THE FERMENTATION PROPERTIES OF NON-*SACCHAROMYCES* WINE YEASTS AND THEIR INTERACTION WITH *SACCHAROMYCES CEREVISIAE*

Alison Soden

Department of Horticulture, Viticulture and Oenology
The University of Adelaide

The Australian Wine Research Institute

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy
July 1998

TABLE OF CONTENTS

ABSTRACT

This thesis is concerned with the contribution of some non-*Saccharomyces* yeasts, particularly strains of *Candida,* to grape juice fermentation. An emphasis was placed upon the investigation of fermentation by non-*Saccharomyces* strains in mixed culture with a winemaking strain of *Saccharomyces cerevisiae.*

A polymerase chain reaction (PCR) method using intron splice site specific primers was developed that permitted rapid identification of a number of species associated with winemaking, and also differentiation of isolates of the same species at the strain level.

For preliminary assessment of their fermentation characteristics and aroma profiles, a number of yeasts were used as pure cultures for winemaking. Differences in fermentative capacity, the effect upon wine composition, aroma description and preference were identified between *S. cerevisiae* and the non-*Saccharomyces* yeasts. Strains within a species also had notably different effects upon fermentation.

As few of these yeasts were able to completely ferment grape juice, acceptable isolates were also assessed for their growth and metabolic activities during mixed culture fermentation of a chemically defined grape juice-like medium, in conjunction with a commercial winemaking strain of *Saccharomyces cerevisiae.* The effect on growth kinetics and wine composition were found to depend upon the relative growth rate and fermentative capacity of the two strains involved. Coinoculation and sequential inoculation strategies were identified that could promote the growth and metabolic activity of a weakly fermentative strain and similarly suppress the strongly fermentative *S. cerevisiae* strain.

Formal sensory analysis was used to describe the aroma of wines made by monocultures of *C. stellata* CBS 2649 and *S. cerevisiae* EC1118, and by coinoculation and sequential inoculation of both of these yeasts. The inoculation protocol was found to have a significant effect upon the fermentation kinetics — particularly the sugar utilisation pattern, the wine composition and the wine aroma. Sequential inoculation, where the fermentation was initiated with *C. stellata* and completed with *S. cerevisiae,* produced a wine that had constitutional and aroma characteristics of both strains, but with significant differences in intensity. This work demonstrates the potential use of selected non-*Saccharomyces* yeasts in commercial winemaking.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this thesis, when deposited in the University Library, being available for loan and photocopying.

ACKNOWLEDGEMENTS

I would like to thank:

My supervisors Dr Paul Henschke, Dr Miguel de Barros Lopes, Dr Vladimir Jiranek and (formerly) Prof. Terry Lee for their guidance during this work and the preparation of this thesis.

Dr Miguel de Barros Lopes and Ms Anna Martens for their essential roles in the collaborative development of a PCR method for yeast identification.

Dr Leigh Francis and Mr Phil Spillman for guidance on the conduct of sensory analysis and for advice on statistics.

Mr Holger Gockowiak and Mr Jeff Eglinton for guidance and advice on just about everything microbiological.

Ms Mariola Kwiatkowski and Dr Leigh Francis for programming the Cobas Fara autoanalyser for glucose and fructose analysis.

Mr Alan Pollnitz for setting up a GC/MS assay for wine volatiles, for running the samples, and for tuition on chromatogram analysis.

Dr Paul Monk and Lallemand Pty Ltd for the provision of yeast isolates, wine bottles and cross-flow filtration services.

Mr Jeff Cohn, Ms Marty Bannister and staff of Vinquiry, California for yeast isolation.

Petaluma Ltd for providing grape juice.

Dr Peter Langridge for the use of laboratory facilities during the PCR work.

The AWRI Analytical Service for the use of their facilities, and in particular Mr Adrian Coulter for HPLC analysis.

The staff and postgraduate students of the AWRI who participated as sensory judges, for their considerable efforts and enthusiasm.

Chapter 1

INTRODUCTION AND AIMS OF THIS STUDY

An understanding of the microbiology of wine production originated with the pioneering studies of Louis Pasteur over 130 years ago (see Amerine *et al.* 1980), which showed that yeasts were responsible for the fermentation of grape juice into wine. Since Pasteur's time, the microbiology of winemaking has been studied extensively, revealing it to be a complex interaction of yeasts and bacteria. During fermentation, yeasts utilise the constituents of grape juice, principally converting sugars into ethanol and carbon dioxide, and generating an array of metabolic products which determine the chemical composition and sensory attributes of the wine. Table 1.1 lists the groups of organisms that interact during wine production.

Table 1.1. The diversity and significance of microorganisms in winemaking

Microorganism	Significance
Yeasts	alcoholic fermentation, autolysis, spoilage
Lactic acid bacteria	malolactic fermentation, spoilage
Acetic acid bacteria	spoilage, stuck fermentation
Other fungi	botrytised wines, mouldiness, cork taint
Other bacteria	spoilage, earthiness and mustiness, inhibition of fermentation
Bacteriophages (viruses)	disruption of malolactic fermentation, killer character

from Fleet (1990)

To fully understand the roles of the different yeasts that can participate in wine fermentation it is essential to determine:
- the taxonomic identity of each species;
- the fermentation growth kinetics;
- the biochemical properties of these yeasts and the chemical changes they impart; and;
- the influence of vinification practices on the growth and metabolism of these yeasts (Fleet and Heard 1993).

1.1. Taxonomy of winemaking yeasts

Yeasts are a phylogenetically diverse group of fungi assigned to either the class Ascomycetes or Basidiomycetes (Kurtzman 1994). Yeast is therefore a term of convenience, describing unicellular, vegetatively dividing fungi (Kurtzman 1988, 1994). The term 'non-*Saccharomyces* yeast' refers to a collective of species other than those of the genus *Saccharomyces*. The register compiled by Barnett *et al.* (1990) lists 590 yeast species of 83 genera. A list of the species associated with winemaking comprises 94 species of 36 genera,

although many of these are isolated only very rarely. The more commonly isolated yeasts are listed in Appendix 1.

The chief characteristics used to separate and identify the yeasts according to Barnett *et al.* (1990) are: Cell morphology; the mode of sexual reproduction; physiological (especially nutritional) characteristics; biochemical characteristics; and genomic (DNA–DNA or DNA–RNA) homology.

1.1.1. Morphology and mode of reproduction
Microscopic observations can be used to classify many yeasts to genus level. Features such as cell size and shape are examined, as is the mode of vegetative division — that is, budding or fission. Certain species form true hyphae or pseudohyphae, the latter being formed by budding cells which do not separate (Kreger-van Rij 1984, Kurtzman 1988). The formation of, and the structure of ascospores (formed by meiotic division), and teliospores (cells of basidiomycetous yeasts in which nuclear fusion occurs), also separate taxa.

1.1.2. Physiological features
Physiological characteristics chiefly used for species differentiation are: Fermentation of key sugars; aerobic growth utilising a single carbon or nitrogen source; growth in the absence of certain vitamins; growth at high osmotic pressure of glucose or sodium chloride; growth at 37°C; growth in the presence of cycloheximide; the ability to split fat compounds; the production of starch-like polysaccharide; urea hydrolysis; and acid formation.

1.1.3. Biochemical characteristics
Biochemical characteristics which influence taxonomic assignment include the chemical composition of the cell wall, and the type of the coenzyme Q respiratory electron acceptor present.

1.1.4. Genome homology
Over the last 35 years techniques for genome comparison have been utilised in yeast classification. Molecular comparison allows the evaluation of current taxonomic assignments on the basis of quantitative genetic differences (Kurtzman 1988, 1994). Early studies compared the guanine + cytosine composition of nuclear DNA, and a difference of 1–1.5% was determined for the delineation of species (Kurtzman 1988).

Nuclear DNA relatedness, measured as the base pair homology between yeasts, can be ascertained by the reassociation percentage of single stranded genomic DNA sequences (Meyer and Phaff 1972). A reassociation efficiency of 80–100% groups organisms into the same species (van der Walt 1987). More recently Kurtzman (1994), has used ribosomal RNA and DNA sequence divergence to examine yeast systematics and phylogeny.

Early genetic studies by Winge and Roberts (1949) showed that a single gene can confer sugar fermentation or assimilation ability. Classification of yeasts may be based upon a difference in only one such attribute, possibly artificially establishing species divisions on the basis of one gene. There is also the accepted practice of defining two yeasts to different genera — not just species — on the basis of sexual reproduction. Two otherwise identical yeasts have different names for the teleomorphic (sexual or perfect) and anamorphic (asexual or imperfect) states. Where a sexual state is known, the name of the teleomorph takes preference, although the description for both states is combined. An anamorphic genus is perhaps a temporary taxonomic resting place until a sexual state is found (Kurtzman 1988). Appendix 2 lists the teleomorphic and anamorphic names of important winemaking yeasts.

The taxonomy of the yeasts is in a constant state of flux as new species are discovered, and as taxonomic principles and methods are modified. Genera and species have even been described and then renamed in the same year, by the same author which serves to confuse rather than clarify the study of yeasts by non-taxonomists (see Barnett *et al.* 1990).

1.1.5. Strain differentiation

It is well established that, for the species *S. cerevisiae*, there exists strains with quite different phenotypes and characteristics (Rankine 1968, Benda 1989, Petering *et al.* 1990). Strain evaluation and differentiation is therefore of importance in the application and commercialisation of *S. cerevisiae* strains (Delteil and Aizac 1988, van der Westhuizen and Pretorius 1992). Within the non-*Saccharomyces* species, strains with differing winemaking characteristics also exist, and some yeast species have been evaluated for the production of important wine constituents during fermentation (section 1.5). A variety of methods have been applied to the differentiation of the commercially important *S. cerevisiae* strains, although only a few researchers have addressed strain differentiation of the non-*Saccharomyces* species (Schütz and Gafner 1993a).

For the differentiation of wine strains of *S. cerevisiae*, Querol *et al.* (1992a) evaluated a number of methods such as whole cell protein electrophoresis, DNA hybridisation, chromosomal patterns, and mitochondrial DNA electrophoresis. Only the last two methods were of value in their studies. Vezinhet *et al.* (1992, 1994) compared more recent molecular methods such as restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (Dubordieu *et al.* 1987, Hallet *et al.* 1990, Querol *et al.* 1992b), and nuclear DNA (Degré *et al.* 1989, Lavallée *et al.* 1994), karyotyping or chromosome electrophoresis (Blondin and Vezinhet 1988, Petering *et al.* 1988, Schütz and Gafner 1993a, Schütz and Gafner 1994a); and the polymerase chain reaction (PCR), method of DNA amplification and fingerprinting (Lavallée *et al.* 1994). In the comparative studies by Vezinhet and coworkers (1992, 1994), electrophoretic karyotyping was found to be the most reliable differentiation technique. This technique is however laborious in comparison to techniques such as the PCR methodologies which are now widely used in the analysis of genetic diversity amongst many

different organisms. Lavallée *et al.* (1994) achieved unambiguous strain identification of commercial *S. cerevisiae* strains using the PCR. Fell (1993) successfully applied the PCR to the identification of several marine yeast species, but the technique did not differentiate strains.

1.2. Survey of the yeast flora of the grape

Grapes are the original and primary source of wine yeasts. Yeast cells adhere to the grape skin, colonising around stomata and the pedicel attachment point, where slight leakage of juice provides nutrition (Belin 1972). Ecological studies of grapevine microflora have been conducted in all of the major wine producing countries. The first systematic survey was by Hansen in 1881 (cited by Martini and Vaughan Martini 1990), who identified yeasts associated with the surfaces of sugary fruits. The findings from many surveys have been extensively reviewed by Kunkee and Amerine (1970), Kunkee and Goswell (1977), Benda (1982), Lafon-Lafourcade (1983), Farkas (1988), Bisson and Kunkee (1991), among others, and therefore specific details are not included here. The validity of many surveys however, has been criticised by Martini and Vaughan Martini (1990), and Martini (1993), because of the lack of quantitative data provided on the populations of individual species. These reports are useful as a summary of species composition, but are somewhat confounded by the variety of isolation methods used. The use of selective enrichment media which can favour the growth of particular species, rather than direct plating methods, biases the results (Martini 1993). These authors also stress that more vigorous dislodgment methods such as sonication must be used for complete isolation of the yeasts present on the grape.

Reliable quantitative data on the microflora of grapes have been collected by researchers including Barnett *et al.* (1972), Davenport (1974), Rosini *et al.* (1982), Goto and Oguri (1983), Goto *et al.* (1984), Parish and Carroll (1985), and Moore *et al.* (1988). The results of these and other studies are summarised by Fleet and Heard (1993), and Bisson and Kunkee (1991), as follows:

- mature, sound grapes inoculate the must with a total yeast count of 10^3–10^5 cells per mL;
- the apiculate yeasts, especially *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*), comprise 50–75% of the grape microflora;
- significant, but present in lower numbers, are species of the genera *Candida* (especially *C. stellata* and *C. pulcherrima*), *Cryptococcus*, *Kluyveromyces*, *Pichia*, *Rhodotorula*, and occasionally, *Brettanomyces*; and;
- *S. cerevisiae* occurs at a low population density, or not at all, on undamaged grapes and is rarely isolated from the vineyard.

1.2.1. Factors affecting the microbial flora of grapes

Different environmental and physical factors affect the species composition and the total number of yeasts present on the grape. These factors include climatic conditions such as temperature and rainfall, as demonstrated by surveys in Italy, Spain and France (Castelli

1957, Kunkee and Goswell 1977, Querol *et al.* 1990, Longo *et al.* 1991). Climate has a particular influence upon *Botrytis cinerea* infection which is responsible for producing the Sauterne style wine which owes much of its character to the fungal infection. Degree of grape maturity at harvest (Goto and Yokotsuka 1977, Rosini *et al.* 1982), physical damage to the berries (Longo *et al.* 1991), fertiliser use (Bisson and Kunkee 1991), and fungicide use (Bureau *et al.* 1982), can all affect the species composition. Fleet and Heard (1993) point out however that quantitative data are lacking in these studies.

Varietal differences such as berry cluster structure can affect both bunch rotting and the composition of microbial flora. The practice of disposing of winery waste in the vineyard presumably also introduces another source of inoculum that can influence the grape microflora composition (Bisson and Kunkee 1991).

1.3. Survey of the yeast flora of grape juice fermentation

The microorganisms resident on the grape are introduced into the must upon crushing of the fruit and the microflora of the associated winery equipment will also inoculate the must. Grape juice is a selective medium which limits the growth of most microorganisms, due to low pH (<3.0–3.9), and high osmotic pressure (commonly 12–27% w/v sugars) (Amerine *et al.* 1980). Once fermentation begins only highly adapted yeasts and bacteria capable of fermenting the substrate under anaerobic conditions, whilst tolerating an increasing concentration of ethanol, will survive and grow.

1.3.1. Factors affecting yeast growth during fermentation

Many vinification practices and winemaking variables affect the fermentation process and the quality of the wine produced. Procedures which influence the initial yeast species composition and population density, and factors which affect yeast growth will all influence the fermentation and the resulting wine.

1.3.1.1. Grape juice composition

Important variables of grape juice composition include the concentration of sugar and nitrogen, vitamin supply, and the presence of insoluble solids and dissolved oxygen (Fleet and Heard 1993). Under most conditions, grape juice contains all of the yeast nutritional factors required for complete fermentation, but there are varietal, vineyard and seasonal differences. To compensate, some European countries permit sugar addition, and in many countries nitrogen, vitamin and trace nutrient additions are also made (Kunkee and Bisson 1993).

The specific nutritional requirements of the non-*Saccharomyces* yeasts have not been reported. For instance, the initial sugar (glucose and fructose) composition of juice inversely affects the growth rate of *S. cerevisiae* and the completeness of fermentation (Lafon-Lafourcade 1983, Monk and Cowley 1984). Glucose is preferentially fermented by *S.*

cerevisiae, but the growth of non-*Saccharomyces* yeasts may change the glucose to fructose ratio, which can in turn affect the fermentation rate (Fleet and Heard 1993, Schütz and Gafner 1993b). *Zygosaccharomyces bailii* ferments fructose before glucose (Sols 1956, Emmerich and Radler 1983), and is also capable of fermenting malic acid (Romano and Suzzi 1992, 1993b), as is *Schizosaccharomyces pombe* (Magyar and Panyik 1989). Interestingly, the pentose sugars found in juice at low concentration are not fermentable by *S. cerevisiae*, but species of *Candida*, *Pichia* and *Metshnikowia* are able to ferment xylose (Rose 1987). Must nitrogen content can also have a direct effect upon fermentation rate (Cantarelli 1957), and different yeasts may have different nitrogen requirements. It is likely that juice composition would impose selection upon the non-*Saccharomyces* yeasts present, and that those yeasts active in the early stages of fermentation would modify the juice, affecting the growth of *S. cerevisiae* which dominates later in fermentation (Herraiz *et al.* 1990).

1.3.1.2. Juice clarification

Clarification treatments such as fining, enzyme treatment, centrifugation and filtration reduce the amount of suspended solids in the juice which is likely to remove the indigenous yeasts still adherent to the grape skins. Cold settling, whilst initially lowering yeast numbers, can actually favour the multiplication of psychrophilic yeasts. Mora and Mulet (1991) observed growth of *Kl. apiculata*, *C. stellata* and *P. membranaefaciens* during the later stages of cold settling. This would presumably allow a significantly greater impact by these yeasts early in fermentation. *S. cerevisiae* number appeared to be reduced by cold settling in this study.

1.3.1.3. Sulfur dioxide

Sulfur dioxide (SO_2) is routinely added during grape crushing to control oxidation of the juice and to restrict the growth of the indigenous flora (Amerine *et al.* 1980). Sulfur dioxide addition can decrease the initial yeast numbers and therefore delay the onset of fermentation, which may also take longer to complete as the rate of fermentation can also be decreased (Fleet and Heard 1993). Sulfur dioxide addition may have a selective effect upon the microbial flora present as the non-*Saccharomyces* yeasts are generally more sensitive to sulfur dioxide than *S. cerevisiae* (Zambonelli *et al.* 1989). This generalisation is questioned however by Heard and Fleet (1985, 1986, 1988a), who showed that sulfur dioxide addition still permitted the growth of *Candida* and *Hanseniaspora* species.

1.3.1.4. Temperature

The importance of temperature control in winemaking has long been recognised (Amerine *et al.* 1980). Temperature affects the rate of yeast growth and metabolic activity, and also the contribution of different yeasts to the fermentation (Fleet and Heard 1993). Generally, white wines are fermented at a temperature of 10–20 °C to retain flavour volatiles and ethanol, whilst red wines are fermented at 20–30 °C to enhance colour and flavour extraction. Sharf and Margalith (1983) examined the effect of fermentation temperature on the growth of mixed cultures of *Kl. apiculata* and *S. cerevisiae*. *Kl. apiculata* dominated the fermentation at

10°C and 20°C, while *S. cerevisiae* dominated at 30°C. These findings were supported by the experiments of Heard and Fleet (1988b) and Gao and Fleet (1988), which demonstrated the domination of fermentation at 10°C by *Kl. apiculata*. Mora and Rossello (1992) noted a similar enhancement of the growth of *P. membranaefaciens* at 10°C as compared to 20°C, during fermentation of grape juice in the presence of *S. cerevisiae*. This is possibly due to the enhanced ethanol tolerance of these yeasts at lower temperature (Heard and Fleet 1988b). Fleet and Heard (1993) therefore suggest that increased growth and metabolic activity of the non-*Saccharomyces* yeasts can be expected at fermentation temperatures below 20°C.

1.3.1.5. pH

Generally grape juice pH varies between 3.0–3.9 (Amerine *et al.* 1980), which can have an effect upon both the species of yeasts present and the population density. Heard and Fleet (1988b) and Gao and Fleet (1988) found that species of *Candida* and *Kl. apiculata* grew better at higher pH. *S. cerevisiae* however, does not appear to be affected within this pH range (Bisson and Kunkee 1991).

1.3.1.6. Yeast killer character

The killer character in yeast was first reported for *S. cerevisiae* in 1963 by Bevan and Makower, and more recently the topic has been reviewed by Young (1987) and Shimizu (1993). Three phenotypes are recognised, killer, sensitive and neutral, where killer types are able to kill sensitive strains and neutral strains exhibit neither killer activity nor sensitivity. Killer strains produce protein or glycoprotein toxins which are lethal to sensitive yeast strains. The phenomemon has since been noted in many other yeast genera including *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Kloeckera, Kluyveromyces, Pichia, Rhodotorula* and *Trichosporon* (Shimizu 1993).

Killer yeasts have been isolated from grapes, juice, fermenting must and wine and indigenous killer strains may alter the ecology of fermentation with subsequent effects upon the wine. Killer strains of *S. cerevisiae* are commercially available and are of oenological interest for their purported ability to suppress indigenous yeasts. Interspecific effects that have been noted include the ability of strains of *Hanseniaspora* and *Pichia* to kill *Saccharomyces* and *Candida* strains (Stumm *et al.* 1977, Sponholz *et al.* 1990). The killer activity of the non-*Saccharomyces* yeasts associated with wine fermentation is therefore of interest.

1.4. Indigenous vs inoculated fermentation

The yeasts that conduct the alcoholic fermentation originate from the grapes, winery equipment and surfaces, and starter cultures. Winemaking originated at least 6000 years ago and was based upon spontaneous fermentation practices (Jackson 1994). In many European wineries uninoculated fermentation practices are still predominantly used, while the newer wine producing countries generally favour the use of a pure yeast starter culture, which is almost always a commercial strain of *S. cerevisiae*. Pure culture inoculation was first

described for brewing by Hansen in 1908 (cited by Martini and Vaughan Martini 1990), and later introduced to winemaking. This practice has advantages such as the rapid initiation of fermentation, which then proceeds quickly and predictably. It is generally presumed that indigenous yeast are suppressed when a high density inoculum culture is added, especially in conjunction with sulfur dioxide addition. Ecological studies of inoculated and spontaneous fermentations however show that the non-*Saccharomyces* yeast can still grow under these conditions (Heard and Fleet 1985, 1986). Indigenous yeast have been attributed with lowered wine quality, uncertain completion of fermentation, and spoilage (Kunkee and Amerine, 1970). Generally, the use of active dry yeast or other pure starter cultures ensures an expected fermentation result, however, the question of which strain to use for which winemaking purpose remains (Rankine 1968, Kunkee and Amerine 1970).

The advantages and disadvantages of using starter cultures have been debated in reviews by Kunkee and Goswell (1977), Benda (1982), Reed and Nagodawithana (1988), and Bisson and Kunkee (1991). According to Kunkee and Bisson (1993), flavour enhancement of wines by the activity of non-*Saccharomyces* yeast has not been demonstrated or documented. In comparisons of indigenous and inoculated fermentations, flavour differences, if not preferences, have however been noted (Dittrich 1978, Edinger and Henick-Kling 1994). There is a strongly held belief by European winemakers that indigenous mixed culture fermentation produces superior wines (Amerine *et al.* 1980). It is also upheld that the aroma of uninoculated wines is more complex than that of wines produced with starter cultures (Benda, 1982), and undeniably, many of the world's great wines are produced without the use of starter cultures. Recently, for a variety of reasons, uninoculated fermentation has become popular amongst some Californian winemakers, with favourable results (Bullard 1994, Goldfarb 1994). Amerine *et al.* (1980) consider however that many purported natural fermentations may use practices such as sulfur dioxide addition or pied de cuve (indigenous yeast starter culture) (Peynaud, 1984) preparation, which impose selective pressures that affect the yeast composition. Certainly the indigenous winery flora will have undergone some selection for adaptation to particular winery practices, and yeasts that can successfully compete with the unadapted grape flora are likely to be present. Indeed Rosini (1984) found that a marked *S. cerevisiae* strain used in a new winery became part of the resident flora, and thereafter participated in all uninoculated fermentations.

1.4.1. Mixed cultures

The use of starter cultures comprised of more than one yeast species or strain received early attention with the classical experiments of Muller-Thurgau in 1896 (as described by Martini and Vaughan-Martini, 1990). The use of non-*Saccharomyces* species as mixed or sequentially inoculated starter cultures has been of interest to some researchers. Herraiz *et al.* (1990) and Zironi *et al.* (1993), found that the use of pure, mixed or sequential cultures of *Kloeckera apiculata, Torulaspora delbrueckii* and *S. cerevisiae* had significant effects upon the volatile composition of the wines produced. Mora *et al.* (1990) found that a

Kluyveromyces thermotolerans starter culture affected both the growth of *S. cerevisiae* during fermentation and the acidity of the resulting wine. Further research into the growth and metabolic activity of the non-*Saccharomyces* yeasts during grape juice fermentation is required to better understand the role of these yeasts in winemaking.

1.5. The effects of yeast on wine composition

The flavour of wine is affected by many variables, with the grape variety and juice composition being of major importance (Noble 1994). Fermentation of juice produces a more complex mixture, the composition of which is principally determined by yeast metabolic activity (Amerine *et al.* 1980). Yeast growth and metabolism have an effect upon wine sensory attributes by the production of specific flavour compounds during the fermentation and modification of the juice substrate. Important substances produced include alcohols, esters, acids, carbonyl compounds, polyols and nitrogenous compounds (Amerine and Joslyn 1970, Benda 1982, Farkas 1988). Controversy lingers however, as to the relative importance of yeast strain to flavour. Bisson and Kunkee (1991) and Kunkee and Bisson (1993) state that there is no substantial evidence linking the *S. cerevisiae* strain used to "special flavour effects", except perhaps for flavour defects such as residual sweetness, oxidation and hydrogen sulfide production, but generally the effect of the yeast upon the sensory character of wine is accepted (Rankine 1968, Curschmann *et al.* 1994).

Different species or strain effects upon the production of a number of important compounds are outlined below. Some specific non-*Saccharomyces* yeasts have been studied, but further quantitative chemical and sensory analysis will contribute to the understanding of the role of these yeasts in wine production. Comparison of the concentration of aroma and flavour compounds in wine to published data on sensory thresholds can indicate likely sensory impact. Complex interactions between compounds may exist however which determine the overall sensory effect (Jackson 1994), and it is therefore necessary to apply sensory analysis to determine the flavour effects of different yeasts in fermentation.

1.5.1. Ethanol

Ethanol is the single most important product of grape juice fermentation by yeast, and it is produced within a range of 6–20% v/v by *S. cerevisiae*. It has a slightly sweet taste, moderates acidity, sweetness and bitterness, and also acts as a solvent or carrier for other compounds (Amerine and Joslyn 1970). Ethanol production is limited by the initial concentration of sugar in the juice, and is affected by fermentation conditions. The yeast species or strain used can have an effect upon the yield of ethanol (Table 1.3). The non-*Saccharomyces* yeasts have notably lower ethanol tolerances than *S. cerevisiae* (D'Amore and Stewart 1987), and this in turn affects ethanol production by these yeasts. The ethanol tolerance of the non-*Saccharomyces* yeasts is affected by the fermentation temperature, with enhanced growth and increased ethanol tolerance of *Kl. apiculata* and *C. stellata,* and also

decreased growth and activity of *S. cerevisiae*, during fermentation below $15°C$ (Gao and Fleet 1988, Heard and Fleet 1988b).

1.5.2. Higher alcohols

Higher alcohols are formed in wine mainly as the anabolic products of sugar metabolism, and the catabolic products of amino acid metabolism by yeasts, often comprising 50% of all volatiles (Etiévant 1991, Rapp and Versini 1991). The characteristic odour and flavour of these compounds at high concentration is described as pungent and objectionable, with a strong, burning taste. At the concentration often found in wine however, higher alcohols can contribute positively to flavour and aroma (Table 1.2) (Margalith 1981).

Table 1.2. Some higher alcohols found in wine

compound	aroma and flavour
n-propanol	spiritous odour
2-methyl-1-propanol (isobutanol)	vinous and heady
2-methyl-1-butanol (active amyl alcohol)	vinous and heady
3-methyl-1-butanol (isoamyl alcohol)	strongest odour and strong, pungent, burning taste
hexanol	woody, green, coconut-like, pungent
2-phenyl ethanol	perfumed, rose-like odour

data from Margalith and Schwartz (1970), Nykänen (1986), Webb (1967), Rankine (1967), Etiévant (1991) Holloway and Subden (1991)

As shown in Table 1.3 *S. cerevisiae* strains and non-*Saccharomyces* species vary in the production of higher alcohols. Some analytical studies have focused on the non-*Saccharomyces* yeasts, and generally it appears that the *S. cerevisiae* strains surveyed produce a wider concentration range of higher alcohols than the non-*Saccharomyces* yeasts studied, except for one *Candida* isolate which produced isobutanol at a concentration of 240 mg/L (Holloway and Subden 1991). This generalisation will perhaps change with the study of more representatives of the non-*Saccharomyces* species.

