mechanism of stress response in yeast cells similar to that protecting them against other stress factors (see Section 5.9).

3.2.1.3 Light and Solar Radiation

A few observations refer to any effect of light on yeast cells, and they point to the possible killing effect of the ultraviolet wavelengths of sunlight. This may explain the relative abundance of pigmented species (e.g., *Cryptococcus* and *Rhodotorula*) on surfaces of plant leaves. A systematic study on phylloplane microbial community suggested that the position of a leaf within an apple tree canopy substantially affects the resident population (Andrews et al., 1980).

Both UV light and radioactive irradiation can be used for the inactivation of yeasts (see Section 6.4).

3.2.2 Chemical Factors

Some of these factors are of physicochemical nature, such as water activity (a_w), pH, and redox-potential (E_h); others are more clearly of chemical character, such as the acidity, the presence or absence of oxygen, and the availability of nutrients. The most straightforward effects are exerted by inhibitory and antimicrobial compounds.

3.2.2.1 Water

Water is an essential requirement of life. Water should be available in fluid and free (not chemically bound) forms. It is also a general solvent of nutrients. In food microbiology, the availability of water for microorganisms is generally expressed in terms of water activity (a_w); the more exact physicochemical term, water potential (ψ), is used less frequently (Marechal et al., 1995). The water activity relates the vapor pressure of a solute (p) to that of the pure water (p_0):

$$a_w = \frac{p}{p_0}$$

and the same relation is expressed in percentage by the equilibrium relative humidity (ERH):

$$\text{ERH}\% = 100 \cdot a_w,$$

which, however, relates to the vapor pressure of air surrounding the food when the two are in equilibrium.

The relation between water potential (ψ) and water activity (a_w) is expressed as follows:

$$\psi = \frac{RT}{V_w} \log a_w,$$

where R is the universal gas constant ($8314\,\text{J}\,\text{k}^{-1}\,\text{mol}^{-1}$), T is the absolute temperature (in degrees K), and V_w is the partial molar volume of water.

Water activity is one of the most important ecological factors affecting the growth of yeasts (and microorganisms in general) in foods. The majority of yeasts are more tolerant to reduced a_w than are most bacteria. Food spoilage yeasts have minimum a_w values of 0.90–0.95 for growth. Several species (e.g., *Zygo. rouxii*) can grow at a_w as low as 0.62. Many other types of yeast are able to grow at low a_w in the presence of high concentrations of either sugar or salt (Table 3.4). This group of yeasts has been referred to as either osmophilic and osmotolerant or xerophilic and xerotolerant (Tilbury, 1980a,b). According to Anand and Brown (1968), the term xerotolerant should be used because these yeasts do not have a general requirement for dry conditions or high osmotic pressure, but merely tolerate low a_w. Tokouka (1993) pointed out that tolerance to low a_w depends on the kind of a_w-controlling solute, and recommended that yeasts be described, respectively, as salt-tolerant and sugar-tolerant.

Early literature reported that some yeasts are capable of growing at a_w as low as 0.62–0.65 (for a survey, see Jermini and Schmidt-Lorenz, 1987a). Later investigations have tended to refute these data. Tokouka et al. (1985) could not detect yeast growth at a_w 0.70 or below. Out of some 140 freshly isolated strains, only four grew better at a_w 0.91 than at higher a_w values. At a_w values less than 0.70 yeast growth was not only inhibited, but also slow death of cells occurred with a decimal reduction time of 57–445 h at a_w 0.625 (Jermini and Schmidt-Lorenz, 1987b). Tokouka and Ishitani (1991) observed that of 35 yeast strains isolated from high sugar foods, one strain of *Zygo. rouxii* had a minimum a_w of 0.67 for growth. Only a few yeasts having a requirement for reduced a_w can justifiably called xerophilic (Koh, 1975; Tokouka et al., 1985). However, many yeasts can be classified as xerotolerant because they can grow at a_w values as low as 0.70.

The principal xerotolerant yeasts species belong to the genus *Zygosaccharomyces.* The most frequent are *Zygo. rouxii*, *Zygo. mellis*, and *Zygo. bisporus*, some strains of which show a minimum a_w of 0.76 for growth but the optimum is above 0.95. Other *Zygosaccharomyces* species show less a_w tolerance, for example, *Zygo. bailii* does not grow below a_w 0.85 (Jermini Schmidt-Lorenz, 1987a). Many xerotolerant yeasts occur in foods with high sugar concentrations (55–65%), among them some strains of *S. cerevisiae*, *Tsp. delbrueckii*, and *Schizo. pombe*. Several yeast species can grow in high-salt foods (15–25% NaCl concentrations), such as *Zy. rouxii* in soy sauce. Other halophilic

TABLE 3.4
Minimum a_w for Growth of Yeasts in Media Adjusted by Different Solutes

	Minimum a_w for Growth Controlled by			
Yeast Species	**Glucose**	**Fructose**	**Sucrose**	**NaCl**
Candida lactiscondensi	0.79	0.78	0.79	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	0.67	0.79	0.86

Source: Adapted from Tokouka, K. and Ishitani, T. (1991) J. Gen. Appl. Microbiol. 37:111–119.

TABLE 3.5
Viability of *Saccharomyces cerevisiae* in Relation to Osmotic Stress

		Cell Viability (% Related to Control)	
Water Activity (a_w)	**Water Potential (MPa)**	**Glycerol**	**Polyethylene-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, P. A., de Maranon, I. M., Molin, P., and Gervais, P. (1995) Int. J. Food Microbiol. 28:277–287. With permission from Elsevier.

or halotolerant species are *Db. hansenii*, *C. versatilis*, *C. halonitratophila*, and *C. lactiscondensi* (Silva-Graca et al., 2003).

Xerotolerant yeasts are of special importance to the food industry for being able to cause spoilage of foods preserved by added sugar or salt. It was found that various processing factors such as temperature, pH, and composition of food interact with water activity on the inhibitory effect of growth (Tokouka, 1993).The minimum a_w value of growth is influenced by the nature of the solute, by temperature or other ecological factors, as well as by the physiological state of the cells (Gervais et al., 1992). Although the mechanism of sugar- and salt-tolerance of yeasts is not completely understood (Larsson and Gustaffson, 1993; Tokouka, 1993), the majority of investigations suggest that yeast cells are capable of adapting to reduced a_w, and the most important criterion in determining osmotolerance appears to be the ability to accumulate high concentration of polyols (Hocking, 1988; van Eck et al., 1993; Gervais and Marechal, 1994). The main solutes accumulated in yeast exposed to osmotic stress are glycerol, arabitol, and mannitol (Spencer and Spencer, 1978). Production of compatible solutes, active pumping out of sodium ions or their exchange for K^+, induction, and differential expression of stress-responsive genes have been suggested to be protective mechanisms; however, different species show contrasting reactions (Ramos, 1999; Hohman, 2002). Extreme osmotic stress can exceed cells' osmoregulatory capacity and cause loss of viability (Table 3.5).

3.2.2.2 Oxygen

Contrary to common belief, yeasts are basically aerobic organisms. Although fermentation is the most noticeable feature for *Saccharomyces* and many other species, about half of all yeast species are strictly nonfermentative aerobes (e.g., the genera *Cryptococcus* and *Rhodotorula*). 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, however, more complex in that in addition to oxygen, the concentration of glucose is also an effector that at high glucose concentrations yeasts start alcoholic fermentation even under aerobic conditions (Crabtree effect; Gancedo, 1998) (see additional discussion in Chapter 4).

In most natural habitats, and also in foods, normal atmospheric conditions prevail with high oxygen and low carbon dioxide concentrations. Carbon dioxide is a metabolic product of various microorganisms including alcoholic fermentation of yeasts. Being easily soluble in water, CO_2 accumulates rarely in inhibitory concentrations. More often, but depending on the pH, the carbon dioxide forms bicarbonate ions that inhibit the growth of yeasts (Curran and Montville, 1989; Dixon and Kell, 1989).

Fruits and vegetables, as well as meat products, can be stored for extended periods under controlled or modified atmosphere, with decreased oxygen and increased CO_2 or N_2 concentrations. This has become an important method of preservation, and it will be discussed in Chapter 6.

3.2.2.3 Acidity and pH

Yeasts, in general, prefer a slightly acidic medium with an optimum pH between 4.5 and 5.5. Yeasts show a remarkable tolerance to pH, and most grow readily at pH values between 3 and 8. Many species, such as *Iss. orientalis*, *P. membranifaciens*, *Dek. intermedia*, and *Kazach. exiguus*, are able to grow at pH values as low as 1.3–1.7 (Pitt, 1974). This tolerance depends on the type of the acidulant, with organic acids being more inhibitory than inorganic acids.

The effectiveness of an acid depends on the reduction of pH and on the specific antimicrobial activity associated with its undissociated form. Consequently, the pH and the pK_a values of an acid strongly influence the antimicrobial activity. Acetic acid is generally more inhibitory than lactic, propionic, citric, and other organic as well as inorganic acids (Moon, 1983; Debevere, 1987). As in the case with other ecological factors, the impact on growth of acidulant and pH is influenced by other factors. For instance, with decreasing water activities, the effect of pH on decreasing growth rate is higher (Table 3.6).

Yeasts tolerate acidic conditions better than alkaline ones; however, alkali tolerance is widely distributed among yeasts. Aono (1990) reported that of 433 strains representing 296 species, 135 strains

TABLE 3.6
Effect of pH and a_w on the Specific Growth Rates of *Zygosaccharomyces rouxii*

	Specific Growth Rate (μ h^{-1}) at a_w Values				
pH	**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

Note: 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.
Source: Adapted from Membré, J.-M., Kubaczka, M., and Chéné, C. (1999) Appl. Environ. Microbiol. 65:4921–4925.

of 86 species were capable of growth above pH 10. Basidiomycetous yeasts (e.g., *Rho. glutinis*, *Rho. mucilaginosa*, *Rho. minuta*, and *Cry. laurentii*) were especially alkali-tolerant, whereas strains belonging to *Dekkera* (*Brettanomyces*), *Saccharomycodes*, and *Schizosaccharomyces* were especially alkali-sensitive and could not grow above pH 8.

The physiological basis of the effect of pH on yeast is not yet completely understood. It is generally believed that the maintenance of a proton gradient across the plasma membrane against a constant intracellular pH of about 6.5 is vital for a yeast cell for optimal activity of critical metabolic processes (Holyoak et al., 1996; Macpherson et al., 2005).

3.2.2.4 Antimicrobial Compounds

The growth of yeasts can be inhibited by a vast range of chemical compounds, some of which occur naturally in foods, while others are added to them deliberately as preservatives (these will be discussed in detail in Chapter 7).

In addition to acetate, lactate, and others, some weak organic acids exert specific inhibitory effects on yeasts, such as benzoic and sorbic acids. These are widely used preservatives in the food industry but 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).

Among others, Conner (1993) and Beuchat (1994) comprehensively reviewed the antimicrobial compounds naturally present in plant and animal tissues. Conner and Beuchat (1984) and Beuchat and Golden (1989) reported that the inhibitory effects of essential oils of allspice, cinnamon, clove, garlic, onion, oregano, savory, and thyme were the most inhibitory to food spoilage yeasts. Yoshida et al. (1987) showed that ajoene, a compound derived from garlic, inhibits yeasts and yeast-like fungi. Middelhoven et al. (1990) observed inhibition of fermentative yeasts in vegetable silage if the crop used contained mustard oils or menthol.

Not all antimicrobial compounds naturally present in plants are associated with essential oils or other lipid fractions. Hydroxycinnamic acids (caffeic, coumaric, and ferulic acids) are among the compounds present in coffee and cocoa beans, tea leaves, and kola nuts known to possess antimycotic activity (Davidson and Branen, 1993; Stead, 1995). Other compounds are not naturally present in plant tissues but are produced in response to injury, microbial infection, or stress. Such compounds are known as phytoalexins, and they develop in various parts of a wide range of plants (Dixon et al., 1983; Whitehead and Threlfall, 1992). Examples are glycinol in soybeans, capidol in bell peppers, and phytotuberin in potatoes, which have antibacterial and antifungal effect. However, the use of active compounds for the purpose of controlling the growth of yeast or other microorganisms in foods is limited by the flavor and aroma characteristics they often impart at low concentrations and the lack of stability at various pH and temperature values.

The main product of alcoholic fermentation of yeasts, ethanol, exerts a toxic effect on various organisms, 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 (Casey and Ingledew, 1986; D'Amore and Stewart, 1987; Fleet and Heard, 1993). Natural residents on grapes such as *Hanseniaspora* (*Kloeckera*) species, which start the spontaneous fermentation of grape juice, are relatively sensitive to ethanol, and die out soon at concentrations of around 5–8% (Table 3.7). Most strains of the true wine yeast, *S. cerevisiae*, can tolerate 13–15% ethanol, and some strains up to 18% or somewhat higher. Some by-products of alcoholic fermentation, for example, diacetyl, may also exert a toxic effect (Jay, 1982).

Tolerance to ethanol is affected by other environmental factors, in particular temperature and pH (Fleet, 2003). Ethanol is thought to increase membrane permeability and thus affect internal pH (Leao and van Uden, 1984; Jones and Greenfield, 1987; Mishra and Prasad, 1989). Gao and Fleet (1988), on the other hand, did not observe a significant reduction in the survival of yeast cells in

TABLE 3.7
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

Note: 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.
Source: Adapted from Membré, J.-M., Kubaczka, M., and Chéné, C. (1999) Appl. Environ. Microbiol. 65:4921–4925.

the presence of ethanol when the pH was decreased from 6.0 to 3.0. The sensitivity of yeasts to ethanol increases as the temperature is increased to 30°C and above or decreased to 10°C or below (van Uden, 1985).

The other end product of alcoholic fermentation, carbon dioxide, also possesses antimicrobial activity. Yeasts, however, are much less sensitive to carbon dioxide than other microorganisms (Jones and Greenfield, 1982; Dixon and Kell, 1989; Slaughter, 1989). *Brettanomyces* species are most tolerant and are the main spoilage yeasts of carbonated beverages. *C. intermedia*, *P. anomala*, and *Zygo. bailii* also tolerated about 0.5 MPa pressure of dissolved CO_2 (Ison and Gutteridge, 1987). Selected strains of *S. cerevisiae* used in champagne production are able to ferment under high pressures of carbon dioxide.

3.2.3 Interactions between Environmental Factors

Under natural conditions, the effects of environmental factors are not isolated from one another, but manifest themselves together and simultaneously, mutually influencing the effect of others (Fleet, 1998). These interactions, moreover, are dynamic and change with time and space. The outcome of interaction is hardly predictable when several factors come into play. For practical applications, the food industry is much interested in the combination of physical and chemical factors in order to apply milder treatments and to better retain the quality and stability of processed foods, while not risking safety (Tapia de Daza et al., 1996). The interaction between temperature, water activity, pH, salt, sugar, and preservatives has been studied in various combinations with different types of food. Of the vast field, 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). The evaluation of interaction between two factors, and finding the synergistic (mutually strengthening) combination is much easier (Table 3.6). However, when several inhibitory factors are considered, the evaluation of their interaction is more difficult (Table 3.8). Extensive experiments have to be carried out, and complex statistical methods as well as predictive mathematical models are applied for their evaluation (Kalathenos et al., 1995). This subject will be treated in more detail in Section 6.8 on combined effects of preservation methods.

3.2.4 Biological Factors

In both natural ecosystems and in foods and beverages, yeasts occur together with other microorganisms and enter into mutual interactions with them. Also, yeasts associated with

TABLE 3.8
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

Note: Selected values from a multifactorial response surface experiment conducted at 25°C in Bacto yeast nitrogen base broth adjusted to various treatment combinations.
Source: Adapted from Kalathenos, P., Baranyi, J., Sutherland, J. P., and Roberts, T. A. (1995) Int. J. Food Microbiol. 25:63–74.

macroorganisms, plants, animals, and humans develop special relations with their hosts. These interactions are mostly mutual, but sometime unidirectional, and can be neutral, synergistic, or antagonistic, and combinations thereof (Lachance and Starmer, 1998).

3.2.4.1 Yeasts and Bacteria

Antibiotic production is one of the best known phenomena for antagonistic relation between bacteria and other organisms. Some polyene antibiotics produced by streptomycetes (e.g., nystatin and amphotericin B) have specific antifungal effects, and have been used in the therapy of human diseases caused by yeasts (e.g., candidiasis; see the section on human pathogens below). Of the other prokaryotes, lactic acid bacteria are known for the production of various bacteriocins, the direct effect of which on yeasts has not been verified (Magnusson et al., 2003). Hydrogen peroxide, often liberated by catalase-negative lactic acid bacteria, may exert a lethal effect on yeasts. In turn, disregarding the inhibitory effect of ethanol, no specific compounds are produced by yeasts being antagonistic to bacteria.

In the association of yeasts with lactic acid bacteria, a number of mutualistic and synergistic interactions are known, particularly in food fermentations. In kefir 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 the 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).

3.2.4.2 Yeasts and Yeasts

The antagonistic (inhibitory) effect of ethanol-producing *Saccharomyces* yeasts on the less alcohol-tolerant yeasts in the fermentation of wine is a commonplace example. Intensively studied are also the specific products of yeasts lethal to other yeasts, called mycocins or killer toxins (Golubev, 2006). 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 impact 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 to the toxin, while other species can be sensitive or neutral. In natural communities, 9–27% of species were shown to produce toxin; in some cases, for example, in fermenting grape juice, toxigenic strains reached 50–75%, whereas the ratio of sensitive yeasts varied between 10% and 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 are sensitive (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).

Killer yeasts often play a role in the competitive interaction between yeast species associated with fruits (Abranches et al., 2001). Killer strains may also facilitate the dominance of wine yeasts during the spontaneous fermentation of grape juice. In other fermentations, 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. In commercial wine production, however, the spontaneous course of events is controlled by the treatment of grape juice with sulfur dioxide and the inoculation of selected wine yeast starter (Fugelsang, 1997).

Predation among yeasts has been considered 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). Yeasts and molds may be prey, attacked by haustoria-like outgrowths that penetrate and kill other cells. The ecological impact of predacious yeasts remains to be assessed; it is believed that it lies in obtaining nutrients.

3.2.4.3 Yeasts and Molds

Some yeast species, in particular *P. guilliermondii*, *P. anomala*, and *Db. hansenii*, inhibit the growth of certain molds attacking fruits and grains. The possible use of antagonistic yeasts to control post-harvest diseases has been reviewed (Wisniewski and Wilson, 1992; Suzzi et al., 1995; Druvefors and Schnürer, 2005). Conversely, a large number of mycelial fungi can attack yeasts. Parasitism is more common among the basidiomycetes than in other fungal groups; nearly 50% of the basidiomycete fungi tested positive (Hutchison and Barron, 1996). Mycoparasitic fungi utilize yeasts as nutrient sources either by lysing yeast cells or by penetrating the cell wall, similar to the way they attack plants and nematodes.

Yeast may rely on nutrients produced by molds, for example, taking up simple sugars liberated by the polysaccharide-splitting enzymes of molds. Longo et al. (1991) demonstrated that a significant increase of yeast species with oxidative metabolism coincided with the proliferation of *Botrytis cinerea* on grapes. Growth of the mold may result in leakage of grape juice, thus enabling yeasts to grow on the surface of berries. As a peculiar case, strict dependence on a type of yeast, *Db. mycophilus*,

has recently been shown on the iron-containing siderophore, a metabolic product of some common soil fungi, *Cladosporium*, *Aspergillus*, and *Penicillium* species (Than et al., 2002).

3.2.4.4 Yeasts, Plants, Animals, and Humans

Yeasts are generally saprotrophic organisms, and their manifold and often intimate relations with flowers, leaves, and fruits of plants have been briefly summarized before. Also, their relations to arthropods have been discussed either serving as their feed or being transmitted by vectors. Mentioned in this section are the antagonistic interactions of yeasts, either as parasites on plants or as pathogens to animals. Typical examples are, respectively, *Nematospora coryli* and *Met. bicuspidata*. A detailed discussion of pathogenicity of yeasts to warm-blooded animals and humans is, however, beyond the scope of this section.

Few yeast species can be considered genuine pathogens to humans; the most important of these are *C. albicans*, *Cry. neoformans*, and *Malassezia furfur* (Hurley et al., 1987; Ahearn, 1998). A number of opportunistic pathogenic yeast species are emerging due to the use of effective antibacterial treatments and immunosuppressive therapy (Hazen, 1995; de Hoog, 1996), and even *S. cerevisiae* has been found in clinical infection (Murphy and Kavanagh, 1999). A number of commensal yeasts can be isolated from the mouth, fingernail, and toenail of healthy hosts, the majority of them belonging to *C. albicans* and *C. parapsilosis* (Kam and Xu, 2002). However, from the food safety point of view, yeasts can be considered harmless organisms in the future too, causing neither food infection nor food poisoning, notwithstanding the sporadic reports that appeared to incriminate some yeasts in diarrhea or allergic responses (Fleet and Balia, 2006).

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4 Metabolism

Metabolic activities of yeasts in foods cause physical, chemical, and organoleptical changes that are related to food spoilage. Fleet (1990, 1992) emphasized the importance of understanding the biochemical and physiological properties of yeasts in assessing their spoilage activities. He also pointed out the existing gap between general knowledge of yeast biochemistry and metabolism and the role that specific metabolic pathways and products play in the chemical and sensory deterioration processes (Table 4.1).

In common with other fungi, yeasts are aerobic organisms that metabolize their nutrients oxidatively, although alcoholic fermentation is the most noticeable feature of a number of yeasts. Contrary to common belief, however, only about half of all yeast species can ferment sugars; these are facultative anaerobic. Interest in yeast metabolism has stemmed largely from the ability of certain species, notably *Saccharomyces*, to ferment sugars and produce ethanol and carbon dioxide. This capability forms the basis for the production of alcoholic beverages and leavened bakery products. Selection of appropriate strains of the baker's, brewer's, and wine yeasts has resulted in a high degree of domestication. Not surprisingly, glycolysis in yeast was one of the first biochemical pathways to be explored; studies on sugar metabolism were later extended to other energy-yielding pathways, intermediary metabolism, and their regulation.

Yeasts have diverse metabolic capabilities. They can utilize a wide range of substrates under a variety of environmental conditions. Sugars represent the main energy and carbon source for yeasts. Degradation of mono-, oligo-, and polysaccharides yields glucose, which enters the glycolytic pathway and tricarboxylic acid (TCA) cycle. These central metabolic pathways are well known, and studies using yeasts have contributed greatly to their understanding (Rose, 1977). Several reviews and books have covered these basic metabolic processes of yeasts (Sols et al., 1971; Fraenkel,

TABLE 4.1
Metabolic Processes of Yeasts Leading to Food Spoilage

Process	Spoilage Characteristic
Energy metabolism	
Fermentation	Ethanol, CO_2, organoleptic volatiles
Respiration	Organic acids, esters, carbonyls
Nitrogen metabolism	
Proteins	Proteolysis products
Amino acids	Higher alcohols, organic acids, amines
Degradation of polysaccharides	
Starch	Attenuation of beer
Pectin	Softening of plant tissues
Degradation of lipids	
Triglyceride hydrolysis	Release of free fatty acids
Sulfur metabolism	
Amino acids, SO_2	H_2S, dimethyl sulfide
Autolysis	
Yeast cell	Fatty acids, thiamin (yeast flavor)

1982; Gancedo and Serrano, 1989; Wills, 1990; Zimmermann and Entian, 1997; Dickinson and Kruckeberg, 2006; Rodrigues et al., 2006).

4.1 NUTRIENTS

The most important nutrients for yeasts are carbohydrates that serve for both carbon and energy sources. Only a few sugars, mostly hexoses and oligosaccharides, can be fermented by yeasts. The range of carbon sources that can be utilized aerobically is much wider, and includes hexoses, pentoses, alcohols, organic acids, and other carbon compounds (Table 4.2). In zymology, the aerobic utilization of a substrate for growth is called assimilation, whereas fermentation refers to the anaerobic metabolism of carbohydrates to form ethanol and carbon dioxide. The differences in the fermentation and assimilation of carbon sources are important diagnostic characters in yeast taxonomy and identification.

Mono- and oligosaccharides are widely utilized by yeasts, although the fermentation of galactose is limited to some species. Not all yeasts are able to metabolize certain di- and trisaccharides (sucrose, maltose, lactose, or raffinose) for lack of the necessary hydrolytic enzymes. Utilization of pentoses is also restricted among yeasts. The fermentation of xylose may be a potentially useful property for the industrial production of ethanol from hydrolysis products of plant hemicelluloses.

The ability of yeasts to metabolize polysaccharides and complex carbohydrates is restricted to relatively few species. Utilization of starch is of particular interest for industrial production of yeast biomass (single-cell protein, SCP) from starchy agricultural wastes. *Db. occidentalis* and *Lipomyces* species are among the few types of yeasts possessing enzymes of various amylase activities. *S. cerevisiae* is unable to hydrolyze starch. One of its special biotypes (formerly recognized as a separate species, *S. diastaticus*) is, however, able to hydrolyze starch (McCann and Barnett, 1986). Some yeast has pectinolytic and xylanolytic enzymes, but only few yeast are known to have cellulolytic capability. Other possible carbon sources for yeasts are hydrocarbons, and several species are capable of growing on these compounds (Phaff, 1986).

Both organic and inorganic nitrogen sources can be utilized by yeasts. Although very few species can hydrolyze proteins extracellularly, short peptides can be transported into the cell and utilized intracellularly (Yamada and Ogrydziak, 1983). Amino acids, amines, and urea are suitable nitrogen sources for practically all yeasts (Large, 1986), as are inorganic ammonium salts. Nitrate utilization, however, is confined to certain species or genera of yeasts, and this is a valuable diagnostic character used for identification purposes.

TABLE 4.2
Examples of Carbon and Energy Sources That Can Be Fermented and/or Assimilated by Some Yeasts

Fermentable substrates
D-glucose, D-galactose, sucrose, maltose, lactose, raffinose (often fermented); trehalose, melibiose, cellobiose, D-xylose, starch, inulin (sometimes fermented)
Substrates assimilated (used aerobically for growth)
D-glucose, D-galactose, L-rhamnose, L-sorbose (hexoses); D-xylose, D-ribose, L-arabinose, D-arabinose (pentoses); sucrose, maltose, cellobiose, trehalose, lactose, melibiose (disaccharides); raffinose, melezitose (trisaccharides); soluble starch, inulin (polysaccharides); α-methyl-D-glucoside, salicin, arbutin (glycosides)
Erythritol, ribitol, D-mannitol, D-glucitol, inositol, glycerol (alcohols)
Citric acid, lactic acid, succinic acid, 2-ketogluconate (organic acids)

Adapted from Kirsop, B.E. and Kurtzman, C.P. (1988) Living Resources for Biotechnology. Yeast. Cambridge Univ. Press. pp. 99–140. With permission.

In addition to basic carbon and nitrogen sources, inorganic microelements and small amounts of complex organic growth factors, mostly vitamins, may be required for the growth of yeasts (Jones and Greenfield, 1984). These requirements are normally fulfilled adequately in natural substrates. Yeasts vary widely in their requirements for minerals and growth factors. Many species synthesize all of the necessary vitamins for growth and propagate vigorously in vitamin-free media. Others require certain vitamins, and this characteristic can be used for identification purposes (Barnett et al., 1983). Biotin appears to be the most commonly required vitamin. Some species require niacin, thiamine, pantothenic acid, folic acid, riboflavin, or myoinositol. Certain yeasts are notable for their exacting properties, for example, *Schizo. octosporus* strains grow poorly in synthetic media unless supplemented with adenine. *Dekkera* (and *Brettanomyces*) species require a high concentration of thiamine for growth.

4.2 SUBSTRATE TRANSPORT

Uptake is the first step by yeast cells in the catabolism of sugars. Transport across the plasma membrane is catalyzed by specific carriers. Several transport carriers have been characterized in yeasts, which are integral parts of the plasma membrane.

In general, two types of transport systems exist, namely, facilitated diffusion and active transport (D'Amore et al., 1989). In general, active transport of monosaccharides coupled to proton symport functions in yeasts (Deák, 1978; Loureiro-Dias, 1989). *S. cerevisiae* and a few other species

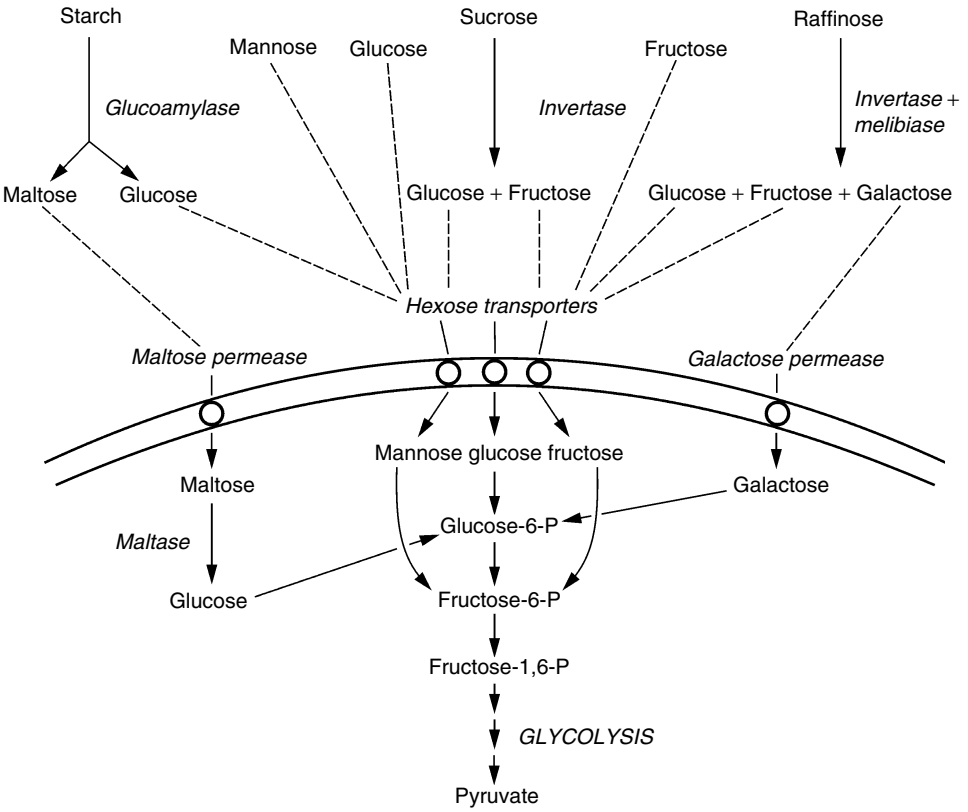

FIGURE 4.1 Schematic outline of the transport and first steps in the carbohydrate metabolism of yeasts. (Modified from Gagiano, M., Bauer, F. F., and Pretorius, I. S. (2002) FEMS Yeast Res. 2:433–470.)

are exceptional in that glucose, fructose, and mannose are transported by a facilitated mechanism. Uptake of galactose is inducible in *S. cerevisiae*, whereas that of maltose is constitutive, and both are carried out by a proton symport mechanism (Lagunas, 1993). Disaccharide transport is generally mediated by proton symport, but in many yeast species, some disaccharides (e.g., sucrose, melibiose, or lactose) are hydrolyzed outside the cytoplasmic membrane (Barnett, 1981).

Hexose carriers have been characterized genetically in *S. cerevisiae*; some 20 genes encode the carriers and related proteins (Boles and Hollenberg, 1997). Two transport systems for glucose have been described in *S. cerevisiae*, one with a high affinity ($K_m \sim 1$ mM) and another with low affinity ($K_m \sim 20$ mM) for the substrate (Bisson and Fraenkel, 1983). Having two systems for glucose transport in *S. cerevisiae* has some practical consequences. The high-affinity system is constitutive, whereas the low-affinity carriers are expressed at higher glucose concentrations, but repressed in the presence of very high-glucose concentrations; for example, at the start of grape fermentation, if the must contains more than 20% sugars, yeast metabolism may be arrested (McClellan et al., 1989).

In baker's yeast, a complexity of transport systems functions. In wheat dough, yeasts utilize the main sugars, that is, glucose, fructose, sucrose, and maltose, as substrates for fermentation. For hexoses, which are present in low concentrations, the high-affinity system is active (Bisson, 1988). Sucrose is first hydrolyzed by yeast invertases located at the cell wall, and the resulting hexoses are taken up. Maltose is incorporated as such and further hydrolyzed by means of intracellular α-glucosidases (Figure 4.1). In bread leavening by yeast, sugar transport appears to be the rate-limiting step (Antuña and Martinez-Anaya, 1993).

4.3 INTERMEDIARY METABOLISM

The common mechanism for metabolizing carbohydrates by yeasts is by the conversion of glucose into pyruvate by the Embden–Meyerhoff–Parnass pathway, also called glycolysis. This pathway consists of 10 enzymatic steps, resulting in net two molecules of ATP per glucose. Other hexoses also enter the pathway after isomerization and phosphorylation by hexokinases.

Glycolysis is not only the source of energy, but also provides intermediates for amino acid biosynthesis. In anaerobiosis, pyruvate will be fermented into ethanol and carbon dioxide; under aerobiosis, it enters the mitochondrium and is oxidized by the TCA cycle and the respiration chain (Figure 4.2).

Metabolic pathways of *S. cerevisiae* are the best understood among yeasts. In fact, "yeast" and *S. cerevisiae* are frequently used as synonymous terms. *S. cerevisiae* is rather exceptional from yeast, in that it shows unusual behavior under both aerobic and anaerobic conditions (Entian and Barnett, 1992). The metabolism of glucose and its regulation can differ greatly among yeasts (Table 4.3). Nearly half of all known yeast species are incapable of fermentation and are strictly aerobic organisms (e.g., the *Rhodotorula* species). A few yeasts are unable to respire (e.g., *C. slooffii*). However, the great majority of yeasts is facultatively fermentative, and can thrive either oxidatively or fermentatively. Fiechter et al. (1981) divided yeasts into three types on the basis of glucose metabolism and its regulation. Crabtree-positive yeasts, like *S. cerevisiae*, are sensitive to glucose concentration and produce ethanol under aerobic conditions in the presence of surplus glucose. In Crabtree-negative yeasts (e.g., *C. utilis*), oxidative metabolism is not repressed by increased concentration of glucose. Obligate aerobic yeasts, like *Rho. glutinis*, degrade glucose exclusively by respiration.

The distinction between fermentative and respirative yeasts applies only to the metabolism of glucose, fructose, and mannose. With other sugars, such as galactose and disaccharides, catabolite repression of respiratory enzymes is not enforced, and respiration is the main pathway of metabolism of these compounds (Sims and Barnett, 1991). This phenomenon is known as the "Kluyver effect."

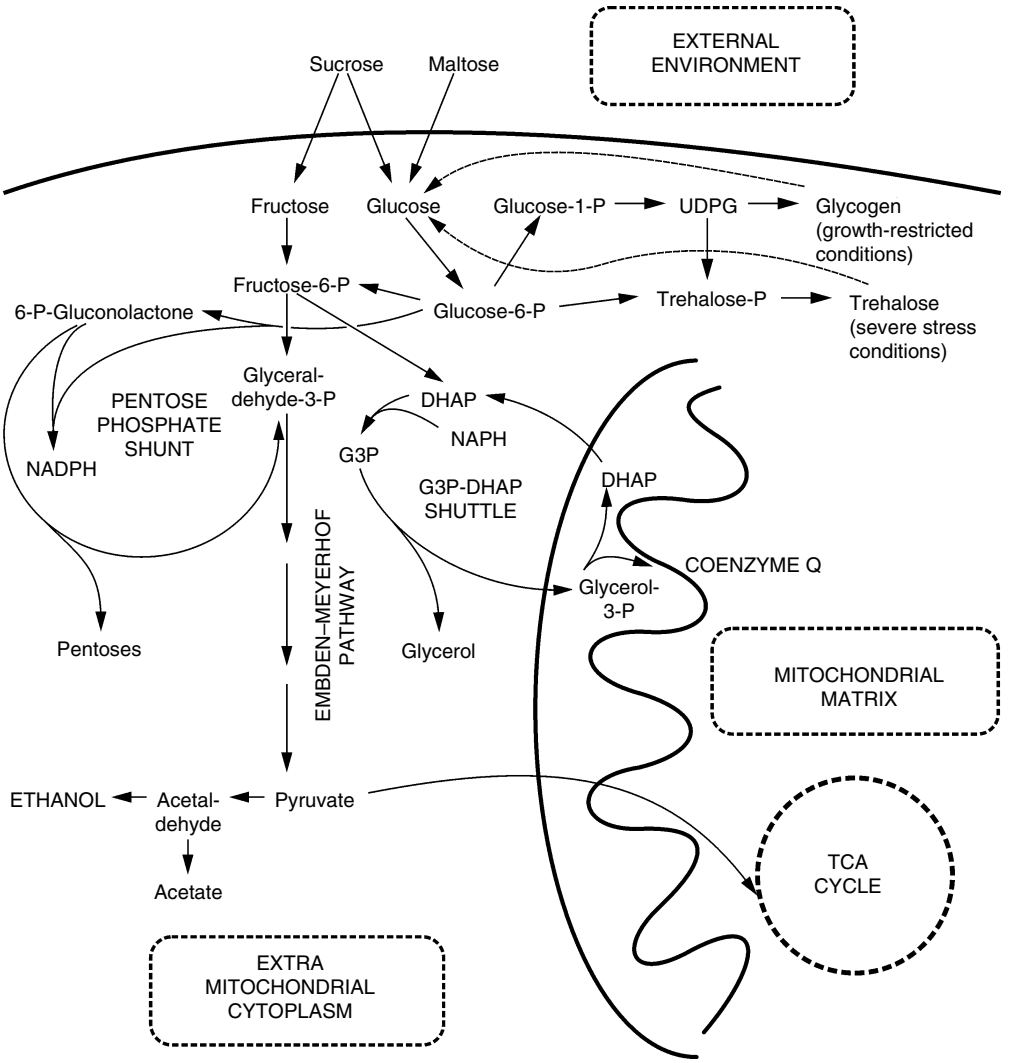

FIGURE 4.2 An outline of the sugar utilization pathways in yeasts. All these pathways are under complex regulation. The majority of the enzymes of the glycolytic (Embden–Meyerhof) and pentose phosphate shunt are synthesized constitutively; the flow of compounds into these pathways is highly controlled. The relative flow of pyruvate to ethanolic fermentation or alternative into the mitochondrial tricarboxylic acid (TCA) cycle and oxidative phosphorylation is governed generally by the availability of oxygen; however, in some yeast such as *S. cerevisiae*, they are governed by the concentration of glucose. Note the important role played by the glycerol phosphate–dihydroxyacetone phosphate shuttle. (From Wills, C. (1990) Crit. Rev. Biochem. Mol. Biol. 25:245–280.)

The regulation of carbohydrate metabolism is peculiar in a group of yeasts—the *Dekkera* and *Brettanomyces* species. Their fermentation is inhibited by anaerobiosis, and glucose is fermented to ethanol and acetic acid only under aerobic conditions ("Custers-effect") (Wijsman et al., 1984).

In recent years, much has been learned at the genetic level on the signal transmission in glucose repression and transcriptional regulation (Banuett, 1998); details on this subject are, however, beyond the scope of the present treatment. Some aspects will be dealt with in Section 5.9.1.

TABLE 4.3
Physiological Classification of Yeasts on the Basis of the Occurrence of Alcoholic Fermentation of Sugars

Classification	Examples
Nonfermentative	*Rhodotorula glutinis*
Facultative fermentative	
a. Crabtree-positive	*Saccharomyces cerevisiae*
	Schizosaccharomyces pombe
b. Crabtree-negative	*Pichia jadinii (Candida utilis)*
	Kluyveromyces marxianus
Obligatory fermentative	*Kazachstania telluris (C. slooffii)*

4.4 ALCOHOLIC FERMENTATION

Under anaerobic conditions and/or at high glucose concentrations, the pyruvate formed in glycolysis is decarboxylated to acetaldehyde, which is then reduced to ethanol. Besides ethanol, a variety of by-products are also produced that influence the sensory characteristics of fermented beverages. Formation of some aroma compounds, such as acetaldehyde, diacetyl, and fatty acid esters, depends on the yeast strains, while others originate from precursors in raw materials (e.g., higher alcohols in grapes) (Cavazza et al., 1989; Lambrechts and Pretorius, 2000; Romano et al., 2003).

Glycerol is one of the most important secondary products of fermentation. The formation of glycerol depends on various factors, such as temperature, pH, sugar concentration, and yeast strain. Glycerol is also important to yeasts for playing a role in the protection of cells from osmotic stress. Beside glycerol, higher alcohols are important by-products of fermentation. Their esters with fatty acids (e.g., isoamyl acetate) usually impart a pleasant taste to wine. Carbonyl compounds, such as diacetyl, on the other hand, are mostly objectionable in the taste of fermented beverages. A variety of other flavor compounds are formed by yeast, whose production is not directly related to alcoholic fermentation. For example, H_2S and organic sulfur compounds are formed in the sulfur and nitrogen metabolism of yeasts. Ethyl carbamate, a suspected carcinogen in fermented beverages, is formed by a chemical reaction between urea and ethanol in aging wine (Ough et al., 1988).

4.5 OXIDATIVE PROCESSES

The oxidative catabolism of pyruvate follows its transport into the mitochondria, conversion to acetyl-CoA and CO_2, and condensation with oxaloacetate to form citrate, the first intermediary of the TCA cycle. In the course of further reactions, the acetate group is fully split to CO_2, and the energy of oxidation is conserved in the form of ATP and reducing power as NADH. ATP is formed by oxidative phosphorylation via the respiratory chain in the inner mitochondrial membrane. When intermediates of the TCA cycle are used for biosynthesis, the cycle is shortcut by the glyoxylate shunt. This is one example of the manifold ways for the integration of metabolism, and the functioning of catabolic and anabolic reactions in unity. Metabolic pathways of carbohydrates are closely connected with those for fatty acids and amino acids; integrated by common intermediaries, the central one of these is pyruvate, as well as the regulation at the levels of enzyme activity and synthesis (Entian and Barnett, 1992).

Growing under aerobic conditions or on nonfermentable carbon sources, yeasts utilize the reverse flow of glycolysis, by circumventing its ATP-producing reactions, to produce building blocks for biosynthesis. This pathway, called gluconeogenesis, directs TCA intermediaries via pyruvate to

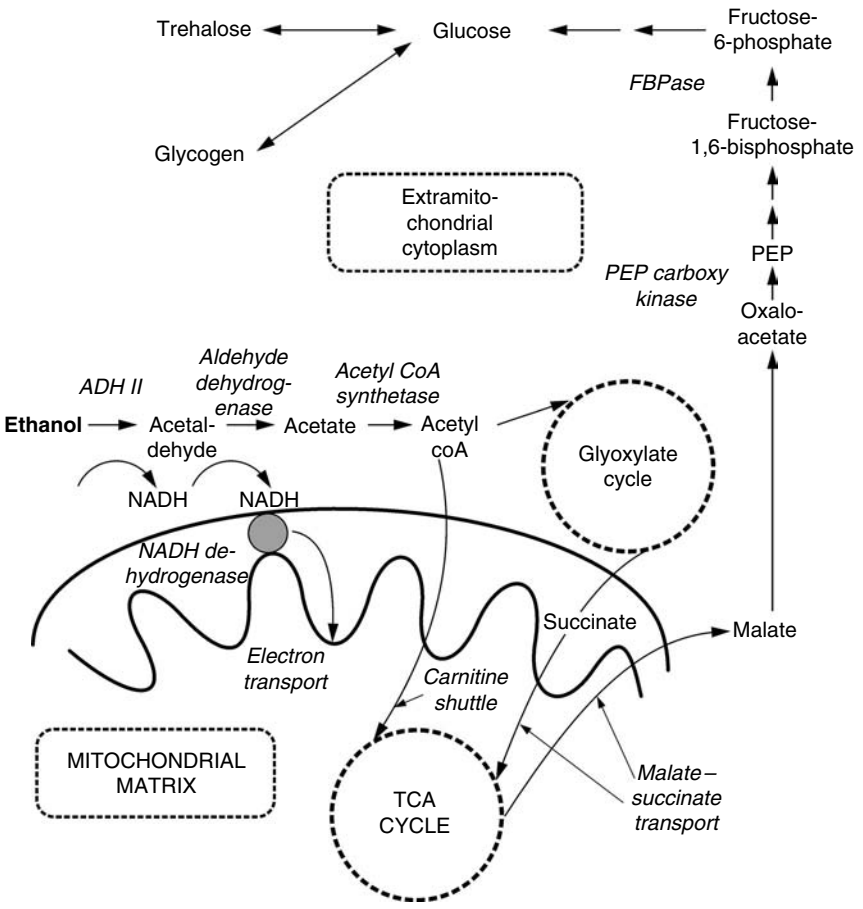

FIGURE 4.3 An outline of the pathways involved in gluconeogenesis. The ATP-generating steps in glycolysis cannot be reversed but are bypassed by alternative enzymatic reactions. The synthesis of sugars from nonfermentable compounds, particularly ethanol, proceeds through at least three routes by which energy from ethanol can generate ATP through mitochondrial activity. These are directly by the production of cytoplasmic NADH, and indirectly through the production of cytoplasmic acetyl CoA or succinate. (From Wills, C. (1990) Crit. Rev. Biochem. Mol. Biol. 25:245–280.)

glucose-6-phosphate, which is then used for the synthesis of reserve carbohydrates and certain amino acids and purine nucleotides (Figure 4.3).

An alternative route of glucose metabolism is the pentose phosphate (PP) pathway, which plays a partial role in catabolism and rather serves for the provision of ribose moiety for the synthesis of nucleotides and some amino acids.

The nitrogen metabolism of yeasts is more diverse than that of carbohydrates, regarding the pathways of both catabolism and synthesis (Messenguy et al., 2006).

Regarding the prominent role of *S. cerevisiae* in both industrial application and basic studies, a vast amount of information is available on its metabolism. Compared with other yeasts, however, *S. cerevisiae* appears to be rather unique and peculiar in many aspects. In recent years, more attention is being paid to non-*Saccharomyces* yeasts (often called "nonconventional" yeasts (Wolf, 1996)). In contrast to *S. cerevisiae*, oxidative catabolism of nutrients (respiration) is prominent in most other yeast. Although the fundamental metabolic pathways are basically similar, considerable differences are found in the starting steps of sugar utilization (transport, hydrolytic enzymes), as well as in the

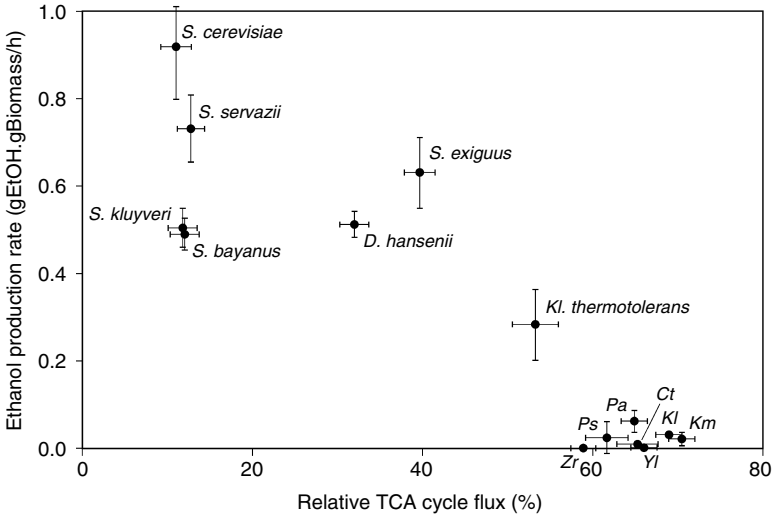

FIGURE 4.4 Correlation between relative TCA cycle flux and ethanol production. Ethanol production rate is highest in *S. cerevisiae* and lower in other respiro-fermentative species such as *S. kluyveri* and *S. bayanus*, indicating the production of additional fermentation by-products such as ethyl acetate. In *Klu. thermotolerans*, a Crabtree-positive yeast, the TCA cycle is only partially repressed and ethanol production is low, whereas ethanol production is nearly absent in fully respiratory yeasts. rate in ascomycetous yeasts. (From Blank, L. M., Lehmbeck, F., and Sauer, U. (2005) FEMS Yeast Res. 5:545–558. With permission of Blackwell Publishing.)

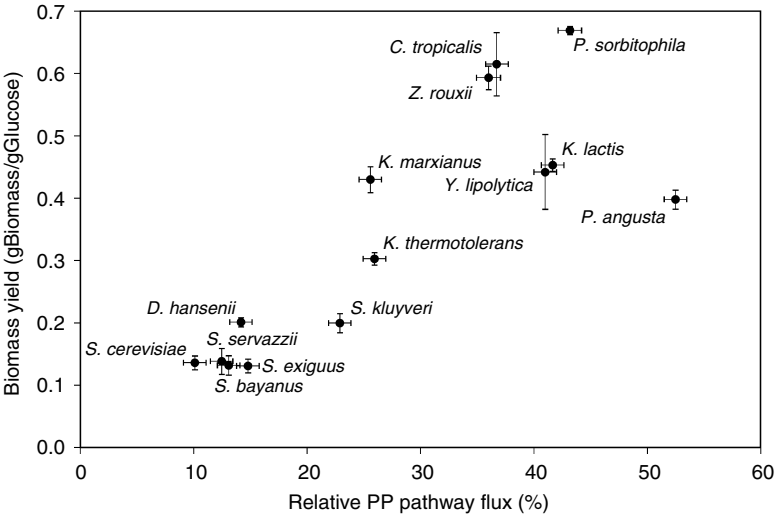

FIGURE 4.5 The relative pentose phosphate (PP) pathway flux as a function of the biomass yield in ascomycetous yeasts. In yeasts the PP pathway activity generally correlates with biomass yield, indicating that the flux through PP pathway is driven by the demand for reduced NADPH used in biosynthesis. Refer to Section 2.4.1 regarding some changes in scientific names. (From Blank, L. M., Lehmbeck, F., and Sauer, U. (2005) FEMS Yeast Res. 5:545–558. With permission of Blackwell Publishing.)

regulation (Flores et al., 2000). In non-*Saccharomyces* yeasts, glucose repression of respiration is less prevailing, and the relative participation in metabolism of TCA cycle is inversely related to the rate of ethanolic fermentation (Figure 4.4), whereas the relative flux through the PP pathway is directly proportional to the biomass yield (Figure 4.5) (Blank et al., 2005).

4.6 REGULATION

Carbohydrate metabolism is effectively regulated in yeasts. The importance of oxygen and glucose in the regulation of metabolism and growth has been recognized since Pasteur's initial studies. The term "Pasteur effect" is generally applied to the phenomenon of decreasing fermentation efficiency in the presence of oxygen (Lagunas et al., 1982). The repression of respiration when yeast is grown in the presence of high levels of glucose has been referred to as the "Crabtree effect" (Fiechter et al., 1981). Although these processes are related, the mechanisms involved are different. Inhibition of fermentation by aerobiosis is explained by a competition for pyruvate between two enzymes, pyruvate decarboxylase and pyruvate dehydrogenase, which direct pyruvate into fermentation and respiration, respectively. The affinity of the latter enzyme for pyruvate is much higher. The inhibition of glycolytic flux involves several rate-limiting and controlling steps, one of the most important being glucose transport (Lagunas, 1986). However, the underlying regulatory mechanisms are more complex and far from being completely understood (Otterstedt et al., 2004; Thierie, 2004).

In recent years, genetic and molecular biology studies have provided a wealth of data on the mechanisms by which yeasts respond to changes in environment, such as glucose concentration, availability of other nutrients, osmotic, heat, and other stresses, or mating pheromones. From these, a general understanding takes form, although the effects are widely different and the details of response pathways vary. By and large, an environmental signal is sensed by a receptor in the cell membrane, being forwarded through a signaling pathway to the nucleus, where it results in altering the transcription of genes, eventually manifested in responses of activation, induction, and repression or derepression of enzymes or other proteins.

In the cytoplasmic membrane, various G (guanine nucleotide binding) proteins are the most frequent signal receptors (e.g., Ras proteins); substrate carriers and other transporters may play a role too (Boles and André, 2004; Holsbeeks et al., 2004). Several signaling pathways have been characterized in yeasts; the protein kinase A-cyclic AMP (PKA-cAMP) and the mitogen-activated protein (MAP) kinase pathways are best known (Geladé et al., 2003). Their compositions are complex and integrated, but often redundant and overlapping (Table 4.4). In responding to nutrients, stresses, and other environmental signals, they play important roles in diverse cellular functions, such as cell cycle, budding, flocculation, differentiation, and mating (Rolland et al., 2002). These will be

TABLE 4.4
Signal Transduction Pathways in *Saccharomyces cerevisiae*

	Response Pathways to Signals					
Kinases	**Mating Pheromone**	**Filamentous Growth**	**Osmotic Stress**	**Nitrogen Starvation**	**Growth Cycle**	**Spore Assembly**
Upstream kinase	Ste20	Ste20	Sho1/Sln1	Ras2	Pkc	Sps1
MAPKKK (MEKK)	Ste11	Ste11	Ste11/Ssk2	Ste11	Bck1	?
MAPKK (MEK)	Ste7	Ste7	Pbs2	Ste7	Mkk1,2	?
MAPK	Fus3	Kss1	Hog1	Kss1/Fus3	Slt2	Smk1

Note: MAP (mitogen activated protein) kinases cascade: MAP kinase kinase kinase (MAPKKK or MEKK) phosphorylates MAP kinase kinase (MAPKK or MEK), which in turn phosphorylates MAP kinase (MAPK). The protein kinases in each pathway are named after the mutants in which they were identified, for example, sterile mutant 20 (ste20) or high-osmolarity growth mutant 1 (Hog1), and so forth; they correspond to genes STE20 and HOG, respectively.

Sources: Adapted from Gustin, M. C., Albertyn, J., Alexander, M., and Davenport, K. (1998) Microbiol. Mol. Biol. Rev. 62:1264-1300; Banuett, F. (1998) Microbiol. Mol. Biol. Rev. 62:249–274; and Gagiano, M., Bauer, F. F., and Pretorius, I. S. (2002) FEMS Yeast Res. 2:433–470.

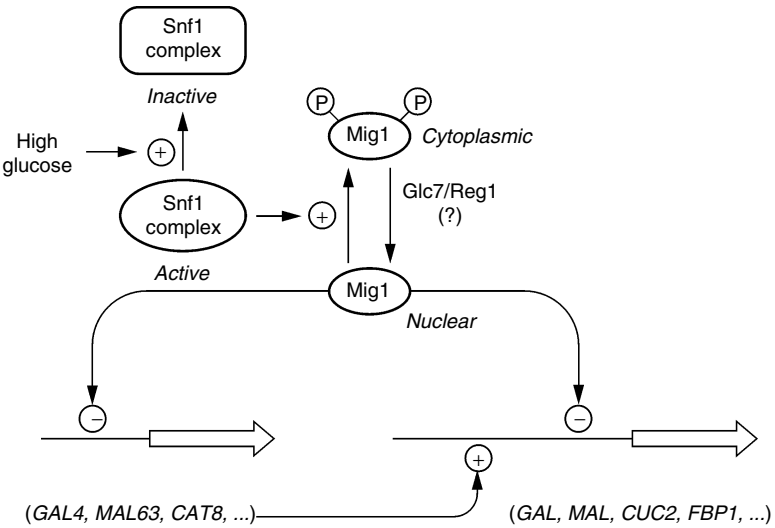

FIGURE 4.6 Schematic view of the mode of action of Mig1 and its regulation. In the presence of glucose, Mig1 is found in the nucleus, where it represses the transcription of genes encoding activators such as GAL4 and MAL63 and of genes whose products are implicated in the metabolism of alternative carbon sources. Glucose removal causes both phosphorylation of Mig1, depending on the Snf1 complex, and its translocation to the cytoplasm. (From Gancedo, J. M. (1998) Microbiol. Mol. Biol. Rev. 63:334–361. With permission of the ASM Journals.)

discussed later in other chapters; here, the metabolic responses to glucose and other nutrients will be outlined.

In *S. cerevisiae*, the sensing of glucose results in the repression of enzymes participating in the metabolism of other carbohydrates; this is the classical carbon catabolite repression (Gancedo, 1998; Carlson, 1999). To distinguish from other glucose signaling routes, it is also called the main glucose repression pathway. Its membrane receptors are intimately associated with the hexokinases, or are the phosphorylating enzymes themselves; the main downstream component of the kinase cascade is the Snf1 protein, whereas Mig1p is the transcriptional repressor. In addition, a number of activators, regulators, corepressors, and other proteins participate in the complex pathway. The repression is a negative regulation process. In the presence of high glucose, Snf1 is inactivated, and does not phosphorylate Mig1, which enters the nucleus and performs the repression of transcription of the transporters and enzymes required in the metabolism of galactose, maltose, and sucrose (Figure 4.6). The pseudohyphal growth regulating MAPK pathway and the PKA-cAMP pathway also respond to glucose and to nitrogen signals as well. In the latter pathway, the main components are the Gpr1p receptor; the Cyr1p adenyl cyclase; the PKA consisting of two subunits, Bcy1 regulatory and Tpk1 catalytic ones; and the repressor Sfl1p. These pathways of glucose regulation contribute to the repression of respiration, of gluconeogenesis (Crabtree effect), and also of stress resistance (Rolland et al., 2002; Schneper et al., 2004; Verstrepen et al., 2004). As we shall see in later chapters on cell growth and physiology, there exists a great deal of cross talk in the pathways regulating metabolic and transcriptional behavior of yeast in responding to the environment.

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5 Growth, Life Cycle, Death

Growth and death are fundamental physiological events; in between them, yeast cells pass over various processes of morphogenesis, cell cycle, aging, injury, and survival. These are determined genetically and expressed in the structural and physiological constitution of cells under the influence of ecological factors of the environment. In the industrial context, for the exploitation of yeasts in fermentation and other applications, the attempt is made to provide the best circumstances for growth and survival, whereas to avoid spoilage, the goal is to inhibit growth and/or destroy and kill cells. To achieve both ends, a thorough understanding of these physiological phenomena is required.

5.1 GROWTH CHARACTERISTICS

Growth of yeasts that mostly propagate vegetatively by budding can be described in a way similar to unicellular, dividing bacteria, in that the increase in cell number or cell mass with time can be characterized by the growth rate (μ) or the generation time (t_g). The number of living cells can be determined by cultivation or microscopic method, or can be indirectly related to cell density with turbidity reading or measurement of dry mass (Chapter 8).

When growth is followed in batch culture by monitoring viable cell count or optical density, characteristic growth phases can be recognized that have counterparts in the traditional bacterial growth curve. The most definite phases are lag, exponential, and stationary, interconnected by transitive parts (Figure 5.1). In aerobic liquid cultures, growth of most yeast can be described by this characteristic S-shaped curve.

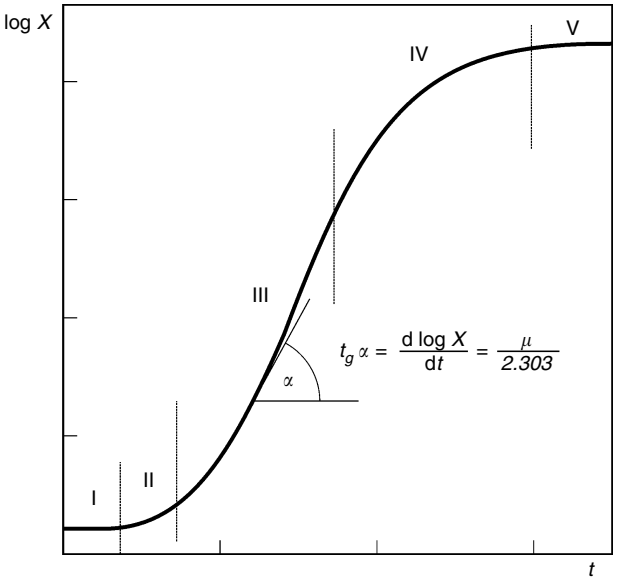

FIGURE 5.1 Growth curve of single-cell microorganisms. Growth phases: I, lag; II, accelerating; III, exponential; IV, declining; V, stationary. X: cell concentration; μ: specific growth rate; t: time.

The growth rate changes with the growth phases; in a given case, it is maximum and steady at the exponential phase. In this stage, the growth rate is directly proportional to the concentration of existing cells (X):

$$\frac{dX}{dt} = \mu t,$$

which gives after integration between 0 and t time

$$X_t = X_0 e^{\mu t},$$

and in logarithmic form

$$\ln X_t = \ln X_0 + \mu t.$$

This is the fundamental equation describing the growth of single-cell organisms.

The specific growth rate is inversely related to the generation time ($\mu = 0.69/t_g$), which is the time elapsed between two cell divisions or buddings, in the case of yeast. Yeast growth can be characterized with a growth rate in the range of 0.17–0.35 h^{-1} (t_g 4 − 2 h), although some yeasts grow much slower; for example, the generation time for some *Zygosaccharomyces* and *Dekkera* species is in the range of 9–20 h. In general, the growth rate of yeasts is slow compared with that of bacteria, while mold growth is much slower than that of yeasts. This gives a certain competitive advantage to bacteria over yeasts in colonization of most foods. However, under certain conditions, for example, in acidic or high-sugar-containing foods, yeasts can easily overgrow bacteria.

The specific growth rate (or the generation time) depends on a number of environmental factors, such as the presence of nutrients, the chemical composition of the growth medium, temperature, pH, a_w, oxygen tension, the presence of growth inhibitors and concentration of excreted metabolites (e.g., ethanol), and many other factors. The size of the population also influences the growth rate; it decreases with increasing concentration of cells. This is described by the following logistic equation:

$$\frac{dX}{dt} \cdot \frac{1}{X} = \mu_{max} \left[1 - \frac{X}{X_v} \right],$$

where μ_{max} is the maximum growth rate and X_v the final cell concentration. This equation also describes a sigmoid-shaped curve, which, after reaching the maximum growth rate, tends to a steady final maximum cell concentration (Figure 5.2). Many other mathematical expressions have been developed in the attempt to describe the growth characteristics of populations. One of them is the Gompertz equation, frequently used in predictive modeling (see below). In addition to the growth rate, the length of the lag phase, that is, the time required for cells to adapt to the prevailing conditions, depends strongly on the growth factors (Medawar et al., 2003). The effects of the environment have been outlined in Chapter 3, and will be discussed in more detail later on.

It should be noted that the preceding description of growth characteristics concerned the propagation of cell populations. In natural conditions, however, microbial populations are composed of heterogeneous cells that differ in their cellular constituents, metabolism, and cell cycle phase. These random variations render the modeling approaches more difficult (Henson, 2003).

The sigmoid-shaped growth curve is exhibited most frequently. In the presence of fermentable substrate this shape is often redoubled. The growth of *Saccharomyces* and other like species is diauxic in nature (Lewis et al., 1993). After an initial lag phase, exponential growth proceeds primarily by fermentation, whereas respiration is substantially repressed. After the exhaustion of the initial fermentable carbon source, cells metabolically adapt to respiration using ethanol during a second (diauxic) lag phase that is followed by a second exponential growth phase, although at a slower rate.

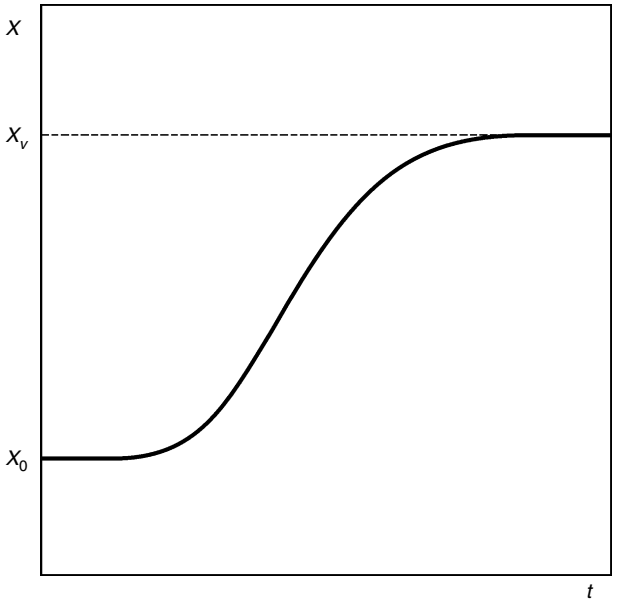

FIGURE 5.2 Change of cell concentration (X) with time (t) according to the logistic equation of growth. X_0: initial cell concentration; X_v: stationary cell concentration.

When cells start growing on nonfermentable substrates (e.g., glycerol or lactate), this second lag and exponential phases do not appear, and the initial exponential growth is immediately followed by the stationary phase. Cells usually enter the stationary phase as a result of carbon starvation, but exhausting other nutrients, including nitrogen, phosphorus, and sulfur, may also force cells to enter the stationary phase (Harder and Dijkhuizen, 1983). Stationary phase cells are characterized by altered physiological properties, for example, they exhibit increased resistance to various stresses. This phase represents a special section of the cell cycle, and will be discussed later.

In biotechnology, for the production of ethanol and other useful metabolites, attempts are made to maintain yeast growth continuously in the exponential phase. It could be done with partial success for a limited period only. In contrast to continuous culture, most industrial propagation is made in fed-batch mode. Under this condition, nitrogen and carbon sources are added at a rate lower than the normal metabolic rate for yeast. The fed-batch process is commonly used in commercial yeast production.

Besides aerobic growth kinetics, the description of fermentation kinetics of yeasts is also of great practical importance. Kinetic models have been developed to account for the ethanol yield, CO_2 production, substrate consumption, and the effect of external parameters (e.g., temperature) on fermentation (Boulton, 1980; Bely et al., 1990; Caro et al., 1991). Although kinetic models can be applied in the practices for the control of some fermentation, alcoholic fermentation appears to be such a complex process that any model has met only partial success. Wine fermentation, for example, is characterized by a combination of parameters not commonly found in other fermentation processes. High concentrations of sugars in grape must cause substrate inhibition, mixed substrates cause competitive inhibition, and generation of ethanol causes product inhibition (Boulton, 1980).

5.2 DEATH AND INACTIVATION

Microbial populations, in the last phase of their growth, begin to decline when the number of cells dying off exceeds that of newly born cells. Death of cells occurs under natural conditions, and can be purposely boosted by adjusting the environmental factors beyond the degree of tolerance of microbial

cells. This is the basis of inactivation methods used for the preservation of foods and many other practical purposes. The safety and quality of processed foods require a thorough knowledge of the rules governing the death of microorganisms.

5.2.1 Death Kinetics

The decline in time for a lethal effect of a yeast population can be expressed, similarly to single-celled bacteria, in terms of first-order kinetics, such as

$$\frac{dN}{dt} = -kN,$$

where N is the number of living (surviving) cells at any moment of time t and k is the death rate constant (in time^{-1} units). Integrating between limits N_0 initial and N_t survivor cell numbers after t exposure time gives

$$N = N_0 e^{-kt}.$$

Using a common logarithm, we obtain

$$\log \frac{N_t}{N_0} = -kt,$$

and plotting N_t survivors against t gives a straight line with a slope k (Figure 5.3).

First applied by Bigelow in 1921 for thermal destruction, the concept of thermal death time (TDT) was introduced with a decimal reduction time D (min), which is the time needed to reduce the population by one log unit at a given constant temperature. The D-value is in inverse ratio to k ($D = 2.3/k$). By plotting D against temperature, a linear relationship is obtained with a slope z-value (in °C) as the temperature increase needed for a tenfold decrease in the D-value (Figure 5.4).

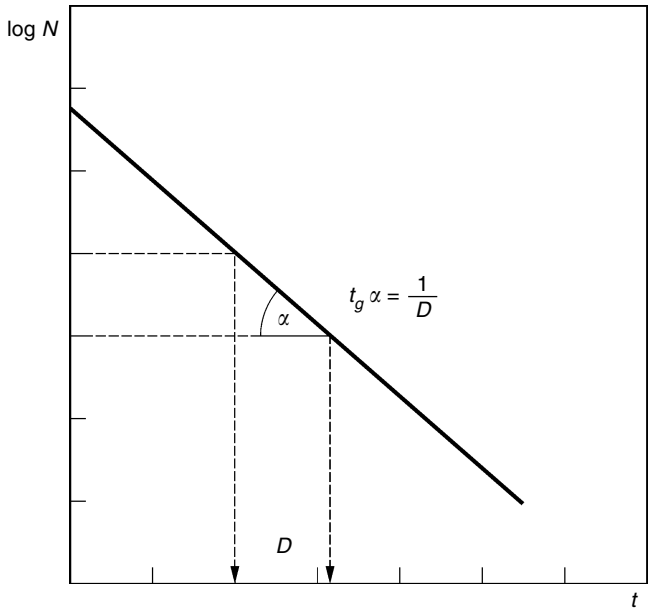

FIGURE 5.3 The survivor curve and the decimal reduction time (D).

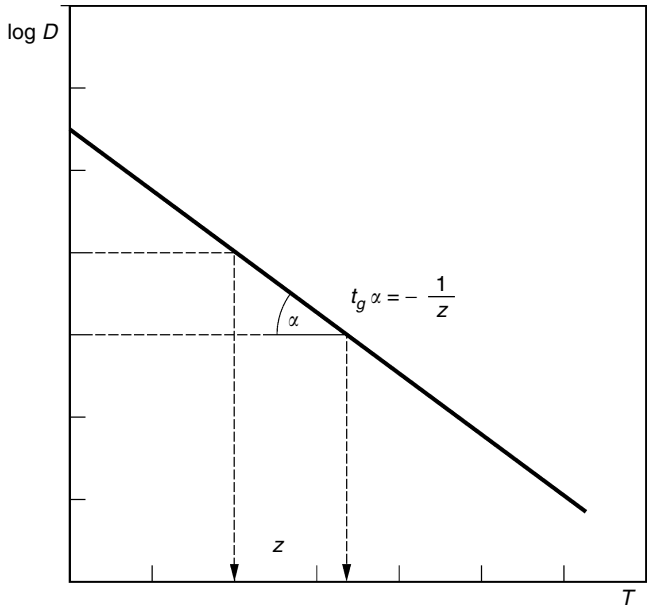

FIGURE 5.4 The thermal death curve and the z-value. D: decimal reduction time; T: temperature.

In general, yeasts can be inactivated at temperatures of over 55°C in few minutes. Measured D-values fall 1–5 min at 55°C, and are less than 0.1 min at 60°C. The thermal death rate increases tenfold at a rise of temperature by 4–5°C (i.e., the z-value is 4–5°C). These are, however, only rough guide values, as they are influenced by various external and internal factors, and also differ according to the nature of the lethal agent, as will be discussed in the next chapter.

Although the assumption of first-order death kinetics has proven reliable in the practice of heat sterilization (Stumbo, 1973), closer inspection of semi-logarithmic survival curves has not always proved to be linear, but instead showed various shapes with shoulder and tail, concave upward or downward (Figure 5.5). A number of mathematical models have been developed to describe these curves, which were interpreted as the manifestation of mixed population or the distribution of resistances or sensitivities to the lethal treatment (Whiting, 1995; Peleg, 1998; van Boekel, 2002).

5.2.2 Modeling Microbial Responses

Microbial kinetics models generally fit the course of either growth or death. Simple primary models express the growth or survival of microorganisms as a response to one or a few environmental factors. However, the number of factors that affect foodborne microbes is large, such as temperature, pH, a_w, redox potential, oxygen, and so forth, and many of them act simultaneously in a combined effect. Secondary models have been developed to include more factors and their interactions, and some other models combine expressions for growth and death into a single equation.

Secondary models are generally of three types: based on the Arrhenius relationship, the square root models, and the polynomial equations using response surface methodology. The Arrhenius-type models describe the relation between the natural logarithm of a rate constant and the reciprocal of the absolute temperature, and has been extended to include terms of pH and a_w (Davey, 1989):

$$\ln k = C_0 + \frac{C_1}{T} + \frac{C_2}{T^2} + C_3 a_w + C_4 a_w^2,$$

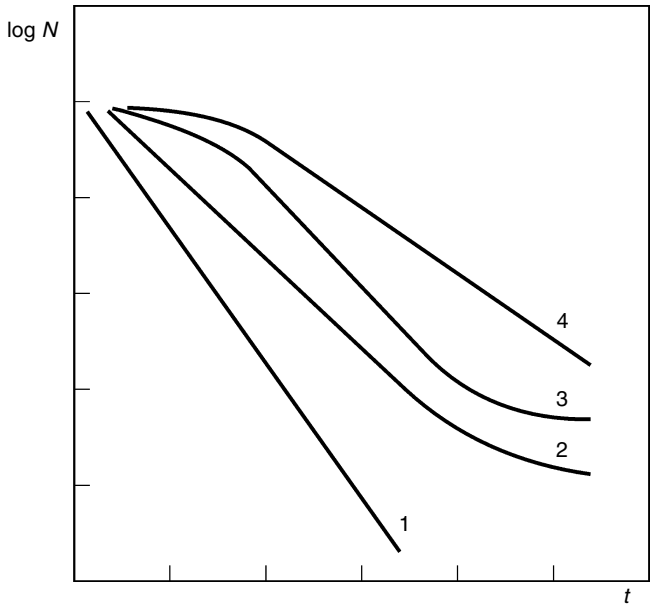

FIGURE 5.5 Commonly observed types of inactivation curves. 1: linear; 2: linear with tailing, concave; 3: sigmoid; 4: linear with shoulder, concave.

where k is the growth rate, T is the temperature, and C_0–C_4 are parameter values. According to the square root model (Ratkowsky et al., 1991) there is a linear relationship between the square root of the rate and the temperature:

$$\sqrt{k} = b(T - T_{\min}),$$

where b is a slope parameter and $T_{\min}$ is the temperature where growth rate attains to zero. This model has also been expanded to include the temperature of maximum growth rate ($T_{\max}$), pH, and a_w (McMeekin et al., 1993):

$$\sqrt{k} = b[(a_w - a_{w_{\min}})(\mathrm{pH} - \mathrm{pH}_{\min})]\,(T - T_{\min})\,[1 - \exp(T - T_{\max})].$$

The response surface approach allows modeling of the effects of several controlling factors at the same time (Buchanan and Phillips, 1990).

Modeling approaches to estimate growth parameters can also be of several types. Kinetic models predict growth rate or lag phase, or death rate (D-values), and are technically more demanding than the probabilistic models, which determine the probability of growth/no-growth or death/survival under defined conditions, or the time-to-grow models based on the time for a microorganism tested to initiate growth under any set of inhibitory or inactivating conditions (Betts and Walker, 2004). Kinetic and probabilistic models can be combined (Ratkowsky et al., 1996), and models expanding all growth and death phases are developed (Ross et al., 2005). Besides, the z-concept has been generalized for growth conditions as well (Pin et al., 2001; Arroyo López et al., 2006). With all these developments, modeling in predictive microbiology has become a powerful tool to quantitatively estimate the fate of microorganisms in foods. Its objective can be either optimizing or preventing their growth, or else inactivating and eliminating microbial activity (McMeekin et al., 1993; Baranyi and Roberts, 2000; Braun and Sutherland, 2006). Examples of applications regarding yeasts will be discussed in Chapter 6, Section 6.8.

5.3 CELL CYCLE

In natural habitats, yeasts are subjected to large fluctuations in environmental conditions. Under favorable conditions, they grow and proliferate in a succession of events forming the cell cycle. The essence of the process is the duplication of the genetic material and its division into two daughter cells (the bud and the mother cell in most yeast). These are the events of DNA synthesis (S-phase) and the mitosis (M-phase), which are separated by gap periods G_1 (growth and preparation for DNA replication) and G_2 (preparation for division). The phases G_1–S–G_2 between two mitosis are called interphase (Figure 5.6).

The basic mechanisms underlying cell cycle and its control appear to be common in all eukaryotic organisms, from yeast to humans. Its understanding is important; for example, cancer may be due to errors in cell cycle regulation. Yeasts have become excellent models in studying cell cycle, and the great progress achieved with them was acknowledged with the awarding of the 2001 Nobel Prize to Lee Hartwell (for work with budding yeast), Paul Nurse (for work with fission yeast), and Tim Hunt (for identifying regulatory proteins, cyclins).

There are as many similarities as differences in the cell cycle between the two types of yeasts; that is, in the budding yeast (*S. cerevisiae*), phases S, G_2, and M are short, and G_1 makes up about two-thirds of the cell cycle, whereas in the fission yeast (*Schizo. pombe*), the M, G_1, and S phases are short, and G_2 comprises the majority of the cell cycle. Isolation of numerous mutants was helpful in deciphering the events and regulation of the cell cycle; most important are the cell division cycle (CDC) mutants.

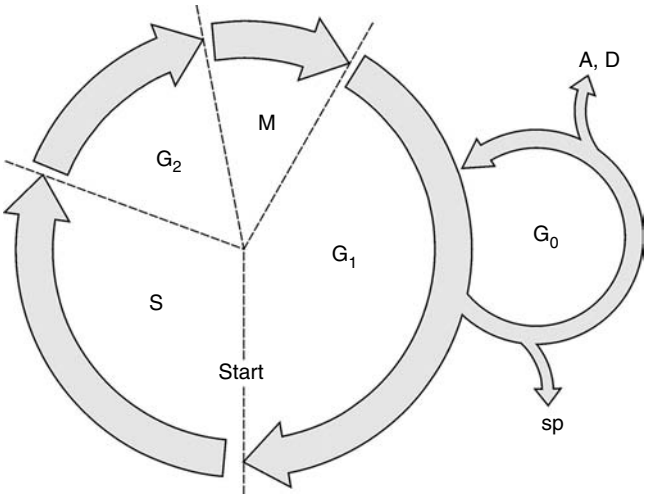

FIGURE 5.6 Phases of the cell cycle. The cell cycle is the succession of events between two cell divisions, during which the processes of DNA synthesis and replications (S phase) and the separation of chromosomes between the mother cell and daughter cell (bud) by mitosis (M phase) occur in temporally distinct order. The S and M phases are, in general, separated by two gaps, known as G_1 and G_2. In G_1, cell increases in size and synthesizes RNAs and proteins. At the end of G_1, there is a checkpoint, a control mechanism to ensure that everything is ready to start DNA synthesis. It must be passed, otherwise the cell arrests in the G_1 phase, or enters into stationary phase (G_0), a stage of no growth where it may reenter the cell cycle if environmental conditions become favorable. Also, from G_1, cells may undergo a process of differentiation, leading to sporulation (sp) or, after aging in G_0, cells eventually die. In G_2, cells continue to grow, and another checkpoint at the spindle assembly determines if cells can proceed to enter mitosis and divide. The control mechanisms are enforced by protein kinases and Cdk/cyclin complexes. Details of the cell cycle vary among yeast species; in *S. cerevisiae*, M, G_2, and S phases are short, and G_1 makes up two-thirds of the cell cycle, whereas in *Schizo. pombe*, S, G_1, and M are short, and G_2 makes up the majority of the cycle.

The cells have several checkpoints and a regulatory system to interrupt the cell cycle. This can be triggered by internal signals (frequent damage in DNA) or by environmental conditions (e.g., lack of nutrients and starvation). Under these circumstances, a cell will leave the cycle temporarily or permanently, and enter a stationary phase, G_0 (zero growth). Stationary phase cells may live for a long time, develop resting states, such as sexual spores, accomplish cell differentiation and morphogenesis, and irreparable DNA damage may even force cell death (apoptosis). Lifespan of cells is determined anyway; aging comes eventually, and leads to death and autolysis.

These processes are among the most exciting areas of cell biology, and great progress has been made in understanding them by studying yeasts using modern methods of molecular biology and genetics. Only some parts of the topic, falling directly under the subject of this book, will be discussed in the following sections.

5.3.1 Stationary Phase

In a response to starvation for lack of nutrients, yeasts stop proliferating and enter a state called the stationary phase, G_0. It is distinct from the G_1 phase, and is characterized by decrease of growth rate, accumulation of storage carbohydrates, thickening of cell walls, as well as increased resistance to environmental stresses (Werner-Washburne et al., 1993, 1996). Cells survive starvation for a long time, as much as a few months, and can resume growth when nutrients become available.

Stationary phase directly follows the diauxic shift (Figure 5.7), and when approaching it, cells accumulate glycogen for reserve carbohydrate and trehalose, which play a role in the increase of resistance to various stresses. Concentration of trehalose directly correlates with the survival at

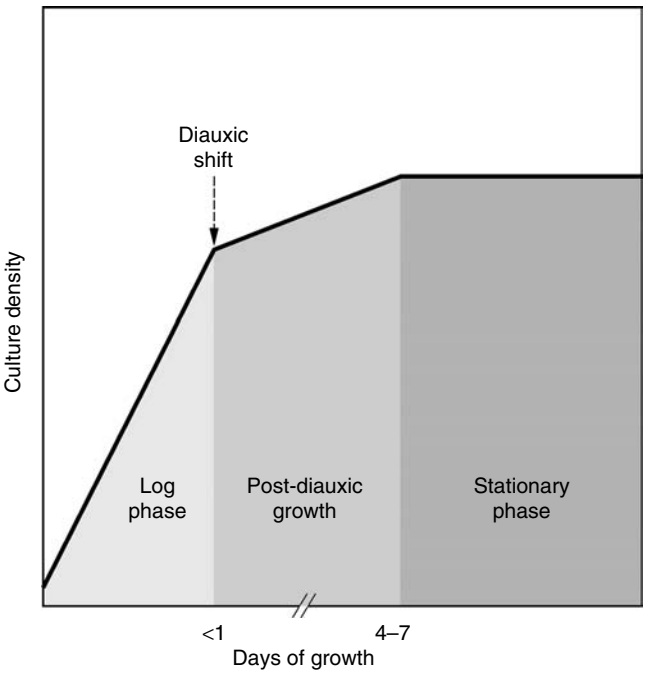

FIGURE 5.7 The diauxic shift in growth curve. During logarithmic growth phase in glucose-containing media, *S. cerevisiae* grows by fermentation. When glucose becomes limiting, the cells transiently arrest growth and switch to a respiratory mode of energy production. This transition period is known as the diauxic shift. During the subsequent post-diauxic growth, cells grow slowly and utilize ethanol produced previously, or alternative carbon sources available. When these sources are exhausted, the cells enter into the stationary phase. (From Herman, P. K. (2002) Curr. Opin. Microbiol. 5:602–607. With permission of Elsevier.)

temperatures of 50–55°C, which are potentially lethal for nonstationary phase cells. Although several events at the molecular level have been identified and about 50 genes expressed specifically with the onset of the stationary phase, and mutants defective in some of these are also known, as yet no specific molecular markers have been found for this particular growth phase. It is known that the entry into stationary phase is regulated by the Ras and Tor signal transduction pathways, both of which play a role in controlling cell growth (Herman, 2002).

From our point of view, acquiring stress resistance in the stationary phase is of primary interest, and a special discussion (Section 5.4) will be devoted to this subject.

5.3.2 Meiosis and Sporulation

Teleomorphic yeast species, such as *S. cerevisiae*, alternate between haplophase and diplophase. The transition between haploid and diploid generation happens through mating, meiosis, and sporulation. Mating type (a or α) of haploid cells is genetically determined by the *MAT* alleles in between the *HML* and *HMR* loci (together forming a mating-type cassette). Expression of *MATa* or *MAT*α phenotype is eventually determined by the *SIR* genes, whereas the *HO* gene mediates the mating-type conversion from a to α and vice versa, enabling mating (conjugation) of opposite mating type. The resulting a/α diploid cell has the potential to sporulate when induced by starvation and other signals (Miller, 1989).

When starved for carbon and nitrogen, almost all diploid cells in the population of *S. cerevisiae* enter meiosis and proceed through ascospore development, resulting in four spores in most cases. The cytological events of meiosis can be followed microscopically and also the pairing of chromosomes by fluorescent *in situ* hybridization (FISH). Analysis of the resulting spores allows us to investigate the outcome of meiotic recombination. Mutants of genes (*RAD*) whose products are responsible for arresting cells in the G_1 phase in response to double-strand breaks provide compelling evidence that breaks in DNA initiate meiotic recombination (Roeder, 1995).

Sporulation in yeasts involves two overlapping processes: the distribution of chromosomes during meiosis and the morphogenesis of spores (Figure 5.8). Concealed behind these is the sequential induction and expression of sporulation-specific genes. At least 150 specific genes have been identified by classical genetic methods as they appear during the early, middle, mid-late, and late phases of sporulation (Mitchell, 1994). However, the use of microarrays showed that the transcription of more than 1000 genes changed in a temporal fashion during sporulation (Chu et al., 1998). Most of the known early genes (about 160) are involved in the prophase of meiosis when the chromosomes first replicate and the homologous pairs align, and then undergo recombination. Another set of about 158 genes play a role in the middle events: the two consecutive meiotic divisions of chromosomes leading to the separation of sister chromatids in parallel with the duplication and separation of spindle pole bodies and formation of the spindle. At the middle–late phase, about 60 genes are induced with the synthesis of prospore membranes and spore walls, whereas only 5 genes are involved in the maturation of the spore wall in the late phase, about 7–12 h after the start of sporulation. Simultaneously with the induction of all these genes, transcription of more than 600 genes becomes repressed.

Beyond genetic determination and environmental induction, sporulation depends on a number of factors, one of which is mitochondrial respiration. Industrial yeast strains usually sporulate poorly and rarely form asci with four spores (Codón et al., 1995).

5.3.3 Morphogenesis, Filamentous Growth, and Flocculation

Dimorphic yeast species, for example, *Ya. lipolytica*, exist as single cells and filaments (hyphae). In addition to true hyphae, individual cells may remain attached together after budding and, having lengthened, form branching chains of cells called pseudohyphae. Morphogenesis for filamentous

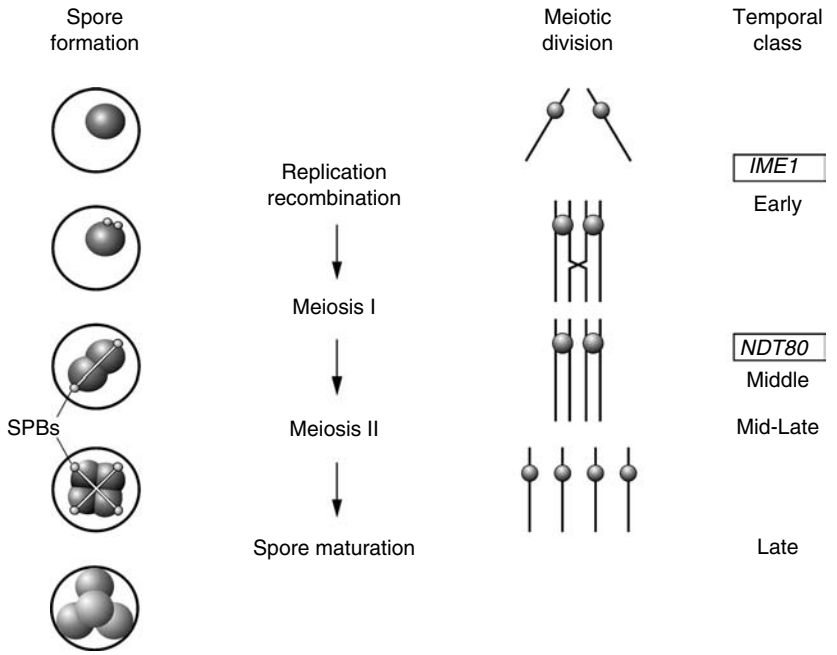

FIGURE 5.8 Events of sporulation. Sporulation involves chromosome distribution and spore morphogenesis. In meiosis, chromosomes first replicate, and then homologous chromosomes align and undergo recombination during prophase. In two consecutive nuclear divisions, chromosomes segregate first (meiosis I), then sister chromatids also separate (meiosis II). Meanwhile, spindle bodies duplicate and separate. During spore morphogenesis, prospore membranes are formed, and maturation of spore walls results in an ascus (sporangium) containing a tetrad of haploid spores. The gene expression program of sporulation involves four temporal classes; transcription of early genes is activated by Ime1 and of some middle genes by Ntd80. (From Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O., and Herskowitz, I. (1998) Science 282:699–705. Reprinted with permission from AAAS.)

growth of yeasts is tightly coupled to the cell cycle (Rua et al., 2001). Some industrial strains of *S. cerevisiae* are characterized by the formation of "flocs," a group of cells adhering together and settling out of suspension, a desired trait in certain types of fermentation. Flocculation is genetically determined (*FLO* genes), and has much in common with filamentous differentiation, such as sharing several features of signaling pathways transmitting environmental factors of induction (Stratford, 1992; see Chapter 7, Section 7.4.1, on brewing).

5.3.4 Aging and Apoptosis

Lifespan of yeasts is determined and finite; the replicative lifespan extends to the end of cells being able to divide (budding), and the chronological lifespan is the time of extended survival in the stationary phase, during which irreversible damages accumulate, leading to death and autolysis (Maskell et al., 2003; McMurray and Gottschling, 2004). These phenomena are best characterized in *S. cerevisiae*, and have also been studied recently in other species, such as *Zygo. bailii*, *Klu. marxianus*, *C. stellata*, and *Klc. apiculata*.

Having divided for a predetermined number of times (marked by the number of bud scars, between 10 and 30 in *S. cerevisiae*), cells enter a state of senescence, accompanied by signs of aging such as altered cell shape, increased vacuole size, decreased protein synthesis and RNA levels, enhanced gluconeogenesis, and others. At the molecular level, concomitant with aging, the silencing proteins (Sir) playing a role in mating switch, redistributed from the chromosomes to the nucleolus,

which enlarges and becomes fragmented. Also, the rDNA repeats from chromosome XII break away and accumulate as extrachromosomal circles in the nucleolar fragments (Sinclair et al., 1998). More than 20 genes have been implicated in aging, some of which are also involved in cell cycle, stress response, sensing, and signaling (Jazwinski, 1999). Eventually, cells die and autolyze, while the cell wall may be preserved; extensive intracellular disorganization takes place, and cell constituents, proteins, lipids, RNA, and DNA degrade (Hernawan and Fleet, 1995; Amrane and Prigent, 1996).

In addition to the normal, physiological aging and death, yeast cells may lose viability in a fast and induced process called apoptosis, also called forced, programmed suicide. This occurs when stationary phase cells are subjected to glucose in the absence of other nutrients, or to low doses of chemical stress, hydrogen peroxide, acetic acid, and preservatives (Granot et al., 2003; Madeo et al., 2004). Apoptosis in yeasts is basically similar to that in metazoans and mammals, and may serve as a good model to better understand cell death mechanisms.

5.4 STRESS RESPONSES

During their lifetimes, yeasts in their habitats are continuously exposed to changes in environmental conditions that they have to respond to in order to survive. These sudden and dramatic, and sometimes slow but extended, changes are called stress effects. In order to cope with the various stresses, yeasts are armored with a complex set of genetic and physiological mechanisms, enabling them to sense and signal stress effects, respond rapidly, and adapt permanently to multiply in the new conditions.

Understanding the physiological and molecular aspects of stress responses is very important for both academic and practical interests. This topic has been the subject of recent reviews (Hohmann and Mager, 2003; Tanghe et al., 2006; Walker and Van Dijck, 2006). The general conclusion of investigations is that yeasts possess general stress responses as well as specific responses to each type of stress (Figure 5.9). This section outlines the general stress mechanisms in yeasts, and responses to individual stresses will be mentioned in relation to the preservative factors to inhibit or inactivate yeasts (Chapter 6). It should be pointed out that although there is much in common in the general stress responses, the routes and mechanisms of which are interwoven and overlapping, there are also as many specific features to individual stress reactions. Moreover, sensitivity and resistance of cells within a population are usually different to a certain degree, and the stress response of a population is heterogeneous. In addition, much of our knowledge about stress responses of yeasts has been accumulated from studies on *S. cerevisiae*, and the increasing number of investigations on other yeast species reveals many differences in stress responses among various yeast species.

5.4.1 General Stress Responses

Yeast respond to a variety of stress factors in a similar mechanism that basically consists of three main parts: receptors for sensing, cascades for transferring the signals, and responding elements at the transcription level. G-proteins and other receptors and signaling pathways have been briefly outlined previously (see Chapter 4, Section 4.6). Here, components at the transcription level will be discussed using the examples of the best-characterized heat responses, which also function in responding to other stresses in general (Estruch, 2000; Trott and Morano, 2003).

Exposing yeast cells to sublethal temperatures for short periods (35–40°C, 30–45 min) leads to protection from death at a subsequent lethal heat treatment (e.g., 52°C). This is called heat-shock response, resulting in the appearance of heat-shock proteins (Hsps). However, a number of other stresses (e.g., oxidative stress, ethanol, nutrient starvation) also induce these proteins; hence, they can be referred to as stress proteins in general. Several types of Hsps exist (e.g., Hsp104, Hsp70, Hsp30, and others), and they act generally as chaperones preventing aggregation of proteins and degrading stress-damaged proteins.

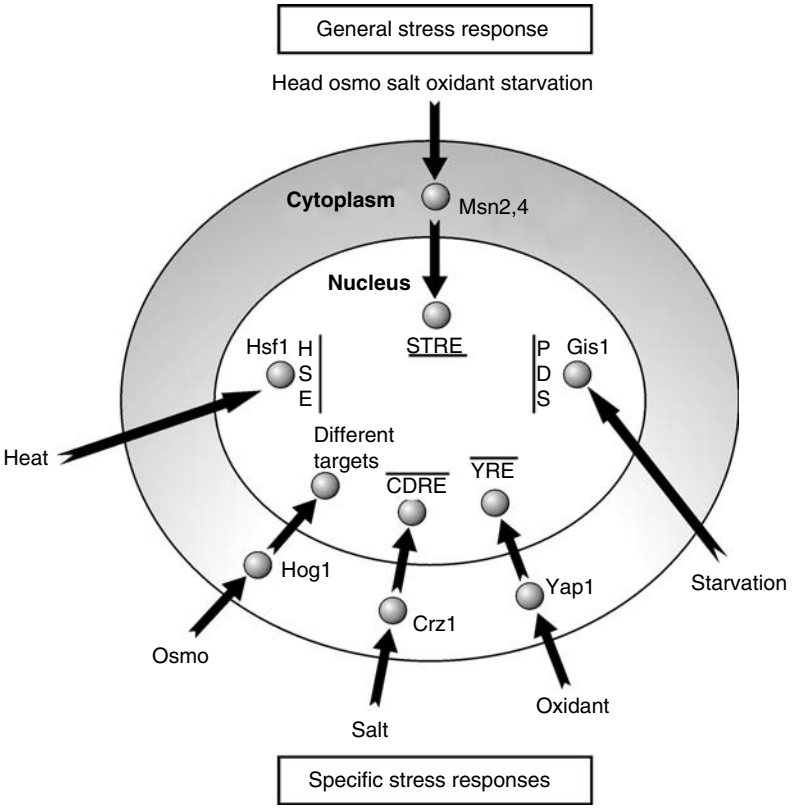

FIGURE 5.9 Stress response mechanisms in *Saccharomyces cerevisiae.* The general stress response is triggered by a variety of stress conditions. It involves the transfer of Msn2 and Msn4 transcription factors to the nucleus, where they bind to stress response elements (STRE) of target genes and induce their transcription. In specific responses to heat, osmotic, salt, oxidation, and starvation stresses, specific factors are activated (hsf1: heat shock, Hog1, Yap1, Crz1, and Gis1 proteins, respectively) that interact with the respective response elements in the nucleus and induce transcription of specific genes in response to stress (HSE: heat shock; CDRE: calcineurin-dependent response; YRE: yap1-response; PDS: postdiauxic shift element). (After Tanghe, A., Prior, B., and Thevelein, J. M. (2006) *In*: Biodiversity and Ecophysiology of Yeasts (eds. Rosa, C. A. and Péter, G.). Springer, Berlin. pp. 175–195. With kind permission of Springer Science and Business Media.)

The stress signal forwarded through one of the cascades eventually reaches some of the transcription factors (e.g., Msn2, Hsf1), which are transferred into the nucleus and interact with the stress-response elements (STRE) or heat-shock elements (HSE) in the promoter of the target genes. These are either induced or repressed as part of the general stress response (Gasch et al., 2000). Examples of genes induced are those involved in the transport and metabolism of nutrients, maintaining interior homeostasis, strengthening cell walls, repairing DNA damage, and many others. Repressed genes are mainly related to growth and protein synthesis.

The consequence of the general stress response is the effect of cross-protection, in that one stress factor (e.g., heat shock) renders cells resistant to other stress conditions, although signal sensors, kinase cascades, and STRE elements may be different. Simultaneous tolerance can be developed to heat, freeze, dehydration, oxidative, and ethanol stress. However, osmotic stress responses are usually different from thermal stress in that they are transmitted by the high-osmolarity glycerol (HOG) pathway (Hohmann, 2000).

Synthesis of molecular chaperones, accumulation of compatible solutes, adaptation of plasma membrane lipid composition, and other phenomena have been recognized as stress protection

mechanisms. One of the most general protective responses is the accumulation of trehalose (Elbein et al., 2003). It is not only a storage carbohydrate and a compatible solute, but also helps protect against stress effect by stabilizing membranes and proteins through maintaining intracellular water relations (Silljé et al., 1999).

5.4.2 Stress Responses in Yeasts

Knowledge of the responses to environmental factors have been gained from studies on populations; hence, they represent the average responses of a heterogeneous assembly of cells.

Heterogeneity is a natural attribute of a cell population; even a laboratory pure culture is, in the best case, a clone, an isogenic population with physiological differences among its members. Heterogeneity manifests itself in differential sensitivity to stress conditions that can be fundamental to the survival of some members of a population (Booth, 2002). Phenotypic heterogeneity comes from the differences in growth phases and rate; cell cycle, age, and size of cells; metabolic regulation; and other properties that show a stochastic distribution (Sumner and Avery, 2002). The technique of fluorescent flow cytometry can detect the heterogeneity of a cell population (Attfield et al., 2001).

Differences in stress resistance occur between strains belonging to the same species, and this has been found repeatedly in wine strains of *S. cerevisiae* (Carrasco et al., 2001; Garay-Arroyo et al., 2004; Zuzuarregui and Olmo, 2004). One reason lies in the genetic segregation under the effect of selective environment (Sipiczki et al., 2004). However, mutation and other genetic changes normally occur in populations and form the basis of natural selection, adaptation, and evolution (Zeyl, 2004).

Most of our knowledge about stress responses relates to *S. cerevisiae*. Recently, other yeast species have been receiving increased attention in terms of biotechnology interest and basic research. From a medical point of view, *C. albicans* is significant, and of food spoilage yeasts, *Zygo. bailii*, *Db. hansenii*, *Schizo. pombe*, *P. anomala*, *Klu. lactis*, and a few others have been investigated for their stress tolerance (Smits and Brul, 2005; Türkel et al., 2006). The general stress resistance mechanism shows analogies to that of *S. cerevisiae*, but specific properties of each toward particular stress factors have evolved in their ecological habitats. For example, in *P. anomala*, contrary to *S. cerevisiae*, invertase synthesis and glucose consumption are not affected by osmotic stress. *Db. hansenii* accumulates trehalose in response to high salt concentration, in line with *S. cerevisiae*, whereas it is expressed highly in the absence of salt in *Zygo. rouxii*, and does not play a role in the weak acid resistance of *Zygo. bailii*.

Obviously, further insight into the stress response of various yeasts will bring important new knowledge for both fundamental and industrial interests.

5.4.3 Injury and Repair

Although yeast cells possess an armory of protective responses to stress factors, they suffer sublethal injury or eventually die on exposure to extreme conditions (Figure 5.10). Loss of vitality may be due to damage of plasma membrane, denaturation of enzyme proteins, breaking of DNA, and blocks in gene transcription, and could be caused by a single event or the sum of all. Explanation of the mechanism of cell death is still a debated issue. An important issue from the practical point of view is the decrease in the fermentative power of baker's yeasts in frozen dough due to freeze–thaw injury. It is suggested that the injury is caused by two main mechanisms, oxidative stress and defects in cell wall biogenesis; and in freeze–thaw tolerance, genes are involved, functioning in vacuolar H^+-ATPase (Ando et al., 2006).

Cells injured by sublethal doses may be able to repair the damage and resume growth under appropriate conditions (Fleet and Mian, 1998). The viable but nonculturable state (VBNC) of cells has been the subject of numerous studies with bacteria. With yeasts, it may be the cause of growth in wine after long periods of noncultivability on plates (Millet and Lonvaud-Funel, 2000). A similar phenomenon also occurred after low-amperage treatment (Guillou et al., 2003). The VBNC state of

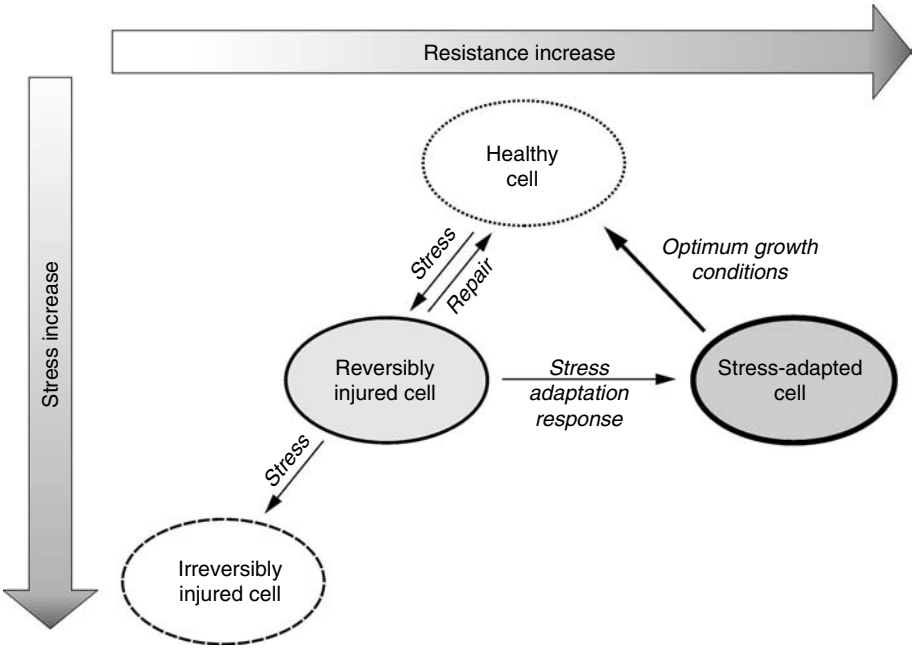

FIGURE 5.10 Microbial stress, injury, adaptation, and resistance to processing. (Reprinted from Lado and Youseff (2002). With permission from Elsevier.)

pathogens after clinical treatment or of spoilage organisms in preserved foods may have practical consequence in their false assessment. If selective media are used for detection without resuscitation, injured cells would not be able to form colonies and hence would produce lower plate counts (Beuchat, 1984).

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6 Preservation: Inhibition and Inactivation of Yeasts

Chapter 3 outlined the main extrinsic and intrinsic ecological factors in relation to the implicit properties of yeasts. Chapter 5 presented the general characteristics of factors affecting growth and death. Here we discuss in more detail the practical application of these factors as preservative treatments aimed at inhibiting and inactivating spoilage yeasts.

6.1 HEAT INACTIVATION

The resistance of yeasts to high temperatures is comparable to that of vegetative bacteria; however, unlike bacterial endospores, the ascospores of yeasts may be only slightly more resistant to heat than vegetative cells (Put and De Jong, 1980; Splittstoesser et al., 1986).

Yeast cells are usually killed within a few minutes at temperatures between 55°C and 65°C (Put and De Jong, 1982a,b; Engel et al., 1994). The decimal reduction time (*D*-value) at 55°C is about 5–10 min, at 65°C, <1 min (Figure 6.1). The death rate increases 10-fold on a rise of temperature by 4–5 degrees (the *z*-value is 4–5°C). These are average values only, as slight differences may occur between yeast species and strains, and the composition of the heating media (foods) exerts a substantial influence on the rate of heat inactivation. For example, the *D*-values for *P. anomala* were about 6 min in fruit juices at pH 3.95, and 2 min at pH 2.62 (Figure 6.2). As referred to earlier

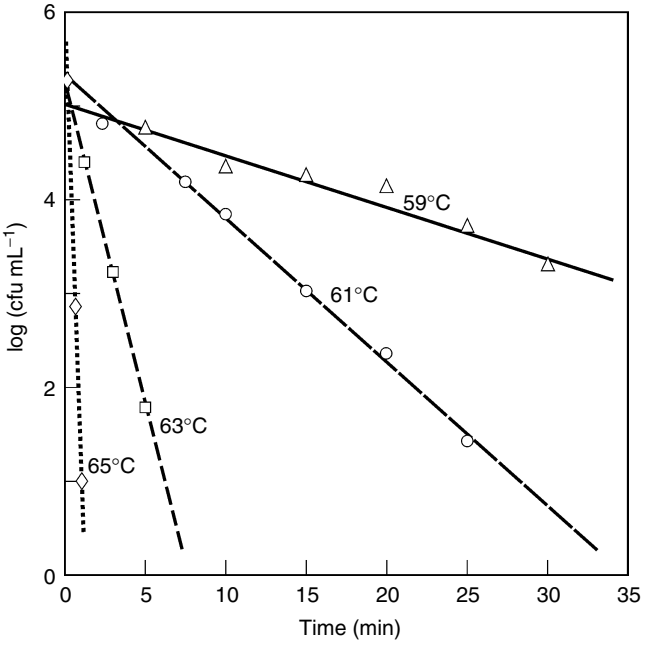

FIGURE 6.1 Survival curves of *Issatchenkia orientalis* (*Candida krusei*) at different temperatures in pH 7.0 buffer. (From Engel, G., Rosch, N., and Heller, K. J. (1994) Kieler Milchwirtsch. Forsch. 46:81–90. With permission of Th. Mann Publishing.)

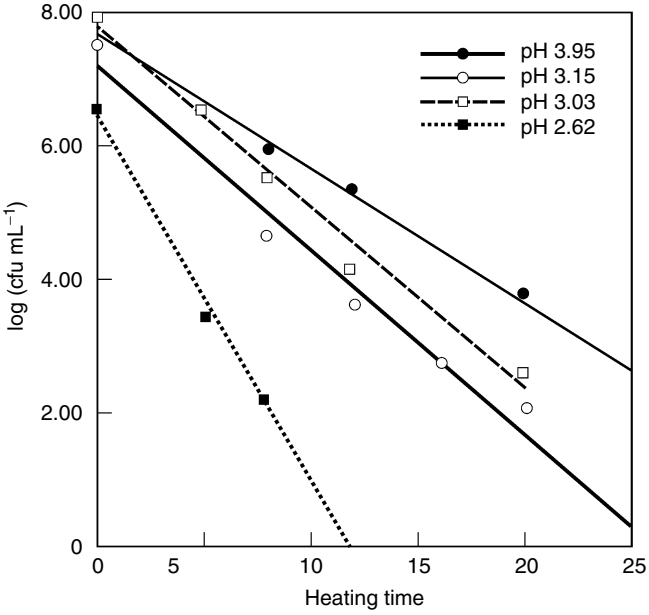

FIGURE 6.2 Survival curves of *Pichia anomala* (*Candida pelliculosa*) in fruit juices of various pH values. (From Tchango, J. T., Tailliez, R., Eb, P., Njine, T., and Hornez, J. P. (1997) Food Microbiol. 14:93–99. With permission from Elsevier.)

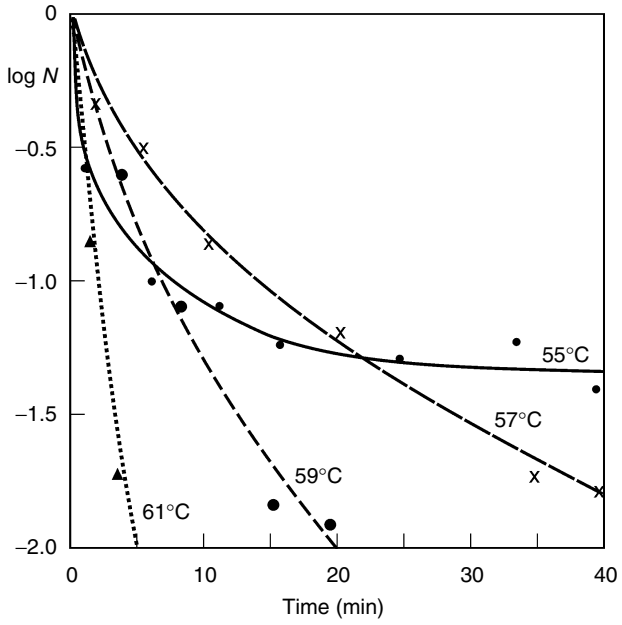

FIGURE 6.3 Survival curves for inactivation of *Zygosaccharomyces bailii* at different temperatures. (From van Boekel, M. A. J. S. (2002) Int. J. Food Microbiol. 74:139–159. With permission from Elsevier.)

(Section 5.2.1), the nonlinear shape of survival curves sometimes renders the calculation of death rate parameters difficult (Figure 6.3).

The heat resistance of yeast cells decreases at acidic pH values, and increases with lowering a_w (Agab and Collins, 1992a). Hence, fruit products, such as juices with a pH of about 3.5 or less, can

TABLE 6.1
Heat Resistance of Vegetative Cells and Ascospores of *Zygosaccharomyces bailii* in Fruit Juices

Juices	pH	$D_{50°C}$ (min) Vegetative Cells	Ascospores
Apple	4.1	3.02	15.6
Orange	3.9	1.97	10.4
Pineapple	3.4	4.48	37.0
Cranberry	3.5	2.04	14.9
Grape	3.0	4.18	33.3

Source: Data from Raso, J., Calderón, M. L., Góngora, M., Barbosa-Cánovas, G. V., and Swanson, B. G. (1998a) J. Food Sci. 63:1042–1044.

be preserved by pasteurization temperatures, whereas the same heat treatment may be insufficient for the preservation of fruit jams of the same pH but containing 55% or more sugar ($a_w < 0.965$) (Corry, 1976). A study on the heat resistance of vegetative cells and ascospores of *Zygo. bailii* in various fruit juices (Raso et al., 1998a) found that the resistance was related not only to pH but also to the composition of juices (Table 6.1). In this study, ascospores exhibited a heat resistance 5–8 times greater than that of vegetative cells. Greater heat sensitivity of *Dekkera* species shown in wines was largely attributed to ethanol, whereas in the range of pH tested (2.4–4.5) neither *D* nor *z*-values changed significantly (Couto et al., 2005).

Conditions prevailing before heat treatment also affect resistance of cells, such as the composition of the growth medium, age, and phase of sporulation (Su and Beuchat, 1984; Su et al., 1985), as well as starvation or aerobic and anaerobic growth (Agab and Collins, 1992b). *D*-values were higher for cells in the exponential phase than for those in the stationary phase; however, this appeared only at lower temperature (45°C) and equalized above 50°C (Couto et al., 2005). Heat resistance of yeasts (*S. cerevisiae*, *Zygo. bailii*) increased when grown in media with reduced a_w or heat treated in the presence of sugars (Stecchini and Beuchat, 1985; Golden and Beuchat, 1992). Protection against heat inactivation was also observed when NaCl was used to reduce a_w (Beuchat, 1981a). Heat tolerance of yeasts increases to a large extent when treatment is made by dry heat. The rate of heat inactivation in an atmosphere containing less than 30% relative humidity decreases to a large extent. At 110°C, *D*-values ranged between 1.25 and 3.63 min for *Db. hansenii*, *Klc. apiculata*, *Lodd. elongisporus*, and *P. anomala*, whereas *C. glabrata* exhibited $D_{127°C}$ value of 0.78 min, and *S. cerevisiae* $D_{135°C}$ ranging from 0.49 to 0.89 min. The *z*-values also increased to 9.1–13.3°C (Scott and Bernard, 1985). These reduction time values in minutes are comparable to those observed at 55–60°C in moist heat.

Factors enhancing heat sensitivity of yeast cells are the pH of the suspending medium and the presence of preservatives. Data on the decrease of heat resistance with decreasing pH are unambiguous (Beuchat, 1981b); however, the type of acidulent and the buffering capacity of foods can have a major influence on the rate of inactivation (Juven et al., 1978; Beuchat, 1983; Torreggiani et al., 1985; Garza et al., 1994). By determining the degree of dissociation of weak acid preservatives, the pH also influences their synergistic action with heat treatment (Tsuchido et al., 1972; Beuchat, 1981b, 1982). Although redox potential is known to be an important factor influencing growth, there are only very few data on the effect of redox potential on heat resistance. Reichart and Mohácsi-Farkas (1994) modeled the kinetics of heat destruction for some bacteria, molds, and yeasts under the combined effect of temperature, a_w, pH, and E_h (150, 240, and 460 mV, corresponding to rH values 13–23) and found, in agreement with other results, that the heat destruction rate increased with decreasing pH and increasing a_w, whereas the effect of the redox potential depended on the kind of microorganisms. Increasing the redox potential of the heating medium accelerated the heat

destruction of lactobacilli; however, it increased the heat resistance of fungi, and also of the yeasts studied (*S. cerevisiae*, *Zygo. bailii*, and *Ya. lipolytica*).

The mechanism of heat inactivation of cells is not well understood, and theories for explanation are equivocal. However, it appears that changes in cellular structures may be largely responsible for causing death, rather than denaturation of enzymes and other proteins or the breaking of nucleic acids.

Török and Reichart (1983) and Török and King (1991) made a detailed analysis of the thermal inactivation kinetics of yeasts, calculating the apparent activation energy and the entropy and enthalpy of activation of the process, and came to the conclusion that damage in higher-order structures (such as ribosomes and mitochondria) must be responsible for the thermal death of yeast cells.

Heat-shock proteins (e.g., Hsp104) protect cells against heat stress. Their level increases in yeast cells growing on nonfermentable carbon sources, suggesting that aerobic metabolism provides more energy for their synthesis. By the blocking of mitochondrial function by inhibitors or in mitochondria-less petite mutants, the induction of Hsp104 synthesis is reduced, providing indirect evidence for the role of intact mitochondria in heat response and thermotolerance (Rikhvanov et al., 2003, 2004). Other macromolecular complexes suspected to be responsible for sensitivity or resistance to heat and other stresses are the production of messenger RNAs and their transport through nuclear membrane pores (Bond, 2006).

6.2 REFRIGERATION AND FREEZING

Yeast cells during storage at 4°C slowly decrease in viability. Cold storage induces deterioration of the physiological state of cells that is marked by the loss of storage carbohydrates, in particular trehalose, followed by the decrease of membrane integrity (Gabier et al., 2005).

Freezing does not cause immediate death of yeast cells, although the number of survivors in a frozen state decreases with time. The degree and rate of death caused by freezing depend on a number of factors, such as the temperature of freezing, the rate of temperature decrease, the time spent in the frozen state, and thawing conditions (Koburger, 1981; Beuchat, 1984). The viability of yeast cells after freezing at −80°C remains higher than those frozen at −20°C. In general, the faster the rate of freezing and thawing, and the lower the temperature of the frozen state, the higher the ratio of survivors. This is due to the formation of ice microcrystals that cause less destruction of cells (Mazur and Schmidt, 1968; Komatsu et al., 1987). Under these conditions, the cell membranes are exposed for a shorter time to the destructive effect of increased osmotic pressure (Gelinas et al., 1991). Hence, in practice, strains in culture collections can be stored at −80°C for a long time, and an even better way to preserve cultures is by very fast deep-freezing at the temperature of liquid nitrogen (−196°C). Protection against inactivation at subfreezing temperatures can be afforded by lowering the a_w of solute in which cells are suspended (Murdock and Hatcher, 1978; Golden and Beuchat, 1992).

6.3 DEHYDRATION (DRYING)

Decreasing of water activity beyond limits permitting growth can be brought about in many ways. Drying is one of the oldest methods of food preservation, and it is still used successfully to inhibit growth of yeasts on fruits and vegetables (e.g., raisins, nuts, and pulses). High concentrations of sugar (50–60%) or salt (5–10%) also bring about binding of free water and prevent yeasts from multiplying (e.g., in jams, syrups, ham, and soy sauce). Some xerotolerant (osmophilic) yeasts, such as several species of *Zygosaccharomyces*, may grow slowly, particularly if moisture is absorbed at the surface of products. Freezing and freeze-drying also result in dehydration of cells; the latter method is widely used for the production of active dry microbial biomass for starter cultures, and for preserving microbial strains in culture collections. Hence, the surviving and preserving of cell viability during dehydration are also of interest. To achieve this goal, several parameters must

be considered, such as the growth phase of cells, the conditions of both dehydration and rehydration, as well as the conditions during storage. Slow rehydration while crossing the a_w range of 0.117–0.445 increases viability, allowing phase transition to take place in membranes (Poirier et al., 1999).

The water activity falls below 0.70 in dried and concentrated foods. A great variety of foods, for example, cheeses, sausages, and bakery products, have a_w values in the range 0.85–0.95. These are called intermediate moisture foods (IMF), and their keeping quality depends on the application of other preservation methods such as chilling, vacuum packaging, modified atmosphere, addition of a preservative, or a combination of these.

A specific molecular mechanism of osmotic stress response called high-osmolarity glycerol (HOG) pathway functions in yeasts, which is different from the general stress responses (Hohmann, 2002). Specific membrane receptors (Sho1, Sln1) activate the MAPK cascade (Table 4.4), and its last phosphorylated member (Hog1) enters the nucleus and regulates the expression of numerous *HSP* and other stress genes, also resulting cell cycle arrest in the G_1 phase, as well as genes controlling glycerol metabolism. Although the HOG pathway plays a key role in osmotic stress response, it is also responsible for the adaptation to citric acid stress (Lawrence et al., 2004), and the MAPK cascades transducer signals not only by osmotic stress but also by other external stimuli.

A high concentration of sugars or salt in the environment causes efflux of intracellular water, reduction of the volume of vacuole, and shrinking of whole cell. The physiological responses of yeasts are twofold: activation of transport systems for expelling ions (Na^+, K^+), and synthesis of compatible solutes to balance interior osmotic pressure (Myers et al., 1997; González-Hernández et al., 2004). Glycerol is the major compatible solute, but trehalose and mannitol are also important in protecting yeasts against osmotic stress. Xerotolerant (osmophilic, halophilic) yeasts have developed other strategies that allow them not only to withstand osmotic stress but also to function better in low-a_w environments. Salt-tolerant yeasts such as *Db. hansenii* and *C. versatilis* maintain a high K^+/Na^+ ratio in the cells, whereas sugar-tolerant yeasts, for example, *Zygo. rouxii*, synthesizes glycerol without stress and are able to retain it to counterbalance osmotic stress (van Zyl et al., 1993; Silva-Graca et al., 2003; González-Hernández et al., 2004).

6.4 IRRADIATION

Various kinds of ionizing radiation such as electron beam, x-ray, and gamma rays of ^{60}Co have high energy and cause extensive death of cells. Irradiation of foods has long been recognized as a potent preservation technique. Extensive research and technical experience have clearly established its benefits and limitations (Farkas, 1998). Its use has been approved in an increasing number of countries, although there remains a certain degree of consumer resistance toward irradiated foods.

No other preservation technology has been evaluated so thoroughly as irradiation, and a large body of literature exists concerning its biological effects and applications (Monk et al., 1995; FAO/IAEA/WHO, 1999). Here, only a small amount of information related to yeasts is provided. The radiation resistance of some yeast is given in Table 6.2. Yeasts are more resistant to ionizing radiation than most vegetative bacteria (Monk et al., 1995). Their decimal reduction value falls in the range of 0.1–0.5 kGy, and a radiation dose of about 5 kGy would reduce their number by 10 log cycles. However, survival curves often show shoulders that render the calculation of the inactivation dose less straightforward (McCarthy and Damoglou, 1996). Some yeast, for example, *Trichosporon* species, and some strains of a given species appear to bear higher radiation resistance.

In commercial application, gamma irradiation at relatively mild doses (1–3 kGy) significantly decreases the yeast contamination of meat and meat products (Lescano et al., 1991; McCarthy and Damoglou, 1993) and nuts (Narvaiz et al., 1992), and increases the storage time of perishable fruits. Irradiation is especially suited for extending the shelf life of soft fruits (e.g., strawberry and raspberry) that are sensitive to other ways of preservation (O'Connor and Mitchell, 1991).

TABLE 6.2
Radiation Resistance of Some Yeast Species

Yeast Species[a]	Irradiation Medium	Irradiation Dose (kGy)[b]
Sporidiobolus pararoseus	Nutrient broth	5
Issatchenkia orientalis	Phosphate buffer	5.5
Debaryomyces hansenii	Grape juice	7.5
Cryptococcus albidus	Grape juice	10
Torulaspora delbrueckii	Grape juice	15
Saccharomyces cerevisiae	Grape juice	18
Guehomyces pullulans	Phosphate buffer	20

[a] Initial cell number 10^6–10^7 per mL.
[b] Dose required for preventing growth for at least 15 days.
Source: Data adapted from Farkas, J. (1999) *In*: High-dose Irradiation: Wholesomeness of Food Irradiated with Doses above 10 kGy (Report FAO/IAEA/WHO study group). WHO, Geneva. pp. 49–77.

6.5 ALTERNATIVE AND NOVEL PRESERVATION TECHNOLOGIES

In recent years, the growing demands for safer, fresher, more nutritious, and novel food products have stimulated research into alternative preservation technologies. Microwave and ohmic heating represent thermal treatment; however, most of the alternative methods apply physical principles other than heat treatment, such as high pressure, pulsed electric field, oscillating magnetic field, and ultrasound, alone or in combination with pasteurization, refrigeration, or freezing. Certain chemical treatments such as the use of chitosans, bacteriocins, and other antimicrobials have been tested as well. So far, experiments have been conducted on a laboratory or pilot scale with little commercial application. The microbiological studies have focused mostly on pathogenic bacteria, but yeasts often serve as eukaryotic model organisms. Several reviews giving an overview of the subject have appeared (Gould, 2000a; Farkas, 2001; Lado and Yousef, 2002; Raso and Barbosa-Cánovas, 2003; Devlieghere et al., 2004; Mañas and Pagán, 2005).

6.5.1 Ohmic and Microwave Heating

Advances in technology have allowed the reduction of excessive heat treatment by the use of high-temperature, short-time (HTST) pasteurization and ultra-high-temperature (UHT) sterilization. Alternative methods to generate heat electrically in products are the microwave and ohmic heating (Farkas, 1997). Use of microwave ovens is widespread in homes but more restricted in industry for food sterilization (Datta and Davidson, 2000), whereas ohmic heating has various potential for unit operations (e.g., extraction, dehydration, and blanching) but not sterilization (Sastry and Barach, 2000). In both cases, inactivation of microorganisms appears to be caused solely by heat without any other specific effect, and products treated by these methods still lack fresh flavor and texture, offering free scope to nonthermal alternative technologies.

6.5.2 High Hydrostatic Pressure

High-pressure processing represents the most advanced alternative technology for the preservation of foods (Smelt, 1998; Farkas and Hoover, 2000; San Martin et al., 2002). High pressure over 100 MPa damages cell membranes and may inactivate vegetative cells, since yeasts are more sensitive than

TABLE 6.3
Decimal Reduction Time (*D*) Values for High-Pressure Inactivation of *Saccharomyces cerevisiae* Ascospores

	D-values (min)			
Pressure (MPa)	**Orange Juice (pH 3.8)**	**Apple Juice (pH 3.9)**	**Model Juice (pH 3.5)**	**Model Juice (pH 5.0)**
500	0.18	0.15	0.14	0.19
450	0.50	0.48	0.38	0.49
400	0.97	0.88	0.72	0.98
350	2.80	2.51	2.15	2.84
300	10.81	9.97	7.21	9.42

Source: Data from Zook, C. D., Parish, M. E., Bradock, R. J., and Balaban, M. O. (1999) J. Food Sci. 64:533–535.

bacteria (Arroyo et al., 1999). Pressures of 400–600 MPa bring about inactivation of yeast cells, most probably by membrane permeabilization, but this is incomplete and some cells may survive injury (Perrier-Cornet et al., 1999). Kinetic analysis of survivor curves suggested a biphasic destruction of cells, consisting of instantaneous death due to the pulse pressure, followed by first-order death rate during holding pressure (Basak et al., 2002). Changes in cell wall structures have also been considered as a possible area of mechanical action of high pressure (Brul et al., 2000). In a recent model study, an explanation was put forward for the inactivation of cells by hydrostatic pressures, both exerting a mechanical stress on cell wall and hydrostatic stress on the interior parts of cell. However, the former action is encountered when pressure load reaches a critical value between 415 and 460 MPa (Hartmann et al., 2006).

Water activity is a critical factor in the inactivation of yeasts, while pH does not influence survival (Table 6.3). A great advantage of high-pressure processing is its antimicrobial efficacy at ambient and even at chilling and freezing temperatures. Inactivation of *S. cerevisiae* was found to be greatest at temperatures above 48°C and below −10°C (Hashizume et al., 1995). Although equipment cost and throughput limit commercial application, the use of high-pressure processing is increasing (Yuste et al., 2001). Application of high-pressure technology is most advanced for preservation of fruit juices, jams, jellies, and sauces—products that are prone to spoilage by yeasts (Parish, 1998; Zook et al., 1999). However, reduction of a_w increases barotolerance in the pressure pasteurization of jams (Oxen and Knorr, 1993; Palou et al., 1997). This effect of a_w causes a larger *D*-value (287 MPa) for *S. cerevisiae* in concentrated orange juice of 42°Bx compared to 135 MPa in single-strength (11.4°Bx) orange juice (Basak et al., 2002).

Apart from sterilization, high pressure can be applied for various other technological purposes, such as homogenization. Guerzoni et al. (1999) found that high-pressure (>100 MPa) homogenization combined with ethanol (≥1%) and elevated temperature (30°C) effectively reduced yeast cell viability by 2–5 log cycles within a contact time of 60 min. On the other hand, development of cross-resistance has been found with pressure on exposure to ethanol, H_2O_2, and cold-shock (Palhano et al., 2004). The combination of temperature and pressure demonstrated a synergetic effect and allows reduction of the level of either agent alone (Donsi et al., 2003).

High-pressure carbonation, a special case of combining pressure and chemical effect, will be discussed later.

6.5.3 Pulsed Electric Field

Electric fields with intensities between 5 and 25 kV/cm, applied for several tens of pulses, each with duration of microseconds, can inactivate microorganisms and can be used for nonthermal

processing of foods placed between two electrodes (for reviews, see Barbosa-Canovas et al., 2000a; Wouters et al., 2001). The formation of pores in the membrane (electroporation) appears to be the main cause of death (Aronsson and Rönner, 2005); however, Harrison et al. (1997) suggest that disruption of cellular organelles, especially ribosomes, is the primary mode of yeast inactivation, with electroporation acting as a secondary mode of inactivation. The growth region of budding was found particularly sensitive to pulsed electric fields (Castro et al., 1993). Yeasts are killed more easily than bacteria by electric fields, and inactivation increases greatly with field strength and the number of pulses (Cserhalmi et al., 2002), while reduced a_w increases resistance (Aronsson and Rönner, 2001). Several other process and product parameters are critical in affecting microbial inactivation by pulsed electric field (Zhang et al., 1994a; Elez-Martinez et al., 2004). The technique has potential in cold pasteurization of liquid foods, such as fruit juices and milk, without causing adverse organoleptic changes (Zhang et al., 1994b; Qin et al., 1996).

Other possible uses of electrical energy include the generation of pulsed shock waves in liquids (Zuckerman et al., 2002), low-amperage electrolysis (Guillou et al., 2002, 2003), and radio frequency electric fields (Geveke and Brunkhorst, 2003), which have been suggested for the inactivation of microorganisms. The efficacy of these treatments in food preservation needs further evaluation.

6.5.4 Oscillating Magnetic Fields

Magnetic fields with intensities high enough to inactivate microorganisms can be generated by electric coils under current. For satisfactory preservation by magnetic fields, foods of high electrical resistivity are the best suited. Successful experiments have been conducted with orange juice and yoghurt inoculated with *S. cerevisiae.* However, more detailed studies are needed before this technology can be developed commercially.

6.5.5 UV Light and Light Pulses

The antimicrobial effect of ultraviolet light results from lethal mutation due to damage of the DNA (Sastry et al., 2000). Generated by mercury lamps, UV-C light between 254 and 280 nm has a germicidal effect on viruses, bacteria, fungi, and parasites (Shama, 1999). Irradiation with long-wave UV (320–400 nm) causes the formation of hydrogen peroxide radicals in the unsaturated fatty acids, which induces changes in membrane permeability. Because UV light has a very low penetration effect, UV irradiation has been used in particular for the sterilization of air and for the treatment of drinking water. It is also commonly used for sterilization of surfaces and packaging materials (Bintsis et al., 2000; Guerrero-Beltrán and Barbosa-Cánovas, 2004). UV light penetrates only 0.1–1 mm into turbid or colored liquids such as fruit juices and milk. These can be treated by UV light in thin films flowing through appropriate devices. Flow rates and UV light doses influence the inactivation of yeast (Guerrero-Beltrán and Barbosa-Cánovas, 2006).

An extension of UV technology using near-infrared region of wavelengths applied in intense short pulses constitutes the basis of a new method that can be used for surface sterilization of foods such as baked goods, seafood and meats, fruits, and vegetables (Dunn et al., 1995; Barbosa-Canovas et al., 2000b). High-intensity light pulses are capable of inactivating microorganisms. In addition, to form pyrimidine dimers in DNA, pulsed light irradiation also causes membrane damage in yeast cells (Takeshita et al., 2003). Surface treatment by laser light (1064 nm of about 10 J/cm energy pulses with 8-ms pulses) caused 10^6 order inactivation of *S. cerevisiae* and *C. albicans* (Ward et al., 1996). Yeast cell counts can be reduced by 5–6 log cycles using full-spectrum light with intensities of 0.1–0.4 J/cm for only 2–4 flashes. Molds and bacteria are apparently more resistant. When dispersed with reflectors to ensure uniform exposure to pulsed UV light, the surfaces of fresh fruits can be disinfected (Lagunas-Solar et al., 2006). This technology is an attractive alternative in aseptic processing.

6.5.6 Ultrasounds

Ultrasound technology (ultrasonics) applies high-energy sound waves of 20,000 or more vibrations per second. High-power ultrasound has microbicide effects caused by intracellular cavitation that disrupts cellular structure and function (Hoover, 2000). Food materials may interfere with sound wave penetration, and ultrasound must be combined with other preservation methods for safe application. Ultrasound combined with heat (thermoultrasonication), pressure (manosonication), or both (manothermosonication) enhance microbial inactivation in foods (Raso et al., 1998b). About 20 kH ultrasound at temperatures between 45° and 55°C decreased the *D*-values for *S. cerevisiae* by third to 30th parts (López-Malo et al., 1999). Ultrasonics appears to have the greatest potential for product decontamination and cleaning of processing equipments. The cleaning action of cavitation appears to remove cells from the surface of raw and minimally processed fruits and vegetables. No commercial products have as yet been released.

6.5.7 Novel Combinations of Preservation Methods

Many of the emerging alternative methods are being developed for use as part of a hurdle technology that involves combining more than one method with synergistic effect.

As in the case of irradiation, alternative physical treatments in combination with other preservation methods can be used in lesser doses than are required alone to obtain optimal product quality and microbiological safety. High-pressure, pulsed electric fields, ultrasounds, and oscillating magnetic fields have been suggested in combination with pasteurization or freezing without decreasing the efficacy of treatment. Combined technologies such as mano–thermo–sonication (pressure + mild heat + ultrasonics) or osmo–dehydro–freezing (sugar addition + air drying + freezing) have been proposed for the protection of fruit juices and fruit slices from yeast spoilage while retaining flavor, color, and functional properties. The utilization of novel interactions to prevent the growth of spoilage as well as of pathogenic microorganisms and to ensure the quality and safety of foods is a complex and challenging task in food technology. Lessons drawn from cross responses to stresses show that a combination of treatments may not be advantageous. Sublethal heating shock raised the resistance of *S. cerevisiae* not only to subsequent treatment at higher temperature, but also to hydrostatic pressure (Iwahashi et al., 1991, 1997).

6.6 CHEMICAL INHIBITION

In the production of foods, a number of chemical preservatives have been used, some of them traditionally for centuries, others introduced recently. They differ widely in chemical nature and origin, and can be organic or inorganic, synthetic or natural, biological products. Comprehensive treatments have been published on chemical preservatives (Russel and Gould, 1991; Gould, 1995), and a recent excellent review discusses current knowledge about mechanisms of action of and microbial stress responses to preservatives (Brul and Coote, 1999).

6.6.1 Acidification

Growth of microorganisms in foods can be inhibited by acidification and decreasing of pH, either by the addition of acids or producing them by fermentation. Acidulents used to adjust acidity and pH are either strong inorganic acids such as hydrochloric (HCl), phosphoric (H_3PO_4), or weak organic acids such as acetic, lactic, and citric acids. Weak organic acids also include those compounds that are used primarily as preservatives, such as benzoic, sorbic, and propionic acids; these will be discussed separately.

In aqueous food systems, strong inorganic acids dissociate nearly completely, and their antimicrobial effect is due to the decrease of pH. Phosphoric acid, used in cola-type drinks, may have

TABLE 6.4
Dissociation of Weak Acid Preservatives as Affected by pH

	pK_a	Undissociated Acid (%) at pH					
Preservative	**2.5**	**3.5**	**4.5**	**5.0**	**5.5**	**6.0**	**7.0**
Acetic acid	4.74	99	95	63	35	14	5.2
Citric acid	3.13	81	30	4.1	1.3	0.4	0.13
Lactic acid	2.74	64	15	1.7	0.5	0.2	0.06
Benzoic acid	4.19	98	83	33	13	4.7	1.5
Sorbic acid	4.76	99	95	65	37	15	5.4
Sulfurous acid (SO_2 in water)	1.81	17	2	0.2	0.06	0.02	0.01

Source: Adapted from De Boer, E. and Nielsen, P. V. (1995) *In*: Introduction to Food-borne Fungi, 4th ed. (eds. Samson, R. A., Hoekstra, E. S., Frisvad, J. C., and Filtenborg, O.). CBS, Baarn, the Netherlands. pp. 289–294.

a specific inhibitory effect as well. Dissociation of weak organic acids depends on their chemical character indicated by the dissociation constant, K_a, or its negative logarithm, pK_a (Table 6.4.). They exert specific antimicrobial effects in undissociated form, and it is then influenced by the pH, the concentration of the acid, and also the buffering capacity of the food. Yeasts in general can grow at much lower pH values than bacteria (see Section 3.2.2.3).

In the pH range of 3.0–3.5, acetate, lactate, or propionate are inhibitory to yeasts in 0.8–1.0% concentration, although tolerance of several yeast species is much higher; for example, *Zygo. bailii* or some strains of *P. membranifaciens* may grow in the presence of 1.5–2.0% acetic acid.

Citric, malic, or tartaric acid are mainly used to adjust the organoleptic property of products; they have little inhibitory effect, which is partly due to their chelating action.

6.6.2 Weak Acid Preservatives

In historic order, propionic, benzoic, and sorbic acids have been applied as preservatives. None of these is used in free acid form because they are all either corrosive or poorly soluble; instead, they are applied as Ca, Na, and K salts, respectively. Esters of *p*-hydroxybenzoic acid (methyl-, ethyl-, and propyl-parabens) are noted for dissociation being independent of pH, whereas efficacy of propionate, benzoate, and sorbate is bound to the undissociated form, and inhibitory action is greatest at acidic pH values below 5.

Propionic acid naturally occurs in Swiss-type cheeses; Ca-propionate as a preservative is effective against molds but has negligible activity against yeasts and bacteria, except for inhibiting rope-causing bacilli. Therefore, the use of propionate is primarily to retard mold growth in cheeses and bakery products (bread, rolls, cakes, etc.). In the latter products, at around pH 6, 0.3–0.4% propionate can be used. Cartons, boxes, and other packaging materials can be impregnated with propionate salts to provide protection against molds.

Benzoic acid can be found in some fruits (cranberries, prunes, etc.). Sodium benzoate is widely used in foods and beverages with pH values below 4, at which point benzoate is most effective against bacteria and yeast but less so in controlling molds. Benzoate is affirmed as a generally-recognized-as-safe (GRAS) preservative in concentrations of up to 0.1%.

Sorbic acid also occurs naturally in some wild plants (Sorbus), and its antimicrobial properties were discovered in the late 1940s. Potassium sorbate is also GRAS, and exhibits broad antimycotic activity against both yeasts and molds; it is less effective against bacteria because lactobacilli are highly resistant toward sorbate. Levels usually not exceeding 0.1% are used in beverages, fruit juices and jams, wines, baked goods, cheeses, margarine, dry sausages, salads, and pickles; if applied, when permitted, in higher concentration, the influence on taste may be sensed.

Parabens are also effective above pH 7, and their solubility increases with the length of alkyl ester. Only the methyl and propyl parabens have GRAS status, and usage is limited to 0.1%. They are relatively ineffective against Gram-negative bacteria. They are used primarily as antimycotics, and also as general preservatives in high-acid foods in which bacterial growth is otherwise controlled, for example, by drying.

Comparison of various yeast species isolated from spoiled food products revealed great differences in their resistance to weak acid preservatives (Pitt, 1974; Liewen and Marth, 1985; Warth, 1986, 1988; Neves et al., 1994). In general, growth of the majority of yeasts could be inhibited by 250–350 mg/L benzoate or sorbate; some yeast, belonging in particular to the genus *Zygosaccharomyces*, show remarkably high resistance in being able to tolerate 800–1500 mg/L concentration of preservatives (James and Stratford, 2003). Of these, *Zygo. bailii* is the most prominent (Thomas and Davenport, 1985), and several other species show similar resistance, such as *Zygo. bisporus*, *Zygo. rouxii*, and *Zygo. lentus*, the latter being described few years ago (Steels et al., 1999). Sometimes, yeasts with unusual resistance to sorbate or benzoate are found, such as strains of *P. membranifaciens* and *C. parapsilosis* growing in the presence of 10–20 g/L sorbate at pH 4 (Deák et al., 1992). Among strains of *S. cerevisiae*, exceptionally high-preservative resistance occurs, especially after adaptation to weak acids. When growing at subinhibitory concentrations of benzoate or sorbate, the tolerance to preservative increases, and growing with one weak acid promotes adaptation to another (Lenovich et al., 1988; Stratford and Lambert, 1999). In addition to pH, which markedly influences the efficacy of weak acid preservatives, other environmental conditions, for example, temperature, concentration of glucose or other nutrient, water activity, aerobiosis, and ethanolic fermentation, affect the degree of inhibition. This can be utilized to increase the efficiency in combination (Beuchat, 1981a, 1994; Wind and Restaino, 1995). The antimicrobial effect of weak acid preservatives on yeasts is usually not lethal, and inhibition of growth may cease and survivors start growing after an extended lag. In combination with mild heating, preservatives bring about inactivation as well (Beuchat, 1981b; Fernandes et al., 1999).

The mechanism of action of weak acid preservatives has been the subject of intensive studies for decades, but it is still not completely clear. Several mechanisms of inhibition have been proposed, including effects on enzymes, uptake of sugars, glycolysis, ATP production, and others. Any hypothesis should also explain the adaptation and resistance to weak acids, as well as the differences in the behavior of various yeasts, in particular between *S. cerevisiae* and *Zygo. bailii*.

Early investigations with sorbate pointed out the inhibition of fermentation by affecting sulfhydryl enzymes (Deák and Novak, 1972b; Sofos and Busta, 1981). Interference with active transport processes was also attributed to both sorbate and benzoate through the uncoupling of transport from energy supply (Deák and Novak, 1970; Warth, 1977). Decreasing the pH in the cytoplasm became favored as a more general mechanism of action for weak acids. Their molecules in undissociated form pass readily through the cell membrane; once inside at neutral pH, they dissociate into protons and anions, and lower the internal pH, thus inhibiting metabolism (Krebs et al., 1983; Cole and Keenan, 1987; Warth, 1991). Dependence of inhibition on the difference between external and internal pH was not unequivocal in some cases; hence, the action of anions was also considered (Eklund, 1983; Russel, 1992), and sorbate showed similar inhibition to alcohols and aldehydes of nonweak acid nature (Stratford and Anslow, 1996, 1998).

The different resistance of *S. cerevisiae* and *Zygo. bailii* to benzoate was also explained by their dissimilar transport. The influx of benzoate into cells of *Zygo. bailii* appears weaker than that of *S. cerevisiae*, and an active mechanism for efflux functions in *S. cerevisiae* but not in *Zygo. bailii* (Warth, 1989; Henriques et al., 1997; Piper et al., 1998; Holyoak et al., 1999). In contrast, *Zygo. bailii*, unlike *S. cerevisiae*, can catabolically degrade weak acids (acetate, benzoate, and sorbate) (Casas et al., 1999, 2004; Mollapour and Piper, 2001a). It was shown long ago that some yeast can utilize and grow on sorbate as a single source of carbon (Deák and Novak, 1972a).

Approaching from a different viewpoint, indirect but conflicting evidence for the involvement of membrane permeability and metabolic energy supply was obtained from the kinetic analysis of

inhibition. Malfeito Ferreira et al. (1997) found that the inhibition kinetics of fermentation can be equally characterized by parameters of exponential inhibitory constant (K_i), minimum and 50% inhibitory concentrations, and they showed a good correlation with the lipid solubility of weak acids, suggesting that they interact with the hydrophobic regions of cell membranes. Leyva and Peinado (2005) calculated the energy requirement in terms of ATP yield and its maintenance coefficient, and found no change in the former but an increase in the latter in the presence of benzoate. This could be interpreted that response to weak acid should be sought in cellular features that would not need extra energy (Quintas et al., 2005). Contrary to these inferences, in terms of μ_{max}, K_S, and K_i, sorbate showed uncompetitive inhibition not interfering membrane transport in *S. cerevisiae* (Han and Floros, 1998), while benzoate in *Zygo. bailii* does not affect consumption rate of glucose (q_S) but decreased growth rate (μ) and yield coefficient (Y_S) on glucose.

In recent years, much progress has been made using molecular techniques, shedding light also on the possible mechanisms of action of weak acid preservatives. It has been confirmed that an active transport mechanism involving membrane H^+-ATPase is induced in *S. cerevisiae* by the presence of sorbate or benzoate (Holyoak et al., 1996); and regulating its activity, two proteins are expressed, a heat-shock protein (Hsp30) and an ATP-binding cassette (ABC) transporter, Pdr12 (Piper et al., 1998, 2001; Holyoak et al., 1999, 2000). Most recently, some details of the acid stress-signaling pathway have been unraveled by identifying specific signaling molecules ($PtdInsP_2$) (Mollapour et al., 2006).

Though differences between *S. cerevisiae* and *Zygo. bailii* in the resistance mechanism to weak acids have been found, the function of H^+-ATPase appears similar in both yeasts, in that it transports protons out in exchange for K^+ (Macpherson et al., 2005). *Zygo. bailii*, in addition, possesses the capacity for catabolism of sorbate and benzoate, and recently, the first gene (*ZbYME2*) contributing to this has been characterized genetically (Mollapour and Piper, 2001b). Deletion of this gene results in loss of ability to degrade preservatives, whereas its heterologous expression confers the capacity for catabolism of sorbate and benzoate in *S. cerevisiae* (Mollapour and Piper, 2001a).

We should recall that stress response mechanisms in yeasts are interwoven, and there is much in common in the pathways to cope with thermal, osmotic, acidic, and other stresses (Brul et al., 2002). Making use of transcriptome and proteome analysis, it was found that sorbate evokes many more stress reactions than the induction of proton and anion pumps (Nobel et al., 2000, 2001). In cells adapting to sorbate, genes encoding specific heat-shock proteins and other stress proteins involved in cell wall maintenance also were induced. This was the basis for starting a quest for membrane-active peptides in combination with cell-wall-degrading enzymes that may constitute a new effective antifungal system. Indeed, it was found that a modified cell wall protein (Cwp2-α-galactoside) incorporates less efficiently into cell walls, and addition of β-1,6-glucan fragments together with a synthetic membrane active peptide synergistically inhibits growth of both *S. cerevisiae* and the spoilage yeast *Zygo. bailii* (Bom et al., 2001).

6.6.3 Sulfur Dioxide

Sulfite has been used for centuries as a sterilizing agent in wine cellars and for reducing color loss of vegetables and meat. Most frequently used in the form of Na or K salts of sulfite ($-SO_3^-$), bisulfite ($-HSO_3^-$), or metabisulfite ($-S_2O_5^-$), all these release free sulfur dioxide (SO_2) in water solution. Dissolved SO_2 exists predominantly as hydrate ($SO_2 \cdot H_2O$) rather than sulfurous acid (H_2SO_3) and dissociates, depending on the pH, to give bisulfite and sulfite; in food systems of pH 3–7, two bisulfite anions often condensate to form metabisulfite:

$$SO_2 \cdot H_2O \leftrightarrow HSO_3^- + H^+ \quad pK_a = 1.86$$

$$HSO_3^- \leftrightarrow SO_2^- + H^+ \quad pK_a = 7.18$$

$$2HSO_3 \leftrightarrow S_2O_5^- + H_2O$$

Various forms of sulfite react easily with food components, in particular, sugars and other aldehydes; hence, a part only of added sulfite remains free SO_2, which is the only form with antimicrobial activity (Rose and Pilkington, 1989; Gould, 2000b).

Sulfite is effective against molds and yeasts, and to a lesser degree against Gram-negative bacteria. Sensitivity of different yeast species to sulfite varies greatly, *Zygo. bailii* and *S'codes ludwigii* being the most resistant (Thomas and Davenport, 1985; Warth, 1985). *S. cerevisiae* is generally more tolerant than *Klc. apiculata* and other non-*Saccharomyces* yeasts occurring in grape must; hence, sulfite is used as a selective agent for enhancing fermentation of wine (Romano and Suzzi, 1993; Henick-Kling et al., 1998). Depending on the concentration and other factors influencing efficacy, sulfite inhibits growth or inactivates microorganisms; its mechanism of action is complex. It behaves similarly to weak acid preservatives as its dissociation is strongly pH-dependent. Entering the cells, sulfite may directly affect various enzymes and be bound to essential components of the plasma membrane and macromolecular assemblies. Resistance of *Zygo. bailii* and *S'codes ludwigii* is caused in part by the production of greater amounts of acetaldehyde (Stratford et al., 1987; Pilkington and Rose, 1988).

In addition to its use as an antimicrobial preservative in wine and cider making, sulfite is used in foods as an antioxidant and reducing agent to prevent rancidity, browning or fading, and loss of vitamins (Gould and Russel, 1991).

6.6.4 Carbon Dioxide

Carbon dioxide developed during fermentation or applied artificially has broad antimicrobial effects that are gaining use as a food processing method. In the inhibition or inactivation of microorganisms by CO_2, several mechanisms may be involved. Indirectly, CO_2 may displace oxygen from the air space, inhibiting the growth of aerobic organisms. This effect is exploited in modified and controlled atmosphere storage. A general effect of CO_2 is the lowering of pH when dissolved in the water phase of foods. When dissolved, CO_2 first forms carbonic acid, which further dissociates to yield bicarbonate and carbonate anions and hydrogen ions, lowering the pH:

$$CO_2 + H_2O \leftrightarrow H_2CO_3$$

$$H_2CO_3^- \leftrightarrow HCO_3^- + H^+ \mathrm{p}K_a = 6.57$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \mathrm{p}K_a = 10.62$$

This same effect can take place in the microbial cells, resulting in the acidification of cytoplasm. Cells invest large amounts of energy in maintaining pH homeostasis by the mediation of H^+-ATPases, either expelling H^+ outside through the plasma membrane, or accumulating excess H^+ in the vacuole (Watanabe et al., 2005). When there is sufficient CO_2 to exceed the cell's buffering capacity, it will inhibit essential enzymes, disturb metabolism, and inactivate microorganisms. Increased carbon dioxide concentration through modified atmosphere packaging (MAP) and storage is a well-proven method for shelf life extension (see Section 6.8), and direct injection of CO_2 into some dairy products is used commercially and is being considered for other uses as well (Hotchkiss et al., 2006).

The toxicity of CO_2 to microorganisms can be enhanced by the application of supercritical CO_2 under pressure, providing a mode of nonthermal pasteurization technology (Damar and Balaban, 2006). *S. cerevisiae* can be inactivated by supercritical CO_2 at 6–21 MPa and 35–45°C, reaching 6–8 log reduction in 20–60 min (Lin et al., 1992; Shimoda et al., 2001). This technology is applicable in fruit juices to inhibit spoilage caused by yeasts (Zook et al., 1999).

6.6.5 Novel Antimicrobial Chemicals

Antimicrobial substances occurring naturally in plant and animal organs and tissues were discussed in Chapter 3. In addition to novel physical methods to inactivate microorganisms in

technological use, the search for new chemical and biochemical compounds is proceeding with promising results. Natural antimicrobials such as lysozyme, nisin, and other bacteriocins demonstrate antagonistic effects on bacteria, but generally have no action against fungi. Reuterin, a metabolite of *Lactobacillus reuteri*, has a broad spectrum of activity and inhibits both Gram-positive and Gram-negative bacteria, as well as fungi. With the exception of nisin that has been used as a biopreservative in a range of foods, no other bacteriocin has yet found application.

Chitosan, the deacylated derivative of chitin, exhibits antimicrobial activity, in particular against filamentous molds and yeasts (Shahidi et al., 1999). Its use in concentrations of 0.3–1.0 g/L has been investigated as a possible means of extending the postharvest shelf life of fresh fruits and vegetables. In fruit juices and mayonnaise-based salads, however, yeast growth was not completely suppressed (Rhoades and Roller, 2000; Roller and Covill, 2000), whereas in apple juice it inactivated spoilage yeasts such as *Klc. apiculata* and *Met. pulcherrima*, but *S. cerevisiae* and *Pichia* spp. multiplied slowly (Kiskó et al., 2005). Chitosan increased the effectiveness of high pressure and ultrasound against *S. cerevisiae* (Papineau et al., 1991; Guerrero et al., 2005). The antifungal activity of chitosan may lie in the plasma-membrane-perturbing effect, inducing a stress response in transcription (Zakrzewska et al., 2005).

Several reports refer to the antimicrobial effects of plant extracts containing various natural substances such as essential oils and oleoresins (Nychas, 1995). Extracts from garlic, onion, and horseradish as well as spices (cinnamon, clove, mustard, oregano, and others) may show antibacterial or antifungal effect or both. However, they impart strong flavor in effective concentrations, which is not compatible with most food products. However, some of them, such as vanillin, can be added to fruit purees, soft drinks, and ice creams, not only as antimicrobials but also for flavoring (Fitzgerald et al., 2004). Tartrate esters of hydroxycinnamic acids occur in wines, and as caffeic, coumaric, and ferulic acids have certain inhibitory effects against yeasts, they may be used alone or by supplementing potassium sorbate in the stabilization of wines (Stead, 1995). The effectiveness of plant extracts can be enhanced when they are used in combination with other preservative factors. For example, Cerutti and Alzamora (1996) demonstrated that vanillin in 0.2% concentration inhibited the growth of important spoilage yeasts (*S. cerevisiae*, *Zygo. bailii*, *Zygo. rouxii*, and *Db. hansenii*) in apple purée at pH 3.5 and a_w 0.95 for 40-day storage at 27°C. Cinnamon and clove oils evaporated into the gas phase of sealed pouches and exerted antimicrobial activity against spoilage yeasts, fungi, and bacteria under conditions of modified atmosphere of low oxygen and high carbon dioxide (Matan et al., 2006). Research on the mechanism of action and to determine the effectiveness (in terms of MIC) of certain essential oils is ongoing (Bennis et al., 2004; Souza et al., 2006).

6.7 SANITIZERS AND DISINFECTANTS

Food safety and quality are ensured largely by controlling the presence and growth of pathogenic and spoilage organisms in and on products, which they get from raw materials, ingredients, and additives, and to a large extent from the surfaces in food processing plants and equipments. Production hygiene is one of the most important factors in preventing microbial hazard and deterioration of foods.

Hence, effective execution of cleaning, sanitation, and disinfection is an inevitable and integral part of food processing.

Antimicrobial compounds are used to eliminate or limit the growth of microorganisms in foods. Their effects, applications, toxicology and safety, and regulatory status are complex issues. Development of resistance and adaptation to some antimicrobials necessitates continued research to identify new antimicrobial compounds and processes. Several recent books and reviews discuss cleaning and sanitation practices (Davidson et al., 2005).

Cleaning of surfaces and disinfection of microorganisms can be accomplished by alkaline detergents, ozone, hydrogen peroxide, chlorinated compounds, peroxyacetic acid, quaternary ammonium

compounds, and numerous commercial disinfectants; new brands appear continuously in the market. It should be noted that clean, hot water is still one of the most effective sanitizing agents, and physical means, such as heating, steaming, UV irradiation, and sonication, can also be applied.

Destruction of vegetative cells of bacteria, yeasts, and molds may not be very difficult, as they are sensitive to heat and chemicals. However, bacterial spores are extremely resistant, and to a lesser degree, fungal conidia and spores. Moreover, many microorganisms excrete extracellular polysaccharides and form biofilms that protect individual cells and can be quite difficult to remove from surfaces.

There exists a wealth of information about the effectiveness of sanitizers and disinfectants concerning bacteria, in particular food-borne pathogens. This holds true for products where yeast is frequently found, such as fresh produce (Beuchat, 2002; Parish et al., 2003; Beuchat et al., 2004). Even with yeasts, interest is focused on pathogenic species, *C. albicans* and few others (Théraud et al., 2004). There are few published studies concerning disinfecting agents against spoilage yeasts. *S. cerevisiae* is used commonly as the test organism in efficacy studies of disinfectants (Collinson and Dawes, 1992; Han et al., 1999; Kim et al., 1999). There is some evidence that ascospores of *S. cerevisiae* are more resistant than vegetative cells to hydrogen peroxide and quaternary ammonium compounds but not to peracetic acid (Romano and Suzzi, 1985; Neumayr et al., 1989; Jones et al., 1991).

In a recent publication (Salo and Wirtanen, 2005), the efficacies were assessed of 17 commonly used disinfectants against 28 strains of various yeast species isolated from dairy, bakery, sugar, beverage, and pickled products, in suspension and carrier (biofilm) tests. Alcohol-based (ethanol, isopropanol) disinfectants were most effective in reducing yeast counts at least four log units; tensides and peroxides were efficient against suspended cells, and foam cleaners also were found efficient in biofilm tests. Disinfectants containing chlorine and persulfate were ineffective in destroying yeasts. In cleaning and disinfecting food processing equipment, the most difficult task is to control developing biofilms and to apply cleaning and disinfecting procedures on microorganisms attached to and embedded in biofilms (Mattila-Sandholm and Wirtanen, 1992). Microbial ecology of biofilms was discussed in general terms in Chapter 3. Studies made in fish-processing plants and brewery bottling plants proved that yeasts in biofilms were more resistant than the bacterial population and remained adhered to surfaces after cleaning and disinfection (Bagge-Ravn et al., 2003; Storgards et al., 2006).

6.8 COMBINED EFFECTS

Preservative factors can be combined using milder treatments to retain the nutritional value and organoleptic quality while ensuring the required safety of products. The combination of preservative factors is often called hurdle technology (Leistner, 1992, 2000). Figure 6.4 illustrates this concept in terms of several hurdles that the microorganisms are not able to overcome. This analogy is inadequate in that "hurdles" are combined simultaneously and interact synergistically. The combination of treatments has resulted in innovative techniques and products, including minimal processing, MAP, and IMF.

Minimally processed foods are mildly heated, contain less acid, salt, and sugar, and in particular, fewer preservatives such as sulfite, nitrite, benzoic, or sorbic acid, but they are usually refrigerated (Ohlsson, 1994). The combination of factors should protect the product from the growth of pathogenic bacteria but may not inhibit spoilage microorganisms, including yeasts. Typical examples are ready-to-use fruits and vegetables processed by trimming, peeling or cutting, washing, disinfecting, and packaging. Blanching may be used to control enzymatic browning and to reduce counts of yeasts, molds, and bacteria (Nguyen-The and Carlin, 1994; Tapia de Daza et al., 1996).

Intermediate moisture foods generally have an a_w below 0.90 and are self-stable when combined with low pH, pasteurization, or refrigeration. Besides various meat products and cheeses, fruit

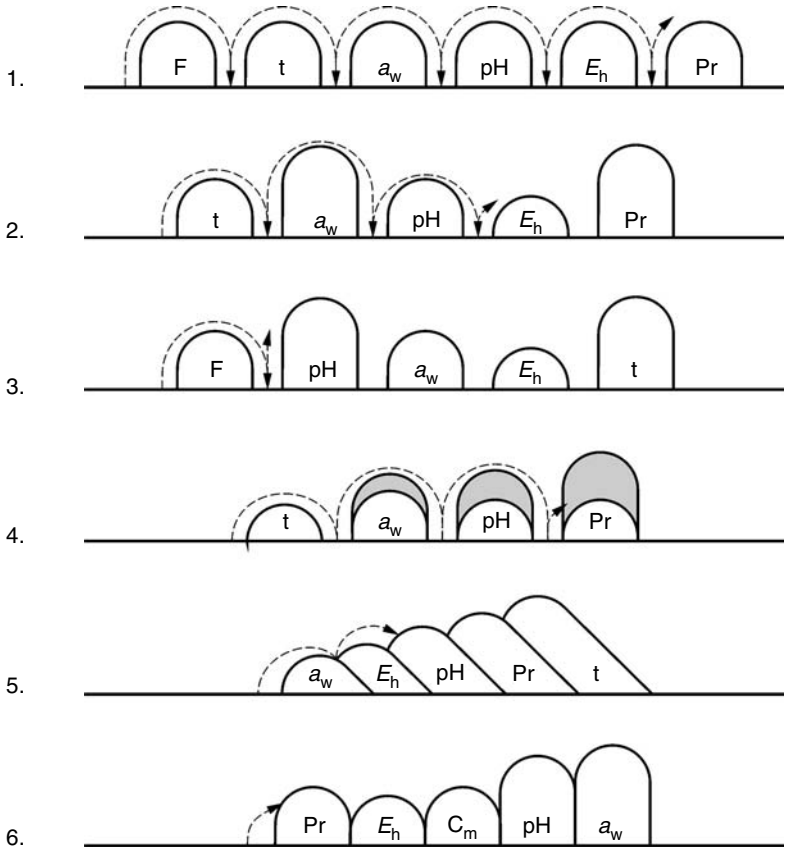

FIGURE 6.4 The hurdle concept of combined preservation. F: high temperature; t: refrigeration; a_w: low water activity; pH: acidification; E_h: low redox potential; Pr: preservatives; and K_m: competitive microbes. Case number 1 shows six antimicrobial hurdles of equal affectivity eventually inhibiting microorganisms. In most cases, one of the factors is stronger and results in inhibition by milder hurdles (2 and 3), in particular when they act in synergism (4). The real situation is more similar to case number 5 when mild effects together build up a strong hurdle, for example, in the ripening of salamis (6) when nitrite and low redoxipotential favor fermentative lactic acid bacteria, thus decreasing pH, while drying reduces a_w. (From Leistner, L. (1992) Food Res. Int. 25:151–158. With permission from Elsevier.)

preserves are good examples of IMF products. Whole fruits or halves, slices, or purees can be preserved by blanching and adjusting the a_w in the range of 0.91–0.98 with sugar. The pH of most fruits is between 3.1 and 3.5, and can be reduced if necessary with the addition of citric acid. Sulphite and sorbate can also be added. Under these conditions, the potential spoilage organisms are xerotolerant yeasts, in particular *Zygo. rouxii*. Its growth was inhibited by $a_w < 0.68$ in the absence of antimicrobials, whereas 1000 mg/L potassium sorbate or 100 mg/L SO_2 (as Na-metabisulfite) was required to retard growth at a_w 0.95 and pH 4 (Tapia de Daza et al., 1995).

Modified atmosphere packaging is frequently used for the preservation of fresh and minimally processed foods. For meat products, reducing the oxygen content within the package can be achieved by applying vacuum, whereas the atmosphere is modified by the ongoing respiration of fresh fruits and vegetables after sealing in semipermeable film or shrink-wrapping. Levels of oxygen may fall to 3–5%, while CO_2 concentration increases to 5–10%. In such an atmosphere, the growth of aerobic

bacteria and molds is greatly reduced, but the activity of yeasts and lactic acid bacteria can continue at a slower rate even if MAP is combined with refrigeration.

Numerous studies have been made to determine the effects of various factors on the growth of microorganisms alone and in combinations, in particular to find synergistic interactions among them in order to develop milder and safer treatment of foods. In previous studies, the change of cell number (cfu/mL) in time and function of various doses were determined in most cases; in more exacting work, growth and death parameters were also calculated. Besides direct cell counting, indirect methods reflecting growth or survival were also applied, such as metabolic CO_2 measurement (Guerzoni et al., 1990), flow cytometry (Sørensen and Jakobsen, 1997), conductimetry (Deák and Beuchat, 1993, 1994), or other techniques. Determination of growth/no growth responses permitted the increase of combination, and using a mixture of species (cocktail) instead of individual strains also proved to be an effective option. The design and mathematical evaluation of experiments only increased the efficiency of these studies, leading to the development of predictive microbiology. In addition to the aforesaid studies, the following examples are intended to give an overview of this exciting area of food microbiology.

Among the most comprehensive and far-reaching investigations are those made by Fleet and coworkers (Praphailong and Fleet, 1997; Charoenchai et al., 1998). Praphailong and Fleet (1997) examined the individual and interactive effects of pH, and concentrations of NaCl, sucrose, sorbate, and benzoate on the growth of 30 strains representing nine important spoilage yeast species, by reading the degree of turbidity of media in microtiter wells. No growth showed *Zygo. bailii* at pH 7.0, *Zygo. rouxii*, *P. membranifaciens*, and *Klc. apiculata* at pH 8.0, whereas only *Klc. apiculata* grew at pH 1.5–2.0. Only *Zigo. rouxii*, *Db. hansenii*, and *P. anomala* grew at 15% NaCl, whereas *S. cerevisiae* and *Klu. marxianus* did not tolerate 7.5% and 10% NaCl, respectively. The greatest salt tolerance occurred at medium pH of 5.0–7.0, but *Zygo. bailii* and *Db. hansenii* tolerated salt well at pH 3.0, and *Klc. apiculata* grew even in the presence of 12.5% NaCl at pH 2.0. All yeasts grew at 50% sucrose, with *Zygo. rouxii*, *Zygo. bailii*, *Db. hansenii*, and *P. anomala* growing at 60% sucrose. pH had little effect on growth with sucrose, but showed stronger interactions with preservatives. *Zygo. bailii* and *Ya. lipolytica* were the species most tolerant of sorbate and benzoate concentrations (750–1200 mg/L) at pH 5.0, the former preservative exerting somewhat stronger inhibition.

In a similar study (Charoenchai et al., 1998) with respect to wine yeasts (*Klc. apiculata*, *Tsp. delbrueckii*, *P. anomala*, *C. stellata*, *C. krusei*, *Db. hansenii*, and *S. cerevisiae*, 22 strains in all), the effects of temperature, pH, and sugar concentration were determined on the doubling time (or specific growth rate) and maximum cell mass during fermentation of grape juice. Growth rates for all species increased with temperature (up to 25°C), and cell biomass decreased with increasing sugar concentration (from 200 to 300 g/L). Variation of pH between 3.0 and 4.0 did not significantly affect either growth parameter. In this study, however, the interaction between ecological variables was not addressed.

Among the first studies using response surface methodology were those made by Cole and coworkers to assess the effect of pH on the growth of *Zygo. bailii*, and the concentrations of fructose, benzoate, sorbate, and sulfur dioxide (Cole and Keenan, 1987; Cole et al., 1987a,b). Measurements were made on the probability of growth (visual inspection of inoculated media in microtiter plate wells for 3 weeks) or calculating generation time and lag period from turbidimetric readings. Strong synergistic effects were found between sugar and week acid concentration that was pH dependent, which significantly affected the length of lag phase.

Guerzoni et al. (1990) applied least-square fit analysis to find synergistic interactions between the effects of a_w, pH, benzoate concentration, and thermal treatment on the stability of fruit-based systems challenged by a spoilage strain of *S. cerevisiae*. The effectiveness of the preservative decreased with reducing a_w and showed only a slight synergistic action with thermal treatment, whereas lowering a_w additively increased the effects of thermal inactivation.

Betts et al. (1999, 2000) studied the effects of various combinations of three factors, temperature, pH, and NaCl concentration, on the time to grow for 13 yeast strains individually, and subsequently in a cocktail of six typical spoilage species in chilled milk and acidic products (such as salad dressing and mayonnaise). In the first study, inoculated microtiter plates were examined visually for turbidity up to 2 months; in the second study, cfu/mL values were determined and the lag time and the time taken to achieve a level of 10^6 cells/mL were calculated from the growth curves. In both studies, a synergistic effect was found between NaCl and pH at the lower temperatures (at or below 8°C). It was also found that inhibition of growth manifested most significantly in the lengthening of the lag phase; under favorable conditions, it constituted 20–30% of the total time to grow, but increased to 45–62% under more extreme conditions (pH $\leq$ 4.5, NaCl $\geq$ 6%, temperature $\leq$ 8°C).

Using the plate count technique, data were transformed and mathematical models developed to predict the probability of spoilage in beverages by Battey et al. (2002). A yeast cocktail of *S. cerevisiae*, *Zygo. bailii*, and *Ya. lipolytica* was investigated under the effect of five variables, each at three levels: pH, titratable acidity, sugar content, and concentrations of benzoate and sorbate. Interactions were found significant for pH and each preservative, but neither titratable acidity nor the sugar content was found significant. Sugar content of beverages in this study (8–16°Bx, corresponding to a_w values of 0.99–0.95) was not a factor in inhibiting growth.