Table 1.3. Concentration of ethanol and some higher alcohols produced by some species of winemaking yeasts

Species	Ethanol (%v/v)	n-Propanol (mg/L)	Isobutanol (mg/L)	Isoamyl alcohol (mg/L)	2-Phenyl ethanol (mg/L)
S. cerevisiae	6–23	9–170	5–78	17–330	5–83
C. krusei	1–6.5	1–23	38–106	22–100	
C. stellata	4.5–6.5	4–8	13–21	6–11	
H'spora guilliermondii	4.3	5.4–16.9	3.8–16.2	9.6–22	
Kl. apiculata	5–6.5	3.9–32	2.9–38	4–17	21–27
M. pulcherrima	2	<1–43	37–123	21–243	22
P. anomala	0.2–4.5	3–15	18–29	11–25	27
Zygosacch. bailii		9–24	3–36	12-125	

Adapted from Heard (1988). Data collated from: Ferreira (1959), Guymon *et al.* (1961), Van Zyl *et al.* (1963), Rankine (1967), Bertrand (1968), Sponholz and Dittrich (1974), Soufleros and Bertrand (1979), Shimazu and Watanabe (1981), Shinohara, (1984), Cottrell and McLennan (1986), Cabrera *et al.* (1988), Herraiz *et al.* (1990), Romano *et al.* (1992), Romano and Suzzi (1993a), Zironi *et al.* (1993)

1.5.3. Esters

Esters are a diverse and abundant group of flavour compounds present in grapes at low concentration, but mainly produced by yeasts (Nykänen, 1986, Etiévant 1991). The sweet, fruity, floral characteristics of esters have a pronounced sensory impact, especially on wine aroma. At a high concentration however, the effect can be overpowering and detrimental to wine sensory character (Amerine and Joslyn 1970, Reed and Peppler 1973). Table 1.4 lists the flavour and odour characteristics of some important esters.

Table 1.4. Odour and flavour of some wine esters

Ester	Odour	Flavour
Ethyl acetate	fruity, brandy- like, nauseating at high concentration	solvent, fruit, perfumed
Ethyl butyrate	fruity, banana, pineapple	papaya, butter, sweetish, apple perfumed
Ethyl hexanoate	fruit, wine, apple, banana, pineapple	apple, fruity, sweetish, aniseed
Ethyl lactate	artificial raspberry and strawberry	buttery
Isoamyl acetate	apple, pear, banana, fruit, sweet, slightly nauseating	banana, apple, solvent
Isobutyl acetate	banana, sweet, fruity	fruity, fermented
Hexyl acetate	sweet, fruity, berry, pear, apple	sweet, aromatic, perfumed
2-Phenyl ethyl acetate	honey, rose-like, cider-like, heady	rose, apple, spoiled fruit, sour, apple peel, sharp, bitter
n-Propyl acetate	fruity, pear-like	solvent, sweetish, perfumed

Data from Williams (1974), Killian and Ough (1979), Etiévant (1991)

Yeast species and strains show considerable variation in the amounts and types of esters produced (Soufleros and Bertrand 1979, Soles *et al.* 1982). Vinification factors that affect yeast presence, growth, and metabolism also influence ester production (Margalith and Schwartz 1970, Soles *et al.* 1982, Nykänen 1986). *S. cerevisiae* would appear to produce the highest concentration of most of the esters measured, with the exception of ethyl acetate and ethyl lactate (Table 1.5). Ethyl acetate in particular is produced at very high concentration by several of the non-*Saccharomyces* strains examined, especially *Hansenula anomala* (Bertrand 1968, Sponholz and Dittrich 1974), and *Hanseniaspora uvarum* (Sponholz *et al.* 1990). This could have a negative impact on the sensory character of wine, but in general esters are desirable and important contributors to wine aroma and flavour, and the role of non-*Saccharomyces* yeast in their production is of importance (Etiévant, 1991).

1.5.4. Acids

The two major types of acid produced by yeast are volatile fatty acids and non-volatile organic acids. The odour of fatty acids varies from vinegar-like to buttery, cheesy and soapy. The relatively high concentration found in wine and the low odour thresholds suggest that these compounds can have a sensory impact. Flavour effects due to acetic, butanoic, hexanoic, 3-methyl butanoic and octanoic acids have been noted in some wines. Acetic acid is the most abundant, and depending upon the wine style, its vinegary aroma can be detrimental at concentrations exceeding 1-3 g/L (Amerine *et al.* 1980, Benda 1982, Nykänen and Suomalainen 1983). Fatty acids occur only in trace quantities in grapes and are largely produced by yeast and bacteria during fermentation (Etiévant 1991). The main non-volatile organic acids occurring in wine, in order of abundance are, malic, tartaric, citric, succinic and lactic acid. Malic and tartaric acid are the major acids of grapes, whereas succinic and lactic acids are products of yeast and bacterial metabolism (Radler 1993). Malic acid is converted to lactic acid by bacteria during malolactic fermentation (Wibowo *et al.* 1985), whilst tartaric acid is only metabolisable by a few species of lactic acid bacteria but not yeast (Radler 1993). Citric acid is both present in grapes and produced by yeasts (Amerine *et al.* 1980). Collectively the organic acids affect wine colour, pH and flavour and their sour, sharp and tart taste imparts a desirable crispness and freshness to wine (Amerine *et al.* 1980).

Yeast metabolism affects the concentration of wine acids by malic acid degradation, acetic acid production, and tartaric acid precipitation during ethanol formation. The concentration of acids found after juice fermentation with different yeasts are compiled in Table 1.6. The data suggest that *S. cerevisiae* produces the highest concentration of most acids. However, *Kl. apiculata, Pichia anomala* and *C. stellata* strains may produce acetic acid at high concentration (Shimazu and Watanabe 1981). The extent of degradation of malic acid by yeasts is species or strain dependent (Fuck and Radler 1972, Shimazu and Watanabe 1981). Utilisation of this acid by some *S. cerevisiae* strains, and species of *Hanseniaspora, Candida,* and *Pichia* occurs, but with little overall effect on acidity (Radler 1993). *Schizosaccharomyces* species and *Zygosaccharomyces bailii* however, are capable of

Table 1.5. The concentration of some esters (mg/L) produced by different species of yeasts associated with winemaking

Yeast species	Ethyl acetate	n-Propyl acetate	Isobutyl acetate	Isoamyl acetate	Hexyl acetate	2-Phenyl ethyl acetate	Ethyl butyrate	Ethyl hexanoate	Ethyl lactate
S. cerevisiae	10–99	0.01–0.3	0.002–0.7	0.13–15.9	0.1–0.5	0.03–5.6	0.02–1.13	0.06–1.89	1–148
C. krusei	220–730			<1					
C. stellata	7–24			0.1–0.3		0.03		0.03–0.15	
H'spora uvarum	36–60			0.2–0.3		0.07–0.13		0.03	14–21
Kl. apiculata	36.9–240		0.002–0.02	0.4–5.0			0–0.01		
M. pulcherrima	150–382		<1						
P. anomala	138–2143			<1.0–10.7					
P. membranaefaciens	16–21			>1.0–5.8					
S'codes ludwigii	26–117		0–0.01	0.1–0.7			0.01–0.02		
Schizo. pombe	11–40		0.01	0.01			0.01–0.02	0.1–0.6	
T'spora delbrueckii	16–74	0.85–2.72	0.4–1.1	0.1–0.8	0.1–0.6	0.03–0.07		0.07–0.24	1.2
Zygosacch. bailii	23–53			0.1–0.6	0.1–0.3	0.06–0.31		0.01–0.16	

Table adapted from Heard, (1988). Data from Bertrand (1968), Sponholz and Dittrich (1974), Killian and Ough (1979), Soufleros and Bertrand (1979), Shinohara (1984), Di Stefano and Ciolfi (1985), Cottrell and McLellan (1986), Nykänen (1986), Cabrera et al., (1988), Sponholz et al. (1990)

Table 1.6. The concentration of some acids in wine produced by different yeast species

| Yeast species | Acid concentration (g/L) | | | | | | | | |
| | organic acids | | | | lactic | volatile fatty acids | | | |
	malic	tartaric	citric	succinic	lactic	acetic	butyric	hexanoic	decanoic
S. cerevisiae	1.0–3.7	2.6–2.8	1.5–2.3	5.8–13.2	0.4–0.7	0.24–0.81	0.11–1.3	0.33–1.76	0.29–1.07
C. krusei	1.99	4.0	0.25	0.33	0.11	1.07			
C. stellata				1.08–1.28					
H'spora guilliermondii					1.15–1				
Kl. apiculata						1.01–1.3	0.09–0.35	0.10–0.46	0.16–0.48
M. pulcherrima					0.13–0.14				
Pichia sp.	2.6								
P. membranaefaciens	1.9	4.05	0.37	0.7	0.01	0.25			
Schizo. pombe	0.26								
T'spora delbrueckii	1.0	2.8	0.20	0.87	0.07	0.11–0.83			
Zygosacch. bailii	1.2–2.6	2.7	0.26	1.61	0.13	0.03-0.31	0.52-1.2	0.24-0.8	0.27

Table adapted from Heard, (1988). Data from Cantarelli (1957), Benda (1970), Goto *et al.* (1978), Shimazu and Watanabe (1981), Caridi *et al.* (1991), Zironi *et al.* (1993).

complete malic acid degradation and have been used for must deacidification (Kuczynski and Radler 1982, van Rooyen and Tracey 1987, Romano and Suzzi 1993b, Yokotsuka *et al.* 1993).

1.5.5. Carbonyl compounds

A large number of aldehydes and ketones have been identified in wines, although most occur in trace amounts (Nykänen 1986, Etiévant 1991). Quantitatively, the major carbonyl compound produced by yeast is acetaldehyde, which is characterised by bruised apple and nutty aromas at low concentration, and a sour, green, fruity odour at higher concentration (Nykänen 1986). From the literature it is apparent that the concentration of acetaldehyde produced in wine varies with yeast species and strain, the stage of fermentation, and conditions that affect yeast activity. *S. cerevisiae* is a significant producer of acetaldehyde (3.4–180 mg/L), as is *Kl. apiculata* (6.1–39 mg/L), *Schizo. pombe* (5.2–158.3 mg/L), and *S'codes ludwigii* (5.2–216.7 mg/L) (Bertrand 1968, Sponholz and Dittrich 1974). Some yeasts are able to produce up to 1000 mg acetaldehyde/L when growing oxidatively as a film on wine, adding to the distinctive character of flor sherry (see Margalith 1981).

The ketone diacetyl is also significant to wine aroma, and is characterised by a sour and buttery odour and flavour, and at high concentration, a sour milk-like odour. The production of diacetyl in wine is mostly due to the activity of lactic acid bacteria during malolactic fermentation, although yeasts can produce detectable amounts of diacetyl (see Margalith 1981).

1.5.6. Glycerol

Glycerol is a major byproduct of yeast metabolism during fermentation. It imparts sweetness to wine at a flavour threshold of 3.8–4.4 g/L, and at a concentration of greater than 25 g/L, increased viscosity is detectable (Noble and Bursick 1984). These sensory effects contribute to the character of botrytised wines, due to the high production of glycerol by the grape pathogen *Botrytis cinerea*. Yeasts produce different amounts of glycerol (Bertrand 1968, Sponholz and Dittrich 1974, Soufleros and Bertrand 1979), and other factors such as grape maturity, fermentation temperature and sulfur dioxide addition also affect its production (Rankine and Bridson 1971, Ough *et al.* 1972). In a study by Sponholz and Dittrich (1974) some strains of *S. cerevisiae* produced between 3.6–8.7 g glycerol/L, whilst *Schizo. pombe* produced 10 g/L, and *Kl. apiculata* up to 11.1 g/L. Due to the sensory and biochemical significance of this compound and the observed effect of yeast species upon its production, further study is warranted.

1.5.7. Nitrogenous compounds

Nitrogen compounds are essential to the growth and metabolism of yeasts, and the nitrogen containing compounds in grape juice are mainly ammonium ions, amino acids, peptides and proteins (Henschke and Jiranek 1993). The nitrogen content of grape juice can be limiting to

yeast growth, and accordingly supplementation to correct deficiencies, or as a precautionary measure, is practised in many countries.

In addition to nitrogen uptake by yeast, the excretion of cellular nitrogen into wine can occur when yeast cells are no longer actively fermenting, and later by autolysis during storage on lees (Joslyn 1955). Products released include proteins, amino acids, nucleotides and fatty acids which can have a sensory impact or be utilised for growth by spoilage or malolactic bacteria (Wibowo *et al.* 1985, Fleet 1990). The release of autolysates is of particular importance to the character of sparkling wine and enhances its aroma and flavour (Feuillat and Charpentier 1982, Charpentier and Feuillat 1993). The contribution of non-*Saccharomyces* yeast species to the autolysis character of wine has not been considered important, even though the early autolysis of the short-lived yeasts presumably supplements the nitrogen pool available later in fermentation (Henschke and Jiranek 1993). Some non-*Saccharomyces* yeasts can also persist well into fermentation and therefore possibly contribute to the autolytic character of the wine (Fleet and Heard 1993).

1.5.8. Extracellular enzymes

The production of extracellular enzymes of oenological importance by the non-*Saccharomyces* yeasts has received some attention. Proteolytic enzymes that break down grape juice proteins increase the reservoir of nitrogen available for yeast growth. There is evidence that some *S. cerevisiae* strains (Feuillat 1984, Sturley and Young 1988, Rosi *et al.* 1987), and some non-*Saccharomyces* yeasts (Fleet 1990, Lagace and Bisson 1990), exhibit proteolytic activity. Proteolytic activity, especially of the non-*Saccharomyces* yeasts, may also play a role in the reduction of wine haze (Lagace and Bisson 1990). Pectinolytic enzymes are often added to degrade cell wall pectins to release more juice and while some strains of *S. cerevisiae* produce polygalacturonases (Fleet 1992), the non-*Saccharomyces* yeasts remain uninvestigated.

Glycosidase enzymes such as ß-glucosidase hydrolyse terpenol flavour precursors in grape juice, liberating wine aroma compounds (Strauss *et al.* 1986). These enzymes occur in grapes and other plants, fungi and yeasts, but are produced by only a few *S. cerevisiae* strains (Kreger-van Rij 1984, Darriet *et al.* 1988). Glycosidase production has been noted however, by species of *Candida, Dekkera, Debaryomyces, Kloeckera* and *Pichia* (Villa *et al.* 1979, Großmann *et al.* 1987, Vasserot *et al.* 1989, Gunata *et al.* 1990, Rosi *et al.* 1994). Although the usefulness of yeasts for the release of bound monoterpenes has been questioned (Strauss *et al.* 1986), the production of glycosidases by the non-*Saccharomyces* yeasts is worthy of further investigation.

1.5.9. Volatile phenols

Volatile phenols are a large group of compounds with characteristic leathery, medicinal, horse-stable odours and flavours, which are appreciated in some wine styles. These

compounds appear in trace amounts in juice and at a greater concentration in wine, as formed via different chemical and metabolic pathways (Etiévant 1991). Some volatile phenols are produced during malolactic fermentation (Etiévant 1991), whilst *S. cerevisiae* and some *Dekkera* and *Brettanomyces* species can also form these compounds in appreciable quantities (Heresztyn 1986, Chatonnet *et al.* 1992). The production of volatile phenols by other non-*Saccharomyces* yeast species is of interest and warrants further investigation.

1.5.10. Sulfurous compounds

Sulfur containing compounds have a significant role in wine flavour because of their high aroma and flavour intensity and volatility (Schutte 1975). Yeasts are able to form volatile sulfurous compounds such as sulfite and hydrogen sulfide from grape derived or added sulfur present in the must. Sulfite is of oenological significance as it binds to acetaldehyde and other compounds, and can contribute to the production of a high concentration of sulfur dioxide in wine (Rankine and Pocock 1969). Some indigenous yeasts have been shown to produce excessive amounts of hydrogen sulfide (Dittrich 1978), the sulfurous, rotten egg odour and flavour of which is detectable at extremely low concentration. Formation of this compound varies with yeast strain and is related to yeast sulfur and nitrogen metabolism (Henschke and Jiranek 1993). Insufficient information is available however, on the production of hydrogen sulfide and other volatile sulfurous compounds by the non-*Saccharomyces* yeasts.

1.6. Wine spoilage by yeasts

Wine spoilage may occur at different stages of the winemaking process, with resulting effects upon the chemical and sensory properties of the wine. Grapes can be infected with undesirable yeasts and bacteria, and fermenting must can support the growth of other undesirable yeast species or strains (Fleet *et al.* 1984, Fleet, 1990). Microbiologically unstable wines can also spoil during storage due to the growth of yeasts and bacteria (Fleet 1992, Sponholz 1993). Overproduction of desirable compounds, such as esters and acids, can make the wine unpalatable and therefore cause spoilage. Some non-*Saccharomyces* species such as *Hanseniaspora uvarum*, *Metschnikowia pulcherrima* and *Pichia anomala* can produce a high concentration of ethyl acetate and acetic acid, and can therefore spoil wine (Tables 1.5 and 1.6) (Sponholz and Dittrich 1974). *Zygosaccharomyces bailii* is a species particularly responsible for the spoilage of grape juice concentrates and wines by the overproduction of esters and polyols, and the reduction of acidity (Goto *et al.* 1978, Sponholz 1993). The properties of this yeast, such as osmotolerance, ethanol tolerance and resistance to preservatives such as sulfur dioxide and sorbic acid, make it a species well adapted to juice and wine spoilage (Sponholz 1993). Strain differences are noted however, with some species and strains of *Zygosaccharomyces* considered to be of potential use for wine production (Romano and Suzzi 1992, 1993b).

A specific spoilage of wine known as mousy taint is characterised by a "mouse-cage" odour and flavour. Several compounds responsible for this taint are produced by strains of lactic acid bacteria and *Brettanomyces/Dekkera* yeast species (Heresztyn 1986), but other yeast species have not been implicated in its production.

1.7. Aims of this study

The work reported in this thesis broadly aims to further the knowledge of the classification, growth, chemical and sensory effects of some non-*Saccharomyces* yeasts associated with winemaking. An emphasis was placed upon the use of mixed culture inoculation to modulate the growth, and hence metabolic activity of these yeasts in grape juice fermentation, in order to effect desirable changes to the chemical and sensory composition of the wine.

Specific aims of these investigations were:

- to differentiate and classify unknown yeasts isolated from grape juice. A polymerase chain reaction (PCR) method for the differentiation of strains was developed as described in Chapter 2;

- to assess the winemaking potential of some non-*Saccharomyces* yeasts by aroma assessment and chemical analysis of wines produced by pure culture fermentation of grape juice. The analysis of Chardonnay wine produced by ten different strains of four species is described in Chapter 3;

- to investigate the effect of mixed culture fermentation upon yeast growth and fermentation kinetics. The effect of inoculation procedure — that is either coinoculation of two strains at different ratios, or sequential inoculation of two strains at different intervals — on yeast growth, sugar utilisation and wine composition was investigated. The effect of different inoculation protocols using selected strains was determined in a synthetic grape juice-like medium, as described in Chapter 4; and;

- to determine the effect of selected yeasts and mixed culture inoculation protocols upon wine composition and aroma. The effect of inoculation with both *Candida stellata* and *Saccharomyces cerevisiae* on the composition and aroma of Chardonnay wine is described in Chapter 5.

Chapter 2

THE USE OF THE PCR FOR YEAST STRAIN DIFFERENTIATION AND SPECIES IDENTIFICATION

2.1. Introduction

The taxonomy of yeast is in a constant state of flux as new species are discovered and as taxonomic principles and methods are revised. Although morphological and biochemical phenotypes are the main criteria for the identification of yeasts (Kreger-van Rij 1984, Barnett, *et al.* 1990), such characters may be determined by only a small fraction of the genome (Winge and Roberts 1949). For example confusing changes have occurred in the taxonomy of the yeast *Saccharomyces cerevisiae*, which had previously been divided into a number of distinct species on the basis of variation in the fermentation and assimilation of different carbon sources (Barnett *et al.* 1990). It is now clear that many of the characters are unstable and due to a single mutation (Scheda and Yarrow 1966).

The phenotypic characters used in yeast systematics have more recently been combined with molecular based criteria. Determination of DNA relatedness by reassociation studies has been of fundamental importance in yeast systematics. By this technique several species of the genus *Saccharomyces*, that could not be differentiated from *S. cerevisiae* on the basis of phenotypic characters have been reclassified (Vaughan Martini and Kurtzmann 1985, Vaughan Martini 1989). More recently, phylogenetic relationships amongst yeasts have been studied by comparing the sequences of the ribosomal RNA genes. The principal advantage of this method is that, since ribosomes share a common evolutionary origin in all organisms, it permits the comparison of both closely and distantly related species (Kurtzman 1992). The latest taxonomic revision of the yeasts and yeast-like fungi (Kurtzman and Fell 1998), describes 800 species — more than in previous treatises — based upon both physiological tests and molecular-based techniques.

Grape must hosts numerous yeast species and strains, especially during the early stages of fermentation. Species identification, and as importantly, the recognition and differentiation of strains are of importance to commercial winemaking and research. Classical techniques are too time consuming to provide species information during the course of a normal fermentation, and offer very limited usefulness for strain differentiation.

The Polymerase Chain Reaction (PCR), is a technique for the *in vitro* synthesis of multiple copies of DNA sequences by primer extension of complementary strands of DNA (Saiki *et al.* 1985). The application of PCR to the discrimination of wine yeast strains (*S. cerevisiae*), by the amplification of random or targeted DNA sequences has been reported (Querol *et al.* 1992a, b). For this work on the discrimination of non-*Saccharomyces* yeasts, a PCR primer with a sequence complementary to yeast intron splice sites was designed. One method of

generating polymorphisms with PCR involves targeting highly conserved sequences which are known to flank variable spacer regions such as consensus sequences for intron splice sites (Hawkins 1988). Introns are present within fungal genes (Scazzochio 1989), and are variable in length and sequence, and as intron splice site sequences appear to be conserved in all fungi, complementary primers should be useful for all yeasts (Jacob and Gallinaro 1989). The design of an intron splice site primer, and the use of a simple method for template DNA preparation allowed rapid differentiation of strains of different non-*Saccharomyces* species associated with winemaking. It was evident that the overall amplification product banding pattern was conserved and recognisable for different strains of a single species, often permitting both strain differentiation and presumptive species identification of uncharacterised isolates in a single reaction.

This work was done in collaboration with Dr Miguel de Barros Lopes of the Australian Wine Research Institute and the Department of Plant Science, the University of Adelaide, and Ms Anna Martens of the Department of Horticulture, Viticulture and Oenology, the University of Adelaide, and Petaluma Ltd, Piccadilly, South Australia.

2.2. Materials and methods

2.2.1. Yeast isolates and media

The yeast strains used in this study are listed in Tables 2.1 and 2.2. Reference strains (Table 2.1), are species type strains obtained from the Centraalbureau voor Schimmelcultures (CBS) culture collection in Delft, The Netherlands. Winery isolates (Table 2.2), were obtained from: The Australian Wine Research Institute (AWRI), culture collection, grape must and winery equipment at Petaluma Ltd, Piccadilly, South Australia (isolated by Anna Martens), and a fermentation at Sonoma-Cutrer Winery, Windsor, California, USA (isolated by Jeff Cohn and staff of Vinquiry, Healdsburg, California, USA).

For identification by physiological techniques, the isolates were sent to the CBS. Yeasts were isolated aseptically from juice and must by sampling and dilution in 0.1% peptone (Oxoid, UK), before plating onto glucose-yeast extract-peptone agar (GYEPA, yeast extract 1% w/v, peptone 0.5% w/v, glucose 4% w/v, agar 2% w/v), and lysine agar (Oxoid, UK). Yeasts were isolated from the surfaces of winery equipment by swabbing with sterile buffered swabs (Disposable Products, Australia). Swabs were then rolled onto the surface of GYEPA plates. Resulting colonies were streaked out onto MYPG (Amyl Media, Australia) plates for the isolation of axenic colonies of different morphology for identification.

Table 2.1. Yeast strains of known identity used in this study

Species Synonym	CBS strain number
Brettanomyces custersianus van der Walt	4805[T]
B. naardenensis Kolfschoten & Yarrow	6042[T]
B. nana Smith *et al.* (formerly *Eeniella*)	1945
Candida stellata (Kroemer & Krumbholz) Meyer & Yarrow	157[T]
C. stellata	843
C. stellata	1713
C. stellata	2649
Dekkera anomala Smith & van Grinsven	8139[T]
B. anomalus Custers	77
B. claussenii Custers	76
D. bruxellensis van der Walt	74[T]
Brettanomyces abstinens Yarrow & Ahearn	6055
B. bruxellensis Kufferath & van Laer	72
B. custersii Florenzano	5512
B. intermedius Krumbholz & Tauschanoff	73
B. lambicus Custers	75
D. intermedia van der Walt	4914
Hanseniaspora guilliermondii Pijper	465[T]
H'spora uvarum (Niehaus) Shehata *et al.*	314[T]
Issatchenkia orientalis Kudryavtsev	5147[T]
Metschnikowia pulcherrima Pitt & Miller	5833[T]
Pichia fermentans Lodder	187[T]
P. membranaefaciens Hansen	107[T]
Saccharomyces bayanus Saccardo	380[T]
S. uvarum Beijerinck	395
S. cerevisiae Meyen *ex* Hansen	1171[NT]
Candida robusta Diddens & Lodder	1907
S. exiguus Reess	379[T]
S. kluyveri Phaff *et. al*	3082[T]
S. paradoxus Bachinskaya	432[NT]
S. pastorianus Reess *ex* Hansen	1538[NT]
S. unisporus Jörgensen	398[T]
Torulaspora delbrueckii Lindner	1146[T]

[T]denotes type strain
[NT]denotes neotype strain

Table 2.2. Winery isolates studied

Species	AWRI strain number	Source[b]
Hanseniaspora guilliermondii [c]	1277	CA
H'spora uvarum [c]	868	NSW
H'spora uvarum	1274	CA
H'spora uvarum	1275	CA
H'spora uvarum	1276	CA
Issatchenkia orientalis [c]	873	NSW
Metschnikowia pulcherrima	1267[a]	SA
M. pulcherrima	1268[a]	SA
M. pulcherrima	1269[a]	SA
M. pulcherrima [c]	1270[a]	SA
Pichia fermentans [c]	1271	SA
P. membranaefaciens [c]	1272	SA
Saccharomyces bayanus	948	SA
S. bayanus [c]	1266	SA
Saccharomyces cerevisiae [c]	870	NSW
S. cerevisiae	871	NSW
S. cerevisiae	1265[a]	SA
Torulaspora delbrueckii [c]	872	NSW

[a] All strains were isolated from grape juice or fermenting must except for the M. pulcherrima isolate and one S. cerevisiae isolate from winery equipment.

[b] SA strains isolated in South Australia and CA strains isolated in California, USA. NSW strains isolated in New South Wales, Australia [Heard, 1985 #14; Petering, 1990 #13].

[c] Isolates independently verified by CBS using standard physiological techniques.

2.2.2. Preparation of DNA template for PCR by freeze-boil method

A rapid cell preparation method for the PCR was an objective of this assay and a freeze-boil method was found to be suitable. Yeast was grown up on MYPG plates for two to three days at 25°C. The plates were then incubated at 4°C for at least 24 hours or until use. It was noted that leaving the plates at 4°C increased the reliability of the PCR, possibly because the cells enter stationary phase, so that the DNA structure is no longer dependent upon the cell cycle. A sample of the colony was then resuspended in 200 µL sterile Milli-Q® water in a microcentrifuge tube. The tube was frozen in liquid nitrogen for 3 min, and then immediately boiled for 10 min in a water bath. The tube was then frozen at -20°C overnight or until ready for use. It was noted that leaving the plates at 4°C A cell suspension of 2 µL (containing approximately 10^4–10^5 cells), was used for each amplification reaction.