In order to include more factors to be studied, the data on growth/no growth can be used rather than the rate of growth, as shown by Evans et al. (2004). Interactive effects of five environmental conditions (alcohol, pH, sucrose, sorbate, and temperature) in 906 combinations were studied in a mixed cocktail of nine yeast strains by determining the time for growth until 150 days. Growth was observed in 29% of the experimental conditions, among which were extreme circumstances, such as at pH value of 2.1, a temperature of 2°C, a sucrose concentration of 55% (w/w), and an ethanol concentration of 12% (v/v). The models used to predict growth or no growth were successful in 94% of the cases, and in about 5% of environmental combinations, growth of spoilage yeasts occurred within 32–150 days, in contrast to previous growth/no growth studies extending for shorter periods (29 or 35 days) (Jenkins et al., 2000; López-Malo and Palou, 2000).

Wide-ranging studies have been carried out on the effect of reduced water activity in combination with other inhibitory factors. The lower a_w values adjusted in peach puree inhibited *Db. hansenii* more strongly than *Zygo. bailii*, and in combination with storage temperature, increased the lag phase stronger than the rate of fermentation (Gardini et al., 1988). In a similar study with *S. cerevisiae* (Guerzoni et al., 1990), it was found that the effectiveness of the preservative benzoate increased with decreasing a_w, in contrast with observations for osmotolerant species, *Zygo. bailii* (Jermini and Schmidt-Lorenz, 1987). In laboratory media, the growth of *S. cerevisiae* was inhibited by a_w (adjusted with glucose) below 0.89; above this value, the generation time increased with increasing sorbate concentration, nearly independent of pH; the effect of pH was more apparent in combination with sorbate and at lower a_w (Cerutti et al., 1990). Growth of fruit spoilage yeasts was inhibited more than fermentation (gas formation) and tolerance to sorbate decreased when the sugar content increased in strawberry homogenizate at pH 4 (Maimer and Busse, 1992). Kalathenos et al. (1995) calculated response surface characterizing the combined effects of pH, ethanol, and fructose concentration on the growth rate and doubling time of *S. cerevisiae*, and concluded that in wine systems (pH 3.2–3.8, sugar 1–4%, ethanol 7–13% v/v), the a_w-lowering effect of ethanol is of significance in addition to its specific toxic effect. Sørensen and Jakobsen (1997) found that the growth parameters of *Db. hansenii* in laboratory medium were markedly influenced at high salt concentrations and at lower temperatures, whereas changes in pH had little effect (Figure 6.5). With bakery products, a_w is the most important factor affecting the growth of xerotolerant molds and yeasts, which are also able to tolerate low-pH values. Indeed, it was shown that the growth rate of *Zygo. rouxii* decreased linearly with sugar concentration from 30 to 90% (corresponding to a_w of 0.957–0.788), without interaction with pH between 3 and 5, although pH 2.5 resulted in a 30% reduction in the growth rate (Figure 6.6) (Membré et al., 1999).

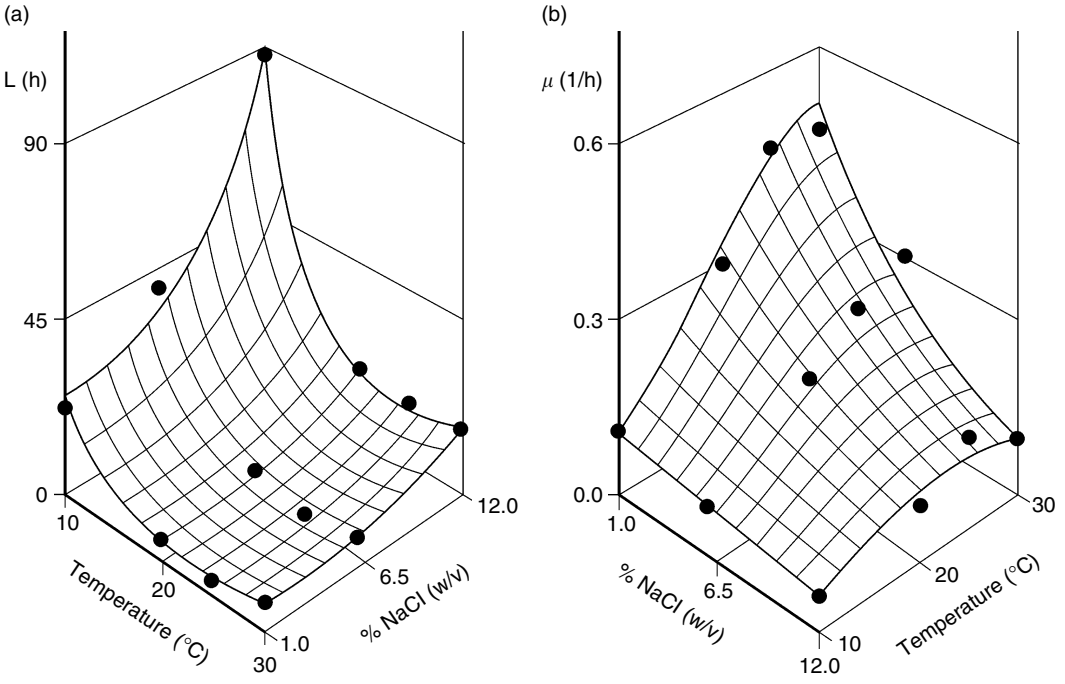

FIGURE 6.5 Three-dimensional response surface plots of the predicted lag phase duration (L), in (a), and maximum specific growth rate (μ), in (b), as a function of temperature and NaCl concentration at pH 5.3. (From Sørensen, B. B. and Jakobsen, M. (1997) Int. J. Food Microbiol. 34:209–220. With permission from Elsevier.)

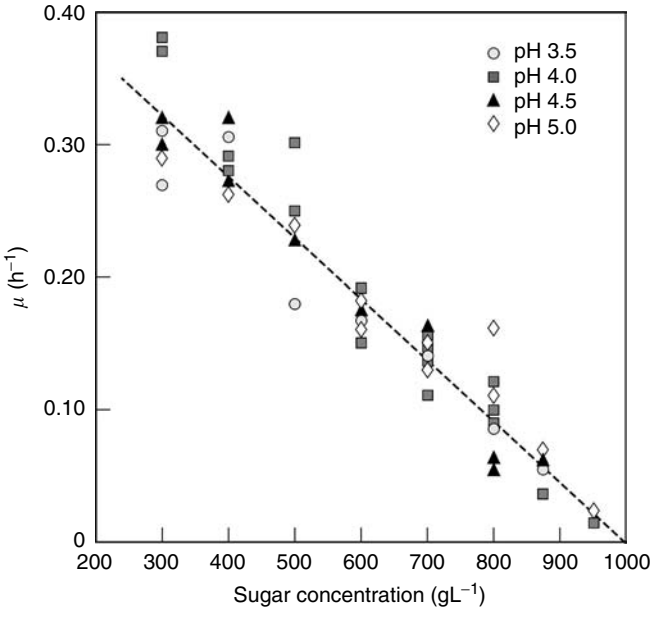

FIGURE 6.6 Linear relationship between specific growth rate (μ) and sugar concentration for the growth of *Zygosaccharomyces rouxii* at 25°C and pH 3.5–5. (From Membré, J.-M., Kubaczka, M., and Chéné, C. (1999) Appl. Environ. Microbiol. 65:4921–4925. With permission of ASM Journals.)

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7 Yeasts in Specific Types of Foods

As discussed in Chapter 3, from an ecological point of view, foods are considered habitats for microorganisms. Whether or not a microorganism will actually grow to the extent that spoilage will result depends on intrinsic and extrinsic factors and the inherent properties of the species. Clearly, a correlation exists between the type of species, the size of populations, and the kind of food. Many yeast species are associated with plant and animal products, and can also be found in food processing environments. Yeasts that get into food from these sources may start growing, and their populations form associations. Such associations can be highly specific, and sharp differences exist between yeast communities in different types of food. However, associations are dynamic and develop in time, in particular, in the course of processing due to changing ecological factors. Eventually, a final spoilage association develops, which is also specific and characteristic to the type of food.

Yeasts display definite preferences for certain environmental conditions prevailing in foods. Surveys of yeasts occurring in a particular food can provide insight as to which species might be encountered in spoilage. Yeasts are most likely to cause spoilage in products such as fruits and soft drinks, which contain sugars and have low pH. Yeasts are also common in high-sugar and/or high-acidity products, which restrict the growth of competing bacteria. It is increasingly recognized that yeasts make up a minor but consistent part of the microbiota of dairy and meat products too, which are proteinaceous, low sugar, low acidity, and prone to bacterial spoilage.

At the time of the writing of the first edition of this book (Deák and Beuchat, 1996), the comprehensive knowledge on the association of spoilage yeasts in a range of food commodities was collected in several reviews and books (Davenport, 1980; Pitt and Hocking, 1985; Beuchat, 1987; Fleet, 1990a,b, 1992; Deák, 1991). Data were available on the effects of single factors, such as temperature, pH, and NaCl concentration, on the growth of yeasts, but relatively little information existed about the dynamic responses of yeasts to changing environment and the combined effectiveness of several factors. Since the appearance of the first edition, enormous amounts of new information have accumulated, filling the gaps in knowledge, and allowing the assessment of the spoilage potential of yeasts in relation to the interaction of ecological factors. Methods for quantitative modeling have been developed to select the most effective factors and to develop alternative product formulations providing enhanced stability.

This chapter gives a compilation of qualitative and quantitative data of yeasts isolated from foods. It provides not only a catalog of yeast species recorded on or in raw and processed foods, but also is intended to contrast and integrate the implicit properties of specific yeasts with the ecological attributes of various foods in terms of their propensity to undergo spoilage. This type of information is valuable to food technologists. This knowledge, together with an emerging picture of frequencies and distribution of species, provides the basis for the development of ecological strategies to prevent food spoilage caused by yeasts.

7.1 FRUITS

Most fruits are marketed after harvesting without excessive handling, or after storage, and there is an increasing trend for produces with some degree of packaging and processing in the form of ready-to-eat, peeled, sliced products, or fresh juices. Processing of pulps and dried and canned fruit products involves intensive preparation and preservation. Yeasts are always associated with fruits

and play a significant role in the spoilage of raw fruits and processed products. Exhaustive reviews have been published on the occurrence of yeasts on fruits and juices, soft drinks, and carbonated beverages, mostly cataloging the diversity of species and focusing on the spoilage aspects (Davenport, 1976, 1981; Fleet, 1992; Thomas, 1993; Tudor and Board, 1993). Recent compilations are centered around ecological considerations on which novel processing techniques and improved methods of investigation and control can be based (Loureiro and Querol, 1999; Loureiro, 2000; Lund and Snowdon, 2000; Stratford et al., 2000; Fleet, 2003; Stratford and James, 2003; Loureiro et al., 2004; Stratford, 2006).

7.1.1 Fresh Fruits

Raw fruits are fresh, living but withering parts of plants, and are rich in nutrients and high in moisture content. When comparing ecological factors setting fruits apart from vegetables, it is the pH, in the range of 3–5 in most fruits, together with high concentrations of soluble carbohydrates, that makes them a primary habitat for yeasts and molds (Table 7.1). Vegetables are characterized by a somewhat higher pH (tomatoes make important exceptions), which renders them more susceptible to bacterial invasion and deterioration (Deák, 1979; ICMSF, 1980; Brackett, 1987).

Yeasts form a part of the natural microbiota of most fruits and vegetables, although populations and the relative proportion of species vary from commodity to commodity, and are influenced by environmental, harvesting, and storage conditions (Dennis and Buhagiar, 1980). The population of yeasts on sound fruits has been the subject of numerous studies. Table 7.2 gives a compilation of yeast species most frequently occurring on fruits.

The microbiota of fruits derives from two sources. The primary (resident) microbiota consists of organisms adhering to the surface of the fruits. The secondary microbiota is carried by vectors,

TABLE 7.1
Composition of Some Fruits and Vegetables

	Component %					
Produce	**Water**	**Carbohydrate**	**Protein**	**Minerals**	**Other**	**pH**
Fruits						
Apple	84.1	14.9	0.3	0.3	0.4	3.2
Apricot	85.4	7.9	1.0	0.6	0.1	3.6
Bananas	74.8	23.0	1.2	0.8	0.2	4.6
Cantaloup	92.1	6.9	0.5	0.3	0.2	6.3
Grapes	81.9	14.9	1.4	0.4	1.4	3.9
Orange	87.2	11.2	0.9	0.5	0.2	3.5
Strawberry	89.9	8.3	0.8	0.5	0.5	4.2
Mean	85.0	13.2	0.9	0.5	0.4	4.1
Vegetables						
Carrots	88.2	9.2	1.3	1.1	0.2	5.0
Cauliflower	91.7	4.9	2.4	0.8	0.2	5.6
Cucumber	96.1	2.7	0.7	0.4	0.1	5.6
French beans	89.9	7.7	2.4	0.8	0.2	5.6
Green peas	81.2	11.4	6.1	0.9	0.4	5.7
Spinach	93.7	3.2	2.3	1.5	0.3	5.1
Tomato	94.1	4.0	1.0	0.6	0.3	4.4
Mean	90.6	6.2	2.3	0.9	0.2	5.4

Source: From Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

TABLE 7.2
Yeasts Frequently Isolated from Fruits

Species	Sources	References
C. citrea	Tropical fruits	28
C. diddensiae	Tropical fruits	29
C. magnoliae	Grapefruit, pineapple, red currant	5, 16, 24
C. maltosa	Tropical fruits	29
C. parapsilosis	Tropical fruits	35
C. sake	Strawberry, figs, tropical fruits	2, 17, 29, 35
C. sorbosa	Tropical fruits	28
C. sorbosivorans	Tropical fruits	35
C. sorboxylosa	Tropical fruits	28
C. spandovensis	Tropical fruits	35
C. stellata	Grape, apple, figs, citrus, red currant, cherries, plumes	1, 4, 8, 21, 22, 26
C. tropicalis	Grapefruit, orange, tropical fruits	16, 19, 32
C. vini	Cherries, plumes	22
C. zeylanoides	Grape, cherries, plumes	5, 22
Clsp. lusitaniae	Orange	32
Cry. albidus	Apple, pear, grape, strawberry, cherries, tropical fruits	1, 2, 4, 5, 7, 9, 29, 30, 36
Cry. humicolus	Apple, grape, tropical fruits	1, 15, 19, 29
Cry. laurentii	Pear, grape, strawberry, tropical fruits, orange	2, 8, 29, 30, 33, 35
Cry. macerans	Orange	33
Db. hansenii	Apple, pear, grape, citrus, tropical fruits	4, 7, 8, 10, 19, 21, 23, 29, 30, 35
Db. vanrijiae	Tropical fruits	29
Geo. klebahnii	Tropical fruits	29
Guehom. pullulans	Cherries	36
Hsp. guilliermondii	Grape, dates, tropical fruits	11, 26, 35
Hsp. uvarum	Apple, grape, citrus, cherries, plums	2, 4, 7, 8, 9, 13, 14, 20, 21, 22, 32, 33, 34
Iss. orientalis	Grape, cherries, plums, tropical fruits	4, 8, 19, 22, 26, 27, 28
Met. pulcherrima	Apple, grape, cherries, plums, pomegranate	1, 4, 7, 10, 14, 20, 22, 34
P. anomala	Apple, grapefruit, cherries, plums, tropical fruits, orange	1, 4, 5, 16, 19, 22, 29, 32, 33, 34
P. fermentans	Apple, citrus, mango, dates	12, 25, 32
P. guilliermondii	Apple, grape, figs, citrus, tropical fruits	1, 8, 21, 26, 29
P. kluyveri	Tropical fruits	28
P. membranifaciens	Grape, citrus, figs, tropical fruits	4, 5, 13, 14, 15, 17, 21, 26, 28, 29, 35
P. pijperi	Tropical fruits	28
Pseudozyma antarctica	Tropical fruits	29, 35
Rho. fujisanensis	Pear	30
Rho. glutinis	Apple, pear, grape, grapefruit, tropical fruits	1, 2, 4, 14, 15, 16, 19, 29, 30, 31, 36
Rho. graminis	Tropical fruits	35
Rho. minuta	Pear	30
Rho. mucilaginosa	Apple, strawberry, cherries, grapefruit, tropical fruits, orange	1, 2, 16, 18, 19, 29, 32, 33, 36
S. cerevisiae	Grape, apple, cherries, grapefruit, tropical fruits, orange	3, 4, 5, 11, 14, 16, 19, 26, 32, 33, 34
Schizo. pombe	Grape	6, 14
Spb. roseus	Apple, pear, strawberry, tropical fruits	1. 2, 4, 7, 19, 30, 31
Tsp. delbrueckii	Apple, grape, grapefruit, cherries	1, 8, 12, 16, 18, 34
Trisp. moniliforme	Apple, grape, citrus	1, 5, 26
Zygo. bailii	Apple, grape	1, 4, 5, 12

Notes: 1: Beech and Carr (1977); 2: Buhagiar and Barnett (1971); 3: Cuinier (1980); 4: Davenport (1976); 5: Deák (1988); 6: Delfini (1985); 7: Doores (1983); 8: Goto and Yokotsuka (1977); 9: Heard and Fleet (1986a); 10: Juven et al. (1984); 11: Kobatake and Kurata (1980b); 12: Kunkee and Goswell (1977); 13: Lachaise (1977); 14: Messini et al. (1985); 15: Parish and Caroll (1985); 16: Parish and Higgins (1990); 17: Pignal et al..(1985); 18: Put et al. (1976); 19: Rale and Vakil (1984); 20: Rosini et al. (1982); 21: Spencer et al. (1992); 22: Stollarová (1976, 1982); 23: Tokouka et al. (1985); 24: Török and King (1991); 25: Vacek et al. (1979); 26: Walker and Ayres (1970); 27: Warnasuriya et al. (1985); 28: Braga et al. (1998); 29: Buzzini and Martini (2002); 30: Chand-Goyal and Spotts (1996); 31: Gildemacher et al. (2006); 32: Heras-Vazquez et al. (2003); 33: Restuccia et al. (2006); 34: Stollárová (1996); 35: Trindade et al. (2002); 36: Venturini et al. (2002).

Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

such as insects, dust, and wind, and can vary dramatically compared with the more stable resident community. The two populations cannot be sharply differentiated. Moreover, the microbiota differs according to geographic area and climatic conditions where fruits are grown, cultivars and horticultural techniques, and stage of ripening, and differences can be found between different areas of the same fruit (Davenport, 1976; Doores, 1983).

Microorganisms are mostly limited to the surface of whole, sound fruits, whereas the internal tissues are free from contamination. If microorganisms occur in the internal tissues, they are limited to a few species (Doores, 1983). Although yeasts, with a few exceptions, are not capable of attacking plant tissues, certain species are associated with the decay of fruits. These, in general, possess pectinolytic enzymes, and also have other hydrolytic enzyme activities (Braga et al., 1998; Buzzini and Martini, 2002; Trindade et al., 2002). Interestingly, yeasts in tropical fruits (pitanga, mangaba, amapa, and others) produced proteinases and lipases as well. Highly proteolytic were, for example, *C. sorbosa*, *C. sorbosivorans*, and *Pseudozyma antarctica*; the latter and *C. maltosa*, *P. anomala*, and *P. membranifaciens* exhibited lipase activity, too (Braga et al., 1998; Buzzini and Martini, 2002; Trindade et al., 2002). *Hsp. uvarum*, *C. stellata*, *Iss. orientalis*, and *S'copsis vini* have been associated with the sour rot disease of grapes (Guerzoni and Marchetti, 1987).

Surveys of the microbiota of apples have focused on three distinct yet interrelated areas, namely, in the orchard, during storage, and within processing facilities (Doores, 1983). The predominant yeasts of sound apples are typified by weakly fermentative species rather than molds and bacteria. Yeast populations range from 10^2 to 10^6 cells per apple and show a seasonal variation reaching a peak count in fall. The main resident species on apple include *Hsp. uvarum*, *Met. pulcherrima*, *Db. hansenii*, *Spb. roseus*, and *Cry. albidus.* Yeasts phases of *Aureobasidium pullulans* and *Cladosporium herbarum* (black yeasts) are also permanent members of the mycobiota. These yeast-like fungi, as well as *Rho. glutinis* and *Spori. pararoseus*, can aggravate russeting of apples, resulting in reduced storability (Gildemacher et al., 2006). Another postharvest disorder, named white haze, has been recently attributed to the intensive colonization of *Tilletiopsis* species, anamorphs of smut fungi (Boekhout et al., 2006). *Aureobasidium pullulans* was present in all apple and pear samples, whereas *Cry. albidus* and *Rho. glutinis* were isolated from 80% of the fruits (Chand-Goyal and Spotts, 1996). In general, 3–7 species colonized pears; with the exception of *Db. hansenii*, these were mostly basidiomycetous yeasts, such as *Cry. infirmo-miniatus*, *Cry. laurentii*, *Rho. aurantiaca*, *Rho. fujisanensis*, *Rho. minuta*, and *Spb. roseus*.

Extensive research has been conducted on the distribution of yeasts on grapes (Davenport, 1974; Goto and Yokotsuka, 1977; Rosini et al., 1982; Messini et al., 1985). Fermentative and oxidative species, represented by *Hsp. uvarum* and *Met. pulcherrima*, respectively, have been commonly detected. Although various other yeasts (e.g., *Cry. albidus*, *Rho. glutinis*, *Db. hansenii*, *C. stellata*, and *P. membranifaciens*) can also be isolated from the skin of the grape berries, *S. cerevisiae* is not among the main resident organisms, and its population increases only after the grapes have begun to maturate. Studies on yeasts on grapes are directed toward the fermentation of must, and will be considered later with wine yeasts (Section 7.4.2).

Berry fruits (strawberries, raspberries, blackberries, and blueberries) are in general prone to fungal contamination, and yeasts follow mold attack. Yeasts were only encountered in 3–5% of berry samples (Tournas and Katsoudas, 2005). Postharvest spoilage is incipient within a week and can be delayed to some degree under storage at low temperatures or controlled atmospheres. Application of fungicides is restricted. A recent study on antifungal coatings with 2% chitosan containing 0.3% potassium sorbate resulted in extending shelf life of strawberries significantly (Park et al., 2005). Strawberries appeared to differ from other fruits in the predominance of cryptococci. Buhagiar and Barnett (1971) found *Cry. albidus* (45%), *Cry. laurentii* (32%), and *Cry. macerans* (7%) among the yeast isolates from sound strawberries, with *C. fragariorum*, *Hsp. uvarum*, *Rho. glutinis* (2–4% each), and five other species found in smaller quantities.

Detailed investigations on the frequency and dominance of yeast species on cherries, plums, and red and white currants grown in temperate zones have revealed that *Hsp. uvarum*, *Met. pulcherrima*,

and *C. vini* are among the permanent and ubiquitous members of yeast associations (Stollarová, 1976, 1982). Beside these, *S. cerevisiae*, *P. anomala*, *Klu. marxianus*, *C. stellata*, *Iss. orientalis*, and *C. zeylanoides* were also frequently isolated. A large-scale study of 715 isolates revealed 16 species belonging to 12 genera on morello cherries (Stollárová, 1996). Permanent components of the yeast biota were *S. cerevisiae*, *Hsp. uvarum*, *Met. pulcherrima*, and *P. anomala.* On black currant, among six different yeast species, and the yeast-like fungus, *Aureobasidium*, the most frequent has been *Met. pulcherrima* (Senses-Ergul et al., 2006). Another extensive study with 442 yeast isolates showed a different picture on sweet and sour cherries in that *Guehomyces pullulans* was the dominant species, with other basidiomycetous yeasts (*Rho. glutinis*, *Rho. rubra*, and *Cry. albidus*) present in lower numbers (Venturini et al., 2002). *Cry. neoformans* was also detected; however, the results of identification by three different methods varied and misidentification might occur. Nevertheless, it is a warning that fresh fruits and vegetables may transmit human pathogenic yeasts. Out of 254 vegetables and 186 fruit samples taken from markets in Delhi, India, 5 contained *Cry. neoformans* (Pal and Mehotra, 1985). *C. albicans* (as its synonym, *C. stellatoidea*) has been reported on grapes (Parish and Carrol, 1985).

Hsp. guilliermondii, *Met. pulcherrima*, and *Db. hansenii* have been described to predominate on pomegranates grown in the Mediterranean area (Juven et al., 1984). This result is similar to those found on fruits of the temperate zone; however, tropical fruits appeared different. Pineapple, banana, kiwi, and papaya are inhabited by a mixed yeast flora consisting of *P. anomala*, *P. guilliermondii*, *P. sydowiorum*, *Tsp. delbrueckii*, *C. versatilis*, and *C. apicola* (Rale and Vakil, 1984; Tokouka et al., 1985; Warnasuriya et al., 1985). Other species such as *S. cerevisiae*, *Hsp. guilliermondii*, *Rho. glutinis*, and *Cry. albidus* are commonly found on other fruits, too. Recent detailed studies on other kinds of tropical fruits revealed a rich and diverse yeast microbiota consisting of 23 species of 8 genera of ascomycetous yeasts and 10 species of 3 genera of basidiomycetous yeasts (Buzzini and Martini, 2002), as well as 42 ascomycetous and 28 basidiomycetous species, 12 and 8 genera, respectively (Trindade et al., 2002). Species not recorded frequently from other fruits were, among others, *C. parapsilosis*, *C. sorbosivorans*, *C. maltosa*, *Geo. klebahni*, and *Pseudozyma antarctica.* Lachaise et al. (1977) isolated from figs a specialized yeast, *C. fructus*, but fermentative spoilage was caused by *P. guilliermondii*, *P. membranifaciens*, *Hsp. valbyensis*, *Hsp. uvarum*, *C. sorboxylosa*, *C. stellata*, and *Iss. orientalis*. Pignal et al. (1985) demonstrated that there is an intimate interaction between the yeast community of figs and that inhabiting drosophilas. Species common to both were *Hsp. valbyensis*, *P. membranifaciens*, *S'copsis vini*, and *C. pseudointermedia.*

Spoilage of fresh fruits usually results from the fermentative activity of yeasts. Nevertheless, a wide variety of weekly or nonfermenting yeasts have been described from rotting oranges, mangoes, and dates (Salik et al., 1979; Vacek et al., 1979; Suresh et al., 1982). Species such as *Met. pulcherrima*, *Db. hansenii*, *Iss. orientalis*, *Hyphop. burtonii*, *Trisp. cutaneum*, and others were frequent; however, the fermentation was caused by *Hsp. uvarum*, *Hsp. valbyensis*, *P. guilliermondii*, *P. fermentans*, *P. kluyveri*, and to a lesser extent, *S. cerevisiae.* Species isolated from rotting citrus fruits (oranges, limes, mandarins, and grapefruits) included *Hsp. uvarum*, *C. guilliermondii*, *C. stellata*, *P. kluyveri*, *P. membranifaciens*, and *Geo. candidum*. Although yeasts were present in considerable numbers in spoiled fruits, they could not be directly implicated in the decay process, although *Hsp. uvarum* appeared to have some pectolytic activity when reinoculated into healthy fruit (Spencer et al., 1992). The most frequent species isolated from fermenting orange fruit and juice was *S. cerevisiae* (35%), followed by *Hsp. uvarum*, *C. tropicalis*, and *Clsp. lusitaniae* (12–17%), whereas *Kazach. unisporus*, *P. anomala*, and *P. fermentans* occurred in 1–2%. *Trisp. ashaii*, a nonfermenting species, also occurred in 10% (Heras-Vazquez et al., 2003).

7.1.2 Stored and Partially Processed Fruits

Yeasts comprising the storage association are directly reflective of harvesting and handling practices. Contaminations are initiated during the growing season, and injuries occur during harvesting and

handling. Yeasts (*Rhodotorula* sp., *Geotrichum* sp., *P. norvegensis*, and *Cry. laurentii*) were detected in small number compared to aerobic bacteria on the surface of sliced watermelon stored at 5°C, but were not primarily responsible for spoilage (Abbey et al., 1988). Golden et al. (1987) detected higher populations of yeasts on individually shrink-wrapped peaches that offered a suitable humid atmosphere for growth. *Cry. laurentii* and *Guehomyces pullulans* were detected in high numbers.

Qualitative analysis of commercially prepared grapefruit sections has indicated that yeasts occur in the range of 3.5–4.3 log cfu mL^{-1} on the surface of the peeled product (Parish and Higgins, 1990). Thirteen yeast species were identified, among them *C. magnoliae*, *C. maltosa*, *C. tropicalis*, *P. anomala*, *Rho. glutinis*, *Rho. mucilaginosa*, *S. cerevisiae*, *Tsp. delbrueckii*, and *Zygo. rouxii*. Maimer and Busse (1992) studied yeasts (*S. cerevisiae*, *Zygo. rouxii*, *P. fabianii*, and *C. parapsilosis*) isolated from processed fruits destined for yoghurt products and concluded that yeast growth could be inhibited by 200 mg L^{-1} sorbic acid at a_w 0.89.

Various techniques have been applied for shelf life extension and disinfection of processed fresh fruits. Citric acid treatment (0.5–1.0% w/v) and cool storage (4–8°C) extended the shelf life of peeled oranges, but this resulted primarily from the inhibition of spoilage bacteria, whereas it allowed the growth of yeasts (*Rho. glutinis*, *Cry. albidus*, *S. cerevisiae*) (Pao and Petracek, 1997). Packaging in polyester films of different permeabilities in normal and modified atmospheres (2.5–10% O_2, 8–20% CO_2) inhibited to various degrees yeast growth in minimally processed orange slices (Restuccia et al., 2006). In samples packaged in normal atmosphere, mainly *Rhodotorula* spp. developed, whereas modified atmosphere resulted in fermentative spoilage due to *S. cerevisiae.* Coating formed by dripping the produce in a chitosan–lactic acid solution and allowing it to develop an equilibrium-modified atmosphere during storage at 7°C maintained strawberries for 12 days, but this failed to control spoilage of lettuce after 4 days (Devlieghere et al., 2004). Surface disinfection by UV light of fresh-cut cantaloupe melon (Lamikanra et al., 2005), or by pulsed UV light of various fresh fruits (apples, kiwi, peaches, raspberries, etc.) (Lagunas-Solar et al., 2006) may provide effective, commercial-scale treatment. In the case of mango pulp, which can be heat treated, steaming followed by γ-irradiation (1.0–2.0 kGy) resulted in an increase of refrigerated shelf life for 270 days compared with 90 days of irradiation without steaming (Youssef et al., 2002). A recent invention is the application of vacuum–steam–vacuum treatment for reducing microorganisms on the surface of produces. Quick steam bursts followed by vacuum to cool the surface by evaporation prevent thermal damage even of whole and fresh-cut cantaloupe (Ukuku et al., 2006).

7.1.3 Beneficial Aspects of Fruit Yeasts

The metabolic activity of yeasts associated with fruits, crops, and seeds are exploited in the spontaneous fermentation of various alcoholic beverages and certain other products, which is by far the most advantageous and economically profitable use of yeasts. These aspects will be treated in detail in separate sections.

In addition to alcoholic fermentation, the enzymatic activity of yeasts involves the processing of fruits of coffee and cocoa trees and the removal of mucilaginous pulps surrounding the beans (Section 7.5.4). Pectinolytic enzymes, detrimental in rotting fruits, can be beneficially used for increasing the yield of juice extraction from fruits, the clarification of juices, and peeling of fruits, instead of mechanical methods (Ben-Shalon et al., 1984; Pretel et al., 1997). In these processes, *Kluyveromyces* species have been applied (Donaghy and McKay, 1994; Moyo et al., 2003).

A novel and expanding beneficial application of yeasts is the biocontrol of fruit spoilage molds. Some yeasts, commonly present on the surface of fruits, show antagonistic action against spoilage fungi. This subject was briefly mentioned in Section 3.2.4.3. Recent reviews of the field were published by Spadaro and Gullino (2004) and Druvefors and Schnürer (2005).

TABLE 7.3
Comparison of the Average Characteristics of Soft Drinks and the Growth Requirements of Yeasts

Parameter	Range	Yeast Requirements
Water (%)	80–99	a_w growth range 0.75–0.99
Sugar (%)	0.5–15.0	Fermentable carbon source
N-content (%)	10^{-4}–10^{-1}	Inorganic nitrogen salts
Vitamin B (%)	traces–10^{-4}	Not required by most strains
Inorganic salts (%)	$10^{-4} - 10^{-1}$	Potassium, phosphate
pH	2.5–4.0	Growth range 1.5–9.0
pCO_2 (kPa)	50–350	Static >800 kPa

Source: From Deák, T. (1980) Acta Alimentaria 9:90–91. With permission.

7.2 FRUIT JUICES AND SOFT DRINKS

Owing to their intrinsic properties, in particular their low pH value and low nitrogen and oxygen contents (Table 7.3), fruit juices and soft drinks impose an adverse environment on most microorganisms. Nevertheless, these beverages are excellent substrates for supporting the growth of some microorganisms such as yeasts, and to a lesser extent, molds, and lactic acid bacteria (LAB) (Berry, 1979; Deák, 1980). Of these beverages, fruit juices contain the highest amount of nitrogenous compounds and vitamins; hence, they are most susceptible to yeast spoilage. Carbonated beverages and drinks not containing fruit juices (e.g., tonic- and cola-type products) are less susceptible to the development of yeasts. In carbonated beverages, the pH, preservative concentration, and the degree of carbonation are three main factors that influence yeast growth. The stability of fruit-based beverages is also influenced by the type of fruit juice or concentrate used in their formulation (Deák et al., 1986). Table 7.4 summarizes the most frequent yeast species occurring in fruit juices and soft drinks.

Freshly squeezed or extracted fruit juices require processing to prevent spoilage. Chilling is most commonly used to extend shelf life of unpasteurized products. Murdock and Hatcher (1975) showed that the rate of yeast growth in orange juice decreased with temperature. *Zygo. rouxii* grew at temperatures between 1.7°C and 10°C, with a generation time of 19.6 to 8.6 h. Arias et al. (2002) found *Hsp. uvarum*, *Hsp. occidentalis*, and *P. kluyveri* and a few other yeast species in freshly squeezed single-strength orange juice, whereas none of these species was identified in pasteurized juice; however, a much broader diversity of yeasts (some 14 different species), apparently due to subsequent recontamination, was found. Casey and Dobson (2004) listed *Zygo. bailii*, *Zygo. rouxii*, *Iss. orientalis*, *Rho. glutinis*, and *S. cerevisiae* as the common spoilage species in fruit juices.

Fruit juices can be chemically preserved, pasteurized, frozen, concentrated, and/or irradiated to prevent spoilage. Preservation of concentrated orange juice can be achieved by the addition of sulfur dioxide (230 mg L^{-1}) or by sorbic acid (800 mg L^{-1}) (Lloyd, 1975). The spoilage yeast, *Zygo. bailii*, often exhibits resistance to benzoic acid. Owing to its low pH, light pasteurization (66°C for 10 s) is sufficient to inactivate most microorganisms in orange juice (Sadler et al., 1992). Other studies have shown that pasteurized juice may undergo spoilage due to yeast or mold growth within 5 weeks of refrigerated storage (Parish and Higgins, 1989). In fresh orange juice, *Hsp. occidentalis* and *Hsp. uvarum* are commonly found; however, the typical species in pasteurized juice are *S. cerevisiae*, *C. intermedia*, and *Tsp. delbrueckii* (Arias et al., 2002; Ros-Chumillas et al., 2005). A factor contributing to the stability of pasteurized orange juice is redox potential, the significance of which is rarely appreciated (Reichart and Mohácsi-Farkas, 1994). Alwazeer et al. (2003) demonstrated that

TABLE 7.4
Yeasts Frequently Isolated from Fruit Juices and Soft Drinks

Species	Sources	References
C. boidinii	Soft drinks	11, 14, 15
C. etchellsii	Soft drinks	2, 18
C. inconspicua	Soft drinks, conc. juices	2, 10
C. intermedia	Fruit juices	22
C. parapsilosis	Fruit juices	22, 24, 25
C. sake	Soft drinks	11, 14, 15
C. stellata	Soft drinks, conc. juices	3, 9, 11
C. tropicalis	Apple juice, soft drinks	1, 2, 11, 14, 16, 22, 25
Clsp. lusitaniae	Fruit juices	22, 25
Db. hansenii	Fruit juices	2, 4, 11, 16
Dek. anomala	Soft drinks	6, 11
Dek. bruxellensis	Soft drinks	11
Hsp. occidentalis	Fruit juices	22
Hsp. uvarum	Soft drinks, fruit juices	2, 4, 16, 19, 20, 22
Iss. orientalis	Soft drinks, fruit juices, conc. juices	2, 3, 8, 10, 14, 16, 25
Lachancea thermotolerans	Soft drinks, fruit juices, conc. juices	3, 10, 11, 25
Lachancea fermentati	Soft drinks	11, 14, 15, 17
Lachancea kluyveri	Soft drinks, conc. juices	15, 17
Lodd. elongisporus	Soft drinks, conc. juices	3, 11, 14, 15, 25
P. anomala	Soft drinks, fruit juices, conc. juices	3, 4, 13, 14, 15, 25
P. fermentans	Soft drinks, apple juice	2, 11, 17
P. guilliermondii	Soft drinks, fruit juices	4, 14, 15, 17
P. kluyveri	Fruit juices	22
P. manshurica	Carbonated orange juice	23
Rho. glutinis	Fruit juices	21
S. cerevisiae	Soft drinks, fruit juices, conc. juices	3, 5, 10, 12, 17, 21, 25
Tsp. delbrueckii	Soft drinks, conc. juices	3, 4, 14, 15, 25
Tsp. microellipsoides	Soft drinks, fruit juices	10, 11, 14, 15, 17
Zygo. bailii	Soft drinks, conc. juices	7, 11, 12, 14, 21, 25
Zygo. rouxii	Conc. juices	3, 25

Note: Barreiro et al. (1981); 2: Beech and Carr (1977); 3: Deák and Beuchat (1993b); 4: Dragoni and Comi (1985); 5: Gardini and Guerzoni (1986); 6: Ison and Gutteridge (1987); 7: Johanssen et al. (1984); 8: Juven (1979); 9: Mauricio et al. (1986); 10: Muzikar (1984); 11: Put et al. (1976); 12: Put and de Jong (1982); 13: Röcken et al. (1981); 14: Sand (1974); 15: Sand et al. (1976a,b); 16: Suresh et al. (1982); 17: Török and Deák (1974); 18: Uchida et al. (1980); 19: Vacek et al. (1979); 20: Walker and Ayres (1970); 21: Casey and Dobson (2004); 22: Arias et al. (2002); 23: Pina et al. (2005); 24: Deák and Beuchat (1993a); 25: Deák and Beuchat (1993c).
Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

oxidizing conditions (Eh 240–360 mV) were most effective for thermal destruction of *S. cerevisiae* in orange juice; on the other hand, reducing conditions (Eh −180 mV) decreased the recovery of heated cells, and were also necessary to stabilize color and ascorbic acid during storage.

The effect of temperature and juice composition on the survival of yeasts has been the subject of numerous studies. The thermal resistance of spoilage yeasts decreases with the addition of citric acid and increases with the concentration of orange juice (Juven et al., 1978; Murdoch and Hatcher, 1978). By combining preservatives and a mild heat treatment, the shelf life of soft drinks can be greatly increased. The heat treatment necessary to achieve pasteurization can be reliably based on the heat resistance of spoilage yeasts, as demonstrated by the intensive studies by Put et al. (1976).

Yeasts are reported to be the only organisms capable of growing in orange concentrate (a_w 0.94) stored at 4°C (Murdock and Brokaw, 1965; Crandall and Graumlich, 1982). Populations of spoilage yeasts (*Iss. orientalis*, *Kazach. exiguus*) decreased progressively during frozen storage at 0°C and −18°C in tomato concentrates (Juven, 1979). *Iss. orientalis* grew in concentrate at 7°C, regardless of the concentration, whereas *Kazach. exiguus* was unable to grow at this temperature in tomato concentrate with 28° Brix and higher. Storage of frozen concentrates is an effective means of preventing yeast spoilage. Deák and Beuchat (1993) studied yeasts in frozen, concentrated orange, apple, cherry, grape, and pineapple juices. The most frequently isolated species were *S. cerevisiae* (24.7% of isolates), *C. stellata* (22.1%), and *Zygo. rouxii* (14.3%); 18 further species were minor participants in the yeast population. In Japan, a novel functional food, fermented extract of honey and a kind of apricot, contains 40–60% of sugars and is stable without pasteurization. However, spoilage may be caused by sugar-tolerant yeasts. About 90% of the isolates belonged to *Zygo. rouxii*, and a few strains identified with *Zygo. bailii*, *C. bombicola*, *and Tsp. delbrueckii* (Taing and Hashinaga, 1997).

The frequent failure of a single physical or chemical method to preserve fruit products has prompted efforts to exploit combined action of pH, a_w, preservatives, refrigeration, and/or pasteurization. Several studies have been conducted to determine the combined effect of two factors, for example, sorbic acid and a_w (Maimer and Busse, 1992), sugars and heat (Stecchini and Beuchat, 1985), solutes and sorbate (Lenovich et al., 1988), and of three factors, for example, heat, organic acids, and sucrose (Torreggiani et al., 1985) or heat, solutes, and sorbate (Golden and Beuchat, 1992) on survival and growth of spoilage yeasts in juices and drinks. Recently, predictive modeling has become a more useful method to simultaneously evaluate several factors at various levels and their interactions. The method has been applied in fruit-based systems (Gardini and Guerzoni, 1986; Cole et al., 1987; Cole and Keenan, 1987; Ison and Gutteridge, 1987; Gardini et al., 1988; Cerutti et al., 1990; Guerzoni et al., 1990; Deák and Beuchat, 1993a), and from these studies, it can be concluded that *S. cerevisiae*, *Dek. anomala*, and *Zygo. bailii* are the most significant spoilage yeasts in nonalcoholic beverages, owing to their high resistance to pH, preservatives, and carbonation.

Zygo. bailii and the whole genus *Zygosaccharomyces* have been viewed as notorious spoilage agents in various foods and juices, and beverages in particular (Thomas and Davenport, 1985; James and Stratford, 2003; Kurtzman and James, 2006). Key physiological characteristics that make these species such problematic spoilage yeasts include xerotolerance and preservative resistance (*Zygo. rouxii*, *Zygo. mellis*), resistance to weak acid preservatives (*Zygo. bailii*, *Zygo. bisporus*, *Zygo. lentus*), growth at low temperature (*Zygo. lentus*), and vigorous fermentation (*Lachancea fermentati*, *Tsp. microellipsoides*). All these cause spoilage in various beverages (Table 7.4).

A novel, natural preservative, chitosan, looks promising for preventing spoilage. In apple juice of pH 3.4 at levels 0.1–0.4 g L^{-1} chitosan inhibited yeast growth at 25°C for 3 weeks (Roller and Covill, 1999). Its initial effect was biocidal, reducing the viable numbers by up to 3 log cycles. Following an extended lag phase, however, some survivors recovered and resumed growth. Interestingly, the most sensitive strain was an isolate of *Zygo. bailii*, usually considered one of the yeasts most resistant to chemical preservatives, whereas *S'codes ludwigii* appeared the most resistant, being inactivated by 5 g L^{-1} chitosan. Although UV light has a very low penetration effect, it could be used with success for the processing of mango nectar when treated for 15 min at 0.07–0.45 L min^{-1} flow rates if flowing was made turbulent so that UV light reached all parts of the liquid. The initial load of yeasts decreased by nearly 2 log cycles, and the nectar maintained without spoilage for 20 days at 3°C (Guerrero-Beltrán and Barbosa-Cánovas, 2006).

Yeast growth in carbonated soft drinks results in the formation of sediment, haze, and off-flavor. If fermentation occurs, it may result in bulging cans or exploding bottles. The cause of spoilage can rarely be ascribed to fruit juice concentrates, syrups, or other ingredients, but more often originates with the processing line. Critical points of contamination are the proportioning pumps, holding tanks, and bottle washers (Sand et al., 1976). Based on 3600 samples analyzed during a 4-year period, Sand (1974) established a relationship between the keeping quality of soft drinks and the

contamination of processing equipment. Yeasts were most frequently detected on bottling lines. Among these isolates were *C. boidinii*, *C. intermedia*, *C. sake*, *C. apicola*, and *C. parapsilosis*, whereas *P. anomala* and *Ya. lipolytica* were isolated frequently from raw materials. *Tsp. delbrueckii*, while occurring consistently in raw materials and on equipment, was rarely encountered in bottled beverages. Spoilage was caused most frequently by *S. cerevisiae*. A recent study for tracing the source of yeast contamination in a production line of carbonated orange juice pointed to insufficient pasteurization as responsible for spoilage (Pina et al., 2005). The contaminant was a single yeast species, identified as *P. galeoformis* (later made a synonym of *P. manshurica*); its ascospores apparently survived the pasteurization in a heat exchanger at 86°C for 60 s.

Sand and Grinsven (1976a,b) made a comprehensive study of yeasts isolated from bottled beverages produced in various countries. Some 30 species were identified, the most frequent ones being *S. cerevisiae*, *Zygo. bailii*, *Zygo. bisporus*, *Zygotsp. florentinus*, *Lachancea fermentati*, *Tsp. microellipsoideus*, *C. stellata*, *C. parapsilosis*, *Bret. naardenensis*, *Dek. Intermedia*, and *P. anomala*. Török and Deák (1974) isolated, in decreasing order of frequency, *S. cerevisiae*, *P. fermentans*, *C. lambica*, and *S. kluyveri* from Hungarian soft drinks; *Zygo. rouxii*, *Tsp. microellipsoideus*, and *P. guilliermondii* were represented by single isolates. Muzikar (1984) summarized the results of studies conducted for several years on nonalcoholic beverages, stating that the most frequent species were *S. cerevisiae* and *S. pastorianus*, and their main sources were the unclean bottles. Spoilage was most often caused by a single species that had adapted to existing conditions. In addition to the *Saccharomyces* species, *Tsp. delbrueckii*, *Tsp. microellipsoideus*, *Db. hansenii*, *C. inconspicua*, *C. vini*, and *Iss. orientalis* were also frequently found. Dragoni and Comi (1985) reported that 40% of juices from peaches, pears, and apricots contained yeasts. The most frequently detected species were *Hsp. uvarum*, *Db. hansenii*, *C. haemulonii*, *P. guilliermondii*, and *P. etchellsii*, while *P. anomala*, *Hyphop. burtonii*, *Tsp. delbrueckii*, *Zygo. rouxii*, *Zygo. bisporus*, and *Zygo. bailii* were isolated occasionally.

7.3 VEGETABLES

Several reviews have been published on the mycology of vegetables (Mundt, 1978; Dennis and Buhagiar, 1980; Brackett, 1987; Dennis, 1987; Bulgarelli and Brackett, 1991). Considerable information has been accumulated on the types and populations of yeasts associated with fresh and processed vegetables (Table 7.5). Compared to molds and bacteria, yeasts generally play only a secondary role in the spoilage of vegetables. However, the activity of yeasts becomes apparent when environmental conditions are favorable, for example, during the lactic acid fermentation of vegetables (Section 7.5.3). In addition, new packaging and storage techniques, minimal processing, and consumer demand for ready-to-use vegetables have resulted in an increased incidence of yeast spoilage in these products.

In contrast to fruits, vegetables are more susceptible to microorganisms and several intrinsic factors predispose them to microbial attack and subsequent spoilage (see Table 7.1). These include high water content, adequate nutrient composition, and near-neutral pH. In addition, the close proximity of vegetables to the soil during development facilitates contamination. Differences between fruits and vegetables in their intrinsic properties are reflected in the general composition of yeast mycobiota. Török and King (1991) investigated 239 yeast strains isolated from partially and fully processed fruits and vegetables. More ascomycetous yeasts were isolated from fruits than from vegetables; in contrast, more basidiomycetous yeasts occurred on vegetables than on fruits. *C. tropicalis* (10%) and *S. cerevisiae* (18%) were the most frequent isolates from fruits, whereas weakly or nonfermenting species such as *P. fermentans* (22%), *Cry. albidus* (10%), and *Trisp. cutaneum* (7%) were dominant among isolates from vegetables.

As a general rule, vegetables become more susceptible to infections as they ripen, and mechanical injuries resulting from harvesting and handling also facilitate entry of invading microorganisms

TABLE 7.5
Yeasts Frequently Isolated from Vegetables and Grains

Species	Sources	References
C. intermedia	Corn, salads	2, 5, 14
C. sake	Carrot, corn	1, 4
C. parapsilosis	Carrot, corn	1, 2, 5
C. tropicalis	Carrot, rice, corn	1, 2, 15
C. versatilis	Corn	5
Cry. albidus	Corn, carrot, cabbage, wheat	1, 5, 6, 9
Cry. humicolus	Fresh-cut salads	18
Cry. laurentii	Corn, cabbage, fresh-cut salads	5, 6, 10, 18,19
Db. hansenii	Corn, barley	5, 13
Gal. geotrichum	Corn, tomato, barley, potato, sprouts	5, 7, 11, 13,16, 20
Iss. orientalis	Corn	2
Kazach. exiguus	Mayonnaise salad	17
Naumovia dairensis	Mayonnaise salad	17
P. anomala	Corn, fresh-cut salads	2, 5,16
P. fermentans	Lettuce, carrot, fresh-cut salads	1, 8, 16, 19
P. membranifaciens	Fresh-cut salads	16
Rho. glutinis	Corn, peas, fresh-cut salads	3, 5, 16
Rho. mucilaginosa	Corn, barley	5, 10, 13
Spb. roseus	Corn, rice, barley, cabbage	6, 10, 12, 13
Tsp. delbrueckii	Lettuce	8
Trisp. cutaneum	Fresh-cut salads	16

Note: 1: Babic et al. (1992); 2: Burmeister and Hartman (1966); 3: Collins and Buick (1989); 4: Deák (1988); 5: Deák and Beuchat (1987); 6: Geeson (1979); 7: de Hoog et al. (1986): 8: King et al. (1991); 9: Kurtzman et al. (1970); 10: Middelhoven and Baalen (1988); 11: Moline (1984); 12: Nakase and Suzuki (1985): 13: Petters et al. (1988); 14: Török and King (1991); 15: Walker and Ayres (1970); 16: Garcia-Gimeno and Zurera-Cosano (1997); 17: Hunter et al. (1994); 18: Jacxsens et al. (2003); 19: Ragaert et al. (2006); 20: Tournas (2005).
Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

(Bulgarelli and Brackett, 1991). Although yeasts are often isolated from vegetables, they typically do not compete well with bacteria and molds. Only rarely have yeasts been implicated as the direct cause of deterioration of vegetables (Moline, 1984; Johnson et al., 1988). Tomatoes may be exceptional, in that yeasts represented nearly 17% of fungal isolates from ripe, damaged, and decayed tomato, and 19% of yeasts showed proteolytic activity, with *Cry. albidus*, *Db. hansenii*, and *Guehomyces pullulans* the most frequently isolated (Wade et al., 2003). A warning observation has been made that growth of a proteolytic *Geo. candidum* in raw tomatoes enhances conditions for the growth of *Salmonella enterica.*

Yeast populations on vegetables are varied and generally range from $<10^3$ to $>10^6$ g^{-1} (Senter et al., 1984, 1987; Deák et al., 1987). Although it is commonly believed that vegetables grown in the soil or close to the ground become easily contaminated, yeast populations in a range of 10^2–10^6 g^{-1} can also occur on hydroponically grown lettuce, but this is nearly two orders of magnitude less than that of bacteria (Riser et al., 1984).

Basidiomycetous yeasts usually dominate the mycobiota on vegetables. From these, several new species have been described, for example, *Bullera* (now *Dioszegia*) *crocea* and *Bullera armeniaca* on cauliflower and cabbage (Buhagiar et al., 1983). Fresh corn has been mainly inhabited by *Rho. ingeniosa*, *Cry. laurentii*, *Spb. roseus*, *Spori. salmonicolor*, and *Rho. rubra*, according to

the study by Middelhoven and van Baalen (1988). In addition to predominating basidiomycetous yeasts (*Rho. rubra*, *Rho. glutinis*, *Cry. albidus*, and *Cry. laurentii*), Deák and Beuchat (1988) also detected a wide range of fermenting ascomycetous yeasts on sweet corn (*Hsp. uvarum*, *C. intermedia*, *C. oleophila*, *P. guilliermondii*, and 10 other species), although these yeasts constituted a minority of the population. In agreement with these observation, Nakase and Suzuki (1985) found ballistospore-forming basidiomycetous species in 86% of yeasts isolated from the leaves and stems of rice plants. *Spb. roseus*, *Bullera crocea*, and *Bullera alba* were the most frequent isolates, with some *Bullera* strains probably representing yet undescribed species.

Storage of vegetables at reduced temperatures helps maintain the quality of fresh produce. Winter white cabbage stored at 2°C for up to 33 weeks has been shown to undergo only minor quantitative and qualitative changes in the mycobiota of the leaf surface (Geeson, 1979). Yeasts were present in populations of 10^2–10^3 cfu cm^{-2}. The most commonly occurring species were *Cry. albidus*, *Cry. laurentii*, *Cry. macerans*, and *Spb. roseus*; a new species was also found, classified recently as *Pseudozyma fusiformata*.

Vegetables appear on the market in fresh, frozen, canned, dried, and fermented forms. The types of treatment and processing to which vegetables are subjected affect yeasts differently. Equipment used in processing is an important source of contamination. Characteristic is the build-up of *Gal. geotrichum* on processing equipment; hence, this yeast being often referred to as machinery mold (Eisenberg and Cichowicz, 1977; Splittstoesser et al., 1977). Yeast counts have been found to increase appreciably during transportation of peas and collards (Senter et al., 1984, 1987). Washing, blanching, and freezing effectively reduce the number of yeasts on these vegetables, but recontamination can occur during subsequent operations. Psychrotrophic yeasts such as *Rho. glutinis* may grow on blanched and frozen peas stored at freezing temperature. A significant increase in yeast population occurred on peas after 12 weeks of storage at −5°C, 20 weeks at −10°C, or 24 weeks at −18°C (Collins and Buick, 1989).

Ready-to-use vegetables such as fresh, washed, peeled, sliced, shredded, or grated produce packed in polymeric film have become increasingly popular for consumers. These products have a shelf life of a week or more if stored at refrigerated temperature (2–4°C). Spoilage is mainly due to psychrotrophic Gram-negative bacteria (*Pseudomonas* spp., *Erwinia* spp.) and LAB, but yeasts are also increasing in numbers with storage time (Garcia-Gimeno et al., 1997). Most frequent yeast species isolated from ready-to-eat vegetable salads were *P. fermentans*, *P. membranifaciens*, and unidentified *Candida* spp. and *Trichosporon* spp. On ready-to-use grated carrots packaged in polymeric film, yeast counts increased up to 10^5–10^6 cfu g^{-1} after 3 days of storage at 10°C. *Cry. albidus* was isolated only in the first days, but *C. lambica*, *C. sake*, *C. tropicalis*, and *C. parapsilosis* were present throughout storage (Babic et al., 1992). Neither the number of yeasts nor the composition of the yeast biota was related to the extent of deterioration of the product, with the exception of CO_2 produced by yeasts. However, in a recent study, correlation between the production of volatile metabolites such as ethanol, ethyl acetate, methyl-butanol, and others, and the counts of the spoilage yeasts, *P. fermentans*, has been established (Ragaert et al., 2006). These compounds were detected when the population of *P. fermentans* exceeded 5.0 log cfu cm^{-2} in mixed lettuce. From a study with modified atmosphere-packed fresh-cut produce, Jacxsens et al. (2003) also reported that microbiological criteria corresponded well with the production of metabolites and detectable changes in sensory properties. Growth characteristics on fresh-cut produce stored under 3% O_2/2–5% CO_2 at 7°C of *C. humicola* and *Cry. laurentii* were, respectively, with lag phase of 15.4 and 73.8 h, and μ_{max} of 0.038 and 0.067 h^{-1}.

Although field contamination is reduced by the removal of the outer leaves and by peeling, partial processing, in general, increases the microbial population in fresh-cut vegetables. Major sources of in-plant contamination are shredders and slicers; nevertheless, yeasts made up only a minor part of the microbial population (Garg et al., 1990). Some ready-to-eat salad vegetables are acidified by the addition of vinegar, mayonnaise, or other salad dressings. In mayonnaise-based salads, the commonest spoilage yeast species were *S. dairensis* and *S. exiguus* (Hunter et al., 1994). Yeasts

are normally present on chill-stored, nonacidified, raw vegetable salads, representing 17% of all isolates (Manwell and Acklund, 1986). In these products, Gram-negative bacteria are dominant, but a large yeast population can also occur (King and Bolin, 1989). Yeast population increased from 2.5–3.2 log cfu g^{-1} to 4.9–6.1 cfu g^{-1} during 15 days of storage at 2.8°C (King et al., 1991). A wide variety of yeast species were isolated from unsealed stored samples. However, in sealed bags, fermentative yeast species such as *P. fermentans* and *Tsp. delbrueckii* prevailed.

Improved market quality may also be achieved through the use of modified or controlled atmospheres. However, modified atmosphere packaging may encourage the growth of yeasts. Brackett (1988, 1989, 1990) reported that yeast populations increased on individually shrink-wrapped tomatoes and bell peppers, but not on broccoli. Golden et al. (1987) and Deák et al. (1987) also observed that yeast developed higher populations on shrink-wrapped bell peppers and sweet corn, respectively. Packaging fresh-cut vegetables under equilibrium-modified atmosphere (EMA) is a new technology for offering a prolonged shelf life of respiring products (Jacxsens et al., 2002). By matching film permeability for O_2 and CO_2 to the respiration rate of the packaged fresh-cut produce, EMA can be established (Day, 1996). Under these conditions, control of storage temperature was found to be of paramount importance. In simulated experiments with cucumber slices, bell peppers and mixed lettuce yeasts proved to be the shelf-limiting group (Jacxsens et al., 2002).

In addition to ready-to-eat salads, various types of sprouts have become very popular in recent years, owing to their nutritional attributes. However, sprouts prepared in a wet atmosphere can be easily contaminated. Tournas (2005) compared mold and yeast contamination in a survey of fresh whole produce, ready-to-eat salads, and fresh sprouts. Although great variation occurred in the contamination levels among samples, yeasts were always the predominant group, with overall counts ranging from <100 to $>10^6$ cfu g^{-1}. They were found in all lettuce, radish, and spinach samples, two-thirds of cucumber and green onion samples, and one-third of green pepper samples. Yeast counts in salads were generally higher, with an average contamination of 8.1×10^5 cfu g^{-1}. This can be explained by the fact that salads undergo extensive handling during shredding, cutting, grating, and packaging. Sprouts were, however, by far the most contaminated, with yeasts reaching numbers as high as 10^8 cfu g^{-1}, especially for bean, alfalfa, and crunchy sprouts. Screening and disinfection of seeds before sprouting would greatly reduce contamination.

7.4 ALCOHOLIC BEVERAGES

Production and consumption of alcoholic beverages began at the dawn of human cultures, and various types of these beverages belong to nearly all civilized and natural communities (Rose, 1977). Beer and wine are perhaps the two alcoholic drinks produced worldwide in the largest volumes. Their annual commercial value is more than that of all other fermentation and biotechnological products combined (including antibiotics, drugs, enzymes, and others) (Hartmeier and Reiss, 2002).

7.4.1 Beer

This chapter focuses on the spoilage aspects of yeasts during the fermentation of beer and in the finished product. However, to fully appreciate the basic function of yeasts in beer production, both beneficial and detrimental roles should be examined. The following is a brief overview of the technology, its control and monitoring, trends of improvement, and possibilities of future development.

Numerous textbooks, chapters, and reviews have dealt with the broad subject of brewing. Authentic historic overviews are given by von Wettstein (1983) and Stewart and Russel (1986). Technology is amply covered by Hammond (1993), Hardwick (1995), Stewart and Russel (1995), Heyse (1995), Lewis and Young (1995), Kunze (1996), Campbell (1997), and Hartmeier and Reiss (2002); brewing microbiology is specially treated by Stewart and Russel (1995), Priest and Campbell (1996), and

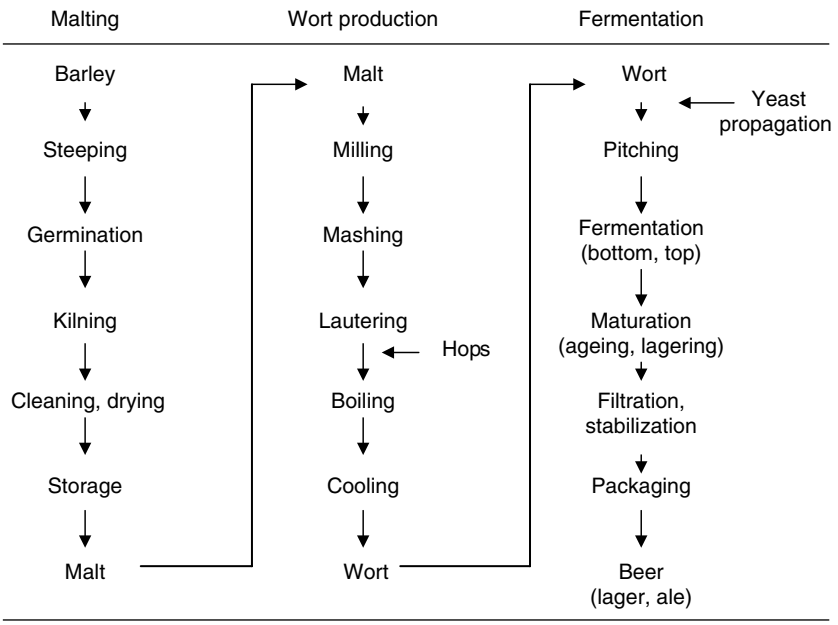

FIGURE 7.1 The brewing process.

Boulton and Quain (2001); whereas both aspects are comprehensively discussed by Dufour et al. (2003).

7.4.1.1 Brewing Technology

Beer is made from barley and/or other starch-containing cereals, hops, and water with yeasts. Brewer's yeast is not able to ferment starch; hence, it must first be decomposed to fermentable sugars. Brewing can be divided into four stages: malting, mashing, fermentation, and final processing (Figure 7.1).

Malting is a process to activate starch-hydrolyzing enzymes in barley grains by germination. In order to do this, grains are soaked in water (steeping) and left germinating for about 6–10 days. After this, the grain is dried by hot air (kilning) in 24–48 h, during which browning reactions occur between sugars and amino acids, flavor components are formed, and microorganisms are partly destroyed. The resulting malt can be stored until use in a brewery. Malting is critical for the quality of beer production.

On dry barley, the number of yeasts has been reported to be $4.7 \times 10^3\ g^{-1}$, which increases up to 1.2×10^6 during the malting process (Petters et al., 1988). Only a few species representing basidiomycetous yeasts, for example, *Rho. mucilaginosa* and *Spb. roseus*, were isolated from dry barley, whereas a greater diversity of yeasts was found in the wet stages of malting, consisting of *C. catenulata*, *C. vini*, *Db. hansenii*, *Ogataea* (*P.*) *polymorpha*, *Hsp. uvarum*, and *Trisp. cutaneum.* In dry barley kernels, *Geo. candidum* occurs rarely but may become one of the dominant yeasts on green malt (Douglas and Flannigan, 1988).

During steeping and germination, a diverse and large population of yeasts develops, which has been recently reviewed by Laitila et al. (2006). Both ascomycetous and basidiomycetous species (25 and 18, respectively) were detected, many of them originating from the barley grains. Several basidiomycetous yeasts (e.g., *Bulleromyces albus*, *Cry. macerans*, and *Filobasidium globisporum*) were active in producing extracellular hydrolytic enzymes (glucanase, cellulase, xylanase, and amylase), although their significance in malting and brewing needs to be confirmed. From a microbiological

point, the most troublesome is the production of mycotoxins by molds. A new trend to biocontrol mold development by the use of *Geo. candidum* culture was added during steeping (Linko et al., 1998; Noots et al., 1999; Foszczynska et al., 2004).

Mashing is the preparation of wort from malt and includes the milling of malt, extraction in hot water, separation of husks (lautering), boiling with hops, removing debris, and cooling. Essentially, during mashing, malt starch is converted into fermentable sugars. On average, wort contains 8% glucose, 55% maltose, and 10% maltotriose, whereas about 20% remains as higher dextrins. Wort also contains amino acids and minerals for yeast growth. The characteristic bitterness of beer is due to the components extracted from hops (humulone and lupulone).

Fermentation starts with the inoculation (pitching) of brewer's yeast. Yeast is usually added to reach a concentration of 10^7 cells mL^{-1}, and during fermentation yeasts multiply only by 3–4 generations. Pitching is made by culture used in previous fermentation; new starter is made only after 10–15 reuses of brewer's yeast, when it becomes physiologically weakened and microbial contaminants increase. Yeast growth starts a few hours after pitching and aeration is needed for enhancing the synthesis of fatty acids and sterols. Nevertheless, respiration is repressed due to the high concentration of sugars (Crabtree effect), and alcoholic fermentation soon commences. In addition to ethanol, yeast produces numerous by-products critical to the flavor of beer. Many of these are advantageous to the quality (higher alcohols, organic acids, and esters), whereas others create undesirable off-flavors (e.g., diacetyl and other carbonyls, sulfur compounds). Details of the sensory aspects of beer quality cannot be dealt with here (consult the references cited above).

In traditional brewing, after the main fermentation, young "green" beer is subjected to maturation. During secondary fermentation, the beer is conditioned. Part of the off-flavoring materials are degraded by the yeasts themselves; carbon dioxide is generated, haze materials precipitate, and yeast cells sediment. Aging and storage (lagering) at low temperatures (0–2°C) for several weeks are typical for one type of beer (lager). Modern technologies, however, restrict maturation to a short time. The beer production is finished by filtration, pasteurization, and packaging.

The large majority of beer produced worldwide is lager beer; the other main type is called ale. The main difference is in the mode of fermentation: lager beer is made by bottom-fermenting yeast while ale by top-fermenting. In both methods, the color of beer produced can be light, pale, or dark brown, depending mainly on the malt used. Applying different fermentation technologies and using additives, a large variety of beer types can be produced.

7.4.1.2 Brewer's Yeast

Over the years, the taxonomic position and names of brewer's yeast have changed several times. Brewer's yeast belongs to the group of *Saccharomyces sensu stricto* formed around the species *S. cerevisiae*; however, brewing strains differ markedly from laboratory to laboratory and from nonbrewing strains of this species due to the selection over the centuries to the specific conditions of beer making (Table 7.6) (Codon et al., 1998). Traditionally, the lager (bottom fermenting) yeast is called *S. carlsbergensis*, and the ale (top fermenting) yeast is *S. cerevisiae*. *S. carlsbergenis* has been put in synonymy with *S. pastorianus*, which proved to be a hybrid species. Recently, *S. carlsbergensis* has again been considered a separate species (Romano et al., 2006). Although the taxonomic classification does not perfectly fit the distinction of lager and ale yeasts, there are several phenotypic differences between them that justify their traditional distinction (Table 7.7) (Hampsey, 1997). Traditional phenotypic methods do not give unequivocal identification of *Saccharomyces* species, and even less differentiation of brewer's yeast. Differentiation of specific brewing strains, that is, typing, is of primary importance to brewers. At least a thousand different brewing yeast strains are used worldwide. Recent molecular methods can be used for a more reliable typing of brewing strains (see Section 9.7).

TABLE 7.6
Differences between Brewing Yeasts and Non-Brewing *Saccharomyces cerevisiae*

Characteristics	Brewing Strains	Laboratory Strains
Ploidity (chromosome copy number)	Aneuploid, polyploid	Haploid, diploid
Sporulation	Poor, viable spores are rare	Abundant viable spores
Mating type	None or rare	Two: a and α
Sexual reproduction	Very rare, in some strains	Common

TABLE 7.7
Differences between Lager and Ale Brewing Yeasts

Characteristics	Lager Strains	Ale Strains
Mode of fermentation	Bottom	Top
Flocculation	Good	Absent or poor
Fermentation at temperature	Below 15°C	Above 15°C
Maximum growth temperature	32–34°C	38–40°C
Utilization of maltotriose	More complete	Less efficient
Utilization of melibiose	Yes	No
Volatile sulfur compounds	More	Less
Fructose transport	Active proton symport	Facilitated diffusion
Sporulation	None	1%–10%

7.4.1.3 Wild Yeasts

Although boiling of wort kills most microorganisms and thence is inoculated by pitching yeast, other kinds of unwanted yeasts can get into beer during fermentation; these yeasts are collectively known as wild yeasts. These wild yeasts are of two kinds: they belong both to *Saccharomyces* and non-*Saccharomyces* genera. Their sources are the brewery environment and the pitching yeast itself, in particular, after several cycles of repeated use.

Any strains of *S. cerevisiae* not being selected as a brewing strain are considered contaminants. Even ale strains among lager yeasts, or wine, distillery, and other *S. cerevisiae* strains are the most dangerous contaminants because their properties are close to brewer's yeast; consequently, their detection is also most difficult (see Section 8.2.4). Some strains of *S. cerevisiae* wild yeast contain an enzyme that decarboxylates wort phenolic acid into compounds causing phenolic off-flavor. Other strains represent a specific biotype possessing glucoamylase, allowing utilizing dextrins and causing excessive attenuation of the beer. These strains are denoted as *S. cerevisiae* var. *diastaticus*, and were once considered a different species. Contamination with strains producing zymocins (killer toxins, see Section 3.2.4.2) may completely eliminate pitching yeast causing stuck fermentation. Killer strains are frequently found among non-*Saccharomyces* wild yeasts, such as *Pichia* and *Candida* species. In a recent study (Jespersen et al., 2000), among *Saccharomyces* contaminants, *S. cerevisiae* has been the most frequent (52%), followed by *S. pastorianus* (26%) and *S. cerevisiae* var. *diastaticus* (13%); only a single strain has been identified with *S. bayanus*.

Wild yeasts were detected in 41% of pitching yeasts investigated (van der Aa Kühle and Jespersen, 1998). Beside the majority of *S. cerevisiae* isolates (57%), *Pichia* spp. (28%) and *Candida* spp. (15%) were found. Of 120 strains of wild yeasts, Back (1987) identified 18 species other than *Saccharomyces* and 2 *Saccharomyces* species other than *S. pastorianus.* The latter were *S.* (*Kazach.*) *exiguus* and *S. cerevisiae* (nonbrewing strains). Other species most frequently isolated

were *P. anomala*, *P. membranifaciens*, *Db. hansenii*, *C. sake*, *Rho. glutinis*, and *Dek. anomala.* In top fermentation, the most frequent wild yeasts observed by Röcken and Marg (1983) were *P. media*, *C. vini*, and *C. ishiwadae*.

Contamination by non-*Saccharomyces* yeast may be responsible for film forming (e.g., *P. membranifaciens*, *P. fluxuum*, and *P. anomala*). Others cause haze and are difficult to filtrate (e.g., *Kloeckera* and *Rhodotorula* species); the presence of still others, in particular *Dekkera* and *Brettanomyces* species, results in off-flavor, although the latter ones contribute to the specific organoleptic properties of certain specialty beers, such as lambic beer (see below).

In draft beer sold unpasteurized in vats or barrels, a broader variety of wild yeasts may develop if the beer is not kept refrigerated. The majority of spoilage yeasts are *Saccharomyces* species. Only 20–30% of wild yeasts belong to other species such as *Dek. anomala*, *C. inconspicua*, *C. vini*, *P. fermentans*, *P. membranifaciens*, *P. jadinii*, *P. anomala*, *Hsp. uvarum*, *Db. hansenii*, and *Tsp. delbrueckii* (Röcken, 1983; Back, 1987; Lawrence, 1988). In addition to yeasts, bacteria are also hazardous beer spoilage organisms (Jespersen and Jakobsen, 1996). The Gram-positive bacteria are mostly lactobacilli and pediococci; among the Gram-negative bacteria *Pectinatus cerevisiiphilus* and *Megasphaera cerevisiae* are specific beer contaminants. Table 7.8 summarizes the most frequently found beer spoilage yeasts.

7.4.1.4 Improving of Brewing Yeast and Technology

The brewing industry strives continuously to improve brewing technology and the properties of brewer's yeast. We mention only some aspects here.

Brewing is traditionally batch fermentation, and efforts have been made to alter it to a continuous process. From the engineering and economic aspects, continuous fermentation may offer many advantages; however, it involves several difficulties and risks related to beer microbiology. On the one hand, continuous fermentation will exhaust the physiological capacity of producing a strain; on the other hand, it can lead to a selection of a deteriorated *Saccharomyces* strain. Moreover, it would also increase the possibility of contamination by wild yeasts. The time needed for maturation also creates an obstacle to continuous operation. An alternative would be conditioning in bottles, a method practiced in the production of specialty beers (Dufour et al., 2003). The use of immobilized yeast has disadvantages similar to continuous fermentation. Immobilized systems have been applied in beer maturation and production of alcohol-free beer, but adaptation to primary fermentation requires further research (Yamauchi et al., 1994).

Over the years, great improvements in brewing yeast performance has been achieved by using classical genetic techniques such as hybridization and mutagenesis followed by selection. Recently, it has become possible to tailor production strains for purpose by methods of recombinant gene technology (genetic engineering). Progress has been made in improving technologically important properties of brewing yeasts, such as carbohydrate utilization, fermentation of dextrins, flocculation and filtration, reduction of H_2S and diacetyl production, and osmotolerance (high-gravity wort). These studies have been reviewed by Hammond (1995), Dequin (2001), Pretorius et al. (2003), and Verstrepen et al. (2001, 2006). However, public concern and legal regulation may create problems in the commercial application of genetically modified organisms and their products. Although a transformed brewer's yeast with glucoamylase for dextrin hydrolysis was approved in 1994 by U.K. authorities, the lack of public acceptance has prevented the industry from commercializing the beer produced with it (Hammond, 1998; Moseley, 1999).

7.4.1.5 Specialty Beers and Other Beer Type Beverages

About 90% of beer produced worldwide is of the lager type resulting from bottom fermentation. However, within lager beers, many variations exist, differing slightly in flavor and color

TABLE 7.8
Yeasts Frequently Isolated from Beers

Species	Sources	References
Bulleromyces albus	Malt	16
C. anglica	Malt	16
C. boidinii	Lager	37
C. inconspicua	Beer	36
C. ishiwadae	Lager	34
C. parapsilosis	Lager	19, 37
C. pararugosa	Malt	16
C. rugosa	Sake, beer	3, 25
C. sake	African beer, lager	2, 37, 39
C. tropicalis	Sorghum beer, lager	25, 37
C. vini	Sorghum beer, lager	12, 13, 25, 34
Cry. macerans	Malt	16
Clsp. lusitaniae	Malt, sorghum beer	16, 25
Db. hansenii	Beer, sorghum, rice beer	2, 19, 24, 25
Dek. anomala	Beer, lambic beer	2, 10, 12, 38
Dek. bruxellensis	Beer, lambic beer	2, 10, 12, 38
Geo. candidum	Malt, pito	8, 16
Geo. capitatum	Sorghum beer	25
Hsp. uvarum	Malt, beer	2, 12, 16, 19
Iss. orientalis	Malt, beer	10, 12, 13, 16, 25, 27
Kazach. exiguus	Beer, sorghum beer	2, 25
Klu. marxianus	Beer, sorghum beer	2, 10, 25
P. anomala	Beer, rice beer	12, 19, 24, 37
P. fabianii	Sorghum beer, rice beer	24, 25
P. fermentans	Malt, lager beer	12, 16, 19, 37
P. guilliermondii	Malt, sorghum beer	16, 25, 37
P. jadinii	Lager	2, 12
P. media	Lager	34
P. membranifaciens	Beer	2, 10, 12, 14, 19, 37
Rho. glutinis	Malt, beer	2, 16, 19
S. bayanus	Beer	10, 15
S. cerevisiae	Sake, beer, sorghum beer	10, 14, 15, 25, 34, 35, 37, 38
S. pastorianus	Beer, lambic beer	15, 26, 38
S'copsis fibuligera	Sorghum beer, rice beer	24, 25
S'codes ludwigii	Beer	2, 19, 25
Schizo. pombe	African beer, pombe	11
Spb. ruberrimus	Malt	16
Tsp. delbrueckii	Beer	10

Notes: 2: Back (1987); 3: Barnett et al. (1983); 8: Demuyakor and Ohta (1991); 10: Dufour et al. (2003); 11: Grieff (1966); 12: Hardwick (1983); 13: Ingledew and Casey (1982); 14: Jespersen and Jakobsen (1996); 15: Jespersen et al. (2000); 16: Laitila et al. (2006); 19: Lawrence (1988); 24: Nout (2003); 25: Nouvellie and Schaepdrijver (1986); 34: Röcken (1983); 35: Röcken et al. (1981); 36: Ruiz et al. (1986); 37: van der Aa Kühle et al. (1998); 38: Verachtert and Dawoud (1984); 39: Walczak et al. (2006).

Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

TABLE 7.9
Specialty Beers and Beer-Type Alcoholic Beverages

Beverage Type	Region	Substrates	Microorganisms
Lambic, gueze	Belgium	Malted barley, wheat, oak	*Saccharomyces*, *Dekkera, Candida*, *Acetobacter, Lactobacillus*, *Pediococcus* species
White beer	Germany	Malted wheat	*S. cerevisiae*, *Dek. bruxellensis*, *Candida* spp., lactobacilli
Kvass	Russia	Barley, rye	*S. cerevisiae* and mixed wild yeast
Bouza	Egypt	Wheat, maize	*S. cerevisiae* and mixed wild yeast
Rice beer	India, East Asia	Rice	*Saccharomycopsis*, *Pichia* species, *Aspergillus*, *Rhizopus*
Sake	Japan	Rice	*Aspergillus oryzae*, *S. cerevisiae*
Sorghum beer	Africa, East Asia, Middle East, South America	Malted and raw sorghum, maize, cereals, fruits	*Saccharomyces*, *Candida* species, *Geo. candidum*, lactic acid bacteria
Pombe	Africa	Millet	*Schizo. pombe*
Tape	Malaysia	Cassava	*Aspergillus*, *Mucor*, yeasts
Pito	Nigeria, Ghana	Sorghum, maize	Yeast, lactic acid bacteria

(e.g., Pilsener, Tuborg, Kulmbacher, Bock, etc.), and the same holds true for ale beers (e.g., Porter, Stout, Gueuze, etc). Some of these beers are produced from barley malt with added wheat, or completely from wheat malt, and are fermented with mixed cultures of yeasts, or yeasts and lactic acid or other bacteria (Table 7.9). A typical example is the Belgian Lambic beer made from malted barley, wheat, and oat with mixed fermentation of *S. cerevisiae*, *Dekkera*, and *Candida* species, as well as *Acetobacter*, *Lactobacillus*, *Pediococcus*, and other bacteria, a spontaneous fermentation often accompanying yeast starters (Verachtert et al., 1990). Other kinds of beer made from normal lager fermentation of light-gravity wort result in developing 0.5% alcohol only ("alcohol-free" beer), or by adding amylolytic enzymes to fermenting wort to further attenuate its carbohydrate content for the production of dietetic beer.

Various other types of alcoholic beverages can be considered beer-like because they are produced from starchy materials such as corn, rice, sorghum, millet, cassava, or others (Hardwick et al., 1995). In the saccharification of starch, enzymes from molds and bacteria play a role, and in addition to, or instead of *S. cerevisiae*, other yeast species contribute, and/or mixed yeast–bacterial fermentation takes place (Novellie and De Schaepdrijver, 1986; Demuyakor and Ohta, 1991). Some of these processes will be discussed in Section 7.4.3.

7.4.2 Wine

Wine microbiology has been extensively studied. Interest has been focused mainly on the composition and succession of yeast biota on grapes as they ripen in the vineyard, in the course of fermenting must, as well as in and around the winery and wine cellars, and finally in the bottled wine. Several reviews and books are available on these subjects. Earlier publications include Kunkee and Goswell (1977), Lafon-Lafourcade (1983), Goswell (1986a,b), and Martini and Martini (1990); more recently updated ones include Fleet (1993, 1997), Kunkee and Bisson (1993), Ciani (2002), Hartmeier and Reiss (2002), and Dequin et al. (2003). A list of yeasts isolated from grapes, must, and winery equipments was compiled by Kunkee and Amerine (1970), Kunkee and Goswell (1977), Lafon-Lafourcade (1983), and Zambonelli et al. (1989), and was recently updated by Martini et al. (1996) and Romano et al. (2006).

7.4.2.1 Wine Production

Wine is made by the alcoholic fermentation of yeasts from fruits, mainly grapes. The basic difference between wines and beers is that fruits contain fermentable sugars and there is no need for previous saccharification as in the case of cereal grains. Consequently, the technology of wine making differs substantially from that of brewing, and is simpler to a certain extent. Nevertheless, technology is responsible for the characteristics of wine, in addition to the properties of grapes and the contribution of yeasts. This chapter will focus on yeasts, and the processes in the vineyard and winery will not be discussed in detail.

Numerous grape varieties are used for making wine. The two main types are white (e.g., Riesling, Traminer, and Chardonnay) and red grapes (e.g., Cabernet Sauvignon, Merlot, and Pinot Noir), and their processing differs to some degree. Harvesting, crushing, and pressing are the basic steps in vinification. However, in the case of white wines, fermentation of pressed juice (the must) starts directly, whereas red wine fermentation is carried out on the skins of grapes to allow extraction (maceration) of color compounds and tannins, and pressing follows after a couple of days. Red wine technology differs further in the use of a smaller amount of sulfur dioxide and the somewhat higher temperature of fermentation, which usually involves malolactic fermentation, too. The fermented wine is then drawn off from the sediment (racking), clarified and stabilized (fining), and aged before or after bottling (Figure 7.2).

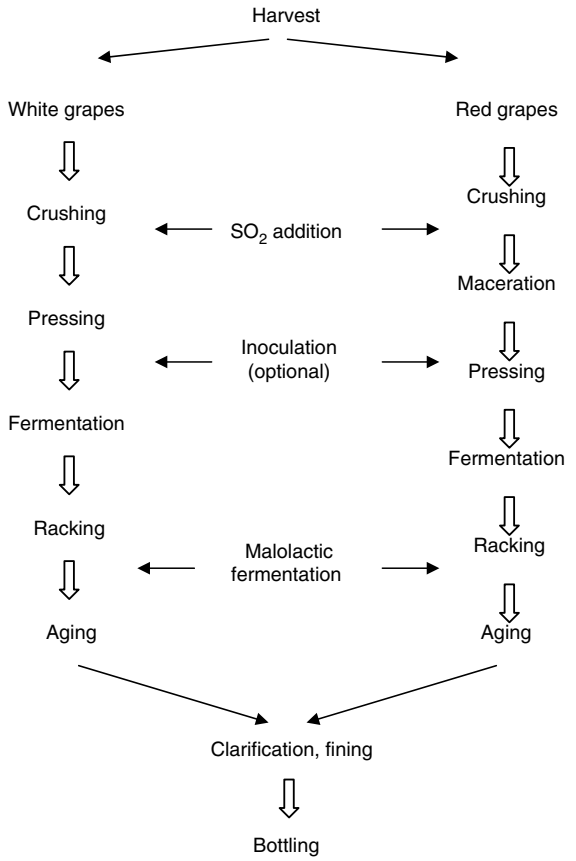

FIGURE 7.2 Scheme of producing white and red wines. (Adapted from Hartmeier, W. and Reiss, M. (2002) *In*: The Mycota X. Industrial Application (ed. Osiewacz, H. D.). Springer Verlag, Berlin. pp. 49–65.)

7.4.2.2 Wine Yeasts

After crushing, the grape must contains a large population of various yeasts, generally about 10^3–10^5 cfu mL^{-1}. The natural sources of these yeasts are the grapes (see Section 7.1.1) and the harvesting and winery equipment (vessels, crushers, pressers, tanks, etc.). This original population of yeasts can bring about the spontaneous fermentation of must. Modern technology applies an artificial addition of pure cultures of the main wine yeast, *S. cerevisiae*, in order to fasten fermentation.

7.4.2.2.1 Indigenous Yeasts

Numerous studies have shown that the early stages of must fermentation are dominated by a mixed biota of non-*Saccharomyces* yeasts indigenous to grapes, such as *Hsp. uvarum*, *C. stellata*, *Iss. orientalis*, *Met. pulcherrima*, and *P. anomala* (Davenport, 1974; Rosini et al., 1982; Fleet et al., 1984; Parish and Caroll, 1985; Heard and Fleet, 1986a; Holloway et al., 1990). *Hsp. uvarum* and other apiculate yeasts predominate in must, making up 40–70% of the population. In addition to apiculate yeasts, *C. stellata* is also a typical and dominant species (13–19%); among other species, *Met. pulcherrima* and *P. membranifaciens* (and its anamorphic state, *C. valida*) are frequently isolated from must as well (Goto and Yokotsuka, 1977; Parish and Caroll, 1985; Mora et al., 1988; Holloway et al., 1990, 1992; Mora and Rosello, 1992). Dissimilarities occur not only between different regions, but also within the same region in different vintages (Rosini et al., 1982; Moore et al., 1988; Mora et al., 1988; Holloway et al., 1990; Querol et al., 1990; Hidalgo and Flores, 1991; Longo et al., 1991). Nevertheless, results of various authors show an overall similarity in composition of the indigenous yeast population in most wine regions of the world (Martini, 1993).

Investigations are also concordant in pointing out that, among the great diversity of species occurring in early must, strains of *Saccharomyces* species are rare and become predominant only during the active alcoholic fermentation (Hidalgo and Flores, 1991; Coratza et al., 1992). In an ecological survey of the yeast flora of grapes by applying a vigorous physical method for the separation of yeast cells, Rosini et al. (1982) showed that, from 1080 single grapes collected during two consecutive vintages, only one culture of *Saccharomyces* was isolated. The origin of *Saccharomyces*-type wine yeast has been the subject of numerous studies (Martini, 1993).

7.4.2.2.2 Origin of S. cerevisiae

Since the time of Pasteur, it has been claimed that *S. cerevisiae* wine strains colonize the grapes and are introduced into must when grapes are crushed (Vaughan-Martini and Martini, 1995). Hansen formulated the idea that *S. cerevisiae* survives during winter in vineyard soil fallen to the ground with ripe grapes, and colonizes grapes again next summer by insect vectors. However, later studies have asserted that *S. cerevisiae* is very infrequent on grapes, leading to claims that it is introduced into must from winery equipment and from the walls of fermentation tanks (Belin, 1979; Cuinier, 1980; Rosini et al., 1982; Martini and Martini, 1990; Martini, 1993).

Applying modern analytical methods and molecular techniques, it became possible to detect the sources and follow the evolution and distribution of *Saccharomyces* strains. Bouix et al. (1981) used electrophoresis of extracellular fractions to distinguish yeast strains. Electrophoretic profiles of strains isolated from the equipments of harvesting and pressing were identical to those found in the alcoholic fermentation. Frezier and Dubourdieu (1992) applied pulsed-field electrophoresis for chromosome karyotyping and mitochondrial DNA restriction enzyme profiles to analyze dominating *S. cerevisiae* strains. Among 260 strains, they found a single stable strain that dominated all fermentations in two consecutive years in a winery. Guillamón et al. (1993a) could distinguish between yeast strains originating from different ecological and geographical regions based on differences between the electrophoretic whole-cell protein patterns; however, they did not find differences between strains from the same cellar.

On the contrary, using a primer from the 2-μm plasmid for PCR-amplification and Chinese hamster embryonal fibroblast (CHEF) karyotyping, Török et al. (1996) demonstrated that *S. cerevisiae*

does occur on grapes, although in very low population (usually less than 0.1% of the resident yeast biota). Mortimer and Polsinelli (1999) also observed that damaged grape berries are rich depositories of *S. cerevisiae* and may serve as inocula of must. Contrary to this, Comitini and Ciani (2006) demonstrated that inoculated *S. cerevisiae* strain persisted, but barely, on both damaged and undamaged grapes. It appears that *S. cerevisiae* may originate from the vineyard, and a few days after the beginning of harvest, it colonizes the winery. In any case, this resident population contributes primarily to the spontaneous fermentation of must (Ciani et al., 2004). Data obtained with pulsed-field gel electrophoresis have also provided evidence for the winery source of *S. cerevisiae*, some strains of which are becoming predominant over consecutive years (Vezinhet et al., 1992; Briones et al., 1996). Querol et al. (1992) showed high similarity in mtDNA patterns in one winery where a single strain of *S. cerevisiae* had become predominant in successive years. A small number of *S. cerevisiae* strains dominated the fermentation in all vats in the same winery independent of grape cultivar and the time of harvest (Frezier and Dubourdieu, 1992). However, data on the geographic diversity of wine yeasts are conflicting. Versavaud et al. (1995) found no correlation between the genetic relatedness and the locality of wineries. The finding that predominant strains were observed over consecutive years in the same cellars has been interpreted as a consequence of their prevalence in the indigenous microbiota present on grapes. On the contrary, Guillamón et al. (1996) established close relationships between strains from the same regions and also by type of wine (white or red). Cappello et al. (2004) observed, however, that *S. cerevisiae* strains were representative of the vineyard area rather than of the variety of grapes. Although wineries could harbor a dominant strain for a while, this dominance appears temporary; hence, the idea of the existence of specific "territorial" yeast does not seem valid (Dequin et al., 2003). In a recent publication (Sturm et al., 2006), yeast populations were studied using the same grape material picked and crushed aseptically directly in the vineyard, and after various crushing, pressing, and maceration processes in the winery. The diversity of yeasts was much greater in the latter cases. *Hsp. uvarum* and *Met. pulcherrima* were common in all cases; however, no *Candida*, *Pichia*, and *Issatchenkia* isolates occurred in the vineyard-fermented sample, in which, on the other hand, *S. cerevisiae* was abundant, suggesting its origin from natural habitats. Other *Saccharomyces* strains (*S. bayanus* or *S. pastorianus*) occurred only in winery fermentations. Intensive processing of grapes increased the initial population of yeasts.

7.4.2.2.3 Succession of Yeasts during Fermentation

Grape must provides a strongly selective milieu for yeasts and other microorganisms. The high sugar content (15–30%), low pH (2.9–3.7), addition of sulfites (40–80 mg L^{-1}), and anaerobiotic conditions are limiting ecological factors that influence the growth and survival of microorganisms. Under these conditions, strong competition develops between them, and the properties of yeasts fit the environment better than most molds and bacteria.

Among yeasts, in particular, *S. cerevisiae* proliferates best due to its high tolerance to ethanol (Figure 7.3). The growth of the non-*Saccharomyces* "wild" yeasts is limited to the first 2–4 days of fermentation, until the ethanol concentration reaches 3–6% (v/v), after which they die off (Fleet et al., 1984; Coratza et al., 1992; Fleet and Heard, 1993; Pina et al., 2004). In addition to the increasing ethanol concentration, lack of oxygen also causes the death of non-*Saccharomyces* yeasts (Holm Hansen et al., 2001). Table 7.10 outlines the succession of yeast species as wine fermentation progresses.

This general sequence does not change substantially, and studies carried out in different parts of the world revealed the same general pattern (Ethiraj et al., 1979; Vojtekova and Minarik, 1985; Heard and Fleet, 1986a; Ruiz et al., 1986; Holloway et al., 1990). However, various factors may affect and modify the natural succession of species during fermentation. As the temperature of fermentation increases above 25°C, *Kazach. exiguus* and *Zygo. bailii* become dominating (Mauricio et al., 1986), and if the temperature decreases to 10°C, the growth and survival of non-*Saccharomyces* yeasts may greatly increase (Heard and Fleet, 1988). Some strains of *C. stellata* or *Klc. apiculata* may occur with higher tolerance to ethanol. Addition of sulfur dioxide brings about a rapid decrease in the total

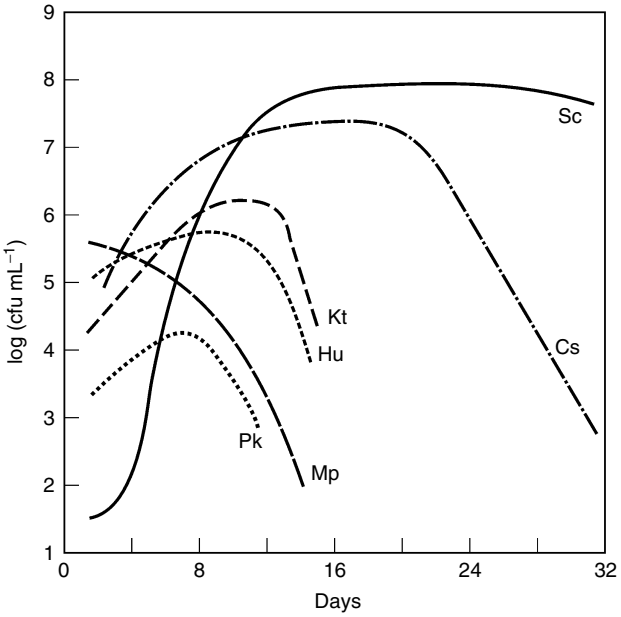

FIGURE 7.3 Composite picture in population changes of various yeast species during spontaneous fermentation of wine. Sc: *Saccharomyces cerevisiae*; Cs: *Candida stellata*; Kt: *Kluyveromyces thermotolerans*; Hu: *Hanseniaspora uvarum*; Mp: *Metchnikowia pulcherrima*; Pk: *Pichia kluyveri*. Data from Fleet, G. H. (1990c) J. Wine Res. 1:211–223; Ciani, M. and Picciotti, G. (1995). Biotechnol. Lett. 17:1247–1250; Lema, C., Garcia-Jares, C., Orriols, L., and Angulo, L. (1996) Am. J. Enol. Vitic. 47:206–216; Cocolin, L., Bisson, L. F., and Mills, D. A. (2000) FEMS Microbiol. Lett. 189:81–87; and Mills, D. A., Johannsen, E. A., and Cocolin, L. (2002) Appl. Environ. Microbiol. 68:4884–4893.

TABLE 7.10
Succession of Yeast Species on Grapes, in Must and Wine

Sources	Yeasts
Where grape must is formed: soil, air, and fresh water	Species with predominant oxidative metabolism; *Saccharomyces* wine yeasts absent
Grape berries	*Kloeckera apiculata*, *Candida stellata*, *Metschnikowia pulcherrima* predominate, *Saccharomyces cerevisiae* absent
Harvesting of grapes: hands of pickers, buckets, and crates	Non-*Saccharomyces* yeast predominates; *Saccharomyces cerevisiae* occurs in low numbers
Fermenting must	*Kloeckera apiculata* initiates fermentation, followed by *Saccharomyces cerevisiae* that terminates the process
Vinery plant: walls, floor, equipment, and various surfaces	*Saccharomyces cerevisiae* predominates; film-forming yeasts are consistently present

population of yeasts and reduces wild yeast species for the most. *S'codes ludwigii* was, however, isolated in high frequencies from sulfited must (Minarik and Navara, 1977; Goto, 1980).

Application of sensitive molecular typing techniques has demonstrated that, during spontaneous fermentation, several different strains of *S. cerevisiae* occur simultaneously, and these can also follow one another successively in the course of fermentation, until one or few become predominant toward the end of fermentation (Frezier and Dubourdieu, 1992; Querol et al., 1992, 1994; Versavaud et al., 1995). The situation becomes more complex when starter strains of *S. cerevisiae* are used as inocula (Heard and Fleet, 1985).

7.4.2.2.4 Inoculated Fermentation

Selected pure strains of yeast are increasingly used to control the course of fermentation. These yeasts have been collected from specific wine-growing regions of the world, purified, characterized, and maintained in culture collections to be supplied for use to wineries. The pure strains have to be scaled up by repeated transfer and propagation. Active dry wine yeast, which can be used in bulk with less or no previous cultivation, has also become commercially available and has excellent storage stability (Reed and Nagodawithana, 1988; Querol et al., 1992). Selected wine yeast strains have specific properties of practical importance, such as tolerance to ethanol, cold, SO_2, sugar concentration, and so on (Zambonelli et al., 1989; Malacrino et al., 2005; Zuzuarregui et al., 2005). It is claimed that their use offers advantages for the production of large and uniform quantities of wine of desired quality.

Inoculated strains will sooner or later replace native yeasts that, however, may survive and contribute to fermentation (Querol et al., 1992; Schütz and Gafner, 1993). A commercial wine strain can establish itself in the winery, becoming predominant, and can continue to be found after several years (Vezinhet et al., 1992; Schütz and Gafner, 1994; Beltran et al., 2002; Santamaria et al., 2005). Large-scale studies over the years have shown, however, that the reverse does not hold; commercial starters did not disseminate from the winery to the vineyard (van der Westhuizen et al., 2000; Valero et al., 2005).

However, there are still opposing views about the application of pure wine yeasts. Indigenous yeasts naturally present in the must may significantly contribute to the fermentation. Heard and Fleet (1985) and Holloway et al. (1990) showed that wild yeasts (*Hsp. uvarum*, *C. stellata*, *Met. pulcherrima*) persisted throughout the fermentation. It is a general belief that fermentations carried out with selected wine yeasts result in wines of lower level of volatile acids and esters and meager organoleptic quality. Wild yeasts, particularly *Hanseniaspora* (*Kloeckera*) species, play an important role in the production of volatile compounds (Holloway et al., 1990; Mateo et al., 1991). On the other hand, if not controlled, indigenous wild yeasts can negatively affect the quality of wine (Holloway and Subden, 1991).

Inoculation can help overcome slow or incomplete (stuck or sluggish) fermentation. Active ready-to-use dry yeasts are recommended to overcome these problems (Kunkee, 1984; Radler and Lotz, 1990). The reasons for difficulties may lie in grape growing or processing (Ingledew and Kunkee, 1985; Strehaiano, 1993), and perhaps the appearance of killer yeasts producing toxins that can destroy wine yeasts. The production of killer toxins occurs primarily among *S. cerevisiae* strains. In laboratory cultures *Pichia*, *Candida*, and *Hanseniaspora* species are also capable of producing killer toxins; such strains were absent in non-*Saccharomyces* species associated with wine (Gutiérez et al., 2001). In the selection of starter strains, resistance to killer toxins is an advantageous feature to be considered.

7.4.2.3 Classification of Wine Yeast

Throughout the previous discussion, the genuine wine yeast has been named *S. cerevisiae.* In most cases, this is true. It is an overall evidence of studies that the species *S. cerevisiae* is the principal and universal wine yeast. *S. bayanus* contributes to a lesser extent in the production of wine, especially in colder climates.

However, as discussed earlier (Section 2.4.1), the taxonomic situation of *S. bayanus* is confused (Sipiczki, 2002). As a member of the *Saccharomyces sensu stricto* group, *S. bayanus* is close to *S. cerevisiae*, but unlike *S. cerevisiae* it is unable to utilize galactose. There are, however, several strains, called *S. uvarum*, that are galactose positive, but considered synonymous with *S. bayanus*. Most of these also ferment melibiose and galactose, and differ from *S. cerevisiae* only in not growing at 37°C. On the basis of molecular evidence, *S. uvarum* has been reestablished recently as a separate species (Nguyen et al., 2000). Reinvestigation of a number of strains isolated from wine, cider, and apple juice showed that these are identical to *S. uvarum*, whereas strains of *S. bayanus* can be

considered partial hybrids between *S. uvarum* and *S. cerevisiae* (Dequin et al., 2003). Thus wine yeasts either belong to *S. cerevisiae* or *S. uvarum* (Demuyter et al., 2004).

In contrast to the domesticated species *S. cerevisiae* and *S. uvarum*, and the hybrids *S. bayanus* and *S. pastorianus*, strains isolated from natural sources have been identified as *S. paradoxus*, considered the natural parent of other species in the *Saccharomyces sensu stricto* group. Hence, several strains that by physiological, biochemical, and molecular tests proved identical to *S. paradoxus* (Redzepovic et al., 2002) were unexpectedly isolated from a Chardonnay grape vineyard. Apparently, this species has a wider ecological niche than previously known.

7.4.2.4 Impact of Yeasts on Wine

The value of wine is attributed to its sensory quality, an extremely complex property determined by a thousand different compounds that are partly derived from grapes and partly produced by yeasts. Both the primary and secondary products of yeast metabolism contribute to the "bouquet" of wine, and include ethanol, glycerol, higher alcohols, organic acids, esters, aldehydes, ketones, amines, and sulfur compounds. In addition, yeasts also enhance the sensory properties of wine by the transformation of precursors originating from grapes. The physiological and biochemical background of sensory attributes of wine has been the subject of extensive studies, details of which are beyond the scope of this chapter; for a comprehensive treatment see other recent publications (Fleet, 1997; Lambrechts and Pretorius, 2000; Dequin et al., 2003; Romano et al., 2006).

Ethanol and glycerol are among the primary products of yeast fermentation, and they determine the body of wine. Among the most important volatile secondary products are the higher alcohols, the acetate esters of which impart pleasant aroma and flavor. Acetoin and phenolic compounds are also important to the taste, odor, and color of wines. Compared with desirable metabolic products, acetic and other acids, diacetyl, aldehydes, and reaction products with sulfur compounds are mainly responsible for the off-flavor (Romano et al., 1997).

For our discussion in this chapter, the points of interest are the species of yeasts and the degree to which they are responsible for the production of favorable and unfavorable sensory determinants. The contribution of *S. cerevisiae*, the main wine yeast, is mostly positive; however, great differences exist between strains. It is a general belief that fermentations carried out with selected wine yeasts result in a wine with a lower level of volatile acids and esters and of meager organoleptic quality. Wild yeasts, particularly several strains belonging to *Hsp. uvarum* and *Hsp. guilliermondii*, have been observed producing pleasant aromas; other species such as *Hsp. osmophila* are considered detrimental for fermentation by-products of acetate, acetaldehyde, and acetoin (Granchi et al., 2002).

Yeast other than *S. cerevisiae* can be used to develop special aromas of wine (Clemente-Jimenez et al., 2004). *Lachancea* (*Klu.*) *thermotolerans* inoculated into grape juice predominated only for 5 days, and was exceeded by *S. cerevisiae* before the fermentation was completed (Mora et al., 1990). *Schizosaccharomyces* species are unusual in that they are able to convert malic acid into ethanol (Taillandier et al., 1988; Redzepovic et al., 2003). This differs from malolactic fermentation carried out by LAB, specifically *Oenococcus oenos*, leading to decarboxylation of malate to lactic acid. This can be advantageous in decreasing acidity and imparting a softer taste to wine, and is often encouraged in red wine. Malolactic fermentation can also be deleterious in less acidic wine and occurs in bottled wine (Henick-Kling, 1993).

7.4.2.5 Spoilage Yeasts

Spoilage yeasts result from the grapes themselves, during fermentation, and after fermentation (Table 7.11). Grapes in the vineyard can rot due to molds (e.g., *Botrytis*) and may be attacked by bacteria such as *Acetobacter* and *Gluconobacter*. Of the spoilage yeasts, grape berries are the primary source of *Dek.* (*Bret.*) *bruxellensis* (Renouf and Lonvaud-Funel, 2007).

TABLE 7.11
Yeasts Frequently Isolated from Wines

Species	Sources	References
C. boidinii	Wine, cider	18
C. cantarelli	Wine, sherry	44, 49
C. ethanolica	Wine	50
C. glabrata	Must, wine	7, 18
C. inconspicua	Must, wine	7, 18, 36
C. norvegica	Cider, winery	3, 31
C. parapsilosis	Must, wine, palm sap	1, 18, 19, 36
C. rugosa	Wine, sake,	3, 18
C. sake	Must, wine, winery	5, 6, 7, 18, 19, 30
C. sorbosa	Must, winery	2, 47
C. stellata	Must, wine, sherry	2, 10, 11, 13, 14, 15, 16, 18, 19, 24, 41, 42, 49
C. tropicalis	Wine, palm sap	1, 7, 18,
C. vini	Wine, winery	7, 18, 19, 23, 25, 39
C. wickerhamii	Wine	44
C. zeylanoides	Wine	7, 18, 23
Cry. laurentii	Grape, must, wine, winery	5, 11, 18, 33
Db. etchellsii	Wine, palm sap	1, 7, 18
Db. hansenii	Must, wine, winery,	2, 5, 7, 18, 19, 36, 42, 44
Dek. anomala	Wine, cider,	4, 32
Dek. bruxellensis	Grape, wine, winery, cider, sherry	4, 12, 18, 19, 25, 31, 35, 49
Hsp. guilliermondii	Must, wine, winery	6, 18, 19, 42
Hsp. osmophila	Wine	45, 46, 48
Hsp. uvarum	Grape,must, wine, winery, cider,	2, 6, 10, 11, 13, 16, 19, 33, 36, 39, 41, 42
Iss. orientalis	Must, wine	6, 11, 18, 19, 25, 27, 41
Iss. terricola	Must	2, 41
Kazach. exiguus	Must, wine	18, 19, 21, 31, 40
Klu. marxianus	Must, wine	18, 36
Lachancea fermentati	Sherry	49
Lachancea kluyveri	Wine	7, 18, 19
Lachancea thermotolerans	Wine	40, 46, 48
Met. pulcherrima	Grape, must, wine, winery, cider	2, 4, 6, 13, 14, 18, 19, 33, 40, 41, 48
P. anomala	Must, winery, sherry	6, 7, 14, 18, 19, 23, 31, 48, 49
P. farinosa	Wine, palm sap	1, 7, 18
P. fermentans	Must, wine,	7, 18, 19, 23, 41
P. guilliermondii	Wine, winery, cider, palm sap	1, 12, 18, 19, 25, 44
P. jadinii	Must, wine	7, 18, 19
P. kluyveri	Grape, wine	33, 46
P. membranifaciens	Must, wine, cider, sherry	4, 6, 7, 18, 19, 23, 28, 30, 40, 48, 49
P. subpelliculosa	Must, winery	5, 18, 33
Rho. aurantiaca	Grape	33
Rho. glutinis	Grape, must, winery, beer, palm sap	1, 5, 6, 18, 19, 33, 36
Rho. mucilaginosa	Wine, winery	5, 6, 7, 18, 19
S. bayanus	Must, wine	8, 9, 17, 26
S. cerevisiae	Must, wine, cider, sake, palm sap	1, 7, 13, 14, 16, 20, 21, 24, 30, 36, 39, 41
S. uvarum	Wine	43
S. paradoxus	Wust	34
S'copsis fibuligera	Wine	7, 18
S'codes ludwigii	Must, wine, cider, palm sap	1, 4, 11, 13, 18, 19, 37
Schizo. pombe	Must, wine, palm wine	1, 8, 18, 37, 48
Spb. roseus	Grape	33
Tsp. delbrueckii	Must, wine, winery	5, 7, 14, 19, 21, 29, 36, 42
Zygo. bailii	Must, wine, cider, palm sap	1, 4, 7, 18, 19, 21, 22, 23, 24, 39, 48
Zygoascus hellenicus	Must	2

Notes: 1: Atputharajah et al. (1986); 2: Beltran et al. (2002); 3: Barnett et al. (1983); 4: Beech and Carr (1977); 5: Belin (1979); 6: Cuinier (1980); 7: Deák (1988); 8: Delfini (1985); 9: Farris and Rugin (1985); 10: Fleet et al. (1984); 11: Goto (1980); 12: Dias et al. (2003); 13: Heard and Fleet (1986b); 14: Heard and Fleet (1986a); 15: Holloway et al. (1992); 16: Khayyat et al. (1982); 17: Kobatake and Kurata (1980b); 18: Kunkee and Goswell (1977); 19: Lafon-Lafourcade (1983); 20: Martini (1993); 21: Mauricio et al. (1986); 22: Minarik (1980); 23: Minarik (1981); 24: Minarik (1983); 25: Martorell et al. (2006); 26: Molina et al. (1992); 27: Moline (1984); 28: Mora and Rosello (1992); 29: Muys (1971); 30: Parish and Carrol (1985); 31: Put et al. (1976); 32: Rosini et al. (1982); 33: Raspor et al. (2006); 34: Redzepovic et al. (2002); 35: Renouf and Lonvaud-Funel (2007); 36: Ruiz et al. (1986); 37: Suarez and Inigo (1982); 39: Vojteková and Minarik (1985); 40: Nurgel et al. (2005); 41: Clemente-Jimenez et al. (2004); 42: Pina et al. (2004); 43: Demuyter et al. (2004); 44: Dias et al. (2003); 45: Granchi et al. (2002); 46: Mills et al. (2002); 47: Sabate et al. (2002); 48: Rojas et al. (2001); 49: Esteve-Zarzoso et al. (2001); 50: Cocolin et al. (2001).

Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

By-products of yeast metabolism during fermentation may cause sensory defects as mentioned above. Both indigenous *Saccharomyces* strains and non-*Saccharomyces* wild yeasts can produce off-flavors (Fleet, 1992; Sponholz, 1993; Boulton et al., 1995; du Toit and Pretorius, 2000). Some strains of *S. cerevisiae* produce hydrogen sulfide far above the flavor threshold. High concentrations of acetaldehyde can be achieved by several *Candida* species (*C. vini*, *C. stellata*, and *C. krusei*) and *S'codes ludwigii*, *P. anomala*, and other *Pichia* species. *Hanseniaspora* (*Kloeckera*) species are also responsible for high levels of acetic acid and its esters. *Dekkera* (*Brettanomyces*) species also produce high amounts of acetic acid, but their most objectionable metabolites are volatile phenols causing mousy off-flavor (Martorell et al., 2006). Another type of yeast capable of producing ethyl phenols in high yields has been identified using *P. guilliermondii* (Dias et al., 2003).

On completion of fermentation, any further yeast activity is harmful to the quality of wine (Sponholz, 1993; Thomas, 1993; Boulton et al., 1995). The majority of yeasts are removed from wine by racking, filtration, and other cellar procedures. If the wine is kept in bulk, spoilage may be caused by *Dekkera* species and *S'codes ludwigii*, both of which impart flavor to wine (Goswell, 1986b). The major spoilage yeast in wine stored in tanks or barrels are film-forming species such as *C. vini*, *C. zeylanoides*, *C. rugosa*, *Iss. orientalis*, and *P. membranifaciens* (Minarik, 1981).

Growth of yeast in bottled wine is unacceptable because it results in haze, cloudiness, sediment, and poor flavor. Winery production lines are often contaminated by various yeasts that can, in turn, contaminate the bottled wine. Common spoilage yeasts in bottled wine include *Zygo. bailii*, *S. cerevisiae*, *C. rugosa*, *P. membranifaciens*, and *C. vini* (Minarik et al., 1983). Many studies have been carried out to characterize the spoilage potential of these yeasts and to assess the minimum acceptable number of yeasts in wine. A major factor inhibiting yeast growth is ethanol content. Wild and wine yeasts are usually inhibited by 12% (v/v) ethanol, but *Zygo. bailii* and some *S. cerevisiae* strains are more tolerant and may cause refermentation of residual sugars, turbidity, and sediment. Moreover, *Zygo. bailii* has an exceptionally high resistance to food preservatives such as sorbate and benzoate (Thomas and Davenport, 1985). *Zygo. bailii* contamination originates partly from habitats in the winery (Minarik, 1983), but mainly from concentrated grape juice used in wine production (Minarik and Hanicova, 1982). This species is considered to be the main cause of serious spoilage of bottled wine (Minarik, 1980). Only a few viable cells in a bottle of wine may be sufficient to cause spoilage (Deák and Reichart, 1986; Andrews, 1992).

7.4.2.6 Specialty Wines

The bulk of wine produced is consumed as table wine, several hundred varieties of which exist. Certain unique types of wine are made by special and additional processes that create distinctive high-quality products. Among these, sparkling, fortified, and botrytized wines are mentioned here. In addition, other wine-type alcoholic beverages are produced in different regions all over the world; these will be treated in a separate section.

Sparkling wines are produced by secondary fermentation in bottles or closed tanks in order to saturate wine with carbon dioxide. By the traditional processing methods of Champagne, carbonation is produced in bottles stored for several months, resulting in a premium-quality product. Pressurized tanks are used for the bulk production of sparkling wine bottled after secondary fermentation. For producing sparkling wine, special strains of *S. cerevisiae* are required that are able to carry out fermentation of added sugar in base wine of high ethanol concentration (10–12% v/v), at low temperature (10–15°C), and under increasing pressure of CO_2 (up to 600 kPa). Eventually, many yeast cells die, and the materials released after autolysis contribute to the aroma and flavor of sparkling wine (Gonzalez et al., 2003).

A special type of wine, sherry, can be produced by holding the wine under a film of yeast (flor) consisting of special biotypes of *S. cerevisiae* (Farris and Rugin, 1985; Goswell, 1986a; Sancho et al., 1986; Charpentier et al., 2004; Esteve-Zarzoso et al., 2004). Flor yeasts are known to have high resistance to ethanol, acetaldehyde, and oxidative stresses (Esteve-Zarzoso et al., 2001;

Aranda et al., 2002). The production of sherry-type wine involves two successive processes, an alcoholic fermentation and a maturation of the wine affected by flor yeasts. The latter are special strains capable of producing wine with higher volatile acidity and high acetaldehyde concentration (Guijo et al., 1986). Before maturation, the base wine is fortified to 15–22% ethanol by the addition of wine distillate. Port-type wines are also prepared by fortification and aging without flor and secondary fermentation.

The mold *Botrytis cinerea* is usually responsible for rotting the grapes. Under certain climatic and cultivation conditions, this rotting is restrained (noble rot), leading to dehydration of grape berries with increased acidity and sugar content (30–40%). Fermentation of must crushed from such grapes results in wine of distinctive "botrytized" character (Sauternes and Tokay types), rich in gluconic acid, keto acids, and specific aroma components (Doneche, 1993). Intensive studies made in the Tokaj wine district, Hungary (Naumov et al., 2002; Bene and Magyar, 2004; Magyar and Bene, 2006), demonstrated prevalence of *Met. pulcherrima* on botrytized grapes, and *C. stellata* and *S. bayanus*/*S. uvarum* in fermenting wine produced from them. Molecular analysis detected among suspected *C. stellata* isolates a novel species, *C. zemplinina* (Sipiczki, 2003). These species are phenotypically very close; similar isolates may occur in Californian botrytized must (Mills et al., 2002).

7.4.2.7 Future Trends in Wine Microbiology

Two main trends are outlined here. One concerns the improvement of wine yeast, *S. cerevisiae*. Commercial starter strains are now commonly used to ensure uniform product and to reduce the risk of spoilage. Classic genetic modification and recent molecular methods are means to increase desirable properties and decrease undesirable ones (Pretorius and Westhuizen, 1991; Querol and Ramón, 1996).

Improvement of technologically important properties in wine making can target, among other features, ethanol tolerance, fermentation capacity, and absence of off-flavors (Table 7.12). These targets have been achieved with some success by the application of classic genetic techniques such as mutagenesis and hybridization, followed by selection, and more recently, protoplast fusion and cytoduction. However, a major limitation of these classical genetic techniques has been in general the difficulty of adding or removing one feature without altering gross performance. In particular, the

TABLE 7.12
Some Desirable and Undesirable Features of Wine Yeasts (Fleet, 1997)

Desirable	Undesirable
High tolerance of alcohol	Production of sulfur dioxide
Complete and rapid fermentation of sugars	Production of hydrogen sulfide
Resistance to sulfur dioxide	Production of volatile acidity
Fermentation at low temperature	High formation of acetaldehyde and esters
Malic acid degradation	Foaming properties
Ferment under pressure	Formation of ethyl carbamate precursors
Production of glycerol	Production of polyphenol oxidase
Production of β-glucosidases	Inhibition of malolactic fermentation
Killer phenomenon	
Good sedimentation	
Production of good flavor and aroma	
Suitability of mass culture and freeze-drying	

Source: Fleet, G. H. (1997) *In*: Food Microbiology: Fundamentals and Frontiers (eds. Doyle, M. P., Beuchat, L. R., and Montville, T. J.). ASM Press, Washington, DC. pp. 671–694.

stable genetic constitution of polyploid/aneuploid industrial strains, lack of mating-type characteristics, and poor sporulation all restricted the possibilities of broad strain improvement. The potential of recombinant gene technology (genetic engineering) has provided more possibilities, and holds much promise for specific modifications. It has resulted in many improved strains, genetically modified at laboratory scale (Pretorius, 2000; Schuller and Casal, 2005; Verstrepen et al., 2006). However, lack of legal approval and resistance to public acceptance have prevented commercial application. The recent appearance on the U.S. market of recombinant wine yeast capable of malolactic fermentation may signal a breakthrough in this respect (Cummins, 2005).

Another direction of interest results from the increasing realization of the role of non-*Saccharomyces* yeasts in the development of the sensory properties of wine. Several species have properties absent from or moderated in *S. cerevisiae*. The use of these species as adjunct cultures may endow wines with unique flavor and character. *C. stellata* has been proposed for use in combination with *S. cerevisiae* cultures (Ciani and Comitini, 2006). When immobilized in high cell concentrations, it improved fermentation at 16°C.

P. fermentans, *C. cantarellii*, and *Klc. apiculata* have been tested in sequential inoculation followed by *S. cerevisiae* and have been shown to increase the flavor composition of wine (Zironi et al., 1993; Toro and Vázquez, 2002; Clemente-Jimenez, 2005). However, apiculate yeasts in mixed culture can increase levels of some sulfur compounds (Moreira et al., 2005). Zohre and Erten (2002) have not observed undesirable levels of volatile compounds in mixed cultures of *Klc. apiculata*, *C. pulcherrima*, and *S. cerevisiae*. Multistarter fermentations of *Hsp. uvarum*, *Tsp. delbrueckii*, and *Lachancea thermotolerans* together with *S. cerevisiae* resulted in analytical profiles comparable to or better than those exhibited by a pure culture of *S. cerevisiae* (Ciani et al., 2006). However, the persistence of non-*Saccharomyces* yeasts at low temperature or high ethanol content can cause stuck fermentation.

7.4.3 Other Alcoholic Beverages

Many alcoholic beverages, mainly regional types, are fermented from fruit juices other than grape must or brewed from grains other than barley. They are called by various names such as sake, ginger, cider, toddy, pulque, and so on. These have been reviewed by Rose (1977), Stewart (1987), and Ng (2004).

7.4.3.1 Beer-Type Beverages

Special beer-type beverages fermented from starch-containing materials were mentioned briefly in Section 7.4.1.5.

Sake is the traditional alcoholic beverage in Japan prepared from a steamed rice mass (moromi) digested first by molds (koji) then fermented by yeasts (Onishi, 1990; Kodama, 1993). The starter (moto) contains a special variety of *S. cerevisiae*, which differs from brewer, wine, distillery, and other industrially used *S. cerevisiae* strains in that it is capable of high (20% v/v) alcohol production at low (15°C) temperature and forms important flavor compounds such as isoamyl acetate (Nunokawa, 1985).

Boiled rice is also used for Indian and Chinese beers saccharified by *Rhizopus*, *Mucor*, and *Aspergillus* molds, and fermented by yeasts. In some products, barley, wheat, or rice bean are also used (Ng, 2004). Sorghum beer (African Kaffir beer) has a sour flavor due to a mixed lactic acid and alcoholic fermentation. The brewing of West African pito uses LAB as well as molds (*Penicillium funiculosum*, *Penic. citrinum*, *Asp. flavus*, and *Rhizopus oryzae*) and yeasts. Amylases from germinated maize or sorghum grains and the molds hydrolyze starch before yeast fermentation starts (Sanni and Lönner, 1993). *Klc. apiculata*, *C. tropicalis*, *Tsp. delbrueckii*, *P. anomala*, *Klc. africanus*, and *Schizo. pombe* occur in the first days, and later *S. cerevisiae* can be isolated almost exclusively (Glover et al., 2005). About 28% of strains has been phenotypically different but still belong to

the *Saccharomyces sensu stricto* complex. Another African beer is Pombe, fermented from millet mainly by *Schizo. pombe*. Tape is a type of beverage brewed in the Far East and African countries from cassava flour and other cereals (Okagbue, 1990).

7.4.3.2 Wine-Type Beverages

A distinguishing feature of these beverages is that the raw materials, mostly various fruits, contain sugars that can be directly fermented by *S. cerevisiae* and other yeasts.

Cider is the fermented juice of apples produced in England and also in some West-European countries, the United States, and Australia. Beech and Carr (1977) and Beech (1993) comprehensively reviewed the role of yeasts in cider making. As in the fermentation of grape juice, yeasts of significance in cider making include weakly fermenting *Hsp. uvarum* and strongly fermenting *S. cerevisiae.* Apparently, however, *S. bayanus* (or *S. uvarum*) is the predominant species in cider (Naumov et al., 2001). In pressed fruit juice and in the early stages of fermentation, other yeasts such as *Met. pulcherrima*, *S'codes ludwigii*, and *Dekkera* species are usually present. If concentrated juice is used, *Zygo. bailii* and *Zygo. rouxii* may also occur (Morrissey et al., 2004; Coton et al., 2006; Suárez Valles et al., 2006). Film yeasts, for example, *P. membranifaciens*, can often be detected on the surface of cider stored in wooden vats. Storage vats of oak wood, on the other hand, positively contribute to the sensory profile of cider (Swaffield et al., 1997). Since cider making is based on spontaneous fermentation, properties may vary greatly according to prevailing ecological conditions (Mafart, 1986; Herrero et al., 2003). Under good manufacturing practices, most spoilage problems can be avoided. However, certain yeasts can still cause disorders, even in the best-managed factories. Carr (1984) pointed out that *Hsp. uvarum* imparts an off-flavor, and *S'codes ludwigii* causes spoilage of bottled cider. *Schizo. pombe* may decompose malic acid (Fournier et al., 1981). Patulin, which may be present in cider made from moldy apples, appears to be decomposed during alcoholic fermentation (Harwig et al., 1973; Stinson et al., 1978).

Swanson et al. (1985) demonstrated that heat-resistant aciduric microorganisms surviving 70°C for 15–30 min were present in apple juice and sweet cider processing environment. Among them were eight species of yeasts. Cabranes et al. (1990) described the dynamics of yeast population during cider fermentation in Asturia, Spain. The species most frequently isolated from fresh juice were *Hsp. uvarum* and *S. cerevisiae*. In contrast to grape wine fermentation, *Hsp. uvarum* remained through the late stages of cider production because of the low alcohol content of the product (Michel et al., 1988).

Several types of palm wines, generally referred to as toddy, are produced in subtropical and tropical regions. Atputharajah et al. (1986) demonstrated that *S. cerevisiae* dominated in coconut palm sap (toddy) fermentation. *P. ohmeri*, *P. guilliermondii*, *P. membranifaciens*, *Schizo. pombe*, *C. parapsilosis*, and *Hsp. occidentalis* were also frequently detected. Yamagata et al. (1980) isolated 925 yeast strains from coconut and nipa palm wine (tuba), 63% of which were *S. cerevisiae.* Oil palm wine has been fermented by the single species *S. cerevisiae*, followed by LAB, and later also acetobacters (Amoa-Awua et al., 2007). In addition to *S. cerevisiae*, a species similar to *C. versatilis* was isolated from Nigerian palm wine (Okagbue, 1988). *Hsp. uvarum* was identified in another study (Owuama and Saunders, 1990), whereas *Schizo. pombe* dominated in Indian palm wine fermentation (Shamala and Sreekantiah, 1988). Okafor (1978) stated that the types and numbers of microorganisms encountered in palm wine fermentation may vary widely with geographic area and palm tree species. In addition to yeasts, lactic- and acetic-acid-producing bacteria also play an important role in developing the traditional taste and flavor of palm wines.

Pulque is made in Mexico from agave juice fermented with a bacterium, *Zymomonas mobilis*, rather than yeasts. Cooked agave is fermented to produce distillates called mescal and tequila. The mixed natural fermentation involves a diverse yeast mycobiota with some characteristic species such as *Clsp. lusitaniae* and *Met. agavea*; however, *S. cerevisiae* soon becomes prevalent. When cooked agave molasses is used, *Schizo. pombe* and *Klu. marxianus* dominate (Lachance, 1995). Sugarcane juice is fermented on a large scale in Brazil, and used for the distillation of a traditional

spirit (aguardente). In juice fermentation, starter ferment is made first, in which *S. cerevisiae* and *Schizo. pombe* are predominant, and fresh sugarcane juice is added to this daily. During fermentation, a succession of yeasts proceeds in a course similar to that in wine fermentation (Pataro et al., 2000).

7.4.3.3 Distilled Spirits

Alcoholic fermentation by *S. cerevisiae* is the base for producing distilled spirits (Watson, 1993). Cereal grains such as malted and unmalted barley, corn, and rye serve as raw materials for the fermentation of whiskys. Scotch whisky is produced from malted barley, whereas Irish whisky is made from unmalted barley. Canadian whisky is made from corn, rye, and barley malt; the American-type whiskys are produced from either rye or corn (Bourbon) (Russel et al., 2003). These spirits must be aged for at least 3 years. Distilleries use specific strains of *S. cerevisiae*.

Rum is fermented from cane juice syrup or molasses, mostly by *S. cerevisiae*; however, for the fermentation of rums with a heavy aroma, *Schizo. pombe* is best suited (Bluhm, 1983). Bacteria (lactobacilli and propionibacteria) are also important in natural fermentation, giving rise to the flavor in the rum distillate (Fahrasmane and Ganou-Parfait, 1998).

Brandy is a distillate from wine, commonly fermented from grapes, but distillates can be made from various fermented fruits or leese (e.g., apple, peach, plum, apricot, and cashew). Cognac and Armagnac are famous brands distilled from wine; spirits from sugar (vodka) and various fruits (palinka, slivovic, etc.) have distinctive features and are important national traditions.

7.5 FERMENTED FOODS

Fermentation is one of the oldest forms of food preservation. Fermented milks, vegetables, and cereals have been consumed since the dawn of the history of human nutrition. As leftovers spontaneously underwent lactic acid and alcoholic fermentation humans acquired knowledge of artisanal production of foods and beverages, without knowing the role of microorganisms. Only by the mid-nineteenth century, with the pioneering work of Pasteur, did we start to understand the microbial nature of fermentation. Since the beginning of microbiology, yeasts have contributed to the development of fermentation into an industrial technology, and more recently to biotechnology (Barnett, 2003).

Fermented foods and beverages have been and still are the most important diets worldwide. Billions of tons are produced and consumed globally, made from cereals, vegetables, milk, and meats. Three main groups of microorganisms are involved in their production: yeasts, LAB, and molds (Campbell-Platt, 1994; Caplice and Fitzgerald, 1999). Throughout the world there are many different types of fermented foods processed from a range of raw materials. The multitude of products can be categorized according to the main ingredients, the consistency (solid products or beverages), the method and technology of production, and the fermenting organisms used, or even the geographic region. However, strict distinction is often difficult: many fermented products consist of various ingredients and result from mixed fermentation by molds, yeasts, and bacteria. In this respect, lager beer or yogurt are exceptional in that they are fermented by single organisms introduced deliberately (starter cultures), whereas in most other cases, a mixed association of fermenting microorganisms develops spontaneously. Cereal-, vegetable-, or meat-based foods are often of solid consistency, in contrast to alcoholic and nonalcoholic beverages. Breads, beers, and cheeses are produced worldwide, at an industrial scale, whereas a large variety of fermented products are made locally, at small scales. These are usually called traditional or indigenous fermented foods, and they are strongly linked to culture and tradition in rural communities. The most important alcoholic beverages are discussed in Section 7.4; leavened breads, fermented dairy, and meat products are the subjects of Sections 7.6, 7.7, and 7.8, respectively. Other fermented products are discussed in the following sections. LAB play a prominent role in the fermentation of most products; however, attention will be focused on those foods and beverages in which yeast contribution is substantial. Table 7.13 lists yeast species associated with fermented and acid-preserved foods.

TABLE 7.13
Yeasts Frequently Isolated from Fermented and Acid Preserved Foods

Species	Sources	References
Arxula adeninivorans	Coffee	30
C. boidinii	Olives	39
C. etchellsii	Pickles, olives, koji	6, 9, 19, 28
C. magnoliae	Pickles, olives, salads	3, 12, 28
C. parapsilosis	Pickles, cocoa, salads, soy sauce	5, 6, 9, 12, 19, 21, 22
C. rugosa	Pickles, olives	8, 10, 14
C. sake	Cocoa, salads, Oriental foods	12, 21, 23, 27
C. tropicalis	Brine, Oriental foods, cocoa	3, 22, 27, 41
C. versatilis	Pickles, koji	9, 19
Db. hansenii	Pickles, olives, salads, cocoa, koji	5, 9, 10, 21, 26, 27, 34, 37, 38
Db. etchellsii	Olives	39
Guehom. pullulans	Cocoa, coffee, Oriental foods, salads	12, 21, 23, 27
Hsp. uvarum	Coffee	31
Hsp. guilliermondii	Cocoa	32, 33, 41
Hyphop. burtonii	Silage, Oriental foods	7, 17, 19, 22
Iss. orientalis	Pickles, cocoa, coffee, Oriental foods	2, 8, 9, 21, 27, 28, 31, 32, 33
Kazach. exiguus	Brine, silage, cocoa, salads, Oriental foods	1, 4, 9, 17, 21
Kazach. servazzii	Pickles	34
Kazach. unisporus	Pickles	35
Naumovia dairensis	Silage, salads	4, 11, 16
P. anomala	Pickles, olives, Oriental foods, cocoa, coffee	3, 6, 7, 9, 10, 14, 19, 21, 27, 31, 39, 40
P. angusta	Pickles, silage, Oriental foods	6, 7, 17
P. guilliermondii	Pickles, brine, olives	3, 8, 10
P. kluyveri	Coffee, cocoa	31, 32
P. membranifaciens	Brine, cocoa, olives, salads	3, 4, 10, 14, 20, 21, 32, 33, 39
P. subpelliculosa	Pickles, Oriental foods	7, 9, 28
S. cerevisiae	Olives, cocoa, salads, Oriental foods	5, 10, 21, 22, 24, 26, 32, 33, 41
S'copsis fibuligera	Cocoa, coffee, ragi, Oriental foods	7, 21, 22, 30
Tsp. delbrueckii	Coffee, pickles, olives	31, 36, 38
Trisp. moniliforme	Fermented cocoa, Oriental foods, salads	5, 12, 21, 26
Ya. lipolytica	Olives, salads	12, 18, 20
Zygo. bailii	Cocoa, pickles, salads, vinegar	1, 2, 9, 13, 20, 21, 25, 28, 29
Zygo. rouxii	Pickles, koji, cocoa, Oriental foods	9, 13, 19, 21, 22
Zygoascus hellenicus	Olives	39

Notes: 1: Baumgart (1982); 2: Buchta et al. (1996); 3: Brackett (1987); 4: Brocklehurst and Lund (1985); 5: Cantoni and Comi (1988); 6: Comi et al. (1981a); 7: Cronk et al. (1977); 8: Deák (1988); 9: Garrido Fernandez et al. (1985); 10: Etchells et al. (1975); 11: Kirsop and Brocklehurst (1982); 12: Kobatake and Kurata (1980b); 13: Kurtzman (1990); 14: Marquina et al. (1992); 15: Middelhoven et al. (1990); 16: Middelhoven and Franzen (1986); 17: Moon and Ely (1979); 18: Muys (1971); 19: Noda et al. (1982); 20: Put et al. (1976); 21: Ravelomana et al. (1985); 22: Sakai et al. (1983); 23: Sandhu and Waraich (1984); 24: Sanchez et al. (1984); 25: Smittle and Flowers (1982); 26: Soni et al. (1986); 27: Venkatasubbaiah et al. (1985); 28: Walker and Ayres (1970); 29: Solieri et al. (2006); 30: Silva et al. (2000); 31: Masoud et al. (2004); 32: Jespersen et al. (2005); 33: Nielsen et al. (2005); 34: Tominaga (2004); 35: Savard et al. (2002); 36: Passos et al. (1997); 37: Tsapatsaris and Kotzekidou (2004); 38: Kotzekidou (1997); 39: Coton et al. (2006); 40: Arroyo López et al. (2006a); 41: Ardhana and Fleet (2003).

Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

Space limitations prevent an in-depth coverage of this varied subject here; comprehensive references are given in Steinkraus (1995), Wood (1998), and Hui et al. (2004).

7.5.1 Soy Products

Fermentation of soybeans is an ancient process that started thousands of years ago in China and spread to East Asia, and more recently to several western countries. The main product is soy sauce, which is made from soybeans and wheat in Japan (shoyu) and mostly soybean in China (sufu). The characteristic feature is the hydrolysis of starch and proteins by molds in a solid-state process known as koji technology. The cooked beans (and wheat grains when used) are inoculated with *Aspergillus oryzae* (mucoraceous molds are also used) and fermented in a high-salt brine together with LAB. In several months, a moldy mass develops (called moromi), to which soy yeast, *Zygo. rouxii*, is added, and alcoholic fermentation starts. During aging, specific flavor compounds develop. After pressing, the raw soy sauce is filtered, pasteurized, refined, and bottled (Hanya and Nakadai, 2003).

In koji, a diverse population of yeasts occurs, including 15–20 different species. The most important soy sauce yeasts are *Zygo. rouxii*, *Db. hansenii*, and *C. versatilis*, all tolerating the high salt concentrations (about 18%) in the brine. *Zygo. rouxii* is a primary producer of fusel alcohols and other aroma components in soy sauce (Jansen et al., 2003). Other yeasts, for example, *P. farinosa*, *P. anomala*, *Cry. albidus*, *C. tropicalis*, and *Hyphop. burtonii* occasionally play a role in the first stage of fermentation (Noda et al., 1982; Mizunoma, 1984; Sugiyama, 1984). Most of them die out in the moromi when the pH decreases and the ethanol content increases. The soy yeast itself subsides above 4% ethanol, and in matured moromi the most salt-tolerant yeasts, *C. versatilis* and *C. etchellsii*, predominate. Film-forming yeasts, for example, *Iss. orientalis*, *Db. hansenii*, and *P. subpelliculosa*, may develop on the surface of moromi, assimilating lactic acid and ethanol and producing undesirable flavors.

Miso, soybean paste, is generally made by a two-phase process (koji and moromi), similar to soy sauce. Unlike liquid soy sauce, miso is thicker, and its composition can be more varied, including rice, barley, and soybean in different ratios (Onishi, 1990).

Tempe is a major fermented soybean product in Indonesia, in which *Rhizopus* species play the role of saccharification (Nout and Kiers, 2005).

7.5.2 Olives

Fermentation and processing of olives is an important branch of agro-industry in the Mediterranean countries and also in California (Brenes, 2004). Two types of olive fermentation are practiced. The naturally ripe, black olives are directly brined in 6–10% salt solution when yeasts are mainly responsible for the fermentation with coexisting LAB. The Spanish-style green, not fully matured olives are first treated in 2% sodium hydroxide (lye) to eliminate bitterness, followed after washing with fermentation in which LAB predominates beside yeasts (Garrido-Fernandez et al., 1997).

A great diversity of yeasts are found in olives; 20 or more species can be detected immediately after brining, yet a few species become predominant, such as *P. membranifaciens*, *P. anomala*, *C. boidinii*, *Db. hansenii*, *Tsp. delbrueckii*, and *S. cerevisiae* (Marquina et al., 1992; Kotzekidou, 1997; Coton et al., 2006). Yeast growth may be responsible for softening and gas pocket formation, and the rise in pH that results from using lactic acid increases the possibility of other malodorous spoilage. Currently, olives are fermented spontaneously on an industrial scale, without the addition of lactic starters. Brine composition can be adjusted by the addition of acetate and/or lactate to control the ecological environment (Tsapatsaris and Kotzekidou, 2004).

Table olives are prepared from brined stocks after packing in 2–5% salt solution, and pasteurized. Chemical preservation can also be applied for both fresh and brined olives. Modeling studies have been made to find the combination of preservative factors in order to inhibit the most resistant yeasts, *P. anomala* and *Iss. occidentalis* (Arroyo López et al., 2006a,b).

7.5.3 Fermented Vegetables

Vegetables and certain fruits are often preserved by salting, acidifying, or by fermentation. The most commonly fermented vegetables in Europe and North America are cabbage, cucumbers, and olives, but a range of other vegetables can be preserved similarly (Fleming, 1982). The lactic acid fermentation takes place in a juice extracted by dry salt or in salt brine, and creates a highly selective environment in which only yeasts can compete with LAB. Yeast activity is common during vegetable fermentation; however, it sometimes leads to spoilage.

In general, two main groups of yeast play a role in fermentation. Some yeast develops in the brine and brings about alcoholic fermentation with desirable flavor by-products as long as fermentable sugars are available. When these are exhausted, other yeasts develop on the surface of brine during the postfermentation stage and oxidize the acids produced by bacterial fermentation. The composition of the yeast biota depends on the method of manufacturing the product. In salt stock pickles with high salt concentrations (up to 15%), the yeast population differs from that of vegetables prepared in low-salt brine (less than 5%). Extensive studies carried out in the 1970s defined the composition and succession of species in these fermentations (Pederson, 1971; Etchells et al., 1975; Fleming, 1982).

The principal successive species of fermentative yeasts in high-salt cucumber brines are *C. versatilis*, *C. lactis-condensii*, *P. subpelliculosa*, *Kazach. exiguus*, *Tsp. delbrueckii*, *Zygo. bailii*, *C. etchellsii*, and *P. anomala*. The major oxidative yeasts that form films on the surface are *Db. hansenii*, *P. ohmeri*, *Zygo. rouxii*, and *Iss. orientalis.* Several other fermentative and oxidative yeasts may be present in smaller numbers. The yeast biota of dill pickles prepared in low-salt brine consists predominantly of *Iss. orientalis* together with *P. jadinii*, *P. guilliermondii*, *P. fermentans*, and *C. rugosa* (Deák, 1988).

Shredded cabbage or other vegetables are mixed with dry salt. Mechanical pressure is applied to expel the juice rich in nutrients and sugars to support acid fermentation. The diversity of yeasts in fermenting sauerkraut and other vegetables is similar to that developing in low-salt cucumber (Figure 7.4). Gas development is tremendous in sauerkraut fermentation, produced by both bacteria

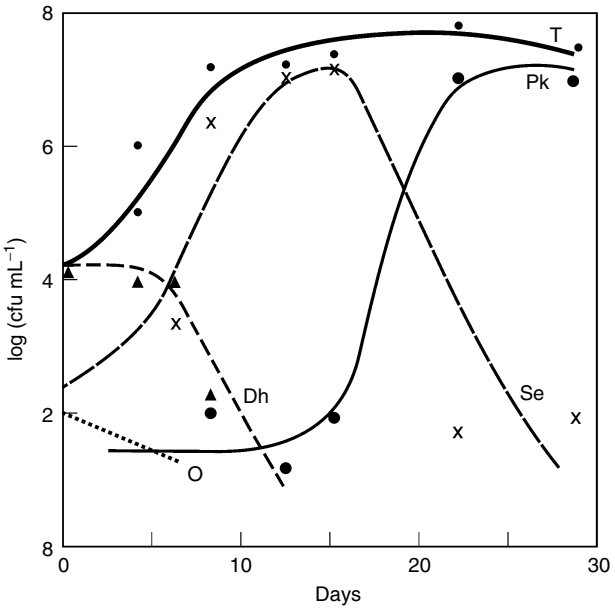

FIGURE 7.4 Population changes of yeasts in sauerkraut fermentation. T: total population; Se: *Saccharomyces* (*Kazachstania*) *exiguus*; Pk: *Pichia kluyveri*; Dh: *Debaryomyces hansenii*; O: other yeasts including *Geotrichum candidum*, *Pseudozyma aphidis*, and *Yarrowia lipolytica.*

and yeasts. In salt-preserved Japanese radish, *S. servazzii* and *Db. hansenii* have been shown to be the dominant yeast species (Tominaga, 2004).

Spoilage of fermented vegetables is often attributed to yeasts (Savard et al., 2002). Spoilage may occur in the form of softening due to the enzymatic breakdown of pectins or by bloating due to gas fermentation. Yeasts do not produce all types of pectinolytic enzymes, although many (e.g., *S. cerevisiae*, *Lachancea kluyveri*, and *P. anomala*) have pectinesterase and polygalacturonase activity that contribute to the breakdown of plant tissues. Oxidative yeasts may grow on the surface of brine where they utilize lactic acid, thus facilitating spoilage by other microorganisms (Etchells et al., 1975). Sorbic acid inhibits the growth of film-forming yeasts (Liewen and Marth, 1985). Gas production by fermentative yeasts is suspected to play a role in bloater spoilage of pickled cucumbers (Vaughn, 1985). However, Daeschel et al. (1985) demonstrated that yeasts do not enter the interior of fermenting cucumbers. In an effort to prevent bloater formation by fermentative yeasts, Daeschel et al. (1988) initiated fermentation by inoculating with mixed cultures of *Lactobacillus plantarum* and strains of *S. cerevisiae* or *Tsp. delbrueckii* that tolerate salt concentration. Starter culture development is most progressed for sauerkraut fermentation (Halász et al., 1999; Gardner et al., 2001; Plengvidhya et al., 2004). However, mixed cultures only ensure acceptable quality and result at best in more rapid pH decrease while the activity of indigenous microorganisms is not eliminated. The use of yeast as adjunct culture may help reduce excessive acidity levels and use up residual sugar, thus avoiding subsequent fermentation and bloater formation in cucumbers (Passos et al., 1997).

Some of the principles concerning fermented vegetables are also applicable to fermenting silage. Used for animal feed, silage consists of grass, green leaves, corn, and plant refuse resulting from lactic acid bacterial fermentation. This inhibits most spoilage bacteria; however, undesirable changes may result from alcoholic fermentation by yeasts. Engel (1986a) reported that the most frequently isolated yeast species in mixed silage were *P. fermentans*, *Iss. orientalis*, *S. cerevisiae*, and *Geo. candidum*, whereas *Trisp. cutaneum* and *P. membranifaciens* were detected less often. The dominant yeasts in corn silage, reported by Middelhoven and van Baalen (1988), are *P. fermentans*, *Iss. orientalis*, *Kazach. exiguous*, and *Naumovia dairensis.* Successively, *Deb. hansenii*, *P. anomala*, and *Geo. candidum* were also encountered in the oxidative breakdown of organic compounds (Middelhoven et al., 1990). The composition of silage may influence the mycobiota. If the vegetation contained mustard oils, nonfermentative yeasts predominated, such as *Db. hansenii*, *Trichomonascus ciferrii*, *Rho. mucilaginosa*, *Rho. rubra*, and *Trisp. cutaneum*. In wheat silage, the yeast biota consisted of *P. holstii*, *C. tenuis*, *P. canadensis*, and *S'copsis selenospora* (Moon and Ely, 1979). Most yeast species present in silages assimilate lactic and acetic acids, and this results in spoilage if the silage is exposed to air (Middelhoven and Franzen, 1986).

7.5.4 Coffee, Cocoa, and Tea

Both cocoa and coffee beans are surrounded by mucilage, pectinaceous pulp that has to be removed before drying and roasting the seeds. This mucilage results from spontaneous mixed microbial fermentation in which yeasts play a prominent role together with bacteria and molds. Several reviews are available on the subject (Lopez and Dimick, 1995; Fowler et al., 1998; Thompson et al., 2001; Schwan and Wheals, 2003, 2004).

The pulp around cocoa beans is rich in fermentable sugars and organic acids, especially citric acid, giving a pH 3.3–4.0, a favorable habitat for yeast growth (Ardhana and Fleet, 2003). Cocoa beans are fermented either in heaps or on trays. Ravelomanana et al. (1985) summarized the traditional cocoa fermentation processes in various cocoa-producing areas of the world. The yeast biota is similar, despite different climatic conditions and variations in processing technology. Yeasts reach high population (10^6–10^7 cfu g^{-1}) during fermentation. *Hsp. uvarum*, *P. membranifaciens*, and *Iss. orientalis* are the dominant species in most fermentation. In addition, *P. anomala*, *P. fermentans*, *P. kluyveri*, *S. cerevisiae*, and *Guehom. pullulans* have been detected frequently in a great diversity

of yeasts. The occurrence of some 30 other species appears to be a matter of chance (Sanchez et al., 1985; Jespersen et al., 2005; Nielsen et al., 2005). Several species with pectinolytic activity are involved in the degradation of the pulp, which can also be due to acid hydrolysis (Sanchez et al., 1984). Attempts have been made to use a cocktail inoculum of three to five principal species with pectinolytic activity involved in the degradation of the pulp to facilitate cocoa fermentation (Schwan, 1988; Schwan and Wheals, 2004).

Coffee can be processed by one of two methods, according to the plant type. Mucilage pulp from the cherries of *Coffea arabica* is removed mechanically, and fermentation in water takes place, followed by drying, whereas the thinner pulp of *Coffea robusta* can be dried directly as fermentation proceeds. The resulting green coffee beans from both methods are then roasted (Fowler et al., 1998).

The mixed fermentation is carried out by bacteria, molds, and yeasts. Yeast populations range from 10^4 to 10^7 cfu g^{-1} during fermentation. The predominant yeast detected are *P. kluyveri*, *P. anomala*, and *Hsp. uvarum*, with *Klu. marxianus*, *Tsp. delbrueckii*, *P. ohmeri*, *Iss. orientalis*, *C. pseudointermedia*, and *S. cerevisiae* occurring in lower counts (Masoud et al., 2004). Great diversity of yeasts has been associated with dry fermented coffee, too (Silva et al., 2000). About 24 different species have been observed, although those belonging to the genus *Pichia* make up 39% of isolates, and neither species dominates. Rather, two otherwise uncommon species, *Arxula adenivorans* and *S'copsis fibuligera*, have been frequently detected. No clear specific differences can be found between the types or regions of processing (Schwan and Wheals, 2003).

Processing of tea leaves is referred to as "fermentation"; in fact, physiological and biochemical changes, but no microbiological changes, take place during drying. However, the traditional Japanese kombucha tea is made by fermentation of sweetened tea with a "tea fungus," which is a symbiotic association including *Acetobacter xylinum* and yeasts (Greenwalt et al., 2000). Yeast species include *Schizo. pombe*, *P. fluxuum*, *S'codes ludwigii*, and others, forming ethanol that *Acetobacter* spp. then convert into acetic acid. Acetate is also produced by *Dek. bruxellensis* (Teoh et al., 2004). A new species, *Zygo. kombuchaenis*, has been described from kombucha tea (Stratford and James, 2003).

7.5.5 Indigenous Fermented Foods

Indigenous or traditional fermented foods constitute a major diet all over the world, and they are produced in particularly great variety in the developing countries of Asia, Africa, and South America. Their preparation still remains an artisanal art done in homes or by small cottage manufacturers, and few products (soy sauce is one) have evolved to commercial production scale (Section 7.5.1). Fermented foods and beverages may include cereals, rice, nuts, cassava, legumes, and other vegetables or fish. Depending on the composition and mode of preparation, molds, LAB, or yeasts dominate fermentation and dictate the character of the finished product. Often a mixed fermentation takes place in which the substrate is first fermented by molds, followed by a secondary lactic acid, alcoholic, or mixed fermentation. Yeasts often play an essential or secondary role in fermentations, or they may be spoilers of indigenous foods.

Though indigenous fermented foods have been produced worldwide for millennia, they have not received the scientific attention they deserve. It is only recently that microbiological changes associated with fermentation have been explored. However, many traditional ethnic foods have not yet been studied. The importance of these foods is increasingly recognized, and this is shown by the growing number of treatises on the subject (Ko, 1982; Beuchat, 1983, 1987; Hesseltine, 1983; Steinkraus, 1995; Wood, 1998; Nout, 2003). Beuchat (1983, 1987, 1995, 1997) followed through comprehensive surveys and the development of knowledge on the microbiology of indigenous fermented foods. Space limitations do not permit us to cover the vast and increasing subject in detail. Table 7.14 presents only selected examples. Treatises often classify indigenous foods and beverages by the regions where they are produced (e.g., soy sauce in the Orient, idli in India, tempeh in Indonesia, ogi and gari in West Africa, and pozol in Mexico). Odunfa (1988), Sanni and Lönner (1993), and Jespersen (2003) reviewed various kinds of African fermented foods and beverages, whereas

TABLE 7.14
Selected Examples of Indigenous Fermented Foods

Product	Substrate(s)	Microorganisms	Use	Regions
Cassava bread	Cassava	LAB, yeasts	Staple food	AS, CA, SA
Chicha	Maize	LAB, yeasts	Food	SA
Dosa	Rice, black gram	LAB, yeasts	Batter cake	IN
Gari	Cassava	LAB, yeasts	Staple food	AW
Idli	Rice, black gram	LAB, yeasts	Food cake	IN
Kenkey	Maize flour	Yeasts, molds	Dough, batter	AW
Kimchi	Cabbage, vegetables LAB, yeasts	Food, condiment	EA	
Kombucha	Tea	Yeasts, acetobacters	Nonalcoholic beverage	EA, EU, NA
Murcha	Rice, wheat	Molds, yeasts	Starter, alcoholic beverage	IN
Ogi	Maize	Molds, yeasts, LAB	Staple food	AS, AW
Oncom	Peanut	Molds, yeasts	Cake	SEA
Pito	Maize, sorghum	Yeasts, molds	Alcolholic beverage	AW
Pozol	Maize	Molds, yeasts	Dough, beverage	CA
Pulque	Agave juice	Yeasts, zymomonas	Alcoholic beverage	CA
Ragi	Wheat, rice	Molds, yeasts	Starter	SEA
Sake	Rice	Yeasts, molds	Alcoholic beverage	EA
Shoyu	Soybean	Molds, yeasts	Condiment	EA
Sufu	Soybean, wheat	Molds, yeasts	Food	EA
Tape	Rice, cassava, maize	Molds, yeasts	Snack food	SEA, EA
Tempe	Soybean	Molds, yeasts, LAB	Food, cake	SEA, SA, NA
Tibi	Sugar cane	LAB, yeasts	Nonalcoholic beverage	CA
Togwa	Maize	LAB, yeasts	Nonalcoholic beverage	AS

Notes: LAB: lactic acid bacteria; AN: Africa North; AS: Africa South; AW: Africa West; CA: Central America; EA: East Asia; EU: Europe; IN: India; NA: North America; SA: South America; SEA: South-East Asia.

Aidoo et al. (2006) published an overview of Asian indigenous foods. Other groupings can be made according to the main substrates, and an attempt is also made here.

The largest group of indigenous fermented food consists of cereal-based products. The main components can be rice, wheat, corn, sorghum, or millet, and similar doughs, pastes, or beverages that can be prepared from cassava, soybean, or other legumes (Blandino et al., 2003).

Idli is a bread-type food made from rice and black gram, fermented and leavened by LAB and yeasts, which also reduces antinutritive phytic acid. Soni et al. (1986) detected *S. cerevisiae*, *Db. hansenii*, and *Trisp. cutaneum* in dosa, while according to Venkatasubbaiah et al. (1985), a more varied yeast biota, namely, *P. anomala*, *C. glabrata*, *C. tropicalis*, *C. sake*, *Db. hansenii*, *Iss. orientalis*, *S. exiguous*, and *Guehom. pullulans*, contributes to the fermentation of idli. Idli batter is consumed after steaming, whereas dosa, a similar product, is fried.

Ogi and togwa are fermented gruels made from corn, sorghum, or millet, whereas kenkey and pozol are maize batters or doughs. They are mostly produced by natural mixed lactic and alcoholic fermentation. Yeasts that occur have been identified as *C. pelliculosa*, *C. tropicalis*, *Iss. orientalis*, and *S. cerevisiae* (Mugula et al., 2002). A probiotic lactic acid starter has been developed for the production of ogi (Okagbue, 1995), and starter cultures have also been tested for togwa and kenkey (Annan et al., 2003; Mugula et al., 2003). Microorganisms in kenkey have been investigated in detail (Halm et al., 1993, 2004; Jespersen et al., 1994). The mixed yeast biota of corn *S. cerevisiae* and *Iss. orientalis* becomes dominating during fermentation.

Ragi is a fermented rice flour cake that contains various molds and yeasts with amylolytic and proteolytic activity. Ragi is not a food itself but is used as a starter for the fermentation of other foods.

Tape ketan, made from rice and cassava by the inoculation of ragi, contains a mold, *Chlamydomucor oryzae* (*Amylomyces rouxii*), and a yeast, *Hyphop. burtonii*, which are the main contributors to fermentation. Other yeasts present in ragi include *S'copsis fibuligera*, *P. canadensis*, *P. anomala*, *P. subpelliculosa*, *S'copsis malanga*, and *C. parapsilosis* (Cronk et al., 1977). Another starter used for Chinese rice pudding fermentation contains a *Rhizopus* species as well as yeasts such as *P. anomala* (Wei and Jong, 1983).

Cassava, a tuber root, is rich in starch; unfortunately, however, it also contains toxic cyanogenic glycosides, and can be consumed only after eliminating these by fermentation. Bacilli, molds, and yeasts (*C. tropicalis* and *Zygotsp. florentinus*) also contribute to softening the texture of products such as gari and others, subsequently prepared (Essers, 1995). Sour cassava starch is widely used in Brazil for traditional baked goods and fried foods. The flour is produced by spontaneous fermentation involving LAB and diversity of yeasts such as *Issatchenkia* spp., a possible new species, *Gal. geotrichum*, *C. ethanolica*, and many others (Lacerda et al., 2005).

Beverages are also fermented from cereals. Beer-like alcoholic beverages from germinated sorghum, corn or millet (e.g., pito), or wheat (bouza), as well as sake and palm wines have been mentioned before (Section 7.4.3). To these can be added various rice wines consumed in India, Nepal, and Thailand. These are produced with amylolytic starters of *Mucor*, *Rhizopus*, and *Aspergillus* molds. One of them is murcha, by which rice starch is saccharified and fermented to alcohol by a mixed population of yeasts that includes both amylolytic species such as *S'copsis fibuligera*, *S'copsis capsularis*, and *Hyphop. burtonii*, as well as strongly fermenting species such as *S. cerevisiae*, *C. glabrata*, and *P. anomala* (Shrestha et al., 2002; Tsuyoshi et al., 2005). Chicha, a drink in South America, is unique in that the corn starch is saccharified by human saliva. Boza is a nonalcoholic, sour beverage produced in Turkey and the Balkan countries. It is fermented from mixed cereals with sugars mainly by LAB; several species of yeasts fermenting poorly have also been isolated, such as *C. tropicalis*, *C. glabrata*, and *Geo. candidum* (Gotcheva et al., 2001).

Cow, sheep, and goat milks fermented spontaneously are produced under various names all over rural Africa and Asia. Unpasteurized or boiled milk is mostly used; in more developed countries UHT milk exists, but starter inoculation is not yet a general practice. Hence, diverse yeasts are found in fermented products (*C. kefyr*, *C. lipolytica*, *C. lusitaniae*, *C. tropicalis*, *Dek. bruxellensis*, *Tsp. delbrueckii*, and *S. cerevisiae*) (Gadaga et al., 2001). A mixed starter of LAB without yeasts has been recommended (Okagbue and Bankole, 1992).

Burong isda is a fermented fish and rice mixture. Sakai et al. (1983) isolated 484 yeast strains of this food, the dominant species being *S. cerevisiae*, *C. tropicalis*, *C. parapsilosis*, *P. strasburgensis*, *P. carsonii*, and *Zygo. rouxii*. The latter two species occurred only in products containing >5% salt. In spite of many studies, little is known about specific microorganisms and their roles in many indigenous fermented foods. Fermentation processes are often brought about by the association and succession of several types of microorganisms, in which yeasts commonly occur. Suzuki et al. (1987) surveyed the yeasts isolated from 29 kinds of Thai fermented foods. Among 19 species identified, the most frequent was *Iss. orientalis.* The distribution of yeasts in 341 samples of 11 indigenous fermented foods was reviewed by Sandhu and Waraich (1984). More than 500 strains of yeasts representing 26 species from the genera *Candida*, *Debaryomyces*, and *Rhodotorula* were isolated. The most frequent isolates were *Iss. terricola* (7.9%), followed by *C. sake* (5.6%), *Guehom. pullulans* (5.0%), and a mold, *Sporothrix*, with a yeast phase called *C. fragicola.*

7.5.6 Vinegar and Condiments

The use of vinegar as a traditional condiment has been mostly displaced by chemically synthesized acetic acid; however, fermented vinegar produced is still essential for delicatessen salads and some other purposes. Vinegar is produced in two stages: an alcoholic fermentation by naturally occurring yeasts is followed by oxidative "fermentation" of ethanol to acetic acid by acetic acid bacteria (Gullo et al., 2006). Wine, cider, malt, and other alcoholic raw materials can be acidified, and depending

on these, vinegar may contain up to 20% acetic acid. It also contains less than 0.5% ethanol and constituents lending aroma and flavor (Ebner and Follman, 1983).

In fermenting vinegar, few types of yeast are able to survive under the circumstances of 3–12% ethanol, 1.2–5.1% titratable acidity, and low pH (2.7–3.5). In a recent study, 10 different yeast species were detected (Solieri et al., 2006). Most strains belonged to the genus *Zygosaccharomyces*; in particular, *Zygo. bailii* was found in 41% of samples, followed by *S. cerevisiae*, *Zygo. pseudorouxii*, and *C. stellata*. Other species recovered were *Zygo. mellis*, *Zygo. bisporus*, *Zygo. rouxii*, *Hsp. valbyensis*, *Hsp. osmophila*, and *C. lactis-condensi*. Contrary to the overall diversity, frequently only one species or only a single strain was detected in an individual sample.

Various condiments, mayonnaise, mustard, and ketchup are preserved by the addition of vinegar or acetic acid as well as preservatives (sorbic acid, benzoic acid). Containing 2.5–3.0% acetic acid and 0.15% benzoate or 0.1% sorbate, these products are usually stable at room temperature. In exceptional cases, however, yeasts may cause spoilage. In particular, *Zygo. bailii*, with excessive tolerance to preservatives, is able to multiply even if a few cells only survive. This causes spoilage in mustard (Buchta et al., 1996).

Mayonnaise and salad dressings are oil-in-water emulsions that are preserved by the addition of acetic acid to give a pH of <4. The water phase contains 5–10% salt, conferring a low a_w. Although other ingredients, particularly spices, may introduce various contaminating microorganisms, these products provide a highly selective environment in which only a few survive (King et al., 1976; Smittle, 1977b; Collins and Buick, 1983). Kurtzman et al. (1971) and Smittle and Flowers (1982) reported that *Zygo. bailii* is the major spoilage yeast in mayonnaise and salad dressing. When oil separates, this may open the way to spoilage. The olive oil itself can be attacked by lipase-producing yeasts such as *Williopsis californica*, *C. rugosa*, and some strains of *S. cerevisiae* (Ciafardini et al., 2006).

7.5.7 Acid-Preserved Foods

Some foods are preserved by vinegar and salt, with or without other preservatives. These include marinated fishes, vegetables, mayonnaise, and salad dressings. LAB and yeasts are the main spoilage microorganisms in these products (Smittle, 1977a,b).

Comi et al. (1981) reported that yeasts were responsible for the deterioration of pickled mushrooms. *P. anomala* represented 95% of the isolates, and *C. etchellsii*, *C. parapsilosis*, and *P. canadensis* were also detected. These yeasts tolerated 20% salt (a_w 0.845). Tomato ketchup and tomato-based sauces at pH 3.5–4.5 can be spoiled by yeasts (Flores et al., 1988). Preservative-resistant *Zygo. bailii*, *P. membranifaciens*, and *Iss. orientalis* are known to tolerate 1% acetic acid, and are involved in the spoilage of low-pH products (Pitt and Richardson, 1973; Draughon et al., 1981; Pitt and Hocking, 1988).

The shelf life of these products is determined mainly by low pH, the use of chemical preservatives (sorbic or benzoic acid), and refrigeration. Salads in dressing prepared from high-quality raw materials under rigorous hygienic manufacturing practices may have a shelf life of 6–8 weeks at 5–7°C. Consumer demand for products without chemical preservatives and with milder acidic taste may reduce shelf life. Careful control of the concentration of acidulent and pH in salad dressings are critical points in preventing the spoilage (Debevere, 1987). Refrigeration temperature is also of prime importance, because the rate of spoilage increases at temperatures above 5°C (Terry and Overcast, 1976). Spoilage may be caused by LAB, molds, and yeasts (Smittle, 1977b; Kirsop and Brocklehurst, 1982; Brocklehurst et al., 1983; Lindroth et al., 1985). The most important spoilage yeasts are *Kazach. exiguus*, *Naumovia dairensis*, *P. membranifaciens*, *Db. hansenii*, *Zygo. bailii*, and *Geo. candidum* (Baumgart, 1977; Smittle, 1977a; Smittle and Flowers, 1982; Brocklehurst et al., 1983; Brocklehurst and Lund, 1984).

Innovative approaches have been taken to extend the shelf life of salad vegetables. One way is to subject them to brief lactic acid fermentation (Bonestroo et al., 1993). Another approach is to

package them under modified atmosphere (Buick and Damoglou, 1989). The use of 80% N_2 and 20% CO_2 in packaged salads results in a 2-week extension of shelf life. *C. lambica*, *Db. hansenii*, and *Ya. lipolytica* were the main spoilage yeasts isolated from gas-packed vegetable salads.

When mayonnaise or salad dressing is used in the preparation of various delicatessen products, the introduction of contaminants with the added ingredients increases the chance of spoilage, particularly as the storage temperature increases above 10°C. Several cases of spoilage caused by yeasts have been described. Brocklehurst et al. (1983) and Brocklehurst and Lund (1985) observed that *Kazach. exiguus*, *Naumovia dairensis*, and *P. membranifaciens* were able to grow and produce gas in mayonnaise-based salads. Baumgart (1982) isolated *Kazach. exiguus*, *C. vini*, and *Zygo. bailii* from spoiled delicatessen foods. Cantoni and Comi (1988) detected *C. inconspicua*, *C. parapsilosis*, *Db. hansenii*, *S. cerevisiae*, *Rho. mucilaginosa*, and *Trisp. cutaneum* in spoiled mayonnaise salads.

A wide range of yeasts have been isolated from chilled potato and macaroni salads and Chinese dumplings (gyoza). Kobatake and Kurata (1980b, 1983b) detected *C. intermedia*, *C. sake*, *Ya. lipolytica*, *Rho. glutinis*, *Rho. minuta*, *Rho. mucilaginosa*, *Trisp. cutaneum*, *Guehom. pullulans*, *Cry. albidus*, *Cry. laurentii*, and *Cysto. infirmo-miniatum* in these products. The majority of these yeasts are psychrotrophic, some of them, for example, *Cysto. infirmo-miniatum*, and *Trichosporon* species are psychrophilic, and many exhibit proteolytic and lipolytic activities.

In contrast to mayonnaise and salad dressings, margarine is a water-in-oil emulsion. The aqueous phase of margarine contains milk solids and salt, making it susceptible to spoilage, most often by yeasts or molds. *Ya. lipolytica* and *Geo. candidum* are common among the yeasts responsible for spoilage (Muys, 1971).

7.6 BREAD AND BAKERY PRODUCTS

Since ancient times, cereals have been the basis of the most important staple foods worldwide. It is assumed that porridges were initially made from ground grains, which were later cooked and baked, while already in 4000 BC, in Egypt, leavened bread was produced. This was probably one of the first uses of fermentation along with making beer. In the history of human nutrition, a diversity of bakery products has been created and continues today (Hammes et al., 2005).

Bread is made basically from flour dough that is allowed to raise (leaven) when baked in the oven. Thus, the making of bread (and other baked goods) requires three main ingredients: flour, water, and yeast.

7.6.1 Flours

Wheat is the most common grain used for the preparation of bread, owing to the high levels of gluten in wheat flour that give the dough sponginess and elasticity. Flours from most other common cereals (rice, corn, rye, barley, oats, sorghum, and millet) and starchy crops (e.g., cassava) can also be used alone or in combination.

Grain kernels are contaminated with airborne microorganisms in the field, and this microbiota is dominated by molds and bacteria. Compared to the significance of molds on grains, which are among the main causes of deterioration leading to great economic losses, the role of yeasts is of lesser importance, although they are a permanent part of the microbiota. In general, yeasts make up only 1% of the microbiota, which consists mostly of bacteria and molds (Legan, 2000). Interestingly, while mold counts are generally higher in the fall and winter months, the peak of yeast population in flour has been recorded in the spring months, reaching more than 10^2 g^{-1} (Richter et al., 1993). Recent progress in the systematics of basidiomycetous yeasts and numerous surveys on rice and various other grains have resulted in the description of several new species (Boekhout et al., 1993; Nakase et al., 1993).

Yeasts are common on all cereals, although they represent only a minority of the microbial population. Their counts range from 10^2–10^4 g^{-1} (Berghofer et al., 2003). According to Richter and

Thalman (1983), about 68% of 178 different grain samples (oats, barley, wheat, corn, and rye) was contaminated with yeasts. During storage of cereals, the population of yeast decreased in the winter. Drying also caused a reduction in the yeast population. Yeasts were detected belonging to the genera *Candida*, *Cryptococcus*, *Pichia*, *Hanseniaspora*, *Rhodotorula*, *Sporobolomyces*, *Saccharomyces*, and *Trichosporon*.

Milling removes the outer parts, hulls, and testae from the kernels, together with the surface microbiota, and results in flours with lower microbial counts. The populations depend on the grade of milling and are higher in lower-grade flour and whole-grain meal. Kurtzman et al. (1970) reported that *Cry. albidus*, a species common in wheat, was not detected in wheat flour. A relatively small diversity of yeast species was detected in wheat kernels and flour. *P. anomala*, *Hyphop. burtonii*, and the yeast-like mold, *Aureobasidium pullulans*, were common to both kernels and flour. Additional species from kernels were *Cry. laurentii*, *Rho. glutinis*, and *Geo. fermentans*, while species detected in flour were *S. cerevisiae*, *P. farinosa*, and an unidentified species of *Candida*.