2.2.3. Yeast DNA purification

Yeast DNA was isolated by standard procedures [Ausubel, 1994   ]. A 10 mL cell suspension from an overnight YEPD culture was resuspended in 200 µL of breaking buffer (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris (pH 8), 1 mM

EDTA (pH 8). The yeast cells were homogenised by vortexing with 0.3 g glass beads and 200 μL of phenol-chloroform-isoamyl alcohol for 3 min. Tris-EDTA buffer (200 μL) was added and the aqueous layer collected after centrifugation. The DNA was precipitated with ethanol. The DNA concentration was determined by measurement of the A_{260} after incubation with RNase and the amount of DNA used in each reaction was 0.5 μg.

2.2.4. Intron splice site primers and PCR conditions

The primer and PCR conditions used throughout this study were designed by Miguel de Barros Lopes. The primer EI1, of sequence CTGGCTTGGTGTATG (de Barros Lopes *et al.* 1996), was used in all of the PCR experiments. The PCR was performed in a 50 μL volume of buffer (Advanced Biotech, USA) with 50 pmol of primer, 2 μL of DNA template, 32 μM of each deoxynucleoside triphosphate, 2.5 mM MgCl and 0.2 units of *Taq* polymerase (Advanced Biotech, USA). The reactions were run on a PTC 100 Programmable Thermal Controller (MJ Research, USA) for 33 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 2 min, and extension at 74°C for 1.5 min. An initial denaturation at 94°C for 3 min and a final extension at 74°C for 5 min were included. Amplified products were resolved on a 2% agarose gel (Agarose NA, Pharmacia, Sweden), stained with ethidium bromide and visualised under UV light. Gels were photographed with the Gel Cam Documentation System (Sony, Japan) and the photographs were scanned to produce a computer image.

2.3. Results

2.3.1. Design and use of intron splice site primers

Introns are not known to be essential and would appear to have evolved with minimal constraint. There are conserved sequence motifs within all introns that are necessary for their removal during the synthesis of mRNA. In the yeast *S. cerevisiae*, the sequence GTATGT almost exclusively defines the splice site of the 5' intron junction cleavage sequence (Woolford 1989). To detect polymorphisms in yeast strains by the PCR a primer complementary to the 5' splice site was designed. The primer was extended at the 5' end by a random sequence to produce a 16-mer oligonucleotide. This sequence was tested for possible secondary structure using the Oligo4 program (National Biosciences, USA). This primer was used singly to obtain amplification between two different intron splice site sequences, although priming of other regions with complementary DNA could have occurred.

During the development of a rapid method for template DNA preparation different boiling and freezing treatments were compared to DNA purification (de Barros Lopes *et al.* 1996). The visualisation of PCR amplification products suggested that simply freezing a yeast colony suspension in liquid nitrogen, followed by boiling for 10 min, produced an amplification pattern that was similar in composition to that of the purified DNA (de Barros Lopes *et al.* 1996). This freeze-boil method was used for all yeast, with the exception of the *Dekkera* and *Brettanomyces* isolates which amplified poorly unless purified template DNA was used in the reaction, although the reason for this was not determined. It was found that

more consistent amplification patterns with a greater number of bands were obtained if yeast cells were transferred to 4°C for at least 24 h prior to the freeze-boil step. A possible explanation for this is that arresting, or retarding the growth of the yeast reduced any nucleic acid-protein interaction, as occurs in growing cells, which may be inhibitory to DNA synthesis.

2.3.2. Differentiation of unknown yeast isolates

In a previous study it was demonstrated that commercial strains of *S. cerevisiae* could be differentiated using the PCR with primers that target intron splice site sequences (de Barros Lopes *et al.* 1996). Figure 2.1 shows that the primer EI1, which is complementary to the yeast intron 5' splice site, is also effective in differentiating yeast isolates from grape juice and winery equipment. Amplification fingerprints of several of the isolates are readily distinguishable although recognizable patterns are observed. Lanes 1–3, 4–5, 8–11 and 13–16 all have amplified fragments in common, but in most cases all of the strains are differentiated by at least one polymorphism. The exceptions are the isolates AWRI 1266 and AWRI 948 in lanes 4 and 5, and AWRI 1267 and AWRI 1268 in lanes 13 and 14 respectively.

2.3.3.   Species possess characteristic amplification fingerprints

Figure 2.1 shows that several yeast isolates produced related amplification fingerprints. In a previous study it was also observed that although polymorphisms exist between *S. cerevisiae* strains, the strains also shared a number of common amplified fragments sequences (de Barros Lopes *et al.* 1996). This finding suggested that individual species produce a characteristic fingerprint. To test this further, a number of type strains for different species obtained from the CBS culture collection were analysed using the PCR method. The results from two genera are shown in Figure 2.2.

Figure 2.2a shows the PCR results for several species of *Saccharomyces*. The type strain for *S. cerevisiae*, CBS 1171[NT], and its asexual anamorph, CBS 1907 (previously *Candida robusta*), are compared to other species of the genus. The two *S. cerevisiae* strains have similar amplification fingerprints that are different from those of distantly related yeasts in the genus, and from closely related species of the *Saccharomyces sensu stricto* group. The PCR fingerprints of two *S. bayanus* strains, the type strain, CBS 380[T], and another strain, CBS 395—formerly the type strain of *S. uvarum*—are compared. The patterns are less conserved than those obtained for *S. cerevisiae*, possibly indicating that *S. bayanus* is less clearly defined. The shared amplification fragments, for example at 860, 550 and 490 bp, still demonstrate a kinship betwen the strains. The *S. bayanus* PCR fingerprint is obviously different to that of the closely related species *S. pastorianus*, CBS 1538[NT], and shows no similarity to the other strains.

Amplification fragment banding patterns for some *Dekkera* and *Brettanomyces* strains are shown in Figure 2.2b. These include the former type strains of a number of species now
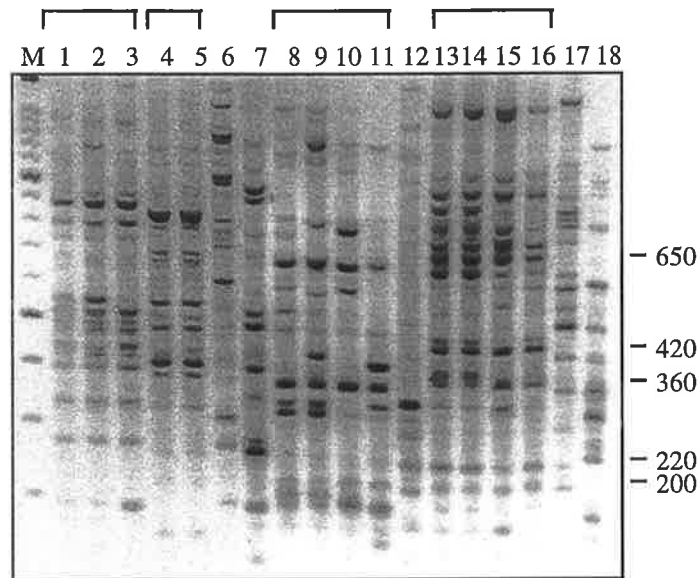
Figure 2.1. Differentiation of indigenous wine yeasts using the PCR with intron primer EI1.

Lanes: 1, AWRI 870; 2, AWRI 871; 3, AWRI 1265; 4, AWRI 1266; 5, AWRI 948; 6, AWRI 872; 7, AWRI 873; 8, AWRI 868; 9, AWRI 1274; 10, AWRI 1275; 11, AWRI 1276; 12, AWRI 1277; 13, AWRI 1270; 14, AWRI 1267; 15, AWRI 1268; 16, AWRI 1269; 17, AWRI 1271; 18, AWRI 1272. The isolates are arranged into groups with similar amplification fingerprints. This is indicated above the lane numbers. The DNA fragments, 650, 420, 360, 220 and 200 bp, discussed in the text in reference to lanes 13–16 are marked.
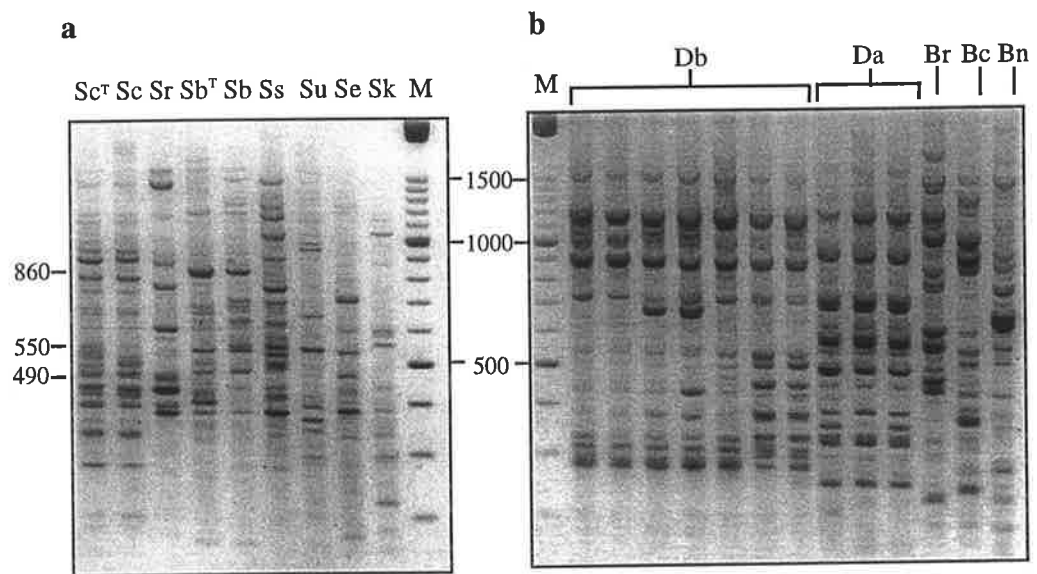
Figure 2.2. Intron primer PCR fingerprints of *Saccharomyces* and *Dekkera/Brettanomyces* yeasts.

a. Genus *Saccharomyces*. Lanes: 1, CBS 1171NT; 2, CBS 1907; 3, CBS 432NT; 4, CBS 380T; 5, CBS 395; 6, CBS 1538NT; 7, CBS 398T; 8, CBS 379T; 9, CBS 3082T. Sc - *S. cerevisiae*, Sr - *S. paradoxus*, Sb - *S. bayanus*, Ss - *S. pastorianus*, Su - *S. unisporus*, Se - *S. exiguus*, Sk - *S. kluyveri*. The 860, 550 and 490 bp DNA fragments discussed in the text in reference to *S. bayanus* are marked.

b. Genus *Dekkera/Brettanomyces*.. Lanes: 1, CBS 74T; 2, CBS 72; 3, CBS 4914; 4, CBS 73; 5, CBS 75; 6, CBS 5512; 7, CBS 6055; 8, CBS 8139T; 9, CBS 76; 10, CBS 77; 11, CBS 6042T; 12, CBS 4805T; 13, CBS 1945T. Db - *D. bruxellensis*, Da - *D. anomala*, Br - *B. naardenensis*, Bc - *B. custersianus*, Bn - *B. nana*.

condensed into two species of *Dekkera* and three species of *Brettanomyces* (Table 2.1). The *Dekkera bruxellensis* strains (lanes 1–7), all have related amplification patterns. The *D. anomala* strains (lanes 8–10), also share similar amplification patterns to each other but are unrelated to the other yeasts in the genus. Each of the fingerprints of *B. naardenensis* CBS 6042^T, *B. custersianus* CBS 4805^T and *B. nana* CBS 1945^T (formerly *Eeniella nana*), are unique (lanes 11, 12 and 13) (Boekhout *et al.* 1994). The results shown in Figure 2.2 indicate that, for *Dekkera/Brettanomyces* and *Saccharomyces* at least, the PCR fingerprints are not only shared by strains of a species, but are also unique to that particular species. Further PCR analysis of further strains of different species will be needed to confirm this observation.

2.3.4. Species identification of indigenous wine yeasts.

By comparing the amplification fingerprints of unknown yeast isolates to those produced by type strains of species associated with winemaking, an effective identification system has been developed (Figure 2.3). Several of the isolates from grape juice have been shown to be strains of *S. cerevisiae*. Lanes 1 and 2 of Figure 2.3 show the PCR fingerprint pattern similarity of the *S. cerevisiae* type strain, CBS 1171^{NT}, and an uncharacterised isolate, AWRI 870. The similarity indicates that AWRI 870, as well as AWRI 871 and AWRI 1265 (Figure 2.1, lanes 1–3), are strains of *S. cerevisiae*. Strain AWRI 1265 produced an identical PCR fingerprint to the commercial strain used in this winery, suggesting that it is more likely to be this inoculated strain than an indigenous yeast (results not shown). Strains AWRI 1266, AWRI 948 and several other yeasts isolated from grape juice were also initially identified as *S. cerevisiae* via physiological tests. Lanes 3 and 4 of Figure 2.3 show that the PCR fingerprint of strain AWRI 1266 is similar to that of the type strain of the related species *S. bayanus*.

The majority of the grape juice isolates from both California and Australia were identified as *Hanseniaspora uvarum* (Figure 2.1, lanes 9 and 10), with more than 11 polymorphic patterns observed using the single primer EI1, four of which are shown in lanes 8–11. The PCR fingerprints obtained for this species are heterogeneous, but fragments of 650, 420, 360, 220 and 200 bp in size are shared, making species recognition possible. Several of the isolates from winery equipment (Figure 2.1, lanes 13–16), produced amplification patterns similar to the type strain of *Metschnikowia pulcherrima* (Figure 2.3, lanes 13 and 14). By visual comparison, strains of the species*Torulaspora delbrueckii* (Figure 2.1, lane 6 and Fig 2.3, lanes 5 and 6), *Issatchenkia orientalis* (Figure 2.1, lane 7 and Figure 2.3, lanes 7 and 8), and *Hanseniaspora guilliermondii* (Figure 2.1, lane 12 and Figure 2.3, lanes 11 and 12), were also putatively identified. The identity of each of the yeasts in Figure 2.3 — with the exception of *S. bayanus* which cannot be distinguished from *S. cerevisiae* by physiological tests — was subsequently verified by classical tests at the CBS yeast identification service.

2.3.5. Heterogeneity within the species *Candida stellata*.

As *Candida stellata* was of interest for further study, four strains were obtained from
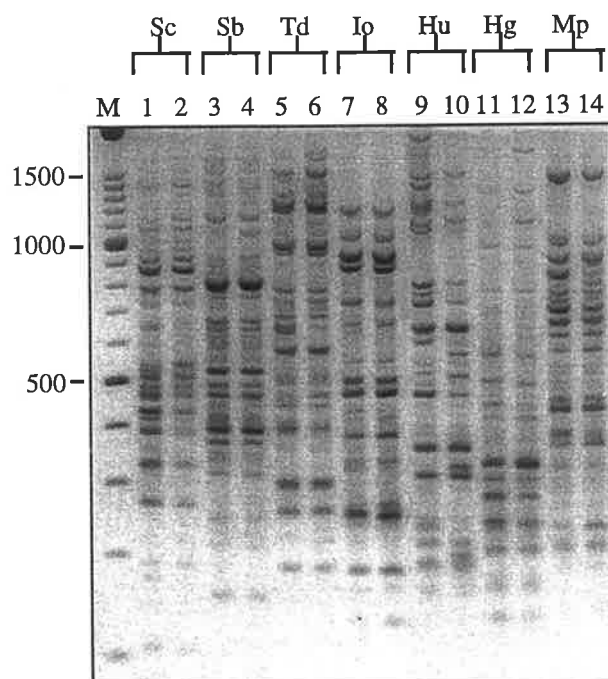
Figure 2.3. Species identification of indigenous wine yeast using the PCR with primer EI1.

Lanes: 1, CBS 1171^{NT}; 2, AWRI 870; 3, CBS 380^T; 4, AWRI 1266; 5, CBS 1146^T; 6, AWRI 872; 7, CBS 5147^T; 8, AWRI 873; 9, CBS 314^T; 10, AWRI 868; 11, CBS 465^T; 12, AWRI 1277; 13, CBS 5833T; 14, AWRI 1267. Sc - *S. cerevisiae*, Sb - *S. bayanus*, Td - *T. delbrueckii*, Io - *I. orientalis*, Hu - *H'spora uvarum*, Hg - *H'spora guilliermondii*, Mp - *M. pulcherrima*.

CBS culture collection and analysed using the PCR (Figure 2.4). The amplification fragment pattern of the type strain CBS 157^T was found to be dissimilar to those of the other strains. The type strain produced a pattern of low intensity bands, but did not exhibit the major bands common to CBS 1713 and CBS 2649. These two strains had four major bands in common and were easily recognised as distinct yet related strains. Strain CBS 843 also amplified weakly but had at least two fragments in common with CBS 1713 and CBS 2649, also with an intense band in common with the type strain.

2.4. Discussion

Many precursor mRNAs in eukaryotes contain intervening sequences, or introns, that are precisely excised by the spliceosome protein during the formation of mature mRNA (Woolford 1989). The reason for the existence and distribution of introns is still debated and their function remains unclear (Fink 1987, Mattick 1994). Many introns are known to be close to selectively neutral, and except for the spliceosome recognition sites, the sequences can be highly variable (Nellen *et al.* 1981, Tarlow *et al.* 1993, Palumbi and Baker 1996). Intron analysis is therefore of use in studies of genome relatedness. Although introns are highly mutable, the splice site sequences are conserved in all yeasts that have been studied to date. The 5' consensus sequence targeted by the primer used in this study is commonly observed in yeast and higher fungi (Johnston and Mortimer 1986).

The PCR, using primers that anneal to plant intron splice site sequences, has been used to map and identify genetic polymorphisms in cereals (Weining and Langridge 1991). In our laboratory, intron primers have been used to detect polymorphisms in commercial winemaking strains of *S. cerevisiae*, and sequencing of the amplified fragments confirmed that splice site sequences were successfully targeted (de Barros Lopes *et al.* 1996). Other specific primers that target the delta elements of transposons have also been used successfully for *S. cerevisiae* strain differentiation (Lavallée *et al.* 1994). These primers may be of little use however for the differentiation of species other than *S. cerevisiae*, which are unlikely to contain these transposable elements (Hawthorne and Philippsen 1994, Pearson *et al.* 1995), whereas the intron 5' splice site primer EI1 was found to be effective for the differentiation of strains of different yeast species associated with winemaking.

Analysis of commercial yeasts showed that *S. cerevisiae* strains generate related PCR fingerprints (de Barros Lopes *et al.* 1996), questioning whether other yeast species produce characteristic amplification patterns. This is indeed supported by the analysis of type strains as shown in Figure 2.2, demonstrating that, at least for the species studied, the amplification pattern is unique. By comparing the PCR fingerprints of unknown isolates to those produced by type strains, it has been possible to identify strains of the species *Hanseniaspora uvarum, H'spora guilliermondii, Issatchenkia orientalis, Metschnikowia pulcherrima, Saccharomyces cerevisiae, S. bayanus* and *Torulaspora delbrueckii* (Figure 2.2).
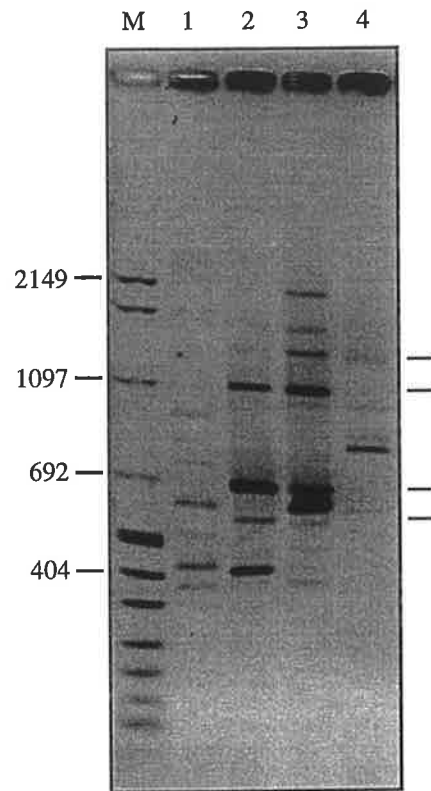
Figure 2.4. Differentiation of *Candida stellata* strains using the PCR.

Lanes: 1, CBS 157ᵀ; 2, CBS 1713; 3, CBS 2649; 4, CBS 843.

Differentiation of the *Saccharomyces* species was also of interest. Phenotypic characters led to the identification of AWRI 1266 and AWRI 948 as *Saccharomyces cerevisiae* or *bayanus*, but the PCR patterns indicated that these isolates were in fact *S. bayanus*. This was an important finding since traditional taxonomic methods do not allow the differentiation of all *Saccharomyces sensu stricto* species, although different molecular methods have been described recently that allow their discrimination (Naumov *et al.* 1992, Cardinali and Martini 1994, Guillamón *et al.* 1994, Lewicka *et al.* 1995). The different amplification fingerprints of the *Saccharomyces* species shown in Figure 2.2 show the discriminatory power of the PCR method. The *Saccharomyces sensu stricto* yeasts were previously considered to be a single species, *S. cerevisiae* (Kreger-van Rij 1984, Barnett *et al.* 1990), but investigation of genome relatedness by DNA reassociation and hybridisation analyses have led to the separation of these yeasts into four distinct species. The sequence similarity between *S. bayanus* and *S. pastorianus* is 72% using reassociation analysis (Vaughan Martini and Kurtzman 1985), which is the upper limit of DNA homology seen for two different species. The PCR amplification fingerprints of the two species are clearly differentiable so it is unlikely that other species will have sufficient homology to produce highly related or undifferentiable amplification patterns.

As for all molecular techniques, the PCR method does not provide a means of differentiating the teleomorph and anamorph states of a species. This is shown for *S. cerevisiae* and its asexual synonym *C. robusta,* and the *Dekkera/Brettanomyces* yeasts in Figure 2.2. Therefore, although the isolates are named as the teleomorphic species as is convention, nothing is known of their sexual reproduction. The results obtained with the *Dekkera/Brettanomyces* yeasts agree with recent systematic changes of the genera. Molecular analysis of these yeasts (Hoeben and Clark-Walker 1986, Boekhout *et al.* 1994), has led to the grouping of the original twelve species into four: *D. anomala, D. bruxellensis, B. custersianus* and *B. naardenensis.* The PCR amplification fingerprints also led to the grouping of these yeasts into the four species (Fig 2b). Mitochondrial DNA analysis (Hoeben et al. 1993), and 26S ribosomal sequence analysis (Boekhout *et al.* 1994), suggest that *Eeniella nana* is derived from within the genus *Brettanomyces.* The PCR amplification fingerprint of this yeast is also included for comparison (Figure 2.2b, lane labelled Bn). Comparison of polymorphisms generated by this method would therefore not appear useful for determination of relatedness between species.

Of the yeasts collected from the two grape musts, the PCR technique was unable to identify two isolates (not shown). Using the phenetic system of classification, these two yeasts were identified as *P. fermentans* and *P. membranaefaciens.* Comparison of the PCR fingerprints with the two type strains for these species showed no kinship. As the *Pichia* genus is known to contain a diverse group of yeasts (Yamada *et al.* 1996), the genotypic dissimilarity between the type strains and the classically identifed isolates, as shown by the PCR results, may be indicative of the current heterogeneity of the genus and its species.

Discrepancies between molecular methods and physiological methods of classification were also observed with other yeasts. Some *Candida stellata* strains studied (Figure 2.4) produced similar PCR fingerprints to each other but dissimilar to that of the *C. stellata* type strain. Sequence comparison of the 26S ribosomal DNA showed the type strain sequence to be markedly different to that of the other three yeasts (de Barros Lopes, pers. comm.). Similarly, several strains isolated from equipment in the South Australian winery were phenetically identified as the ubiquitous yeast *Rhodotorula mucilaginosa*. The intron primer produced unrelated PCR fingerprints for these isolates, none of which resembled the fingerprint of the type strain of *R. mucilaginosa* (results not shown).

The presence of multiple strains of different yeast species in grape juice fermentation has been demonstrated by Schütz and Gafner (1993a, 1994a, b) and Gafner *et al.* (1996). For the yeast isolated from grape juice investigated in this study, amplification fragment variation, especially amongst the predominant *Hanseniaspora* isolates, was evident. As the PCR method and intron primer can achieve strain differentiation, the composition of grape juice fermentation at both the species and strain level can potentially be revealed by the same reaction, making it a useful tool for ecological studies.

The main advantages of the intron-based PCR method described here is that it is rapid, simple and relatively inexpensive. In all of the yeast isolates studied to date — with the exception of the *Dekkera bruxellensis* strains — suitable PCR fingerprints could be obtained without first isolating the DNA. The method suffers from the same disadvantage as DNA reassociation experiments, that is, that similarities can only be observed between closely related yeasts. The DNA reassociation method although definitive, is of little use for routine yeast identification as hybridisations must be performed repeatedly until the analogous type strain is found. Although the information obtained using PCR is clearly not as precise as sequencing, and it is not useful for determining phylogenetic relationships, an advantage of the use of this single primer, however, is that different loci across the genome are compared. This reduces the risk of erroneous results as can occur during the analysis of a single genetic locus (see Oosthuizen *et al.* 1987).

2.5 Conclusion

A method based on the PCR using an intron primer has been developed that permits both yeast species identification and strain differentiation. It is not envisaged that this method could replace existing techniques in yeast taxonomy, but it has value as a supplementary tool. The method can assist taxonomists in rapidly detecting unrelated yeasts in a heterogenous species, as was observed with *Candida stellata* (Figure 2.4), and *Pichia* isolates. In this study the method was applied to the indigenous yeast of grape must and wine, and it may be useful for other yeast ecological studies especially for the grouping of uncharacterised isolates by

amplification pattern commonality — and therefore potential conspecificity. By comparing the PCR amplification pattern of isolates to a data base of type strain fingerprints (where appropriate), it may be possible to quickly identify a completely uncharacterised yeast.

The power of such a system will be determined by the analysis of a large number of different strains within a species. How closely different strains match to the type strain will be dependent upon current genetic diversity within that species as determined by changes in classical taxonomy that affect species groupings and definitions (Kurtzman *et al.* 1980).

Chapter 3

THE EFFECT OF FERMENTATION WITH SELECTED NON-*SACCHAROMYCES* YEASTS UPON WINE COMPOSITION, AROMA AND PREFERENCE

3.1. Introduction

Despite opposing views as to whether the grape variety (Noble 1994) or the fermentation (Benda 1970), is the most important factor in determining wine flavour, a significant contribution to the aroma of wine is clearly due to the metabolic activity of yeasts (Amerine and Joslyn 1970, Margalith and Schwartz 1970, Benda 1982, Rapp and Versini 1991). Yeasts utilise grape juice constituents during fermentation, producing metabolites that contribute to the chemical composition and sensory attributes of the wine. The study of the ecology of traditional fermentation practices in Bordeaux, revealed probably for the first time, the extent of growth of yeasts, other than species of *Saccharomyces*, during fermentation (Fleet *et al.* 1984). As the growth of these yeasts was quantitatively significant, the species involved — principally *Hanseniaspora guilliermondii* and *Candida stellata* — were considered to be likely to have an effect upon wine composition..

At least 600 volatile compounds including alcohols, esters and acids determine wine aroma, and many studies have analysed and quantified the often high production of these by the non-*Saccharomyces* yeasts (Van Zyl *et al.* 1963, Soufleros and Bertrand 1979, Millán and Ortega 1988, Mateo *et al.* 1991, Romano *et al.* 1997). For example, Mateo *et al.* (1991) showed the importance of low fermentative power yeasts, especially the apiculate species, on the production of volatile compounds that impacted upon wine aroma. Total volatile production by several of the non-*Saccharomyces* isolates studied was greater than that of the *S. cerevisiae* strains.