Spicher and Mellenthin (1983) compared the yeast biota of wheat and rye grains to the flours milled from them. Although a greater number of species were detected on wheat compared to rye kernels, the opposite was observed in flours. Out of 14 species, 7 were isolated from both cereal grains and flours (*Cry. albidus*, *Rho. ingeniosa*, *Rho. glutinis*, *P. membranifaciens*, *P. anomala*, *Trisp. cutaneum*, and *Hyphop. burtonii*). Four species (*Cry. laurentii*, *C. stellata*, *P. canadensis*, and *Spb. salmonicolor*) occurred only on kernels, while *C. zeylanoides*, *S. cerevisiae*, and *Guehom. pullulans* were detected only in flours. These yeasts originated as contaminants, most probably from the milling machinery.

Cassava flour constitutes an important source of food in many African countries. Its yeast biota appears to differ from that of wheat flour in that it contains a mixed population ranging between 10^2 and 10^6 g^{-1}, with *Iss. orientalis* representing the majority (57%) of isolates. Among 10 other species, *S. cerevisiae* also occurred (Okagbue, 1990).

7.6.2 Baker's Yeast

The importance of yeasts in bakery products has been reviewed by Seiler (1980), Ponte and Tsen (1987), Legan and Voysey (1991), and Bonjean and Guillaume (2003).

The technology of baker's yeast production has been reviewed (Trivedi et al., 1986; Beudeker et al., 1990; Deák, 1993, 2003; Rose and Vijayalakshmi, 1993).

On the technological level, the most important properties of baker's yeast include (1) the ability to hasten leavening of the dough; (2) the ability to adapt to different carbon sources, by expressing invertase and maltase activities; and (3) stress resistance, particularly cryo- and osmo-tolerance (Oda and Oichi, 1989). Yeast should contribute to the flavor of the baked products as well.

During propagation, storage, and use, baker's yeasts have to withstand, adapt to, and function in the most stressful conditions. During production, yeasts must adapt to low sugar and high aeration and repression of fermentation to produce large biomass (Figure 7.5). Cells are then maintained in dry or frozen state until use, when rehydration or thawing and inoculation cause osmotic shock in a new environment that requires the induction of enzymes for maltose utilization under semian-aerobic conditions. Thus, the ability of baker's yeast to cope with stress conditions is an essential physiological requirement (Attfield, 1997).

The baker's yeast strains used currently have been developed as a result of centuries of experience and purposeful selection and are best suited for bread making. Nevertheless, research to improve strains continues. Although methods of classical genetics (selection, mutation, and hybridization) are still very useful, novel methods such as protoplast fusion and genetic engineering have resulted in baker's yeast strains with even better technological properties (Angelow et al., 1996; Linko et al., 1997; Randez-Gil et al., 1999). Genetic analysis with sophisticated techniques, such as microarray analysis, has helped improve baker's yeast performance (Shima et al., 2005).

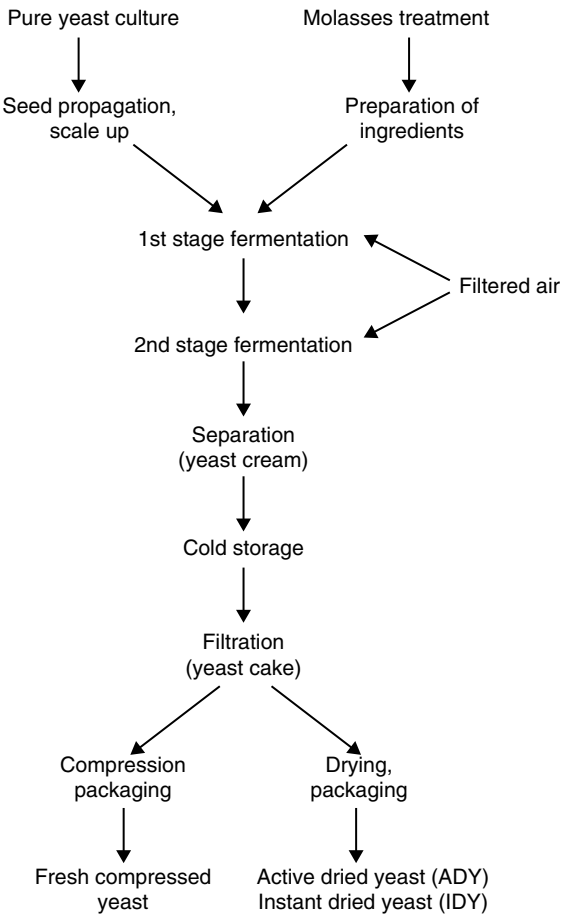

FIGURE 7.5 Flow chart showing the production of baker's yeast. *Source:* From Deák, T. (2003) *In*: Encyclopedia of Food Sci. Nutr., 2nd ed. (ed. Trugo, L.). Academic Press, London. pp. 6233–6240. With permission of Elsevier.

Baker's yeast is marketed either as fresh or preserved products. For direct industrial use, liquid yeast cream or compressed yeast is available; the dry matter content of the former is about 20%, the latter concentrated by filter press and/or centrifugation to 30–34%. Both have to be stored at 4°C to maintain activity. For long-term storage, yeast cell mass is dried. According to the drying method, active dry yeast or instant dry yeast can be produced. Both have a shelf life of 1–2 years. Active dry yeast has to be rehydrated before use, whereas instant dry yeast can be mixed directly in dough where it becomes active again (Spicher, 1983). Another mode of permanent storage is in the form of frozen dough (Hernandez-Lopez et al., 2003). However, baker's yeast cells in frozen dough suffer freeze–thaw damage during processing, and intensive research is under way to clarify its mechanism and improve stress resistance (see Section 5.4.3).

Certain spoilage agents may be present in commercial baker's yeasts. According to a study by Viljoen and Lues (1993), wild yeasts may make up 10% of compressed yeast block samples. The majority of contaminants belong to the genus *Saccharomyces*, but *P. anomala, Hyphop. burtonii*, and *Zygo. bailii* have also been present (Table 7.15).

Although *S. cerevisiae* strains are generally used as baker's yeast, the quest for alternative yeast species in bread making continues. *Tsp. delbrueckii* has been reported to be more freeze-tolerant than *S. cerevisiae*, and the selection of cryoresistant strains potentially applicable in baking has been

TABLE 7.15
Yeasts Frequently Isolated from Flour and Bakery Products

Species	Sources	References
C. glabrata	Sourdough	15, 21
C. humilis	Sourdough	12, 13, 14, 21
C. parapsilosis	Bread	9
C. tropicalis	Wheat flour, dough, baker's yeast	3, 8, 10
C. vini	Baker's yeast, dough	3, 4
Cry. albidus	Wheat flour	5, 10
Cry. laurentii	Wheat flour	5, 10
Db. hansenii	Baker's yeast, dough, bread	1, 3, 4, 16, 21
Hyphop. burtonii	Wheat flour, bread	5, 6, 9, 10, 22
Iss. orientalis	Sourdough	12, 14, 17, 21
Kazach. exiguus	Sour dough, bread	4, 7, 8, 9, 12, 14, 17
Kazach. servazzii	Sourdough	16, 21
Klu. marxianus	Dough, baker's yeast	4, 18, 21
P. anomala	Wheat flour, sour dough, filled cake	5, 6, 10, 17, 20, 21
P. fermentans	Sourdough	15, 21
P. guilliermondii	Dough, bakery goods	4, 6
P. membranifaciens	Flour, bread	9, 10, 15
Rho. glutinis	Wheat flour, baker's yeast, dough	3, 4, 5, 10
S. cerevisiae	Wheat flour, dough, bread	2, 8, 9, 10, 12, 13, 14, 15, 21
S'copsis fibuligera	Sour dough, bread	4, 6, 8, 9, 19, 21, 22
Schizo. pombe	Filled bakery goods	6, 11
Tsp. delbrueckii	Sour dough, bread	1, 4, 9, 15, 21
Tsp. microellipsoides	Flour	5
Trisp. moniliforme	Flour, baker's yeast	3
Zygo. bailii	Bread	6, 9
Zygo. bisporus	Filled bakery goods	6, 11
Zygo. rouxii	Filled bakery goods	6, 11

Notes: 1: Azar et al. (1977); 2: Barber et al. (1983); 3: Fowell (1965); 4: Infantes and Schmidt (1992); 5: Kurtzman et al. (1970); 6: Legan and Voysey (1991); 7: Ng (1976); 8: Salovaara and Savolainen (1984); 9: Spicher (1986); 10: Spicher and Mellenthin (1983); 11: Seiler (1980); 12: De Vuyst and Neysens (2005); 13: Foschino et al. (2004); 14: Pulvirenti et al. (2004); 15: Succi et al. (2003); 16: Meroth et al. (2003); 17: Gobbetti (1998); 18: Caballero et al. (1995); 19: Nielsen and Rios (2000); 20: Lanciotti et al. (1998); 21: Hammes et al. (2005); 22: Suhr and Nielsen (2004).
Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

made (Oda and Tonomura, 1993; Almeida and Pais, 1996). Attempts have been made to evaluate *Klu. marxianus* as baker's yeasts for the ability of this species to utilize lactose and grow in whey, a waste by-product in dairy industry (Caballero et al., 1995).

7.6.3 Bread Making

The production of bread, rolls, and some sweet goods includes leavening, which can be achieved by fermentation employing LAB and yeasts or baker's yeast alone. Dough also can be raised by producing CO_2 in a chemical reaction or by physical means, evaporating water or forcing air into certain products only (Spicher and Brümmer, 1995; Linko et al., 1997; Hammes and Gänzle, 1998).

The traditional way of leavening is a spontaneous fermentation of dough with indigenous microorganisms in flour. LAB soon becomes predominant through the selective effect of lactic acid. Heterofermentative LAB also produces carbon dioxide and leavens the dough. This is the sourdough

process that can be applied by saving a part of the leavened dough and using it for the next batch (backslopping). Although traditional sourdough fermentation has remained in use, in particular for rye and mixed grains bread, selected starter cultures also are used frequently (Stolz, 1999; De Vuyst and Neysens, 2005).

In sourdough, yeasts occur together with LAB (Gobetti, 1998). The most common species are *S. cerevisiae*, *Kazach. exiguus* (and its anamorph, *C. holmii*), *C. milleri* (syn. *C. humilis*), *Tsp. delbrueckii*, and *Iss. orientalis* (Spicher et al., 1979; Meroth et al., 2003; Pulvirenti et al., 2004). However, a much greater diversity of additional species have been isolated from sourdough (Rossi, 1996; Mantynen et al., 1999; Hammes et al., 2005). Yeast species may vary in sourdough starters used in various regions. In the special "San Francisco sourdough," *Kazach. exiguus* dominates, with *S. cerevisiae* playing a minor role (Sugihara et al., 1971; Ng, 1976). In sourdough prepared for a typical Swedish rye meal, only one yeast species closely resembling *Tsp. delbrueckii* was detected (Lönner et al., 1986). In South African wheat dough, 79% of yeast was identified as *Saccharomyces*, 16% *Zygosaccharomyces*, and 5% *Torulaspora* species (Viljoen and Lues, 1993). Leavening of sourdough in Finland has been attributed to *S. cerevisiae*, *Kazach. exiguus*, *C. stellata*, *Kazach. unisporus*, and *S'copsis fibuligera* (Salovaara and Savolainen, 1984), whereas in Iran *Tsp. delbrueckii* and *Db. hansenii* were detected (Azar et al., 1977). The yeast biota of French natural sourdoughs is dominated by *S. cerevisiae* (74%); in a few sourdough samples, individual species has been found, such as *Kazach. exiguus*, *P. anomala*, and *Tsp. delbrueckii* (Infantes and Schmidt, 1992). In Italian wheat sourdough, *S. cerevisiae* also dominates (77%), and in addition *P. fermentans*, *P. membranifaciens*, *Iss. orientalis*, and *C. glabrata* have been isolated (Succi et al., 2003).

In sourdough microbiota, the population of LAB (10^8–10^9 cfu g^{-1}) surpasses 100 times that of yeasts, and a stable association between the two groups of microorganisms develops (Vogel, 1997). Metabolic interactions between LAB and yeasts contribute not only to leavening activity but also lead to the production of volatile metabolites that create the special character of sourdough bread (Lues et al., 1993; Hansen and Hansen, 1994; Damiani et al., 1996; Gobetti, 1998).

Isolation, selection, and large-scale propagation of specific yeast have made it possible to use baker's yeast alone for leavening. Today about two-thirds of breads are prepared with special selected strains of industrially produced baker's yeast. It is used in the form of liquid yeast cream, compressed yeast cake, or active and instant dry yeast, which are usually added directly to the flour (straight dough process). Wild yeasts always occur in small number in dough, but rarely they cause problems because of the overwhelming abundance of baker's yeasts. The main fermentable sugar in the sponge dough is maltose, liberated from starch by amylase in flour. The leavening ability of baker's yeast is related to its maltose fermentation capability, which is regulated by one or more genes coding for α-glucosidase and maltose permease. Some strains produce these enzymes constitutively, whereas in others they are induced by maltose in the dough (Oda and Ouchi, 1990b). The invertase activity of *S. cerevisiae* may not influence the leavening activity in sweet dough (Oda and Ouchi, 1990a). Microorganisms originating from flour and dough are normally inactivated during baking. Microbial spoilage of baked goods is mainly due to contamination after baking. Sliced bread is particularly susceptible to contamination (Seiler, 1980). Internal spoilage of bread is mainly caused by bacilli producing ropiness or by molds developing dark spots (Legan and Voysey, 1991). Yeasts and yeast-like organisms are responsible for the development of white spots in the crumb (known as "chalky bread"). This type of spoilage can be caused by *S'copsis fibuligera*, *Hyphop. burtonii*, or *Zygo. bailii* (Spicher, 1984; Legan and Voysey, 1991). Other yeast species, such as *S. cerevisiae*, *Tsp. delbrueckii*, *P. membranifaciens*, and *C. parapsilosis*, may also occur (Spicher, 1986). Modified atmosphere packaging inhibited most molds but not *S'copsis fibuligera*. Effects of essential oils and oleoresins were tested in the gas phase. The yeast growth was retarded by allyl isothiocyanate; however, for sensory reasons, it can be applied only in rye bread and hot-dog bread (Nielsen and Rios, 2000).

7.6.4 Bakery Products

In bakery products with coatings, fillings, or ingredients such as nuts, raisins, jam, or jelly that may be added after baking, contamination with *S. cerevisiae* and more often with xerotolerant yeasts (*Zygo. rouxii*, *Zygo. bisporus*, *Schizo. pombe*, *Schizo. octosporus*) may cause fermentative spoilage (Seiler, 1980; Legan and Voysey, 1991). Apple turnovers (a_w 0.93, pH 5.0) packaged under modified atmosphere with reduced oxygen may overgrow fermentative spoilage by *S. cerevisiae* (Smith et al., 1987). Reduction of a_w failed to inhibit growth of *S. cerevisiae* at ambient temperature, but growth was completely suppressed by ethanol vapor (0.56–1.57%, v/v) at a_w 0.85–0.90. In cream-filled cakes in which *P. anomala* caused spoilage, the source of contamination was linked to hazelnut paste (Lanciotti et al., 1998).

Pizzas are popular products in many countries. Yeasts and LAB have been identified as the main spoilage microorganisms causing acidification and blowing of fresh ham pizza stored in modified atmosphere (20% CO_2) packaging. Nisin inhibited only LAB (Cabo et al., 2001). Potassium sorbate was effective in preventing fungal spoilage in intermediate moisture bakery products (a_w 0.80–0.90), whereas calcium propionate and sodium benzoate were effective only at low a_w levels (Guynot et al., 2005).

Although less effective than sorbate or benzoate, calcium propionate in a concentration of 0.3%, at $a_w < 0.95$ and pH < 4.8, inhibited growth of moulds and the yeasts *Hyphop. burtonii* and *S'copsis fibuligera* for a 2-week period in bakery products (Suhr and Nielsen, 2004).

7.7 HIGH-SUGAR PRODUCTS

Dehydrated vegetables, grain flours, pasta, milk powder, and other dried foods generally contain less than 25% moisture and have an $a_w < 0.60$. Such products are microbiologically stable for indefinite periods of time, provided they remain dry. Intermediate moisture foods (IMF) vary widely in terms of a_w (0.60–0.90) and moisture content (10–50%), and the addition of preservatives or other hurdles are required for preservation. This category includes many foods containing 50–70% sugar, in which bacteria are inhibited but some molds and yeasts may grow at $a_w > 0.70$. High-sugar foods are usually stable at room temperature for long periods. Listed among these foods are honey, syrups, jam, jelly, fudge, marmalade, marzipan, dried fruits, and fruit concentrates. Other high-sugar products, such as cookies, toffees, candies, chocolates, and refined sugars, contain only 15–20% moisture, and they usually are also stable at ambient temperature.

The stability of high-sugar products depends primarily on a_w, combined with other factors such as pH, presence of preservatives, temperature, mode of packaging, and conditions of storage. Spoilage may be caused by xerotolerant fungi, also referred to as osmophiles (Tilbury, 1980a,b; Corry, 1987). At a_w values in the range of 0.70–0.85, the growth rate of these organisms is very slow, and hence spoilage in products may become apparent only after many months (Jermini and Schmidt-Lorenz, 1987). If, however, high-sugar products are stored in an atmosphere of high relative humidity, the a_w increases due to hygroscopicity, and this may permit a significantly faster growth.

Earlier investigators reported that strains of many yeast species were capable of spoiling products with a_w as low as 0.61 (Onishi, 1963). As noted by Jermini et al. (1987) and Jermini and Schmidt-Lorenz (1987a), this was due to the uncertainty of measuring and expressing the degree of sugar tolerance. The nomenclature and taxonomy of these yeasts also created confusion. Yeasts are rarely able to grow at a_w lower than 0.70, and the most frequently isolated species from high-sugar products are those presently classified in the genus *Zygosaccharomyces* (James and Stratford, 2003). Section 3.2.2.1 described a_w relation of yeast growth, and Table 7.16 lists yeasts most frequently isolated from high-sugar products.

Tokouka et al. (1985) and Jermini et al. (1987) isolated exclusively *Zygo. rouxii* from honey. Poncini and Wimmer (1986) also detected *Zygo. rouxii* strains, as well as *Tsp. delbrueckii*, *S. cerevisiae*, *Schizo. octosporus*, and *Met. reukaufii* in honey. Jermini et al. (1987) isolated 28 strains of

TABLE 7.16
Yeasts Frequently Isolated from High-Sugar Products

Species	Sources	References
C. apicola	Refined sugar, raisins	5, 7, 16
C. etchellsii	Raisins, raw sugar, dried fruits, syrup	11, 20
C. lactiscondensi	Candied fruit, dried fruits, refined sugar	7, 11, 16, 17, 18
C. versatilis	Raw sugar, concentrated juice	17, 20
Db. hansenii	Jam, syrup, raw sugar, molasses, confectionery	1, 3, 6, 13, 16, 17
Hsp. guilliermondii	Fruit concentrates, raisins	5, 20
Klu. marxianus	Sugar cane, dried figs, molasses	1, 2, 13, 20
Lachancea thermotolerans	Raisins, raw sugar, marzipan	16, 17, 18, 20
P. anomala	Jam, raisins, dried fruits, confectionery, molasses	11, 13, 17
P. fermentans	Raisins, molasses, raw sugar	5, 13, 20
P. membranifaciens	Cane sugar, molasses, raisins	1, 5, 13
S. cerevisiae	Honey, candied fruit, raisins, confectionery	5, 12, 14, 17, 18
Schizo. octosporus	Honey, candies, molasses, dried fruit	14, 17, 19
Schizo. pombe	Raisins, cane sugar, molasses	5, 13, 17, 18, 19, 20
Tsp. delbrueckii	Honey, candied fruit, raw sugar, molasses, raisins	6, 13, 14, 17
Zygo. bailii	Fruit concentrates, molasses, dried fruits	5, 8, 13, 16, 20
Zygo. bisporus	Jam, syrups, raw sugar, molasses	3, 15, 16, 17, 20
Zygo. mellis	Honey, syrups, salted beans	9, 10, 20
Zygo. rouxii	Honey, syrups, concentrated juices, molasses, raisins, confectionery, soy sauce, miso	3, 4, 6, 12,13, 14, 17, 18, 20

Notes: 1: Anderson et al. (1988); 2: Bärwald and Hamad (1984); 3: Cantoni and Comi (1988); 4: Comi et al. (1982); 5: Deák (1988); 6: Jermini et al. (1987); 7: Kreger-van Rij (1984); 8: Kunkee and Goswell (1977); 9: Kurtzman (1990); 10: Lodder (1970); 11: Madan and Gulati (1980); 12: Országhová and Kieslingerová (1984); 13: Parfait and Sabin (1975); 14: Poncini and Wimmer (1986); 15: Put et al. (1976); 16: Tilbury (1976); 17: Tokouka et al. (1985); 18: Tokouka and Ishitani (1991); 19: Vaughan Martini (1991); 20: Walker and Ayres (1970).
Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

xerotolerant yeasts from spoiled honey, fruit juice concentrates, marzipan, syrups, and other high-sugar products. All strains were identified with *Zygo. rouxii*, with the exception of two strains of *Zygo. bailii* and one strain each of *Tsp. delbrueckii* and *Db. hansenii* that originated from orange concentrates, fitness drinks, and canned figs of relatively higher a_w (0.835–0.991). Though strains of *Zygo. rouxii* were isolated from products with a_w as low as 0.631, they did not grow below a_w 0.76. Tokouka et al. (1985) demonstrated that only strains of *Zygo. rouxii*, *Zygo. Bisporus*, and *C. lactis-condensi* grew better at a_w 0.91–0.95 than at 0.986, and they considered these yeasts to be osmophilic. Jermini et al. (1987) reported only a single strain of *Zygo. rouxii* that was osmophilic.

Tokouka et al. (1985) isolated several xerotolerant yeasts from high-sugar products. *Zygo. rouxii* was most commonly found in sugar and molasses. Single strains of *Schizo. octosporus*, *Schizo. pombe*, *Tsp. globosa*, and *C. mannitofaciens* were also isolated from these products. In jams, *Db. hansenii*, *P. anomala*, *C. silvicola*, and *Rho. mucilaginosa* occurred; *Zygo. rouxii*, *C. lactis-condensi*, *Tsp. delbrueckii*, and *S. cerevisiae* were detected in candied fruits; and *P. anomala*, *Tsp. delbrueckii*, and *C. tropicalis* were isolated from confectionery products. Tokouka and Ishitani (1991) isolated 35 strains of yeasts from high-sugar foods and related materials. Only five strains were of *Zygo. rouxii*; the rest represented various other species. One strain of *Z. rouxii* was able to grow at a_w 0.67 in a fructose-containing medium, whereas seven *Candida* spp. had a minimum a_w for growth of about 0.79.

Országhová and Kieslingerová (1984) isolated only *Zygo. rouxii* from defective confectionery products and fermenting candied fruits. These strains as well as collection strains of *Db. hansenii* and *S. cerevisiae* grew well at an a_w of 0.75. Xerotolerant strains can be isolated from dried fruits, and nonxerotolerant yeasts originating from the fresh fruit. Madan and Gulati (1980) detected *P. angusta*, *P. ciferrii*, *P. subpelliculosa*, *P. polymorpha*, *P. anomala*, *S. cerevisiae*, *C. lactis-condensi*, and *C. etchellsii* on raisins, dates, and cashew nuts. Tokouka et al. (1985) isolated *Lachancea thermotolerans*, *Spb. roseus*, and *Schizo. pombe* from raisins and dried dates.

Refined sugar generally contains only very low numbers of microorganisms. White crystalline sugar may contain 100–200 bacteria and only 1–3 yeasts in 10 g (Klaushofer et al., 1971). Microbial growth occurs, however, during the production of both cane and beet sugar. Yeasts find optimum conditions for growth in the juices at various stages of production. Although both sugarcane and sugar beets are highly contaminated raw materials, most microorganisms are destroyed during processing. Raw sugar may become contaminated with sugar-tolerant yeasts that may develop biofilms on equipment surfaces during the continuous operation of mills (Tilbury, 1980b). The a_w of raw sugar may vary from 0.575 to 0.825, and both fructophilic and xerotolerant (osmophilic) yeasts (*Zygo. rouxii*, *Db. hansenii*, *C. etchellsii*, *C. versatilis*, *C. gropengiesseri*), as well as nonxerotolerant yeasts (*P. anomala*, *P. farinosa*, *S. cerevisiae*) can be found in raw cane sugar (Tilbury, 1980b).

Bärwald and Hamad (1984) isolated 23 strains of yeasts from juice samples taken from processing lines in two cane sugar factories. These strains belonged to 9 genera and 11 species; however, only 7 strains of *Klu. marxianus* present in nearly all samples were of practical importance because of their heat tolerance at 55°C. The other yeasts were either not thermo- or xerotolerant or did not assimilate sucrose. Garcia and Alcina (1981) also noted *Kluyveromyces* and *Brettanomyces* strains that were able to grow at 40°C. The importance of these yeasts in sugar production, however, cannot be compared to potential problems associated with thermophilic bacteria or dextran-forming *Leuconostoc* strains. Anderson et al. (1988) isolated thermotolerant yeasts from sugar cane that were able to ferment sucrose at temperatures above 40°C. The majority (80%) of strains were *Klu. marxianus* and *Klu. lactis*, some of which grew up to 47°C. A few strains of *P. angusta*, *S. cerevisiae*, *Db. hansenii*, *P. membranifaciens*, *Geo. capitatum*, and *C. oleophila* also occurred.

Honey, with a sugar content of more than 80% corresponding to a water activity 0.6 or less, and at a low pH (3.9, range 3.4–5.5), is usually protected from microbial spoilage. Moreover, honey has some antimicrobial properties, the most important being the glucose oxidase system producing hydrogen peroxide. Only two groups of microorganisms, yeasts and spore-forming bacteria, are of concern in honey (Snowdon and Cliver, 1996). In a few cases, honey caused infant botulism; it can be fermented by xerophilic yeasts if it absorbs water. Honey may contain microorganisms in counts of about 10^2 cfu g^{-1} which may be in inactive, nongrowing, or even VBNC state. The primary sources of microbial contamination are nectar, pollen, and honeybees themselves; however, only *Saccharomyce*s and *Candida* species have been associated with bees. A larger diversity of yeasts, comprising at least ten genera, occurs in honey, originating from secondary sources after harvesting the honey.

7.8 DAIRY PRODUCTS

Yeasts are important in the dairy industry for several reasons. They play an essential role in the preparation of certain fermented products and in the ripening of certain cheeses. Yeasts also can be used to ferment whey, a major by-product of cheese making (Marth, 1987). However, yeasts can also cause spoilage of milk and dairy products. The significance of yeasts in dairy products is increasingly recognized. Several reviews have discussed the harmful and beneficial roles of yeasts in these products (Fleet and Mian, 1987; Marth, 1987; Fleet, 1990b; Seiler and Busse, 1990; Varnam, 1993; Jakobsen and Narvhus, 1996; Viljoen, 2001; Hansen and Jakobsen, 2004).

Dairy products, as varied as they are, offer a special ecological niche that selects for the occurrence and activity of certain main yeast species. Fleet (1990b) summed up some key properties of yeasts

TABLE 7.17
Yeasts Frequently Isolated from Dairy Products

Species	Sources	References
C. catenulata	Cheese	32, 36, 37, 40, 41
C. intermedia	Cheese	27, 34, 37
C. parapsilosis	Cheese, yoghurt, butter	6, 16, 18, 19, 27, 40, 41
C. rugosa	Cheese, yoghurt	1, 12, 16, 20, 27,40
C. tropicalis	Cheese, yoghurt	6, 16, 18, 25, 38
C. silvae	Cheese	27
C. zeylanoides	Cheese	27, 31, 34, 40, 41
Cry. albidus	Cheese	35, 37
Cry. curvatus	Cheese	34
Cry. laurentii	Cottage cheese, butter	1, 3
Db. hansenii	Milk, yoghurt, cheese, ice cream	2, 5, 7, 8, 10, 11, 15, 15a, 16, 17, 18, 20, 21, 22, 27, 29, 29, 30, 31, 32, 33, 34, 35, 39, 41
Dek. bruxellensis	Yogurt	38
Gal. geotrichum	Milk, yoghurt, cheese	6, 7, 8, 15, 24, 25, 28, 29, 31, 33, 39, 41
Hyphop. burtonii	Cheese, dairy products	4, 22, 25
Kazach. unisporus	Cheese	27
Klu. lactis	Cheese, yoghurt, cream	2, 4, 15a, 20, 21, 24, 25, 27, 31, 32, 33, 39
Klu. marxianus	Cheese, yoghurt, kefyr	4, 6, 8, 9, 11, 12, 15a, 17, 19, 23, 27, 32, 33, 35, 37, 40, 41
Naumovia dairensis	Yogurt	38
P. anomala	Cheese, yoghurt	22
P. fermentans	Milk, cheese	15, 23, 31
P. membranifaciens	Cheese	8, 15, 39
Rho. rubra	Cheese	33,34,35
S. cerevisiae	Yoghurt, kefyr, cheese, ice cream	4, 5, 9, 12, 18, 19, 20, 21, 27, 32, 37, 38
Tsp. delbrueckii	Cheese, yoghurt	4, 14, 25, 32, 35, 38
Trisp. moniliforme	Milk, butter, cheese	7, 13, 19
Ya. lipolytica	Milk, cheese, yoghurt	1, 3, 4, 9, 14, 16, 20, 26, 30, 33, 35, 38, 41
Zygo. rouxii	Ice cream	5

Notes: 1: Banks and Board (1987); 2: Besancon et al. (1992); 3: Brocklehurst and Lund (1985); 4: Comi et al. (1981b); 5: Comi et al. (1992); 6: El Bassiony et al. (1980); 7: Engel (1986a); 8: Engel (1986b); 9: Engel et al. (1986); 10: Fleet et al. (1984); 11: Fleet and Mian (1987); 12: Green and Ibe (1987); 13: Gueho et al. (1992); 14: McKay (1992); 15: Nunez et al. (1981); 15a: Schmidt and Lenoir (1980); 16: Seham et al. (1982); 17: Rohm et al. (1992); 18: Seiler (1991); 19: Seiler and Busse (1990); 20: Suarez and Inigo (1982); 21: Suriyarachi and Fleet (1981); 22: Tilbury et al. (1974); 23: Tzanetakis et al. (1987); 24: Walker and Ayres (1970); 25: Flórez and Mayo (2006); 26: De Wit et al. (2005); 27: Callon et al. (2006); 28: Decker and Nielsen (2005); 29: Arfi et al. (2005); 30: Das et al. (2005); 31: Fadda et al. (2004); 32: Romano et al. (2001); 33: Cosentino et al. (2001); 34: Pereira-Dias et al. (2000); 35: Welthagen and Viljoen (1999); 36: van den Tempel and Jakobsen (1998); 37: Roostita and Fleet (1996); 38: Gadaga et al. (2001); 39: Vasdinyei and Deák (2003); 40: Cocolin et al. (2002); 41: Lopandic et al. (2006).
Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

that determine their dominance in dairy products. These include the fermentation and assimilation of lactose; proteolytic and lipolytic activity; assimilation of lactic acid and citric acid; growth at low temperature; and salt tolerance. Best matching these attributes, the most frequently occurring species in dairy products are *Db. hansenii*, *S. cerevisiae*, *Klu. marxianus*, *Ya. lipolytica*, and *Trisp. cutaneum*, although a diversity of other yeasts have been isolated from them (Table 7.17). Large-scale studies serve as examples. Seiler (1991) differentiated 59 species among 2664 isolates from cheese brines,

cheese quarg, yoghurt, and fruit preparations. *Db. hansenii* was most frequently isolated (16%), followed by *S. cerevisiae*, *C. parapsilosis*, and *C. tropicalis.* Rohm et al. (1992) described a diversity of yeasts in fermented milk products, cheese brines, and whey. Of 1013 isolates representing 37 species, *Db. hansenii* and *Klu. marxianus* were most frequent, although the former did not occur among 84 isolates from kefyr. The incidence of species showed a wide variation in the dairy products; intraspecific physiological differences were also observed among strains isolated from different products.

7.8.1 Milk

Milk is an excellent substrate for growth of many microorganisms, including yeasts. Fresh, raw milk contains varying populations of yeasts, depending on the milking hygiene. Raw milk held at refrigerator temperature allows the growth of psychrotrophic strains. Spoilage is generally caused by bacteria; souring by LAB is common in nonpasteurized milk. Yeasts also occur in raw and pasteurized milk (Fleet, 1990b).

Engel (1986a,c) reported that all of 128 samples of raw milk contained yeasts in low number (10^2–10^4 mL^{-1}). *Cry. curvatus* was isolated most frequently, followed by *Geo. candidum*, *Trisp. cutaneum*, and *Db. hansenii*. *P. membranifaciens* and *Ya. lipolytica* also occurred, and *Klu. marxianus*/*Klu. lactis* represented only 5% of yeast population.

Pasteurization of milk destroys most microorganisms except thermoduric bacteria. Yeasts in pasteurized market milk originate from secondary contamination. In a survey of pasteurized milk samples, Fleet and Mian (1987) detected *Db. hansenii*, *Klu. marxianus*, *Cry. flavus*, and *Cry. diffluens.* Cocolin et al. (2002) using molecular techniques, identified 12 species of yeasts from raw milk: the major isolates, in order of frequency of occurrence, were *Klu. marxianus* (20%), *C. pararugosa* (19%), *C. parapsilosis* (13%), *Cry. curvatus* (11%), *C. catenulata* (9%), and *C. zeylanoides* (9%).

7.8.2 Fermented Milk Products

Two types of dairy products in which yeast activity plays a major role are fermented milk products and cheeses. Some fermented milk products, for example, kefyr and kumiss, result from a mixed fermentation of LAB and lactose-fermenting yeasts. Kefyr is cultured by inoculation of grains that contain a symbiotic mixture of LAB and yeasts (Marshall, 1986). *Klu. lactis* (*C. kefir*) has been isolated from kefyr grains, but nonlactose fermenting species such as *Kazach. unisporus*, *S. cerevisiae*, and *Kazach. exiguus* also occur (Engel, 1984; Engel et al., 1986).

Yoghurt is produced all over the world and, although it is prepared only by lactic acid starters and may be frozen or pasteurized, it often contains yeasts that may be troublesome. The spoilage of yogurt is well documented (Suriyarachchi and Fleet, 1981; Green and Ibe, 1987; Salji et al., 1987; McKay, 1992). *Db. hansenii* was most frequently isolated from spoiled items (Jordano et al., 1991). Rohm et al. (1990) detected a broader spectrum of spoilage yeasts in yogurt. Among 233 isolates, 13 species in decreasing order of frequency were *Db. hansenii*, (22%), *Rho. mucilaginosa* (18%), *Ya. lipolytica* (15%), *Met. reukaufii* (13%), *C. zeylanoides* (9%), *C. tropicalis* (8%), and *C. parapsilosis* (6%). *P. anomala*, *Klu. marxianus*, and *Tsp. microellipsoides* have been detected in specific cases of spoilage of plain yogurts (Caggia et al., 2001; Cappa and Cocconelli, 2001; Mayoral et al., 2005).

In recent years, the addition of fruit and flavors to yoghurts has amplified the risk of spoilage yeasts. Tilbury et al. (1974) observed that *Db. hansenii*, *C. versatilis*, *C. intermedia*, and *P. anomala* occurred most frequently in various flavored yogurts. In a study by Suriyarachchi and Fleet (1981), *Db. hansenii* and *Klu. marxianus* were the most frequently isolated species, followed by *S. cerevisiae*, *Rho. rubra*, *Klu. lactis*, *C. versatilis*, *P. toletana*, and *Iss. orientalis*. In another survey (Green and Ibe, 1987), the species occurring in highest count and incidence were *Clavisp. lusitaniae*, *Klu. lactis*, and *Iss. orientalis*, while *S. cerevisiae*, *C. rugosa*, and *Rhodotorula*, *Sporobolomyces*, and

Debaryomyces species were isolated less frequently. *S. cerevisiae* has also been responsible for the spoilage of fruit yogurt (Fleet and Mian, 1987).

In many regions, both kefyr and yogurt are produced by spontaneous fermentation in homes (Loretan et al., 2003; Mufandaedza et al., 2006). Naturally fermented products are considered organoleptically superior to commercially produced cultured products; however, pathogenic bacteria may survive and grow in fermented milk, presenting health risks. Some strains of LAB and also yeast (*C. kefyr*, tel. *Klu. marxianus*) have been observed to inhibit pathogens and can be recommended for use as a starter culture (Gadaga et al., 2001; Mufandaedza et al., 2006).

Consumption of fermented milk products has long been considered to benefit human health. Bio-yogurt and similar products are produced carrying probiotic lactobacilli and bifidobacteria, and recently also a yeast, "*S. boulardii*," claimed to be of probiotic property. However, survival of probiotic strains in yoghurt may be temporary (Lourens-Hattingh and Viljoen, 2001). Potential for probiotic application has been tested among various dairy yeast strains showing *Klu. lactis* with attractive properties (Kumura et al., 2004).

7.8.3 Cheeses

Yeasts contribute to the ripening of some types of cheeses by metabolizing lactic acid and raising the pH, thus enabling the growth of proteolytic bacteria. Yeast activity is important both within and on the surface of soft cheeses (blue cheese, Gorgonzola, Camembert, etc.). Nunez et al. (1981) made a thorough study of the blue cheese manufacturing and ripening. *P. fermentans*, *Kazach. unisporus*, *Geo. capitatum*, and *Geo. candidum* were isolated from milk and curd. During ripening, *P. membranifaciens* and *P. fermentans* dominated the interior of cheeses, while *Db. hansenii* and *Geo. candidum* were dominant on the surface. Of the great diversity of yeasts in brine used for the production of blue cheese, *Db. hansenii* (am. *C. famata*) and *C. catenulata*, with at least ten species, predominant (van den Tempel and Jakobsen 1998). In a recent study, *Zygosaccharomyces*, *Pichia* and *Rhodotorula* species, *Geo. candidum*, and *Klu. lactis* have been identified as dominant yeasts in blue-veined cheese (Flórez and Mayo, 2006).

Seiler and Busse (1990) identified 23 species among 365 yeast strains isolated from the brines of soft, semihard, and hard cheeses from different manufacturers. Dominant species were *Db. hansenii* and *Trisp. cutaneum.* Other species such as *C. tenuis*, *C. intermedia*, *Klu. marxianus*, *S. cerevisiae*, and *C. parapsilosis* were detected in one dairy plant, suggesting that the yeast biota of different dairies may be quite specific and probably contributes to differences in sensory quality of cheeses. Diversity of yeasts is particularly broad in traditional and artisanal products and in cheeses still made from raw milk in some regions (Pereira-Dias et al., 2000; Cosentino et al., 2001; Romano et al., 2001; Fadda et al., 2004; Callon et al., 2006). In addition to yeasts frequently encountered (*Klu. lactis*, *Klu. marxianus*, *Db. hansenii*, *C. zeylanoides*), less common species are also detected, including *S. cerevisiae*, *S. unisporus*, *C. parapsilosis*, *C. intermedia*, *C. rugosa*, *C. silvae*, *P. fermentans*, and *P. guilliermondii*.

Yeasts play an important role in the ripening of Camembert cheese. They are part of a complex association of fungi that develops on the surface and contributes to ripening by producing proteases and lipases as well as utilizing lactic acid (Lenoir, 1984). Schmidt and Lenoir (1980) did not find differences between the inner and outer yeast population of Camembert cheese. A total of 193 strains were isolated from fresh cheese. *Klu. marxianus* (am. *C. kefyr*) and *Klu. lactis* (am. *C. sphaerica*) dominated, but *S. cerevisiae*, *Db. hansenii*, and *C. versatilis* were also present. After ripening, 482 strains were isolated. The major species were *Klu. lactis*, *Klu. marxianus*, capable of fermenting lactose, and *Db. hansenii*.

Yeasts also contribute to the production of Roquefort cheese by forming a slime on the surface (Besancon et al., 1992). *Db. hansenii* has been dominant, with *Klu. lactis* occurring less frequently. Many isolates tolerate 15–20% NaCl, assimilate lactose and lactic acid, and have lipolytic activity. All these properties contribute to the development of cheese aroma.

Application of molecular techniques has enabled to follow the succession of microbial association at both species and strain levels during ripening of cheeses (Petersen et al., 2001). Yeasts are dominant in the first days of ripening and include *Db. hansenii* and, depending on the variety of cheese, *Ya. lipolytica*, *Klu. lactis*, and *C. zeylanoides*. After about 5 days, the yeast population decreases with a concomitant increase of bacteria. This is made possible by the utilization of lactic acid and increase of pH (Valdés-Stauber et al., 1997; Petersen et al., 2002).

Recently, attempts have been made to use cheese-specific yeasts as adjunct cultures to accelerate ripening and flavor development. Because of their proteolytic and lipolytic activity, the yeasts *Db. hansenii*, *Ya. lipolytica*, and *Geo. candidum* may be used as starters coinoculated with LAB (Van den Tempel and Jakobsen, 2000; Ferreira and Viljoen, 2003; Boutrou and Guéguen, 2005; De Wit et al., 2005). Although *Geo. candidum* contributes to the proteolysis of soft cheese (Boutrou et al., 2006), it may weaken the inhibitory effect of *Penicillium camemberti* on mold contaminants (Decker and Nielsen, 2005). *Db. hansenii* and *Klu. lactis*, when grown in association with *Brevibacterium linens*, greatly improved the aromatic potential of the bacterium during cheese ripening (Arfi et al., 2005). Yeasts such as *Db. hansenii* and *Ya. lipolytica* also enhanced the activity of *Propionibacterium freudenreichii* ssp. *shermanii* in dry-salted cheese; in addition, they produced esters that impart a fruity flavor (Das et al., 2005).

Yeasts are frequent contaminants in many types of cheese. On cheddar, cream, and cottage cheeses, Tilbury et al. (1974) detected *Db. hansenii* most frequently, accompanied by *C. versatilis*, *C. intermedia*, and *P. anomala*. Suarez and Inigo (1982) isolated *C. rugosa*, *Geo. capitatum*, *Tsp. delbrueckii*, *S. cerevisiae*, *C. inconspicua*, and *Klu. marxianus* from Mahon cheese. In a Greek cheese, *P. membranifaciens* and *P. fermentans* were most frequently isolated, along with only a few isolates of *Klu. marxianus* and *S. cerevisiae* (Tzanetakis et al., 1987). From French-type soft cheese, *Geo. candidum* and *Ya. lipolytica* have been isolated (Senses-Ergul et al., 2006). Camembert and blue-veined cheeses have unique physical and chemical properties that select for the prevalence of a rather similar population of yeasts (Roosita and Fleet, 1996). In a survey including various dairy products from cottage cheese to hard cheeses from 14 different factories, although 26 yeast species have been identified, the two species that dominated all types were *Db. hansenii* and *Geo. candidum* (Vasdinyei and Deák, 2003). From the large number of studies, an overall conclusion can be drawn that the different types of cheeses still represent a specific ecological habitat in which a generally similar diversity of yeasts develops (Viljoen and Greyling, 1995; Welthagen and Viljoen, 1998, 1999; Westall and Filtenborg, 1998a).

Although yeasts can be beneficial in the ripening of cheeses, contributing to their flavor and aroma, excessive growth may cause undesirable sensory changes and lead to softening. Romano et al. (1989) suggested that yeasts were responsible for early blowing during ripening of Parmesan cheese. Swelling has also been caused by yeasts in soft cheese packaged in modified atmosphere (Westall and Filtenborg, 1998b). Moreover, under poor hygienic conditions, *C. albicans*, an opportunistic pathogenic yeast, may occur in cheese (El-Bassiony et al., 1980). *Geo. candidum* has been implicated in impaired taste and flavor when its population exceeded 10^4 g^{-1} in a German fresh-cheese type (Engel, 1986b). Other yeasts responsible for undesirable sensory changes include *Klu. marxianus*, *P. membranifaciens*, *Ya. lipolytica*, and *Db. hansenii*. In spoiled cottage cheese varieties stored at 7°C, *Db. hansenii*, *C. sake*, *C. zeylanoides*, *Cry. albidus*, *Cry. laurentii*, and *Ya. lipolytica* have been detected; the latter species grew in samples containing 500–600 mg L^{-1} sorbic acid (Brocklehurst and Lund, 1985). *Ya. lipolytica* and *Cry. laurentii* were the most common species, whereas *Spb. roseus* and *Guehom. pullulans* were responsible for the development of surface film.

7.8.4 Butter and Cream

Butter, spreads, and margarine are water-in-oil emulsions, with intrinsic factors limiting the growth of most microorganisms. High fat content, addition of salt and preservatives, and the aqueous phase comprising separated minor droplets control microbial activity (Delamarre and Batt, 1999). Few

data exist on yeasts in these dairy products. It was reported that spoilage of butter by yeast is rare (Fleet and Mian, 1987; Fleet, 1990b); however, in a recent study (Lopandic et al., 2006), a variety of yeasts were detected in spoiled butter, with the most frequent isolates belonging to *C. parapsilosis*, *C. zeylanoides*, *Ya. lipolytica*, and *Geo. candidum*. With sour cream, only *Klu. lactis* was associated.

7.8.5 Other Dairy Products and Eggs

Dairy products other than those discussed above are not free from yeasts. Ice cream and similar frozen dairy desserts readily support microbial growth. Cream desserts normally contain yeasts, of which *P. anomala* and *C. versatilis* are frequently isolated (Tilbury et al., 1974). Xerotolerant yeasts may cause spoilage in ice cream mix. Comi et al. (1992) isolated *Zygo. rouxii* most frequently from spoiled products, with *Zygo. bailii*, *Zygo. bisporus*, *Db. hansenii*, and *S. cerevisiae* occurring less frequently.

The disposal of cheese whey is a growing problem in the dairy industry. There have been suggestions for using whey for single-cell protein (SCP) and lactase production. Selected strains of *Klu. marxianus* (am. *C. kefyr*) and *Klu. lactis* appear most appropriate for these purposes (Gomez and Castillo, 1983; Moulin et al., 1983; Decleire et al., 1985; for a review, see Berry et al., 1987).

Shell eggs have been studied extensively from the bacterial point of view, with less attention focused on yeasts. Yeasts can grow in or on eggs and cause spoilage, particularly when packed and stored (Beuchat and Cousin, 2001). Levels of yeasts on unwashed eggs may reach log 2.0 cfu per piece but washing removes 30–40% of contamination (Jones et al., 2004; Musgrove et al., 2005). *Debaryomyces hansenii* (and its anamorph *C. famata*) is associated most commonly with eggs; however, a mixed and varied mycobiota occurs on them, comprising both ascomycetous and basidiomycetous yeast species.

7.9 MEAT, POULTRY, FISH, AND SEAFOODS

Most of the major intrinsic ecological determinants of microbial growth, namely, nutrients, moisture, and pH, are provided at near optimum levels in fresh meat, poultry, fish, and seafoods, as well as products prepared from them (Table 7.18). However, under these conditions, bacteria prevail rather than yeasts. Refrigeration is an indispensable condition for storage of meat and meat products and is the most important extrinsic factor in controlling the growth of microorganisms on these foods (McMeekin, 1982).

Numerous studies have been conducted on yeasts in meat, frankfurters, poultry, and shrimp, and these have been reviewed by Jay (1987), Fung and Liang (1990), and Samelis and Sofos (2003). Several surveys have been made to determine yeast species most frequently found on meat (Table 7.19), and these will be considered here.

TABLE 7.18
Meat as a Medium for Yeast Growth

Rich in nitrogenous substrates (proteins, amino acids, vitamins)
Rich in lipids (triglycerides, phosphatides)
Glycogen the main carbohydrate
High a_w (0.99) in fresh meat; reduced a_w (0.94–0.97) in cured and processed meat
pH 5.6–6.8 in fresh meat; pH < 5.5 in cured and fermented meat products
Temperature of chilled storage −5°C to 5°C

TABLE 7.19
Yeasts Frequently Isolated from Meat and Meat Products

Species	Sources	References
C. albicans	Beef, shellfish	3, 11, 14, 26
C. catenulata	Beef, sausage, poultry, shellfish	3, 11, 20, 33
C. diddensiae	Beef, fish, shellfish	3, 16, 17
C. glabrata	Poultry, fish, shellfish	3, 17
C. inconspicua	Beef, fish	11, 24, 27
C. intermedia	Beef, sausage, poultry, fish, shellfish	5, 6, 7, 12, 13, 14, 16, 17, 22, 23, 25, 32
C. norvegica	Beef, shellfish	3, 11, 26
C. parapsilosis	Beef, sausage, poultry, fish, shellfish	4, 5, 6, 10, 14, 15, 17, 18, 19, 20, 32
C. rugosa	Beef, sausage, poultry	11, 17, 20, 26
C. sake	Beef, shellfish	3, 11, 14, 15, 18, 26
C. tropicalis	Beef, fish, shellfish	3, 7, 17, 26
C. versatilis	Beef	7, 11, 16, 26
C. zeylanoides	Beef, sausage, lamb, fish	1, 7, 11, 12, 13, 16, 18, 19, 21, 24, 28, 30, 32
Cry. albidus	Beef, sausage, seafood	7, 11, 17, 18, 19, 20, 24
Cry. humicolus	Beef, seafood	7, 11
Cry. laurentii	Beef, lamb, ham, seafood	11, 12, 13, 14, 15, 16, 18, 19, 21, 23
Cystofilob. infirmominiatum	Beef, lamb, seafood	7, 12, 13, 18, 19, 21
Db. hansenii	Beef, sausage, ham, fish, shellfish, poultry	3, 4, 5, 6, 8, 9, 10, 11, 24, 27, 28, 29, 30, 31, 32, 33
Gal. geotrichum	Sausage, ham	4, 5, 8
Guehom. pullulans	Beef, sausage, lamb, poultry, fish	3, 6, 7, 8, 10, 11, 16, 18, 19, 20, 21
Hyphop. burtonii	Beef, ham, seafood	7, 8, 19
Kazach. exiguus	Beef, poultry, shellfish	3, 7, 17, 27
Klu. marxianus	Ham	29
P. anomala	Sausage, ham, fish, shellfish	3, 17, 20, 23, 27
P. guilliermondii	Sausage, seafood, shellfish	3, 19, 20
P. membranifaciens	Beef, fish	11, 17, 24
Rho. glutinis	Beef, poultry, fish, seafood, shellfish	1, 7, 9, 11, 15, 15a, 16, 17, 18, 19, 24, 27
Rho. minuta	Beef, sausage, poultry, fish	2, 7, 11, 16, 17, 18, 20, 24
Rho. mucilaginosa	Beef, sausage, ham, fish, shellfish	4, 7, 9, 11, 12, 13, 15, 15a, 16, 19, 20, 23, 24, 30, 31
Trisp. moniliforme	Beef, sausage, poultry, fish	4, 5, 7, 8, 12, 13, 17, 22
Ya. lipolytica	Beef, sausage, lamb, seafood, poultry	1, 11, 12, 13, 14, 16, 18, 19, 20, 25, 29, 30, 31, 32, 33

Notes: 1: Banks and Board (1987); 2: Bärwald and Hamad (1984); 3: Buck et al. (1977); 4: Comi and Cantoni (1980a); 5: Comi and Cantoni (1980b); 6: Comi and Cantoni (1980c); 7: Comi and Cantoni (1985); 8: Comi and Cantoni (1983); 9: Comi et al. (1984); 10: Comi et al. (1983); 11: Dalton et al. (1984); 12: Dillon et al. (1991); 13: Dillon and Board (1991); 14: Dillon and Board (1990); 15: Fleet (1990a); 15a: Hood (1983); 16: Hsieh and Jay (1984); 17: Jay (1987); 18: Johanssen et al. (1984); 19: Kobatake and Kurata (1980a); 20: Leistner and Bem (1970); 21: Lowry and Gill (1984); 22: McCarthy and Damoglou (1993); 23: Molina et al. (1990); 24: Ross and Morris (1965); 25: Viljoen et al. (1993); 27: Walker and Ayres (1970); 28: Cocolin et al. (2006); 29: Sanz et al. (2005); 30: Andrade et al. (2006); 31: Gardini et al. (2001); 32: Encinas et al. (2000); 33: Viljoen et al. (1998).

Source: Updated from Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL.

7.9.1 Red Meat and Meat Products

Yeasts contribute a small but permanent part of the natural microbiota on meat. The ability of some yeasts to grow at low temperature, high salt concentration, and under reduced oxygen tension enables them to proliferate in refrigerated, cured, and vacuum-packed meat and meat products. Yeasts, however, are not considered to be of great importance in the spoilage of these foods (Jay, 1987; Dillon and Board, 1991).

Dillon and Board (1991) and Dillon et al. (1991) surveyed the diversity of yeasts in pastures, on fleece and carcass surfaces, and in ground lamb and lamb products. Pigmented yeasts (*Rho. rubra* and *Rhosp. infirmo-miniatum*) occurred in pastures and on the fleece but remained consistently low on carcass surfaces and meat products. Yeast populations on carcasses are similar to those recovered from other meats and slaughterhouse environments (Baxter and Illston, 1976; Dalton et al., 1984; Lowry and Gill, 1984). *Db. hansenii*, *C. zeylanoides*, and *Cry. laurentii* dominated, whereas *Ya. lipolytica* and *Trisp. cutaneum* appeared only in ground lamb and lamb products. These investigations demonstrated a route of yeast contamination from the field via the slaughterhouse to the processing plant. It is evident from the literature that yeasts are an indigenous component of the microbial association of meats and meat products.

In a detailed study, Dalton et al. (1984) showed that *Db. hansenii*, *C. zeylanoides*, *C. vini*, *Cry. curvatus*, and *Rho. mucilaginosa* occurred in the slaughterhouse, in the air, on equipment, and in chilled storage rooms, as well as in fresh sausage and ground beef. *Pi. membranifaciens*, *Cry. laurentii*, *Leucosp. scottii*, and *Cry. humicolus* were detected in smaller numbers. *Trisp. cutaneum*, *Rho. glutinis*, *Rho. mucilaginosa*, and *Cry. laurentii* occurred mainly on slaughter equipment and in the air, while *C. mesenterica*, *P. carsonii*, and *Ya. lipolytica* appeared to get into the sausage through the addition of ingredients. Viljoen et al. (1993) described yeasts associated with Vienna sausage. The most prevalent isolates belonged to the genera *Candida* and *Debaryomyces*.

Yeasts have been shown to be an important component of the microbial association of British fresh sausage, because sulfite preservative selects for them (Dalton et al., 1984). Yeasts also dominate the microbiota of ground lamb products (Dillon and Board, 1990). The yeast biota recovered from ground beef consists of *Cry. laurentii*, *C. sake*, *C. zeylanoides*, and *C. parapsilosis*, and to a lesser extent, *Cry. albidus*, *Db. hansenii*, and *Ya. lipolytica* (Johannsen et al., 1984). After 14 days of storage at 4°C, the first three species persisted, but *Db. hansenii* became dominant. Baxter and Illston (1976) identified *Cry. laurentii*, *Db. hansenii*, and *P. membranifaciens* as psychrotrophic yeasts on meats, while Barnes et al. (1978) described a similar mycobiota on stored turkey carcasses; the population of psychrotrophic yeasts (*Cry. laurentii*, *C. zeylanoides*, and *Guehom. pullulans*) increased during refrigerated storage.

Banks and Board (1987) reported that, while the composition of the yeast population in meat products was similar, the size of the population differed. Low populations (10^2–10^4 cfu g^{-1}) were recovered from bacon and some types of fresh sausages, and yeasts attained high counts (10^4–10^6 cfu g^{-1}) in ground meat, poultry, and fresh sausages. The most frequent psychrotrophic yeasts isolated from these chilled foods were *C. zeylanoides*, *Db. hansenii*, *Ya. lipolytica*, *C. rugosa*, *Cry. humicolus*, *Cry. laurentii*, and *Rho. glutinis.* Comi and Cantoni (1985) reported that the low populations of yeasts in various meat products increased to 10^5–10^6 cfu g^{-1} in 7 days and 10^6–10^7 cfu g^{-1} in 14 days. *Candida* spp. (35%) and *Trichosporon* spp. (25%) dominated the yeasts in fresh meat. Their relation changed during refrigerated storage (*Trichosporon* 60% and *Candida* 20%), and population of *Cryptococcus* and *Rhodotorula* species increased. *Ambrosiozyma platypodis* and *Lipomyces starkeyi* present in fresh meat were absent from refrigerated samples. Compared to red meat, no consistent differences has been observed in yeast populations in poultry (Jay, 1987). Lin and Fung (1987) isolated 56 yeast strains from beef and ham products; *Ya. lipolytica* (48%), *Db. hansenii* (14%), and *C. zeylanoides* (11%) were the most frequent. In another study, Fung and Liang (1990) collected 41 yeast strains from various meats. Contrary to the earlier findings, *Ya. lipolytica* was not isolated. The identified species included *Db. hansenii*, *P. membranifaciens*, *S. cerevisiae*, *Tsp. delbrueckii*, and *C. versatilis*.

An increase in yeast population during refrigerated storage indicates that yeasts contribute to changes in the composition of meat that may lead to spoilage, although yeasts are rarely the direct cause and determining factor in the spoilage of meats and meat products (Dillon, 1998). Characteristics of yeasts recovered from fresh and spoiled ground beef appear similar (Jay and Margitic, 1981; Hsieh and Jay, 1984). On spoiled ground beef, a broad spectrum of yeasts occurs, but indigenous species such as *C. zeylanoides*, *P. fermentans*, and *Ya. lipolytica* remain dominant. About 60–80% of recovered strains belonged to the genus *Candida*, and some 20 species were represented by one or few isolates. *Db. hansenii*, *Ya. lipolytica*, and *Klu. marxianus* have been associated with the spoilage of commercial sliced and vacuum-packed ham (Sanz et al., 2005).

Surface desiccation inhibits bacterial growth on meat stored at refrigeration temperatures down to −5°C, and yeast may develop under these conditions. On lamb loins wrapped in plastic film and stored at −5°C, a population of yeasts developed, consisting of *Cry. laurentii*, *Rhosp. infirmominiatum*, *Guehom. pullulans*, and *C. zeylanoides* (Lowry and Gill, 1984). *Cr. laurentii* made up 90% of the yeast population.

Packaging operations may increase contamination by yeasts. *Ya. lipolytica*, *C. zeylanoides*, and *Db. hansenii* were isolated most frequently from vacuum-packaged Vienna sausage (Viljoen et al., 1993). Equipment surfaces were mainly responsible for the contamination. In contrast to vacuum packaging, nitrogen gas packaging was particularly effective in controlling the growth of yeasts on frankfurters stored at 3–7°C (Simard et al., 1983). Yeast counts were higher on nontenderized ham compared to tenderized ham, whereas vacuum packaging had no significant effect (Kemp et al., 1986).

Irradiation greatly reduces the incidence of yeasts on meat products. After gamma irradiation (1.5–3.0 kGy) of British fresh sausage, populations of *Tsp. delbrueckii* and *Db. hansenii* decrease substantially. *C. zeylanoides* and *Trisp. cutaneum*, in particular, appear to have high resistance to irradiation (McCarthy and Damoglou, 1993).

7.9.2 Fermented and Cured Meat

Meat fermentation is an ancient practice used to extend the shelf life of perishable raw meat. Addition of salt, nitrate, and nitrite initiate complex biochemical and microbiological changes. The ecological conditions favor the growth and activity of LAB. These play a leading role in the fermentation of meat by producing lactic acid and decreasing the pH to about 5.3. During fermentation, proteolytic and lipolytic reactions and other biochemical processes change the characteristics of meat. In addition to LAB, enterococci and coagulase-negative cocci also contribute to the production of aromatic substances resulting in the ripening of meat (Lücke, 1985; Rantsiou and Cocolin, 2006).

Curing of ham and bacon brings about substantial changes in the chemical composition of meat and also in the microbiota. Processing often results in increases in population of yeasts on meat products. During the preparation of sausages, various ingredients contaminated with yeasts may alter the original microbiota. Yeasts occur frequently on fermented sausages and country-cured hams and may either contribute to improved flavor or spoilage of these products. *Db. hansenii* occurs most frequently and *C. rugosa*, *C. catenulata*, and *Ya. lipolytica* are also often recovered from cured meat products (Leistner and Bem, 1970; Monte et al., 1986; Coppola et al., 2000). Comi and Cantoni (1980b, 1983) attributed some importance to the proteolytic and lipolytic activities of certain yeast species in the curing of ham and ripening of sausages. *Db. hansenii*, *Hyphop. burtonii*, *Trisp. cutaneum*, and *Guehom. pullulans* exhibited the highest activity. Molina et al. (1990) identified 120 yeast isolates from dry cured ham; 67% belonging to species similar to *P. anomala*, 19% to *Rhodotorula* spp. and the remainder to *Cryptoccus* and *Debaryomyces*. From Iberian dry-cured ham, in decreasing order of frequency, *Db. hansenii*, *Ya. lipolytica*, *C. zeylanoides*, *Db. polymorphus*, *P. carsonii*, *S. cerevisiae*, and *Rho. mucilaginosa* have been isolated (Núñez et al., 1996; Andrade et al., 2006). With the exception of *Db. hansenii*, which has been consequently detected in all six lots of different European dry sausages, other yeast species, for example, *C. guilliermondii*, *Iss. orientalis*, *C. parapsilosis*, *C. zeylanoides*, *Trisp. beigelii*, *Geo. capitatum*, and *Geo. candidum*, have occurred

only in one or two types of product; in one case *C. albicans* has also been observed (Lopez et al., 2001).

Microbial coverage that consists of molds and yeasts develops on the surface of some fermented sausages and yeasts. Salt-tolerant yeasts (*Db. hansenii*, *C. parapsilosis*, and *Rho. mucilaginosa*) can be detected, but probably do not exert a great impact on the ripening of dry sausages (Comi and Cantoni, 1980a). Grazia et al. (1989) observed, however, that the yeast biota of salami (82% of which consisted of *Db. hansenii*) influenced red color formation and improved product quality. This has been confirmed by several studies (Lücke, 1986; Grazia et al., 1989; Encinas et al., 2000; Gardini et al., 2001). *Db. hansenii* plays a dominant role in the ripening of dry sausage and raw ham, and many other types of yeast can also be isolated, such as *C. zeylanoides*, *P. triangularis*, *Met. pulcherrima*, and *Ya. lipolytica* (Baruzzi et al., 2006; Cocolin et al., 2006).

Fermented sausages are mostly prepared by natural, spontaneous process. Use of starter cultures that initiate rapid acidification and lead to desirable sensory quality of safer and healthier products has nevertheless been limited (Samelis et al., 1998). Attempts to develop new starter cultures with more functional properties are in progress (Leroy et al., 2006). In addition to LAB and cocci, certain yeast strains with a well-developed flavor, such as *Db. hansenii* and *C. utilis*, have also been considered to be included in the starter mixture (Sørensen, 1997; Olesen and Stahnke, 2000; Durá et al., 2004; Flores et al., 2004; Martin et al., 2006).

7.9.3 Poultry

The spoilage of poultry meat is largely attributed to bacteria (Jackson et al., 1997), but yeasts are also regularly present in the microbiota. Yeast population in the range of 10^2–10^4 cfu g^{-1} has been reported on fresh chicken carcasses, levels that may increase to 10^6 cfu g^{-1} during refrigerated storage (Gallo et al., 1988; Geornaras et al., 1994; Hinton et al., 2002). Diriye et al. (1993) detected very low populations of yeasts on the surface of frozen fish and chicken. Viljoen et al. (1998), in a detailed study including 159 isolates from fresh and spoiled chicken carcasses, identified 20 yeast species representing seven genera. *C. zeylanoides* (35–45%) and *Db. hansenii* (15–27%) have been most frequent; *Rho. mucilaginosa*, *Cry. laurentii*, and *C. blankii* were isolated from fresh samples only, whereas *Trisp. beigelii* and *Trisp. cutaneum* were isolated from spoiled samples only. Poultry spoilage is mainly restricted to the surface of the carcass, and the processing equipment is the major source of contamination. Laubscher et al. (2000) established the presence of high and diverse populations of yeasts in the trachea of chickens, already adapted to the habitat, and they may contribute to the spoilage of poultry meat after slaughtering.

Ismail et al. (2000) isolated 152 strains in 12 species from 50 commercial chicken and turkey products, including whole and ground, fresh and roasted items, as well as in liver, heart, and gizzard samples. *Ya. lipolytica* (39%) and *C. zeylanoides* (26%) were predominant, and most strains showed proteolytic and lipolytic activities. Basidiomycetous species, *Rho. mucilaginosa* in majority, have mostly occurred in ground poultry meats. Although some isolates of *Ya. lipolytica* and *C. zeylanoides* have been characterized by molecular tests (ITS-PCR, RAPD, PFGE; Deák et al., 2000), follow-up studies including DNA sequencing revealed differences in strains identified as *Ya. lipolytica*, resulting in the description of a novel species, *C. galli* (Peter et al., 2004). Immersion and spraying with chlorinated water are techniques commonly used in poultry processing; however, immersion treatment using various chemicals in model experiments with *Ya. lipolytica* to decrease the level of contamination were only partially successful (Ismail et al., 2001). Decoctions of basil, marjoram, sage, and thyme significantly reduced the population of *Ya. lipolytica*, but did not control its growth during storage at 5°C.

7.9.4 Fish and Seafoods

Fish and shellfish are excellent substrates for the growth of microorganisms. Typically, bacteria dominate the microbiota. The incidence of microorganisms in seafoods is influenced mainly by the extent

of pollution of the water from which harvest is made and by the temperature of storage. Pagnocca et al. (1989) demonstrated that yeast species in water and sediment are similar to those of shrimps taken from them. *Db. hansenii* was the most frequent species isolated, followed by *C. parapsilosis* and *C. intermedia.* The extent of water pollution strongly influenced the yeast population of white shrimp.

Though smaller in number than bacteria, yeasts are widely distributed in both seawater and freshwater (Morris, 1975; Hagler and Ahearn, 1987). A large number of species have been isolated from marine and freshwater animals (Jay, 1987). Although some marine yeast is thought to be pathogenic to their host, it appears that most are saprobionta.

There is no indication that characteristic yeast biota would associate with different types of fish. Ross and Morris (1965) reported that yeast population was similar on 16 species of fish, with *Db. hansenii* dominating (50%), followed by *C. inconspicua* and *C. parapsilosis.* Yeast species frequently isolated from fish and shellfish reflect species prevalent in water from which they are taken. Included are red-pigmented yeasts (*Rho. glutinis*, *Rho. mucilaginosa*, and *Rho. pallida*), other basidiomycetous yeasts (*Cry. albidus*, *Rhosp. infirmo-miniatum*, *Trisp. cutaneum*, *Guehom. pullulans*, and *Cry. humicolus*), and also some ascomycetous yeasts such as *C. parapsilosis*, *C. zeylanoides*, *Ya. lipolytica*, and *P. guilliermondii* (Kobatake and Kurata, 1980a; Hood, 1983). Buck et al. (1977), in a broad survey on shellfish, noted the presence of more than 30 yeast species, among which were several species known to be associated with human infection (*C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*).

There is little evidence to suggest that yeast play a significant role in the spoilage of stored fish and shellfish. Some reports, however, indicate that psychrotrophic yeasts may contribute to certain deleterious changes (Comi et al., 1983, 1984; Kobatake and Kurata, 1983a). Kobatake et al. (1992) reported that proteolytic psychrotrophic yeasts are widely distributed in raw seafoods. In particular, *Ya. lipolytica*, *Guehom. pullulans*, and *Leucosporidium scottii* were active species at low (0–5°C) temperatures. *Ya. lipolytica* possesses strong proteolytic and lipolytic activities and may contribute substantially to the spoilage of chilled raw seafoods (Kobatake et al., 1988).

Fresh and frozen oysters may undergo pink discoloration due to the occurrence of red-pigmented yeasts. *Rho. rubra* and *Rho. glutinis* make up 32% of the yeast population, together with *C. stellata*, *C. sake*, *C. parapsilosis*, and *Cry. laurentii* (Fleet, 1990a).

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8 Detection and Enumeration

Determination of the extent of yeast contamination in foods and beverages is an essential part of quality assurance and quality control in the food industry. Likewise, enumeration of desirable yeasts and detection of undesirable ones associated with fermented foods and beverages are important in quality management programs. Cultivation using growth media is the traditional method for enumeration and detection of yeasts. The ideal media for these purposes should repress the growth of bacteria and molds and be nutritionally adequate to support all yeast species, including fastidious types. Although media with all these characteristics do not exist, several media have been formulated that satisfy general-purpose use, and several others have been devised to select specific groups or types of yeasts. Mycological media are also used in isolation and identification of yeasts.

Earlier literature on these media was referred to in the first edition of this handbook, including proceedings of international workshops on the methods of mycological examination of foods, which provide extensive information on cultivation media and methods (King et al., 1986; Samson et al., 1992). Recent reviews and chapters provide comprehensive coverage on the subject (Pitt and Hocking, 1997; Beuchat, 1998; Beuchat and Cousin, 2001; Deák, 2003a; Loureiro et al., 2004).

Traditional cultivation methods and newer techniques for detecting and enumerating yeasts in foods are presented here; formulas for the media most commonly used in the food industry are given in the appendix.

8.1 CONVENTIONAL ENUMERATION TECHNIQUES

The procedure for the detection and enumeration of yeasts from food usually involves a number of steps after the preparation of media and equipment, such as obtaining and homogenization of the sample, serial dilution of the suspension, inoculation and incubation of media, and the assessment of the original population size in the primary sample (enumeration, counting). These steps are followed by the isolation and purification of individual strains that can be subjected to a process of identification and typing by testing the morphological, physiological, biochemical, and molecular characteristics of the culture. These procedures are discussed separately in Chapter 9.

8.1.1 Preparation of Samples

For reliable microbiological analysis of foods, proper sampling procedures are essential. A sample should be representative of the whole lot from which it is taken. To this end, statistical sampling plans are recommended for the microbiological qualification of various foods (ICMSF, 1986). Aseptic techniques must be followed during collection and transportation of samples.

In the laboratory, subsamples are removed and prepared for analysis. Food samples may be solid or liquid, and samples can also be derived from the food environment, from the air, and from the surface of processing equipment. A common requirement for all samples is to bring them into suspension aseptically. Solid samples need to be homogenized with a sterile blender or in a sterile plastic bag when using a stomacher (Deák et al., 1986a). In most cases, the initial suspension of food samples is prepared in a 1:10 dilution, which can be further diluted when required.

Peptone water (0.1% solution of peptone) is commonly used for the preparation and dilution of samples (Samson et al., 1992). However, in certain cases, other diluents should be used to achieve

maximum recovery. This is often the case when high-sugar foods are analyzed for the occurrence of xerotolerant yeasts. In order to minimize osmotic shock, a buffered solution of diluents should be used (Beuchat and Hocking, 1990; Corry et al., 1992).

Beuchat and coworkers have evaluated combinations of diluents and cultivation media for their efficacy in enumerating *Zygo. rouxii* in intermediate a_w foods. Abdul-Raouf et al. (1994) evaluated combinations of five diluents (0.1% peptone water, 40% and 50% glucose, and 18% and 26% glycerol) with three media (tryptone glucose yeast extract agar, TGY; dichloran 18% glycerol agar, DG18; and malt extract yeast extract 50% glucose agar, MY50G) for enumerating *Zygo. rouxii* in sugar, corn, chocolate, fruit syrups, condensed milk, and pastry fillings. Hernandez and Beuchat (1995) tested diluents consisting of 0.1% peptone water and up to 50% glucose or 35% glycerol on various enumeration media for enumerating *Zygo. rouxii* in blueberry syrups (a_w 0.818, 0.870, and 0.921). The overall conclusion was that the use of 40% or 50% glucose diluent in combination with MY50G agar could be recommended for enumerating xerotolerant yeasts from high-sugar, intermediate a_w foods.

Sometimes, instead of dilution, concentration of cell suspensions may be necessary. This can be achieved by centrifugation or filtration. The need for concentrating arises if too few cells are present to be countable after inoculation of the medium. This is often the case with the investigation of samples of food subjected to heat treatment. Membrane filtration is one of the best methods for concentrating microbial cells.

8.1.2 General-Purpose Media

Numerous media have been developed for the detection, enumeration, and isolation of yeasts from foods (King et al., 1986; Fleet, 1990; Deák, 1991, 2003a). Pitt (1986) lists the multiple requirements for an ideal fungal cultivation medium, which include being nutritionally adequate to support growth and permit maximal recovery from all foods while suppressing bacterial growth completely and restricting the spread of molds to facilitate enumeration. No single medium exists to meet all requirements, and an informed choice should be made to apply the best purpose-fitting medium among those currently available. Table 8.1 compiles the types of media used for the detection, enumeration, and isolation of yeasts from foods.

TABLE 8.1
Media for the Detection, Enumeration, and Isolation of Yeasts from Foods

- General, basal media
  - Malt extract agar (MEA)
  - Sabouraud glucose agar (SGA)
  - Potato dextrose agar (PDA)
  - Tryptone glucose yeast extract agar (TGY)
- Selective media for fungi
  - Acidified
    - Potato dextrose agar (APDA)
    - Tryptone glucose yeast extract agar (ATGY)
  - Antibiotic supplemented
    - Oxytetracycline glucose yeast extract agar (OGY)
    - Chloramphenicol tryptone glucose yeast extract agar (TGYC)
  - Retarding moulds
    - Rose Bengal chloramphenicol agar (RBC)
    - Dichloran Rose Bengal chloramphenicol agar (DRBC)

Traditionally, acidified media have been used for the cultivation of yeasts, such as malt extract with a pH of 3.5 to inhibit the growth of most bacteria. Acidified malt extract and tryptone glucose yeast extract broth or agar, as well as potato dextrose agar, are commonly used general-purpose media today. The pH should be adjusted with appropriate amounts of 10% tartaric acid before pouring the agar medium. Acidified media are suitable for analyzing yeasts associated with high-acid foods such as fruit purees, pickles, and kefyr (Beuchat and Nail, 1985; Deák, 1992); however, acidified media are generally inferior to antibiotic-supplemented media for the enumeration of yeasts in products whose pH is in the range 5.4–6.8 (Beuchat, 1979; Thomson, 1984; Deák et al., 1986b). Comparing 10 media, Welthagen and Viljoen (1997) demonstrated that antibiotic-supplemented media resulted in higher counts than acidified media in dairy products of neutral pH, whereas no difference has been observed between the two types of media with cheeses and yoghurt at low pH.

Antibacterial antibiotics, such as oxytetracycline, gentamicin, and chloramphenicol, can be used alone or in combination (Mossel et al., 1970, 1975; Koburger and Rodgers, 1978). Use of chloramphenicol is convenient because it is heat stable and can be added together with other ingredients before autoclaving (Samson et al., 1992). These antibiotics in a concentration of 100 mg L^{-1} are usually not inhibitory to yeasts; however, Banks and Board (1987) observed that gentamicin prevented the growth of several yeasts in a concentration of 50 mg L^{-1}.

On acidified media or antibacterial antibiotic-supplemented media, yeasts and molds can develop simultaneously. In food microbiology, these fungi are often considered indicators of overall contamination, expressed in terms of "yeast and mold count." Considering the great differences in physiology, growth, and metabolism, the joint count of yeasts and molds has no real meaning for assessing their role in foods (Deák, 2003b). Although molds, in general, develop colonies more slowly than yeasts, certain zygomycetous fungi (mostly *Mucor* and *Rhizopus* species) produce excessive mycelium that easily covers the surface of media, making enumeration and isolation difficult. Attempts have been made to allow the enumeration of yeasts separately from molds and to control the growth of spreading molds. Rose Bengal and dichloran tend to restrict spreading growth of molds, and thus facilitate the counting of yeasts. King et al. (1979) described a medium containing these compounds as well as chloramphenicol for the inhibition of bacteria, and this dichloran Rose Bengal chloramphenicol (DRBC) agar has become one of the most commonly used media in food mycology. However, on exposure to light, Rose Bengal becomes cytotoxic for yeasts (Banks et al., 1985; Chilvers et al., 1999); hence, the media containing it should be stored in the dark.

Auremine, gentian violet, or malachite green may also serve as a media supplement for inhibiting the growth of fungi (Bragulat et al., 1995). An inter-laboratory evaluation of 11 different selective media indicated that DRBC, DG18 (see below), and MEA supplemented with NaCl and oxytetracycline ranked equally in recovering yeasts in the presence of molds and bacteria from blue-veined cheese. Biphenyl inhibited molds effectively.

8.1.3 Cultivation and Enumeration Methods

When agar media are used, viable counts are estimated by either the pour plate or the spread plate technique. One advantage of the pour plate method is the somewhat greater sensitivity by inoculating a 1 mL aliquot sample in contrast to the 0.1 mL that can be spread on the surface of agar in a standard Petri dish. However, plating is achieved by pouring molten agar media, heated to about 45°C, which imposes a heat stress on yeast cells (Kennedy et al., 1980). Beuchat et al. (1992b) indicated that spread plating results in better recovery of yeasts than pour plating. From comparative studies, it can be concluded that spread plating is preferable to pour plate methods for giving significantly higher counts (Ferguson, 1986; Deák et al., 1986b; Seiler, 1992a).

The temperature of incubation can also influence the development of yeast colonies. In general, a temperature between 25°C and 28°C is appropriate for most yeasts, and even an ambient room temperature suffices in most cases. As discussed before (Section 3.2.1.1), no true thermophiles exist

among yeasts, but an incubation temperature of 37°C can be of value for distinguishing between some species. On the other hand, psychrotrophic and psychrophilic yeasts are common in some foods, and for the enumeration of these, an incubation temperature lower than 25°C must be applied. At this temperature, the length of incubation is longer, and 5–7 days is recommended for the maximum recovery of yeasts from chilled meat and dairy products (Banks et al., 1987). Cultivation of xerotolerant yeasts and development of colonies on selective media also require longer incubation time (Beuchat, 1998). Five days at 25°C can be considered a standard incubation regime for yeasts (Beuchat et al., 1991; Hocking and Pitt, 1992).

Enumeration is often made by counting the colonies developed on plates. It is assumed that each colony arises from a single cell originally inoculated into the medium. However, this is not always the case. Even after thorough homogenization, chains or clumps of cells may remain and produce a single colony. Hence, instead of cell counts, the term colony-forming units (CFUs) per g or mL is commonly used in food microbiology.

The most probable number (MPN) method is used in liquid media, inoculating 3–5 tubes of broth per dilution. Statistical tables are used to convert the turbidity data generated into viable count per mL. Compared to plate counts, MPN often results in higher counts, but this is due to the lower level of statistical significance (Koburger and Norden, 1975; Deák et al., 1986b). The MPN method allows a larger volume of inocula to be used and can be applied for enumeration of low populations. Membrane filtration is a more appropriate method when low numbers of cells are to be enumerated (Deák et al., 1986b).

Indirect estimates of cell populations can be made using various correlations of cell number with turbidity, metabolic activity, or dry mass. In order to find this correlation, the actual cell numbers should first be determined by the plate count method at various degrees of turbidity, enzyme activity, or cell mass. For example, absorbance values, measured using a spectrophotometer, can be converted to cell numbers with the aid of standard calibration curves. However, direct proportionality between absorbance and cell mass occurs only at low cell concentrations. Gas production is used to estimate the raising power of baker's yeast in dough.

8.2 SELECTIVE AND DIFFERENTIAL MEDIA

The type of growth medium used and the conditions of incubation will affect the result of cultivation. Hence, plate count methods can be used very flexibly with respect to the kind of organisms and the type of foods. With the appropriate media and incubation types, various groups or species of yeasts can be detected in food and beverage samples. Selective media permit the development of specific yeasts while controlling the growth of most others, whereas differential media permit the growth of several yeast species that can, however, be distinguished by the different color, shape, or size of colonies. Several media with these properties have been formulated (Beuchat, 1993; Loureiro et al., 2004).

8.2.1 Xerotolerant Yeasts

Detection and enumeration of xerotolerant yeasts is of importance, for these are capable of developing and causing spoilage in intermediate- and low-moisture foods (a_w 0.85–0.65). This property serves as the basis for formulating media selective for this group of yeasts.

General-purpose media adjusted to low a_w by adding high amounts of sugar ($\geq$50%) can be used for selective detection of xerotolerant yeasts; however, for satisfactory results, the medium and the diluents should be osmotically balanced (Beuchat and Hocking, 1990; Cava and Hernandez, 1994; Hernandez and Beuchat, 1995). Senses and Özbas (2004) showed that a malt-yeast extract basal medium supplemented with 25% glucose and 25% fructose, giving an a_w of 0.95,

completely inhibited the growth of *S. cerevisiae*, decreased that of *Z. bailii* somewhat, and did not exert any effect on *Zygo. rouxii*, whereas PDA supplemented with 10% glucose and 40% sucrose (a_w 0.92) permitted the growth of both *Zygosaccharomyces* species and inhibited *S. cerevisiae* only partially.

Hocking and Pitt (1980) developed a medium for the recovery of xerophilic fungi containing 18% glycerol (a_w 0.955), dichloran, and chloramphenicol (DG18). It has proved suitable for the enumeration of yeasts from low-a_w products (Cava and Hernandez, 1994; Braendlin, 1996). DG18 performed equally well with malt extract yeast extract agar with 30% glucose (MY30G) and tryptone yeast extract agar with 10% glucose (TY10G) to recover xerotolerant yeasts from concentrates (Andrews et al., 1997). Beuchat et al. (1998a) compared DG18, TG10Y, and MY50G in combination with various diluents (0.1% peptone water, 40% glucose, and 30% glycerol) and found that all combinations, with the exception of 0.1% peptone water, performed well in the recovery of *Zygo. rouxii* from a wide range (a_w 0.73–0.85) of intermediate-moisture foods. Owing to its good performance and the convenience of using a commercial product, DG18 has become widely used not only for detecting xerotolerant fungi but also as a general-purpose medium for the enumeration of molds and yeasts (Dijkman, 1986; Seiler, 1992b). It has been demonstrated, however, that commercial batches of DG18 may perform differently (Frändberg and Olsen, 1999) and moreover that DG18 may retard colony growth and even inhibit some yeasts (Beckers et al., 1986; Deák, 1992; Nuñez et al., 1996). Beuchat et al. (2001), using various media in a collaborative study, observed significantly lower counts on DG18 even in comparison with acidified potato dextrose agar. Another collaborative study (Deák et al., 2001) demonstrated that compared to DRBC and TGYC, DG18 is inferior in terms of performance and cannot be recommended as a general-purpose enumeration medium. It does perform well in its original purpose—detecting xerotolerant molds and yeasts.

8.2.2 Preservative-Resistant Yeasts

Another group of food spoilage yeasts, such as *Zygo. bailii*, *P. membranifaciens*, *Iss. orientalis*, is notable for high resistance to preservatives and acids (Deák et al., 1992). Acidified media are generally recommended for detecting and enumerating these yeasts. Basal media, MEA, or TGY, supplemented with 0.3–0.5% acetic acid, is selective for preservative-resistant yeasts (Makdesi and Beuchat, 1996a,b). In a comparative study (Hocking, 1996), ATGY (0.5% acetic acid) proved to be the most effective selective medium for these yeasts. A strongly selective medium containing 0.5% acetic acid and 0.1% potassium sorbate permits only the development of *Zygo. bailii* (Erickson, 1993).