Sensory analysis has a pivotal role in yeast evaluation as it permits characterisation of important wine properties that can not be elucidated by instrumental analysis. Studies of the sensory impact of the non-*Saccharomyces* yeasts have mostly compared 'spontaneously' fermented wines — where the yeast composition is largely unknown, and the relative contribution of individual strains cannot be determined — to those inoculated with *S. cerevisiae*. Such studies have not demonstrated a positive effect of uninoculated fermentation upon wine sensory character (Bisson and Kunkee 1991, Bisson quoted in Ross, 1997), although this practice is increasingly used in wine production in the US and Australia with favourable results (Goldfarb 1994, Ramey 1995, Price 1996, Ross 1997).

Sensory characterisation of individual non-*Saccharomyces* yeasts would appear to have received little attention since 1956 when Malan noted the flavour of wines fermented by pure cultures of *S. cerevisiae* and *H'spora uvarum.* Presumably the lack of interest derives from

the perceived limited application of these often weakly fermentative yeasts as pure cultures for winemaking; and because the potential for overproduction of acetic acid, sulfides and other volatile compounds by these yeasts can negatively impact on wine flavour.

This study aimed to compare the sensory properties and composition of wines made with selected strains, irrespective of their ability to complete fermentation. It is worth noting that yeasts present for even a short time during fermentation could impact on the fermentation and subsequently wine flavour. In addition to the production of secondary metabolites of importance to wine aroma and flavour, non-*Saccharomyces* yeasts active early in fermentation will deplete nutrients and alter the composition of the juice. The effect of this on the growth of yeasts active later in fermentation should also be considered (Fleet 1990).

Ten yeast strains, representing four species, were used to vinify a sterilised grape juice, in order to select strains with positive or novel fermentation properties and sensory impact. Fermentation was completed to varying extents by the different strains, and wines were appraised for aroma characteristics and overall preference by an expert panel. On these bases, acceptable yeasts were selected for mixed culture fermentation studies as described in Chapter 4. Although the aroma characteristics of juice partially fermented by a particular strain may be altered in mixed culture fermentation, it was necessary to assess yeasts as pure cultures for preliminary assessment of winemaking potential.

3.2. Materials and methods

3.2.1. Juice and winemaking

The 1996 Barossa Valley Chardonnay juice (S. Smith & Sons, Pty Ltd, Australia) used for fermentation was of the following composition: pH 3.39; free sulfur dioxide, 6 mg/L; total sulfur dioxide, 36 mg/L; sugar concentration, 220 g/L. The juice was supplemented with 200 mg/L Di-ammonium phosphate (Sigma, Australia).

The juice was filtered through a Seitz pad filter EF 30/30, fitted with clarification grade D4, and sterilisation grade Z8 Ekwip filter pads, before a final filtration through a 0.2 μm pore size Sartobran PH capsule (Sartorius, Germany). The filtration unit, pads and capsule were rinsed with citric acid solution, then with reverse osmosis purified water, and finally flushed with nitrogen gas before use. All juice manipulations were carried out under nitrogen gas pressure to minimise air contact.

A 1.5 L volume of juice was decanted into autoclaved (121°C, 15 min), 2 L glass bottles fitted with rubber stoppers modified to hold an airlock, gas inlet, filling and sampling tubes. The gas port was fitted with a 0.2 μm pore size membrane filter (Gelman Sciences, USA) and the sampling port was fitted with a sterile multidirectional stopcock (Braun, Germany) and Luer lock (Braun, Germany). Prior to filling, the fermentation vessels were flushed with nitrogen gas sterilised via a 0.2 μm pore size membrane (Schleicher & Schuell, Germany).

Fermentations were conducted at 18°C, on an orbital shaker operated at 100 opm, in order to prevent any yeast sedimentation which may have led to slow or incomplete fermentation. When gas evolution slowed, ferments were sampled daily by aseptic withdrawal of a 1 mL sample. Prior to this procedure, the vessels were gassed with sterile nitrogen to prevent the ingress of air. When the refractive index of the sample remained constant for three successive days, the fermentation was deemed finished, irrespective of the residual sugar concentration. Fermentations of lower residual sugar (< 2%) were also monitored for sugar depletion by the Clinitest assay (Ames, Miles Inc., USA). After 14 days, the rate of fermentation of six of the treatments was minimal (<0.1°Bx/d), therefore these wines were bottled.

After fermentation had ceased, the wines were stored at 4°C for cold stabilisation and yeast sedimentation. The airlocks were sealed, and sterile nitrogen gas was supplied at low pressure to prevent the ingress of air. After seven days the wines were filtered through a 0.22 μm pore size membrane (Gelman Sciences, USA). Filtration proceeded under nitrogen gas pressure, with wine being collected in a sterilised, 2 L glass bottle sealed with a rubber stopper fitted with a filling tube and a gas inlet and outlet, both fitted with a 0.2 μm pore size membrane (Schleicher & Schuell, Germany). After filtration the gas outlet was stoppered and the bottle stored under nitrogen gas pressure. The wines were further cold stabilised at 4°C for five to seven days, during which time sulfur dioxide, as a sterile solution of potassium metabisulfite (BDH, UK), in wine, was added incrementally to achieve a concentration of 8–15 mg free sulfur dioxide/L. Without further adjustment the wines were aseptically decanted into autoclaved, nitrogen flushed, 375 mL bottles and closed with crown seals. Bottles were flushed with sterilised nitrogen gas prior to filling. As most of the wines had a high residual sugar content, all filtration and bottling procedures were carried out aseptically to prevent post-bottling fermentation. Wines were stored at 4°C for four weeks prior to analysis.

3.2.2. Yeast strains and fermentation treatments

The yeast strains used in this experiment were received from: The Centraalbureau voor Schimmelcultures (CBS), The Netherlands; The Food Science and Technology Department, The University of New South Wales (UNSW); and Lallemand Inc. Australia; as indicated in Table 3.1. Yeasts were maintained on cryopreservant beads (Protect Bacterial Preservers, Technical Services Consultants, Ltd, UK), in the gaseous phase of liquid nitrogen.

This study originated as an evaluation of the winemaking potential of two species of *Candida*. Two strains curated as *C. stellata* (AWRI 860 and AWRI 872), were later identified as *T'spora delbrueckii* by PCR (method described in Chapter 2, but results not shown), and by classical techniques at the CBS yeast identification service.

Yeast treatments consisted of single, pure culture fermentations of the juice by each of the strains listed in Table 3.1. As a suitable reference wine for some of the partial ferments, an

EC1118 wine with a high residual sugar content was made by arresting fermentation at 12°Bx. This was achieved by chilling the wine at 4°C for 48 h followed by sterile filtration.

Table 3.1 Yeast strains and fermentation treatment codes

Treatment code	Yeast species	Strain number	Source
Cs 843	*C. stellata*	CBS 843	CBS, *ex* wine grapes, Germany
Cs 861	*C. stellata*	AWRI 861	UNSW, *ex* wine, Australia
Cs 1713	*C. stellata*	CBS 1713	CBS, *ex* wine, Italy
Cs 2649	*C. stellata*	CBS 2649	CBS, *ex* grape juice, France
Ck 573	*C. krusei*	CBS 573[T]	CBS, not of wine origin[1]
Ck 863	*C. krusei*	AWRI 863	UNSW, *ex* wine, Australia
Ck 873	*C. krusei*	AWRI 873	UNSW, *ex* wine, Australia
Td 860	*T'spora delbrueckii*	AWRI 860	UNSW, *ex* wine, Australia
Td 872	*T'spora delbrueckii*	AWRI 872	UNSW, *ex* wine, Australia
EC1118	*S. cerevisiae*	EC1118	Lallemand Pty Ltd, Australia
EC1118-12[2]	*S. cerevisiae*	EC1118	Lallemand Pty Ltd, Australia
Mix[3]			

[1]CBS 573[T] is the type strain for *C. krusei*
[2]Fermentation ceased at approximately 12 °Brix
[3]Fermentation initiated with the nine non-*Saccharomyces* strains and superinoculated with EC1118 after 48 h

To simulate a spontaneous fermentation, a mixture of all of the non-*Saccharomyces* starter cultures, inoculated to give an initial concentration of 1×10^5 cells/mL of each of the nine strains, was used to initiate one fermentation which was then superinoculated after two days with EC1118 at an inoculum density of 1×10^6 cells/mL. This fermentation was therefore conducted by a mixed culture. Viable plating onto selective Lysine agar (Oxoid, UK) (Radler *et al.* 1985) showed that non-*Saccharomyces* yeasts were present at least until day five of fermentation; otherwise the ecology of this fermentation was not further investigated.

3.2.3. Starter culture preparation

Starter cultures of each strain were prepared in grape juice diluted 1:1 with MilliQ® purified water (Millipore Australia Pty. Ltd.). A single cryopreservant bead was incubated statically at 25°C for 24–48 h in a tube containing 10 mL of YM medium (Amyl Media, Australia). A loopful of the inoculated broth was also streaked onto YM agar and incubated to check for purity, as inferred from homogeneity of colonial morphology. A 200 µL aliquot of the broth culture was used to inoculate 30 mL of a starter culture medium, consisting of the Chardonnay juice diluted 1:1 with sterile MilliQ® water. This culture was incubated aerobically at 18°C, by agitation at 200 rpm in a cotton-plugged, baffled, conical flask. When the density of this culture had reached 12×10^8 cells/mL, in 2–4 days, depending upon the strain, the juice was inoculated with the volume of culture required to give an initial density of 1×10^6 cells/mL.

3.2.4. Chemical analysis

Free and total sulfur dioxide was determined by the aspiration method (Rankine and Pocock 1970). The concentration of glucose and fructose was determined enzymatically using a Boehringer kit (Boehringer Mannheim, Germany) as per the manufacturer's instructions. The concentration of organic acids, glycerol and ethanol was determined by High Performance Liquid Chromatography (Frayne, 1986). All analytical results are expressed as the means of duplicate determinations.

For the quantification of esters, alcohols and fatty acids, the following extraction and gas chromatography (GC) methods were used. A 25 ml wine sample, to which the internal standards methyl octanoate (0.5 mg/L), and nonanoic acid (1.0 mg/L), were added was extracted successively with 20, 10 and 5 mL of redistilled pentane-dichloromethane (2:1). The extract was dried with anhydrous magnesium sulfate (BDH, UK) and concentrated by evaporation under a stream of nitrogen gas. A 2 μL sample was analysed using a Hewlett-Packard 5890A Series II gas chromatograph fitted with a 30 m x 0.25 mm J&W fused silica capillary column DB-1701, 0.25 μm film thickness. The oven was started at 50°C, held at this temperature for 1 min, increased to 250°C at 20°C/min, and held at this temperature for 20 min. The injector was held at 220°C and the transfer line at 275°C. The splitter, at 30:1, was opened after 36 s in the splitless/split runs. Positive ion electron impact spectra at 70 eV were recorded in the range m/z 40-300 on the Hewlett-Packard 5971 Mass Selective Detector. The approximate concentration of the compounds of interest was calculated by comparison of peak area to that of the corresponding internal standard. These calculations assume a one to one response ratio for the analyte and its corresponding internal standard.

3.2.5. Preference testing

The wines were characterised by a panel of judges for aroma description and overall preference. It was considered that the varied residual sugar content of the wines would not unduly affect aroma assessment. The varying ethanol concentration might have however, as ethanol has intrinsic aroma characterisitics and acts as a carrier for other volatiles (Amerine *et al.* 1980). Nevertheless, it was considered most appropriate to present the wines unadjusted with respect to ethanol content, as this would have required excessive dilution of the less fermented juices, which would have affected aroma.

As the fermentation treatments were unreplicated and wine volume was limited, rigorous sensory testing requiring panel training was considered to be inappropriate for this assessment. A judging panel of 12 staff and students of the AWRI, experienced in the sensory assessment of wine, were selected for the task. Although the exercise took place in a group setting, the judges worked in isolation after an explanation of the tasks. The 12 wine samples were coded and simultaneously presented in XL5 glasses with plastic lids, in a complete block design (Meilgaard *et al.* 1991), for appraisal on a single occasion. The samples were presented to each judge in a different, random order. The wines were ranked by each judge in the order of

preference, as can be expedited by an untrained panel (Meilgaard *et al.* 1991). A preference rank of 1 (most preferred) to 12 (least preferred), was assigned to each wine by each judge, with tied ranks impermissible. Judges were also asked to describe any "off-aromas" or faults, and award a score to each wine as follows: 0, no faults; 1, just detectable; 2, moderate; 3, strong; 4, very strong. Although the judging panel was not trained for fault recognition and intensity scoring for these particular wines, this was generally a familiar exercise for them. Each judge also provided as many descriptive terms for the aroma of each wine as they considered appropriate.

3.2.6. Data analysis

Sensory analysis data were interpreted by using the JMP version 3.10 statistical software (SAS Institute Inc.). A Friedman analysis of variance (ANOVA) for ranked data was performed to assess the effect of yeast treatment on wine preference. In order to determine if the ranks of two wines were significantly different from each other a least significant ranked difference (LSRD) test was used (O'Mahoney 1986, Meilgaard *et al.* 1991, Lawless and Heymann 1998). The significance of differences in fault intensity score was determined by a oneway ANOVA. To determine which wines were significantly different each pair was compared using an honestly significant difference (HSD) multiple comparison test (O'Mahoney 1986). The association between wine preference and other variables was measured by the Spearman rank correlation coefficient (O'Mahoney 1986, Lawless and Heymann 1998).

3.3. Results

3.3.1. Fermentation kinetics and wine composition

Fermentation was completed to different extents by the yeasts, as is apparent from the concentration of residual sugar and ethanol for each treatment shown in Table 3.2. Although some fermentations may have proceeded further given more time, by 14 days sugar consumption was minimal and the wines were bottled.

Fermentation was completed in seven days by both *S. cerevisiae* EC1118 and *C. stellata* CBS 843, which produced wines with a residual sugar concentration of 3–4 g/L. The fermentative capacity and ethanol yield of the *C. stellata* strain was greater than is expected for this species (Soufleros and Bertrand 1979, Benda 1982). For the three wines of low residual sugar content (< 4 g/L), the highest ethanol yield was by the Cs 843 and Mix treatments, which both produced 14.1% ethanol v/v as compared to 13.7% for EC1118. Further fermentation trials and analysis would be necessary however for the determination of the fermentation efficiency values for these yeasts.

The *C. krusei* strains CBS 573[T] and AWRI 873 were the most weakly fermentative yeasts, and it was also noted that excessive amounts of foam were produced during active fermentation by these strains. This is a highly undesirable property, thought to be due to the

Table 3.2. Yeast strain fermentative capacity

Treatment code	Fermentation time (days)	Concentration of residual sugar (g/L)	Concentration of ethanol (% v/v)
EC1118 -12	3	79.8	9.0
EC1118	7	3.8	13.7
Cs 843	7	3.4	14.1
Cs 861	12	40.8	12.1
Cs 1713	12	64.0	10.2
Cs 2649	14	86.7	8.7
Ck 863	14	43.8	11.4
Ck 873	14	156.8	4.3
Ck 573	14	204.6	2.6
Td 872	14	35.7	12.2
Td 860	14	49.8	11.8
Mix	14	3.4	14.1

reduced ability of such yeast to produce hydrophobic proteins on the surface of the cell wall (Dittrich and Wenzel 1976).

3.3.2. Wine composition

The wines differed in concentration of a number of components of importance to sensory character (Table 3.3); however compositional data on the juice was unavailable. Citric acid varied in concentration between 0.4–2.0 g/L in the wines with lowest values noted for the Td 872 and Mix treatments, and highest values for Cs 1713 and Cs 2649 treatments.

Succinic acid is the main carboxylic acid produced by yeast, and in these wines the concentration varied between 0.2–1.4 g/L. Formation varied with strain, and strains of *C. krusei* produced both the highest and lowest concentration of this acid. The concentration of malic acid in the wines ranged between 3.4–5.7 g/L. The least fermented juice, in treatment Ck 573, had the highest concentration of malic acid at 5.7 g/L, whilst the lowest concentration of 3.4 g/L was evident in the wine produced by *C. stellata* AWRI 861. Acetic acid concentration varied within a range of 0.1–1.4 g/L for the strains studied here, with both the lowest and highest production by strains of *C. stellata*. Lactic acid is produced in large amounts by only a few yeasts (Radler 1993) and although there was some formation of this acid by two *C. stellata* strains and EC1118, the amounts were considered minimal and non-discriminatory.

The concentration of glycerol in the wines varied widely, with two of the *C. stellata* wines containing around 17 g/L. This was far in excess of the range of 1.4–9.9 g/L reported for Australian wines (Amerine *et al.* 1980).

Table 3.3. Concentration of organic acids, residual sugars and glycerol (g/L) in the wines[1]

Treatment code	citric acid	tartaric acid	malic acid	succinic acid	lactic acid	acetic acid	glucose	fructose	glycerol
EC1118-12	0.6	3.7	4.9	0.8	0.1	0.4	24.0	55.8	5.8
	0	0.28	0.28	0	0.07	0	0.57	1.34	0.14
EC1118	0.7	2.6	4.2	1.0	0.1	0.3	0.3	3.6	7.2
	0.07	0.07	0.14	0	0	0.01	0.07	0.07	0.42
Cs 843	0.5	2.0	3.9	0.9	0.1	0.1	0	3.4	6.8
	0	0	0	0	0	0	0	0	0
Cs 861	0.5	2.3	3.4	0.5	0.1	0.5	17.3	23.5	6.4
	0	0.14	0.07	0	0	0	0.91	0.99	0.2
Cs 1713	1.7	2.9	4.3	1.1	0	1.4	64.0	0	17.0
	0	0.07	0.07	0	0	0.07	0.71	0	0.28
Cs 2649	2.0	3.8	4.5	0.7	0	1.2	86.7	0	17.4
	0.07	0.07	0.14	0	0	0.05	1.77	0	0.4
Ck 863	0.5	2.5	4.8	1.4	0	0.6	16.4	32.3	4.6
	0.28	0.42	0.57	0.14	0	0	3.39	3.54	0
Ck 873	0.6	3.7	5.2	0.2	0	0.8	63.5	93.4	4.0
	0.07	0.14	0.28	0	0	0	0.49	2.19	0.28
Ck 573	0.6	4.0	5.7	0.2	0	0.6	93.9	110.7	2.9
	0.07	0.21	0.21	0	0	0	4.24	3.04	0
Td 872	0.4	2.3	4.2	1.1	0	0.3	10.8	24.9	4.4
	0.07	0	0.07	0	0	0	0	0.14	0.07
Td 860	0.5	2.7	4.4	1.2	0	0.4	16.6	31.2	4.6
	0.07	0.35	0.49	0.14	0	0	0	2.9	0.8
Mix	0.4	1.9	4.0	0.9	0	0.3	0	3.4	8.1
	0	0	0	0	0	0	0	0	0

[1] composition of juice prior to yeast inoculation not available
mean
standard deviation

As well as large variation in the total sugar content of the wines, the ratio of glucose to fructose remaining also varied. Two of the *C. stellata* strains, CBS 1713 and CBS 2649, were exceptional in that all of the fructose was depleted to leave 65 and 88 g glucose/L respectively. The preferential uptake of fructose by *C. stellata* has also been noted by Minárik *et al.* (1978), while *S. cerevisiae* usually depletes glucose preferentially.

Analysis of some fatty acids, esters and alcohols, also revealed differences in composition between the wines (Table 3.4). None of the higher alcohols measured exceeded in concentration the range reported for table wines (Amerine *et al.* 1980). The fatty acids octanoic and decanoic acid were produced at a much higher concentration by EC1118 than by the non-*Saccharomyces* yeast strains. Production of dodecanoic acid however, was highest by two of the *C. stellata* strains CBS 1713 and CBS 2649. The significance of this is unknown as few studies have dealt with the sensory contribution of fatty acids, and the aroma of dodecanoic acid has not been determined (Etiévant, 1991). Production of esters

Table 3.4. Approximate concentration of some volatile compounds extracted from the wines (mg/L)

Compound	EC1118-12	EC1118	Cs 843	Cs 861	Cs 1713	Cs 2649	Ck 863	Ck 873	Ck 573	Td 872	Td 860	Mix
2-methyl-1-propanol	1.6	2.1	2.6	5	6.8	7.1	8.0	6.7	1.8	2.7	5.1	4.6
2- & 3-methyl-1-butanol	39.6	59.0	69	44.2	10.2	14.7	83.0	44.3	19.4	22.6	83.0	75.9
2- & 3-methyl butyl acetate	0.2	0.2	0.2	0.8	0.1	0.2	0.3	2.5	1.3	0.3	0.3	0.7
ethyl hexanoate	0.5	1.3	0.4	0.1	-	-	0.1	-	-	0.1	0.2	0.7
hexyl acetate	0.1	0.3	0.3	-	-	-	-	-	-	-	-	0.3
hexanoic acid	0.1	4.0	2.8	0.5	0.1	0.5	0.7	0.7	0.7	0.2	0.1	1.7
ethyl octanoate	0.5	1.2	0.4	0.2	-	-	0.1	-	-	0.1	0.2	0.5
phenyl ethyl alcohol	9.2	10.7	-	17.7	9.5	13.4	23.9	12.1	11.6	24.0	25.8	13.0
octanoic acid	3.9	4.9	1.5	0.3	0.1	0.1	0.4	0.2	0.2	0.5	0.6	1.7
decanoic acid	0.7	0.9	0.5	0.7	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3
dodecanoic acid	0.1	0.1	0.2	0.1	0.4	0.4	0.1	0.1	0.1	0.1	0.1	0.1
Σ esters	1.3	2.8	1.3	1.1	0.1	0.2	0.5	2.5	1.3	0.5	0.7	1.9
Σ higher alcohols	50.4	72.8	80.8	66.9	26.5	35.2	114.9	63.1	32.8	49.3	113.9	83.5
Σ acids	1.2	9.9	5.0	2.0	0.7	1.1	1.3	1.1	1.1	0.9	0.9	3.8

- not detected

was generally highest by *S. cerevisiae* EC1118, with the exception of the combined production 2- & 3-methyl butyl acetate at a much higher concentration by four strains and the Mix treatment. These amyl acetate esters are redolent of pear, apple and banana, and would have contributed to the aroma of the wine at the concentration measured (Etiévant 1991).

3.3.3. Sensory analysis

The aroma preference ranks assigned to each wine by the 12 judges were summed and the order of wine preference determined (Table 3.5), although it is important to note that relative rank does not indicate the degree of difference between samples. The significance of the differences in preference rank for each wine was assessed by applying an analysis of variance (ANOVA). A Friedman ANOVA for ranked data (Meilgaard *et al.* 1991, Lawless and Heymann 1998), showed that the preference ranking did differ significantly for the 12 wines ($p < 0.001$) (data not shown). It was notable that the most preferred wine was that made using EC1118. In order to determine which wines were ranked significantly differently from the most preferred, a least significant ranked difference (LSRD) test was used (O'Mahoney 1986, Meilgaard *et al.* 1991, Lawless and Heymann 1998). This showed that the EC1118 wine was not significantly preferred to the wines ranked second to eighth (Table 3.5).

Table 3.5. Wine aroma assessment by preference rank and fault score

Preference rank	Treatment code	Sum of preference rank	Mean fault score
1	EC1118	39[a]	0.4[a]
2	Td 860	52[a]	0.6[a]
3	Ck 863	57[a]	0.9[a]
4	Cs 2649	61[a]	0.8[a]
5	EC1118-12	62[a]	0.5[a]
6	Cs 843	64[a]	1.2[a]
7	Td 872	73[a]	0.7[a]
8	Cs 861	78[a]	1.1[a]
9	Mix	88[b]	1.7[b]
10	Cs 1713	116[b]	2.8[b]
11	Ck 873	122[b]	3.0[b]
12	Ck 573	124[b]	2.8[b]

[a]represents treatments not significantly different in preference or fault score from EC1118 ($p < 0.001$)

[b]represents treatments significantly different in preference or fault score from EC1118 ($p < 0.001$)

The judges were not specifically trained for the recognition or scoring of aroma fault intensity in these wines, although this was generally a familiar exercise for them. The aroma faults scored were generally described as hydrogen sulfide or sulfide, and oxidised or

aldehydic. The EC1118 wine was scored lowest in faults, and this was not significantly different from the group of eight wines that were indistinguishable on the basis of aroma preference (Table 3.5), as determined by a oneway ANOVA. The wines ranked ninth to twelfth by preference were also found to be significantly higher in intensity for aroma faults than EC1118, as determined by a HSD multiple comparison test (O'Mahoney 1986). The association between preference rank and fault score was measured for wine aroma using the Spearman rank correlation coefficient (O'Mahoney 1986, Lawless and Heymann 1998). As might be expected, the two were significantly associated (p< 0.001), such that as the fault score increased the preference rank decreased.

3.3.4. Description of wine aroma.

A complete list of aroma descriptors for the wines is compiled below, with the wines listed in order of preference (see Table 3.5) and identified by the yeast that conducted the fermentation. Although aroma preference for the first eight ranked wines did not differ significantly, the aromas were quite varied as portrayed by the descriptive terms. If a term was used by more than one judge this frequency is noted in brackets. The following abbreviations are used: sl., slight/slightly; VA , volatile acidity; v., very.

1. *S. cerevisiae* EC1118

ester (6), bubblegum (3), banana lolly (2), grassy (2), pineapple (2), apple, nutty, aniseed, asparagus, rich floral, v. fruity, leesy, sweaty socks, sl. VA.

2. *T'spora delbrueckii* AWRI 860

ester (4), lolly (3), melon (2), peach (2), pineapple (2), dried pear (2), floral (2), sl. dusty (2), grape juice, tropical, quince, tropical fruit, strawberry ester, toffee apple, sl. ethyl acetate, honey, malty, bready, yeasty, wet paper, flyspray, weakly acetic.

3. *C. krusei* AWRI 863

ethyl acetate (3), toffee (2), cardboard (2), tropical, tinned pineapple, green fruit, peach, dried pear, fig, raisin, non-vinous, strawberry ester, lolly, nutty/oxidised, cashew, bready, dusty, sweaty, plasticine.

4. *C. stellata* CBS 2649

stewed fruit (2), cooked apple (2), green fruit (2), sulfide/eggy (2), tropical, lucerne, sweet, stone fruit, citrus peel, cooked orange, grape juice, tomato juice, stalky, nutty, cooked juice, oxidised juice, salami, herbaceous, bready, sl. mouldy, solvent-like, sweaty.

5. *S. cerevisiae* EC1118 -12

tropical fruit (5), grape juice (3), pineapple (2), grassy (2), aldehydic (2), buttery (2), vinous, mango, green, pear, ethyl acetate, banana lolly, ester, butterscotch, sl. butyric, bready/yeasty, sharp, earthy note.

6. *C. stellata* AWRI 843

isoamyl acetate (8), ester (5), ethyl acetate (4), bubblegum (3), fruit salad, passionfruit, sl. grapy, strawberry, VA, lolly, acetaldehyde, fermentation bouquet, sweaty, sl. H_2S/garlic, sl. vegetal, earthy, rubbery, intense.

7. *T'spora delbrueckii* AWRI 872

boiled sweets (3), ester (3), neutral grape juice (2), fruity (2), sweaty (2), cooked fruit, sl. apple, cider, toffee apple, apricot, strawberry essence, sweet, botrytised, caramel, sl. nutty, interesting, sl. oxidised, dull, stale, solvent-like.

8. *C. stellata* AWRI 861

fruity (3), caramel (3), cooked juice (2), honey (2), grassy (2), hessian/mouldy (2), peach, guava, dried pear, ester, jam, subdued, vegetal, nutty/oxidised, microbial, butyric, bread, dough.

9. Mixture

oxidised/aldehydic (8), apples (5), banana ester (4), feijoa, green, apple cider, nutty, ethyl acetate, bubblegum, confectionary, toffee, dough, bread, yeast.

10. *C. krusei* AWRI 873

high ethyl acetate (8), nectarine, rotten fruit, cut grass, cat's urine, vegetal, solvent, pungent, sickly, pungent sulfide, burnt, earthy, dusty, leathery, dirty, sweaty, microbial, yeasty, spoilt.

11. *C. stellata* CBS 1713

hydrogen sulfide (4), cabbage (3), vile (3), yeasty (3), rotten fruit (2), stewed (2), compost, dirty, underarm, paper, mercaptan, tarry, rubbery, non-vinous, green fruit, autolysed, ester, volatile.

12. *C. krusei* AWRI 573

ethyl acetate (4), fruity (2), hydrogen sulfide (2), volatile (2), faecal (2), sweaty (2), cheesy (2), vomit, sickly, butyric, off, vegetal, cat's urine, honeyed, over-ripe fruit, nectarine, ripe melon, wood-like, earthy, bready, meaty, unusual, plastic, chocolate.

3.4. Discussion

S. cerevisiae, although regarded as the principal wine yeast, is not the only species that contributes to fermentation. Non-*Saccharomyces* yeasts play a significant role in both traditional winemaking and inoculated fermentation, and introduce ecological and biochemical diversity to winemaking (Fleet and Heard 1993). This study used ten yeasts to vinify a Chardonnay juice, in order to assess the effect upon the chemical composition and aroma profile of the resulting wines.

Fermentation was completed to varying extents by the strains studied (Table 3.2). Complete fermentation of sugar was achieved by *S. cerevisiae* EC1118, *C. stellata* CBS 843 and the mixed yeast culture. The incompleteness of fermentation by the non-*Saccharomyces* yeasts is generally attributed to low ethanol tolerance, which affects cell viability and fermentative capacity (Casey and Ingledew 1985). The non-*Saccharomyces* yeasts are also generally thought to produce less ethanol and more secondary metabolites than *S. cerevisiae* (Fleet 1990), although this could not be confirmed for the strains investigated in this study. The *C. krusei* type strain CBS 573[T], that was not of wine origin had the lowest fermentative capacity, producing only 2.6% ethanol.

Unusual sugar fermentation patterns were also noted for the *C. stellata* strains CBS 1713 and CBS 2649, which exhibited a strong preference for fructose. The preferential uptake of fructose by *C. stellata* has also been noted by Minárik *et al.* (1978), although not to this extent, while *S. cerevisiae* usually depletes glucose preferentially. Sluggish or stuck fermentation can result in, or perhaps be caused by, an excess of fructose due to preferential uptake of glucose by yeast (Schütz and Gafner 1993b). This situation could possibly be prevented by the involvement of such a fructophilic yeast in fermentation.

The production of glycerol also varied with strain, with particularly high production by the *C. stellata* strains CBS 1713 and CBS 2649. Ciani and colleagues (1996, 1998), also found that high glycerol production by strains of *C. stellata*, but not as high as 17 g/L as noted for these ferments. An increase in sweetness due to glycerol would be perceptible in these wines given a flavour threshold of 5.2 g/L. Glycerol can also increase wine viscosity, although the concentration in these wines was below the only reported sensory threshold value of 26 g/L (Noble and Bursick 1984).

From the analysis of some representative compounds of importance to wine aroma, it was evident that production varied with yeast strain, irrespective of the extent of fermentation. Acetic acid is a normal byproduct of alcoholic fermentation and varied within a range of 0.1–1.4 g/L for the strains studied here, with both the lowest and highest production by strains of *C. stellata*. Spoilage can be evident at 0.6–0.9 g/L depending upon the wine (Amerine *et al.*

1980), but acetic acid or volatile acidity character was rarely noted during sensory analysis of these wines.

The concentration of malic acid in the wines ranged between 3.4–5.7 g/L. As the composition of the juice was not determined, the differences noted between the treatments may represent decomposition, fermentation or uptake by the yeast. The least fermented juice had the highest concentration of malic acid at 5.7 g/L. The lowest concentration was evident in the wine fermented by *C. stellata* AWRI 861, which probably represented the greatest decomposition of malic acid by any of the yeast strains, despite the incomplete fermentation of sugar.

Succinic acid is the main carboxylic acid produced by yeast, and in these wines the concentration varied between 0.2–1.1 g/L. Formation varied with strain, and strains of *C. krusei* were both the highest and lowest producers. Of the yeast strains tested by Shimazu and Watanabe (1981), all of the non-*Saccharomyces* species, including *C. krusei,* were found to be low producers of succinic acid in comparison to *Saccharomyces* species. The non-volatile succinic acid was present in all wines at well above its flavour threshold value of 35 mg/L in water (Etiévant 1991), and would perhaps therefore have contributed a characteristic bitter and salty taste to the wines (Ribéreau-Gayon and Peynaud 1975).

The concentration of citric acid in the wines varied between 0.4–2.0 g/L with lowest values for the Td 872 and Mix treatments, and highest values for the Cs 1713 and Cs 2649 treatments. Although formation of this acid by yeast is considered minor (Radler 1993), a concentration of 2.0 g/L is much greater than the range of 0–0.7 g/L reported in must and wine (Amerine 1980).

Variation noted in the concentration of tartaric acid was most likely due to the precipitation of tartrate salts, as metabolism of this grape acid by yeast has not been reported (Radler 1993). The solubility of tartaric acid is determined by a number of factors, and those influenced by yeast metabolism are pH and the concentration of ethanol, which would most likely account for the variation observed.

Acetate esters and ethyl esters of fatty acids are microbial products considered to be of major importance to wine aroma. Firstly, they are major constituents of the volatile fraction of wine, and, secondly, their fruity odours are often used in the description of wine (Etiévan, 1991). The production of esters was generally highest by *S. cerevisiae* EC1118 (Table 3.4), with the exception of the esters 2- and 3-methyl butyl acetate which were produced at 4–12.5 times the concentration by the Cs 861, Ck 873, Ck 573 Td 860 and Mix treatments. These amyl acetate esters are redolent of pear, apple and banana, and would have contributed to the aroma of the wine produced by these yeast treatments (Etiévant 1991).

Higher alcohols comprise a group of compounds important to the complexity of wine aroma, which may benefit positively from their presence (Margalith 1981). Higher alcohol production was greatest by the non-*Saccharomyces* strains, and varied independently of the extent of fermentation. Phenyl ethyl alcohol (phenylethanol), which has a distinct rose-like aroma, and textural properties that may contribute to wine character (Etiévant 1991), was produced at the highest concentration by strain AWRI 860. The concentration of phenyl ethyl alcohol in these wines was low in comparison to the reported range of 4–197 mg/L. Odour thresholds determined in wine however range between 7–200 mg/L (Etiévant 1991) so prediction of the impact of phenyl ethyl alcohol on these wines is unclear. The alcohols 2-methyl-1-propanol (isobutanol) and 2- & 3-methyl-1-butanol (active and isoamyl alcohols respectively) ranged in concentration from 1.6 to 8 mg/L in these wines. The sensory characters are described as "harsh, pungent and repulsive" and these compound are considered to make a significant contribution to wine aroma and flavour, albeit negatively at high concentration (Etiévant 1991). The reported odour threshold values in wine for 2 methyl-1-propanol range between 300–750 mg/L, far in excess of the concentration measured in these wines. A combined odour threshold of 60 mg/L for 2- & 3-methyl-1-butanol (Etiévant 1991), would however suggest a noticeable contribution to the aroma of some of the non-*Saccharomyces* wines.

The fatty acids octanoic and decanoic were produced at a much higher concentration by EC1118 than the non-*Saccharomyces* yeasts, although both acids were present at a concentration below their respective aroma thresholds of 10 and 6 mg/L (Etiévant 1991). Although these acids are considered to be inhibitory to yeast growth (Lafon-Lafourcade *et al.* 1984), production by the non-*Saccharomyces* yeasts was low or not detectable, and therefore probably not of sensory or biochemical importance in these ferments. Production of dodecanoic acid was highest by two *C. stellata* strains, CBS 1713 and CBS 2649. The significance of this is unknown as few studies have dealt with the sensory contribution of fatty acids, and the aroma of this acid, and its threshold concentration have not been determined (Etiévant 1991).

The differences in chemical composition of the wines were accompanied by aroma differences as were evident from the judges' descriptions. There is a wealth of analytical data on wine composition as determined by yeast metabolic activity. Comparison of the concentration of individual compounds to published aroma and flavour threshold data can also permit inference of their likely sensory impact. The aroma of wine however, is determined by complex interactions of many volatile compounds, and is not just the sum of its parts (Etiévant 1991). For example, a mixture of esters was found to impart a different intensity and quality to wine than the individual esters were (van der Merwe and van Wyk 1981). The role of sensory analysis for the selection of yeast strains in this study was of

particular importance considering the unknown impact of these strains upon wine aroma and flavour.

Sensory evaluation is a very useful set of tools for the evaluation of the how complex mixtures such as wine are perceived. Analytical results regarding the presence, type and magnitude of aroma, flavour, colour and tactile (mouthfeel) differences can be obtained. Certainly where the consumer is of importance, hedonic questions regarding quality and preference are best answered by sensory analysis. This study provides a preliminary survey of the aroma of wines made by pure cultures of some non-*Saccharomyces* yeast. The results provide some comprehensive information on the aroma character and preference of some wines made without the involvement of the ubiquitous wine yeast *S. cerevisiae*. Formal difference testing and descriptive analysis of these wines was not considered necessary, given the evident differences between the wines, and the preliminary nature of this exercise.

Preference ranking of the wines by aroma assessment showed that the EC1118 wine was the most preferred, although statistically the wines ranked first to eighth were not different in preference. This less than discriminatory result may have been due to the conservative nature of the LSDR test (O'Mahoney, 1986). It is also important to bear in mind that the judges were asked to distinguish some atypical wines on the basis of personal preference. Ideally preference testing is carried out by a large number of consumers familiar with the product category (Meilgaard *et al.* 1991, Lawless and Heymann 1998), but given the atypical nature of these wines, this may have proved difficult.

The wines ranked ninth to twelfth were significantly less preferred than EC1118. These wines were rated significantly higher in fault intensity, which varied in nature from aldehydic, sulfidic and ethyl acetate tainted. The lowest ranked wine was made by *C. krusei* strain CBS 573$^{\text{T}}$, that fermented only approximately 20 g sugar/L, to produce objectionable ethyl acetate and sulfide characters. This yeast strain was not of wine origin, and it was clearly apparent that it was neither adapted for the fermentation of grape juice, nor acceptable to human consumers because of the unpleasant aroma of the secondary metabolites. The domesticated yeast *S. cerevisiae* has been carefully selected for character traits that are more advantageous to winemakers and consumers than to the yeast itself, such as decreased production of aroma volatiles that are considered objectionable. It is therefore hardly surprising that the EC1118 wine was ranked as both the most preferred and lowest in aroma faults.

The mixed culture wine (Mix) was significantly less preferred than the EC1118 wine and significantly higher in faults, principally described as aldehydic. The growth and fermentation activity of the inoculated strains was not elucidated beyond the observation that non-*Saccharomyces* yeast initiated fermentation prior to inoculation with *S. cerevisiae* on day 3, and were viable until day 5 of fermentation as determined by plating onto Lysine medium.

The aroma descriptors generated by the judges were varied and quite useful for discrimination of the wines. The aromas of the partially and completely fermented *S. cerevisiae* EC1118 wines (treatments EC1118-12 and EC1118), were described somewhat similarly. The pronounced ester character of the most fermented wine (treatment code EC1118), was described by a number of judges as 'ester', 'banana lolly', and 'bubblegum'. The diversity of aroma terms generated for the non-*Saccharomyces* wines was high. For the preferred wines, novel aroma descriptors, for young unwooded Chardonnay wine at least, such as 'malty', 'flyspray', 'citrus peel', 'tomato juice', 'salami', 'cider', 'caramel', 'toffee', 'dried pear' and 'guava' were reported. The ninth to twelfth ranked wines, which were significantly lower in preference to EC1118, were described mostly by negative terms. The Mix wine was mostly noted for an 'aldehydic' and 'oxidised' fault. The yeasts *C. krusei* AWRI 873 and AWRI 573, and *C. stellata* CBS 1713 produced aromas described as 'sulfidic', 'sweaty', 'dirty', 'vegetal', 'cabbage', 'compost', 'mercaptan', 'faecal', 'vomit' and 'meaty' which are indicative of the production of a variety of sulfurous volatiles (Etiévant 1991). The least preferred wines were considered repugnant and unacceptable by many of the judges, attesting that the relative preference rank did not indicate the magnitude of the difference in preference.

3.5. Conclusion

This study partially characterised the fermentative capacity, sugar utilisation, wine composition, aroma profile and preference of two *T'spora delbrueckii*, three *C. krusei* and four *C. stellata* strains. Great diversity in winemaking properties exists amongst these non-*Saccharomyces* species which are commonly associated with grape juice fermentation. Certainly some potentially exploitable traits, such as different sugar utilisation properties, the production of glycerol at high concentration and the production of novel aromas, not commonly associated with *S. cerevisiae*, were identified during this study.

A group of yeasts that produced wines that did not differ significantly in aroma preference from that of the commercial yeast EC1118 was identified. Four yeast treatments of significantly lower preference, and higher fault score to EC1118 were also identified. On the basis of preference rank, aroma description and differences in composition, three yeast strains were selected for mixed species fermentation studies (Chapters 4 and 5). These were *T'spora delbrueckii* AWRI 860, and the *C. stellata* strains CBS 2649 and CBS 843. The use of such non-*Saccharomyces* strains in conjunction with commercial yeasts offers potential for utilising the novel properties in a controlled fermentation of predictable outcome. It becomes necessary therefore to determine how culture in the presence of a commercial strain, as may be necessary to complete the fermentation of sugar, affects the growth and metabolic activity of different non-*Saccharomyces* strains (Chapter 4)

Chapter 4

THE EFFECT OF INOCULATION PROTOCOL UPON STRAIN GROWTH AND WINE COMPOSITION IN MIXED YEAST SPECIES FERMENTATION

4.1. Introduction

Many studies of the yeast ecology of grape juice fermentation, as reviewed by Kunkee and Amerine (1970), and Fleet and Heard (1993) among others, have shown that different species are present at different stages of the fermentation. Yeasts significant during the early and mid stages of fermentation include species of *Hanseniaspora/Kloeckera*, *Candida* and *Pichia*, which are succeeded by *Saccharomyces cerevisiae*. Yeasts indigenous to the vineyard and grapes, and resident on winery equipment inoculate the must at a density of 10^3–10^5 colony forming units (CFU) per mL (Fleet 1990). The growth of particular strains in fermentation appears to be limited by a number of factors including cold settling (Mora and Mulet 1991), the concentration of sulfur dioxide (Heard and Fleet 1988a), sugar, oxygen and ethanol (Dittrich 1977), and the pH and temperature (Sharf and Margalith 1983, Gao and Fleet 1988, Heard and Fleet 1988b).

Further quantitative analysis of the growth of individual species during fermentation is necessary for a better understanding of the microbiology of vinification and the factors affecting it (Fleet 1990). Quantitative studies have shown that the survival of non-*Saccharomyces* yeasts in fermentation — even when inoculated with *S. cerevisiae*, is greater than is generally assumed (Fleet *et al.* 1984, Heard and Fleet 1985, Martínez *et al.* 1989, Pardo *et al.* 1989, Mora *et al.* 1990).

Indigenous yeasts have been shown to play a role in the production of volatile compounds that may impact upon wine aroma (Holloway *et al.* 1990, Mateo *et al.* 1991). There is a perception that the indigenous non-*Saccharomyces* yeasts improve wine sensory character and complexity in spontaneous fermentation, although such winemaking practices can be risky as the fermentation result is unpredictable. If not controlled, the indigenous yeasts can negatively affect the composition of wine and its sensory properties. There is potential therefore for exploitation of the novel properties of non-*Saccharomyces* yeasts under conditions that better control and predict their contribution to fermentation (Bisson and Kunkee 1991). This could be achieved by using multiple strains for fermentation under conditions that modulate the growth and metabolic activity of the often weakly fermentative non-*Saccharomyces* yeasts, but in the presence of a *S. cerevisiae* strain to ameliorate or complete fermentation as necessary.

Generally, the successful establishment of a yeast (usually *S. cerevisiae*) in fermentation relies upon the vigour of the starter culture and the achievement of numerical dominance over the indigenous population (Delteil and Aizac 1988). It is widely accepted that the addition at high density of a selected yeast will ensure its dominance in fermentation (Rankine and Lloyd 1963). The work of Heard and Fleet (1985) first demonstrated that non-*Saccharomyces* yeast could still be numerically significant in such inoculated fermentations, although in a study by Martínez *et al.* (1989), inoculation with *S. cerevisiae* allowed only partial growth of the indigenous non-*Saccharomyces* yeast. The ecology and population dynamics of such fermentations are no doubt influenced by a number of factors.

Some studies have investigated the effect of combinations of different yeasts in fermentation, either as mixed inocula to initiate fermentation, or as the sequential addition of yeasts during fermentation. The sensory character of wine was reportedly improved by coinoculation of multiple *S. cerevisiae* strains (Verona and Castelli, 1955, cited by Kunkee and Amerine, 1970), and Schütz *et al.* (1995) demonstrated that cofermentation with four *S. cerevisiae* strains produced a wine of "greater aroma complexity" than that produced by fermentation with a single strain. Kir'yalova (1958, cited by Kunkee and Amerine, 1970), reported improved "quality" of fruit wines by fermentation with mixed cultures of *Torulopsis* (*Candida*) and *S. cerevisiae*. Romano *et al.* (1993), and Schütz and Gafner (1993a) have suggested that the use of selected apiculate (*Hanseniaspora/Kloeckera*) yeasts in fermentation with *S. cerevisiae* could alter its growth. Sponholz *et al.* (1990), found that yeast growth and ester formation during cofermentation by *H'spora uvarum* and *S. cerevisiae*, was influenced by the killer status of both strains, as well as their relative inoculation densities. An interesting use of the novel properties of *Schizosaccharomyces pombe* was by Magyar and Panyik (1989), who successfully deacidified grape juice by initial fermentation with this malate utilising yeast, followed by the addition of *S. cerevisiae* to complete sugar fermentation.

A thorough understanding of the effect of the type of inoculation procedure upon the conduct of fermentation is an essential prelude to the controlled use of non-*Saccharomyces* yeasts in commercial winemaking. In this study strains of the species *Torulaspora delbrueckii* and *Candida stellata*, selected on the basis of winemaking potential and differences in fermentation efficiency, as reported in Chapter 3, were used to determine the effect of different coinoculation ratios upon yeast growth. The effect of sequential inoculation at different times was also compared to the coinoculation treatments for one strain of *C. stellata*. Strains were selected on the basis of differences in fermentation activity, in order to assess the importance of such strain differences upon mixed culture fermentation. This study investigated how different inoculation strategies impacted upon fermentation by the quantitative analysis of yeast growth, sugar depletion, and the composition of the wine produced. Due to the inherent variability in composition of different grape juices a synthetic, chemically defined fermentation medium was used in these studies to ensure reproducibility.

4.2. Materials and methods

4.2.1. Yeast strains and inoculation protocol

The yeast strains used in this study were received from the Centraalbureau voor Schimmelcultures (CBS) culture collection, The Netherlands; The University of New South Wales (UNSW) culture collection, Sydney, Australia; and Lallemand Pty Ltd, Australia (Table 4.1). Yeasts were maintained in the AWRI culture collection on cryopreservant beads (Protect Bacterial Preservers, Technical Services Consultants, Ltd, UK), in the gaseous phase of a liquid nitrogen cryovessel.

Table 4.1. Yeast strains and origins

Species	Strain number	Origin
T'spora delbrueckii	AWRI 860	*ex* grape juice, Australia, (UNSW)
C. stellata	CBS 843	*ex* grape juice, Germany, (CBS)
C. stellata	CBS 2649	*ex* grape juice, France, (CBS)
S. cerevisiae	EC1118	*ex* dried yeast, Lallemand Pty Ltd

Coinoculation treatments consisted of concurrent inoculation of one of the non-*Saccharomyces* strains with EC1118 at different ratios. Strain EC1118 was always inoculated at a density of 1×10^5 cells/mL and the ratio of inoculation density of the non-*Saccharomyces* strain relative to EC1118 varied from 1:10 to 50:1 as outlined in Table 4.2.

Table 4.2. Coinoculation protocol

Treatment	Ratio of non-*Sacch.*: EC1118	Inoculation density (cells/mL) non-*Sacch.*	EC1118
A[1]	non-*Saccharomyces* monoculture	1×10^5	0
B	EC1118 monoculture	0	1×10^5
C	1: 10	1×10^4	1×10^5
D	1: 1	1×10^5	1×10^5
E	10: 1	1×10^6	1×10^5
F[2]	50: 1	5×10^6	1×10^5

[1] T'spora delbrueckii AWRI 860, C. stellata CBS 843 and CBS 2649
[2] This ratio investigated for CBS 2649 only

4.2.2. Chemically defined grape juice medium

Experiments were conducted in a synthetic medium modelled on grape juice (Henschke and Jiranek 1993), the composition of which is detailed in Table 4.3.

Table 4.3. Composition of chemically defined grape juice-like medium

Compound	amount per litre	Compound	amount per litre
Carbon sources	(g)	**Vitamins**	(mg)
D-glucose	100	myo-Inositol	100
D-fructose	100	Nicotinic acid	2
		Pyridoxine. HCl	2
Acids	(g)	Ca Pantothenate	1
$KHC_4H_4O_6$	2.5	Thiamine. HCl	0.5
L-malic acid	3.0	*p*-aminobenzoic acid.K	0.2
citric acid	0.2	Riboflavin	0.2
		Biotin	0.125
Salts	(g)	Folic acid	0.2
K_2HPO_4	1.14		
$MgSO_4.7H_2O$	1.23	**Lipids**	
$CaCl_2.2H_2O$	0.44	Ergosterol	10 mg
		Tween 80	0.5 mL
Nitrogen source	(g)		
NH_4Cl	1.7	**pH**	3.2
Trace elements	(mg)		
$MnCl_2.4H_2O$	200		
$ZnCl_2$	135		
$FeCl_2$	30		
$CuCl_2$	15		
H_3BO_3	5		
$Co(NO_3)_2.6H_2O$	30		
$NaMoO_4.2H_2O$	25		
KIO_3	10		

All compounds were of analytical grade, water was MilliQ® grade, pH adjustment was with KOH and HCl solutions. The medium was sterilised by filtration through a 0.22 µm pore size membrane. Lipids were dissolved in ethanol, and added after filtration

4.2.3. Starter culture preparation

A single yeast cryopreservant bead was placed in a tube containing 10 mL of YM broth (Amyl Media, Australia) and incubated at 25°C for 24–48 h. A volume of 300 µL of this culture was then added to 30 mL of starter culture medium consisting of the chemically defined grape juice medium altered to contain 50 g glucose/L and 50 g fructose/L. Starter cultures were incubated aerobically in cotton-plugged, baffled, conical flasks, shaking at 200 rpm in an orbital shaking water bath (Paton Scientific, Australia) at 18°C. After 2–4 d, when the culture had reached a density of 1–2 $\times 10^8$ cells/mL, cells were enumerated to determine the appropriate inoculation volume. It was noted that the two *C. stellata* strains formed cell aggregates, the size of which was estimated during cell counts. This aggregation led to a

discrepancy between cell number and cell viability at the start of fermentation as determined by plate counts. For the sequential inoculation treatments, which necessitated inoculation on different days, a new starter culture of EC1118 or CBS 2649 was prepared daily as necessary, so that a culture in exponential growth phase was always available.

4.2.4. Fermentation and monitoring

Triplicate fermentations of 150 mL volume were conducted in autoclaved, 250 mL conical flasks modified to fit a water-filled airlock and a side arm fitted with a rubber septum (Suba Seal, USA). Flasks were incubated at 18°C in an orbital shaking water bath operated at 100 rpm. To prevent the ingress of air through the fermentation lock, flasks were gassed with membrane sterilised nitrogen gas prior to sampling procedures. Flasks were also gassed when carbon dioxide evolution slowed during the latter stages of fermentation.

Fermentations were sampled aseptically by needle and syringe withdrawal of 1 mL of culture through the rubber septum. The viable starting population was determined by sampling 30 min after inoculation, after which sampling was performed at approximately 24 h intervals. The total viable colony count was determined by serial dilution and plating of duplicate aliquots onto YM agar (Amyl Media, Australia). The viable colony count of CBS 2649 was similarly determined by plating onto Lysine medium (Oxoid, UK). Lysine agar does not support the growth of *S. cerevisiae* beyond a petite colony size (Radler *et al.* 1985), thereby allowing differentiation and enumeration of the larger CBS 2649 colonies. Plates were incubated at 25°C and colonies were counted after 3–4 d. *S. cerevisiae* was enumerated by subtracting the non-*Saccharomyces* colony count from the total yeast count. Fingerprinting of representative colonies from different stages of fermentation using the PCR method described in Chapter 2 confirmed the accuracy of strain differentiation using the two media (results not shown).

The progression of fermentation was followed by measurement of the refractive index. When this no longer decreased the residual sugar concentration was estimated by Clinitest reagent tablets (Ames, Miles Inc., USA). When fermentation was considered finished, the airlock was stoppered, and the flask left at 4°C for 48 h to sediment the yeast, after which the wine was decanted and centrifuged, and the supernatant stored at -20°C before analysis.

4.2.5. Chemical analysis of wines

The concentration of glucose and fructose in the ferment samples was determined using an enzyme kit (Boehringer Mannheim, Germany). A Cobas Fara automatic analyser (Roche Instruments, Switzerland), was programmed to perform the analyses according to the kit manufacturer's instructions. The concentration of organic acids, glycerol and ethanol was determined by High Performance Liquid Chromatography (Frayne 1986).

4.3. Results and discussion

4.3.1. COINOCULATION OF *T'SPORA DELBRUECKII* AWRI 860 AND *S. CEREVISIAE* EC1118

4.3.1.1. Cell growth and sugar consumption

The effect of coinoculation treatment upon growth of yeasts and sugar depletion during fermentation is shown in Figure 4.1a–e. For all of the fermentations data are graphed as means from duplicate determinations of three treatment replicates. A summary of the growth kinetics of each treatment is presented in Table 4.4.

Table 4.4. Growth kinetics of *S. cerevisiae* EC1118 and *T'spora delbrueckii* AWRI 860 in mixed culture at different starting ratios

Treatment	Strain	Initial population (CFU/ml)	Duration of fermentation (d)	Max. population (CFU/ml)	Min. doubling time (h)	Glucose utilisation max. rate (g/L/d)	Fructose utilisation max. rate (g/L/d)
A	AWRI 860	5.1×10^3	14+	2.3×10^8	4.5	25	19
B	EC1118	2.6×10^4	7	1.9×10^8	5.8	25	29
C 1:10[1]	AWRI 860	6×10^2	7	4.4×10^7	4.4	27	26
	EC1118	2.4×10^4		1.3×10^8	5.9		
D 1:1	AWRI 860	5.9×10^3	8	1.1×10^8	4.8	27	26
	EC1118	3.5×10^4		7.6×10^7	6.1		
E 10:1	AWRI 860	4×10^4	9	2.0×10^8	4.1	24	19
	EC1118	4.4×10^4		7.4×10^7	5.3		

[1]ratios in treatments C, D and E represent AWRI 860:EC1118, based upon microscopic cell counts

It was noted that the viable population shortly after inoculation was lower than expected from the intended inoculation ratios. This discrepancy can be attributed to the viable plating method which does not account for cell clumping, and to the calculation of required inoculum volume based upon 100% viability of the starter cultures. A reduced viability of the AWRI 860 starter culture would account for the lower than expected population density seen after inoculation for this yeast in all treatments.

In treatment A, AWRI 860 did not exhibit a lag phase and grew with a fast doubling time of 4.5 h to reach a maximum population of 2.3×10^8 CFU/mL in three days. The viable population declined to 6×10^6 CFU/mL at day 14 when fermentation of sugar had ceased. The sugars were initially consumed rapidly (44 g/L/d), although this slowed as the stationary phase population declined, to leave a concentration of residual sugar of 17 g/L. This decline in number may have been due to ethanol toxicity or related to nutrient depletion.