8.2.3 Wild Yeasts

In the fermentation industry, all species other than the producing strain (starter, mostly specific brewing, wine, or baking strains of *S. cerevisiae*) are considered contaminants and are collectively called "wild yeasts" (see Section 7.4.1). The presence of wild yeasts among pitching yeasts is a serious problem in a brewery. Their detection is a difficult task because species of *Candida*, *Dekkera*, *Pichia*, and other genera (so-called non-*Saccharomyces* wild yeasts) as well as strains of *S. cerevisiae* and other *Saccharomyces* species may be present that are very similar to the brewing strain (Back, 1987; Campbell, 1996). A variety of selective and differential media have been developed based on nonutilized substrates, inhibitors, and dyes (Jespersen and Jakobsen, 1996; Dull et al., 1998; Van der Aa Kühle and Jespersen, 1998) (Table 8.2).

A related problem exists in the baking industry—in detecting wild yeast contaminants that make up about 7% of baker's yeast (Viljoen and Lues, 1993). In wine making, indigenous yeast species inevitably occur on grapes and winery surfaces, whether or not *S. cerevisiae* wine strains

TABLE 8.2
A Compilation of Various Media for Detection of Wild Yeasts in Breweries

Medium	Selective component
Lysine	Nitrogen source
CLEN	Cadaverine, lysine, ethylamine, and nitrate
XMACS	Xylose, mannitol, adonitol, cellobiose, and sorbitol
Actidione	Inhibitor
Copper	Inhibitor
Lin's LWYM	$CuSO_4$
Schwartz	Fuchsin sulfate
Crystal violet	Dye
Dextrin	Carbon source

Source: Adapted from Jespersen, L. and Jakobsen, M. (1996) Int. J. Food Microbiol. 33:139–155; Van der Aa Kühle, A. and Jespersen, L. (1998) Int. J. Food Microbiol. 43:205–213; Deák, T. (2003a) *In*: Yeasts in Foods. Beneficial and Detrimental Aspects (eds. Boekhout, T. and Robert, V.), Behr's Verlag, Hamburg. pp. 39–68.

are inoculated. Detection of non-*Saccharomyces* species is somewhat easier than the typing of *S. cerevisiae* strains, which can be performed effectively only by molecular tests (see Section 9.7) (Kish et al., 1983; Heard and Fleet, 1986; Jiranek et al., 1995).

8.2.4 Media for Specific Yeasts

In clinical diagnosis, a number of media have been developed for the direct detection and presumptive identification of pathogenic yeasts *Cry. neoformans* and *C. albicans.* These usually include dyes or chromogenic substrates facilitating differentiation by producing an easily recognizable product on the action of certain specific enzymes (Chaskes and Tyndall, 1978; Kwon-Chung et al., 1982; Rouselle et al., 1994; Willinger et al., 1994; Baumgartner et al., 1996; Fricker-Hidalgo et al., 2001; Eraso et al., 2006). Recently, a chromogenic medium, CHROMagar Candida, has been commercialized that successfully differentiates *C. albicans* and other *Candida* species of clinical significance (Odds and Bernaerts, 1994; Pfaller et al., 1996; Aubertine et al., 2006). Trials have been made to exploit the discriminatory power of CHROMagar in other fields, including food mycology (Bovill et al., 2001; Tornai-Lehoczki et al., 2003). In some cases, closely related species of food-borne yeasts show different color on this medium, separating, for example, *Zygo. bailii* from *Zygo. rouxii* and *Klu. lactis* from *Klu. marxianus*.

Earlier attempts to develop media for detecting yeasts in specific food products (other than fermentations, mentioned earlier) resulted in partial success. Inhibitors and substrates giving a color reaction are included in media intended to be applied in the enumeration of yeasts in meat products (Dillon and Board, 1989; Fung and Liang, 1990), dairy products (Welthagen and Viljoen, 1997; Viljoen et al., 2004), and tropical fruits (Rale and Vakil, 1984).

Recently, significant developments have been achieved in devising specific media for several food-borne yeast species (reviewed by Loureiro et al., 2004). Various strategies have been applied, such as differential dyes, unique carbon sources, and enzyme activities in media specific for *Ya. lipolytica*, *Zygo. bailii*, *Tsp. delbrueckii*, *Db. hansenii*, and *Dekkera* and *Kluyveromyces* species (Table 8.3).

TABLE 8.3
Species-Specific Media

Species	Selective Agent	Reference
Candida albicans	Aniline blue	Goldschmidt et al. (1991)
	CHROMagar	Odds and Bernaerts (1994)
Cryptococcus neoformans	Aminophenols	Chaskes et al. (1978)
Food-borne yeasts	CHROMagar	Tornai et al. (2003)
Debaryomyces hansenii	β-Glucosidase	Siloniz et al. (2000)
Dekkera spp.	4-Ethylphenol, acetate	Rodrigues et al. (2000)
Kluyveromyces marxianus	β-Galactosidase, X-gal	Valderrama et al. (1999)
K. marxianus, K. lactis	X-gal	Nguyen et al. (2000)
Saccharomyces cerevisiae	Eosin, methylene blue	Siloniz et al. (2000)
S. cerevisiae, H_2S-producing	Bismuth sulfite	Jiranek et al. (1995)
Torulaspora delbrueckii	Acetate, tellurite	Siloniz et al. (1999)
Zygosaccharomyces bailii	Acetate, sorbate	Ericksson (1993)
Zygo. bailii, Zygo. bisporus	Formic acid	Schuller et al. (2000)
Yarrowia lipolytica	Crystal violet	Fung and Liang (1990)
	Tyrosin	Carreira and Luoreiro (1998)

8.3 EVALUATION OF MEDIA

Media used in the quality assurance of foods and beverages should be subjected to quality control themselves. Evaluation of media is necessary to assess their performance and test their suitability, and in order to validate and standardize their use. In the laboratory, the sensitivity and accuracy of every new batch prepared or purchased are to be tested and controlled. Within the framework of ICFMH, the Working Party on Culture Media has been established to focus special attention on this important subject, organize meetings, and publish monographs (Baird et al., 1995; Corry et al., 1995; Corry, 1998). Reviews have also been dedicated to the evaluation of media (Wenk, 1992; Curtis and Beuchat, 1998). Although interest is focused on bacteriological media (only three media, OGY, DRBC, and DG18, used for yeasts are dealt with in these monographs), the importance of quality control of media is being increasingly recognized in food mycology (Deák, 2003a).

The recovery of stressed microorganisms and toxicity of selective media to injured cells are particularly relevant issues (Beuchat, 1984, 1986). Golden and Beuchat (1990) reported that the recovery of sublethally heat-injured cells of *Zygo. bailii* is affected by the type of solute in the medium. At equal a_w of 0.936, glucose allowed better recovery than sucrose, sorbitol, or glycerol. Fleet and Mian (1998) observed that after freezing and thawing, 5–20% of injured yeast cells are not detected on antibiotic-containing selective media, even after resuscitation for 3 h at 25°C in 2% malt extract broth. Desiccation and long-term storage at freezing and chilling temperatures cause injury and decrease recovery on the selective media DRBC, APDA, and DG18 to a larger extent than on TGYC (Beuchat et al., 2001).

8.4 RAPID AND ALTERNATIVE METHODS

Food microbiology has traditionally relied on cultivation techniques in assessing the safety, stability, and quality of products. Although these techniques are flexible and inexpensive and provide both qualitative and quantitative data on the types and numbers of microorganisms, they are labor intensive (media preparation, dilution, plating, incubation, counting, isolation, and further characterization of isolates) and slow, requiring several days to obtain results, thus providing retrospective information. Moreover, the results are less reliable, because they are influenced by the cultivation conditions

TABLE 8.4
Limitations of Cultivation Techniques

Sampling: attachment of cells to surfaces, biofilms
Dilution and plating: osmotic shock, heat stress
Minor species lost on dilution
Inappropriate growth medium
Aerobic incubation
Viable but noncultivable cells
Long time to obtain results

TABLE 8.5
Devices and Instruments Accelerating Conventional Cultivation Methods

Technique	Application
Sampling	
Stomacher	Homogenization
Gravimetric diluter	Dilution
Counting	
Spiral plater	Enumeration
Petrifilm	Plate counts
Dipslides	Colony counts
DEFT	Microscopic counts
HGMF	Microscopic counts

(Table 8.4). The food industry requires adequate data at the appropriate time for ensuring the safety, quality, and shelf life of products and for monitoring and control of processing. To achieve these goals, in past decades, several alternative, rapid, and automated methods have been developed based on novel physical, chemical, immunological, and molecular principles. This section deals with these techniques as they apply to the mycological investigation of foods. The subject has been reviewed several times over the years by experts (Fung, 1991, 2002; Gourama and Bullerman, 1995; van der Vossen and Hofstra, 1996; de Boer and Beumer, 1999; Loureiro et al., 2004) and by this author (Deák, 1994, 1995, 2001, 2003a,b,c).

8.4.1 Modified Cultivation Methods

Improvements of conventional cultivation methods have been made in media preparation, plating techniques, and counting methods by using devices and automated instruments for saving time and providing convenience (Table 8.5). Sample preparation can be facilitated with the use of a gravimetric diluter, stomacher, and pulsifier. The spiral plater spans 3–4 decimal dilution steps by distributing a small sample volume on the surface of a Petri dish. Petrifilm and Simplate replace agar plates with ready-to-use prepared media on membranes and plastic devices, respectively (Beuchat et al., 1998b). Both Petrifilm and Simplate produce results comparable to traditional plating in enumerating yeasts and molds from various foods (Beuchat et al., 1990, 1991, 1992b; Vlaemynck, 1994; Spangenberg and Ingham, 2000; Taniwaki et al., 2001).

The hydrophobic grid membrane filter (HGMF) technique, commercialized as the IsoGrid system, works on the MPN principle, confining the growth of microorganisms to 1600 grid cells fabricated on a membrane. Positive grids are counted under a microscope. The large number of grid cells allows counting in a 3–4 log range without dilution (Brodsky et al., 1982; Erickson, 1993; Entis and Lerner, 1996). Colony counters eliminate operator fatigue in microscopic and manual counting but may result in errors due to differences in colony size and the difficulty of differentiating colonies from sample particles.

8.4.2 Direct Counting

Direct microscopy, such as the Howard mold count, has been used for a century to detect mold contamination in tomato products. Methylene blue staining has also been used for a long time to discriminate between living and dead cells, for example in compressed baker's yeast (Smart et al., 1999).

An alternative microscopic method is the direct epifluorescent filter technique (DEFT), which also differentiates between live and dead cells stained with a fluorescent dye (Kiskó et al., 1997). Enumeration under a fluorescent microscope is fast; however, it also causes operator fatigue. An instrument coupling DEFT with image analysis offers a fully automated but more expensive counting system (Pettipher et al., 1992).

Flow cytometry is an instrumental method with far more capability than microscopic techniques; it has the potential to enumerate yeast counts in various foods and beverages (Jespersen et al., 1993; Joosten et al., 1996; Bouix et al., 1999) and to analyze single cells as well as heterogeneous microbial populations (Davey and Kell, 1996). Combined with various fluorescent staining and molecular labeling techniques, flow cytometry has been used to assess yeast viability (Millard et al., 1997; Abe, 1998; Deere et al., 1998) and to monitor fermentation activity in brewing, wine making, and baking (Guldfeldt et al., 1998; Attfield et al., 2000; Malacrino et al., 2001). The large capacity of commercially available instruments with automated sample processors permits data to be generated for predictive modeling (Sørensen and Jakobsen, 1997). Coupling it with molecular probes has extended the power of flow cytometry to the identification of yeasts (Page et al., 2006).

8.4.3 Biochemical Methods

ATP is produced by all living organisms, and an ATP assay provides a good estimate of metabolic activity. The assay is based on the production of light (bioluminescence) due to the utilization of ATP in the luciferin–luciferase system. Yeast population size shows good correlation with ATP content in beverages and dough (Autio and Mattila-Sandholm, 1992; Seiler and Wendt, 1992). However, for specific application, a major problem is the separation of ATP from yeasts and from other microorganisms and plant and animal cells in food particles. Moreover, ATP may derive from live or dead cells. Hence, ATP bioluminescence is most applicable for assessing general contamination. As the detection time is very short (<10 min), the method fits HACCP schemes and real-time monitoring (Hawronskyj and Holah, 1997). Table 8.6 shows that ATP bioluminescence is the fastest of the rapid methods available.

Long-chain fatty acid composition has been suggested as a method to differentiate and identify yeast species (Augustyn et al., 1992). Recently, Loureiro and coworkers (Malfeito-Ferreira et al., 1989, 1997; Sancho et al., 2000; Loureiro et al., 2004) revived the subject, demonstrating that culture conditions do not significantly influence the fatty acid composition of cells. Based on the presence or absence of 18:2 and 18:3 polyunsaturated fatty acids, three groups of species have been identified. Group 1 (characterized by the absence of C18:2 and C18:3 fatty acids) includes strongly fermentative yeasts (*Hanseniaspora*, *Saccharomyces*, and *Schizosaccharomyces* species) that can cause spoilage in wine, fruit juices, and soft drinks. To group 2 (with C18:2 but without C18:3) belong species

TABLE 8.6
Comparison of Detection Time and Sensitivity of Some Rapid Methods

Method	Detection Time	Sensitivity (log cfu g^{-1})	Specificity
ATP bioluminescence	5–10 min	3–4	No
DEFT	30 min	3–4	No
Flow cytometry	30 min	2–3	Good
Immunology	1–2 h	4–5	Good
Chitin assay	6–8 h	4–5	Moderate
Electrometry	6–24 h	1–2	Moderate
Petrifilm	24–48 h	2–3	Good

such as *Zygosaccharomyces*, *Dekkera*, *Torulaspora*, and *Yarrowia*, which are the most dangerous spoilage yeasts in all branches of the food industry. Group 3 (containing both C18:2 and C18:3 fatty acids) includes a large number of weakly or nonfermentative yeasts (members of the genera *Candida*, *Pichia*, *Debaryomyces*, *Rhodotorula*, and others) that can be regarded as indicators of poor hygiene and lack of good manufacturing practice. This "zymological indicator" concept awaits further confirmation, easier methods for fatty acid profiling, and development of a large database before it gains widespread acceptance in the food industry.

8.4.4 Physical Methods

Metabolic breakdown of substrates during the growth of microorganisms results in changes in the electrical resistance of the medium that can be measured. By the monitoring of impedance, capacitance, or conductance changes, metabolic activity can be estimated in terms of detection time, which in turn can be calibrated against the population size. Thus, electrometry can be used for estimating the number of viable cells. Electrometry has already become an accepted method for monitoring microbial activity. Changes in impedance and conductance can be measured in commercially available automated instruments that have large sample capacity. Detection of yeasts by impedimetry or conductimetry has found wide application in the beverage and fruit juice industries and also for testing various other foods (Evans, 1982; Zindulis, 1984; Connolly et al., 1988; Henschke and Thomas, 1988). The technique can be adapted to detection of specific yeasts and used in direct or indirect mode (Owens et al., 1989; Deák and Beuchat, 1994). Indirect conductimetry has proved more sensitive than the direct method, has been applied to detect yeasts in fruit juices and carbonated beverages (Deák and Beuchat, 1993a,b,c, 1994), and is sensitive enough to detect very low populations of yeasts in beverage concentrates (Deák and Beuchat, 1993d, 1995).

Near-infrared spectroscopy (NIR) and Fourier transform infrared spectroscopy (FTIR) provide rapid, reagentless, nondestructive screening methods for the analysis of fungal contamination on solid material such as grains (Greene et al., 1992). Specific FTIR spectra allow rapid and reliable identification of yeasts (Henderson et al., 1996; Kümmerle et al., 1998). Application of the method requires standardized sample preparation for cell mass estimation and a reference spectrum database for identification.

In recent years, efforts have been made to estimate fungal contamination by the detection of volatiles with an electronic nose system (Dickinson et al., 1998; Schnürer et al., 1999; Magan and Evans, 2000). Volatile production patterns can be specific to a species, and electronic nose systems can be developed for qualitative analysis of spoilage fungi and pathogenic yeasts (Casalinuovo et al., 2006). The system has been used for detection of spoilage yeasts in milk (Magan et al., 2000).

8.4.5 Immunological Methods

Whereas immunological tests and commercial kits are widely used in food bacteriology and, to some degree, in the detection of mycotoxins (Li et al., 2000), their application in the detection of food spoilage yeasts is lagging far behind. Although many types of yeasts produce thermostable antigens (Midelhoven and Notermans, 1988, 1993), growth conditions influence the amount of antigens excreted. Antibodies to detect these antigens are not available commercially. Enzyme-linked immunosorbent assay (ELISA) has been developed for only a few yeast species (*S. cerevisiae*, *Brettanomyces* spp., and *Candida* spp.) (Kuniyuki et al., 1984; Yoshida et al., 1991; Middelhoven and Notermans, 1993). Although immunoassays are rapid, specific, and sensitive, they have not yet received their due place in the study of food-borne yeasts.

8.4.6 Molecular Techniques

Molecular techniques have revolutionized biology and biotechnology and gained wide application in all fields of food microbiology, including the study of yeasts. Various molecular methods are common tools in research and are promising tools in the routine analysis of clinical and food samples. However, the high specificity and sensitivity of molecular methods predetermine their use for the detection, identification, and typing of microorganisms rather than for quantitative enumeration. Molecular techniques are discussed in the next chapter.

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9 Identification

The importance of yeasts in the spoilage of foods is being increasingly recognized (Beuchat, 1987; Deák, 1991; Fleet, 1992; Boekhout and Robert, 2003). Accurate identification of yeast isolates is an essential part of the analysis of yeast associated with a food product. Identification of species allows technologists to evaluate the spoilage potential of yeasts by relating their physiological properties to the ecological attributes of the food system. It is obvious that proper identification of yeasts occurring in foods would form a sound ecological basis for the improvement of processing technologies and the assessment of product quality. Hence, it is imperative for the food laboratory to have the capacity to identify yeast isolates rapidly and accurately as a routine part of process control and quality assurance.

The traditional methods for identifying yeasts rely on morphological, physiological, and biochemical characters (investigation of phenotypic properties) and require in-depth expertise in conducting and evaluating some 90 or more diagnostic tests. These methods are complex, expensive, and time consuming. Various approaches have been tried to facilitate the identification of yeasts. These include miniaturized and simplified techniques, commercially available manual kits and automated systems, and nontraditional techniques. The introduction of molecular methods has radically altered the method of identification and provided a sound basis for the recognition of species.

9.1 PHENOTYPIC IDENTIFICATION PROCEDURES

Many criteria are used in the classification and standard description of yeasts. The presence or absence of morphological and physiological characteristics has traditionally served for the identification of unknown isolates. Standard procedures and tests used for the characterization and identification of yeasts are described in detail by Lodder (1970) and van der Walt and Yarrow (1984), and in manuals and practicals (Barnett et al., 1983, 1990a; Kreger-van Rij, 1987; Kurtzman, 1988; Smith and Yarrow, 1988; Kurtzman et al., 2003).

Traditional identification procedures rely heavily on morphological criteria of vegetative and sexual reproduction, including ultrastructural studies of cell walls, septae, and spores. Tests involving electron microscopy are not, however, routinely used in yeast identification. The standard identification procedures include several physiological and biochemical tests to determine the fermentation of sugars and the assimilation of (aerobic growth on) carbon and nitrogen compounds. In addition, the conditions for growth and several other characteristics are important in identification. Table 9.1 summarizes the major phenotypic criteria traditionally used.

Many yeasts reproduce sexually; others reproduce asexually. Classification of taxa at the highest level has been based on the formation of sexual states (Ascomycota or Basidiomycota); in their absence, an artificial taxon (Deuteromycota) was formed. Further differences in sexual and vegetative reproduction are used to define families and genera (see Table 2.2). Classification based on molecular phylogeny has altered yeast taxonomy; however, the mode of reproduction has remained an important trait in phenotypic identification.

The detection of sexual forms and spores often requires crossing complementary mating types from separate colonies or involves microscopic examination of cells grown on several different media to induce sporulation during a lengthy incubation period. Hence, finding sexual spores can be very time consuming and is often uncertain. Sometimes, ascospores are abundantly present in fresh isolates. When the shape and structure of spores, the persistence of asci, and the occurrence of conjugation are observed, the identification of an isolate is more easily achieved.

Various forms of vegetative reproduction in yeasts are also important in their identification. Modes of budding (multilateral, bipolar), splitting cells, formation of arthroconidia and ballistoconidia, and some other microscopic features of morphology (e.g., shape and size of cells, development of true hyphae or pseudohyphae), as well as characteristics of macroscopic growth (colony type, color, formation of pellicle on liquid medium, etc.), are to be noted and recorded for the purpose of identification.

Standard physiological and biochemical tests are primarily used in the identification process. The ability to ferment certain sugars is tested using the Durham tube method to detect whether carbon dioxide production occurs. However, under semianaerobic test conditions, carbon dioxide development does not strictly correlate with the ability of yeast to produce ethanol anaerobically (van Dijken et al., 1986).

The standard method for testing assimilation of carbon and nitrogen compounds consists of cultivation in liquid medium at 30°C for 3 weeks. Quicker results can be obtained by using shaken cultures or by the auxanographic method in agar medium. The results of assimilation are influenced by the method of testing. Auxanography is not considered to be as reliable as cultivation in broth. Nevertheless, with the auxanographic methods, results can be obtained within 2 or 3 days, and contaminants are more readily detected.

Other physiological tests of importance in identification include growth at 37°C in vitamin-free medium containing 50% glucose or 10% sodium chloride. Some physiological characteristics, such as growth in the presence of cycloheximide or at 37°C, can be used widely in yeast identification, while others, such as growth in a vitamin-free medium or in the presence of 1% acetic acid, are useful for the identification of certain species.

Two biochemical tests are of great value for differentiating ascomycetous and basidiomycetous yeasts. These are the diazonium blue B (DBB) color test and the urease reaction. The DBB test is positive only with basidiomycetous yeasts. The quick (4 h) positive urease reaction in rapid urea broth correlates well with the presence of basidiomycetous yeasts; however, exceptions do occur. The most notable exception is the genus *Schizosaccharomyces*. Also, extended incubation of *Ya. lipolytica* and possibly a few other strongly proteolytic yeasts may give false-positive urease tests (Péter and Deák, 1991).

The choice and number of tests used in the standard description vary among species and genera (Kreger-van Rij, 1984; Kurtzman and Fell, 1998). Moreover, experts describing various groups of yeasts in taxonomic treatises have used slightly different methods. Data presented in the manuals by Barnett et al. (1983, 1990a) cover some 90 characters, and these books are good sources of valuable and comparable information for identifying yeasts.

Once morphological and physiological data have been determined, the identity of a yeast isolate can be established using identification keys based on the description of the recognized species. Traditional identification keys are hierarchical and first require a generic assignment based on of life cycle, sexual propagation, and other morphological data. To avoid difficulties in determining these characters, keys have been devised using mostly or exclusively physiological responses. Barnett et al. (1983, 1990a) published dichotomous keys of this kind that require knowledge of some 60–90 characters, among them results of some tests used infrequently, such as requirements for individual vitamins.

Performing the traditional identification procedure requires considerable experience and skill in the execution and evaluation of a large number of specified and standardized phenotypic tests. Traditional methods are also complex, time consuming, and expensive. The determination of some 60–90 characteristics is hardly a feasible task for routine identification of hundreds of isolates in an industrial laboratory. In recent years, much effort has been put into facilitating the identification of yeasts by improving and simplifying traditional methods, introducing nontraditional techniques, developing commercial identification systems, and applying computerized identification programs and databases.

9.2 IDENTIFICATION KITS AND SYSTEMS

Early approaches in the methodology of yeast identification were directed toward the improvement of conventional identification methods. Assimilation reactions were critically studied, and redundancies due to common pathways in the utilization of certain substrates were revealed (Fiol, 1975; Barnett, 1977; Novak et al., 1977). The technique of auxanography has been improved, and the basal medium has been modified (Land et al., 1975; Michelsen et al., 1977). Rapid methods for testing nitrate assimilation have been described (Hopkins and Land, 1977; Pincus et al., 1988), and a rapid test for urease reaction has been introduced (Roberts et al., 1978). Efforts have been made to enhance the detection of sporulation (Su et al., 1985). Proposals have been made to increase the efficacy of carbon assimilation tests for discriminating among yeast species by introducing new substrates (Golubev and Blagodatskaya, 1978; Seiler, 1991). Although these approaches contributed significantly to the improvement of techniques for the identification of yeasts, they remain within the limits of conventional physiological methods. Another line of research has led to the miniaturization of identification tests in an attempt to develop rapid and simple techniques. Manual methods make use of the technical advantage of serological microtiter plates to accommodate small amounts of substrates used in traditional fermentation and assimilation tests. This technique has found application in the identification of food-borne yeasts (Lin and Fung, 1987a; Seiler and Busse, 1988; Heard and Fleet, 1990). Recently, a new system called YeastIdent-Food/ProleFood has been developed for the identification of yeasts from foods (Velázquez et al., 2001). It is based on 24 physiological and biochemical manual tests, and the evaluation is supported by computer software comprising a database of 168 species.

These improvements paved the way for the development of commercial ready-to-use systems in various microwell formats and provided a stimulus for further development of automated identification systems (Fung, 1982; McGinnis, 1982). The impetus for advancement has come largely from interest in the identification of clinically important yeasts. Several miniaturized kits and systems have been developed and marketed over the past decades. Some are designed to be used manually, while others are automated to various degrees (Table 9.1). API 20C, Uni-Yeast Tec, Abbott Quantum II, Vitek ATB32, Automicrobic, and similar systems are growth based; that is, they require inoculation of pure isolates and provide results after 2–3 days of incubation. Several systems require additional tests or morphological investigations; without extra tests, they provide low-degree (50–80%) accuracy of identification (Land et al., 1991a; Stager and Davis, 1992; Paugham et al., 1999).

Some commercially available systems provide accurate and reliable results, giving 90% or more agreement with data obtained by traditional methods. The API 20C system is probably the most widely used and has often been considered a reference method for evaluating other systems (Salkin et al., 1987; St. Germain and Beauchesne, 1991). Compared to the traditional method or to the API 20C manual kit, automated systems are less accurate; however, updated versions of the AutoMicrobic and VITEK systems compare favorably with the reference method (El Zaatari et al., 1990; Aubertine et al., 2006). Automated systems still require time for preparing and incubating identification panels, but subjectivity in reading and evaluating the results is eliminated (Lin and Fung, 1987a). The semiautomated, computer-linked Biolog system is based on 94 biochemical tests arranged in a microtiter tray. It is aimed at rapid identification of clinical yeasts against a large database (267 species). However, less than one-third of substrates are usually used for the identification of yeasts, and in evaluating the system with food and beverage isolates, only 68% of them were correctly identified (Praphailong et al., 1997).

Novel approaches use enzyme-based rapid reactions (Salkin et al., 1987; Land et al., 1991b) (Table 9.1). Chromogenic substrates (nitrophenyl-linked carbohydrates and amino acid-β-naphthylamides) are used to assess the presence and activity of intracellular enzymes in various yeasts. These systems provide very rapid results that can be read within 4 h. Of the commercialized systems, the recent version of the MicroScan panel appears to be reliable for identification

TABLE 9.1
Characteristics of Some Commercial Yeast Identification Systems

System	Method	No. of Tests	No. of Species in Database	Time (h) Required for Result	Accuracy (%)	References
Growth-based systems						
API 20C	Manual	20	42	72	99	St. Germain and Beauchesne (1991)
ATB 32ID	Manual/automated	32	63	48	91	Rohm and Lechner (1990)
AutoMicrobic	Automated	30	62	24	83–97	Pfaller et al. (1988), El-Zaatari et al. (1990)
Auxacolor	Manual	15	26	48	91	Campbell et al. (1999)
Microring YT	Manual	6	18	48	53	Shankland et al. (1990)
Minitek	Manual	12	28	72	97	Lin and Fung (1987b)
Quantum II	Automated	20	34	24	82–86	Pfaller et al. (1988), Salkin et al. (1985)
Uni-Yeast-Tek	Manual	15	42	48	40	Salkin et al. (1987)
Biolog	Semiautomated	96	267	48	52	Praphailong et al. (1997)
VITEK 2	Automated	46	53	15	89–99	Aubertine et al. (2006), Graf et al. (2000)
Enzyme-based systems						
Microscan	Manual/automated	27	42	4	85	St. Germain and Beauchesne (1991), Land et al. (1991b)
YeastIdent	Manual	20	42	4	55–60	El-Zaatari et al. (1990), Salkin et al. (1987)
RapID Yeast	Manual	18	42	4	96	Espinel-Ingroff et al. (1998)

Source: Updated from Deák, T. (1993) Int. J. Food Microbiol. 19:15–26. With permission from Elsevier.

TABLE 9.2
Application of Commercial Systems for the Identification of Food-Borne Yeasts

System	Accuracy (%)	No. of Species Not in Database	References
API 20C	74	13	Subden et al. (1980)
	30	9	Deák and Beuchat (1988)
	33	26	Török and King (1991)
Minitek	97	5	Lin and Fung (1987b)
AutoMicrobic	90	7	Lin and Fung (1987b)
ATB 32 ID	50	25	Rohm et al. (1990)
YeastIdent	20	9	Deák and Beuchat (1988)
	19	26	Török and King (1991)
Biolog	52	0	Praphailong et al. (1997)

Source: Updated from Deák, T. (1993) Int. J. Food Microbiol. 19:15–26. With permission from Elsevier.

(St. Germain and Beauchesne, 1991). The VITEK 2 system is a fully automated instrument dedicated to the identification of clinical yeasts with fluorescent or chromogenic biochemical tests, allowing results to be obtained in 15 h (Graf et al., 2000; Aubertine et al., 2006).

All of these commercially available identification systems were designed to meet the needs of clinical diagnosis. For this reason, their databases are restricted to 60 or fewer yeast species of clinical importance. Efforts to apply these techniques to the identification of food-borne yeasts remain only partially successful (Table 9.2). The most reliable commercial systems could be used for the identification of food-borne yeasts if their databases are extended and certain additional tests are performed (Lin and Fung, 1987b; Deák and Beuchat, 1988, 1993a).

9.3 SIMPLIFIED IDENTIFICATION SCHEMES

In food microbiology, the main disadvantage of many identification methods is that several phenotypic physiological tests require a long time to perform, causing delay in the process of evaluation, which is not acceptable to most food processors. Food mycologists have attempted to elaborate simple and rapid methods dedicated to the identification of yeasts isolated from foods. Various identification schemes make use of morphological observations using selective media and integrate selected determinative tests and miniaturized traditional methods (Davenport, 1981; Lin and Fung, 1987a; Smith and Yarrow, 1988; Heard and Fleet, 1990). These methods and keys, though specifically designed for food-borne yeasts, consider only a small number of the most important species and rely on only a few tests. Hence, their potential for identification of food isolates is limited. As an extreme example, Rodriguez (1987) described an identification scheme for wine spoilage yeasts based only on five tests. For monitoring processing technologies, assessing the quality of products, or conducting ecological surveys of foods, one is interested in the general profile of a yeast population and the dominant species whose physiological attributes determine the keeping quality of a particular food under given storage conditions (Deák, 1979). Although foods represent widely diverse niches from an ecological point of view, the occurrence in foods of many types of yeasts that have adapted to other specific habitats such as ambrosia beetles or necrotic cacti is highly improbable (Davenport, 1977; Phaff and Starmer, 1980). In turn, each type of food is likely to be inhabited by yeast species that fit or readily adapt to the specific ecological conditions inherent in that food. Thus, it is possible to target yeasts that have been found in foods, or even in a specific

TABLE 9.3
Calculated Prior Probabilities of Occurrence (%) of Most Frequent Food-Borne Yeasts[a]

Species	All Foods	Fruits, Beverages, Wine, Beer	Meat, Dairy Products	Low-a_w Products	Low-pH Products
S. cerevisiae	7.04	6.40	6.70	7.53	7.37
Db. hansenii	6.2	4.61	8.65	5.64	4.73
P. anomala	4.56	4.25	4.19	3.53	5.52
P. membranifaciens	4.32	4.43	3.35	3.53	3.95
Rho. glutinis	4.17	2.58	5.86	1.64	1.84
Rho. mucilaginosa	3.78	3.45	3.63	0.94	2.10
Tsp. delbrueckii	3.64	4.68	1.95	7.53	2.10
Klu. marxianus	3.36	2.21	3.91	4.11	9.79
Iss. orientalis	3.20	3.23	2.56	1.76	3.95
Zygo. bailii	3.05	4.76	1.14	4.94	5.52
C. parapsilosis	2.80	1.38	4.42	1.12	5,26
Zygo. rouxii	2.66	3.20	1.67	9.41	4.21
C. guilliermondii	2.52	2.40	2.23	1.41	2.37
Cry. albidus	2.33	1.45	2.44	0.82	1.84
C. tropicalis	2.08	1.85	2.05	1.41	1.58
Kazach. exiguus	1.76	1.11	2.42	1.88	2.63
P. fermentans	1.60	1.60	1.30	1.88	1.05
Guehom. pullulans	1.54	0.46	3.16	0.47	2.10
Hsp. uvarum	1.34	3.20	—	0.59	—
C. zeylanoides	1.14	0.37	2.42	0.47	0.53

Note: [a] Data from Table 7.5.
Source: Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL. p. 210.

type of food, and devise a simplified identification scheme confined to these yeasts by selecting those phenotypic diagnostic tests that are most efficient for discriminating this particular group of yeasts.

As presented in the survey of the literature on yeast species isolated from the main types of foods in Chapter 7, it appears that some 50–100 species may commonly occur, depending on the type of food. The greatest number of yeasts reside on fruits, fruit products, and beverages, namely, soft drinks, fruit juices, and wine, in which the occurrence of about 100 species has been described. Some 60–70 species may occur on vegetables and dairy and meat products, whereas only about 30–40 species have been found in foods that offer a more restricting environment such as salted, brined, and fermented products and those containing high sugar concentrations. Barnett et al. (1990a) presented a specific key to identify food-borne yeasts, considering the sources of isolates recorded in the original description, which include 111 yeast species. Further keys include 42 species for soft drinks, 94 species for wine, and 42 species for beer. Deák and Beuchat (1987) compiled a list of 215 food-borne yeasts from a survey of the literature published since the 1950s. From these data, Deák (1993) estimated the frequencies of food-borne yeasts, presented in more detail by Deák and Beuchat (1996) and tabulated and discussed previously in Chapter 7. Table 9.3 summarizes calculated frequencies of the 20 most common food-borne yeast species. In contrast to the diversity of incidental yeast contaminants, yeast biota, consisting of a moderate number of species, can be expected to occur in samples from any type of food. About 15–20 species can generally be detected in a given food product, of which only two or three species will be dominant, and the majority will be represented by one or two strains (Table 9.4).

TABLE 9.4
The Composition of Yeast Biota in Various Foods

Food	Total Number of Species	Number of Dominant Species[a]	Number of Minor Species[b]	Reference
Fruits	18	1	14	Deák (1988)
Decaying citrus	11	4	1	Spencer et al. (1992)
Sweet corn	20	2	13	Deák and Beuchat (1988)
Carrots	11	2	6	Babic et al. (1992)
Orange concentrate	12	3	5	Deák and Beuchat (1993b)
Must	11	3	6	Moore et al. (1988)
Must	13	2	3	Holloway et al. (1990)
Winery plant	23	3	13	Deák (1988)
Spoiled bottled wine	7	1	4	Deák (1988)
Sugar cane	8	1	7	Anderson et al. (1988)
Cassava flour	10	1	9	Okagbue (1990)
Pickles	5	1	3	Deák (1988)
Olive brine	13	2	11	Marquina et al. (1992)
Vegetable salad	5	2	1	Buick and Damoglou (1989)
Oriental fermented food	19	2	13	Suzuki et al. (1987)
Cheese brine	17	2	0	Seiler and Busse (1990)
Kefyr	4	1	0	Rohm et al. (1992)
Minced lamb	15	3	Not stated	Dillon and Board (1991)
Vienna sausage	22	2	12	Viljoen et al. (1993)

Notes: [a] Represented in >10% of isolates.
[b] Represented by one or two isolates.

Accurate identification for taxonomic purposes must be based on standard methods; in ecological studies, simplified methods can be used. In an industrial setting, where hundreds of strains isolated from a range of food samples are to be identified routinely, efforts are made to save time and labor by applying simplified identification methods. It is usually acceptable if a few isolates are identified only presumptively or even incorrectly, because such misidentification does not distort the overall picture of the yeast biota (Barnett et al., 1990a). Simplified identification keys can be constructed by selecting the most effective diagnostic tests and restricting for food-borne yeasts. Keys of these kinds concerning specific habitats—for example, wines, strawberries, or meat products—have been constructed and successfully applied (Buhagiar and Barnett, 1971; Lin and Fung, 1987a; Heard and Fleet, 1990; Kotzekidou, 1997). Deák (1986) proposed a simplified identification key for yeast species associated with food. The key was extended by Deák and Beuchat (1987) to cover a wide range of food-borne yeasts (over 200 species). Later, improved versions were elaborated, under the name SIM (for simplified identification method), which restricted the number of yeasts to 80 of the most frequently occurring food-borne species (Deák, 1991, 1992). SIM has been tested and compared with traditional methods and commercial identification systems (Deák and Beuchat, 1988, 1993a; Deák, 1992). The major advantage of SIM is that it usually requires only 15–20 tests, and identification can be attained within a week. Saving labor, materials, and time, SIM allows food microbiologists to cope more easily and rapidly with the formidable task of yeast identification.

Deák and Beuchat (1993b) compared two commercial kits (API 20C and Yeast ID32C) and SIM for their performance in identifying 166 yeast isolates from concentrated fruit juices and beverages. In this study, 25 species representing 15 genera were identified. The percentage of isolates correctly identified by SIM was 91%, whereas the API 20C and ID32C systems correctly identified 86% and 76%, respectively. The number of species not included in the databases was 4 in SIM, 10

in API 20C, and 7 in ID32C. Discrepant test reactions and errors in databases were responsible for failed and false identification. Nevertheless, the accuracy of individual test reactions was high, giving only 1.6% (SIM), 2.5% (API 20C), and 1.7% (ID32C) discrepant results. Both commercial kits, if supplemented with a few tests necessary for the identification of yeasts, can be easily applied to the SIM database developed for food-borne yeasts, thus offering a convenient and accurate identification procedure.

SIM has also been evaluated by peers and, in general, has been judged acceptable and reliable (Golden et al., 1987; Abbey et al., 1988; Fleet, 1990; Molina et al., 1990; Okagbue, 1990; Demuyakor and Ohta, 1991; Török and King, 1991; Venturini et al., 2002; Senses-Ergul et al., 2006). SIM has also received criticism, mainly for not considering the full range of variations within certain species (Rohm and Lechner, 1990; King and Török, 1992). Indeed, for the sake of simplicity and rapidity, in devising SIM, accuracy of identification has been sacrificed to some degree by disregarding less frequent variations within species. Rohm and Lechner (1990) examined the reliability of the 1987 version of SIM, which covers over 200 species (Deák and Beuchat, 1987), by subjecting it to a computerized identification program (Barnett et al., 1985). The program's database, different from that of SIM, considers several variations that were omitted from SIM in order to simplify it, and, in light of this, in a number of cases, accurate identification could not be achieved. On the basis of these experiences and critical suggestions, in the first edition of this handbook (Deák and Beuchat, 1996), a revised and improved version of SIM was described, including 99 yeast species that occur most frequently in various foods. The criteria for selecting diagnostic tests have been described there and in the earlier versions of SIM (Deák, 1986; Deák and Beuchat, 1987), and will not be recapitulated here. An updated and extended version of SIM is presented in Appendix 2.

9.4 COMPUTER-ASSISTED IDENTIFICATION

Parallel to the rapid developments in computer technology generally, applications to the identification of microorganisms have also been developed. Computer programs have been devised not only for the storage and retrieval of data matrices but also for the selection of the most effective diagnostic characters and for the construction of identification keys and diagnostic tables. A further prerequisite for the use of computers in microbial identification is the accumulation of large data banks for taxons and an elaboration of principles of numerical taxonomy. This has been achieved for bacteria (Willcox et al., 1980; Williams, 1992), and the same principles can be easily adapted for yeasts, as discussed by Robert (2003).

Barnett and coworkers were the first to construct computerized identification keys for yeasts (Barnett and Pankhurst, 1974; Barnett et al., 1983), and the program has been commercialized (Barnett et al., 1985, 1990b, 2000). Although dichotomous keys make use of carefully selected and weighted characters, such keys are sequential; they are based on positive and negative reactions of single tests at each stage. Hence, an aberrant result at any stage can lead to misidentification. A further disadvantage of these computerized keys lies in the large number of tests (50–60) required before the application of the key.

Simultaneous with technical developments associated with new and improved identification techniques, many commercial manual kits and automated systems are now supplemented by computerized identification schemes, offering rapid and objective results. In conjunction with commercial identification systems, the numerical codes and profile numbers have become well known and widely used. Character data for the unknown isolate are first determined and then converted into a code number (e.g., 1, 2, 4), the sequence of which (a numerical profile) is then compared against the identification database. For example, the API 20C kit for yeast generates a seven-digit number from a set of 20 tests (API, 1982). A printout of the profile index serves as a full identification matrix, since each profile represents a unique pattern of species included in the database. Automated systems usually provide an online database and identification matrix (e.g., Microlog YT for Biolog, ID-YST

for Vitek 2). Manufacturers of manual identification kits also offer access to computerized profile indices.

Robert (2003) provides an extensive review of computerized identification systems for yeasts and also introduces polyphasic identification systems called ETI and BioloMICS, developed by experts working at the University of Amsterdam and CBS, respectively (Robert, 2000; Boekhout et al., 2002).

The most advanced computer programs are based on probabilistic data matrices. These express the identification characters in frequency percentages. The product of any set of characters provides a probabilistic score for each taxon. A set of characters for the unknown isolate is then compared to these probabilities. The computer program defines the overall similarity of the unknown against taxons included in the database, and the unknown is then assigned to the taxon with the highest probability (Willcox et al., 1980). Probabilistic programs have been developed and made available for the identification of yeasts (Compass, 1986; ASM-CAIM, 1989; Barnett et al., 1990b, 2000). The experience from using these programs is that mechanized application may lead to erroneous identification (Rohm and Lechner, 1990), and computerized programs and systems can yield reliable results only in the hands of experienced workers (King and Török, 1992). These authors warn against the assumption that high similarity scores always mean correct identification. One reason for discrepancy is that the probability method involves an erroneous assumption, namely that the frequencies of occurrence of various taxa (called prior probabilities) are equal. Certainly, this does not hold when any food sample, clinical specimen, or other material is being analyzed. The mathematical method of Bayesian analysis takes into account the different prior probabilities of occurrence, that is, the prevalence of different species in a particular type of material, and enables the calculation of the so-called posterior probabilities that would give the highest rate of correct identification (Berger, 1990). In practice, however, it is difficult to estimate prior probabilities. Based on the compilation of a large amount of published data, the prior probability of occurrence of yeast species in food materials was presented for the first time by Deák (1993). An extensive list of calculated frequencies is presented in Table 9.5, whereas Table 9.3 shows a shorter list of frequencies of yeasts most commonly found in foods. Deák (1993) demonstrated how the identification scores changed when prior probabilities are considered. Using the data from an earlier version of SIM (Deák, 1993), a computerized program has been developed that applies a system designed by Reva et al. (2001) for the identification of aerobic spore-forming bacteria.

Computers also have applications in the evaluation of data obtained by some nontraditional techniques. It is extremely difficult, if not impossible, to compare the large set of peaks or bands obtained by gas chromatography of fatty acids or by electrophoresis of proteins and DNA fragments. A variety of computer programs have been devised to facilitate their analysis, starting from the scanning of gels or printouts in a densitometer to remove background noise and to normalize fingerprints. Computation has now an essential and indispensable role in the analysis of molecular data and offers great potential for application in identification. Several databases are available through the Internet, for example, for sequence comparison at GenBank (National Center for Biotechnology Information), EMBL (European Molecular Biology Laboratory), or DDBJ (DNA Databank of Japan). In the GenBank database are accessible the D1/D2 sequences (about 600 bp) of the large subunit ribosomal RNA gene of over 1000 ascomycetous and basidiomycetous yeast species (Kurtzman and Robnett, 1998; Fell et al., 2000), which currently represent the largest dataset available for purposes of taxonomy and identification (see Section 9.6.4).

9.5 NONTRADITIONAL IDENTIFICATION TECHNIQUES

To overcome the inherent slowness of traditional cultivation methods, alternative rapid and instrumental methodologies have been developed based on the analysis of proteins, isoenzymes, and fatty acids.

TABLE 9.5
Calculated Frequencies (%) of Yeasts in Foods

Species	All Foods (99 species)	Fruits, Beverages, Wine, Beer (96 species)	Meat, Dairy Products (74 species)	Low-a_w Products (56 species)	Low-pH Products (62 species)
Bret. naardenensis	0.05	0.09	—	—	—
Bulleromyces albus	0.09	—	0.21	—	—
C. albicans	0.54	0.37	1.40	—	0.53
C. apicola	0.52	0.65	0.56	1.41	0.39
C. boidinii	0.55	0.43	0.56	—	1.05
C. cantarellii	0.15	0.23	0.14	0.35	—
C. catenulata	0.45	0.28	0.52	0.39	—
C. diddensiae	0.20	0.03	0.42	—	0.26
C. diversa	0.10	0.14	—	—	—
C. etcellsii	0.45	0.48	0.35	1.06	1.58
C. glabrata	0.72	0.86	0.35	0.70	1.05
C. inconspicua	0.58	0.41	0.73	—	0.79
C. intermedia	0.84	0.74	0.84	0.35	0.39
C. lactiscondensi	0.15	0.49	0.14	0.94	0.53
C. magnoliae	0.66	0.46	0.84	0.70	1.18
C. maltosa	0.09	0.14	—	—	—
C. mesenterica	0.09	—	0.21	—	—
C. norvegica	0.45	0.46	0.37	—	0.53
C. oleophila	0.09	0.09	0.07	—	0.79
C. parapsilosis	2.80	1.38	4.42	1.12	5.26
C. rugosa	0.60	0.18	1.26	—	1.18
C. sake	1.32	1.72	0.74	0.47	2.10
C. stellata	0.95	1.66	0.21	0.35	0.39
C. tenuis	0.12	0.09	0.14	—	—
C. tropicalis	2.08	1.85	2.05	1.41	1.58
C. versatilis	0.95	0.92	0.84	0.35	0.79
C. vini	0.84	1.11	0.42	0.35	0.39
C. zeylanoides	1.14	0.37	2.42	0.47	0.53
Citerom. matritensis	0.26	0.14	0.42	0.70	0.39
Clsp. lusitaniae	0.22	0.28	0.14	0.70	—
Cry. albidus	2.33	1.45	2.44	0.82	1.84
Cry. curvatus	0.07	0.09	0.10	—	—
Cry. humicolus	0.45	0.35	0.52	—	0.39
Cry. laurentii	1.58	0.92	2.33	1.12	0.66
Cys. infirmominiatum	0.22	0.06	0.28	—	0.26
Db. etchellsii	0.36	0.35	0.31	—	0.39
Db. hansenii	6.72	4.61	8.65	5.64	4.73
Db. polymorphus	0.37	0.37	0.31	0.70	0.39
Dek. anomala	0.21	0.65	—	—	—
Dek. bruxellensis	0.14	0.43	—	—	—
Dipodascus ingens	0.12	0.09	0.14	—	—
Filobasidiella neoformans	0.16	0.14	—	—	—
Filobasidium capsuligenum	0.10	0.06	0.09	—	—
Gal. geotrichum	0.60	0.11	1.57	—	0.66
Geo. fragrans	0.19	0.09	0.31	0.35	—
Guehom. pullulans	1.54	0.46	3.16	0.47	2.10

(Continued)

TABLE 9.5 (Continued)

Species	All Foods (99 species)	Fruits, Beverages, Wine, Beer (96 species)	Meat, Dairy Products (74 species)	Low-a_w Products (56 species)	Low-pH Products (62 species)
Hsp. guilliermondii	0.45	1.01	—	1.88	—
Hsp. osmophila	0.14	0.41	—	—	—
Hsp. uvarum	1.35	3.20	—	0.59	—
Hsp. valbyensis	0.21	0.41	—	—	—
Iss. orientalis	3.20	3.23	2.56	1.76	3.95
Iss. terricola	0.15	0.28	—	—	0.39
Kazach. exiguus	1.76	1.11	2.42	1.88	2.63
Klu. lactis	0.60	0.28	0.98	0.47	0.53
Klu. marxianus	3.36	2.21	3.91	4.11	0.79
Lachancea fermentati	0.15	0.46	—	—	—
Lachancea kluyveri	0.45	0.65	0.19	0.47	0.53
Lachancea thermotolerans	0.66	0.98	0.28	1.41	—
Lodd. elongisporus	0.18	0.55	—	0.35	—
Leucosporidium scottii	0.12	0.12	0.09	—	—
Met. pulcherrima	1.06	2.46	—	—	—
Met. reukaufii	0.19	0.37	—	—	—
Naumovia dairensis	0.11	—	0.42	—	1.18
P. angusta	0.75	0.58	0.87	1.76	1.97
P. anomala	4.56	4.25	4.19	3.53	5.52
P. burtonii	1.78	0.55	3.63	2.11	2.16
P. farinosa	0.45	0.46	0.56	0.47	1.05
P. fermentans	1.60	1.60	1.30	1.88	1.05
P. guilliermondii	2.52	2.40	2.23	1.41	2.37
P. jadinii	0.50	0.74	0.19	0.47	0.53
P. membranifaciens	4.32	4.43	3.35	3.53	3.95
P. nakasei	0.09	0.21	—	—	—
P. ohmeri	0.24	0.23	0.21	0.70	0.79
P. subpellicilosa	1.56	1.29	1.67	2.11	2.37
Rho. glutinis	4.17	2.58	5.86	1.64	1.84
Rho. minuta	1.35	0.55	2.30	—	1.58
Rho. mucilaginosa	3.78	3.45	3.63	0.94	2.10
S. bayanus	0.60	1.85	—	0.59	0.66
S. cerevisiae	7.04	6.40	6.70	7.53	7.37
S. pastorianus	0.30	0.92	—	—	—
S'codes ludwigii	0.33	1.01	—	—	—
S'copsis fibuligera	0.36	0.25	0.46	0.94	1.58
Schizo. octosporus	0.18	0.35	—	1.06	—
Schizo. pombe	0.98	1.69	0.23	2.98	—
Spori. salmonicolor	0.12	0.12	—	—	—
Spb. roseus	0.55	0.83	0.19	0.47	—
Trichomonascus ciferrii	0.14	0.09	0.14	—	0.79
Trisp. moniliforme	0.94	0.38	1.74	—	2.63
Tsp. delbrueckii	3.64	4.68	1.95	7.53	2.10
Tsp. globosa	0.09	0.12	—	—	—
Tsp. microellipsoides	0.50	1.11	—	0.47	—
Wickerhamiella domercqiae	0.18	0.18	0.14	0.23	—

(Continued)

TABLE 9.5 (Continued)

Species	All Foods (99 species)	Fruits, Beverages, Wine, Beer (96 species)	Meat, Dairy Products (74 species)	Low-a_w Products (56 species)	Low-pH Products (62 species)
Ya. lipolytica	1.14	0.37	2.09	—	1.58
Zygoascus hellenicus	0.14	0.23	0.09	—	0.26
Zygo. bailii	3.05	4.76	1.14	4.94	5.52
Zygo. bisporus	0.36	0.92	0.14	2.11	0.39
Zygo. mellis	0.11	0.18	—	3.53	—
Zygo. rouxii	2.66	3.20	1.67	9.41	4.21
Zygotorulasp. florentinus	0.12	0.38	—	—	—

Note: Compiled from published data on (1) the number of strains isolated from foods or listed in standard descriptions, (2) the number of types of food in which a given species is detected, and (3) the number of times a given species occurred in various foods. The estimated frequency is expressed as the percentage of products for all species considered (Deák and Beuchat, 1987; Deák, 1991, 1993).

Source: Deák, T. and Beuchat, L. R. (1996) Handbook of Food Spoilage Yeasts. CRC Press, Boca Raton, FL. pp. 210.

9.5.1 Protein Electrophoresis

Separation of soluble proteins using polyacrylamide gel electrophoresis (PAGE), with or without sodium dodecyl sulfate (SDS), is a well-established biochemical technique. In microbiology, PAGE has also been used to distinguish strains within one species or to compare strains of closely related species. Bruneau and Guinet (1989) applied electrophoretic protein patterns for the identification of medically important yeasts and concluded that the method allowed good species discrimination, but the preparation of extracts was time consuming. Electrophoretic protein patterns have been used to differentiate wine yeasts (van Vuuren and van der Meer, 1987) and brewing yeasts (Casey et al., 1990). Dowhanick et al. (1990) were able to distinguish between ale and lager yeast strains and nonbrewing yeast contaminants on the basis of protein fingerprints. Querol et al. (1992), however, using SDS–PAGE, did not observe differences between wine yeast strains belonging to the species *S. bayanus* and *S. cerevisiae.*

Vancanneyt et al. (1991) showed that intraspecies variations are mainly due to quantitative differences in protein patterns. Analysis of whole-cell protein patterns enabled Guillamón et al. (1993) to distinguish among strains of *Saccharomyces* species from different ecological and geographical wine districts. These somewhat contradictory results on the feasibility of the technique of protein electrophoresis may be partly due to the fact that the results of protein electrophoresis are dependent on growth conditions (Degré et al., 1989). The reproducibility of protein patterns requires rigidly standardized methods, and the evaluation and comparison of numerous bands call for the normalization of densitometric tracings and computer-assisted numerical analysis.

Multilocus enzyme electrophoresis (MLEE) may provide more distinctive features among yeasts than whole-cell protein patterns. However, in a study, no correlation between MLEE clusters and the currently defined species of the *Saccharomyces sensu stricto* group could be detected (Lewicka et al., 1995). In contrast to this result, distinctive electrophoretic isoenzyme profiles have been obtained for *Saccharomyces sensu stricto* species (*S. cerevisiae*, *S. bayanus*, *S. pastorianus/carlsbergensis*, and *S. paradoxus*) by analyzing the electrophoretic mobilities of nonspecific esterases, acid phosphatase, lactate dehydrogenase, and glucose-6-phosphate dehydrogenase isoenzymes (Duarte et al., 1999).

9.5.2 Fatty Acid Analysis

Gas chromatography of cellular volatile fatty acids requires relatively expensive apparatus and lengthy preparatory work. Under standardized cultivation and analytical conditions, volatile fatty

acid analysis (VFAA) proved to be a reliable method for characterizing microorganisms. Yeast identification using VFAA has been the subject of thorough studies and has become a promising technique for industrial application (Botha and Kock, 1993). VFFA has been used for the characterization of brewing yeasts (Oosthuizen et al., 1987) and wine yeasts (Tredoux et al., 1987; Augustyn and Kock, 1989; Rozes et al., 1992). The technique, however, cannot differentiate between the three major *Saccharomyces* species in fermentation, *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* (Augustyn et al., 1992). On the other hand, the method has potential for differentiating fermenting wine yeasts from spoilage yeasts (Rozes et al., 1992).

The fatty acid composition of yeast cells is strongly influenced by cultivation conditions (Baleiras Couto and Huis in't Veld, 1995). Moreover, it is not possible to cultivate all yeasts under any particular standardized cultivation regime. Hence, the method does not appear to be a generally applicable technique for yeast identification (Augustyn et al., 1992). Nevertheless, a commercial identification system based on fatty acid profiles and supported by an extensive computerized database has been developed for bacteria and yeasts and is claimed to provide reliable results (MIDI, 1992). In a survey of 69 strains representing 19 species of lipomycetaceous yeasts, van Rensburg et al. (1995) concluded that fatty acid analysis is useful to discriminate between taxa. The distribution of unsaturated fatty acids corresponds to chromosomal DNA patterns (karyotypes) and Coenzyme Q composition.

The development of zymological indicators is also based on the analysis of yeast long-chain fatty acid composition and is claimed to have technological significance (see Section 8.3.3).

9.6 MOLECULAR TECHNIQUES

Recent progress in molecular biology has led to the development of new techniques for the detection, identification, and typing of microorganisms, among them food-borne yeasts.

Compared with phenotypic properties, the genotype represented in nucleic acid sequences is more stable because it is not changed by environmental influences during growth. Nevertheless, DNA molecules are subject to mutations and sequence rearrangements that result in differences (polymorphisms) between closely related species and even strains belonging to the same species. Microorganisms can be reliably characterized on the basis of differences in DNA or RNA sequences. Moreover, changes of genetic material during evolutionary time allow the discrimination of organisms at different taxonomic levels and permit insight into their phylogenetic relations.

During the past decade, molecular techniques have become the most important tools of investigation and have revolutionized all fields of microbiology as well as food mycology. No attempt can be made here to review comprehensively the vast literature about the progress made in recent years. Selected examples will be cited to give an overview of and update on the use and application of molecular techniques for the detection, identification, and typing of yeasts associated with foods (for reviews, see Deák, 1995; Querol and Ramón, 1996; van der Vossen and Hofstra, 1996; Smole Mozina and Raspor, 1997; van der Vossen et al., 2003; Fernández-Espinar et al., 2006).

Techniques for the detection of differences at nucleic acid levels can be broadly categorized into four groups: (1) direct detection using gel electrophoresis, (2) detection based on hybridization, (3) detection after amplification, and (4) sequencing. From another viewpoint, molecular techniques can be discussed in terms of their use to delineate higher taxa, to identify species, and to differentiate at the subspecies level (typing of strains).

9.6.1 Direct Electrophoretic Methods

Nucleic acids, primarily DNA, have to be extracted from organisms for taxonomic studies or identification and typing. Many protocols have been developed for the isolation and purification of DNA from a culture of cells, to separate mitochondrial DNA from the bulk nuclear DNA, or to isolate RNAs (Holm et al., 1986; Mann and Jeffery, 1989; Querol and Barrio, 1990; Kurtzman et al., 2003). With special methods, DNA can be directly extracted from natural samples or food products (Dickinson et al., 1995; Ros-Chumillas et al., 2005).

Determination of nuclear DNA base composition (expressed as G+C mol%) was the first method used in molecular taxonomy (Nakase and Komagata, 1968; Price et al., 1978). Yeast strains differing by 1–2 mol% are considered separate species. The taxonomic significance of DNA composition has been eclipsed by hybridization and sequencing methods. Two other direct methods based on the electrophoretic separation of DNA fragments and chromosome-sized DNA molecules are commonly used in identification and typing.

9.6.1.1 Restriction Endonuclease Analysis

Restriction endonuclease analysis (REA) was among the first DNA-based methods developed. It is a simple technique in which DNA is cleaved by restriction endonucleases and the resulting fragments are separated by agarose gel electrophoresis. Ethidium bromide–stained bands are visualized under UV light. Restriction analysis of whole genomes often results in very complex patterns, rendering evaluation difficult. Restriction fragment length polymorphisms (RFLPs) can be better observed after transferring the DNA fragments from the gel onto membrane filters (Southern blotting) and hybridizing them with labeled DNA probes. Nowadays REA of genomic DNA is rarely used without probe hybridization (Soll, 2000).

In yeasts, multiple copies of the rDNA sequences and, to a lesser extent, those of the mitochondrial genome (mtDNA) produce the majority of intense bands in RFLP patterns. mtDNA polymorphism has been used extensively to characterize yeast species, in particular wine yeasts (Querol and Ramón, 1996; Fernández-Espinar et al., 2001; López et al., 2001; Martorell et al., 2005a) (Table 9.6). mtDNA-RFLP has been applied both as a rapid fingerprinting method for typing of strain and also for species identification, even in taxonomic studies, for example in the genera *Metschnikowia*

TABLE 9.6
Selected Direct Electrophoretic Techniques Used for Identification and Typing of Yeasts from Foods and Beverages

Method	Yeast Species	Food, Beverage	Reference
mtDNA-RFLP	*S. cerevisiae*	Wine	Comi et al. (2000)
RFLP	*S. cerevisiae*	Wine	Fernández-Espinar et al. (2001)
	S. cerevisiae	Sherry	Ibeas et al. (1997)
	S. cerevisiae	Wine, bread, beer	López et al. (2001)
	S. cerevisiae	Wine	Querol and Ramón (1996)
	Zygo. bailii, Zygo. rouxii	Nougat	Martorell et al. (2005a)
	Klu. marxianus, Klu. lactis	Cheese	Suzzi et al. (2000)
	S. cerevisiae, C. humilis	Sourdough	Foschino et al. (2004)
	C. zeylanoides, Db. hansenii	Cheese	Romano et al. (1996)
	Saccharomyces, Kloeckera, Candida	Wine	Cocolin et al. (2002a)
PFGE	*Saccharomyces* spp	Brewing	Jespersen et al. (2000)
	Indigenous yeasts	Must	Povhe Jemec et al. (2001)
	S. cerevisiae	Sherry	Ibeas et al. (1997)
	S. cerevisiae	Maize dough	Hayford, Jespersen (1999)
	S. cerevisiae	Beer	Tornai-Lehoczki and Dlauchy (2000)
	*Ya. lipolytica, C. zeyl*anoides	Poultry	Deák et al. (2000)
	Indigenous yeasts	Cocoa fermentation	Jespersen et al. (2005)
	Saccharomyces spp	Must	Antunovics et al. (2005)
	Brettanomyces, Dekkera	Wine	Mitrakul et al. (1999)
	S. cerevisiae	Wine	Schuller et al. (2004)

(Giménez-Jurado et al., 1995) and *Zygosaccharomyces* (Guillamón et al., 1997) and in *Saccharomyces sensu stricto* (Nguyen et al., 2000a). The specificity of mtDNA–RFLP can be increased using DNA probes (Querol et al., 1992), while certain regions of the mitochondrial genome are suitable for developing DNA markers such as the ori-rep-tra sequence (Piskur et al., 1995) or the cox1 gene (López et al., 2003).

9.6.1.2 Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) is another direct way to analyze nucleic acids. PFGE enables the separation of large (chromosome-sized) DNA molecules using continuous reorientation of electric fields, which results in differences of migration on the gel, depending primarily on the size of DNA molecules (Figures 9.1 and 9.2). Originally, the method was developed to study the number and size of chromosomes of yeasts and other eukaryotic organisms (karyotyping), and it has become an effective taxonomic tool (Boekhout et al., 1993; Vaughan-Martini et al., 1993). Karyotype analysis demonstrated a high level of chromosome polymorphism in most species of yeasts and revealed high-intraspecific variability (Naumov et al., 1995; Tornai-Lehoczki and Dlauchy, 1996; Belloch et al., 1998; Gente et al., 2002) (Figure 9.3). The resolution can be increased by digesting chromosomal DNA with rare-cutting endonucleases and also by applying specific probes (Török et al., 1992; Jespersen et al., 2000). This proved to be a useful method for differentiating brewing and wine yeast strains (Guillamón et al., 1996; Yamagishi and Ogata, 1999; Tornai-Lehoczki and Dlauchy, 2000). PFGE has been applied to the characterization of various yeast species associated with foods and beverages (Table 9.6).

9.6.2 DNA Hybridization Methods

On thermal denaturation, the two strands of DNA separate (by melting), and the complementary strands reassociate again when allowed to cool. When mixtures of DNA molecules are treated likewise, hybrid double-stranded DNA may form if two single strands are complementary enough.

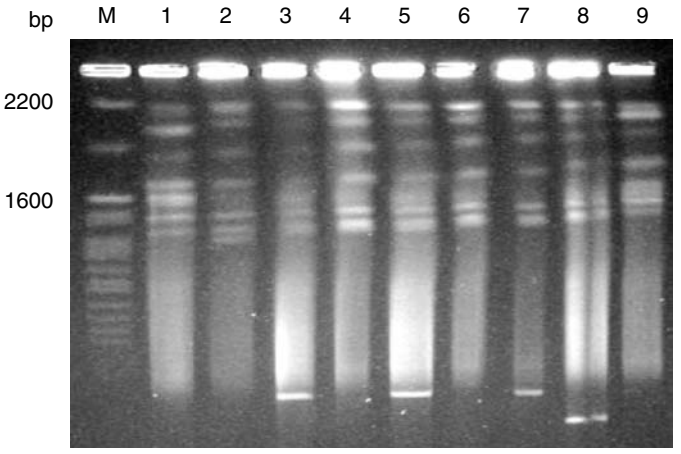

FIGURE 9.1 Pulsed-field gel electrophoresis of chromosomal DNA from *Candida zeylanoides*. CHEF-DR II apparatus (Bio-Rad); electrophoretic conditions: 100 V for 15 h with 120 s pulse time, followed by 180-s pulses for 33 h at 14°C in 1% agarose gel. Isolates: 1 and 9 from *C. zeylanoides*-type strain; 2 and 8 from turkey sausage; 3 and 5 from chicken breast; 4, 6, and 7 from smoked turkey. (From Deák, T., Chen, J., and Beuchat, L. R. (2000) Appl. Environ. Microbiol. 66:4340–4344. With permission of ASM Journals.)

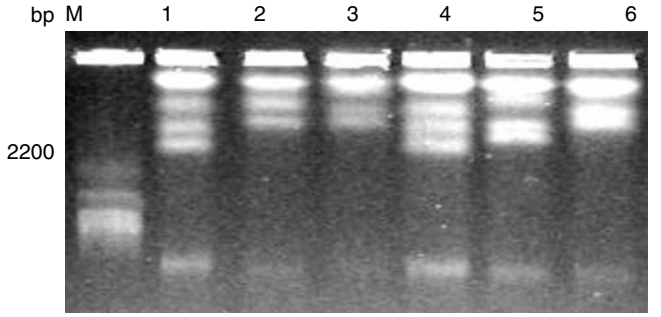

FIGURE 9.2 Pulsed-field gel electrophoresis of *Yarrowia lipolytica* chromosomal DNA. CHEF electrophoretic conditions: 50 V for 36 h with 1200-s pulse time followed with 1800-s pulses for 36 h at 14°C in 0.75% agarose gel. Isolates: 1, 4, and 5 from chicken liver; 2, 3, and 6 from chicken breast. (From Deák, T., Chen, J., and Beuchat, L. R. (2000) Appl. Environ. Microbiol. 66:4340–4344. With permission of ASM Journals.)

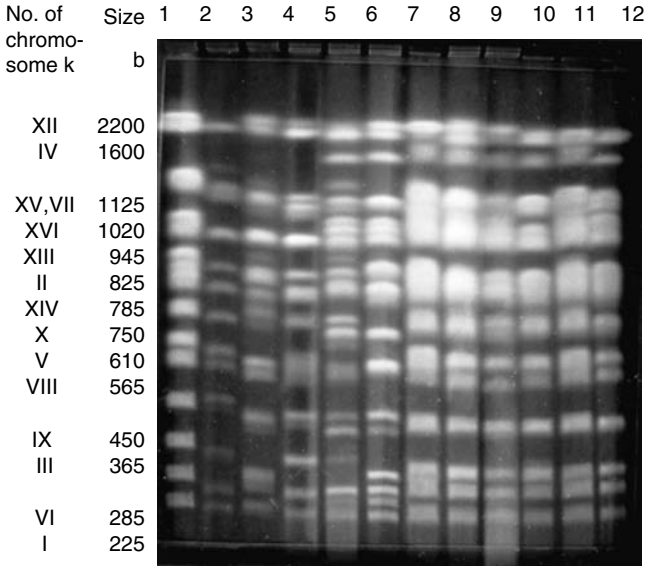

FIGURE 9.3 Karyotypes of *Saccharomyces sensu stricto* species. (From Tornai-Lehoczki, J. and Dlauchy, D. (2000) Int. J. Food Microbiol. 62:37–45. With permission from Elsevier.)

Thus, nucleic acid hybridization permits the genetic relatedness between different organisms to be estimated. Measuring reassociation of nuclear DNA (DNA–DNA homology) has formed the basis of determining specific relations between yeast strains (Kurtzman and Phaff, 1987; Kurtzman et al., 2003). Strains that show at least 80% DNA relatedness are considered to belong to the same species (Martini and Phaff, 1973). Although experimental conditions greatly influence duplex formation, and several exceptions show various degrees of sexual compatibility (mating, sporulation) at very low (20–50%) DNA homology, the technique of hybridization has remained fundamental to taxonomy (Kurtzman, 2006).

Hybridization is widely used with DNA probes. In this technique, single-stranded nucleic acid molecules are treated with a short, labeled oligonucleotide sequence (DNA probe) and allowed to anneal, forming a hybrid. Hybridization can be detected directly using radioactive or fluorescent labels, or indirectly with enzyme reporters. DNA probes have become effective tools for the rapid

detection of microorganisms, also providing specific identification and subtyping, depending on the specificity of the oligonucleotide sequence used as a probe (Soll, 2000). Specific DNA probes can be based on conserved coding sequences such as rRNA genes and also on variable sequences such as insertion elements, repetitive microsatellite or minisatellite sequences, or even synthetic oligonucleotides. These methods are useful for typing, and are usually termed DNA fingerprinting (Baleiras-Couto et al., 1996a,b). Multiple copies of rRNA genes are excellent targets for binding specific DNA probes. The ribotyping method is commonly applied in the identification and typing of bacteria, and an automated instrument (RiboPrinter®) has been developed for diagnostic use. It has been applied only recently to yeasts (Arvik et al., 2005). A version of the method has become widespread since the amplification of rDNA sequences in polymerase chain reactions (PCRs) has become possible.

9.6.3 Amplification Techniques

The advent of PCR has led to the development of new molecular techniques. PCR is a powerful tool that allows exponential amplification of specific DNA sequences by *in vitro* DNA synthesis in a thermocyclic process consisting of three steps repeated many times (Saiki et al., 1988). The detailed protocols have been reviewed [e.g., Bridge et al. (1998); for yeasts, a specific source is White et al. (1990); see also Kurtzman et al. (2003)].

The specificity of the reaction is determined primarily by the sequence of the oligonucleotide primer hybridized (annealed) to the denatured single-strand DNA. As in the case of DNA probes, primers can be based on either single-copy or multicopy sequences. Multicopy sequences, when used as primers, offer more sensitivity. Mitochondrial DNA and ribosomal DNA sequences (PCR-ribotyping) are used most commonly (Giesendorf et al., 1996).

The simplest method for the detection of amplified products is ethidium bromide staining of PCR products separated on agarose gel. PCR amplicons can be further analyzed by RFLP or hybridization with probes or both (Mitchell et al., 1994; Shin et al., 1999). Size determination based on agarose gel electrophoresis is not precise enough to confirm species identification. Amplicons can be rapidly and accurately sized with capillary electrophoresis (Turenne et al., 1999). Various post-amplification methods exist with increased potential for discriminating strains and species, the most precise method being the sequencing of amplified DNA.

Several variations of the basic PCR technique have been developed, and most have been applied for yeasts (Table 9.7); some of these are discussed here. Fernández-Espinar et al. (2006) provide a recent and excellent overview of PCR methods for species identification and intraspecies typing.

9.6.3.1 PCR Fingerprinting

PCR primers can be devised for the amplification of repeated genomic elements of known sequences (REP–PCR, interrepeat PCR). Microsatellite fingerprinting has been widely used for typing yeast strains (Baleiras-Couto et al., 1996a; Gallego et al., 1998). Intron splice sites, δ-elements, and synthetic repeats such as $(GAC)_5$ or $(GTG)_5$ are used to characterize yeast strains (de Barros Lopes et al., 1996; Fernández-Espinar et al., 2001; Caruso et al., 2002). Even prokaryotic repeated elements can be used for the amplification of fungal DNA, such as the enterobacterial repetitive intergenic consensus (ERIC) sequence (Deák et al., 2000).

A simple PCR method called random amplified polymorphic DNA (RAPD) analysis or arbitrary primed PCR (AP–PCR) makes use of short, synthetic primers with no prior sequence information (Figure 9.4). In contrast to the standard PCR, RAPD analysis employs a single primer with arbitrary nucleotide sequence and amplifies complementary sequences throughout the whole genome at random. A large variety of 10-bp-long (decamer) oligonucleotides are available commercially. RAPD is a very popular method for its simplicity; however, because it uses nonspecific primers at low-stringency conditions, the reproducibility of the method is low (Power, 1996). RAPD methods

TABLE 9.7
Restriction Analysis (RFLP) of PCR Techniques Used for Yeasts

Polymerase chain reaction	PCR
Restriction enzyme analysis of PCR products	PCR–RFLP
PCR of repetitive elements	rep–PCR
Amplified ribosomal DNA restriction analysis	ARDRA
PCR of rRNA gene sequences	PCR ribotyping
Random amplified polymorphic DNA (analysis)	RAPD
Arbitrary primed polymerase chain reaction	AP–PCR
PCR of rDNA internal spacer regions	ITS–PCR
Ribosomal intergenic spacer analysis (by PCR)	RISA
Amplified fragment length polymorphism (analysis)	AFLP
Single-strand conformational polymorphism	SSCP
Denaturing gradient gel electrophoresis	DGGE
Temperature gradient gel electrophoresis	TGGE
Terminal restriction fragment length polymorphisms	T–RFLP
Nucleic acid sequence–based amplification	NASBA
Reverse transcriptase polymerase chain reaction	RT–PCR

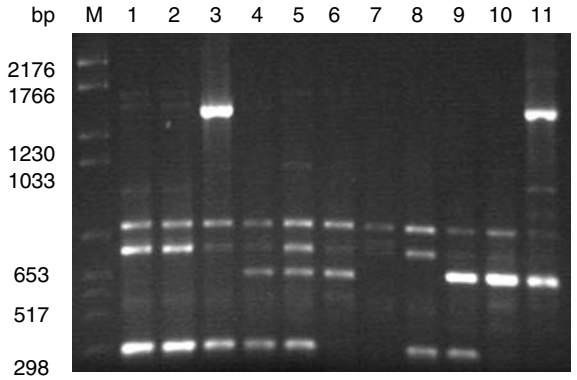

FIGURE 9.4 RAPD-PCR amplification of *Geotrichum candidum* strains with OPE 16 primer. Isolates: 1, Camembert cheese; 2, Roquefort cheese; 3–9 and 11, various cottage cheese samples; 11, goat cheese.

are particularly useful to determine relationships below the species level (subtyping) and may also help to distinguish species (Romano et al., 1996; Andrighetto et al., 2000) (Table 9.8).

9.6.3.2 PCR Ribotyping

For species identification, the restriction analysis (RFLP) of PCR-amplified rDNA is used most frequently. This PCR–RFLP technique is also known as amplified rDNA restriction analysis (ARDRA). With various primers (White et al., 1990), a part or whole of the rRNA genes or the intergenic regions (ITS, ETS, NTS) can be amplified (Figure 9.5; see also Figure 2.1). By amplifying the conservative as well as variable rDNA regions and using various restriction enzymes (e.g., HaeIII, HinfI, RsaI, and others) for analysis of amplicons, the differentiation of organisms at various taxonomic levels is possible.

The most frequently used regions are the ITS1 and ITS2 regions flanking the 5.8S rRNS gene (Guillamón et al., 1998; Fernández-Espinar et al., 2000; Caggia et al., 2001; Redzepovic et al.,

TABLE 9.8
Selected Examples of PCR-Fingerprinting Techniques Used for Food-Borne Yeasts

Method	Yeasts	Food	Reference
Minisatellites	Non-*Saccharomyces*	Wine	Capece et al. (2003)
	S. cerevisiae, *Klc. apiculata*	Wine	Caruso et al. (2002)
	S. cerevisiae	Wine	Marinangeli et al. (2004)
δ-Elements	*S. cerevisiae*	Wine	Legras et al. (2003)
	S. cerevisiae, non-*Saccharomyces*	Wine	Schuller et al. (2004)
	S. cerevisiae	Wine	Ciani et al. (2004)
REP ERIC, intron splice sites	Indigenous yeasts	Wine	Hierro et al. (2004)
	C. zeylanoides, *Ya. lipolytica*	Poultry	Deák et al. (2000)
	S. cerevisiae	Starters	De Barros Lopes et al. (1996)
RAPD	*Hanseniaspora* spp.	Fruits, must	Cadez et al. (2002)
	Various yeasts	Dairy	Andrighetto et al. (2000)
	Dekkera spp.	Wine	Mitrakul et al. (1999)
	Wild yeasts	Brewing	Barszczewski and Robak (2004)
	S. cerevisiae, *S. pastorianus*	Beer	Tornai-Lehoczki and Dlauchy (2000)

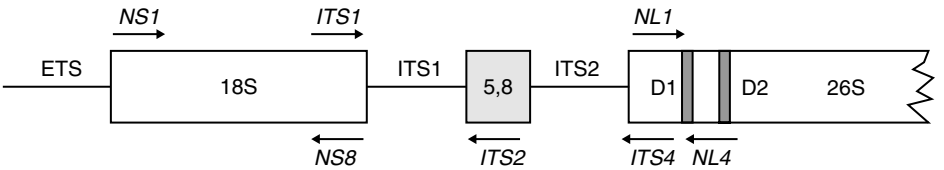

FIGURE 9.5 Some primers used for amplification of various rDNA sequences. *NS1–NS8*: whole 18S rDNA; *NS1–ITS2*: 18S–ITS1 sequence; *ITS1–ITS4*: ITS1-5.5S rDNA-ITS2 sequences; *NL1–NL4*: D1/D2 region of 26S rDNA.

2002; Esteve-Zarzoso et al., 2004). Esteve-Zarzoso et al. (1999) built up a database of this region comprising 132 yeast species (http://yeast-id.com). This method has been used for the taxonomic study of species in various yeast genera such as *Debaryomyces* (Ramos et al., 1998), *Hanseniaspora* (Bujdosó et al., 2001; Cadez et al., 2002), *Metchnikowia* (Valente et al., 1997), *Dekkera* (Egli and Henick-Kling, 2001), *Zygosaccharomyces* (Esteve-Zarzoso et al., 2003), and *Saccharomyces* (Fernández-Espinar et al., 2000; Naumová et al., 2003). It has been applied for the characterization of yeasts associated with various foods and beverages such as wine, beer, fruit juice, yoghurt, and dairy products (Figure 9.6). Dlauchy et al. (1999) developed a database for 128 species using RFLP analysis of an rDNA region including most of the 18S gene and the ITS spacer. This method has allowed the differentiation of *S. cerevisiae* and *S. paradoxus* (Redzepovic et al., 2002). Numerous other studies amplified different rDNA regions (e.g., NTS, 18S-NTS, ITS2, 18S, and 26S) and used various restriction enzymes (Table 9.9). Although the potential resolution of these PCR-RFLP methods is comparable to that obtained from large subunit domains D1/D2 (Berbee et al., 2000; Scorzetti et al., 2002), and sometimes even surpasses it (Fell and Blatt, 1999; Kurtzman, 2005), the lack of a standard methodology and a unified database is a great disadvantage for general use.

Primers are known to amplify mt-rDNA sequences in addition to the nuclear rRNA genes. RFLP of the whole mtDNA is a widely used indicator of differences below the species level in yeasts (Belloch et al., 1997; Guillamón et al., 1997; Fernández-Espinar et al., 2001).

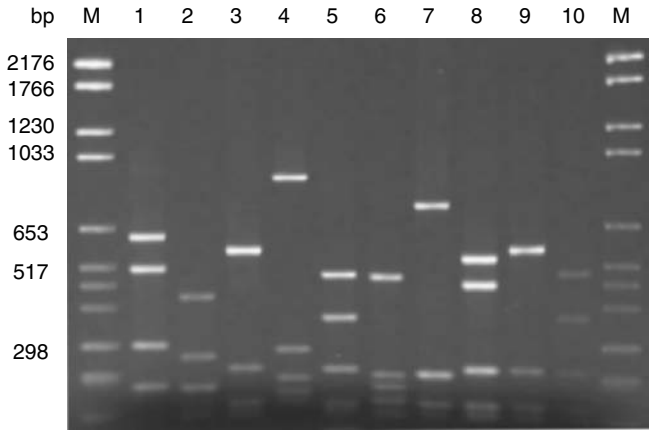

FIGURE 9.6 PCR-RFLP patterns of various yeast species. Amplified 18S–ITS1 region restricted with HaeIII enzyme and separated in agarose gel. Columns: 1, *Trichosporon inkin*; 2, *Pichia membranifaciens*; 3 and 9, *Candida glabrata*; 4, *Kazachstania exiguus*; 5 and 10, *Candida parapsilosis*; 6, *Metschnikowia reukaufii*; 7, *Clavispora lusitaniae*; 8, *Torulaspora delbrueckii*; M, DNA size marker.

TABLE 9.9
PCR-RFLP Methods Used for the Study of Food and Beverage Yeasts

Amplified rDNA	Yeasts	Food	Reference
ITS1-5.8S-ITS2	Lager yeast	Beer	Jespersen et al. (2000)
	S. cerevisiae, *S. pastorianus*	Beer	Tornai and Dlauchy (2000)
	Non-*Saccharomyces*	Wine	Fernández et al. (2000)
	Non-*Saccharomyces*	Wine	Capece et al. (2003)
	S. cerevisiae	Sherry	Esteve-Zarzoso et al. (2004)
	Non-*Saccharomyces*	Orange juice	Arias et al. (2002)
	C. humilis	Sourdough	Gullo et al. (2003)
18S-ITS2	*S. cerevisiae*, *S. paradoxus*	Wine	Redzepovic et al. (2002)
18S-NTS2	Non-*Saccharomyces*	Wine	Capece et al. (2003)
	Klu. lactis	Dairy	Nguyen et al. (2000b)
ITS1-25S	*S. cereviaie*, *S. bayanus*	Beer, wine	Josepa et al. (2000)
18S	*C. milleri*, many others	Sourdough	Mäntynen et al. (1999)
26S	*Saccharomyces*, *Torulaspora*	Various	Smole Mozina et al. (1997)
D1/D2 26S	*Dek. bruxellensis*, *Dek. anomala*	Wine	Cocolin et al. (2004)

9.6.3.3 PCR Variations and Alternative Amplification Techniques

The sensitivity and specificity of the amplification can be improved by various modifications of the standard protocol. One technique is termed nested PCR, in which the product of the first PCR amplification is subjected to a second round of PCR using primers internal to the sequence of the first product. The nested PCR method has been used for the detection of *Dekkera* (*Brettanomyces*) strains in sherry (Ibeas et al., 1996).