In monoculture EC1118 completed fermentation in seven days (treatment B). The doubling time was 5.8 h, slower than for AWRI 860, and a lesser population of 1.9×10^8 CFU/mL was reached, although sugar was more rapidly depleted, at 54 g/L/d, than by AWRI 860.
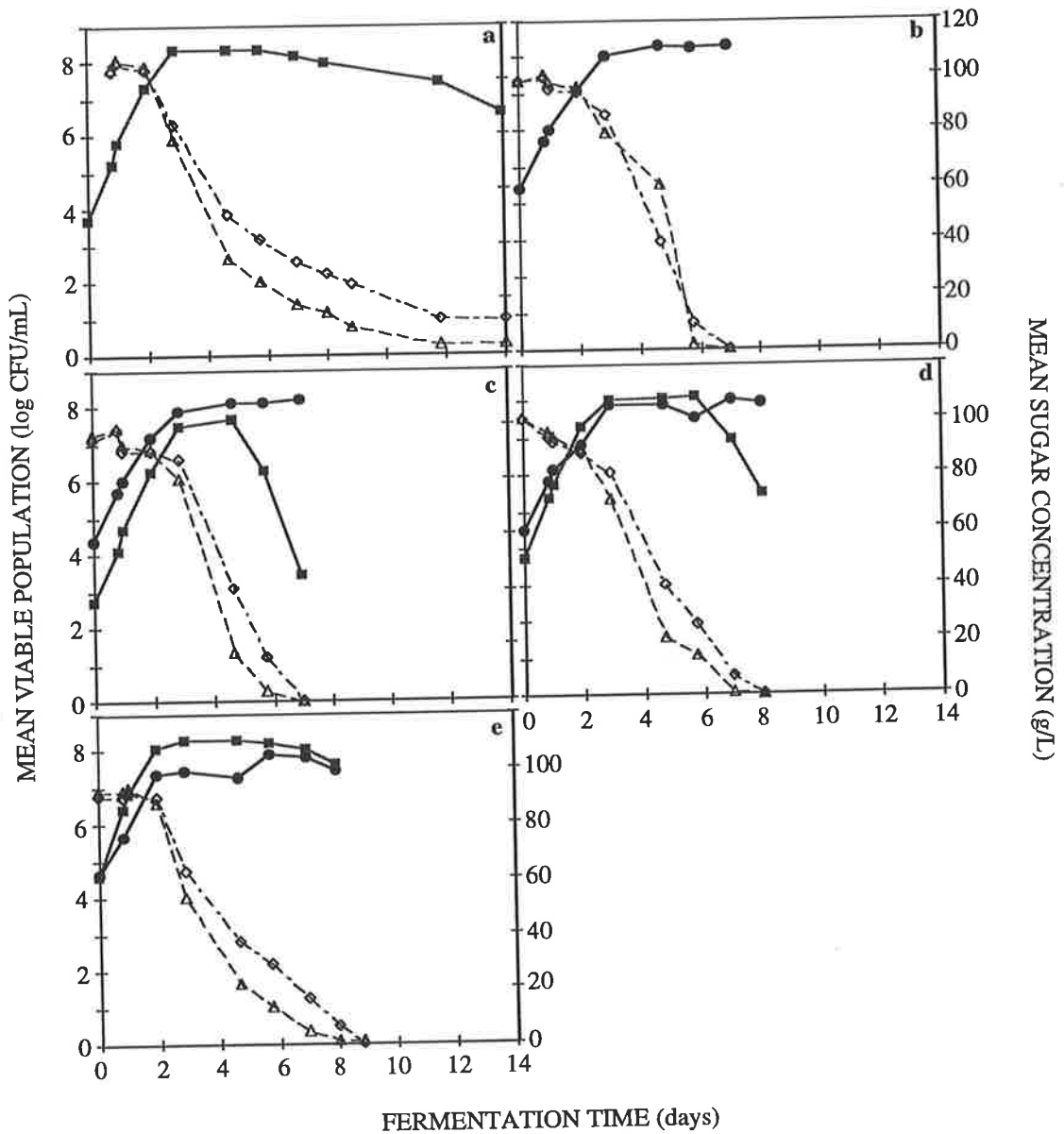
Figure 4.1. Effect of coinoculation ratio of *T'spora delbrueckii* AWRI 860 and *S. cerevisiae* EC1118 upon yeast population and sugar depletion during fermentation

a. Treatment A. *T'spora delbrueckii* AWRI 860 monoculture
b. Treatment B. *S. cerevisiae* EC1118 monoculture
c. Treatment C. AWRI 860: EC1118; 1:10
d. Treatment D. AWRI 860: EC1118; 1:1
e. Treatment E. AWRI 860: EC1118; 10:1

——■——AWRI 860 ——●——EC1118 — -▲- - glucose - - ◇ - - fructose

For treatment C, where coinoculation occurred at 1:10 (AWRI 860: EC1118), fermentation was completed in the same time as for the EC1118 monoculture. The maximum population of AWRI 860 was reduced to 20% of that attained in monoculture, although the doubling time was similar at 4.4 h (Table 4.4). EC1118 reached a maximum population that was 68% of that attained in monoculture, at a similar doubling time of 5.9 h. The maximum, viable population was 1.7×10^8 CFU/mL in total, of which 75% was EC1118. A notable decrease of the AWRI 860 population occurred by day 6, whilst EC1118 number did not decrease during fermentation. So, when AWRI 860 started fermentation at one tenth of the concentration of EC1118, its growth was suppressed as compared to monoculture, but with little effect on EC1118 growth.

For treatment D a coinoculation ratio of 1:1 was intended, but plate counts showed that the EC1118 population after inoculation was approximately six times that of AWRI 860, apparently due to a reduced viability of the AWRI 860 starter culture or clumping of cells that would have reduced the count attained by viable plating. The doubling time for EC1118 was 6.1 h, similar to that observed in previous treatments, and the maximum population of 7.6×10^7 CFU/mL attained represented a decrease of 40% compared to growth in monoculture. AWRI 860 however, grew to a maximum population of 1.1×10^8 CFU/mL, and numerical dominance over EC1118, within two days. After day six however, the viable population decreased to finish fermentation at a density of 3×10^5 CFU/mL. Despite this reduction in cell yield, AWRI 860 was competitive against EC1118 when inoculated at this ratio, representing no less than 58% of the combined viable yeast population of 1.9×10^8 CFU/mL, for at least four days out of the eight day fermentation.

When inoculated at an intended ratio of 10:1 (AWRI 860: EC1118), in treatment E, the viable population of AWRI 860 was, as for the previous treatments, less than expected, only equalling that of EC1118. Nevertheless this yeast reached a population of 2.0×10^8 CFU/mL in three days, which was maintained with only a slight decline noted at the end of fermentation. EC1118 however, was only able to reach a maximum population of 7.4×10^7 CFU/mL two days before the end of fermentation. A minimum doubling time of 5.3 h was achieved during the first two days of fermentation, after which the growth rate slowed. AWRI 860 remained numerically dominant throughout fermentation, representing at least 74% of the cell population at any time. It was noted that fermentation time was extended to nine days, more than for EC1118 in monoculture, but less than for AWRI 860 in monoculture. The decline of AWRI 860 at the end of fermentation observed in previous treatments was not observed. This may be related to the comparatively lower ethanol concentration at the end of fermentation in treatment E. Also possible is that the population decrease noted in treatments C and D was attributable to nutrient depletion or the production of inhibitory substances by *S. cerevisiae* EC1118. This may not have occurred to the same extent in this

treatment (AWRI 860: EC1118, 10:1) where EC1118 was numerically inferior to AWRI 860 which was inoculated at a higher density than in the previous treatments.

4.3.1.2. Wine composition

The concentration of residual sugar, organic acids, glycerol and ethanol in the wines is shown in Table 4.5. Analyses were duplicated for each of the triplicate fermentations of a treatment and mean values and standard deviations are reported.

Table 4.5. Composition of wines made by coinoculation of *T'spora delbrueckii* AWRI 860 and *S. cerevisiae* EC1118 (g/L except ethanol, %v/v)

Treatment	citric acid	tartaric acid	malic acid	succinic acid	lactic acid	acetic acid	glycerol	glucose	fructose	ethanol
unfermented medium	0.2	2.5	3.0	0	0	0	0	100	100	0
A AWRI 860	0.2 0	1.9 0.17	2.5 0.23	0.6 0.06	0 0	0.5 0.07	5.9 0.36	3.6 1.6	13.9 3.5	10.6 0.25
B EC1118	0.2 0	1.8 0.06	2.5 0.1	0.3 0.06	0.1 0.02	0.4 0.05	6.0 0.06	0.1 0.17	0 0	12.4 0.2
C 1:10[1]	0.2 0.01	1.8 0	2.5 0	0.3 0.06	0.1 0.01	0.3 0.02	5.9 0.16	0 0	0 0	12.1 0.1
D 1:1	0.2 0	1.9 0.21	2.5 0.26	0.4 0.06	0 0.01	0.2 0.04	6.5 0.7	0.1 0.12	0 0	11.8 0.2
E 10:1	0.2 0	1.8 0.15	2.4 0.15	0.5 0.06	0 0.02	0.3 0.09	6.5 0.62	0.3 0.58	0.4 0.75	10.6 0.9

mean
standard deviation
[1] Treatments C, D and E ratios represent AWRI 860: EC1118

The *T'spora delbrueckii* AWRI 860 monoculture (treatment A) did not complete fermentation, leaving 17.5 g sugar/L to yield 10.6% ethanol. The EC1118 monoculture in treatment B was similar in composition to treatment A, although complete fermentation of sugar in this case produced a wine of 12.4% v/v ethanol. There were no treatment differences for the concentration of glycerol and most of the organic acids, except for the concentration of succinic acid produced by AWRI 860 (treatment A), which at 0.6 g/L was double that for the EC1118 treatment (B), but within the range of 0.07–1.61 g/L reported for different yeast species (Fuck and Radler 1972, Shimazu and Watanabe 1981). The concentration of malic acid also did not vary significantly between the monocultures, or any of the treatments, and was therefore not depleted differentially by the two yeast strains studied. *S. cerevisiae* generally utilises only a small amount of the available malic acid, although some non-*Saccharomyces* species can effectively ferment this acid. The concentration of glycerol did not vary significantly between any of the treatments.

The coinoculation treatments produced wines of similar composition to the monocultures. The concentration of succinic acid however, increased as the relative inoculation density of AWRI 860 increased, and may have been indicative of the involvement of this strain in fermentation. Differences in the concentration of ethanol were also noted. When AWRI 860 was inoculated at 10:1, the concentration of ethanol was 10.6% v/v, which was significantly lower than for EC1118 in monoculture, and the same as for the incomplete fermentation by AWRI 860. From these data it would appear that some differences in wine composition were attributable to the extent of growth of AWRI 860 in fermentation as was modulated by the inoculation protocol.

4.3.1.3. Conclusion

In monoculture *T'spora delbrueckii* AWRI 860 grew rapidly to a high population, although the utilisation of sugar during stationary phase occurred more slowly than for EC1118, and the population declined such that fermentation was incomplete after 14 days. Upon coinoculation, this strain was very competitive with EC1118 — depending upon the relative initial cell density. When inoculated at a ratio of 1:10, AWRI 860 did not achieve the cell density of monoculture before undergoing a population decrease in the later stages of fermentation. When inoculated at the ratio of 1:1 or 10:1 the strain became increasingly competitive, and at the higher ratio was able to achieve complete numerical domination of the fermentation. It would appear that the growth of this strain, especially in the earlier stages of fermentation with EC1118, was markedly influenced by its relative inoculation density. The fermentation time was also extended in association with the relative inoculation density of AWRI 860, suggesting that sugar depletion and fermentation rate were being limited somewhat by this slower growing strain. Of the wine components measured, the metabolic activity of AWRI 860 would appear to have only affected the production of succinic acid in those coinoculation treatments that favoured its growth. Analysis of other compounds of sensory importance such as esters and higher alcohols however, suggested further differences in wine composition as influenced by the fermentation treatment (results not shown).

4.3.2. COINOCULATION OF *C. STELLATA* CBS 843 AND *S. CEREVISIAE* EC1118

4.3.2.1. Cell growth and sugar consumption

The effect of coinoculation ratio upon yeast growth and sugar depletion during fermentation is shown in Figure 4.2. Some aspects of these graphs are summarised in Table 4.6.

For CBS 843 in treatment A, a 48 h lag phase was followed by a 48 h exponential growth phase to achieve a population of 5×10^7 CFU/mL in a minimum doubling time of 8.9 h (Table 4.6). The population then slowly increased to reach a maximum of 1.2×10^8 CFU/mL by day eight. This strain was fructophilic, depleting all of the fructose by day 10 to leave 4 g glucose/L at the end of fermentation on day 18. In Chardonnay juice this strain was able to
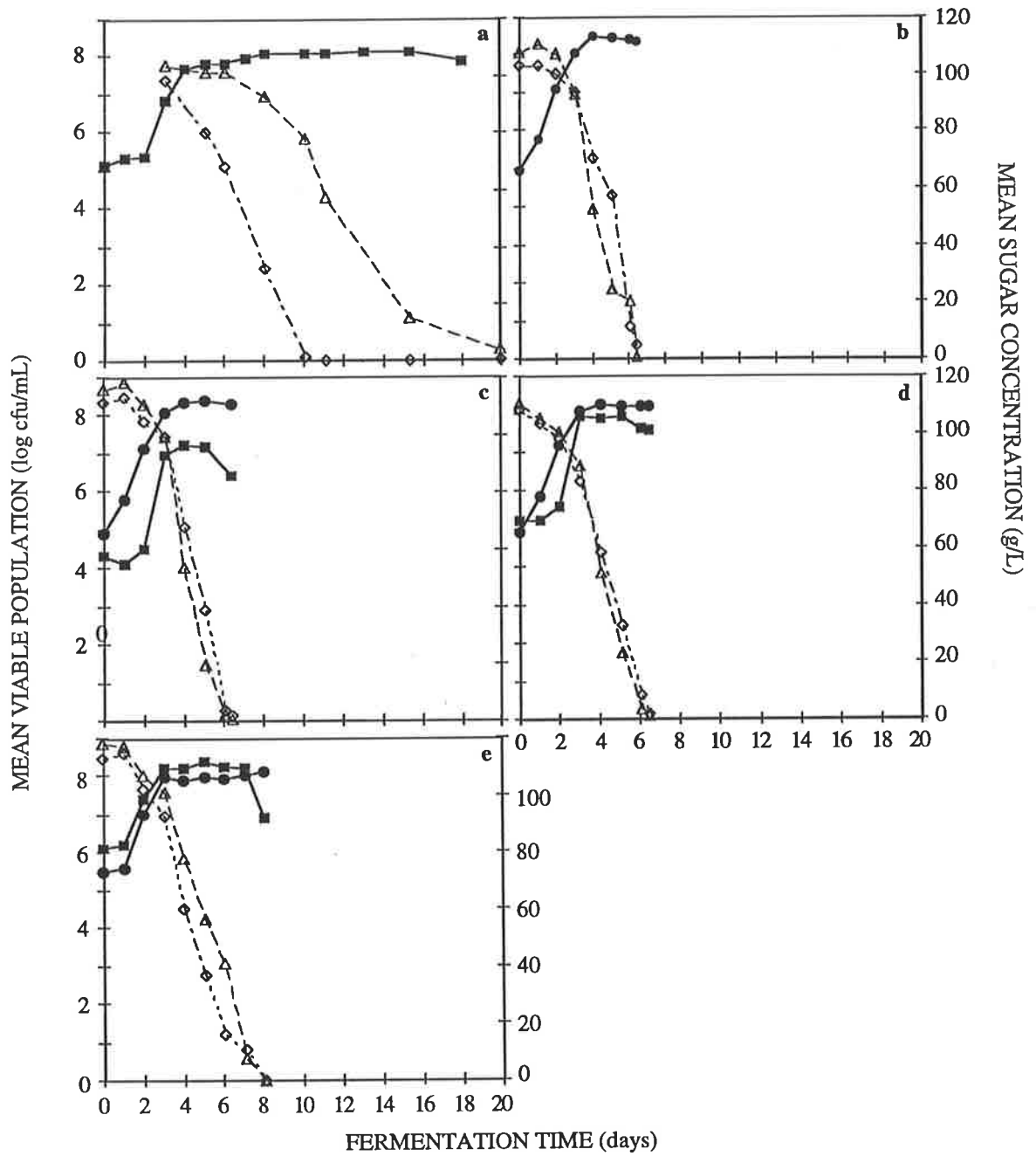
Figure 4.2. Effect of coinoculation ratio of *C. stellata* CBS 843 and *S. cerevisiae* EC1118 upon yeast population and sugar depletion during fermentation

a. Treatment A. *C. stellata* CBS 843 monoculture
b. Treatment B. *S. cerevisiae* EC1118 monoculture
c. Treatment C. CBS 843: EC1118, 1:10
d. Treatment D. CBS 843: EC1118, 1:1
e. Treatment E. CBS 843: EC1118, 10:1

———■———CBS 843   ———●———EC1118   — -▲- -glucose   -- ◇-- fructose

Table 4.6. Growth kinetics of *S. cerevisiae* EC1118 and *C. stellata* CBS 843 in mixed culture at different coinoculation ratios

Treatment	Strain	Starting population (CFU/mL)[1]	Duration of fermentation (d)	Max. population (CFU/mL)	Min. doubling time	Glucose utilisation max. rate (g/L/d)	Fructose utilisation max. rate (g/L/d)
A CBS 843	CBS 843	1.3×10^5	18	1.2×10^8	8.9	12	14
B EC1118	EC1118	9.4×10^3	6.4	2.8×10^8	8.3	27	26
C 1:10²	CBS 843	2.2×10^4		1.6×10^7	2.9		
	EC1118	8.5×10^4	6.4	2.4×10^8	7	32	32
D 1:1	CBS 843	1.8×10^5		1.3×10^8	3.1		
	EC1118	2.5×10^5	6.4	1.9×10^8	6.9	28	25
E 10:1	CBS 843	1.3×10^6		1.7×10^8	6.1		
	EC1118	9.7×10^4	8.1	1.1×10^8		23	25

[1]The initial viable population is consistently lower for CBS 843 than expected due to cell aggregation (see 4.2.3)
[2]The ratios in treatments C, D and E represent CBS 843:EC1118, based upon microscopic cell counts

complete fermentation of 20g sugar/L in seven days, suggesting that the synthetic medium may have imposed some limitation upon the growth of this strain, given that the other conditions such as the temperature of fermentation and the agitation rate of the vessels were the same.

The preferential uptake of fructose by *C. stellata* has also been noted by Minárik *et al.* (1978). Monosaccharide uptake by *S. cerevisiae* is considered to be rate limiting in sugar fermentation (Gancedo and Serrano 1989), and although sugar uptake mechanisms have been studied in some species of *Candida* (Cartwright *et al.* 1989), there is no information specifically on *C. stellata*. The faster fermentation of fructose by the Sauternes yeast *Zygosaccharomyces bailii* however, was found by Sols (1956), to be due to transport of glucose and fructose into the cell by a permease with a higher affinity for fructose. Contradictary to this finding, Emmerich and Radler (1983) showed that other *Zygosacch. bailii* isolates exhibited preferential uptake of fructose mediated by separate carriers or uptake mechanisms. The biological significance of the preferential fermentation of fructose is unknown, but these authors speculated that it may be linked to the osmotolerance of this species, although a reason for this was not given. Coincidentally, *C. stellata* has been reported to be tolerant of high sugar content grape juice (Lafon-Lafourcade, 1983) and juice concentrates (Deak and Beuchat 1993). The almost exclusive uptake and fermentation of fructose by CBS 843 in the presence of glucose is a very interesting trait worthy of further study.

EC1118 (treatment B) grew without a lag phase to reach a maximum population of 2.8×10^8 cells/mL in four days, in a minimum doubling time of 8.3 h. Glucose and fructose were

depleted at similar rates with a combined maximum of 53 g/L/d — double that of CBS 843 — to complete fermentation in 6.4 d.

In treatment C, where EC1118 was inoculated at ten times the density of CBS 843 — or four times the density as suggested by the viable plating results — fermentation was completed in the same time as for EC1118 in monoculture. CBS 843 grew to reach a maximum population 1.6×10^7 cells per mL which represented only 13% of the growth achieved in monoculture. Interestingly, after a lag phase of two days, exponential growth of this strain occurred at a very rapid doubling time of 2.9 h which was sustained for two further days, followed by a decrease in the population of CBS 843 occurring at 6.4 d. EC1118 reached a maximum population that was 84% of that attained in monoculture, and the minimum doubling time was decreased to 7 h. EC1118 dominated this fermentation numerically, representing no less than 94% of the total population at any point. Fermentation was completed in 6.4 d as by EC1118 in monoculture.

Interestingly, when inoculated at a ratio of 1:1 with EC1118 (treatment D), CBS 843 exceeded its maximum population in monoculture by 14%, achieving this a day sooner. EC1118 was reduced to 66% of its maximum population in monoculture. CBS 843 again exhibited a 2 d lag followed by rapid growth with a doubling time of 3.1 h, whilst at its fastest rate EC1118 doubled in population every 6.9 h. Although the acute decrease in population of CBS 843 seen in treatment C,was not noted, a steady population decline was evident. Overall CBS 843 was more competitive at this ratio than at 1:10, in terms of growth rate and population some suppression of EC1118 was evident.

At an inoculation ratio of 10:1 (treatment E), the lag phase for CBS 843 was reduced from two days, as in Treatments C and D, to one day. CBS 843 exceeded its maximum population in monoculture by 48% achieving this in a doubling time of 6.1 h. This population increase suggested that a limiting factor in the synthetic medium was being furnished by EC1118 which improved the growth of CBS 843 when it was inoculated at a competitive ratio. The CBS 843 population decreased on the final day of fermentation which may be due to ethanol intolerance. EC1118 exhibited a one day lag phase not observed at the other inoculation ratios and only reached 38% of its monoculture population maximum. Throughout fermentation EC1118 was numerically dominated by CBS 843, and represented 60% or less of the total population, up until the last day of fermentation. The rate of growth of EC1118 and the population maximum was effectively reduced by the presence of CBS 843 under these conditions. The sugar utilisation curves also showed that at any time point there was less fructose than glucose present in the medium, as seen for sugar utilisation by CBS 843. The duration of fermentation for this treatment was extended by at least one day to eight days.

Table 4.7. Composition of wines made by coinoculation of *C. stellata* CBS 843 and *S. cerevisiae* EC1118 (g/L except ethanol, %v/v)

Treatment	citric acid	tartaric acid	malic acid	succinic acid	lactic acid	acetic acid	glycerol	glucose	fructose	ethanol
unfermented medium	0.2	2.5	3.0	0	0	0	0	100	100	0
A CBS 843	0.3	0.5	1.7	0.3	0	0.3	10.0	4.0	0	11.3
	0	0.22	0.08	0	0	0.01	0.84	3.1	0	0.56
B EC1118	0.2	0.4	2.1	0.3	0.1	0.3	5.9	0	0	11.8
	0	0.06	0.06	0	0	0.02	0.13	0	0	0.38
C 1:10[1]	0.2	0.5	2.1	0.3	0.1	0.3	6.4	0	0	11.9
	0.01	0.06	0	0.05	0	0.01	0.09	0	0	0.08
D 1:1	0.2	0.5	2.1	0.3	0.1	0.3	7.3	0	0	12.1
	0	0.12	0	0.05	0	0.02	0.15	0	0	0.13
E 10:1	0.2	0.6	2.0	0.3	0	0.4	7.8	0	0	12.2
	0	0.05	0.05	0	0	0.07	0.31	0	0	0.29

mean
standard deviation
[1] ratios in treatments C, D and E represent CBS 843: EC1118

4.3.2.2. Wine composition

The effect of cofermentation of CBS 843 and EC1118 upon wine composition is shown in Table 4.7. Firstly it was notable that CBS 843 almost completed fermentation (4 g glucose/L remaining), and exhibited greater production of ethanol at 11.3% v/v, than the 7–10% range reported for this species (Benda 1982). Comparison of the two monocultures (treatments A and B) showed some differences in composition for the wine components under consideration. The most notable difference was in glycerol concentration which was considerably higher at 10.0 g/L for CBS 843, than for EC1118 which produced 5.9 g/L.

Differences in the concentration of organic acids were not considered significant between the monocultures, and acetic acid in particular was produced at 0.3 g/L by both species. This was of note considering the differences in glycerol production and the empirical relationship between glycerol and acetate production (Gancedo and Serrano 1989). Although acetic acid is known to be formed in high amounts by some species of *Candida* (Shimazu and Watanabe 1981), similarly low production was evident for the *C. stellata* strain studied by Ciani and Picciotti (1995) in low sugar grape juice fermentation. Comparisons are tenuous however as many fermentation variables such as temperature, pH, and the initial concentration of sugar or nitrogen in the medium affect acetic acid production (Zoecklein *et al.* 1995).

Differences in wine composition were also evident between the coinoculation treatments although differences in the concentration of the organic acids were not generally significant for any of the treatments. Residual sugar differences were also minor. It is noteworthy that CBS 843 was almost able to completely ferment 200 g sugar/L with 4 g glucose/L remaining

under these conditions — albeit slowly. The production of glycerol however may be an indicator of the involvement of CBS 843 in fermentation, as its concentration of 10.0 g/L in monoculture (treatment A) was higher than that of EC1118 (treatment B) at 5.9 g/L. Glycerol concentration increased with the increase in inoculation ratio, to reach 7.8 g/L at 10:1 (CBS 843: EC1118). As glycerol is produced mostly at the beginning of fermentation as a response to hyperosmotic stress (Cartwright *et al.* 1989, Bisson 1993, Mager and Varela 1993), the treatment differences may relate more to the initial population density of CBS 843 than to its growth later in fermentation. The production of ethanol appeared to increase under coinoculation conditions, as compared to either species in monoculture (treatments A and B), but the variability in the analytical data for this compound makes the significance of this unclear. A recent study of *S. cerevisiae* mutants showed that the overproduction of glycerol was related to the decreased production of ethanol (Michinik *et al.* 1997). The metabolism of high glycerol producing non-*Saccharomyces* strains, may also result in a lower ethanol yield (Heard 1988), which has implications for the use of such strains in winemaking. Further, Ciani and Ferraro (1996) also found increased production of glycerol and decreased production of ethanol in *C. stellata* fermentations. The apparent overproduction of ethanol by treatments C–E, as compared to EC1118 in monoculture, probably relates more to the difficulty of accurately measuring this compound given the standard deviations of the data.

4.3.2.3. Conclusion

Generally, the coculture of CBS 843 with EC1118 improved its growth relative to monoculture, except at a low starting density and ratio (843: EC1118, 10^4:10^5 cells/mL). This may suggest that some factor necessary for maximum growth of this strain, which was absent or limiting in the synthetic grape juice medium, was being furnished by EC1118. At the highest ratio (843: EC1118, 10:1), the growth of CBS 843 exceeded that of EC1118, effectively introducing a lag phase for EC1118 and reducing the maximum population attained. The growth of strain CBS 843 would appear to be influenced by the cell number present at the beginning of fermentation, such that the higher the inoculation density, the greater the impact on fermentation with regard to the suppression of growth of *S. cerevisiae* EC1118.

4.3.3. COINOCULATION OF *C. STELLATA* CBS 2649 AND EC1118

4.3.3.1. Cell growth and sugar consumption

The effect of coinoculation treatment upon yeast growth and sugar depletion during fermentation is shown in Figure 4.3a–f. Data are graphed as means from duplicate determinations of three treatment replicates and some graphical data are summarised in Table 4.8.

Table 4.8. Growth kinetics of *S. cerevisiae* EC1118 and *C. stellata* CBS 2649 in mixed culture at different starting ratios

Treatment	Strain	Initial population (CFU/ml)	Duration of fermentation (d)	Max. population (CFU/ml)	Min. doubling time (h)	Glucose utilisation max. rate (g/L/d)	Fructose utilisation max. rate (g/L/d)
A CBS 2649	CBS 2649	6.5×10^4	25	3.1×10^7	9.6	4.2	26
B EC1118	EC1118	8.0×10^4	7	1.7×10^8	6.9	25	29
C 1:10[1]	CBS 2649	8.6×10^3	7	3.7×10^7	6.0	38	30
	EC1118	7.0×10^4		1.7×10^8	6.2		
D 1:1	CBS 2649	6.6×10^4	7	5×10^7	8.6	25	24
	EC1118	6.9×10^4		1.7×10^8	7.2		
E 10:1	CBS 2649	4.5×10^5	7	6.3×10^7	11.6	22	19
	EC1118	2.7×10^5		1.5×10^8	6.4		
F 50:1	CBS 2649	2.6×10^6	7	4.0×10^7	17.8	25	22
	EC1118	?[2]		1.0×10^8	6.7		

[1] ratios in treatments C-F represent CBS 2649: EC1118, based upon microscopic cell counts
[2] initial viable count results for EC1118 in treatment F unavailable due to systematic plating error

In the monoculture treatment (A), CBS 2649 was a slow growing strain that took 7 d to reach a low maximum population of 3.1×10^7 CFU/mL with a doubling time of 9.6 h during the exponential growth phase. This strain was able to ferment 98% of the available sugar (200g/L), to yield 11.3% ethanol v/v in 25 days. When this strain was used to ferment Chardonnay juice as described in Chapter 3, fermentation ceased after 14 days producing a wine of 8.7% ethanol v/v. The greater ethanol production and tolerance by this strain in the synthetic medium may be attributable to pH or nutritional differences which could have affected the fermentation capacity of CBS 2649 (Casey and Ingledew 1985). In treatment A, a population decrease however was noted two days before the completion of fermentation that may have been due to ethanol intolerance. As for the previous *C. stellata* strain studied, CBS 843, fructose was used almost exclusively for exponential growth, being depleted at a maximum rate of 26 g/L/d, whilst glucose utilisation was minimal. After exhaustion of the available fructose, glucose utilisation occurred at a low rate of 4.2 g/L/d — about one sixth the rate of fructose utilisation — such that fermentation took 25 days to complete.