In multiplex PCR, more than one pair of primers are applied simultaneously under the same PCR conditions. Mannarelli and Kurtzman (1998) used four primers for the identification of human pathogenic yeasts. Two universal primers were developed from the 18S rRNA gene, and two specific primers were obtained from the variable D1/D2 region of the 26S rRNA gene. Six primers for introns

in the cox1 (cytochrome oxidase subunit 1) gene in the mtDNA were used in multiplex PCR for monitoring wine fermentation (López et al., 2003). Fujita et al. (2001) simultaneously applied primers for the ITS-5.8S and ITS2 rDNA regions to discriminate between medically important *Candida* and *Trichosporon* species.

A specific modification of PCR–RFLP is the technique of amplified fragment length polymorphism (AFLP) analysis, which amplifies DNA fragments randomly chosen from restriction digest. The amplified products are separated by denaturing PAGE and produce a specific fingerprint of high resolution (de Barros Lopes et al., 1999). The complex technique has found application in the clinical identification of yeasts (Theelen et al., 2001; Borst et al., 2003), in identifying brewer's yeast strains (Perpéte et al., 2001), and also in studying phylogenetic relationships (Lopandic et al., 2005).

In addition to PCR, several other amplification methods have been developed, among them the ligase chain reaction (LGC), nucleic acid sequence-based amplification (NASBA), Qβ replicase amplification, strand displacement amplification (SDA), rolling circle amplification, and several others. These have been reviewed by Schweitzer and Kingsmore (2001). Few have been used with yeast so far. LGC was used to distinguish food spoilage species *Zygo. bailii* and *Zygo. bisporus* (Stubbs et al., 1994). NASBA appeared to be a good alternative to PCR for the detection of medically important *Candida* species (Widjojoatmodjo et al., 1999; Borst et al., 2001; Loeffler et al., 2003).

Reverse transcription PCR (RT–PCR) is a method for the amplification of RNAs. In contrast to DNA, mRNA turnover time is short; transcribed mRNAs have a half-life of a few minutes. Hence, the detection of mRNA is a better indicator of cell viability than DNA-based methods. PCR detection of RNAs can be accomplished by reversing into DNA; however, it is a complex and sensitive method. The primary application of RT–PCR is for the study of RNA viruses, but it has been extended to cellular organisms as well. Recently, it has been used to detect viable yeasts in food (Bleve et al., 2003; Cocolin et al., 2004).

9.6.3.4 Post-Amplification Techniques

In addition to the methods for the detection of amplified products mentioned above, a variety of other post-amplification analytic methods have been developed for high-resolution electrophoresis, allowing the detection of polymorphisms in amplicons that are of the same size. Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) separate the amplification products on the partial denaturation of DNA helices, whose electrophoretic mobility will differ depending on their sequences (Muyzer, 1999). These methods have been used to profile yeast populations in raw milk (Cocolin et al., 2002b), during the fermentation of wine (Cocolin et al., 2001; Manzano et al., 2005), and in sourdough bread (Meroth et al., 2003). Minority species in mixed populations could be detected using DGGE in fermenting coffee and on grapes (Masoud et al., 2004; Prakitchaiwattana et al., 2004).

Single-stranded conformational polymorphism (SCCP) analysis also takes advantage of sequence-dependent differences between reannealed single-stranded PCR products, which also result in changes in electrophoretic mobility. The SSCP method has been used for the diagnosis of medically important yeasts and opportunistic fungi (Walsh et al., 1995; Hui et al., 2000).

Terminal RFLP (T–RFLP) analysis employs fluorescently tagged primers in PCR, followed by digestion with appropriate restriction enzymes to distinguish the terminal restriction fragment (Marsh, 1999).

9.6.4 Sequencing

rDNA sequences are the most important characteristics used for identification, taxonomic, and phylogenetic studies. The advantages of rDNAs are that they are present in all living organisms, have common evolutionary origin, occur in multiple copies, and have conserved and variable parts

available both for delineating higher taxa and differentiating among strains belonging to a species (Kurtzman, 2006).

For taxonomic purposes and establishing phylogenetic relationships, two regions of rDNA are most commonly used, the partial and whole sequences of the 18S rDNA of the small ribosomal subunit and the D1/D2 domain of about 600 nucleotides of the large subunit ribosome. Kurtzman and Robnett (1998) determined the D1/D2 sequences of all ascomycetous yeast species, and Fell et al. (2000) published these sequences for all basidiomycetous yeasts.

In the past, molecular sequencing with cloning was a technically demanding, expensive, and time-consuming procedure. With the emergence of PCR-based direct DNA sequencing technologies using automated instruments (e.g., the ABI Prism capillary electrophoresis sequencer) and gene bank databases for comparison of sequences, this technique has become a common, routine tool in molecular studies. In two decades, sequencing has become the most reliable aid to the identification of species. Complete and partial sequences of rRNA genes are most widely used in taxonomic studies (Valente et al., 1999) and for establishing phylogenetic relations (Kurtzman and Robnett, 2003) (Table 9.10). Currently, the classification of yeasts is based on the analysis of rDNA sequences (Kurtzman and Robnett, 1998; Fell et al., 2000; Kurtzman and Fell, 2006).

9.6.5 Recent Developments

PCR technology and instrumentation are being advanced continuously. Developments in the field not only open up novel methods of application but also contribute to solving some problems encountered in conventional analysis such as quantitative detection and the discrimination of DNA from live and dead cells. Novel techniques and instrumentation as well as future trends have been reviewed elsewhere (Meldrum, 2000; Schneegass and Köhler, 2001; Schweitzer and Kingsmore, 2001; Heller, 2002).

TABLE 9.10
Sequenced Regions and Their Use in the Classification and Phylogeny of Yeasts

	Range of Discrimination				
DNA Region	**Higher Taxa**	**Genus**	**Species**	**Strains**	**Reference**
Ribosomal DNA					
5S rDNA	+				Fan et al. (1995)
5.8S rDNA	+				Mitchell et al. (1992)
18S rDNA, partial	+	+	+		Kurtzman (1992)
18S rDNA, total	+	+	+		Tehler et al. (2000)
25S rDNA, partial	+	+	+		Berbee et al. (2000)
25S rDNA, D1/D2	+	+	+		Fell et al. (2000)
ITS1, ITS2			+	+	Cappa et al. (2001)
ITS, total			+	+	Oda et al. (1999)
IGS (NTS)			+	+	Fell and Blatt (1999)
mtDNA					
cox1 gene		+	+	+	Belloch et al. (2000)
NAD5 gene	+				Paquin et al. (1995)
Nuclear genes					
MET2	+	+	+		Masneuf et al. (1996)
RNA polymerase II		+			Liu et al. (1999)
Actin		+			Daniel and Meyer (2003)

9.6.5.1 Real-Time PCR

A significant innovation in PCR technology has been the monitoring of the amplification reaction in real time. The principle of the method is to apply a fluorescent dye such as SYBR Green intercalating into dsDNA and to monitor this process during each PCR cycle. This makes the electrophoretic detection of amplicons unnecessary. The amplified product can be further characterized by the analysis of its melting curves. Moreover, if a template of known concentration is amplified together with the sample DNA, the quantification of the latter is also possible (Wilhelm and Pingoud, 2003).

A further development has been the use of fluorescent probes and the integration of the capillary thermal cycler with the fluorometer. The automated TaqMan system (Perkin-Elmer Applied Biosystems) is based on the 5′ exonuclease activity of *Taq* polymerase and the use of a fluorogenic probe that is labeled at the 5′ end with a reporter dye (e.g., FAM) and at the 3′ end with a quencher dye (TAMRA). During PCR, the probe hybridizes to the target and is cleaved by the *Taq* polymerase, releasing the reporter dye from the quencher. This results in an exponential increase in fluorescence that is proportional to the amount of DNA amplified (Guiver et al., 2001). The system has found increasing application in the detection of yeasts in food and beverages (Phister and Mills, 2003; Casey and Dobson, 2004; Martorell et al., 2005b).

Another probe system, the Light Cycler (Roche Diagnostics), is based on fluorescence resonance electron transfer between two specific hybridization probes, one labeled with a fluorescein dye and the other with a fluorophore. When both probes are hybridized during annealing to the target, a transfer of excitation energy to the fluorophore results in emission of light, which can be measured. The emitted fluorescence is proportional to the amount of DNA generated during the PCR process, allowing detection in real time. Quantification is also possible, and the amplicons can be identified by melting point analysis (Loeffler et al., 2000).

9.6.5.2 Peptide Nucleic Acid Probes

Peptide nucleic acid (PNA) is a pseudopeptide in which the sugar–phosphate backbone of DNA is replaced by a polyamide chain. To this, the nucleotide bases are bound in the same spacing as in DNA (Stender et al., 2002). Owing to its uncharged backbone, the PNA exhibits favorable hybridization characteristics such as higher specificity and more rapid hybridization than traditional DNA probes. Similar to the latter, PNA probes can be labeled with fluorescent dyes. Perry O'Keefe et al. (2001) described specific PNA probes used in a standardized fluorescence *in situ* hybridization (FISH) method for the detection of all bacterial species and the yeast *S. cerevisiae.*

9.6.5.3 Fluorescence *In Situ* Detection

FISH combines the sensitivity and precision of molecular techniques with the direct visualization of microorganisms within their natural habitat without cultivation. FISH is based on the hybridization of specific nucleic acid probes labeled with fluorescent dyes to target sequences within intact cells. Fluorescent signals are detected using epifluorescent microscopy, a flow cytometer, or a laser scanner (Moter and Göbel, 2000).

FISH has been widely applied to study bacterial diversity in environmental samples and for the population analysis of microbial communities. Far fewer studies have been made to date with fungi (Li et al., 1997). The technique has been applied to the detection of pathogenic yeasts (Lischewski et al., 1997). More recently, FISH–PNA has been used for the identification of *Dekkera-Brettanomyces* wine spoilage yeasts (Stender et al., 2001).

9.6.5.4 Flow Cytometry of Fluorescent Probe Hybridization

A recently developed multiplex technology makes use of fluorescent probe hybridization combined with flow cytometric detection and evaluation. Species-specific DNA capture probes are bound to fluorescent beads, and the hybridization of target amplicons is detected in a flow cytometer. Simultaneously, 100 different sets of specific probes can be analyzed in each well of the common 96-well plate format in less than an hour after amplification. The method is an adaptation of a commercial technology (Luminex) and has been applied for the study of clinical yeasts (Diaz and Fell, 2004; Page and Kurtzman, 2005).

9.6.5.5 Green Fluorescence Protein

Analysis of specific gene function was made possible by introducing reporter genes such as the green fluorescence protein (GFP) isolated from the jellyfish *Aequorea.* GFP expression can be detected using confocal and fluorescence microscopy or flow cytometry. GFP has been used for the quantification of gene expression in *S. cerevisiae* (De Wulf et al., 2000), and for relating the growth dynamics and glucoamylase excretion of individual *S. cerevisiae* cells (Porro et al., 2000).

9.6.5.6 DNA Microarrays

Microarray technology provides powerful, high-throughput tools and unprecedented opportunities to explore genome differences and gene expression profiles. DNA microarrays (DNA chips) contain thousands of oligonucleotides from a reference genome bound to a microchip, where they can be hybridized to fluorescently labeled DNA from the genome of interest. The loci at which the two genomes differ, either in DNA sequence or in gene expression, can be counted and mapped. This allows a parallel analysis of DNA homology/differences and transcriptional profiles to study functional genomics (Gingeras and Rosenow, 2000; Ye et al., 2001). The completion of the yeast genome project has given a great impetus to the improvement of DNA microarray technology (Brown and Botstein, 1999). With the increasing availability of complete microbial genomes, the use of DNA microarrays is an emerging technology in many areas of molecular research, including genetics, physiology, ecology, pathogenesis, and others. It has as yet unexplored potential as a diagnostic tool as well. For species identification, unique sequences of rRNA genes and many key functional genes for most representative species should be arranged in a hybridization array and then probed with DNA from the test organism (Cho and Tiedje, 2001).

Currently, the application of microarrays in mycology has been in the study of genomics and gene expression (Williams, 2002). However, this technique has the potential to be a powerful tool for the rapid genetic identification and study of community structure and diversity. In the past few years, several studies have been made to investigate, with DNA chips, the gene expression of wine yeasts, and these have been reviewed by Perez-Ortin and colleagues (2002). Expression patterns under fermentation conditions and between wild and industrial yeast strains revealed significant differences that correlate with physiological properties and can be exploited for the development of wine technology.

Further advancement in miniaturized molecular analytical technology is anticipated. Microarrays, microchips, bioelectronic chips, and other devices will provide new detection methods and smaller dimensions (nanotechnology), and will integrate multiple steps of complex analytical procedures (lab-on-a-chip tools) (Kricka, 2001). An automated microchip electrophoresis instrument has already been used in a multiplex PCR for the rapid detection of yeasts (Fujita et al., 2001). Biochip technology will be applied beyond genomics to proteomic-based testing (Jain, 2002). Inevitably, developments will extend to the field of food mycology too. The accelerated pace of progress brings the future into sight.

TABLE 9.11
Selected Molecular Methods Used for Studying Wine Yeasts

Method	Reference
RFLP	Vezinhet et al. (1990)
+ probes	Querol et al. (1992)
mtDNA	Guillamón et al. (1996)
PFGE	Tornai-Lehoczki and Dlauchy (1996)
+ FLP	Versavaud and Hallet (1995)
+ probes	Oda and Tonomura (1995)
PCR fingerprinting	Lavallée et al. (1994)
Microsatellite	Gallego et al. (1998)
δ-Transposon	Fernández-Espinar et al. (2001)
Intron sites	de Barros Lopes et al. (1996)
RAPD	Quesada and Cenis (1995)
PCR ribotyping	Valente et al. (1996)
ITS1-ITS2-RFLP	Esteve-Zarzoso et al. (1999)
ITS2-RFLP	Fernández et al. (2000)
NTS-RFLP	Caruso et al. (2002)
18S-RFLP	Capece et al. (2003)
AFLP	de Barros Lopes et al. (1999)
Nested PCR	Ibeas et al. (1996)
Multiplex PCR	López et al. (2003)
PCR–DGGE	Cocolin et al. (2001)
PCR–TGGE	Manzano et al. (2005)
PNA *in situ* hybridization	Stender et al. (2001)
DNA chips	Perez-Ortin et al. (2002)

In summary, the enormous capability and flexibility of molecular techniques are amply demonstrated by their various applications in studying wine yeasts (Table 9.11).

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10 Outlook

This book is largely devoted to discussing the role of yeasts in the spoilage of foods and beverages. This adverse activity of yeasts is, however, counterbalanced and far compensated for by the diverse beneficial contributions of yeasts to human life. Since the beginning of civilization yeasts have been intimately associated with human activities, by bringing about leavening of bread and fermentation of alcoholic beverages. The production of these foods and beverages is an essential part of our everyday life, and the yeasts used in these processes are the most domesticated microorganisms.

In the nineteenth century, the discipline of biochemistry took shape with the discoveries of the fermentative activity of cell-free yeast extract and the enzyme systems responsible for alcoholic fermentation. In a recent and wider sense, the fermentation industry has extended to production of various metabolites, cell constituents, and cell mass, the bioconversion of organic substances, in which yeasts play a significant role. With the application of genetic and molecular techniques, industrial fermentation has been transformed into biotechnology. Yeasts have also contributed to a large extent to the development of molecular biology, and they are key players in the science of the twenty-first century, genomics.

Thus, in the final chapter of this book, it is appropriate to take a brief look at the beneficial role of yeasts in industrial applications and basic science. Two aspects are outlined—the exploitation of yeasts in biotechnology and the potential value of the yeast genome. These topics are covered in more detail in a number of recent books and reviews (Demain et al., 1998; Walker, 1998; Buzzini and Vaughan-Martini, 2006).

10.1 POTENTIAL EXPLOITATION OF YEASTS BEYOND MAKING BREAD, BEER, AND WINE

This was the title of a keynote lecture presented at the twentieth International Symposium on Food Microbiology and Hygiene (Deák, 2006), and it also fits here. Some examples are given to illustrate the current and future trends of exploitation of yeasts in five major fields: (1) food and beverage fermentation, (2) products of cell mass and cell constituents, (3) bioethanol, (4) pharmaceutical and bioactive substances, and (5) other uses. The potential uses of existing yeast biodiversity and the genetic improvement of production strains will be emphasized.

Table 10.1 lists yeast species currently used on an industrial scale and those showing potential value in biotechnological application.

10.1.1 Food and Beverage Fermentation

The traditional use of yeasts for the production of bread, beer, and wine has been comprehensively reviewed (Pretorius, 2000; Bonjean and Guillaume, 2003; Dequin et al., 2003; Dufour et al., 2003). Since the beginning of the twentieth century, the application of yeast starters has become a standard practice in the industrial fermentation of these products.

In brewing, where the malt is fermented practically by the starter alone, the pivotal role of *S. cerevisiae* as the pitching yeast is unquestioned. Efforts have been directed only to improve the performance of this starter, as will be discussed later. The use of other starters at the previous stage of steeping and malting has been considered only recently. Sometimes the barley is heavily contaminated by mycotoxin-producing fusaria. It has been shown that a *Geo. candidum* starter culture can be used

TABLE 10.1
Yeast Species of Current and Potential Use in Biotechnology

Species	Application
C. milleri	Sourdough
C. shehatae	Bioethanol
C. sake	Biocontrol
C. oleophila	Biocontrol
C. maltosa	SCP on hydrocarbons
Db. hansenii	Cheese, sausage ripening, and proteases
Db. (*Schwanniomyces*) *occidentalis*	Amylase
Eremothecium ashbyi	Riboflavin
Geo. candidum	Cheese ripening
Hsp. uvarum	Wine fermentation
Klu. marxianus	Milk fermentation, SCP from whey
Klu. lactis	Milk fermentation, SCP from whey
Pachysolen tannophilus	Bioethanol
Phaffia rhodozyma	Astaxanthin
P. angusta (*Hansenula polymorpha*)	Bioethanol
P. anomala	Biocontrol
P. jadinii (*C. utilis*)	Feedstock
P. (*Komagataella*) *pastoris*	Heterologous proteins
P. stipitis	Bioethanol
Pseudozyma flocculosa	Biocontrol
Rho. glutinis	Carotene
Schizo. pombe	Cider fermentation
S. cerevisiae	Brewer's, baker's, wine yeast; bioethanol, invertase, and heterologous proteins
Kazach. exiguus	Sourdough
S. "*boulardii*" (*S. cerevisiae*)	Probiotics
S'copsis fibuligera	Amylase
Tsp. delbrueckii	Sourdough
Zygo. rouxii	Soy sauce

Source: Data from Walker, G. M. (1998) Yeast Physiology and Biotechnology. Wiley, Chicester, UK; Demain, A. L., Phaff, H. J., and Kurtzman, C. P. (1998) *In*: The Yeasts. A Taxonomic Study, 4th ed. (eds. Kurtzman, C. P. and Fell, J. W.). Elsevier, Amsterdam. pp. 13–19; Abbas, C. A. (2006) *In*: Yeasts in Food and Beverages (eds. Querol, A. and Fleet, G. H.). Springer Verlag, Berlin. pp. 285–334; Buzzini, P. and Vaughan-Martini, A. (2006) *In*: Biodiversity and Ecophysiology of Yeasts (eds. Rosa, C. A. and Péter, G.). Springer, Berlin. pp. 533–559.

for the protection of barley, and its presence also increases the enzymatic potential of malt (Linko et al., 1998; Foszczynska et al., 2004).

The situation is different in the fermentation of must and in the leavening of bread, where the pure starter is not used alone but in association with other yeasts, often together with lactic acid bacteria and also accompanied by other bacteria and molds. In enology, it has been long debated to what degree the autochthonous yeasts may contribute in the fermentation to the aroma and "bouquet" of the wine. Some even question the use of the starter strain and prefer the indigenous yeasts to maintain the specific character of the "terroir." Experiments on a lab scale and by mini-vinification have been conducted to show the potential role of yeasts other than *S. cerevisiae* in wine making. Among these, *Hsp. guilliermondii* and its anamorphs *Klc. apiculata*, *P. fermentans*, *C. stellata*, and others have been suggested as novel adjuncts in simultaneous or sequential mixtures with *S. cerevisiae* (Clemente-Jimenez et al., 2005; Moreira et al., 2005).

Starters are extensively used in the leavening of bread and various other baked goods. The baker's yeasts also belong to the species *S. cerevisiae*; they are special strains. Lactobacilli also play

an important role in sourdough and are often associated with yeasts. In addition to *S. cerevisiae*, which can be considered as baking yeast, at least 25 different yeast species have been described from sourdough (Meroth et al., 2003), among which *C. milleri*, *C. glabrata*, and *C. krusei* may become dominant. However, further studies are necessary to determine their importance in the fermentation and to select appropriate species for use as a starter culture (Vogel, 1997). Currently, *Kazach. exiguus* and *C. humilis* are being considered in commercial sourdough preparations (De Vuyst and Neysens, 2005; Hammes et al., 2005).

Over the years, the use of yeasts for the production of food and beverages has been broadened to include dairy, meat, and bakery products and spirits and alcoholic beverages other than wine (Fröhlich-Wyder, 2003; Samelis and Sofos, 2003; Hammes et al., 2005). The role of yeasts in the fermentation of some of these products has been well known for a long time, for example, in kefyr and sourdough. In most cases, however, yeasts in these products were considered to be spoilage organisms, or, in the best case, innocuous, allochthonous members of the microbiota. Studies revealing the rich biodiversity of yeasts in many of these products have also revealed that certain yeasts may play a beneficial role in fermentation and ripening.

In the fermentation of dairy products and some types of meat products, starters are used as well. In these starters, the dominant microorganisms are lactic acid bacteria; however, yeasts and other microbes may join them, contributing to the development of flavor and texture. In kefyr grains, *S. cerevisiae*, *Klu. marxianus*, and *Tsp. delbrueckii* live in strong symbiotic association with lactic acid bacteria (Narvhus and Gadaga, 2003). Although lactic acid starters are used primarily for the fermentation of cheeses, adventitious yeasts always participate in their ripening and maturation (Ferreira and Viljoen, 2003; Das et al., 2005; Leroy et al., 2006). In addition to *S. cerevisiae*, owing to their proteolytic and lipolytic activity, *Db. hansenii* and *Ya. lipolytica* are regarded as good candidates for ripening agents in soft cheeses (Guerzoni et al., 2001; Suzzi et al., 2001; Ferreira and Viljoen, 2003; van den Tempel and Jakobsen, 2003), and *Geo. candidum* in the production of Camembert cheese (Molimard et al., 1994; Boutrou and Guéguen, 2005). Of these, *Db. hansenii* has already been commercialized as a potential adjunct culture (Durá et al., 2004; Flores et al., 2004). Less is known about the involvement of yeasts in the ripening of sausages. *Db. hansenii* or other lipolytic yeasts may be considered commercial starter cultures (Olesen and Stahnke, 2000).

In the fermentation of dairy and meat products, yeasts are only second to lactic acid bacteria, whereas in the alcoholic fermentation of various beverages other than wine, yeasts play a leading role. In many of them, for example cider, sake, tequila, rum, and others, beyond *S. cerevisiae*, other yeasts can be dominant in developing the characteristic flavor and aroma.

In recent years, the rich and varied microbiota participating in various other food and beverage fermentations has been the subject of detailed studies, and it has emerged that some species may be applied as adjuncts to improve the quality of the product. Several yeast species are among the potential candidates. In pickled cucumbers, mixed fermentation using *S. rosei* (now *Tsp. delbrueckii*) with lactic acid bacteria has been considered (Passos et al., 1997). The fermentation of coffee, cocoa, cider, olives, and a number of traditional indigenous products has recently been the subject of intensive studies that shed light on the complex microbial interactions and the most important species (Jespersen et al., 2005). Among them are several yeast species with the potential to be developed into a starter culture (Schwan and Wheals, 2003; Coton et al., 2006). The participation and role of yeasts in mixed fermentation, such as in soy sauce, Oriental products, coffee, and cacao, requires further exploratory studies. Of these fermentations, the microbiology of soy sauce production is perhaps best known. The process is controlled by the starters of the koji mold *Aspergillus oryzae* or *Asp. sojae* and the moromi yeast, *Zygo. rouxii* (Hanya and Nakadai, 2003). The "soy yeast," *Zygo. rouxii*, is undoubtedly one of the main producers of aroma compounds; however, little is known about the contribution of some 20–25 other yeast species isolated from various stages of soy sauce production. The microbiota of indigenous (traditional and oriental) fermentations is so variable that no definite picture can be drawn of the yeasts (and other microorganisms) present in these products. The preparation of most indigenous fermented products is still a traditional small-scale art rather than

a controlled process. At least 20–30 yeast species may participate to some degree in the development of the characteristic quality of these naturally fermented foods (Sanni and Lönner, 1993; Narvhus and Gadaga, 2003), while in the fermentation of several commodities, it is the lactic acid and other bacteria or molds that play the determining role.

10.1.2 Yeast Cell Mass and Commodity Products

After the main fermented foods and beverages, the second major group of commodities include those made from yeast cell mass or cell-derived products. Among these are pressed baker's yeast and active dried yeast, food and feed yeasts, yeast autolysates and extracts, as well as cell components such as enzymes, vitamins, carotenoids, lipids, steroids, polysaccharides, glucans, nucleotides, flavors, and many others. Several of these are important ingredients and adjuncts in the production of food and beverages, whereas others find application in chemical, pharmaceutical, cosmetic, and other industries. It would be far beyond the scope of this chapter to give an overview of all these, and reference is made to the comprehensive reviews that have appeared elsewhere (Halasz and Lasztity, 1991; Reed and Nagodawithana, 1991; Abbas, 2006).

P. jadinii, better known in anamorphic form as *C. utilis*, is the most widely used species for the production of cell mass for animal feed. It grows abundantly on molasses, and to a certain degree also on agricultural and industrial wastes (wood hydrolysate, sulfite liquor). Research has been ongoing to find or develop a yeast species or strain able to directly utilize lignocellulosic materials for the bioconversion of renewable agricultural products and residues to feedstock or industrial fuel. Although a few yeast species have the metabolic capability to hydrolyze starch (e.g., *Db. occidentalis*, *S'copsis fibuligera*) and to utilize cellobiose and xylose after the partial hydrolysis of woody materials (e.g., *C. shehatae*, *P. stipitis*), an economically feasible solution has not yet been achieved, even with genetically engineered strains (see below) (Jeffries and Kurtzman, 1994; Leathers, 2003).

A large number of yeast species have been recognized for their ability to utilize hydrocarbons as their sole carbon and energy sources (e.g., *C. maltosa*, *C. tropicalis*, *Ya. lipolytica*, and many others) (Tanaka and Fukui, 1989; Fickers et al., 2005). In the 1970s, large industrial plants were set up to produce single-cell protein from this source. After the hike in oil prices, this technology became unprofitable and ceased. Nowadays, hydrocarbon-utilizing yeasts can be used for the degradation of oil spills and environmental remediation. A large group of yeasts, however, are capable of utilizing methanol, which could serve as an inexpensive source of single-cell proteins from the anaerobic decomposition of agricultural wastes.

Baker's yeast is a primary product as well as a source of many derived products, together with spent brewer's yeast. However, beyond *S. cerevisiae*, an increasing list of other yeast species are being exploited in producing and manufacturing these commodities. Whey is a major waste product in the dairy industry, and lactose-utilizing yeasts, such as *Klu. marxianus*, can be used for the production of protein-rich cell mass, as well as valuable bioingredients, oligonucleotides, and flavor enhancers (Belem and Lee, 1998). Further examples are *C. utilis* for feed; *Klu. lactis* for aromas and lactase; *Rho. glutinis*, *Spori. pararoseus*, and *Phaffia rhodozyma* for carotenoids and colorants; *Rho. glutinis* also for lipids; *Db.* (*Schwanniomyces*) *occidentalis* for amylase; *Eremothecium ashbyi* for riboflavin; *Ya. lipolytica* for citric acid and lipase; and *Spori. salmonicolor* for flavor compounds (Dufossé et al., 2002).

Pectinolytic enzymes are important in the food industry for improving juice extraction and clarification. Instead of adding pectinases, *S. cerevisiae* wine strains can be transformed to constitutively overexpress their own endopolygalacturonase gene (Fernández-González et al., 2005). Production of pectinases is not uncommon among yeasts; 7% of species belonging to six genera isolated from tropical fruits secreted pectinolytic enzymes (da Silva et al., 2005).

10.1.3 Bioethanol

In terms of the exploitation of yeasts beyond the field of food and beverages, the most important biotechnological application is the production of bioethanol as a gasoline additive or even substitute. In some countries, particularly Brazil, and also in the United States and Canada, a considerable amount of ethanol is fermented from cane juice or other sugar-rich agricultural raw materials (Wheals et al., 1999). *S. cerevisiae* is used for this purpose, and current interest is directed to the improvement of fermentation technology and to the utilization of cheap agro-industrial by-products or wastes. In this regard, the conversion to ethanol of lignocellulosic hydrolysates using yeasts such as *P. stipitis*, *C. shehatae*, or *Pachysolen tannophilus*, which can ferment cellobiose and xylose, is of primary concern. The ethanol yield is far less than in the case of *S. cerevisiae*, and research has been conducted into the genetic transformation of *S. cerevisiae* with genes for xylose fermentation (Kuyper et al., 2005). A multitransformant strain containing not less than four foreign genes was engineered that was capable of directly degrading cellulose (van Rensburg et al., 1998). The wild type of a methylotrophic yeast, *P. angusta* (*Hansenula polymorpha*), is able to ferment cellobiose and xylose to ethanol (Ryabova et al., 2003). *Klu. marxianus* can be used to ferment inulin and produce ethanol from many plant feedstocks. Recently, a strain of *Klu. marxianus* has been used for bioethanol production from cheese whey (Kargi and Ozmichi, 2006).

10.1.4 Pharmaceutical and Bioactive Products

As a further biotechnological extension, yeasts can be utilized for the production of compounds of pharmaceutical value. A few of these can be obtained from natural strains of *S. cerevisiae* or nonconventional yeasts, and more from genetically modified (GM) strains expressing heterologous proteins. Since the early 1980s, genes for the production of a number of vaccines, antigens, hormones, and other biotherapeutic compounds have been cloned into yeasts and expressed at laboratory scale, and some of these (e.g., insulin, interferon, hepatitis A antigen) have reached commercial production. GM yeasts will be discussed below in more detail. In biotherapy the potential use of yeasts as probiotics should be mentioned. Compared with the widely accepted probiotic activity of lactic acid bacteria and bifidobacteria, yeasts are less recognized, although some strains of *S. cerevisiae*, referred to as "*S. boulardii*," have been used to control gastrointestinal disorders (McFarland et al., 1993). More recently, it has been shown that viable and dead cells, in particular cell wall preparations (glucomannans), can be applied to bind and remove mycotoxins from the intestine of poultry and also from juices (Bejaoui, 2004; Yiannikouris, 2004; Basmacioglu, 2005); moreover, a new yeast species, *Trisp. mycotoxinovorans*, has been described with the ability to degrade mycotoxins (Molnar et al., 2004).

10.1.5 Other Uses of Yeasts

Several other potential applications of yeasts relate to both foods and other biotechnological products and processes. Yeasts with a certain capability for biodegradation have been considered for bioremediation and action in environmental protection. Hydrocarbon-assimilating yeasts may be useful for the degradation of oil spills, yeast cells as a biosorbent can be used for the removal of heavy metals and radioactive isotopes, and strains of *Trisp. cutaneum* and the yeast-like *Aureobasidium pullulans*, which are able to degrade phenols and other aromatic compounds, can be used for their removal from industrial effluents. Olive oil manufacture results in large quantities of black wastewaters due to phenolic compounds that could be decolorized by depolymerization of the phenolics by *Geo. candidum* (Ayed et al., 2005). Another example is the reduction of residues of the pesticide glyphosate in wheat flour during proofing of yeasted dough (*S. cerevisiae*), as demonstrated by Low et al. (2005).

An area attracting growing interest is the application of yeasts for biocontrol. Some yeast species, in particular *P. guilliermondii*, *P. anomala*, and *Db. hansenii*, inhibit the growth of certain molds attacking fruits and grains. The possible use of antagonistic yeasts to control post-harvest diseases and the production of mycotoxins has been reviewed elsewhere (Wisniewski and Wilson, 1992; Suzzi et al., 1995; Druvefors and Schnürer, 2005).

10.2 IMPROVEMENT OF YEAST STRAINS USED IN PRODUCTION

Since the creation of the first pure cultures of yeast, intensive research has been carried out, leading to the production of industrial strains with improved properties, first by mutation, selection, and hybridization, and later by protoplast fusion and cytoduction, and from the 1980s on, by genetic engineering. Excellent reviews of these studies have been made by Pretorius and Westhuizen (1991), Hammond (1995), Dequin (2001), and Schuller and Casal (2005).

In the field of traditional food and beverage fermentation, only a few examples will be mentioned for the broad purposes of improvement in technologically important properties. Among these are, in brewing, carbohydrate utilization, fermentation of dextrins, flocculation and filtration, reduction of H_2S and diacetyl production, and osmotolerance (high-gravity wort); in baking, fast dough raising, organic acid resistance, and rehydration tolerance; and in wine making, ethanol tolerance, fermentation capacity, and absence of off-flavors. Goals have been achieved with some success. A major limitation of these classical genetic techniques has been, in general, the difficulty of adding or removing one feature without altering gross performance. In particular, the stable genetic constitution of polyploid/aneuploid industrial strains, lack of mating-type characteristics, and poor sporulation all restricted the possibilities of broad strain improvement. Recombinant gene technology (genetic engineering) has provided more possibilities and holds much promise for specific modifications.

The principal aim of genetic modification is the transformation of the host cell by the introduction of foreign genes. It is beyond the scope of this chapter to describe in detail the techniques for transformation and cloning [from the extensive list of reference manuals, see Broach et al. (1991), Jones et al. (1992), Evans (1996), and Pringle et al. (1997)]. Briefly, the major steps are (1) identifying the target gene and obtaining the DNA fragment from a genomic cDNA library or by PCR amplification; (2) creating a suitable plasmid vector; (3) joining the DNA fragment to the vector DNA, generating a recombinant DNA molecule; (4) inserting the recombinant molecule into the host cell; and (5) screening transformed cells and selecting the target gene using the appropriate marker system.

Yeasts are excellent hosts for the production of recombinant proteins, offering ease of genetic manipulation and cultivation to high cell density with a fast growth rate. Moreover, yeasts are able to perform complex eukaryotic-type posttranslational modification and produce proteins similar to proteins of mammalian origin. *S. cerevisiae*, the best genetically characterized organism, is the host used most frequently for transformation. However, the *S. cerevisiae* transformation system has some limitations, in that the proteins are often overglycosylated and may contain a terminal group suspected to be allergenic; the yield of recombinant proteins is relatively low, and the narrow substrate specificity of the species limits fermentation design. Some of the nonconventional yeasts, such as *P.* (*Komagataella*) *pastoris*, *P.* (*Ogataea*) *angusta* (*Hansenula polymorpha*), and others, may be more advantageous hosts, although the number of cloned genes, the availability of molecular genetic tools, and the understanding of metabolic regulation are limited compared with the case of *S. cerevisiae* (Cereghino and Cregg, 2000).

The primary efforts have been directed at the genetic improvement of the production characteristics of *S. cerevisiae* starter strains used in brewing, wine making, and baking. Table 10.2 lists some examples of these. Note that, in several cases, the genetic modification is achieved by self-cloning; that is, the GM strain does not contain foreign genes from organisms other than *S. cerevisiae*.

TABLE 10.2
Genetically Modified *Saccharomyces cerevisiae* Starters for Brewing, Baking, and Wine Making

Improvement	Proteins, Genes	Sources
Wine yeast		
Clarification, no haze	Pectate lyase *pelA*	*Erwinia chrysanthemi*
	Endopolygalacturonase *PGU1*	*S. cerevisiae*
Flocculation	Flocculin *FLO1*	*S. cerevisiae*
Flor formation	Adhesin *FLO11*	*S. cerevisiae*
Stress tolerance	Trehalose *TPS1,2*	*S. cerevisiae*
Ethanol tolerance	Sterols *SUT1*	*S. cerevisiae*
Glycerol overproduction	Glycerol-P-dehydr. *GPD1*	*S. cerevisiae*
Resveratrol production	β-Glucosidase *bglN*	*C. molischiana*
Malolactic fermentation	Permease *mae1*	*Schizo. pombe*
	Malic enzyme *mleS*	*Lactococcus lactis*
Brewer's yeast		
Dextrin fermentation	Glucoamylase *STA2*	*S. cerevisiae* var. *diastaticus*
	Amyloglucosidase *AMG*	*Aspergillus awamori*
Flocculation	Glucanase *EG1*	*Trichoderma reesii*
Diacetyl elimination	Acetolactate decarboxylase *ALDC*	*Enterobacter aerogenes*
Reduced H_2S production	Sulfuhydrase *MET25*	*S. cerevisiae*
Acetate esters production	Acetyltransferase *ATF1*	*S. cerevisiae*
Antibacterial property	Pediocin *pedA*	*Pediococcus cerevisiae*
	Leucocin *lcaB*	*Leuconostoc carnosum*
Baker's yeast		
Melibiose utilization	α-Galactosidase *MEL*1	*S. bayanus*
Maltose utilization	Stronger promoter *ADH*	*S. cerevisiae*
Cryoresistance	Aquaporin *AQY1*	*Schizo. pombe*
Osmotolerance	Glycerol synthesis *GPD1*	*S. cerevisiae*

Source: Data from Hammond, J. R. M. (1995) Yeast 11:1613–1627; Randez-Gil, F., Sanz, P., and Prieto, J. A. (1999) Trends Biotechnol. 17:237–243; González-Candelas, L., Gil, J. V., Lamuela-Raventós, R. M., and Ramón, D. (2000) Int. J. Food Microbiol. 59:179–183; Dequin, S. (2001) Appl. Microbiol. Biotechnol. 56:577–588; Pretorius, I. S. (2000) Yeast 16:675–729; Pretorius, I. S., du Toit, M., and van Rensburg, P. (2003) Food Technol. Biotechnol. 41:3–10; Schuller, D. and Casal, M. (2005) Appl. Microbiol. Biotechnol. 68:292–304; Panadero, J., Randez-Gil, F., and Antonio Prieto, J. (2005) J. Agric. Food Chem. 53:9966–9970; Verstrepen, K. J., Chambers, P. J., and Pretorius, I. S. (2006) *In*: Yeasts in Food and Beverages (eds. Querol, A. and Fleet, G. H.). Springer Verlag, Berlin. pp. 399–444.

Examples of the expression of heterologous genes in *S. cerevisiae* and other yeast hosts are listed in Tables 10.3 and 10.4. The development of transgenic strains has been extended to the broader field of biotechnology, in particular for the production of bioethanol and pharmaceuticals. The sequential introduction of multiple genetic alterations into a single host genome is now not exceptional. Examples are the total biosynthesis of the steroid hydrocortisone, which involves as many as 13 engineered genes (Szczebara et al., 2003), and a cellulose-fermenting yeast containing genes from four different organisms (van Rensburg et al., 1998). In this regard, it is worth mentioning that screening among yeast isolates from natural sources revealed rich sources of cellulose-decomposing strains, several of which turned out to be novel species (Nakase et al., 1994; Buzzini and Martini, 2002; Carreiro et al., 2004). Potential bioetanol and pharmaceutical producing strains may well be found among these isolates.

Genetic modification of microorganisms and, in particular, crop plants has been the subject of much controversy and is being debated both in scientific circles and by the general

TABLE 10.3
Genetic Modification of *Saccharomyces cerevisiae* Expressing Foreign Genes

Foreign Gene	Donor Species	Result
β-Galactosidase	*Kluyveromyces lactis*	Lactose utilization
L-Galactose dehydrogenase	*Arabidopsis thaliana*	Ascorbic acid (vitamin C)
α-Amylase + glucoamylase	*Lipomyces kononenkoa S'copsis fibuligerae*	Starch fermentation
Xylose isomerase	*Piromyces* sp. fungus	Xylose fermentation
α-Glucuronidase	*Aureobasidium pullulans*	Xylan degradation
Cellobiase + endo-β-glucanase + cellobiohydrolase + cellodextrinase	*S'copsis fibuligera, Butyrivibrio fibrinosolvens Phaanerochaete chrysosporiums, Ruminococcus flavefaciens*	Cellulose degradation
Pectate lyase	*Fusarium solani*	Pectin hydrolysis
Eight foreign genes, disruption five host genes	Mammalian	Hydrocortisone

Source: Data from Van Rensburg, P., van Zyl, W. H., and Pretorius, I. S. (1998) Yeast 14:67–76; Rubio-Texeira, M., Arevalo-Rodriguez, M., Lequerica, L., and Polaina, J. (2000) J. Biotechnol. 84:97–106; Szczebara, F. M., Chandelier, C., Villeret, C., Masurel, A., Bourot, S., Duport, C., Blanchard, S., Groisillier, A., Testet, E., Costaglioli, P., Cauet, G., Degryse, E., Balbuena, D., Winter, J., Achstetter, T., Spagnoli, R., Pompon, R., and Dumas, B. (2003) Nat. Biotechnol. 21:143–149; Sauer, M., Branduardi, P., Valli, M., and Porro, D. (2004) Appl. Environ. Microbiol. 70:6086–6091; Knox, A. M., du Preez, J. C., and Lilian, S. G. (2004) Enzyme Microbial Technol. 34:453–460; Kuyper, M., Hartog, M. M. P., Toirkens, M. J., Almering, M. J. H., Winkler, A. A., van Dijken, J. P., and Pronk, J. T. (2005) FEMS Yeast Res. 5:399–409; de Wet, B. J. M., van Zyl, W. H., and Prior, B. A. (2006) Enzyme Microb. Technol. 38:649–656; Gonz'alez-Candelas, L., Cortell, A., and Ramon, D. (1995) FEMS Microbiol. Lett. 126:263–270; Stephen, R., Hamilton, S. R., Bobrowicz, B. et al. (2003) Science 301(5637):1244–1246.

TABLE 10.4
Examples of the Production of Foreign Proteins in Nonconventional Yeasts

Yeast	Protein	Year of Publication
Schizo. pombe	Invertase from *S. cerevisiae*	1985
	α-Amylase from *Db. occidentalis*	1989
	Glucoamylase from *S*. "*diastaticus*"	1986
Pichia (*Komagataella*) *pastoris*	β-Galactosidase	1987
	Hepatitis B antigen	1987
	Bovine lysozyme	1989
	Human epidermal growth factor	1990
Pichia angusta (*Hansenula polymorpha*)	β-Lactamase	1988
	Glucoamylase	1991
	Human serum albumin	1990
Kluyveromyces lactis	Prochymosin	1990
	Human serum albumin	1991
	α-Amylase from *Db. occidentalis*	1989
Yarrowia lipolytica	Porcine α-interferon	1990
	Bovine prochymosin	1988
	Human proinsulin	1993
Zygo. bailii	Lactate dehydrogenase	2004

Source: Data from Romanos, M. A., Scorer, C. A., and Clare, J. J. (1992) Yeast 8:423–488; Madzak, C., Gaillardin, C., and Beckerich, J. M. (2004) J. Biotechnol. 109:63–81.

public. The great developments and achievements already made in the field should be taken seriously, and the issues arising from technological, environmental, economic, social, ethical, and political viewpoints should be discussed critically and rationally (Pretorius, 2000; Schuller and Casal, 2005; Verstrepen et al., 2006). Concerns about GMOs and GM products are beyond the scope of this review. Regarding microorganisms, it should be realized, however, that vaccines, drugs, and enzymes produced by genetically engineered strains have been on the market for years and are not only beneficial but also indispensable. Several of them are produced by GM yeasts, such as interferons, somatostatin, insulin, chymosin, and others. The issue is different, however, when not the purified product but the organism containing foreign genes itself is included in consumables or foods. Baker's yeast with high maltase activity, brewer's yeast with glucoamylase for dextrin hydrolysis, and a sake yeast with enhanced ethyl caproate flavor (Akada, 2002) have been approved by regulatory authorities. However, none of these has yet been commercialized, because the lack of public acceptance has made the industry refrain from using them (Moseley, 1999). The appearance in the U.S. market of recombinant wine yeast capable of malolactic fermentation may signal a breakthrough in this respect (Cummins, 2005).

10.3 GENOMICS

Genomics is the study of an organism's genome and the use of its genes. It deals with the systematic use of genomic information, combined with other data, to provide answers in biology, medicine, and industry. Genomics has the potential of offering new therapeutic methods for the treatment of some diseases, as well as new diagnostic methods. Other important applications are in the food and agriculture sectors.

10.3.1 Genome Sequences

The first genome to be sequenced in its entirety was that of bacteriophage ϕX174 in 1977. The sequencing of the first bacterium followed in 1995, and since then genomes have been sequenced at a rapid pace. *S. cerevisiae* was the first eukaryote to have its genome sequenced, in 1996 (Goffeau et al., 1996). A rough draft of the human genome was completed by the Human Genome Project in early 2001. The complete sequence was announced on the fiftieth anniversary of the publication of structure of DNA, in 2003. In recent years, the genome sequences of many prokaryotic microorganisms and higher eukaryotes have been completed, and large numbers of microbial genome sequencing projects are under way.

Following the deciphering of the *S. cerevisiae* genome, the next yeast species to be fully sequenced was the genetic model organism fission yeast, *Schizo. pombe*, in 2002. In 2000, the French consortium Génolevures started a program to explore, by partial "shotgun" sequencing, the genome of 13 ascomycetous yeasts (Souciet et al., 2000). From this and other projects, to date the complete sequences of 14 yeast species are known, and 10 partial sequences are available or sequencing is in progress (Dujon, 2005; Sherman et al., 2006) (Table 10.5). The size of yeast genomes falls in the range 9–20 Mb, and the number of genes (supposed genes, open reading frames, ORFs) varies from 4700 in the smallest genome (*Eremothecium gossypii*) to 6700 in the largest one (*Ya. lipolytica*). The best known is that of *S. cerevisiae*, first described and continually annotated (*Saccharomyces* Genome Database; Fisk et al., 2006).

The total sequence of the chromosomal DNA of *S. cerevisiae* constitutes 12 Mb. This is divided into a haploid set of 16 chromosomes, ranging in size from 200 to 2200 kb. The genome contains about 6300 potential genes (ORFs), of which the majority are protein-coding genes. However, the different databases give an estimation of ORFs between 6142 and 6340, and approximately 5800 are expressed as proteins. The number of known and characterized protein genes increases steadily as research progresses. In 2002, only 3289 protein genes were annotated (52%), which increased to 4004

TABLE 10.5
Yeast Species Sequenced Completely or Partially up to 2005

Species	Number of Chromosomes	Genome (Mb) Partial **	Genome (Mb) Complete	Reference
*Saccharomyces cerevisiae**	16		12.1	Goffeau et al. (1996)
*Saccharomyces paradoxus**	16		12.0	Kellis et al. (2003)
*Saccharomyces mikatae**	16		12.0	Kellis et al. (2003)
Saccharomyces kudriavzevii	16		12.0	Cliften et al. (2003)
*Saccharomyces uvarum**	16	4.7		Bon et al. (2000a)
Saccharomyces exiguus	14–16	2.4		Bon et al. (2000b)
Saccharomyces servazzii	7–12	2.4		Casaregola et al. (2000a)
*Saccharomyces castellii**	8		11.4	Cliften et al. (2003)
Saccharomyces kluyveri	8	2.3		Neuvéglise et al. (2000)
*Saccharomyces bayanus**	16		12.1	Kellis et al. (2003)
*Candida glabrata**	13		12.3	Dujon et al. (2004)
*Candida tropicalis**	12	2.3		Blandin et al. (2000a)
			14.5	Broad Inst.
Candida parapsilosis	14		10.1	Logue (2005)
*Candida albicans**	8		14.9	Jones et al. (2004)
*Candida dubliniensis **	10		14.5	Sanger Inst. (2004)
*Candida guilliermondii**	8		12.0	Broad Inst. (2004)
*Candida lusitaniae**	9		16.0	Broad Inst. (2005)
Zygosaccharomyces rouxii	7	4.5		Montigny et al. (2000a)
Kluyveromyces thermotolerans	7	2.4	10.6	Malpertuy et al. (2000)
*Kluyveromyces lactis**	6	5.6		Bolotin-Fukuhara et al. (2000)
			10.6	Dujon et al. (2004)
Kluyveromyces marxianus	10	2.3		Llorente et al. (2000)
*Kluyveromyces waltii**	8		10.7	Kellis et al. (2004)
Lodderomyces elongisporus	10		15.4	Broad Inst. (2006)
*Eremothecium gossypii**	7		9.2	Dietrich et al. (2004)
*Debaryomyces hansenii**	7		12.2	Dujon et al. (2004)
		2.6		Lépingle et al. (2000)
Pichia sorbitophila	7	4.4	13.9	Montigny et al. (2000b)
Pichia angusta	6	4.7		Blandin et al. (2000b)
Hansenula polymorpha	6		9.5	Ramezani-Rad et al. (2003)
*Pichia stipitis**	8		15.4	Joint Genome Inst. (2006)
*Yarrowia lipolytica**	6	4.5	20.5	Casaregola et al. (2000b)
*Cryptococcus neoformans**	14		17.8	Loftus et al. (2005)
*Schizosaccharomyces pombe**	3		12.5	Wood et al. (2002)

Notes: *Complete or nearly completed sequences.

**Génolevures project, partially sequenced (Souciet et al., 2000; Dujon, 2005).

References: Sanger Institute (2004) http://www.sanger.ac.uk/Projects/C_dubliniensis/; Broad Institute (2006) http://www.broad.mit.edu/annotation/fgi; Joint Genome Institute http://genome.jgi-psf.org/Picst3/Picst3.info.html; others in the list of references at the end of the chapter.

(63%) by 2005. Ribosomal RNA is coded in about 120 copies in tandem arrays on chromosome XII; there are more than 260 tRNA genes, and the rest is noncoding sequence, introns, and transposons. The extranuclear part of the yeast genome constitutes the mtDNA (only 86 kb), the 2-μm circle plasmid, and dsRNA viruses that occur in almost all strains.

10.3.2 Functional Genomics

Originally, the word "genome" referred to the complete genetic makeup of an organism. Because of the success of large-scale genome sequencing and molecular biology projects connected to it, the term has been extended to other contexts. The subset of genes transcribed in a given organism is called the transcriptome; the proteome is the collection of proteins found in a particular cell type under a particular set of environmental conditions; and the metabolome is the set of all metabolites and small molecules produced in a cell under given conditions. The terms genomics, transcriptomics, proteomics, and metabolomics have been coined to describe the study and the information obtained on genes, proteins, and metabolites (Figure 10.1).

Understanding the function of genes and other parts of the genome at the molecular level is known as functional genomics. Functional genomics uses various molecular tools to examine how organisms utilize their genome in different environments. It aims to determine patterns of gene expression and interaction in the genome, based on the knowledge of a large part of or the complete genomic sequence of an organism. It can provide an understanding of how microorganisms respond to environmental influences at the genetic level (i.e., by expressing or repressing specific genes) and reorganize genomic expression to adapt to stress conditions (Johnston, 2000).

Genome microarrays (DNA chips) are the method of choice to study functional genome expression (Lashkari et al., 1997; Bond and Blomberg, 2006). By monitoring the cellular RNA content, it is possible to observe the expression of many genes simultaneously, even those with unknown biological functions, as they are switched on and off during normal development or while an organism attempts to cope with changing environmental conditions. RNA profiling, or transcriptomics, can also shed light on genetic differences allowing some strains to perform better than others.

Gasch and Werner-Washburne (2002) review the functional genomics of yeast responses to environmental stress and starvation. Comparative analysis of the transcriptome in response to diverse environmental changes revealed that the expression of roughly 900 genes (around 14% of all yeast genes) is altered following stressful environmental transition; transcription of about two-thirds of them decreased (repressed genes), whereas transcription of around 300 genes increased (induced genes). More than 70% of repressed genes are involved in protein synthesis; in contrast, the induced genes are involved in a variety of cellular processes, including DNA repair, protein folding, cytoskeletal reorganization, and other processes.

Moving from the gene to the protein level, proteomics provides a fundamentally different yet complementary view of cellular metabolism. Proteomics is an approach that aims to identify and characterize complete sets of proteins and protein–protein interactions in a given species. To understand cellular functioning, it is also necessary to determine the localization and distribution in different compartments of the cellular content of proteins (the proteome).

Protein localization data are valuable in elucidating protein function. Kumar et al. (2002) reported the first proteome-scale analysis of protein localization within any eukaryote, *S. cerevisiae*. They determined the subcellular localization of 2744 yeast proteins and, extrapolating these data, defined

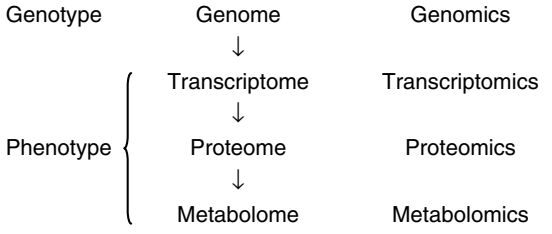

FIGURE 10.1 Flow of information in cells.

TABLE 10.6
Protein Function Categories (%) in the Yeast Protein Database

Protein Function	Ratio
Metabolism and energy	12.0
Protein transactions	11.4
RNA transactions	10.3
DNA transactions	4.9
Solute transport	4.6
Membrane transactions	4.8
Cell division	3.5
Cell wall structure	2.8
Signal transduction	1.6
Unknown	41.1

Source: http://www.proteome.com/database/YPD/ (2000).

the subcellular distribution of all 6100 yeast proteins. From this analysis, the yeast proteome has been estimated to encompass approximately 5100 soluble proteins and more than 1000 transmembrane proteins. About 47% of yeast proteins are cytoplasmic, 13% mitochondrial, 13% exocytic (including proteins of the endoplasmic reticulum and secretory vesicles), and 27% nuclear; of the latter proteins, 38% were found to be associated with chromosomal DNA. The distribution of yeast proteins in various functional categories is shown in Table 10.6. According to data from 2000, the function of more than 40% of proteins was unknown; currently this figure is about 28%.

Metabolomics is the next level of functional genomics. It is the study of all metabolites (intermediates and products) under a given set of conditions and their changes in response to environmental stresses. It also provides information as to which metabolic pathways are used by an organism and how regulation affects enzyme activities and metabolite concentrations. Analysis of the metabolic flux profiles of cells (fluxomics) represents one step further toward understanding cellular metabolism (Sanford et al., 2002). Different levels of genomics from genes to proteins and further to metabolites generate an avalanche of data. This necessitated the development of bioinformatics techniques that facilitate the interpretation of complex data sets. Integration of computerized information at different levels resulted in the *in silico* reconstruction of genome-scale metabolic networks that can model the real metabolic fluxes in yeast cells (Förster et al., 2003).

The functional modification and flexibility of the yeast genome to adapt to environmental changes have been demonstrated in numerous studies with yeasts. One of the best-known cases of genomic expression responses is the diauxic shift to adapt to a secondary growth substrate, which induces the expression of thousands of genes involved in transport, respiration, fatty acid metabolism, glyoxylate cycle, and other processes. Most of them subside as the cells enter the stationary phase (De Risi et al., 1997). Functional genomic analysis demonstrated similar responses in wine yeasts under differing nitrogen conditions and during the shift from fermentation to respiration (Backhus et al., 2001; Pérez-Ortin et al., 2002).

10.3.3 Comparative Genomics

The availability of a large number of genome sequences from various hemiascomycetous and other yeasts has allowed genome-wide comparison of different species to explore the evolutionary history of genomes and the general mechanisms of their development. Comparison of sequence data

among species can reveal phylogenetic relations and evolutionary processes that lead to genome diversification and speciation.

The availability of the complete genome of *S. cerevisiae* has inspired random sequencing efforts of related yeasts and resulted in partial coverage of more than 10 other yeast species within the frame of the Génolevures project (Génolevures Consortium, 2000). In comparison with *S. cerevisiae*, the genome of the closely related *S. bayanus* var. *uvarum* contains 2780 homologous ORF sequences, that of *Klu. lactis* also shares more than 2600 ORFs with *S. cerevisiae*, and even the more distantly related *Ya. lipolytica* has in common over 1000 homologous ORFs (Bon et al., 2000a,b; Bolotin-Fukuhara et al., 2000; Casaregola et al., 2000b).

One immediate impact of genome comparison has been the significant improvement of sequence annotation and the discovery and identification of genes (Brachat et al., 2003; Kellis et al., 2003). It was postulated several years ago that whole-genome duplication occurred in ancient *S. cerevisiae* (Wolfe and Shields, 1997). This can be a major force in biological evolution when the two copies follow separate development paths and evolve to fulfill different functions. Comparison of homologous chromosomal regions in closely and distantly related yeast species has confirmed this hypothesis (Dujon et al., 2004; Kellis et al., 2004; Bolotin-Fukuhara, 2006). Pieces of duplicated genome subsequently translocated to different chromosomes, and the majority (about 92%) of the genes were lost from one or the other duplicated copy.

Other modes of molecular evolution include single chromosome duplication (aneuploidity), duplication of a single gene or a segment of adjacent genes, chromosomal rearrangements, loss of genes, and acquisition of new genes through recombination. Recently, Barrio et al. (2006) reviewed the different molecular mechanisms that can play a role in the adaptive evolution of yeasts. Unlike in bacteria, horizontal gene transfer from another species is limited in yeasts (Hall et al., 2005). Interspecific hybridization, on the other hand, appears to be a mechanism of acquisition of new genes. Among *Saccharomyces sensu stricto* species, hybrids are easily formed. The brewer's yeast, *S. pastorianus*, is a well-characterized hybrid between *S. cerevisiae* and a *S. bayanus*-like yeast, inheriting chromosomes from both parents and mtDNA from the latter (Casaregola et al., 2001). *S. bayanus* itself appears to be a hybrid (Nguyen and Gaillardin, 2005), and hybrids have been described between *Zygosaccharomyces* species as well (James et al., 2005). It appears that natural interspecific hybridization is an important mechanism in the evolution of yeasts, providing new gene combinations (Bond and Blomberg, 2006).

Genomics studies combined with DNA microarray technology permit adaptive evolution within identical populations to be examined. In one experiment, Ferea et al. (1999) set up three replicate, clonal populations from a vegetatively reproducing *S. cerevisiae* strain. Propagation continued under identical conditions for 250 and 300 generations, at the end of which about 10% of the genes in microarrays showed evolutionary changes in comparison with the parent strain.

Sequence differences among the genomes of individual strains of a species can be revealed from global comparison using whole-genome DNA microarrays. This allows the detection of a variety of single base-pair substitutions, deletions, and insertions, and single nucleotide polymorphisms exist between two *S. cerevisiae* strains (Gresham et al., 2006). Evolutionary forces acting on the genome produce significant genetic variation in populations occupying different habitats. *S. cerevisiae* carrying about 30,000 single nucleotide polymorphisms is a powerful model for ecological and evolutionary genomics studies (Landry et al., 2006).

10.3.4 Application of Genomics

Sequence information on prokaryotic and eukaryotic organisms is increasing dramatically. After the sequencing of the first eukaryotic organism, the yeast *S. cerevisiae*, the completion of the human genome sequence represents another milestone in the science of the twenty-first century, genomics. We are now in the postgenomic era, when the development and implementation of new genomics techniques are leading to the exploitation of sequence information. With increasing application of

the potential implied in transcriptomics, proteomics, and metabolomics, new branches of "-omics" are emerging, developing a new "omics era" (Ward and White, 2002).

Genome sequences can be obtained directly from environmental samples without cultivation and isolation of individual strains. This has resulted in the emergence of metagenomics (also referred to as environmental or community genomics) as a powerful tool for the genomic analysis of natural microbial populations (Handelsman, 2004; Rodriguez-Valera, 2004; Allen and Banfield, 2005). Microorganisms that have not yet been cultured represent the vast majority of life in most environments. Metagenomics facilitates the study of the ecology and metabolic activity of microbial communities, and reveals the heterogeneity of environmental populations and the processes of adaptation governing changes and evolution. The knowledge gained from these studies provides valuable information on metabolic pathways and biosynthetic routes and enables screening of thousands of clones, offering the possibility of finding biotechnology applications (Steele and Streit, 2005). Although metagenomics is already used extensively in exploring natural communities of bacteria, it is yet to be adapted for studying fungal biota. An example is the study of the human yeast oral flora (Xu and Mitchell, 2003).

Genomics techniques are applied on a large scale by the pharmacological industry for the discovery of potential drugs, for the identification of drug targets, and for finding markers for various diseases ("pharmacogenomics") (Martin et al., 2000; McCarthy and Hilfiker, 2000). Another term, nutrigenomics, refers to the study of the influence of diet on genes—how our health is influenced through interactions of foods with our personal genetic make-up. Nutrigenomics explores the way various food constituents affect particular genes by increasing the risk of diseases such as diabetes, obesity, heart disease, and some cancers. A broader term, "nutritional genomics," covers nutrigenomics, which explores the effects of nutrients on the genome, proteome, and metabolome, and nutrigenetics, the major goal of which is to elucidate the effect of genetic variation on the interaction between diet and disease (van der Werf et al., 2001; Weimer and Mills, 2002).

Today genomics is also used in food production and processing (Pridmore et al., 2000; De Vos, 2001; Verrips et al., 2001; Abee et al., 2004). Genomic sequencing and genome-wide analysis of the transcriptome and proteome have been applied for the characterization of industrial yeasts such as lager, ale, wine, and baker's strains. These molecular tools also allow the comprehensive analysis of industrial fermentations to understand the complexity of environmental conditions as stress factors in industrial yeasts. Bond and Blomberg (2006), in a recent review, offer a broad survey of the principles and applications of genomics for the analysis of industrial yeasts, with particular emphasis on the characterization of yeast strains using the techniques of proteomics and transcriptomics. Comparative proteomics is an efficient tool for the analysis of lager, ale, wine, and baker's yeasts under the conditions of fermentation and for understanding gene expression and stress responses during the production of beer, wine, and yeast cell mass (Gasch et al., 2000; Causton et al., 2001; Erasmus et al., 2003; Higgins et al., 2003; James et al., 2003; Rossignol et al., 2003).

The use of selected strains of *S. cerevisiae* has yielded tremendous advantages in traditional fermentation and novel biotechnology industries. Although they have already resulted in numerous innovations and improvements in the properties of traditional starters, the methods of conventional breeding, hybridization, and selection are nevertheless somewhat limited in their capacity. The application of molecular techniques and recombinant gene technologies as further possible means for the development of novel starters will require serious consideration in the future. The introduction of foreign genes into baking, brewing, and wine yeasts and into a number of nonconventional yeast species has resulted in many improved strains genetically modified at the laboratory scale. Only a few of them have legal approval, but the lack of public acceptance has made the industry refrain from use in commercial applications. Hence, the exploration of the rich and only partially known biodiversity of natural ecosystems, among them indigenous fermentations, is a promising and challenging path in the quest for novel potential starters and adjuncts not only in the production of food and beverages, but also across various biotechnology sectors, from bioenergy and pharmaceuticals to bioremediation and environmental protection.

Today, by developing new tools and techniques and generating basic understanding of biological processes, genomics is leading to tremendous advances in biotechnology. As a result of genomics data and information, the genes for desirable traits can be rapidly identified and used to create new biotechnology products. The combination of microbial genomics and biotechnology is leading to the development of new diagnostic tools, better vaccines, and improved treatments for disease, better detection of pollutants, herbicide-resistant and insect-resistant crops, and cleanup of contaminated environments. The rapidly increasing genomics information has great potential to enhance the assessment of risks to human life and the environment and provide unprecedented opportunities for the amalgamation of the biological sciences with physics, computer information, and engineering technology.

The exploration of the genomes of a number of yeast species in increasing detail reveals the phylogeny and evolutionary history of this diversified group of eukaryotes, uncovers new dimensions in our understanding of the functions of genes and genomes, and opens up effective tools to explore them. In addition, as demonstrated in the previous chapters of this book, among yeasts there are undesired spoilage and even potentially pathogenic species that may affect the quality and safety of foods. Protection from and defense against these species are also enhanced by progress in genomics.

Genomic information provides insight into the metabolic capacity of microorganisms, leads to the discovery of new enzymes and metabolic pathways, contributes to our understanding of the growth and survival of pathogens and spoilage microbes through the food-processing chain, and enhances the development of advanced production strains for industrial fermentation. Yeasts have always been in the forefront of basic and applied research; they are maintaining and broadening their role in the genomics era, and will continue to be beneficial in human life.

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Appendices

A.1 MEDIA FOR DETECTION, ENUMERATION, AND IDENTIFICATION OF FOOD-BORNE YEASTS

Listed here are formulas for the most common media used for the detection, enumeration, and identification of food-borne yeasts. For detailed discussions on the use of these and other mycological media, the reader is referred to Samson et al. (1995), Yarrow (1998), Barnett et al. (2000), Deák (2003), and Kurtzman et al. (2003).

Chemicals should be of high quality, and distilled water should be used. Unless otherwise stated, all ingredients in a formula are given for 1 L of medium; they should be combined and dissolved by heating, and then sterilized by autoclaving at 121°C for 15 min.

A.1.1 General Media for Detection and Enumeration

A.1.1.1 Tryptone Glucose Yeast Extract Agar (TGY)

Tryptone 5 g, glucose 100 g, yeast extract 5 g, agar 15 g, pH 6.0.

TGY agar is a general-purpose medium. If used for testing food products loaded with bacteria that may overgrow yeast colonies, chloramphenicol (0.1 g) should be included (TGYC agar). To inhibit most of the bacteria or to select for preservative-resistant yeasts, TGY can be acidified with 1% glacial acetic acid added to molten and tempered (50°C) agar just before plates are poured; pH should be about 3.5 (ATGY agar).

A.1.1.2 Dichloran Rose Bengal Chloramphenicol Agar (DRBC)

Glucose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, Rose Bengal (5% solution, w/v) 0.5 mL, chloramphenicol 0.1 g, dichloran (0.2% w/v in ethanol) 1 mL, agar 15 g, pH 5.6.

DRBC agar is a commonly used general-purpose agar inhibiting growth of most bacteria and spreading molds. The original formula (King et al., 1979) contained chlortetracycline, which is heat labile and has been replaced with chloramphenicol, which can be added before autoclaving. Prepared agar should be stored away from light.

A.1.1.3 Dichloran 18% Glycerol Agar (DG18)

Glucose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, chloramphenicol 0.1 g, dichloran (0.2% w/v in ethanol) 1 mL, glycerol 220 mL, agar 15 g, pH 6.5.

Dissolve all ingredients except glycerol, make up the volume to 1 L, then add glycerol to give a final concentration of 18%, and sterilize. Commercial product is available. DG18 agar is recommended for testing xerotolerant (osmophilic) yeasts in intermediate-moisture foods; it is not to be used as a general-purpose medium.

A.1.1.4 Malt Agar (MA)

Malt extract powder 100 g, agar 30 g, pH 5.4.

Traditionally used for cultivating yeasts; its partial selectivity is due to low pH; because of this, however, apply milder autoclaving or steaming, and use higher amount of agar.

A.1.1.5 Sabouraud Glucose Agar

Glucose 20 g, peptone 10 g, agar 15 g, pH 7.0.

Another traditional medium used for cultivation of yeasts; without agar, it can also be used as broth.

A.1.1.6 Yeast Extract Malt Extract Medium (YM)

Yeast extract 3 g, malt extract 3 g, peptone 5 g, glucose 10 g, pH 6.5.

A general-purpose medium that can be used as broth or agar (20 g); also available commercially.

A.1.1.7 Wallerstein Laboratory Nutrient Medium (WL)

Yeast extract 4 g, tryptone 5 g, glucose 50 g, KH_2PO_4 0.55 g, KCl 0.425 g, $CaCl_2$ 0.125 g, $FeCl_3$ 0.025 g, $MnSO_4$ 0.025 g, bromcresolgreen 0.022 g, pH 5.5.

Slightly selective and differential medium for brewing and industrial fermentation. Commercially available.

A.1.2 Selective and Differential Media

A.1.2.1 Wild Yeast Media

(i) $CuSO_4$-MYGP

Malt extract 3 g, yeast extract 3 g, glucose 10 g, peptone 5 g, $CuSO_4 \cdot 5H_2O$ 312 mg, agar 20 g, pH 6.2.

The addition of $CuSO_4$ to commonly used general media is one of the several formulations to detect non-*Saccharomyces* and *Saccharomyces* wild yeasts in brewing yeast (Jespersen and Jakobsen, 1996).

(ii) Lysine agar

Yeast carbon base 11.75 g, L-lysine · HCl 2.3 g, agar 20 g.

A.1.2.2 *Brettanomyces* Enrichment Medium (EBB)

Grape juice 200 mL, ethanol 40 mL, malt extract 1.5 g, yeast extract 1.5 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4$ 0.2 g, Tween 80 2 mL, biphenyl 0.2 g, chloramphenicol 0.05 g, pH 5.0.

For specific enrichment of *Dekkera* (*Brettanomyces*) *bruxellensis* from grapes (Renouf and Lonvaud-Funel, 2006).

A.1.2.3 CHROMagar *Candida*

Peptone 10 g, chromogenic mixture 22 g (proprietary substance), chloramphenicol 0.5 g, agar 15 g.

Commercial product, developed for rapid presumptive clinical diagnosis of *Candida albicans*, but can be applied for differentiation of several types of food-borne yeasts (Tornai-Lehoczki et al., 2003).

A.1.2.4 *Yarrowia lipolytica* Differential Medium

Peptone 5 g, yeast extract 5 g, L-tyrosine 10 mM, $MnSO_4 \cdot 5H_2O$ 1 mM, lactate 50 mM, agar 20 g, pH 6.2.

Ya. lipolytica is recognized by the production of strong brown pigment from tyrosine within 24 h at 25°C (Carreira and Loureiro, 1998).

A.1.2.5 *Kluyveromyces* Differential Medium (KDM)

Peptone 3 g, malt extract 3 g, glucose 10 g, chloramphenicol 0.5 g, agar 20 g, pH 7.0; X-Gal 0.8 g, IPTG 0.1 g.

X-Gal (5Br-4Cl-3indoxyl-β-D-galactopyranoside; Sigma) and IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma) are added to the autoclaved and cooled (45°C) medium. Only two yeast species, *Klu. marxianus* and *Klu. lactis*, with both β-galactosidase and β-glucosidase activities, produce deep-blue-colored colonies (Valderrama et al., 1999).

A.1.2.6 *Debaryomyces hansenii* Differential Medium

Peptone 3 g, malt extract 3 g, glucose 10 g, chloramphenicol 0.5 g, agar 20 g, pH 7.0; Salmon-Gluc 0.15 g, OMe-Gluc 0.1 g.

Salmon-Glc (6Cl-3-indoxyl-β-D-glucopyranoside; Biosynth) and its inducer 1-*O*-methyl-β-D-glucopyranoside; Biosynth) are added to the autoclaved and cooled (45°C) medium. All *Db. hansenii* strains produce salmon-colored colonies. Some *Hanseniaspora* species may also produce pink-to-salmon colonies, but could easily be distinguished from *Db. hansenii* by characteristic apiculate cell morphology (Siloniz et al., 2000).

A.1.2.7 Eosin-Methylene Blue Differential Medium

Peptone 3 g, malt extract 3 g, glucose 10 g, chloramphenicol 0.5 g, methylene blue 0.065 g, triphenyltetrazolium 0.1 g, eosin 0.4 g, agar 20 g.

This medium can differentiate between some common food-borne yeast species by different colony color. *Zygo. bailii* and *Zygo. rouxii* develop black to violet colonies; *Tsp. delbrueckii*, *Db. hansenii*, and *Iss. orientalis* produce violet colonies; whereas those of *S. cerevisiae* are metallic green. When the medium is supplemented with 0.2 g K-tellurite and 5 g acetic acid, it becomes selective for *Iss. orientalis* and *Tsp. delbrueckii* (Siloniz et al., 1999).

A.1.3 Media for Identification

A.1.3.1 Acetate Broth (1%)

Basal medium with 5 g glucose and 10 mL glacial acetic acid. Add acetic acid to sterilized medium.

A.1.3.2 Basal Medium

Tryptone 5 g, yeast extract 5 g.

A.1.3.3 Benzoic Acid/Sorbic Acid Broth

Basal medium with 5 g glucose, pH adjusted to 3.5 with 0.1 M HCl. Add and dissolve 470 mg sodium benzoate or 400 mg potassium sorbate (equivalent to 400 mg/L benzoic acid or 300 mg/L sorbic acid, respectively).

A.1.3.4 Corn Meal Agar

Commercial dried medium is best used. If not available, add 60 g finely ground yellow corn meal into 900 mL water and heat (60°C) and extract for 1 h; filter through cheesecloth and bring the filtrate to 1 L; add and dissolve 20 g agar; distribute 5 mL portions into tubes. After sterilization, make slants.

A.1.3.5 Cycloheximide Broth

Basal medium with 10 g glucose. Add filter-sterilized cycloheximide stock solution (0.1 g in 100 mL) to sterilized medium to give a final concentration of 0.01%.

A.1.3.6 Disks for Nitrogen and Carbon Assimilation Tests

In performing a simple auxanographic test to determine the ability of yeasts to assimilate various carbon and nitrogen sources, it is customary to place a few milligram substrates on the surface of the agar plate, close to the brim of Petri dish. This procedure is, however, time consuming and does not apply uniform amounts of substrate. For routine application of a large number of auxanograms, it is practical to prepare filter paper disks impregnated with various substrates used in assimilation tests.

Filter paper disks (0.5–0.8-mm diameter) are sterilized by dry heat and saturated with 10× stock solution of substrates. From carbon sources, 500 mM solutions are prepared and filter sterilized. From nitrogen sources, only 10 mM stock solutions are used to avoid toxic concentrations of ethylamine, cadaverine, or nitrate. The impregnated disks are dried at 105°C and can be stored for months in hermetically sealed bottles. Disks containing certain carbon sources are commercially available (e.g., those used with the Minitek yeast kit).

A.1.3.7 Fermentation Broth

Basal medium with 20 g test sugar (e.g., glucose, sucrose, and maltose) and 2.5 mL bromthymol blue (1% solution in 80% ethanol). Distribute in Durham tubes (with a small inverted tube). After autoclaving and cooling, the insert tube should be completely filled with broth.

A.1.3.8 60% Glucose Agar

Basal medium with 20 g agar. Glucose added in 60% (w/w) to the melted agar. After steaming for 30 min, slants are prepared.

A.1.3.9 Maintenance Agar

Basal medium with 10 g glucose and 20 g agar. Use slants.

A.1.3.10 Malt Extract Yeast Extract Medium

Malt extract 3 g, yeast extract 3 g, peptone 5 g, glucose 10 g. Use as broth or solidified with 25 g agar.

A.1.3.11 Morphology Media

Basal medium with 5 g glucose or malt extract yeast extract broth can be used for studying cell morphology and pellicle formation or determining growth temperature.

For demonstrating the formation of hyphae or ballistoconidia, slide cultures on potato glucose agar or corn meal agar can be used. These are prepared by pipetting 0.5 mL molten agar on a sterile glass microscope slide to form a thin layer. Inoculate the center of the layer lightly with a straight

wire. Place the slide on a U-shaped glass rod in a Petri dish. Put a piece of wet cotton in the Petri dish to avoid drying during incubation.

A.1.3.12 Potato Glucose Agar

Commercial product (PDA—potato dextrose agar) is best used. If not available, wash, peel, and use finely ground white potato tubers. Soak 100 g in 300 mL water overnight in a refrigerator. Autoclave and filter through cheesecloth. Mix 230 mL potato extract with 770 mL water, and add 20 g glucose and 20 g agar; heat to dissolve.

A.1.3.13 Rapid Urea Broth

Bacto yeast extract 0.1 g, KH_2PO_4 0.091 g, Na_2PO_4 0.095 g, urea 20 g, phenol red (1% solution) 1 mL, pH 6.9.

A.1.3.14 16% Salt Glucose Agar

Basal medium with 50 g glucose and 20 g agar. Dissolve 16% (w/w) NaCl in molten agar and steam for 30 min. Use slants.

A.1.3.15 Vitamin-Free Broth

Best used is Bacto vitamin-free yeast base (1.67 g L^{-1}). If not available, use a broth containing glucose 10 g, $(NH_4)_2SO_4$ 5 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g.

A.1.3.16 Vitamin Stock Solution

A 100× stock solution contains (per L): biotin 0.2 mg, Ca-pantothenate 40 mg, folic acid 0.2 mg, inositol 200 mg, niacin 40 mg, *p*-aminobenzoic acid 20 mg, pyridoxine-HCl 40 mg, riboflavin 20 mg, and thiamin 100 mg. Sterilize by filtration. It can be prepared from commercial Bacto yeast nitrogen base without amino acids and ammonium sulfate (17 g L^{-1}) or may be substituted with 2% yeast extract solution.

A.1.3.17 Yeast Carbon Base

Commercial Bacto Yeast Carbon Base is best used. Dissolve 11.7 g and solidify with 20 g agar. It may be substituted with the following medium: glucose 10 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 20 g. Yeast carbon base is used for nitrogen assimilation tests; see A.1.3.b.

A.1.3.18 Yeast Nitrogen Base

Commercial Bacto Yeast Carbon Base is best used. Dissolve 6.7 g and solidify with 20 g agar. If not available, it may be substituted with the following medium: $(NH_4)_2SO_4$ 5 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 20 g. It is used for carbon assimilation tests; see A.1.3.b.

A.2 SIMPLIFIED IDENTIFICATION METHOD FOR THE MOST COMMON FOOD-BORNE YEASTS

A.2.1 The Simplified Method

On the basis of previous experience and criticism (discussed in Section 9.3), an improved version of the simplified method (SIM) has been developed and is presented here. The current key includes

120 yeast species that occur most frequently in various foods. The simplified method applies only to a selected number of tests that are most effective in the identification of a restricted domain of food yeasts. The diagnostic characters of the species considered were collected from the literature (Kreger-van Rij, 1987; Barnett et al., 1990, 2000; Kurtzman and Fell, 1998) and expressed as probabilities in terms of percentage of positive reactions, instead of the customary +, −, ±, or v (variable), w (weak), or d (delayed) denotations. These are summarized in Table A.1. In constructing the identification keys, tests were used in which yeast species give 85–99% unequivocal (positive or negative) responses.

In all, 30 tests are applied in the identification keys (Table A.2). The majority of tests (18) are conventional carbon- and nitrogen-source assimilations performed by the auxanographic method. In addition, the fermentation of glucose, growth in the presence of 0.01% cycloheximide, and the urease reaction are always tested, while some other physiological tests (growth at 37°C, without added vitamins, in 60% glucose or 16% NaCl) are to be tested only for the differentiation of certain otherwise similar yeasts. These physiological and biochemical tests are further supplemented by morphological observations such as the shape of cells and the occurrence of true or pseudohyphae or arthroconidia. No specific procedures are used to demonstrate the formation of sexual spores, as this trait is not used in the key, and the species are always named according to the teleomorphic form whenever it is known. If the presence of spores or conjugating cells is noted, these are valuable pieces of information in confirming species assignment. In addition to microscopic morphology, macroscopic properties of growth are sometimes also considered in the key, and these can be ascertained without further tests (e.g., color of colony and formation of pellicle). Of all these tests, only 17 are included in the standard identification regime of SIM and performed with each isolate to be identified, avoiding at least two-thirds of the tests required for the traditional identification method. Only three test tubes and three Petri dishes are necessary to perform the standard set of tests, thus saving materials. The arrangement of standard tests is shown in Figure A.1. The procedures for identification tests and the suggested regime for the application of SIM are described below, and the formulas for media and reagents used are given in Appendix 1.

A.2.2 The SIM Procedure

In the mycological investigation of foods, yeast strains are often isolated from selective media formulated to suppress the growth of bacteria or to retard the spreading of molds. This does not guarantee the purity of yeast colonies developed on these media. Identification should never be initiated with colonies picked directly from such selective or general-purpose media used for plating food samples. Purification of strains must be achieved by repeated streaking on nonselective general-purpose media (e.g., tryptone glucose agar and potato dextrose agar), and a well-isolated colony similar in appearance to other colonies that have been subcultured on solid media for 24–48 h should serve as a starting inoculum for identification.

Observation of the morphology of colonies and cells is of great value in identification. Morphological investigations should begin with the observation of colonies at the first isolation and during the purification of yeast cultures. The color and the appearance of colonies provide useful information. Whether colonies are smooth, shiny or rough, dull, wrinkled, creamy, slimy or mucous in appearance on solid media and the formation of film or pellicle on the surface of broth cultures can aid identification. Observation of wet mounts under light microscopy may reveal unique cell morphology such as splitting, lemon-shaped or conjugating cells, long filaments or cylindrical pseudohyphal cells, or septate hyphae. Sometimes, spores can also be observed. Although microscopy can be tedious, it is worth doing frequently in the course of identification. Data on microscopic morphology can be collected from young agar cultures at the time of purification and also later from broth media and potato agar slide culture. Spores can often be revealed in older cultures. When examining slide cultures, care should be taken to distinguish true and pseudohyphae. Three criteria distinguish between these forms of filaments: (1) cross walls (septae) can be regularly found in true hyphae; (2) the

TABLE A.1
Diagnostic Characteristics of Food-Borne Yeasts: Probabilities (%) of Positive Reactions

Species	Ure	Nt	Et	Cyc	Ce	Mt	M	R	G	Me	Mg	Kg	Cd	Ct	It	D	vit	37	L	Te	Km	lA	Rm	X	Hy	Ph	Pe	A	sp	red
Bret. naarde nensis	1	1	1	99	99	90	40	1	90	1	1	40	99	1	1	99	1	1	1	99	75	1	50	99	1	99	1	1	1	1
Bulleromy- ces albus	99	1	40	50	99	90	99	99	90	80	99	90	99	70	99	1	1	1	75	99	75	99	99	99	50	1	50	*B*	1	1
C. albicans	1	1	1	99	1	99	99	1	99	1	50	99	99	99	1	99	15	99	1	85	1	50	1	99	50	1	99	1	1	1
C. apicola	1	1	1	1	1	99	1	99	1	1	1	70	99	70	1	85	50	1	1	1	1	1	1	80	1	50	1	1	1	1
C. boidinii	1	99	99	99	1	99	1	1	1	1	1	15	99	1	1	99	1	20	1	1	1	20	1	99	1	99	99	1	1	1
C. cantarellii	1	1	99	99	1	99	1	1	15	1	1	99	1	1	1	99	99	1	1	99	1	1	1	25	1	1	1	1	1	1
C. catenulata	1	1	1	99	1	99	75	1	99	1	1	30	99	99	1	50	1	70	1	65	70	1	1	70	1	99	20	1	1	1
C. diddensiae	1	1	99	1	99	99	99	1	99	1	65	1	99	90	1	85	1	99	1	85	1	99	1	99	1	99	1	1	1	1
C. diversa	1	1	1	1	1	99	1	1	1	1	1	1	99	99	1	99	1	40	1	1	1	1	1	50	1	15	1	1	1	1
C. etchellsii	1	99	1	1	1	50	80	1	85	1	1	65	50	30	1	70	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C. glabrata	1	1	1	1	1	1	1	1	1	1	1	20	1	1	1	99	1	99	1	85	1	1	1	1	1	1	1	1	1	1
C. inconspicua	1	1	1	1	1	1	1	1	1	1	1	1	99	80	1	1	1	99	1	1	1	1	1	1	1	30	1	1	1	1
C. intermedia	1	1	1	15	99	99	99	99	99	1	85	99	99	85	1	99	1	1	99	99	70	30	30	99	1	99	99	1	1	1
C. lactis- condensi	1	99	1	15	1	1	1	85	1	1	1	1	1	1	1	99	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C. magnoliae	1	99	1	15	40	99	1	50	70	1	1	85	99	90	1	99	1	70	1	20	1	1	1	20	1	1	15	1	1	1
C. maltosa	1	1	1	99	80	99	99	1	99	1	99	99	99	60	1	99	1	99	1	99	1	1	1	99	1	99	1	1	1	1
C. mesenterica	1	1	99	1	85	85	99	1	1	1	99	99	99	85	1	15	1	1	1	85	1	1	1	15	99	99	50	1	1	1
C. milleri	1	1	1	50	1	1	1	99	99	1	1	1	1	1	1	99	1	1	1	99	1	1	1	1	1	1	1	1	1	1
C. norvegica	1	99	1	15	99	99	1	1	1	1	1	1	99	75	1	85	1	1	1	1	1	1	70	90	1	30	1	1	1	1
C. oleophila	1	1	1	99	99	99	99	1	99	1	50	99	99	99	1	99	1	1	1	99	20	1	1	99	1	99	99	1	1	1
C. parapsilosis	1	1	1	50	1	99	99	1	99	1	99	99	99	99	1	99	15	99	1	99	1	99	1	99	1	99	1	1	1	1
C. rugosa	1	1	1	1	1	80	1	1	85	1	1	1	99	30	1	1	1	99	1	1	1	10	1	50	1	99	80	1	1	1
C. sake	1	1	1	1	40	99	99	1	99	1	50	99	99	50	1	99	50	1	1	99	1	1	1	85	1	99	30	1	1	1
C sorboxylosa	1	1	1	1	1	1	1	1	1	1	1	1	99	99	1	30	1	50	1	1	1	1	1	99	1	99	1	1	1	1
C. stellata	1	1	1	1	1	1	1	99	1	1	1	1	1	1	1	99	1	1	1	1	1	1	1	1	1	1	1	1	1	1
C. tenuis	1	1	85	30	99	99	99	1	99	1	99	99	99	99	1	85	10	10	80	99	60	50	99	99	10	99	1	1	1	1

(Continued)

TABLE A.1
(Continued)

Species	Ure	Nt	Et	Cyc	Ce	Mt	M	R	G	Me	Mg	Kg	Cd	Ct	It	D	vit	37	L	Te	Km	lA	Rm	X	Hy	Ph	Pe	A	sp	red
C. tropicalis	1	1	1	99	90	99	99	1	99	1	80	99	99	80	1	99	10	99	1	99	99	1	1	99	50	99	1	1	1	1
C. vartiovaarae	1	99	1	1	99	99	99	1	1	1	99	1	99	70	1	99	99	1	1	99	1	1	1	99	1	50	50	1	1	1
C. versatilis	1	99	1	70	90	90	90	70	99	75	15	99	99	70	1	99	1	1	60	99	1	10	1	10	1	1	1	1	1	1
C. vini	1	1	1	1	1	99	1	1	1	1	1	25	99	1	1	10	1	1	1	1	1	1	1	1	1	99	50	1	1	1
C. zeylanoides	1	1	1	99	10	99	1	1	10	1	1	99	80	99	1	15	15	15	1	99	1	1	1	1	1	99	30	1	1	1
Citero. matritensis	1	99	1	1	1	99	99	99	1	1	99	99	99	70	1	99	99	1	1	99	1	1	1	1	1	1	1	1	R	1
Clavisp. lusitaniae	1	1	1	1	85	99	99	1	70	1	85	99	99	50	1	99	30	99	1	99	1	30	85	99	1	99	1	1	O	1
Cry. albidus	99	99	15	1	99	85	85	90	50	30	60	99	99	50	85	1	1	15	70	70	50	85	50	85	1	1	1	1	1	1
Cry. curvatus	99	1	80	70	99	60	75	99	99	1	50	99	99	99	85	1	30	15	85	85	70	30	50	99	99	99	50	1	1	1
Cry. diffluens	99	99	15	1	99	85	85	90	1	1	50	99	99	99	90	1	1	1	1	70	50	85	50	85	1	1	1	1	1	1
Cry. humicolus	99	1	99	90	70	99	99	85	99	90	85	99	90	70	99	1	15	15	99	99	50	70	70	99	99	99	99	1	1	1
Cry. laurentii	99	1	75	50	99	99	99	99	99	99	99	99	85	80	99	1	50	15	99	99	70	99	85	85	15	15	50	1	1	15
Cysto. infirmomin	99	99	15	50	99	99	99	99	70	1	1	99	99	99	99	1	1	1	30	85	99	99	50	85	15	15	1	1	1	99
Db. carsonii	1	1	1	1	50	99	99	50	99	50	99	99	99	99	1	1	1	30	1	99	99	20	50	90	1	99	50	1	R	1
Db. etchellsii	1	1	1	1	99	99	99	1	99	1	99	99	99	99	1	85	1	99	1	99	1	40	1	99	1	99	1	1	R	1
Db. hansenii	1	1	85	50	85	99	99	99	99	70	99	99	99	70	1	30	15	50	50	99	70	85	50	99	1	15	50	1	R	1
Db. occidentalis	1	1	1	99	99	99	99	99	99	50	50	99	99	99	1	99	1	50	50	99	99	50	1	99	1	1	1	1	R	1
Db. polymorphus	1	1	99	99	99	99	99	99	99	85	99	99	99	99	1	60	99	40	75	99	99	30	1	85	1	50	99	1	R	1
Dekkera anomala	1	85	1	99	99	85	85	50	99	1	75	1	99	1	1	99	1	85	85	85	1	1	1	1	50	99	20	1	H	1
Dekkera bruxellensis	1	60	1	99	50	1	85	15	70	1	75	20	99	1	1	99	1	85	1	85	1	1	1	1	50	99	50	1	H	1
Dipodascus ingens	1	1	1	99	1	1	1	1	99	1	1	1	99	1	1	1	70	1	1	1	1	1	1	1	1	99	99	*A*	R	1
F'ella neoformans	99	1	50	1	85	99	99	70	99	1	99	99	30	70	99	1	1	70	1	99	70	85	70	99	50	1	1	1	1	1
Filob. capsuligenum	99	1	1	15	85	99	99	1	85	1	99	1	99	99	99	99	1	1	1	99	99	30	1	70	50	50	30	1	1	1
Gal. geotrichum	1	1	1	50	1	70	1	1	99	1	1	1	50	50	1	15	50	1	1	1	1	1	1	99	99	1	99	*A*	R	1
Geo. fermentans	1	1	1	99	99	99	1	1	99	1	1	1	99	99	1	90	99	30	1	1	1	50	1	99	99	1	99	*A*	1	1
Geo. fragrans	1	1	1	99	1	70	1	1	99	1	1	1	99	15	1	85	1	1	1	1	1	1	1	1	99	1	99	*A*	1	1
Guehom. pullulans	99	99	99	15	99	99	85	99	99	85	70	99	50	99	70	1	70	1	85	99	99	70	85	70	99	70	85	*A*	1	1
Hsp. guilliermondii	1	1	1	99	99	1	1	1	1	1	1	99	99	1	1	99	1	1	1	15	1	1	1	1	1	15	1	1	H	1
Hsp. occidentalis	1	1	1	1	99	1	1	1	1	1	1	1	99	1	1	99	1	1	1	1	1	1	1	1	1	1	30	1	1	H	1

Hsp. osmophila	1	1	1	1	99	1	99	1	1	1	1	1	99	1	1	99	1	1	1	15	1	1	1	1	1	85	1	1	R	1
Hsp. uvarum	1	1	1	99	99	1	1	1	1	1	1	99	99	1	1	99	1	1	1	15	1	1	1	1	1	30	1	1	R	1
Hsp. valbyensis	1	1	1	99	99	1	1	1	1	1	1	1	99	1	1	99	1	1	1	15	1	1	1	1	1	1	1	1	H	1
Hsp. vini	1	1	1	1	99	1	85	1	1	1	1	1	99	1	1	99	1	1	1	1	1	1	1	1	1	1	1	1	R	1
Issat. orientalis	1	1	1	1	1	1	1	1	1	1	1	1	99	85	1	99	99	99	1	1	1	1	1	1	1	99	99	1	R	1
Issat.. terricola	1	1	1	1	1	1	1	1	1	1	1	30	99	85	1	85	1	70	1	1	1	1	1	1	1	99	99	1	R	1
Kazach. exiguus	1	1	1	85	1	1	1	85	99	1	1	1	1	1	1	99	1	1	1	99	1	1	1	1	1	1	1	1	R	1
Kazach. unisporus	1	1	1	99	1	1	1	1	99	1	1	1	99	1	1	99	1	1	1	50	1	1	1	1	1	1	1	1	R	1
Kluyv. lactis	1	1	1	99	85	99	70	85	99	1	75	30	99	20	1	99	1	70	85	70	1	1	1	50	1	50	50	1	R	1
Kluyv. marxianus	1	1	1	99	65	60	1	99	99	1	1	1	99	20	1	99	1	99	70	15	1	50	1	85	1	50	15	1	R	1
Lachancea. thermotolerans	1	1	1	1	1	85	99	99	85	1	99	15	99	1	1	99	1	30	1	99	1	1	1	1	1	50	1	1	R	1
Lachancea cidri	1	1	1	99	1	99	99	99	99	99	99	99	50	1	1	99	1	1	1	99	1	1	1	50	1	1	1	1	R	1
Lachancea fermentati	1	1	1	99	70	85	99	99	99	30	99	50	99	1	1	99	1	99	1	99	1	1	1	50	1	50	1	1	R	1
Lachancea kluyveri	1	1	1	1	15	80	85	85	99	99	99	15	99	1	1	99	1	99	1	99	1	1	1	1	1	25	1	1	R	1
Leucosp. scottii	99	99	1	50	85	99	99	99	99	50	1	85	99	70	50	1	85	1	50	99	1	50	85	99	30	70	1	1	1	1
Lipomyces lipofer	1	1	99	99	99	99	99	99	99	99	99	99	50	99	1	1	30	1	50	99	99	50	1	50	1	1	1	1	R	1
Loddero. elongisporus	1	1	1	99	1	99	99	1	99	1	99	99	99	99	1	99	15	99	1	99	1	1	1	70	1	99	1	1	O	1
Metsch. pulcherrima	1	1	1	1	99	99	99	1	99	1	99	99	99	99	1	99	1	15	1	99	1	1	1	85	1	30	1	1	N	1
Metsch. reukaufii	1	1	1	1	99	99	99	1	75	1	70	99	99	15	1	99	1	15	1	99	1	1	1	50	1	30	1	1	N	1
Naumovia dairenensis	1	1	1	70	1	1	1	1	99	1	1	1	1	1	1	99	1	15	1	30	1	1	1	1	1	1	1	1	R	1
P. angusta	1	99	99	99	80	99	99	1	15	1	80	1	99	80	1	99	1	99	1	99	1	1	50	70	1	1	15	1	H	1
P. anomala	1	99	99	1	85	99	99	80	70	1	99	1	99	99	1	99	99	50	1	85	99	15	1	50	1	70	50	1	H	1
P. burtonii	1	1	99	1	99	99	99	85	99	1	85	99	99	99	1	85	70	1	1	99	99	50	1	85	99	99	85	*A*	H	1
P. fabianii	1	99	1	10	99	99	99	99	1	1	99	1	99	99	1	99	1	99	1	90	99	1	1	99	1	50	99	1	H	1
P. farinosa	1	1	99	15	70	99	1	1	99	1	1	1	99	99	1	85	50	99	30	50	1	15	15	50	1	99	99	1	R	1
P. fermentans	1	1	1	1	1	1	1	1	1	1	1	1	99	99	1	99	1	99	1	1	1	1	1	99	1	99	99	1	H	1
P. guilliermondii	1	1	1	99	99	85	99	99	99	85	85	99	99	99	1	85	15	99	1	99	1	99	50	99	1	99	50	1	H	1
P. holstii	1	99	30	99	99	99	99	1	90	1	99	90	99	99	1	99	1	85	1	99	99	99	99	99	99	99	50	1	H	1
P. jadinii	1	99	1	1	99	85	99	99	1	1	85	15	99	99	1	99	99	99	1	99	1	1	1	85	1	99	50	1	H	1

(Continued)

TABLE A.1
(Continued)

Species	Ure	Nt	Et	Cyc	Ce	Mt	M	R	G	Me	Mg	Kg	Cd	Ct	It	D	vit	37	L	Te	Km	lA	Rm	X	Hy	Ph	Pe	A	sp	red
P. kluyveri	1	1	1	1	1	1	1	1	1	1	1	1	99	50	1	99	1	50	1	1	1	1	1	1	1	99	99	1	H	1
P. membrani-faciens	1	1	1	1	1	1	1	1	1	1	1	1	99	1	1	15	50	30	1	1	1	1	1	30	1	99	99	1	R/H	1
P. nakasei	1	1	1	1	1	1	1	1	1	1	1	1	99	20	1	99	1	1	1	1	1	1	1	30	1	99	1	1	H	1
P. ohmeri	1	1	1	50	99	99	99	99	99	1	99	99	99	99	1	99	1	99	1	99	1	1	1	15	1	99	99	1	R/H	1
P. subpelliculosa	1	99	99	1	70	99	99	99	50	1	99	1	99	85	1	99	15	70	1	99	70	15	1	85	70	99	30	1	H	1
Rho. glutinis	99	99	1	50	90	90	99	85	85	1	85	85	70	70	1	1	50	50	1	99	1	50	50	85	1	1	1	1	1	99
Rho. minuta	99	1	1	50	70	85	1	15	15	1	15	90	15	30	1	1	30	30	50	70	1	85	1	70	1	1	1	1	1	99
Rho. mucilaginosa	99	1	1	50	80	85	85	99	80	1	30	15	60	50	1	1	30	30	1	99	1	70	30	99	1	15	1	1	1	99
S. bayanus	1	1	1	1	1	40	70	70	70	15	50	1	1	1	1	99	70	50	1	70	15	1	1	1	1	50	1	1	R	1
S. cerevisiae	1	1	1	1	1	1	70	70	70	50	50	1	1	1	1	99	15	50	1	50	30	1	1	1	1	50	1	1	R	1
S. pastorianus	1	1	1	1	1	1	70	70	70	70	50	1	1	1	1	99	15	1	1	70	15	1	1	1	1	25	1	1	R	1
S'codes ludwigii	1	1	1	1	99	1	1	70	1	1	1	1	99	1	1	99	1	30	1	1	1	1	1	1	1	50	1	1	R	1
S'copsis fibuligera	1	1	70	99	99	70	99	70	1	1	99	15	60	50	80	85	1	90	1	50	99	1	1	1	99	50	30	1	H	1
S'copsis vini	1	1	1	99	10	70	50	70	1	1	50	30	1	1	1	10	1	1	1	30	1	1	1	1	99	1	99	1	R	1
Schizo. japonicus	99	1	1	1	1	1	20	90	1	1	1	1	1	1	1	99	1	99	1	1	1	1	1	1	99	1	1	*A*	R	1
Schizo. octosporus	99	1	1	1	1	1	70	1	1	1	1	1	50	1	1	99	1	60	1	1	1	1	1	1	1	1	1	*A*	R	1
Schizo. pombe	99	1	1	50	1	1	99	99	15	1	50	50	70	1	1	99	1	99	1	1	1	1	1	1	1	1	1	*A*	R	1
Sporid. pararoseus	99	1	1	1	99	70	99	90	70	1	90	30	99	50	1	1	85	1	1	99	50	1	50	30	99	1	99	1	1	99
Sporid. salmonicolor	99	99	1	99	30	99	30	70	30	1	30	1	1	50	1	1	99	1	1	85	1	15	1	50	50	50	50	*B*	1	99
Sporob. roseus	99	70	1	15	85	85	99	99	50	1	50	1	50	30	1	1	30	1	1	70	85	30	1	20	15	15	1	*B*	1	85

Stephanoascus ciferrii	1	1	99	1	85	85	99	99	99	85	15	99	99	50	99	1	1	99	1	99	50	99	85	99	99	30	50	1	H	1
Tsp. delbrueckii	1	1	1	1	1	70	30	50	50	1	50	99	1	1	1	99	30	30	1	85	1	1	1	15	1	50	1	1	R	1
Tsp. globosa	1	1	1	99	1	50	1	99	1	1	1	99	1	1	1	99	99	99	1	15	1	1	1	1	1	1	1	1	R	1
Tsp. microellipsoides	1	1	1	1	1	50	1	99	99	99	1	50	99	1	1	99	15	1	1	15	1	1	1	15	1	50	1	1	R	1
Trichosp. moniliforme	99	1	99	99	99	99	85	85	99	85	70	99	99	70	70	1	1	1	99	75	70	70	70	90	99	70	85	*A*	1	1
Wickerh. domercqiae	1	99	1	1	1	99	1	1	70	1	1	99	99	99	1	1	1	15	1	1	1	1	1	15	1	1	1	1	R	1
Williopsis californica	1	99	1	1	99	99	50	1	1	1	50	1	50	70	1	99	1	1	1	50	1	1	50	99	1	1	50	1	S	1
Williopsis saturnus	1	99	1	1	99	50	50	99	1	1	50	1	99	1	1	99	99	50	1	50	1	1	50	99	1	1	99	1	S	1
Yarrowia lipolytica	1	1	99	99	15	99	1	1	15	1	1	15	99	85	1	1	1	15	1	1	1	1	1	1	85	99	50	30	R/H	1
Zygoascus hellenicus	1	1	1	99	85	99	99	70	99	1	15	1	99	30	99	99	1	99	1	99	99	99	50	99	99	99	30	1	H	1
Zygo. bailii	1	1	1	1	1	85	1	30	50	1	1	50	99	1	1	99	1	50	1	70	1	1	1	1	1	50	1	1	R	1
Zygo. bisporus	1	1	1	1	1	50	1	1	50	1	1	15	99	1	1	99	1	1	1	1	1	1	1	30	1	1	1	1	R	1
Zygo. mellis	1	1	1	1	1	70	50	1	50	1	1	15	99	1	1	99	15	50	1	50	1	1	1	1	1	50	15	1	R	1
Zygo. rouxii	1	1	1	1	1	80	50	1	50	1	1	10	99	1	1	99	15	50	1	40	1	1	1	1	1	50	15	1	R	1
Zygotorulasp. florentinus	1	1	1	99	1	85	70	99	70	99	99	50	99	1	1	99	30	1	1	99	1	1	1	30	1	50	1	1	R	1
Zygotorulasp. mrakii	1	1	1	99	1	99	1	99	99	99	1	99	50	1	1	99	99	1	1	1	1	1	1	1	1	1	1	1	R	1

Note: see abbreviations for generic names in the front of this book.

Abbreviations: ure, urease reaction. Assimilation tests: lA, L-arabinose; Cd, cadaverine; Ce, cellobiose; Ct, citrate; Et, erythritol; It, inositol; Kg, 2-ketogluconate; L, lactose; M, maltose; Me, melibiose; Mg, α-methylglucoside; Mt, mannitol; Nt, nitrate; Rm, rhamnose; St, starch; Te, trehalose; X, xylose. Growth tests: Cyc, 0.01% cycloheximide; vit, without vitamins; 37, at 37°C. Fermentation: d, glucose. Morphology: Hy, true hyphae; Ph, pseudohyphae; Pe, pellicle; A, arthroconidia; B, ballistoconidia; sp, spores; R, round; O, oval; H, hat-shaped; N, needle-shaped. S, Saturn-shaped; Red, color of colony.

Values mark positive responses, 99 (always positive) and 1 (always negative) are used only for computational purposes instead of 100 and 0, respectively.

TABLE A.2
List of Tests Used in SIM

Standard Tests	Supplementary Tests
Urease reaction	Growth at 37°C
Nitrate assimilation	Growth without vitamins
Growth with 0.01% cycloheximide	Xylose assimilation
Fermentation of glucose	Trehalose assimilation
Cadaverine assimilation	Lactose assimilation
Cellobiose assimilation	Rhamnose assimilation
Mannitol assimilation	L-Arabinose assimilation
Erythritol assimilation	Sucrose assimilation
2-Ketogluconate assimilation	Fermentation of sucrose
α-Methyl glucoside assimilation	Growth with 1% acetate
Raffinose assimilation	Growth with 16% NaCl
Maltose assimilation	Growth with 60% glucose
Melibiose assimilation	
Galactose assimilation	Microscopic morphology
Starch assimilation	
Citrate assimilation	Wet mount preparate
Lysine assimilation	Slide culture

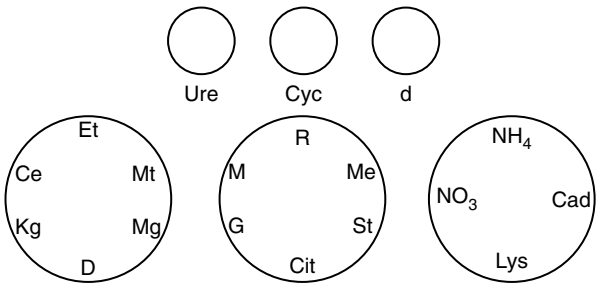

FIGURE A.1 Arrangement of standard tests used in SIM. Ure: urease test; Cyc: growth in the presence of 0.01% cycloheximide; d: fermentation of glucose. Assimilation substrates on plates: Et, erythritol; Ce, cellobiose; Mt, mannitol; Kg, 2-ketogluconate; Mg, methyl-α-glucoside; D, glucose (control); R, raffinose; M, maltose; Me, melibiose; G, galactose; St, soluble starch; Cit, citrate; NH_4, ammonium sulfate; NO_3, nitrate; Cad, cadaverine; Lys, lysine.

tips of pseudohyphal cells are rounded, whereas no constriction can be seen in true hyphae; and (3) the terminal cells of true hyphae are always considerably longer than the adjacent cell, whereas in pseudohyphae terminal cells are shorter than, or equal in length to, the preceding cell. When septate, branching hyphae are formed, the presence or absence of clamp connections close to septae should be determined. Clamps can be observed only with basidiomycetous (urease positive) yeasts and may signal the presence of a sexual form.

Production of ballistoconidia may be evident from the development of smaller satellite colonies around an isolated single colony formed earlier. The mirror image of colonies can be formed on the lid of a Petri dish if it is kept upside down for long enough. Ballistoconidia are produced on short stalks or sterigmata, which can be observed on cells under a microscope. Freshly isolated strains that appear to be *Rhodotorula* or *Cryptococcus* should be examined for the production of ballistoconidia, because this ability may be lost after some time of subculturing.

Formation of starch is a useful diagnostic characteristic for some basidiomycetous yeasts. Although it is not among the standard or supplementary tests of SIM, starch formation can be easily observed by flooding a culture with iodine solution. Starch production may be pH dependent, giving a positive reaction only at low pH (3.5–4.0).

For routine purposes, the following regime of work has been found practical.

1. *Preparation of media and glassware and accurate labeling*.

Melt and temper at 50°C 20 mL volumes of yeast nitrogen agar base for sugar assimilation tests and yeast carbon base for nitrogen assimilation tests. Substrates for assimilation should be carefully marked on the bottom of Petri dishes according to the scheme on Figure A.1. Appropriate labeling of isolates should be carefully done on all tests. Also, pour tempered (50°C) potato dextrose agar (0.5 mL) on sterile microscopic slides and allow it to solidify before inoculation test for investigating filamentous growth.

2. *Inoculations from agar slant culture*.

Inoculate potato agar slides and urea broth directly from young (1–2 days) agar cultures. Slides should be inoculated lightly using a straight wire, and a loopful of cells should be suspended in 0.5 mL of rapid urea broth. Results of the latter test can be recorded within 4 h of incubation at 37°C.

3. *Preparation of cell suspension*.

A cell suspension of visible turbidity (about 10^7 cells mL^{-1}) is prepared in sterile water. Nitrogen assimilation medium (yeast carbon base) is inoculated with 0.5 mL of this suspension. Vitamin-free broth, when applied, is also inoculated very lightly with one loopful of this suspension. Before inoculating yeast nitrogen base for sugar assimilation and glucose broth for fermentation tests, respectively, supplement the cell suspension with 2–3 drops of sterile 2% yeast extract solution as a vitamin source.

4. *Glucose fermentation*.

This test involves the observation of gas formation in 2% glucose broth in Durham tubes as a positive sign of fermentation. Broth is inoculated with 0.1 mL of vitamin-supplemented cell suspension.

5. *Nitrogen and carbon source assimilation*.

Auxanograms are prepared by pouring melted agar base media inoculated with 0.5 mL of cell suspension into Petri dishes. The inoculum should be thoroughly mixed with the medium. After solidification, place filter paper disks impregnated with appropriate nitrogen or carbon sources on the agar surface according to the labeling (Figure A.1). Nitrogen assimilation can be recorded after 48 h of incubation, while sugar assimilation plates should be incubated for up to 1 week.

6. *Incubation*.

Incubate all inoculated media (except rapid urea broth) at 25–28°C. Readings are made on the second, fourth, and seventh days. Slow and latent reactions that may be positive after 2 or 3 weeks are considered negative for the purpose of SIM.

Not all tests used in traditional identification procedures should be done for the SIM procedure. On the contrary, only a few of them are included in the standard set of tests regularly performed in SIM. Sometimes not even all of these are necessary. For example, if the microscopic investigation reveals lemon-shaped, bipolarly budding cells, only one sugar assimilation plate should be prepared, with cellobiose, maltose, raffinose, sucrose, and 2-keto-gluconate disks, because these would suffice for the differentiation of suspected *Hanseniaspora* or *Saccharomycodes* species. If the rapid urease test turns positive within 4 h, suggesting a basidiomycetous yeast, include inositol and lactose in place of citrate and soluble starch in the auxanographic tests, because assimilation of the former two substrates provides better discrimination for basidiomycetous yeasts.

When using the SIM key, great importance is given to the sugar and nitrogen assimilation tests, particularly to those on which the master key is based. The accuracy of identification depends mainly on the certainty of results in these tests. An erroneous reading of the result will lead to a group within which the identification of the isolate is false or impossible. It must be noted that in constructing the keys, use was made of tests in which yeast species give 85–99% unequivocal responses. Hence,

there is a certain probability that, even if the test result is correct, it would not fit the identification scheme. Therefore, species identification should never be based only on those features included in the keys.

Having performed the standard set of tests, the amount of data available always exceeds that considered in the keys. To confirm or reject presumptive identity, all data available should be compared to the characters of the likely identified species. To this end, after all test results have been recorded, consult Table A.1, which summarizes the most probable characters of species expressed in percentages of positive reactions. Section A.2.4 describes the main groups of the SIM key, pointing out the most distinctive properties of species and calling attention to the main differences between species of similar characteristics.

A.2.3 Identification Keys in SIM

The current version of SIM employs selected tests, six of which form a master key, leading to seven groups. Within each group, a dichotomous key is given, which eventually leads to species level. The master key (Table A.3) is arranged in such a way that the first three groups are split on the basis of positive urease reaction and the assimilation of nitrate and erythritol, respectively. Responses of yeasts to these tests are very stable. None of the species included in the key gives a variable urease reaction, and only two are variable in the two assimilation tests. The remaining species are further divided by other effective tests, such as resistance to cycloheximide and assimilation of cellobiose and mannitol.

Individual identification keys for each of the seven groups formed by the master key are arranged in a familiar dichotomous fashion (Tables A.4 through A.10). Physiological and morphological characteristics are listed (left column) for which the results can be positive (middle column) or negative (right column); in either case, the answer may be a number referring to another entry in the key, or it may be the name of a species as which the unknown isolate can be identified with high probability. Selected tests in which yeasts give 85–99% unequivocal (positive or negative) responses only are included in these keys.

A.2.4 Description of Main Groups of Yeast Species Included in SIM

A.2.4.1 Group 1: Urease-Positive Yeasts

The first group includes urease-positive yeasts, among them all basidiomycetous species considered in the SIM. Some of these yeasts occur quite frequently in foods; however, from the spoilage point of view, they have little practical importance to the food industry. Group 1 also includes the fission yeasts

TABLE A.3
Master Key of SIM

Character	Positive	Negative
1. Urease reaction	Group 1	2
2. Nitrate assimilation	Group 2	3
3. Erythritol assimilation	Group 3	4
4. Growth with cycloheximide	5	6
5. Cellobiose assimilation	Group 4	Group 5
6. Mannitol assimilation	Group 6	Group 7

TABLE A.4
Group 1: Urease-Positive Yeasts

22 Species (no variable)

Bulleromyces albus	*Filob. capsuligenum*	*Schizo. octosporus*
Cry. albidus	*F'ella. neoformans*	*Schizo. pombe*
Cry. curvatus	*Leucosp. scottii*	*Spori. salmonicolor*
Cry. diffluens	*Moniliella suaveolens*	*Spori. pararoseus*
Cry. humicolus	*Rho. glutinis*	*Spb. roseus*
Cry. laurentii	*Rho. minuta*	*Trisp. moniliforme*
Cystofilob. infirmo-miniatum	*Rho. Mucilaginosa*	*Guehom. pullulans*
	Schizo. japonicus	

Test	Positive	Negative
1. Arthroconidia	2	4
2. Ferm. glucose	–*Moniliella suaveolens*	3
3. Nitrate	–*Guehom. pullulans*	–*Trisp. moniliforme*
4. Ferm. glucose	5	8
5. Raffinose	6	7
6. Hyphae	–*Schizo. japonicus*	–*Schizo. pombe*
7. Mannitol	–*Filob. capsuligenum*	–*Schizo. octosporus*
8. Ballistoconidia	9	12
9. Red colony	10	–*Bulleromyces albus*
10. Nitrate	–*Spori. pararoseus*	11
11. Cycloheximide	–*Spori. salmonicolor*	–*Spb. roseus*
12. Red colony	13	16
13. Nitrate	14	15
14. Inositol	–*Cystofilob. infirmominiatum*	–*Rho. glutinis*
15. Raffinose	–*Rho. mucilaginosa*	–*Rho. minuta*
16. Nitrate	17	19
17. Inositol	18	–*Leucosp. scottii*
18. Galactose	–*Cry. albidus*	–*Cry. diffluens*
19. Hyphae	20	21
20. Melibiose	–*Cry. humicolus*	–*Cry. curvatus*
21. Melibiose	–*Cry. laurentii*	–*F'ella. neoformans*

Notes: Substrates assimilated: L-arabinose, cadaverine, citrate, erythritol, galactose, inositol, 2-ketogluconate, lactose, lysine, maltose, mannitol, melibiose, nitrate, raffinose, rhamnose, sucrose, starch, trehalose, xylose.

Abbreviations: ferm.: fermentation of sugars; 37°C: growth at this temperature; acetate: growth in medium with 1% acetic acid; NaCl 16: growth in medium with 16% NaCl; cycloheximide: growth in medium with 0.01% cycloheximide; vitamin: growth in vitamin-free medium.

(genus *Schizosaccharomyces*), whose uniqueness among yeasts is reflected in their urease-positive character (Vaughan Martini, 1991).

The urease test has become a very important identification tool because it distinguishes yeasts related to basidiomycetous fungi from those that are ascomycetous. The ability to split urea correlates very closely with the Diazonium blue B (DBB) color reaction used in the traditional identification method to differentiate between these two large groups of yeasts (Hagler and Ahearn, 1981). Because of its ease and rapidity of use, the urease reaction is included in SIM. Revealing the basidiomycetous character of yeasts, a positive urease reaction often coincides with the formation of carotenoid pigments, mucous colonies, ballistoconidia, and the lack of fermentation. Only a few basidiomycetous yeasts are able to ferment; two of these exceptions are included, namely,

Filobasidium capsuligenum and *Moniliella suaveolens*, which ferment glucose slowly. On the basis of these traits, the basidiomycetous yeasts can be easily recognized even if sexual forms are not evident.

The Group 1 key (Table A.4) first separates some *Moniliella* and *Trichosporon* species characterized by a budding yeast phase as well as true hyphae breaking into arthroconidia. It should be noted that morphologically similar members of the genus *Geotrichum* belong to ascomycetous fungi and are distinguished by a negative urease reaction and by the lack of budding cells. *Trisp. moniliforme* and *Trisp. pullulans* (now placed in a separate genus, *Guehomyces*) are fairly widespread in various foods. They possess active extracellular enzymes, proteases, and lipases and often contribute to the spoilage of proteinaceous foods; in fruit products, their large population can be considered a sign of unsatisfactory plant hygiene, similar to the build-up of "machinery mold" (*Geotrichum*) on improperly cleaned processing equipment. The two species noted here can be easily differentiated by the assimilation of nitrate.

Schizosaccharomyces species differ from other urease-positive yeasts by their ability to ferment glucose vigorously. In this respect, they are similar to the classical fermenting yeasts, *Saccharomyces*, from which they differ by their peculiar mode of vegetative reproduction: fission. Cells are separated by cross-wall formation, and budding never occurs. They require vitamins for growth and often develop poorly on many media and in assimilation tests. Spores are formed after the conjugation of cells, and their development is accompanied by the synthesis of starch, the presence of which in mature sporangia can be demonstrated by iodine solution. Two species, *Schizo. octosporus* and *Schizo. japonicus*, produce 6–8 spores per cell; the third one, *Schizo. pombe*, produces only four. *Schizosaccharomyces* species are xerotolerant. *Schizo. octosporus*, in particular, causes spoilage in syrups and fruit concentrates. More common is *Schizo. pombe*, which may occur in wine and fruit juices and is somewhat resistant to preservatives.

The red-colored and often mucoid colonies of *Rhodotorula* species are often striking in appearance. Perhaps for this reason, they may be over-represented among yeasts isolated from foods. By all accounts, *Rho. glutinis* and *Rho. mucilaginosa* (syn. *Rho. rubra*) are among the most commonly observed yeasts in foods; a third species, *Rho. minuta*, is less frequent. Their natural habitats are soil, fruits, and other plant surfaces. Not being able to ferment, they are rarely associated with the spoilage of foods, although they survive in reduced a_w and low-pH environments and are capable of growing at refrigeration temperatures. The rhodotorulas assimilate a broad range of substrates used in identification tests. Unfortunately, many assimilation reactions are variable. For this reason, *Spb. roseus*, also a common resident of plant surfaces, can be mistaken for *Rho. glutinis*. However, *Spb. roseus* does not grow at temperatures above 25°C, and most strains assimilate soluble starch, unlike *Rho. glutinis*. The most conclusive test for the identification of *Sporobolomyces* species is the demonstration of the formation of ballistoconidia. This is also an important criterion in recognizing *Spori. salmonicolor* and *Bulleromyces albus*. The former usually develops large hyphal and pseudohyphal cells and may form pink colonies, for which the latter is negative.

The *Cryptococcus* species develop white colonies, although some (e.g., *Cry*. [tel. *Cysto.*] *infirmominiatum*) that are infrequently found in foods may produce yellow or pink colonies. Cryptococci are characterized by the assimilation of inositol and glucuronate, and this can be used to confirm identity. The most frequently observed species included in the key are *Cry. albidus*, *Cry. laurentii*, *Cry. humicolus*, and *Cry. curvatus*, which can be identified using the standard tests of SIM. *Cry. humicolus* (recently *Pseudozyma humicola*) develops both true hyphae and pseudohyphae. *Cry. curvatus* (syn. *C. curvata*) forms only long, wavy pseudohyphae. Teleomorphic states of *Cysto. infirmominiatum*, *Filob. capsuligenum*, and *Filobasidiella neoformans* can also produce hyphae and even clamp connections; however, these develop rarely, so these species are more commonly observed in their anamorphic states, as *Cry. infirmominiatus*, *C. japonica*, and *Cry. neoformans*, respectively. These yeasts share plant habitats with *Rhodotorula* species, where they can

gain access to fruit and vegetable products. Many strains are psychrotrophic and are able to grow at 0–5°C. A typical example is *Leucosp. scottii* (am. *C. scottii*), which does not grow at temperatures above 25°C.

A.2.4.2 Group 2: Nitrate-Assimilating Yeasts

Assimilation of nitrate is used for separating the second group of yeasts in SIM (key in Table A.5), as it is a very stable property among yeasts. Nevertheless, peculiar species of the *Dekkera* (anamorph *Brettanomyces*) genus are variable in this trait, and, for this reason, they are also included in Groups 4 and 5.

A remarkable property of *Dekkera* species is their ability to produce acetic acid, which results in an easily recognizable odor in young cultures. These yeasts ferment glucose aerobically and grow very slowly in all media, developing tiny colonies that are short lived due to acidification. Their habitats are confined to beverages. *Dek. anomala* is more frequent in beer, whereas *Dek. bruxellensis* is

TABLE A.5
Group 2: Nitrate-Positive Species

19 Species (2 variable)

C. boidinii	*C. versatilis*	*P. fabianii*
C. etchellsii	*Citerom. matritensis*	*P. jadinii*
C. lactiscondensi	*Dek. anomala* (4)*	*P. subpelliculosa*
C. magnoliae	*Dek. bruxellensis* (4)	*Williopsis californica*
C. norvegica	*P. angusta*	*Williopsis saturnus*
C. vartiovaarae	*P. anomala*	*Wickerhamiella domercqiae*
	P. holstii	

Tests	Positive	Negative
1. Erythritol	2	5
2. Cycloheximide	3	4
3. Cellobiose	*–P. holstii*	*–C. boidinii*
4. Vitamin	*–P. anomala*	*–P. subpelliculosa*
5. Cycloheximide	6	10
6. Mannitol	7	9
7. Erithritol	*–P. angusta*	8
8. Methylglucoside	*–P. holstii*	*–C. versatilis*
9. Lactose	*–Dek. anomala*	*–Dek. bruxellensis*
10. Cellobiose	11	18
11.Vitamin	12	14
12. Raffinose	13	*–C.vartiovaarae*
13. Citrate	*–P. jadinii*	*–Williopsis saturnus*
14. Ketogluconate	*–C. magnoliae*	15
15. Raffinose	*–P. fabianii*	16
16. Sucrose	*–Williopsis californica*	*–C. norvegica*
17. Maltose	18	19
18. Raffinose	*–Citerom. matritensis*	*–C. etchellsii*
19. Ferm. glucose	20	*–Wickerham. domercqiae*
20. Cellobiose	*–C. magnoliae*	*–C. lactiscondensi*

Notes: See Table A.4.
*Numbers in parentheses refer to the group of the key into which variable species also fall.

common in wine. The two species can be differentiated by the ability of *Dek. anomala* to assimilate lactose and succinate (Smith et al., 1990).

Nitrate assimilation was once the sole differentiating character for the former genus *Hansenula*, which has now been merged with the genus *Pichia* (Kurtzman, 1984). In foods, *P. anomala* and *P. subpelliculosa* are among the most frequent yeasts. They share similar habitats such as fruits, juices, wine, and pickled vegetables; both are moderately xerotolerant and may occur in fruit concentrates and other high-sugar products. Both species assimilate erythritol, and they can be distinguished from each other by the ability of *P. anomala* to grow in vitamin-free medium.

Two other former *Hansenula* species, now *P. jadinii* and *P. angusta* (*Ogataea polymorpha*), occur less frequently in nonalcoholic beverages but are more common in wine and foods of low a_w. The former is better known in its anamorphic form, *C. utilis*, and the latter by its former name, *Hansenula polymorpha*. *C. utilis* is an important industrial yeast, produced on large scales for fodder. Both species can be easily identified using the standard tests of SIM. Other ex-*Hansenula* species, now *P. holstii* and *P. fabianii*, as well as *Williopsis saturnus* and *Will. californica*, are normal inhabitants of coniferous trees and also occur on other plants, in soils, and in water. The former are usually isolated in the anamorphic state (*C. silvicola* and *C. fabianii*, respectively); the latter produce characteristic Saturn-shaped spores.

The rest of the nitrate-assimilating yeasts included in Group 2 belong to the genus *Candida.* Two of them, *C. domercqiae* and *C. globosa*, are also known in teleomorphic form as *Wickerhamiella domercqiae* and *Citerom. matritensis*, respectively. Spore formation is rare, however, and the anamorphs occur more frequently. Several common food-borne species are among these yeasts, and most, with the exception of *C. boidinii* and *C. norvegica*, possess some degree of xerotolerance in that they grow in products containing 50–60% (w/w) glucose or 10–15% (w/w) NaCl. Consequently, they can often be isolated from foods such as fruit concentrates, syrups, and brines. In the majority of cases, their identification is straightforward, following the standard regime of SIM, with no additional tests needed to assign species identity. *C. boidinii* is erythritol positive; it frequently occurs in soft drinks, wine, and beer. Its additional distinguishing property is the long cylindrical shape of cells that form well-developed pseudohyphae. *Wic. domercqiae* is the only nonfermenting, nitrate-assimilating yeast in Group 2. Its cell size is very small, only 2–3 μm in diameter. Neither *C. versatilis*, *C. etchellsii*, *C. magnoliae*, *C. lactiscondensi*, nor *Citerom. matritensis* (am. *C. globosa*) produce pseudohyphae; these species were previously classified in the genus *Torulopsis*. Formation of pseudohyphae was considered an unstable property, so the genus *Torulopsis* was merged with the genus *Candida.*

A.2.4.3 Group 3: Erythritol-Assimilating Yeasts

The third group of SIM includes yeasts that assimilate erythritol, a fairly stable property; of 120 species considered, only *Db. hansenii* and *S'copsis fibuligera* are variable in this trait. The Group 3 key is shown in Table A.6.

The characteristic mucous colonies of the species *Lipomyces* can easily be recognized. They are typical soil yeasts and notable for a specific mode of sporulation. Four species, *Hyphop. burtonii*, *S'copsis fibuligera*, *Trichomonascus ciferrii*, and *Ya. lipolytica*, produce true hyphae and budding cells, sometimes also pseudohyphae. Only the first two ferment glucose. *Hyphop. burtonii* (syn. *P. burtonii*, *C. variabilis*) and *Ya. lipolytica* (*C. lipolytica*) are more common in foods than the other two. *Hyphop. burtonii* is widespread in bakery, meat, and dairy products as well as in salads and oriental foods, whereas *Ya. lipolytica* occurs primarily in meat and dairy products. *S'copsis fibuligera* is found mainly on grapes and may be present in grape juice and wine, whereas the occurrence of *Trichomonascus* (formerly *Stephanoascus*) *ciferri* is confined to meat products and fermented vegetables.

Db. polymorphus and *Db. hansenii* ferment weakly or not at all, and they usually form a thick pellicle on the surface of liquid media, although they do not produce well-developed pseudohyphae. Unfortunately, many characteristics of *Db. hansenii* are variable, and this renders the

TABLE A.6
Group 3: Erythritol-Assimilating Yeasts

13 Species (2 variable)		
C. cantarellii	*Db. hansenii* (4,5,6)*	*Hyphop. burtonii*
C. diddensiae	*Db. polymorhus*	*P. farinosa*
C. mesenterica	*Lipomyces lipofer*	*S'copsis fibuligera* (4)
C. tenuis	*Lipomyces starkeyi*	*Trichomon. ciferrii*
		Ya. lipolytica
Tests	**Positive**	**Negative**
1. Mucous colony	2	3
2. Cycloheximide	*–Lipomyces starkeyi*	*–Lipomyces lipofer*
3. Maltose	4	11
4. Galactose	5	10
5. Raffinose	6	9
6. Hyphae	7	8
7. Ferm. glucose	*–Hyphop. burtonii*	*–Trichomon. ciferrii*
8. Vitamin	*–Db. polymorphus*	*–Db. hansenii*
9. Ketogluconate	*–C. tenuis*	*–C. diddensiae*
10. Ferm. glucose	*–S'copsis fibuligera*	*–C. mesenterica*
11. Hyphae	*–Ya. lipolytica*	12
12. Cycloheximide	*–C. cantarellii*	*–P. farinosa*

Notes: See Table A.4.
*Numbers in parentheses refer to the group of the key into which variable species also fall.

identification of this very common yeast rather difficult. *Db. hanseniii* is one of the most frequent food-borne yeasts, commonly occurring in all types of foods, especially in salted and cured meat and fermented dairy or vegetable products. *Db. polymorphus* is far less common. In recognizing *Db. hansenii* and differentiating it from a number of yeasts with similar properties, it is important to observe some distinguishing characteristics. Colonies of *Db. hansenii* are usually dull, often wrinkled, and sporulated colonies turn brown. It has spherical cells and the spores, if formed, are also spherical, without ridges, and only one develops per cell, filling it nearly full. *Db. hansenii* often occurs in its anamorphic form, *C. famata*. The species usually assimilates a large number of substrates, among them always raffinose, mannitol, and citrate, while many similar yeasts fail to assimilate one or more of these. It rarely grows without vitamins, unlike the closely related species *Db. polymorphus*, which has ovoid to cylindrical cells. *Db. polymorphus* also ferments glucose more consistently and develops pseudohyphae more frequently than does *Db. hansenii*.

The few erythritol-positive *Candida* species (*C. cantarellii*, *C. diddensiae*, *C. mesenterica*, *C. tenuis*) and *P. farinosa* can be readily identified using standard SIM tests. *C. diddensiae* is notable for the variable shape and size of its cells. Neither of these yeasts occurs frequently in foods.

A.2.4.4 Group 4: Cycloheximide-Resistant Cellobiose-Assimilating Yeasts

After separating the urease-positive nitrate- and erythritol-assimilating yeasts, three further characteristics are used for the primary grouping of the remaining yeasts. These are the ability to grow in the presence of 0.01% cycloheximide and to assimilate cellobiose and mannitol. Properties such as peculiar cell morphology, assimilation of organic nitrogen sources (cadaverine, lysine), and some carbon

sources (raffinose and citrate) are very useful in differentiating species. In addition to the complete set of standard SIM tests, in certain cases supplementary tests are required for the identification of physiologically similar species.

Cycloheximide is a specific growth inhibitor for many types of yeasts, among them the industrially important *Saccharomyces* species. Nevertheless, approximately one-third of yeasts are resistant to this compound. Of these, the cellobiose-assimilating species are included in Group 4, and cellobiose-negative species are in Group 5 of SIM. The Group 4 key is shown in Table A.7.

Characteristic cell morphology (apiculate and lemon-shaped cells) easily distinguishes those yeasts that produce buds bipolarly. Three species of *Hanseniaspora* key out to this group, while the remaining cycloheximide-sensitive species belong to Group 7. Slight differences exist among *Hanseniaspora* species, and all have an anamorphic state, *Kloeckera*. They ferment glucose strongly and are most frequently found at the initiation of grape must fermentation. The most common

TABLE A.7
Group 4: Cycloheximide-Resistant, Cellobiose-Assimilating Yeasts

19 Species (7 variable)

Bret. naardenensis	*Dek. anomala* (2,5)*	*Klu. lactis*
C. maltosa	*Dek. bruxellensis* (2,5)	*Klu. marxianus* (5)
C. oleophila	*Hsp. guilliermondii*	*P. guilliermondii*
C. tropicalis (5)	*Hsp. uvarum*	*S'copsis fibuligera* (3)
C. zeylanoides (5)	*Hsp. valbyensis*	*Zygoascus hellenicus*
Db. hansenii (3,5,6)	*Geo. fermentans*	*Lachancea fermentati*
Db. occidentalis		

Tests	Positive	Negative
1. Mannitol	6	2
2. Bipolar budding	3	5
3. Ketogluconate	4	*–Hsp. valbyensis*
4. 37°C	*–Hsp. guilliermondii*	*–Hsp. uvarum*
5. Lactose	*–Dek. anomala*	*–Dek. bruxellensis*
6. Hyphae	7	10
7. Arthroconidia	*–Geo. fermentans*	8
8. Galactose	9	*–S'copsis fibuligera*
9. Raffinose	*–Zygoascus hellenicus*	*–C. tropicalis*
10. Raffinose	11	17
11. Ketogluconate	12	15
12. Citrate	13	*–Lachancea fermentati*
13. Pseudohyphae	*–P. guilliermondii*	14
14. 10% NaCl	*–Db. hansenii*	*–Db. occidentalis*
15. Methylglucoside	16	*–Klu. marxianus*
16. Lactose	*–Klu. lacti*	*–Lachancea fermentati*
17. Ferm. glucose	18	*–C. zeylanoides*
18. 37°C	19	20
19. Starch	*–C. tropicalis*	*–C. maltosa*
20. Citrate	*–C. oleophila*	*–Bret. naardenensis*

Notes: See Table A.4.

*Numbers in parentheses refer to the group of the key into which variable species also fall.

species, *Hsp. uvarum* (*Klc. apiculata*), also occurs on fresh fruits, in juices, soft drinks, and beer. The same habitats are shared by other *Hanseniaspora* species, although with various frequencies of occurrence. The salient properties of *Hanseniaspora* species and *S'codes ludwigii*, which possesses similar apiculate cells, are summarized in Table A.11.

In Group 4, a few filamentous types of yeasts are included. *Zygoascus hellenicus* and *S'copsis fibuligera* produce abundant true and pseudohyphae, while hyphae of *Geo. fermentans* split into arthroconidia. Occasionally, *C. tropicalis* may also form true hyphae, but it differs from the former species in several assimilation tests. The long filamentous pseudohyphal cells of *Dek. anomala* may be mistaken for true hyphae.

The ability to assimilate raffinose divides the remaining yeasts in Group 4 into two subgroups. Vigor of fermentation and formation of pseudohyphae and pellicles are useful distinguishing characteristics in the identification of these species.

Members in the genera *Kluyveromyces* and *Zygosaccharomyces* strongly ferment glucose. Spores of *Kluyveromyces* species are mostly bean shaped and liberate easily from the sporangium. Heterothallism often precludes spore formation, and the anamorphs are known as *Candida* species. The classification of the genus *Kluyveromyces* has undergone several revisions (see Section 2.4.1). Of the species currently recognized, those important in foods are *Klu. marxianus* (syn. *Klu. bulgaricus*, *Klu. fragilis*, anamorph *C. kefyr*, and syn. *C. pseudotropicalis*), *Klu. lactis* (syn. *Klu. drosophilarum* and anamorph *C. sphaerica*), and *Klu. thermotolerans* (reassigned to *Lachancea* and anamorph *C. dattila*). The first two species are considered here; *Klu. marxianus* will also key out in Group 5, because its cellobiose assimilation is variable, whereas *Lachancea* (*Klu.*) *thermotolerans* falls into Group 6, because it is sensitive to cycloheximide. For their identification, it is helpful to note that *Klu. lactis* assimilates α-methylglucoside but not 2-ketogluconate, whereas *Klu. marxianus* assimilates neither of these compounds nor maltose. Both species are capable of utilizing lactose, a rare property among yeasts. Hence, they are most often associated with dairy products and less common in other foods, although *Klu. marxianus*, being moderately xerotolerant and rather heat resistant, may occur in syrups and concentrates.

Most *Zygosaccharomyces* species are sensitive to cycloheximide and will be treated in Groups 6 and 7. Two species, however, are able to grow in the presence of 0.01% cycloheximide. Of these, *Zygo.* (now *Lachancea*) *fermentati* is cellobiose positive, whereas *Zygo.* (now *Zygotorulaspora*) *florentinus* is not. Consequently, they key out in Groups 4 and 5, respectively. *Lachancea fermentati* is rather similar to *Klu. lactis* in its physiological properties, although it is unable to utilize lactose. Conjugating cells are often observed, facilitating recognition of *Lachancea fermentati*. However, some strains of *Klu. lactis* may be lactose negative, whereas some strains of *Lachancea fermentati* do not show conjugation. Fermentation of maltose, positive for the latter, will always differentiate between the two species.

Well-developed pseudohyphae are one of the distinguishing characteristics for seven species included in Group 4. Of them, *C. zeylanoides* usually does not ferment glucose, whereas *P. guilliermondii* (*C. guilliermondii*) assimilates raffinose, but the remaining species do not. *C. tropicalis* and *C. maltosa* grow well at 37°C, whereas *C. oleophila* and *Bret. naardenensis* do not. *P. guilliermondii* is the most frequent species, occurring in a wide range of foods, such as fruits, soft drinks, wine, and syrups. *C. tropicalis* also commonly occurs in fresh fruits, fermented juices, beverages, and meat and dairy products. Only the assimilation of soluble starch differentiates between *C. tropicalis* and *C. maltosa*, although the former may develop true hyphae as well. *Bret. naardenensis* differs in many characteristics from the *Candida* species mentioned and has physiological properties similar to *Dekkera* species. However, *Bret. naardenensis* is a less active acetic acid producer and assimilates mannitol, unlike the *Dekkera* species. *Bret. naardenensis* appears to be confined to soft drinks and carbonated beverages, in which it is a notorious spoilage yeast.

Db. hansenii strains that fail to assimilate erythritol may appear in this group or in Group 5.

A.2.4.5 Group 5: Cycloheximide-Resistant Cellobiose-Negative Yeasts

Table A.8 presents the key for Group 5. Some species in this group are easily distinguishable morphologically. *Geo. fragrans* and *Geo. candidum* (its teleomorphic form is *Galactomyces geotrichum*) produce true hyphae that break into arthroconidia but never budding cells. *Trichosporon* species have similar morphology and also possess budding cells, and they are basidiomycetous yeasts, while the *Geotrichum* species are ascomycetous fungi, as revealed by their negative urease reaction. *Gal. geotrichum* occurs mostly in vegetables and dairy products and sometimes in fruits; *Geo. fragrans* is infrequently found in beverages. Closely related to these species is *Dipodascus ingens* (*C. ingens*), which possesses unusually large and wide cells but does not produce septate hyphae. *S'copsis fibuligera*, on the other hand, produces both true and pseudohyphae as well as budding cells.

TABLE A.8
Group 5: Cycloheximide-Resistant, Cellobiose-Negative Yeasts

22 Species (10 variable)

C. albicans	*Dek. anomala* (2,4)*	*Naumovia dairensis* (7)
C. catenulata	*Dek. bruxellensis* (2,4)	*Kazach. exiguus*
C. milleri	*Dipodascus ingens*	*Kazach. unisporus*
C. parapsilosis (6)	*Gal. geotrichum* (6)	*S'copsis vini*
C. tropicalis (4)	*Geo. fragrans*	*Tsp. globosa*
C. zeylanoides (4)	*Klu. marxianus* (4)	*Lachancea cidri*
Db. hansenii (3,4,6)	*Lodd. elongisporus*	*Zygotsp. florentinus*
		Zygotsp. mrakii

Test	Positive	Negative
1. Hyphae	2	5
2. Arthroconidia	3	*–S'copsis vini*
3. Ferm. glucose	4	*–Gal. geotrichum*
4. Vitamin	*–Dipodascus ingens*	*–Geo. fragrans*
5. Melibiose	6	9
6. Citrate	*–Deb. hansenii*	7
7. Maltose	8	*–Zygotsp. mrakii*
8. Lactose	*–Lachancea cidri*	*–Zygotsp. florentinus*
9. Cadaverine	10	20
10. Mannitol	11	17
11. Raffinose	*–Klu. marxianus*	12
12. Methylglucoside	13	16
13. Starch	14	15
14. Ferm. sucrose	*–C. tropicalis*	*–C. albicans*
15. L-arabinose	*–C. parapsilosis*	*–Lodd. elongisporus*
16. Maltose	*–C. catenulata*	*–C. zeylanoides*
17. Maltose	18	19
18. Lactose	*–Dek. anomala*	*–Dek. bruxellensis*
19. Raffinose	*–Klu. marxianus*	*–Kazach. unisporus*
20. Raffinose	21	*–Naumovia dairensis*
21. Ketogluconate	*–Tsp. globosa*	22
22. Spores	*–Kazach. exiguus*	*–C. milleri*

Notes: See Table A.4.
*Numbers in parentheses refer to the group of the key into which variable species also fall.

The former species can be found in wine cellars, the latter on grapes, but they rarely cause spoilage in wine or other beverages.

Db. hansenii and *Klu. marxianus* are variable in the assimilation of cellobiose and were noted in Group 4. Mention was also made of *Zygotorulaspora* (formerly *Zygo.*) *florentinus*, a fairly common yeast in beverages. All of these species are able to assimilate raffinose, a feature distinguishing them from raffinose-negative, but otherwise physiologically similar, species. Identification requires supplementary tests, such as the assimilation of soluble starch and L-arabinose, as well as the fermentation of sucrose and maltose. The species concerned are *C. albicans*, *C. tropicalis*, *C. catenulata*, *C. parapsilosis*, *C. zeylanoides*, and *Lodd. elongisporus*. Some of these yeasts, being variable in key characters, also occur in Groups 4 and/or 6. The salient features of these raffinose-negative yeasts are summarized in Table A.12.

C. albicans is the most common human pathogenic yeast. It can be identified by the formation of germ tubes and chlamydospores. This species now includes the former *C. claussenii* and *C. stellatoidea*, which are mainly saprobiontic. Hence, records of *C. albicans* in foods are not unusual. It has been found in fruits, soft drinks, must, wine, and various other foods. It is not easy to differentiate *C. albicans* from the far more common food-borne yeast *C. tropicalis*. The latter usually ferments sucrose, while *C. albicans* does not; both species ferment maltose.

C. catenulata is a less frequent yeast that occurs in meat products and alcoholic beverages. It is not able to ferment sucrose or maltose, and even glucose is fermented only weakly. *C. parapsilosis* is very widespread in foods. Physiologically, it is very similar to *Lodd. elongisporus*. The two species differ in that only *C. parapsilosis* can assimilate L-arabinose.

The remaining species in Group 5 are characterized by the inability to utilize cadaverine as a source of nitrogen, a rare property among yeasts, although common among *Saccharomyces* species (see Group 7). Two former *Saccharomyces* species, *S. dairensis* and *S. exiguus* (now *Naumovia* and *Kazachstania*, respectively), possess some degree of resistance to cycloheximide and key out in this group. *Kazach. exiguus* is a fairly common spoilage yeast, while *S. dairensis* is less frequent. *C. milleri* is basically similar in all characteristics to *Kazach. exiguus*, except for not forming spores. *Tsp. globosa* is also cadaverine negative and can be easily identified using standard tests.

A.2.4.6 Group 6: Cycloheximide-Sensitive Mannitol-Assimilating Yeasts

This group (key in Table A.9) includes yeasts such as the *Zygosaccharomyces* species and *Tsp. delbrueckii* that are of particular importance in food spoilage. These species were at one time considered to be members of the genus *Saccharomyces sensu lato*, including the classical fermenting yeasts (Vaughan Martini and Martini, 1989; Barnett, 1992). Later, the genus was split, and *Saccharomyces sensu stricto* retained the primarily diploid species, whereas the genera *Zygosaccharomyces* and *Torulaspora* were created for yeasts with a primarily haploid life cycle. In *Saccharomyces*, conjugation takes place immediately at spore germination, and the resulting diploid cells later directly transform into sporangia. In *Zygosaccharomyces*, conjugation between independent cells usually precedes spore formation, whereas in *Torulaspora* conjugation mainly occurs between mother cell and bud. If any of these events can be observed, identification is greatly facilitated. Cells bearing protuberances signify possible conjugation. In the absence of sexual reproduction, identification must be based only on physiological criteria. Unfortunately, identification is difficult in this way because most of these criteria became variable after amalgamating a number of species on the grounds of DNA homology, especially into *S. cerevisiae* and *Tsp. delbrueckii* (see Table 2.7 and 2.8). Identification often requires supplementary tests, such as growth on lysine, in the presence of 1% acetic acid, 60% glucose, or 16% NaCl. Although *Zygosaccharomyces* species and *Tsp. delbrueckii* are usually able to assimilate mannitol, sometimes they may fail and consequently fall into Group 7.

TABLE A.9
Group 6: Cycloheximide-Sensitive, Mannitol-Assimilating Yeasts

23 Species (10 variable)

C. apicola	*Db. carsonii*	*Lachancea kluyveri* (7)
C. diversa	*Db. etchellsii*	*Tsp. delbrueckii* (7)
C. intermedia	*Db. hansenii* (3,4,5)	*Zygo. bailii* (7)
C. parapsilosis (5)*	*Gal. geotrichum* (5)	*Zygo. bisporus* (7)
C. rugosa	*Lachancea thermotolerans*	*Tsp. microellipsoides* (7)
C. sake	*Met. pulcherrima*	*Zygo. mellis* (7)
C. vini	*Met. reukaufii*	*Zygo. rouxii* (7)
Clsp. lusitaniae	*P. ohmeri*	

Tests	Positive	Negative
1. Cadaverine	2	*–Tsp. delbrueckii*
2. Arthroconidia	*–Gal. geotrichum*	3
3. Ferm. glucose	4	5
4. Raffinose	8	15
5. Maltose	6	7
6. Gluconate	*–Db. hansenii*	*–Db. carsonii*
7. Galactose	*–C. rugosa*	*–C. vini*
8. Maltose	9	13
9. Citrate	10	12
10. Pseudohyphae	11	*–Db. hansenii*
11. 37°C	*–P. ohmeri*	*–C. intermedia*
12. Melibiose	*–Lachancea kluyveri*	*–Lachancea thermotolerans*
13. Melibiose	*–Tsp. microelliposoides*	14
14. Trehalose	*–Zygo. bailii*	*–C. apicola*
15. Cellobiose	16	20
16. 37°C	17	18
17. Rhamnose	*–Clsp. lusitaniae*	*–Db. etchellsii*
18. Pseudohyphae	*–C. sake*	19
19. Citrate	*–Met. pulcherrima*	*–Met. reukaufii*
20. Ketogluconate	21	22
21. 37°C	*–C. parapsilosis*	*–C. sake*
22. Citrate	*–C. diversa*	23
23. Acetate	24	25
24. Trehalose	*–Zygo. bailii*	*–Zygo. bisporus*
25. NaCl 16%	*–Zygo. mellis*	*–Zygo. rouxii*

Notes: See Table A.4.
*Numbers in parentheses refer to the group of the key into which variable species also fall.

Inability to grow on cadaverine immediately sets *Tsp. delbrueckii* apart from other species in Group 6. *Tsp. delbrueckii* is variable in most standard tests; however, assimilation of 2-ketogluconate and growth on lysine enhance its identification. This species commonly occurs in fruits, soft drinks, wine, concentrates, and some other high-sugar products, as well as in a wide variety of other foods. Its anamorph is known as *C. colliculosa*. *Gal. geotrichum*, when it fails to grow in the presence of cycloheximide, may key out here. Although it assimilates cadaverine, it differs sharply from *Tsp. delbrueckii* by having true hyphae and arthroconidia.

Two former *Zygosaccharomyces* species, *Lachancea fermentati* and *Zygotorulaspora florentinus*, have been treated in Groups 4 and 5, respectively. The remaining food-borne *Zygosaccharomyces*

species are considered here or, if they fail to utilize mannitol, in Group 7. Delimitation of these species on the basis of sugar utilization is uncertain. In general, they assimilate only a few carbon sources and usually not maltose and citrate (Table A.13). *Torulaspora* (*Zygo.*) *microellipsoides* can be set apart from other *Zygosaccharomyces* species for being able to assimilate melibiose. Special tests must be done to distinguish between the most common *Zygosaccharomyces* species. *Zygo. bailii* and *Zygo. bisporus* are able to grow in the presence of 1% acetic acid; the former assimilates trehalose, but the latter does not. *Zygo. rouxii* and the recently reestablished species *Zygo. mellis* can grow in media containing 60% (w/w) glucose; however, this is not characteristic of these species alone. Many strains of *Zygo. bailii* and *Zygo. bisporus* as well as several other yeast species, such as *C. apicola*, *Db. hansenii*, *P. etchellsii*, *P. farinosa*, *P.* (*Kodamaea*) *ohmeri*, and *Tsp. delbrueckii*, to mention only those in Group 6, can also grow in substrates containing 60% glucose. The only trait that differentiates *Zygo. rouxii* from *Zygo. mellis* is the ability of the former to grow on media containing 16% NaCl and 5% glucose (Kurtzman, 1990). A species described recently, *Zygo. lentus* (not considered in the key), isolated from spoiled beverages, is similar to *Zygo. bailii* but fails to grow in the presence of 1% acetate (Steels et al., 1999).

The outstanding property of *Zygo. bailii* is its high tolerance and even adaptation to preservatives, which makes this organism a notorious spoilage agent in chemically preserved foods (Thomas and Davenport, 1985). Most strains of *Zygo. bailii* grow in media containing 400 mg L^{-1} benzoic acid or 300 mg L^{-1} sorbic acid at pH 3.5, whereas most strains of *Zygo. bisporus* do not. The highly xerotolerant *Zygo. bisporus* is most commonly found in high-sugar products but also causes spoilage of beverages. *Tsp. microellipsoides* is less frequently encountered, although it occurs in soft drinks and wine. *Zygo. rouxii* is undoubtedly the most important and most common xerotolerant yeast, often isolated alone from high-sugar products such as syrups, fruit concentrates, and honey. It also frequently occurs in foods with higher a_w, for example fruit juices. In the past, no distinction was made between *Zygo. rouxii* and *Zygo. mellis*, although the latter is thought to actually occur in high-sugar products, whereas *Zygo. rouxii* is more likely to be confined to high-salt foods such as soy sauce (Kurtzman, 1990).

Utilization of raffinose serves as a good differentiating character in Group 6. *P.* (*Kodamaea*) *ohmeri* and *C. intermedia* also assimilate galactose and citrate, whereas *Lachancea* (syn. *S.*) *kluyveri* and *Lachancea* (syn. *Klu.*) *thermotolerans* do not. Both *P. ohmeri* and *C. intermedia* produce pellicles on the surface of liquid media. They differ, among other ways, in growth temperature. The former tolerates low a_w and can be found in fruit concentrates, while the latter occurs more often in fruit juices.

S. kluyveri is unique among *Saccharomyces* species in that it can utilize cadaverine and lysine; it appears to be distantly related to the genus (Vaughan-Martini and Kurtzman, 1988) and has recently been reassigned to *Lachancea* (Kurtzman, 2003). *Lachancea kluyveri* may occur in soft drinks and alcoholic beverages. As noted above (Group 4), *Klu. thermotolerans* (now also in *Lachancea*) differs from other *Kluyveromyces* species in being sensitive to cycloheximide. It is also unable to assimilate lactose and hence occupies different food niches than *Klu. marxianus* and *Klu. lactis*, but shares similar food environments with *Tsp. delbrueckii*, although it is found less frequently. *C. apicola* (formerly *Torulopsis apicola*) does not produce pseudohyphae and assimilates only a few carbohydrates. In turn, it grows well at 60% (w/w) glucose and, accordingly, is frequently implicated in spoiled high-sugar products.

Several raffinose-negative species also belongs to Group 6 of the SIM key. Of these, *C. diversa* can be distinguished for not assimilating maltose, and *C. rugosa* as well as *C. vini* for their inability to ferment glucose. In turn, *C. sake*, *C. parapsilosis*, *Clsp. lusitaniae*, and *Db. etchellsii* are rather similar to the raffinose-negative yeasts discussed previously in Group 5. Their identification poses great difficulties (Table A.13). Several yeast species in Group 6 can be frequently found in fruit products and alcoholic beverages. These include *C. vini*, *C. rugosa*, *C. sake*, *Db. carsonii*, and *Db. etchellsii*. *C. diversa* and *Clsp. lusitaniae* are less frequent but do occur on fresh fruits and in fruit juices.

Rudimentary or even no pseudohyphae set *Met. pulcherrima* and *Met. reukaufii* apart from other species in Group 6. Long, needle-shaped spores are a key characteristic for the genus *Metschnikowia*; however, spores are rarely observed. Their anamorphs are classified as *C. pulcherrima* and *C. reukaufii*. The former species is more common and can be isolated easily from grapes and must, and also from other fruits. The two species are very similar. *Met. pulcherrima* assimilates trehalose and forms large lipid-containing chlamydospores on aging, when colonies turn reddish-brown.

A.2.4.7 Group 7: Cycloheximide-Sensitive Mannitol-Negative Yeasts

Yeasts falling in Group 7 of SIM are negative in all characteristics used in the master key. Some are also negative in most standard physiological tests, which renders their identification rather difficult. Morphological properties and growth characteristics play important roles in their identification (Table A.10).

Several species of bipolarly budding yeasts from the genus *Hanseniaspora* and *S'codes ludwigii* key out here. They strongly assimilate cellobiose, similar to the other *Hanseniaspora* species that are resistant to cycloheximide (see Group 4). Similarly to those, both *Hsp. osmophila* and *S'codes ludwigii* are confined to fruits, soft drinks, cider, and wine. Cells of the latter species are unusually large (more than 10 μm in diameter); this and the assimilation of raffinose differentiate this yeast from the *Hanseniaspora* species. Table A.11 summarizes the salient properties of all lemon-shaped yeasts included in the SIM keys.

All five *Zygosaccharomyces* species from Group 6 may also show up in this group when they fail to assimilate mannitol. They have been discussed in detail earlier.

Very restricted assimilation spectra and fermentative capabilities characterize several common food-borne yeasts (Table A.14). *P. membranifaciens* (am. *C. valida*) and *C. inconspicua* ferment glucose very weakly or not at all. The former produces pseudohyphae and pellicles; it is widespread in nearly all types of food, especially in beverages, wine, and fermented vegetables (Mora and Rosello, 1992). *C. inconspicua* is less common in foods. *P. fermentans* (am. *C. lambica*) and *P. nakasei* (am. *C. citrea*), as well as *Iss. orientalis* (am. *C. krusei*) and *Iss. terricola* (anamorph not known), ferment glucose, assimilate citrate, and produce pseudohyphae and, with the exception of *P. nakasei*, pellicles. The only tests differentiating between these species are the assimilation of xylose and the ability to grow in vitamin-free medium. *Iss. orientalis* and *P. fermentans* are the most common among these species, occurring in many types of food. *Iss. orientalis* is fairly resistant to preservatives and is highly salt tolerant.

The most important food-borne yeasts, the domesticated *Saccharomyces* species, comprise the last subgroup of yeasts to be discussed. Their main characteristic is the vigorous fermentation of sugars. For identification purposes, however, this property is of little practical value. Formerly, some 20 *Saccharomyces* species were distinguished primarily on the basis of differences in sugar fermentation, which proved to be inconsistent (Vaughan Martini and Martini, 1989). Moreover, based on a high degree of DNA homology, many former *Saccharomyces* species were merged into a single one, *S. cerevisiae*, with the consequence that most physiological properties of this species became equivocal. Recently, *S. bayanus* and *S. pastorianus* (syn. *S. carlsbergensis*) have been reestablished, and new species have been added to the genus *Saccharomyces* (see Section 2.4.1).

For the purpose of identification, it is practical to separate *Saccharomyces* species from other species in Group 7 based on their inability to grow on cadaverine and lysine (Table A.15). A few more yeasts from Groups 3, 5, and 6 also fall into the cadaverine-negative category, such as *Tsp. delbrueckii*, *Tsp. globosa*, *C. catenulata*, *C. stellata*, *C. glabrata*, and *C. cantarellii.* Table A.15 summarizes the characteristics that can be used for their identification. Yeasts resistant to cycloheximide form a subgroup comprising *Kazach. exiguus*, *Naumovia dairensis*, *Tsp. globosa*, *C. cantarellii*, and *C. zeylanoides.* Among them, species differentiation can be made using the assimilation of 2-ketogluconate, erythritol, and raffinose.

TABLE A.10
Group 7: Cycloheximide-Sensitive, Mannitol-Negative Yeasts

26 Species (9 variable)

C. glabrata	*Iss. orientalis*	*Naumovia dairensis* (5)
C. inconspicua	*Iss. terricola*	*Lachancea kluyveri* (6)
C. milleri (5)*	*P. nakasei*	*S. pastorianus*
C. sorboxylosa	*P. fermentans*	*Tsp. delbrueckii* (6)
C. stellata	*P. kluyveri*	*Zygo. bailii* (6)
Hsp. osmophila	*P. membranifaciens*	*Zygo. bisporus* (6)
Hsp. occidentalis	*S'codes ludwigii*	*Tsp. microellipsoides* (6)
Hsp. vineae	*S. bayanus*	*Zygo. mellis* (6)
	S. cerevisiae	*Zygo. rouxii* (6)

Tests	**Positive**	**Negative**
1. Cadaverine	2	20
2. Bipolar budding	3	6
3. Maltose	4	5
4. 34°C	*–Hsp. osmophila*	*–Hsp. vineae*
5. Raffinose	*–S'codes ludwigii*	*–Hsp. occidentalis*
6. Melibiose	7	8
7. Methylglucoside	*–Lachancea kluyveri*	*–Tsp. microellipsoides*
8. Ferm. glucose	11	9
9. Pellicle	*–P. membranifaciens*	10
10. Xylose	*–C. sorboxylosa*	*–C. inconspicua*
11. Xylose	12	13
12. Pellicle	*–P. fermentans*	*–C. sorboxylosa*
13. Citrate	14	15
14. Vitamin	*–Iss. orientalis*	*–Iss. terricola*
15. Pellicle	16	17
16. Glucoseamine	*–P. kluyveri*	*–P. nakasei*
17. Acetate	18	19
18. Trehalose	*–Zygo. bailii*	*–Zygo. bisporus*
19. NaCl 16%	*–Zygo. mellis*	*–Zygo. rouxii*
20. Lysine	21	22
21. Ketogluconate	*–Tsp. delbrueckii*	*–C. stellata*
22. Maltose	23	25
23. Vitamin	*–S. bayanus*	24
24. 37°C	*–S. cerevisiae*	*–S. pastorianus*
25. Galactose	26	*–C. glabrata*
26. Raffinose	*–C. milleri*	*–Naumovia dairensis*

Notes: See Table A.4.
*Numbers in parentheses refer to the group of the key into which variable species also fall.

The remaining cadaverine-negative yeasts are also cycloheximide sensitive. *Tsp. delbrueckii* and *C. stellata* can be differentiated by their ability to grow on lysine; they differ from each other in the assimilation of 2-ketogluconate. *C. glabrata* may be separated from *Saccharomyces sensu stricto* species on the basis of its much smaller cell size (less than 5 μm in diameter) and its ability to assimilate only trehalose, and not sucrose, raffinose, maltose, or galactose. *C. glabrata* occurs in alcoholic beverages and meat products as well. *C. stellata* is predominant on grapes and at the start of must fermentation (Mora et al., 1988; Holloway et al., 1992).

TABLE A.11
Salient Properties of Bipolarly Budding Species Included in SIM

Species	Kloeckera anamorph	Characteristics*					
		cyc	Kg	M	S	R	37
Hsp. guilliermondii	*Klc. apis*	99	99	1	1	1	99
Hsp. occidentalis	*Klc. javanica*	1	1	1	99	1	1
Hsp. osmophila	*Klc. corticis*	1	1	1	99	1	1
Hsp. uvarum	*Klc. apiculata*	99	99	99	1	1	1
Hsp. valbyensis	*Klc. japonica*	99	1	1	1	1	1
Hsp. vineae	*Klc. africana*	1	1	99	50	1	1
S'codes ludwigii	None	1	1	1	99	70	30

*All assimilate cellobiose and cadaverine and ferment glucose; all are negative in urease reaction and assimilation of nitrate, erythritol, and mannitol.
Abbreviations: cyc, growth with 0.01% cycloheximide; Kg, assimilation of 2-ketogluconate; M, maltose; S, sucrose; R, raffinose; 37, growth at 37°C.

TABLE A.12
Salient Properties of Raffinose-Negative Species Included in SIM*

Species	Characteristics**										
	cyc	Ce	M	G	Mg	cit	S	St	Rm	37	psh
Bret. naardenensis	99	99	99	50	85	1	1	1	85	50	1
C. albicans	99	1	99	99	50	99	70	99	1	99	99
C. catenulata	99	1	70	99	1	99	1	70	1	70	99
C. maltosa	99	85	99	99	99	50	99	1	1	99	99
C. oleophila	99	99	99	99	50	99	99	1	1	99	99
C. parapsilosis	50	1	99	99	99	99	99	1	1	99	99
C. sake	1	50	99	99	50	50	99	1	1	1	99
C. tropicalis	99	70	99	99	50	70	50	99	1	99	99
C. zeylanoides	85	30	1	30	1	99	1	1	1	15	99
Clsp. lusitaniae	1	85	99	50	85	50	99	1	99	99	99
Db. etchellsii	1	99	99	99	99	1	99	1	1	99	99
Lodd. elongisporus	99	1	99	99	99	99	99	1	1	99	99
Met. pulcherrima	1	99	99	99	99	99	99	1	1	1	1
Met. reukaufii	1	99	99	85	99	1	99	1	1	50	1

*All are also negative in urease reaction and assimilation of nitrate and erythritol, but all assimilate mannitol (except *Bret. naardenensis*, which is variable).
**Assimilation tests: Ce, cellobiose; M, maltose; G, galactose; Mg, methyl glucoside; cit, citrate, S, sucrose; St, starch; Rm, rhamnose; growth tests: cyc, 0.01% cycloheximide; 37, at 37°C; morphology: psh, pseudohyphae.

Cells of *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* are usually larger than 5 μm in diameter, and they assimilate one or more of the sugars listed in the preceding text. Much effort has been made to find conventional physiological tests that would correlate with DNA homologies used for the delimitation of *Saccharomyces* species. It appears that growth at 37°C and without added vitamins

TABLE A.13
Salient Properties of *Zygosaccharomyces* Species Included in SIM

	Characteristics**										
Species*	**cyc**	**Ce**	**Mt**	**M**	**R**	**Te**	**Me**	**AcA**	**60**	**16**	**SA/BA**
Zy. bailii	1	1	85	1	30	99	1	99	99	1	85
Zy. bisporus	1	1	50	1	1	1	1	99	99	1	1
Zy. cidri[1]	99	1	99	99	99	99	99	1	1	1	1
Zy. fermentati[1]	99	99	85	99	99	99	15	1	1	1	1
Zy. florentinus[2]	99	1	85	85	99	99	99	1	1	1	1
Zy. microellipsoides[3]	1	1	50	50	99	15	99	1	1	1	1
Zy. mellis	1	1	70	70	1	50	1	1	99	99	1
Zy. mrakii[2]	99	1	99	1	99	1	99	1	1	1	1
Zy. rouxii	1	1	70	70	1	50	1	1	99	1	1

Current assignments: [1]*Lachancea*, [2]*Zygotorulaspora*, and [3]*Torulaspora*.
*Not considered in SIM: *Zy. cidri* and *Zy. mrakii*.
**Assimilation tests: Ce, cellobiose; Mt, mannitol; M, maltose; R, raffinose; Te, trehalose; Me, melibiose; growth tests: cyc, 0.01% cycloheximide; AcA, 1% acetate; 60, glucose 60% (w/w); 16, NaCl 16% (w/w); SA, sorbic acid 300 mg L^{-1}; BA, benzoic acid 400 mg L^{-1}.

TABLE A.14
Salient Properties of Yeasts Negative in All Tests of SIM Master Key

	Characteristics*										
Species	**cad**	**R**	**Mt**	**G**	**X**	**Te**	**d**	**vit**	**37**	**psh**	**pel**
C. glabrata	1	1	1	1	1	99	99	1	99	1	1
C. inconspicua	99	1	1	1	1	1	1	1	99	70	1
C. sorboxylosa	1	1	1	1	99	1	50	1	50	99	1
C. stellata	1	99	1	1	1	1	99	1	1	1	1
Iss. orientalis	99	1	1	1	1	1	99	99	99	99	99
Iss. terricola	99	1	1	1	1	1	99	1	70	99	99
P. fermentans	99	1	1	1	99	1	99	1	99	99	99
P. kluyveri	99	1	1	1	1	1	99	1	50	99	99
P. membranifaciens	99	1	1	1	30	1	15	50	50	99	99
P. nakasei	99	1	1	1	15	1	99	1	1	99	1
Naumovia dairensis	1	1	1	99	1	30	99	15	1	1	1
Zygo. rouxii	99	1	70	50	1	50	99	15	50	50	15

*Assimilation tests: cad, cadaverine; R, raffinose; Mt, mannitol; G, galactose; X, xylose; Te, trehalose; fermentation: d, glucose; growth tests: vit, vitamin-free medium; 37, at 37°C; morphology: psh, pseudohyphae; pel, pellicle.

could be used to this end (Vaughan Martini and Martini, 1993). Only *S. bayanus* grows in vitamin-free medium, and most *S. cerevisiae* strains grow at 37°C, whereas *S. pastorianus* does not.

S. cerevisiae is undoubtedly the most common and widely distributed yeast in foods. It is intimately associated with many food processing environments and is considered a highly domesticated species (Martini, 1993). Conversely, *S. cerevisiae* is also an active spoilage yeast in various foods.

TABLE A.15
Salient Properties of *Saccharomyces* Species and Some Cadaverine-Negative Yeasts*

	Characteristics**										
Species	**cad**	**lys**	**cyc**	**Mt**	**Kg**	**R**	**M**	**G**	**S**	**Te**	**37**
C. cantarellii	1	50	99	99	1	1	1	15	1	99	30
C. glabrata	1	15	1	1	50	1	1	1	1	70	99
C. lactiscondensi	1	50	15	1	1	70	1	1	99	1	1
C. stellata	1	99	1	1	1	99	1	1	99	1	1
C. zeylanoides	50	50	99	99	99	1	1	30	1	99	15
S. bayanus	1	1	1	1	1	70	70	70	70	50	50
S. cerevisiae	1	1	1	1	1	70	70	70	70	50	85
Naumovia dairensis	1	1	70	1	1	1	1	99	1	30	1
Kazach. exiguus	1	1	70	1	1	70	1	99	30	30	1
Lachancea kluyveri	99	99	1	50	15	70	70	99	99	99	99
S. pastorianus	1	1	1	1	1	70	70	70	70	70	1
Tsp. delbrueckii	1	99	1	70	99	50	30	50	50	85	30
Tsp. globosa	1	50	99	50	99	85	1	1	99	15	99

*All are budding, nonhyphal, urease- and nitrate-negative yeasts; all are erythritol negative (except *C. cantarellii*); all are citrate negative (except *C. zeylanoides*); all ferment glucose (except *C. zeylanoides*).
**Assimilation tests: cad, cadaverine; lys, lysine; Mt, mannitol; Kg, 2-ketogluconate; R, raffinose; M, maltose; G, galactose; S, sucrose; Te, trehalose; growth tests: cyc, 0.01% cycloheximide; 37, at 37°C.

On the positive side, *S. cerevisiae* is indispensable to breadmaking and is the genuine wine yeast, while *S. bayanus* mainly contributes to the production of special types of wine. *S. pastorianus* is brewing yeast, indispensable in the production of lager beers.

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Scientific Name Index

D

E

F

G

H

I

K

L

M

N

O

P

R

S

T

U

V

W

X

Y

Z

Subject Index

D

E

F

G

H

I

K

L

M

N

O

P

R

S

T

U

V

W

X

Y

Z

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