In monoculture *S. cerevisiae* EC1118 (treatment B) completed fermentation in 7 d and attained a higher maximum population, 1.7×10^8 CFU/mL, than CBS 2649, with a faster doubling time of 6.9 h. Glucose and fructose were depleted at similar rates, 29 and 25 g/d respectively, during this exponential growth phase as is usual for *S. cerevisiae*.
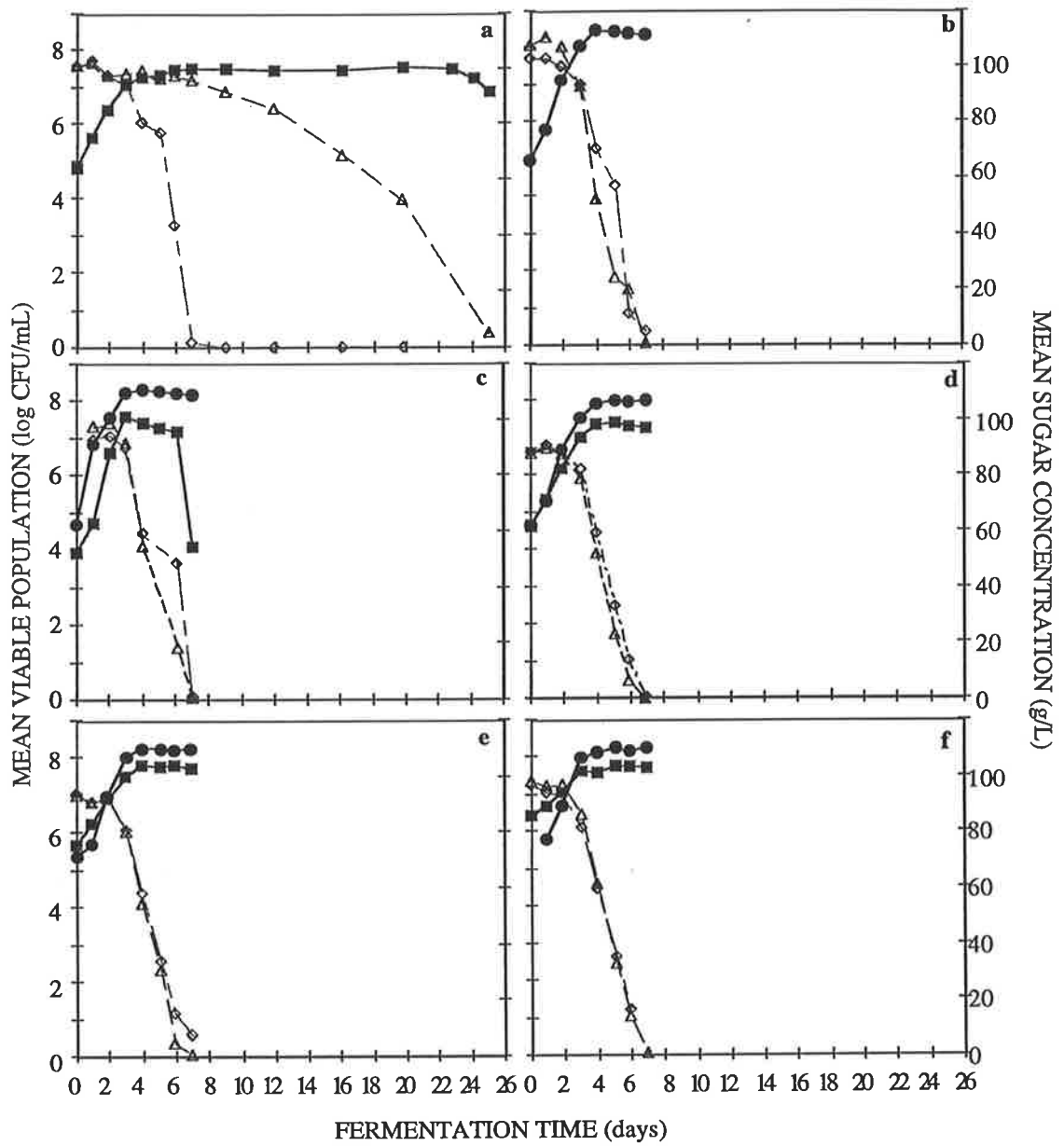
Figure 4.3.   Effect of coinoculation ratio of *C. stellata* CBS 2649 and *S. cerevisiae* EC1118 upon yeast population and sugar depletion

a. Treatment A. *C. stellata* CBS 2649 monoculture
b. Treatment B. *S. cerevisiae* EC1118 monoculture
c. Treatment C.   CBS 2649: EC1118, 1:10
d. Treatment D.   CBS 2649: EC1118, 1:1
e. Treatment E.   CBS 2649: EC1118, 10:1
f. Treatment F.   CBS 2649: EC1118, 50:1

——■——CBS 2649 ——●——EC1118   —–△—-glucose   – – ◇ – – fructose

At the 1:10 coinoculation ratio (CBS 2649: EC1118, treatment C), fermentation was completed in 7 d, as quickly as for EC1118 in monoculture (treatment B). Interestingly, the maximum population of 3.7 x10^7 CFU/mL reached by CBS 2649 was similar to that of the monoculture, but was reached a day sooner despite its low inoculation density relative to EC1118. This may suggest that some factor necessary for optimum growth of this strain, that was deficient in the synthetic grape juice medium, was being provided by EC1118. The presence of CBS 2649 did not however affect the growth kinetics of EC1118 at this relatively low inoculation ratio, despite the multiplication of *C. stellata* to establish a notable population. A slight decrease in doubling time to 6.2 h was noted for EC1118, but the monoculture maximum cell yield of 1.7 x10^8 CFU/mL was attained. After CBS 2649 reached stationary phase, a steady decline in viable population was noted, with a sharper decrease on the last day of fermentation. This cell death may not have been due to ethanol toxicity alone, as it did not occur in the other coinoculation treatments (D—F) which each produced a similar amount of ethanol.

Unlike the results for AWRI 860 and CBS 843 at the same ratio (see 4.3.1 and 4.3.2), the presence of EC1118 didn't suppress the growth of CBS 2649, as compared to its growth in monoculture. A slight enhancement of CBS 2649 growth, that is, increased viable cell yield and decreased doubling time as compared to monoculture, in the presence of a numerically dominant EC1118 population, may have been an effect of this treatment. This must be interpreted cautiously as it may be that the monoculture growth conditions were limiting, even though fermentation was completed, and does not represent the potential growth of this yeast as occurred in mixed culture. For consistency however the growth kinetics of this strain in mixed culture are still compared to the monoculture growth kinetics.

When the two strains were inoculated at the same concentration (treatment D), CBS 2649 again exceeded its maximum population in monoculture (by 64%), The ten-fold increase in inoculation density of CBS 2649, as compared to the previous treatment (1:10), resulted in a greater cell yield for this strain, albeit at a slower doubling time of 8.6 h. EC1118 equalled its monoculture maximum of 1.7 x10^8 CFU/mL at the slightly protracted doubling time of 7.2 h, suggesting little compromisation of growth.

When inoculated at ten-fold the density of EC1118 (treatment E), CBS 2649 obtained a maximum population of 6.3 x10^7 CFU/mL that was higher than for the previous coinoculation treatments. The doubling time for CBS 2649 during exponential phase increased to 11.6 h, which was slower than in monoculture. The maximum population of EC1118 was 12% lower than in monoculture, and the doubling time during exponential growth was similar to that of the monoculture, so growth was not considered to be compromised.

Suppression of *S. cerevisiae* was only observed in treatment F where the inoculation density of CBS 2649 was 50-fold that of EC1118. The maximum population of EC1118 was reduced to 1×10^8 CFU/mL — 58% that of monoculture — although a similar doubling time of 6.7 h was observed during exponential growth. This suggested that the competitive effect of CBS 2649 in fermentation did not affect the generation time of EC1118 despite a purported slight suppression of the overall cell yield. CBS 2649 achieved a maximum population of 4×10^7 CFU/mL which was within the range of maxima for both the 1:1 and 10:1 coinoculation treatments, however the generation time was 17.8 h, six hours more than for the 10:1 treatment. Fermentation was nevertheless completed in seven days, although the total viable yeast population reached was 1.4×10^8 CFU/mL, 71% of which was CBS 2649. The other coinoculation treatments reached greater populations of around 2.1–2.2×10^8 CFU/mL, suggesting that the 50:1 ratio of CBS 2649 to EC1118 was perhaps slightly limiting to the growth of both strains. It is perhaps possible that a nutrient became depleted in these fermentations due to improved growth of the non-*Saccharomyces* yeast, which seemed to benefit from the presence of EC1118, as apparent at the 1:10 ratio. It was observed that as the cell density of CBS 2649 inoculated increased, so did the cell generation time, perhaps suggesting that the medium is less than optimum for the growth of this strain. Also, as the initial cell number of EC1118 decreased relative to CBS 2649, the supplementary effect of the presence of this strain may have been diminished.

4.3.3.2. Wine composition

On the basis of the constituents measured, the two monocultures produced distinctly different wines (Table 4.9). The CBS 2649 wine was higher in glycerol at 10 g/L, and perhaps lower in ethanol, at 11.3% v/v, than the EC1118 wine. Production of acetic acid by the two species did not vary significantly in these fermentations and was around 0.3 g/L. This was notable given the difference in the concentration of glycerol between the two monocultures and the usually empirical relationship between production of these two compounds.

Differences in wine composition were also evident between the coinoculation treatments. Glycerol production appeared to be a good indicator of the involvement of CBS 2649 in fermentation as its concentration was high in monoculture at 10.0 g/L, as compared to EC1118 at 6.1g/L; and increased with the increase in inoculation ratio to attain a concentration of 7.7 g/L at the 50:1 ratio. As glycerol is produced mostly at the beginning of fermentation (Cartwright *et al.*), the differences in concentration noted may relate more to the initial population density and metabolic activity of CBS 2649 than to its growth later in fermentation. Differences in the concentration of ethanol apparent between the cofermentation treatments were difficult to interpret due to the analytical variability of this volatile compound. An increased concentration of ethanol in the coinoculated wines, in comparison to the EC1118 monoculture wines (treatment B), was suggested for treatments C–F, but no

Table 4.9. Chemical composition of wines made by coinoculation and sequential inoculation of *C. stellata* CBS 2649 and *S. cerevisiae* EC1118 (g/L except ethanol, % v/v)

Treatment	citric acid	tartaric acid	malic acid	succinic acid	lactic acid	acetic acid	glycerol	glucose	fructose	ethanol
unfermented medium	**0.2**	**2.5**	**3.0**	0	0	0	0	**100**	**100**	0
A CBS 2649	**0.3**	**0.5**	**1.7**	**0.3**	0	**0.3**	**10.0**	**3.9**	0	**11.3**
	0.02	0.22	0.08	0	0	0.01	0.84	3.08	0	0.56
B EC1118	**0.2**	**0.4**	**2.1**	**0.3**	**0.1**	**0.3**	**6.1**	0	**0.4**	**12.0**
	0.02	0.06	0.16	0.04	0.03	0.13	0.32	0	0.80	0.60
C 1:10[1]	**0.2**	**2.2**	**2.5**	**0.4**	**0.1**	**0.5**	**6.7**	**0.8**	**1.1**	**12.5**
	0	0.21	0	0.07	0.03	0.37	0.42	0.90	0.64	0.07
D 1:1	**0.2**	**1.1**	**1.9**	**0.3**	**0.1**	**0.2**	**7.1**	0	0	**12.1**
	0	0.29	0.05	0	0.02	0.06	0.67	0	0	0.36
E 10:1	**0.2**	**0.9**	**2.0**	**0.3**	**0.1**	**0.3**	**7.6**	0	0	**12.7**
	0.01	0.16	0	0.06	0.03	0.05	0.29	0	0	0.05
F 50:1	**0.2**	**1.3**	**2.0**	**0.3**	**0.1**	**0.3**	**7.7**	0	0	**12.3**
	0.01	0.40	0.04	0.05	0.02	0.03	0.19	0	0	0.57

mean
standard deviation
[1] ratios in treatments C-F represent CBS 2649: EC1118, based upon microscopic cell counts

particular trend was evident and some standard deviations were high (Table 4.9).

Generally, differences in the concentration of the organic acids did not vary greatly between treatments. Differences in the concentration of tartaric acid probably related more to precipitation of tartrate during storage than to yeast growth as this acid has not been reported as a fermentation substrate for any yeast (Radler, 1993). Differences in the deposition of tartrate in identical samples can occur during storage at -20°C. Differences in the concentration of residual sugar were considered minor. It is noteworthy that CBS 2649 was able to almost completely ferment 200 g sugar/L under these conditions — albeit slowly.

4.3.3.3. Conclusion

This experiment showed that a weakly fermentative *C. stellata* strain, CBS 2649, could completely ferment a synthetic grape juice medium. The extent of growth of this strain, in the presence of a fast fermenter, the commercial *S. cerevisiae* strain EC1118 was very much dependant upon the inoculation protocol. By conferring a numerical advantage at inoculation, CBS 2649 was able to grow appreciably and affect the chemical composition of the wine. Coinoculation conditions of increasing density of CBS 2649 relative to EC1118 improved its growth and contribution to fermentation, but suppression of EC1118 was not noted except where CBS 2649 was inoculated at a 50-fold higher density. It is important to note however, that inoculation with EC1118, even when CBS 2649 was inoculated at only one tenth of the cell density, did not inhibit the multiplication of the *C. stellata* strain. This concurs with the

findings of Heard and Fleet (1985,1986) that the growth of non-*Saccharomyces* yeast was not suppressed in inoculated fermentation.

4.3.4. SEQUENTIAL INOCULATION OF *C. STELLATA* CBS 2649 AND *SACCH. CEREVISIAE* EC1118

4.3.4.1. Cell growth and sugar consumption

Sequential inoculation treatments consisted of inoculation with CBS 2649 and *S. cerevisiae* at different intervals, that is: inoculation with *S. cerevisiae* one day before, the same time as, and one, two and three days post-inoculation with CBS 2649. Reference wines were made by monocultures of each of the two yeasts (Table 4.10). The effect of inoculation treatment on the growth of the yeasts and sugar depletion during fermentation is shown in Figure 4.3g–m and summarised in Table 4.11.

Table 4.10. Sequential inoculation protocols for mixed culture fermentation with CBS 2649 and EC1118

Treatment code	Protocol and time of inoculation of EC1118 relative to CBS2649
G[1]	CBS 2649 monoculture
H	EC1118 monoculture
I	1 day earlier
J	same day
K	1 day later
L	2 days later
M	3 days later

[1]Intended inoculation density of CBS 2649 and EC1118 was 1×10^6 cells/mL for all treatments

Sequential inoculation demonstrated the effect of the timing of inoculation upon the growth of the strains EC1118 and CBS 2649. When CBS 2649 (treatment G), was inoculated at a viable density of 9.5×10^4 cells/ mL, fermentation was completed in 12 d. As noted previously the viable population of CBS 2649 was substantially lower than the intended inoculation density, due to the clumping nature of this yeast, and a lesser than 100% viability of the starter culture as assumed for calculation of the necessary amount of inoculum. The maximum population of 9.9×10^7 CFU mL was reached in 5 d, with a population decrease noted on the last day of fermentation. Growth was increased and fermentation time decreased as compared to the lower density coinoculation monoculture (treatment A, see section 4.3.3.1), probably because the starting population was tenfold higher, enabling a greater maximum population to be achieved under conditions that would appear to be less than optimal.

The EC1118 monoculture (treatment H) took six days to complete fermentation at an initial viable density of 9.1×10^5 cells/mL, as compared to 7 d for the lower density monoculture,
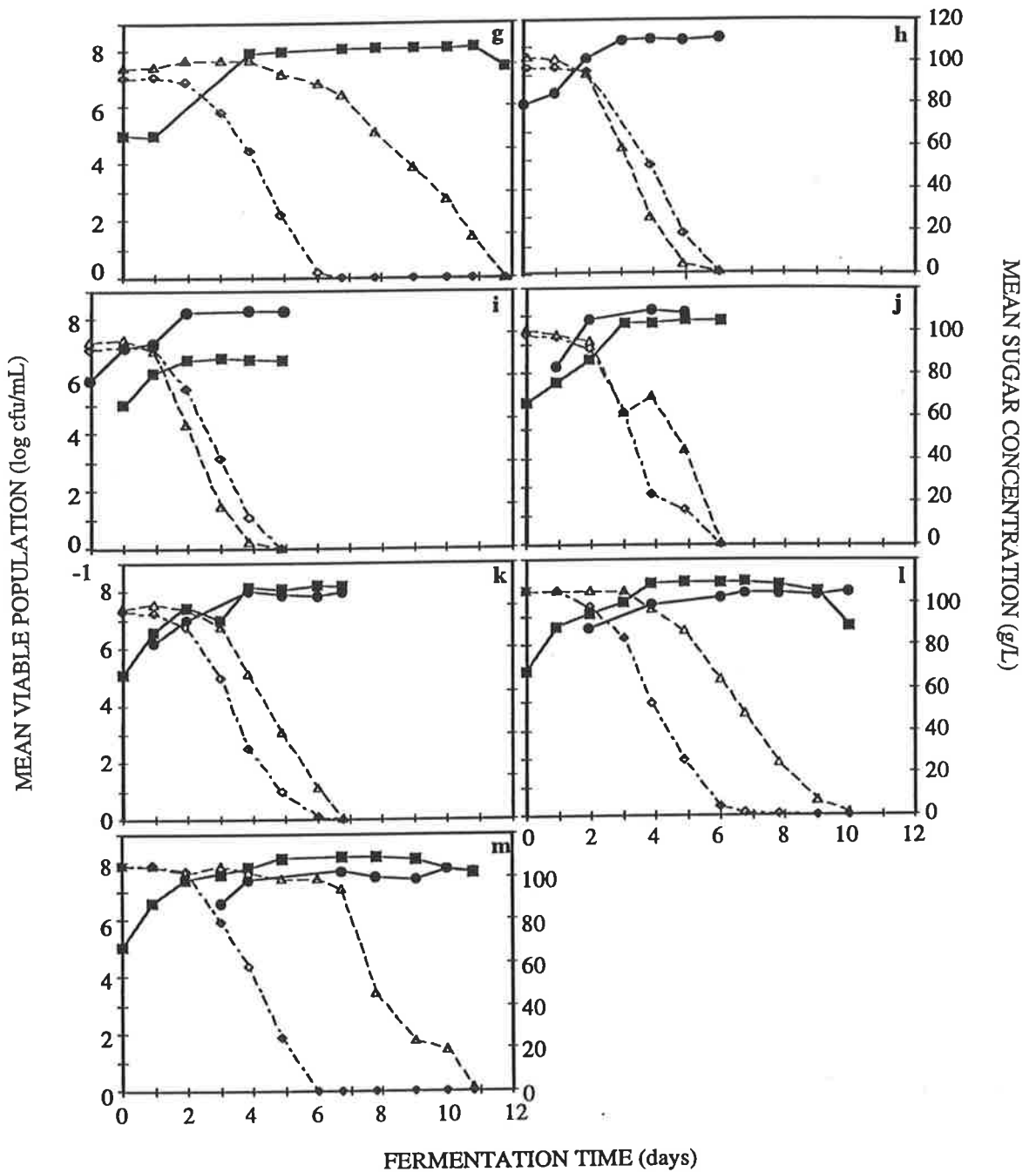
Figure 4.3.   Effect of sequential inoculation interval of *C. stellata* CBS 2649 and *S. cerevisiae* EC1118 upon yeast population and sugar depletion

g. Treatment G.   *C. stellata* CBS 2649 monoculture

h. Treatment H.   *S. cerevisiae* EC1118 monoculture

i. Treatment I.   EC1118 inoculated at day -1 and CBS 2649 inoculated at day 0

j. Treatment J.   CBS 2649 and EC1118 both inoculated at day 0

k. Treatment K.   CBS 2649 inoculated at day 0, and EC1118 inoculated at day +1

l. Treatment L.   CBS 2649 inoculated at day 0, and EC1118 inoculated at day +2

m. Treatment M.   CBS 2649 inoculated at day 0, and EC1118 inoculated at day +3

———■——CBS 2649 ——●——EC1118   — -▲- -glucose   -- ◇ -- fructose

Table 4.11. Growth kinetics of *C. stellata* CBS 2649 and *S. cerevisiae* EC1118 in sequential fermentation

Treatment (day of EC1118 inoculation)	Strain	Starting population (CFU/mL)	CBS 2649 pop. when EC1118 inoc. (CFU/mL)	Time to complete fermentation (d)	Max. population reached (CFU/mL)	Min. doubling time	Glucose utilisation max. rate (g/d)	Fructose utilisation max. rate (g/d)
G	CBS 2649	9.5×10^4	-	12	9.9×10^7	7.1	16.4	22.3
H	EC1118	9.1×10^5	-	6	1.7×10^8	9.1	35.0	31.4
I (-1)[1]	CBS 2649 EC1118	9.6×10^4 6.6×10^5	0	6	3.6×10^6 1.8×10^8	6.3 8.8	24.7	26.7
J (0)	CBS 2649 EC1118	8.7×10^4 nd	8.6×10^4	6	5.8×10^7 1.5×10^8	7.7 4.4	23.3	35.1
K (+1)	CBS 2649 EC1118	1.2×10^5 2.0×10^6	3.8×10^6	7	1.5×10^8 1.1×10^8	4.5 11.6	25.1	29.4
L (+2)	CBS 2649 EC1118	1.2×10^5 2.1×10^6	2.5×10^7	10	1.5×10^8 7.6×10^7	9.0 21.7	17.6	23.2
M (+3)	CBS 2649 EC1118	1.1×10^5 3.1×10^6	4.3×10^7	10	1.4×10^8 5.2×10^7	6.0 23	23.0	25.4

[1] day of inoculation of EC1118 relative to CBS 2649
nd, not determined

treatment B, although the same maximum population of 1.7 $\times 10^8$ CFU/mL was reached at a slower doubling time of 9 h. As expected the sugars were fermented at similar rates.

In treatment I, EC1118 was inoculated 24 hours prior to CBS 2649. Fermentation was completed in 6 d, as for the EC1118 monoculture, treatment H. CBS 2649 reached a maximum population that represented only 4% of that population achieved in monoculture. So, prior inoculation with EC1118 resulted in a profound inhibition of growth of CBS 2649 not seen for coinoculation (see 4.3.3). The ameliorative effect of cofermentation with EC1118 upon the growth of CBS 2649 (see section 4.3.3.1) did not occur in this treatment. The competitive disadvantage conferred to CBS 2649 by inoculation in the presence of the actively growing, strongly fermentative *S. cerevisiae* strain may have been due to nutrient depletion and substrate modification by EC1118. Even so, CBS 2649 still multiplied to reach and maintain a viable population of 3.6 $\times 10^6$ CFU/mL throughout fermentation.

In treatment J, CBS 2649 and EC1118 were inoculated simultaneously at the same intended density, although due to a plating error the actual implanted population of EC1118 was not determined, and as noted previously the density of *C. stellata* is probably underestimated by viable plating due to its tendency to clump. In three days, EC1118 reached 88%, and CBS 2649 reached 59%, of their respective monoculture population maxima. CBS 2649 exhibited much greater growth than in the previous treatment although its growth was not greater than in monoculture, as for the other simultaneously inoculated treatment (D), in the coinoculation experiment, perhaps due to the 10-fold increase in the inoculation density of both strains.

In treatment K, EC1118 was inoculated 24 hours after CBS 2649, when its population had reached 3.8 $\times 10^6$ cells/mL. CBS 2649 attained 150% of its maximum population in monoculture in about 4 d, again exhibiting the enhancement of growth in the presence of EC1118 seen for the coinoculation treatments, although this was not the case for the simultaneous inoculation treatment (J). The growth of EC1118 was partially suppressed, as this strain reached only 65% of its monoculture maximum in 3–4 d, with a slower doubling time of 11.6 h. Throughout fermentation, the CBS 2649 population exceeded that of EC1118, with the exception of one anomalous data point (Fig 4.3k). Fructose was utilised at a faster rate (29.4 g/L/d), than glucose (25.1 g/L/d), earlier in fermentation, and was completely exhausted a day sooner than glucose was, presumably indicative of the preferential fructose consumption by CBS 2649. It would seem that prior inoculation by one day conferred a distinct competitive advantage to the weakly fermentative strain CBS 2649.

In treatment L, EC1118 was inoculated 2 d after CBS 2649, which had by then reached a population of 2.5 $\times 10^7$ CFU/mL. The growth of CBS 2649 was very similar to that of treatment K, with no alteration to the maximum population reached, or in the time taken to attain this. A slight decline in population occurred however on the final day of

fermentation. Strain EC1118 was numerically dominated until the final day of fermentation, and its exponential growth rate and maximum population were further reduced as compared to the previous treatment, although slight growth was noted for the duration of fermentation. The fermentation time was extended to 10 days. Faster fructose uptake was again noted with this sugar being depleted more than 3 d before glucose exhaustion, indicative of sugar uptake by CBS 2649 and therefore suggestive of greater metabolic activity of this yeast as compared to the glucophilic strain EC1118 in this fermentation.

In treatment M, EC1118 was inoculated 3 d after fermentation commenced, by which time the CBS 2649 population had reached 2.5×10^7 CFU/mL, before continued growth to a maximum of 1.5×10^6 CFU/mL. The EC1118 population reached 5.2×10^7 CFU/mL, its lowest value for any of the mixed culture treatments, representing 30% of its monoculture maximum. The maximum total yeast population was 1.9×10^8 CFU/mL, which was maintained for the duration of fermentation, with CBS 2649 representing 73% of this population. This three day inoculation interval treatment therefore most favoured the growth of CBS 2649 and partially suppressed the growth of EC1118. Fermentation time was extended to 11 d and the sugar uptake curves were the same as the CBS 2649 monoculture (treatment G). That is fructose was exhausted by day 6, and glucose five days later, suggestive of greater CBS 2649 activity.

Overall the generation time of EC1118 increased in proportion to its delay in inoculation. When EC1118 was inoculated 24 h before CBS 2649 its doubling time was 8.8 h, which increased to 23 h when EC1118 was inoculated 3 d after CBS 2649. Also in treatments L and M, EC1118 was present for eight days before the completion of fermentation, which inferred decreased metabolic activity as compared to fermentation in monoculture (treatment H), where this yeast completed fermentation in just six days.

4.3.4.2. Wine composition

The composition of the wines produced by the sequential inoculation treatments is shown in Table 4.12. For the wine produced by the monoculture of CBS 2649 (treatment G), the concentration of glycerol was greater, and the concentration of acetic acid slightly lower, than for the wine in treatment A. As the production of glycerol takes place mostly at the beginning of fermentation (Cartwright *et al.*1989), the increased production in treatment G may be related to the ten fold higher initial yeast population present here as compared to treatment A. It was not possible to determine by comparison of treatments A and G if the increased production of glycerol related to a decrease in ethanol concentration, as some variability in ethanol measurement was evident. Other differences in composition between the monoculture treatments A and G were insignificant, except for a difference in the concentration of tartaric acid related to precipitation rather than yeast activity. Such differences in tartrate deposition in otherwise similar wines are often seen and perhaps relate to differences in the presence of particles that act as nuclei for crystal formation.

Table 4.12. Chemical composition of wines made by sequential inoculation of *C. stellata* CBS 2649 and *S. cerevisiae* EC1118 (g/L except ethanol, % v/v)

Treatment	citric acid	tartaric acid	malic acid	succinic acid	lactic acid	acetic acid	glycerol	glucose	fructose	ethanol
unfermented medium	0.2	2.5	3.0	0	0	0	0	100	100	0
G CBS 2649	0.3 0.02	1.9 0.10	1.9 0	0.4 0.05	0 0	0.1 0.02	11.6 0.17	0 0	0 0	11.2 0.15
H EC1118	0.2 0.01	0.7 0.06	1.9 0.06	0.2 0.04	0.1 0	0.4 0.02	6.8 0	0 0	0 0	12.0 0.06
I -1 d[1]	0.2 0.01	0.7 0.05	1.9 0.06	0.2 0.07	0.2 0	0.4 0.02	7.0 0.19	0 0	0 0	12.3 0.28
J same d	0.2 0.01	1.1 0.12	2.0 0.06	0.2 0	0.1 0	0.4 0.04	8.0 0.13	0 0	0 0	12.2 0.19
K +1 d	0.2 0.01	1.0 0.18	2.0 0.05	0.2 0.06	0.1 0.05	0.2 0.01	9.6 0.22	0.3 0.37	0 0	12.1 0.18
L +2 d	0.2 0.01	1.0 0.29	2.2 0.05	0.3 0.06	0.1 0.05	0.3 0.01	10.4 0.29	0.2 0.28	0 0	11.9 0.26
M +3 d	0.3 0.02	1.2 0.06	2.2 0.06	0.3 0.06	0 0	0.4 0.01	11.6 0.21	0.8 0.95	0 0	11.7 0.15

mean
standard deviation
[1] timing of inoculation of CBS 2649 relative to that of E1118

The composition of the wine made by Treatment I, where EC1118 was inoculated 24 h prior to CBS 2649, showed only minor differences to that of the EC1118 monoculture, and on the basis of glycerol production, which was not significantly greater than for treatment H, minimal metabolic activity of CBS 2649 may be inferred. The sugar depletion curves also do not demonstrate the fructophilic trend evident of CBS 2649 activity.

The concentration of glycerol increased with the increase in interval between CBS 2649 and EC1118 inoculation, from 7 g/L when EC1118 was inoculated a day earlier than CBS 2649, to 11.6 g/L when CBS 2649 was inoculated 3 d before EC1118. This would appear to be indicative of the impact of CBS 2649 metabolic activity, at least in the early stages of fermentation when glycerol production is highest (Cartwright *et al.* 1989, Bisson 1993). The glycerol content of treatment G wine was 11.6 g/L, as high as for any of the mixed culture ferments, suggesting that, of the coinoculation and sequential inoculation protocols tested for this yeast, preinoculation by three days most favoured the metabolic activity of CBS 2649. As the concentration of glycerol increased it might be expected that the concentration of ethanol would have shown a concomitant decrease (Michinik *et al.* 1997), as may have occurred for treatments K–M. Analytical standard deviations however, make confirmation of this observation necessary. In the least it would appear that the treatments I (EC1118 preinoculated by one day), and M (EC1118 postinoculated by 3 d), differed significantly in the yield of ethanol, which was 12.3% and 11.7% v/v respectively.

When CBS 2649 was preinoculated by 2 and 3 days in treatments L and M respectively, growth and sugar consumption by the non-*Saccharomyces* yeast had occurred by the time of inoculation of EC1118, and a change in the composition of the medium, in terms of nutrient depletion and metabolite production also occurred. This could perhaps account for the diminished growth of EC1118, and the resulting differences in wine composition. It has been found that the fermentation activity of non-*Saccharomyces* yeast is likely to influence the medium composition and affect the growth and biochemical behaviour of *S. cerevisiae* (Herraiz *et al.* 1990). Zironi *et al.* (1993), also found that inoculation of *S. cerevisiae* into a must partially fermented by apiculate yeasts significantly modified its metabolism with respect to higher alcohol production. Preinoculation of the *C. stellata* CBS 2649 in these experiments also affected *S. cerevisiae* EC1118 activity.

4.3.4.3. Conclusion

Sequential inoculation of two yeasts at the same density offered an effective and predictable way to modulate the growth of both of the participating strains. Preinoculation by one day of the faster growing strain *S. cerevisiae* EC1118 partially suppressed the growth and metabolic activity of CBS 2649, despite the *C. stellata* strain being inoculated at a high concentration of 1 $\times 10^6$ cells/mL (9.6 $\times 10^4$ CFU/mL). Conversely, initial inoculation with CBS 2649, followed by inoculation with EC1118 at increasing intervals favoured the growth and expression in fermentation of the slow growing strain CBS 2649. The longer the interval before inoculation with EC1118, the greater the fermentation activity of CBS 2649, and the suppressive effect upon EC1118 later in fermentation. The improved growth of the non-*Saccharomyces* yeast also affected the composition of the wine, especially with respect to glycerol concentration.

Bisson and Kunkee (1991) have suggested that the growth rate of yeasts early in fermentation is of minor importance, and that growth rates later in fermentation, and differences in cell concentration are more useful predictors of strain involvement, as these relate to competition for nutrients, and inhibitory effects of compounds such as ethanol. Some nutrients such as oxygen and nitrogen are depleted early in fermentation, when some compounds such as glycerol and acetic acid are formed (Whiting 1976). It is suggested in these experiments however, that the impact of a yeast can occur early in fermentation, and may not relate only to its overall persistence.

4.4. OVERALL CONCLUSION

In these experiments three non-*Saccharomyces* yeasts, *T'spora delbrueckii* AWRI 860, and *C. stellata* strains CBS 2649 and CBS 843, exhibited different growth and fermentation properties but were all able to yield up to 11.3% ethanol v/v, albeit more slowly than EC1118. The two *C. stellata* strains were characterised by preferential uptake of fructose and production of a high concentration of glycerol, which was useful for inferring the extent of the involvement of these strains in cofermentation.

Coinoculation modulated the growth of the participating yeasts depending upon the relative inoculation density and fermentative capacity of these strains. The growth and metabolism of the non-*Saccharomyces* yeasts could be enhanced — to the detriment of EC1118 — to achieve quantitative dominance in fermentation. The effect of a particular coinoculation ratio was strain dependent, relating to the growth and fermentation rate of the participating non-*Saccharomyces* yeast. For instance, the slowest fermenter studied, *C. stellata* CBS 2649, showed improved growth in the presence of EC1118 at a relative inoculation density of 1:10, which was not noted for the two other non-*Saccharomyces* strains studied. CBS 2649 grew best and was also able to slightly suppress the growth of EC1118, only when inoculated at a 50-fold greater density than the *S. cerevisiae* strain, whereas the more rapid fermenter *T'spora delbrueckii* AWRI 860 was able to achieve domination of fermentation at the lower coinoculation density of 10:1 (AWRI 860: EC1118). Depending upon the non-*Saccharomyces* strain used for fermentation and the inoculation treatment, the wine composition and the fermentation time were also affected.

The effect of coinoculation upon fermentation would therefore appear to depend upon the fermentation vigour of the two participating strains, and the choice of a suitable coinoculation protocol would therefore depend upon the yeast strain combination to be used. Importantly, fermentation conditions that influence the growth of the non-*Saccharomyces* yeasts such as temperature would also affect the outcome (Fleet *et al.* 1989). Determination of the optimal coinoculation protocol, under the appropriate conditions for particular strain combinations however, would lead to the increased growth and metabolic activity of non-*Saccharomyces* yeasts during fermentation, with concomitant effects upon the yield of important metabolites and the sensory character of the wine.

Sequential inoculation either greatly suppressed or enhanced the growth of the slow fermenter CBS 2649, depending upon the timing of inoculation. Preinoculation of EC1118 severely inhibited the growth of CBS 2649, although this did not occur during coinoculation. Inoculation of CBS 2649 one day before EC1118 however improved its growth to the detriment of growth of the *S. cerevisiae* strain, and increasing this interval to three days resulted in severe suppression of EC1118 growth and domination of fermentation by the weakly fermentative *C. stellata* strain. So, even for the slow growing non-*Saccharomyces* yeast, CBS 2649, as well as a commercial *S. cerevisiae* strain, preinoculation resulted in significant and predictable effects upon yeast growth. The inoculation of faster growing strains using this protocol would surely emphasise the advantage conferred by preinoculation.

In winemaking by sequential culture, initial inoculation with a non-*Saccharomyces* yeast would allow uninhibited growth of this strain early in fermentation, with the option of inoculation after an appropriate interval with a *S. cerevisiae* strain to achieve additional sensory effects and ensure the completion of fermentation. It would however be necessary to

select strains that are able to effectively initiate fermentation under the required conditions. The timing of the second inoculation would also have to allow potentially for continued growth of the first strain. Sequential inoculation very late in fermentation, when a high concentration of ethanol has been attained, may not be successful if the latter yeast was not first acclimatised to the conditions.

Of potential value to winemakers is the use of fructophilic yeast strains such as *Candida stellata* CBS 2649 in sluggish or stuck fermentations. In some cases such problem fermentations are due to an alteration of the ratio of residual glucose and fructose, such that fructose may be present at a greater relative concentration (Schütz and Gafner 1993b). Should the fructophilic yeast be sufficiently ethanol-tolerant its inoculation as a "rescue" strain to consume fructose and complete fermentation could conceivably be successful. Otherwise competitive inoculation of less ethanol-tolerant, fructophilic strains at the beginning of fermentation may also prevent such problems from occurring.

This study, although preliminary, has highlighted the effect of inoculation protocol, be it inoculation ratio, or timing of inoculation of two strains, on yeast growth, sugar depletion, fermentation kinetics and the concentration of some wine components. Further studies would need to confirm the effects of different combinations of strains, and different fermentation conditions, upon the outcome of particular inoculation protocols. In this way conditions can be further established for the exploitation of the biochemical and sensory diversity of non-*Saccharomyces* yeast in a controlled fermentation of predictable outcome.

Chapter 5

THE EFFECT OF MIXED YEAST SPECIES FERMENTATION ON THE AROMA AND COMPOSITION OF CHARDONNAY WINE

5.1. Introduction

The composition and sensory character of wine is determined by grape variety, viticulture and the metabolism of yeasts during fermentation (Amerine and Joslyn 1970, Margalith and Schwartz 1970, Benda 1982). The diversity of metabolic activities and products of the non-*Saccharomyces* yeasts can impact upon the kinetics of fermentation and the composition of the resulting wine as reported in Chapters 3 and 4.

Although chemical analysis has identified and measured many yeast metabolites considered to influence wine 'quality' and aroma (Van Zyl *et al.* 1963, Soufleros and Bertrand 1979, Millán and Ortega 1988, Mateo *et al.* 1991, Romano *et al.* 1997), formal sensory studies of wines made with non-*Saccharomyces* yeasts would appear to be less prevalent. Sensory evaluation of wine allows the accurate and reproducible measurement of complex wine sensory attributes by human judges. Although judges are highly prone to variability and bias in their responses, they are the only instruments capable of measuring a number of important wine attributes (Meilgaard *et al.* 1991). Sensory testing has therefore developed as a formalised, structured discipline to minimise tasters' variability and bias.

In this study the technique of descriptive analysis of wine aroma was used to describe and measure differences in wine aroma. Descriptive analysis, or quantitative descriptive analysis is a scoring method used for the evaluation of sensory aspects of food and beverages. A group of judges together develop a vocabulary for attributes that describe and differentiate the samples. Judges are then trained in the recognition and rating of the intensity of these attributes, often with the use of a suitable reference to define the attribute (Cairncross and Sjöström 1950, Stone *et al.* 1974).

Sensory studies concerned with the role of non-*Saccharomyces* yeasts have mostly compared 'spontaneously' fermented wines to those made with *S. cerevisiae* under similar conditions. In such spontaneous fermentations the yeast composition may be diverse, but is largely unknown, and the relative importance and contribution of individual strains cannot be determined. Such studies have not demonstrated a positive effect of indigenous yeast fermentation upon wine sensory character (Bisson and Kunkee 1991, Bisson quoted in Ross 1997), although, for a number of reasons, this traditional winemaking practice is increasingly being used in commercial production in the USA and Australia with favourable results (Goldfarb 1994, Ramey 1995, Price 1996, Ross 1997). An alternative however to this often unpredictable fermentation technique would be the use of multiple, selected strains under

controlled inoculation conditions. The effect of yeast inoculation protocol upon fermentation kinetics and wine composition was investigated in Chapter 4, demonstrating that a suitable inoculation procedure can promote the growth, and participation in fermentation, of even a weakly fermentative non-*Saccharomyces* yeast in the presence of a commercial wine yeast that would be well adapted for the fermentation of grape juice.

From the sensory evaluation of some non-*Saccharomyces* yeasts reported in Chapter 3, a strain of *C. stellata*, CBS 2649, was selected for further sensory assessment. The species *C. stellata* is commonly isolated from grape must and has been found to be competitive and persistent in fermentation (Heard 1988, Mora *et al.* 1990, Mora and Mulet 1991), and can produce and tolerate up to a 9% v/v concentration of ethanol (Holloway *et al.* 1992). In this study the strain CBS 2649, in conjunction with the commercial *S. cerevisiae* strain, EC1118, was used to vinify a sterilised grape juice. Two mixed culture inoculation protocols identified as having different effects upon fermentation kinetics (Chapter 4) were selected for this study. That is coinoculation of CBS 2649 at ten times the inoculum density of EC1118; and sequential inoculation of EC1118 after the initiation of fermentation with CBS 2649. In addition reference wines were made by monocultures of each of the two yeasts. Yeast growth studies and profiling of the wines by chemical and sensory analysis detailed the contribution of CBS 2649 to these fermentations.

5.2. Materials and Methods

5.2.1. Juice and winemaking

Chardonnay juice from the Adelaide Hills 1997 vintage was provided by Petaluma Pty. Ltd. (Piccadilly, South Australia). The juice was diluted with Milli-Q® water (Millipore Aust. Pty. Ltd.) to reduce the concentration of sugar to 200 g/L as used in the mixed culture fermentation experiments (Chapter 4). The final composition was: pH, 3.27; free SO_2, 15 mg/L; total SO_2, 25 mg/L; titratable acidity (TA), 5.5 g tartaric acid/L; concentration of sugar, 205 g/L. Di-ammonium phosphate (Sigma, Australia), was added to give a final concentration of 200 mg/L. The juice was clarified by cross-flow filtration through a membrane of 0.45 μm pore size (Gilbert's Refrigeration and Airconditioning Services, Lonsdale, South Australia), and then sterile filtered into fermentation vessels via a 0.2 μm Sartobran PH capsule (Sartorius, Germany). All juice decanting was carried out under carbon dioxide or nitrogen gas pressure to exclude air and prevent oxidation. Twelve litre Pyrex glass bottles fitted with rubber stoppers modified to hold an airlock, gas inlet, filling port and sampling tube were used for fermentation. The gas port was fitted with a 0.2 μm membrane filter (Gelman Sciences, USA) and the sampling port was fitted with a sterile multidirectional stopcock (Discofix, Braun, Germany), and Luer lock (Braun, Germany). The stoppers were autoclaved (121°C, 20 min), and the fermentation vessels were sterilised by prolonged exposure to 70% v/v ethanol, thoroughly drained and then flushed with sterile CO_2 gas to exclude air.

5.2.2. Yeast strains

The yeast strains used in this experiment were *C. stellata* CBS 2649 (ex grape juice, France), received from the Centraalbureau voor Schimmelcultures (CBS) at Delft, The Netherlands, and *S. cerevisiae* EC1118 from Lallemand Pty Ltd Australia. Both yeasts were maintained in the AWRI culture collection on cryopreservant beads (Protect Bacterial Preservers, Technical Services Consultants, Ltd, UK) in the gaseous phase of a liquid nitrogen cryovessel.

5.2.3. Starter culture preparation

To propagate the yeast cultures a single cryopreservant bead was placed in a tube containing 10 mL of YM broth (Amyl Media, Australia) and incubated at 25°C for 24–48 h. A volume of 5 mL of this culture was then added to 500 mL of starter culture medium consisting of the Chardonnay juice which had been diluted one in two with sterile MilliQ® water. Starter cultures were incubated at 18°C for two to four days, and continuously sparged with sterile air through a submerged gas diffuser, until the culture had reached a density of 1–2 x10^8 cells/mL. For the sequential inoculation treatment, new starter cultures of EC1118 were prepared daily by subculture of the previous culture (10% v/v), into fresh medium so that a yeast culture in exponential growth phase was always available.

5.2.4. Fermentation treatments

Duplicate 10 L fermentations of the following four treatments (A–D) were conducted. The inoculum density for each yeast was 5 x10^6 cells/mL, except for treatment C, strain EC1118 which was inoculated at 5 x10^5 cells/mL. Treatment A: *C. stellata* CBS 2649 monoculture. Treatment B: *S. cerevisiae* EC1118 monoculture. Treatment C: Coinoculation of CBS 2649: EC1118 at a relative cell density of 10:1. Treatment D: Sequential inoculation whereby CBS 2649 was inoculated first, and, when fermentation activity ceased, EC1118 was superinoculated. The juice was inoculated with the required volume of starter culture by aseptic injection into the filling port. Fermentations were mixed with magnetically coupled stirrers operated at approximately 200 rpm, in an 18°C temperature controlled room. Vessels were gassed with filter sterilised nitrogen when carbon dioxide evolution by the ferments slowed, or during sampling procedures, to prevent the ingress of air through the fermentation lock.

The viable yeast population was determined by viable plating 30 minutes after inoculation, and at approximately 12 or 24 hour intervals thereafter. Fermentations were sampled aseptically by syringe withdrawal of 1 mL of culture. The total viable colony count was determined by serial dilution of the culture in 1% peptone (Amyl media, Australia) and plating of duplicate 25 µL aliquots of a range of dilutions onto YM agar (Amyl media, Australia). The viable colony count for CBS 2649 was determined in the same manner using Lysine medium (Oxoid, England). Lysine agar does not support the growth of *S. cerevisiae* beyond petite colonies (Radler 1985), which facilitated the differential enumeration of CBS 2649 in the presence of *S. cerevisiae*. Plates were incubated at 25°C and colonies were

counted after three or four days. A colony count for EC1118 was obtained by subtracting the CBS 2649 count from the total count. The accuracy of colony and strain differentiation was verified by PCR fingerprinting of colonies of each type as described in Chapter 2 (results not shown).

When the refractive index of the sample remained constant for three successive days, the fermentation was deemed finished, irrespective of the residual sugar concentration. Ferments of lower residual sugar (< 2%) were also able to be monitored for sugar depletion by the Clinitest assay (Ames, Miles Inc., USA). Treatment A did not complete fermentation, and was considered finished when the refractive index had not decreased for five successive days. The ferments were then stored at 4°C for seven days to sediment the yeast. The airlocks were sealed, and sterile nitrogen gas was supplied at low pressure to prevent the ingress of air. The wines were filtered through a Seitz pad filter EF 30/30, fitted with Ekwip filter pads, clarification grade D4, and sterilisation grade Z8, before sterile filtration through a 0.2 μm Sartobran PH capsule (Sartorius, Germany). The filtration unit, pads and capsule were rinsed with citric acid solution, followed by RO water, and then flushed with nitrogen gas before use. Between treatment lots the unit was rinsed with RO water, drained and regassed. The filtrate was tasted off before collection to avoid dilution of the wine. Filtration proceeded under nitrogen gas pressure with wine being collected in a sterilised, 12 L Pyrex glass vessel sealed with a bung fitted with a filling tube and gas inlet and outlet, fitted with 0.2 μm disposable filters (Schleicher & Schuell, Germany). After filtration the gas outlet was stoppered and the collection vessel pressurised with nitrogen gas. The wines were cold stabilised at 4°C for seven days, during which time a sterile solution of potassium metabisulfite (BDH, UK) in treatment wine, was added incrementally to achieve a concentration of free sulfur dioxide of 25–30 mg/L. Without further adjustment, the wines were aseptically decanted into sterilised, nitrogen-gassed, 780 mL bottles and crown sealed. Wines were stored at approximately 15°C for 4–6 weeks prior to chemical and sensory analysis.

5.2.5. Chemical analysis

The concentration of free and total sulfur dioxide was determined by the aspiration method (Rankine and Pocock 1970). The concentration of ethanol was determined by Near infra red spectrometry (Bran & Luebbe Infra-alyzer, USA). The pH and TA were determined with a autoburette and titrator (Radiometer, USA), according to Amerine and Ough (1980). The concentration of glucose and fructose was determined by enzyme kit (Boehringer Mannheim, Germany). The assay was facilitated by using a Cobas Fara automatic analyser (Roche Instruments, Switzerland), programmed to perform manipulations according to the enzyme kit manufacturer's instructions. The concentration of organic acids and glycerol was determined by High Performance Liquid Chromatography (Frayne 1986). The concentration of ethyl acetate and acetaldehyde in the wines was determined by Capillary Gas Chromatography (GC) (Amerine and Ough 1980).

5.2.6. Quantitative descriptive analysis

For quantitative descriptive analysis (QDA) of the aroma of the wines, 20 staff and postgraduate students of the Australian Wine Research Institute were selected as judges on the basis of availability and enthusiasm for the task. Of the 14 males and six females who participated, all but three were familiar with wine assessment and had previously participated in QDA studies. The age range of the judges was 22–60 years.

A number of training and discussion sessions, comprising both isolated booth sessions and group discussions were conducted in order to develop both a vocabulary of aroma descriptors and familiarity with the testing procedure. Sessions one and two comprised aroma and palate description of one wine from each treatment. Session three was a group presentation of three of the wines and individual selection by the judges of useful aroma descriptors from the complete list generated in the first two sessions. Session four was a group presentation of 23 aroma standards and one wine from each of the four treatments. On an individual basis the judges selected the standards that they considered were most appropriate for description of the wine aroma, and nominated which wines the terms were most useful for. The aroma intensity and character of the standards were also assessed. The judges chose to have the aroma standards presented as solids (eg fruit) or as aqueous solutions rather than as wine-based mixtures. Sessions five and six were group presentations of a smaller set of refined aroma standards together with wines from all four treatments. The judges noted which standards were the most useful for discriminating between all of the wines, and continued to comment on their composition. Session seven involved the group of judges reaching consensus on the final list of eight aroma descriptors and their formulation. A recognition test of these aroma standards in a neutral white wine base was successfully completed by each judge.

Four practice rating sessions in isolated booths were held to familiarise judges with the scoring system. The intensity of each of the eight aroma attributes was rated using a 10 point category scale where 0 was not detectable, 1 was just detectable, and 9 was of high intensity. Four wines were presented per session, with each judge receiving the same wines. Minor adjustment of the composition and concentration of some of the aroma standards continued according to judges' feedback.

Three formal rating sessions of the wines were held in which 14 judges each evaluated four different samples (wines). The final 14 judges were selected from the original pool of 20 on the basis of performance in the practice sessions. An incomplete block design for eight samples (Cochran and Cox 1957, plan 11.10), in which four samples were presented in 14 blocks, was repeated on three concurrent mornings. Wine samples (25 mL), from freshly opened bottles, were presented in XL5 glasses with petri dish covers. Judges worked in isolated booths under red lights. Each booth contained a set of the eight aroma standards which were smelled before a wine was presented, and could be referred to throughout the

session. The aroma standards and their composition are listed in Table 5.1. Water was also provided for sniffing as required. Judges evaluated four samples, presented one at a time to reduce intrablock correlation. When a judge had finished with a sample, it, and the score sheet were removed and a new sample and score sheet presented. Unique glass codes were used throughout the sessions, and the allocation of sample numbers to the wines, and the presentation order of the samples within a block (set), were randomised. Presentation of a set of samples to a judge was also at random.

Table 5.1. Aroma reference standards and composition

Aroma standard	Composition
Tropical fruit	Berri tropical fruit juice 80% v/v
Apricot	Goulbourn Valley fruit in juice 80% v/v
Banana	Ripe banana, 1 cm slice
Lime	Fresh lime juice 20% v/v + Bickford's lime juice cordial 20% v/v + 2 cm x 2 cm piece of lime peel and zest
Honey	Leabrook Farms pure honey 3.5% v/v
Rose	Queen natural rosewater 0.14% v/v + 1 cm x 1 cm piece of fresh rose petal
Ethyl acetate	Ethyl acetate 0.04% v/v
Sauerkraut	Gee Vee canned sauerkraut, liquid 1% v/v

All standards were presented as solids alone, or in MilliQ® water where dilution is indicated. For liquid standards approximately 15 mL was presented. All standards were presented in covered XL5 glasses.

5.2.7. Difference testing by duo-trio analysis

Fermentation duplicates were compared by duo-trio analysis (Amerine *et al.* 1965) to assess whether aroma differences existed within a treatment. Two treatments that were not distinguishable by descriptive analysis were also compared by this method of difference testing. The tests were conducted in balanced reference mode where the two samples being compared were both used as references, and sets were presented to the judges at random. Within each set the reference wine was identified and the order of presentation of the two sample wines was randomised (Meilgaard *et al.* 1991). Samples (20 mL) were presented in coded, covered, XL5 glasses under red lights in isolated tasting booths. Four sets were presented to each judge in each of two sessions. The judging panels numbered 23 and 21 staff and students of the Australian Wine Research Institute respectively, almost all of whom had previously participated in wine sensory testing. A judge evaluated each comparison once only.

5.2.8. Data analysis

The quantitative descriptive analysis data were interpreted by using the JMP version 3.10 statistical software (SAS Institute Inc.). A two-way analysis of variance (ANOVA), was

performed on each term rated by the judges to assess the main effects of wine sample and session. In addition, two sample t-tests were carried out on individual treatments to assess for variation between replicates for each attribute. A further one way ANOVA was performed for the effect of treatment for each attribute. A Principal Components Analysis (PCA), was performed on the mean scores for each of the aroma attributes for the eight wines using the correlation matrix. The duo-trio test results were interpreted using binomial probability tables, where p= 1/2, one tailed (Amerine *et al.* 1965).

5.3. Results

5.3.1. Yeast growth during fermentation

Figure 5.1 shows the yeast growth and sugar depletion during fermentation for each treatment. Generally, curves for each replicate were close and mean data are shown. For treatment D, however, one replicate showed an extended yeast growth lag phase and overall fermentation time, as the mean data would have been misrepresentative one replicate only is shown.

In monoculture (treatment A), CBS 2649 showed an immediate and significant drop in population after inoculation, the reason for this decline was not investigated but may be attributed to the presence of sulfur dioxide in the juice albeit at a free concentration of 15 mg/L. The yeast however recovered to stabilise at a maximum population of 5 $\times 10^7$ CFU/mL after nine days, during which time little sugar was utilised. Fermentation then proceeded slowly for 12 days, at which point all of the fructose had been utilised. Sugar uptake ceased at this point, even though the glucose concentration had not decreased since the beginning of fermentation. The EC1118 monoculture (treatment B), grew without exhibiting a lag phase to a viable population of almost 1 $\times 10^8$ CFU/mL in three days. The population then slowly declined as fermentation progressed to completion in 16 days. Glucose was utilised preferentially to fructose as has been previously discussed for this strain.

In treatment C, where the two strains were coinoculated, the initial decrease of viability of CBS 2649 — as seen in treatment A — did not occur. Possibly the presence of another yeast buffered CBS 2649 against the element that caused its death in monoculture. However, growth of this yeast did not occur, possibly due to nutrient competition or the production of inhibitory substances by EC1118, although this was not investigated. The growth of EC1118 was not affected by the presence of CBS 2649, and the rate and preference of sugar uptake was similar to the treatment B monoculture (EC1118).

In the sequential inoculation treatment (D), growth of CBS 2649 initially proceeded as for the monoculture treatment (A), Fermentation proceeded until sugar utilisation by CBS 2649 ceased, at which point all of the fructose had been consumed as for treatment A. At this point EC1118 was inoculated, and the viable population achieved was higher than expected, although the explanation for this is unknown. The viable CBS 2649 population was
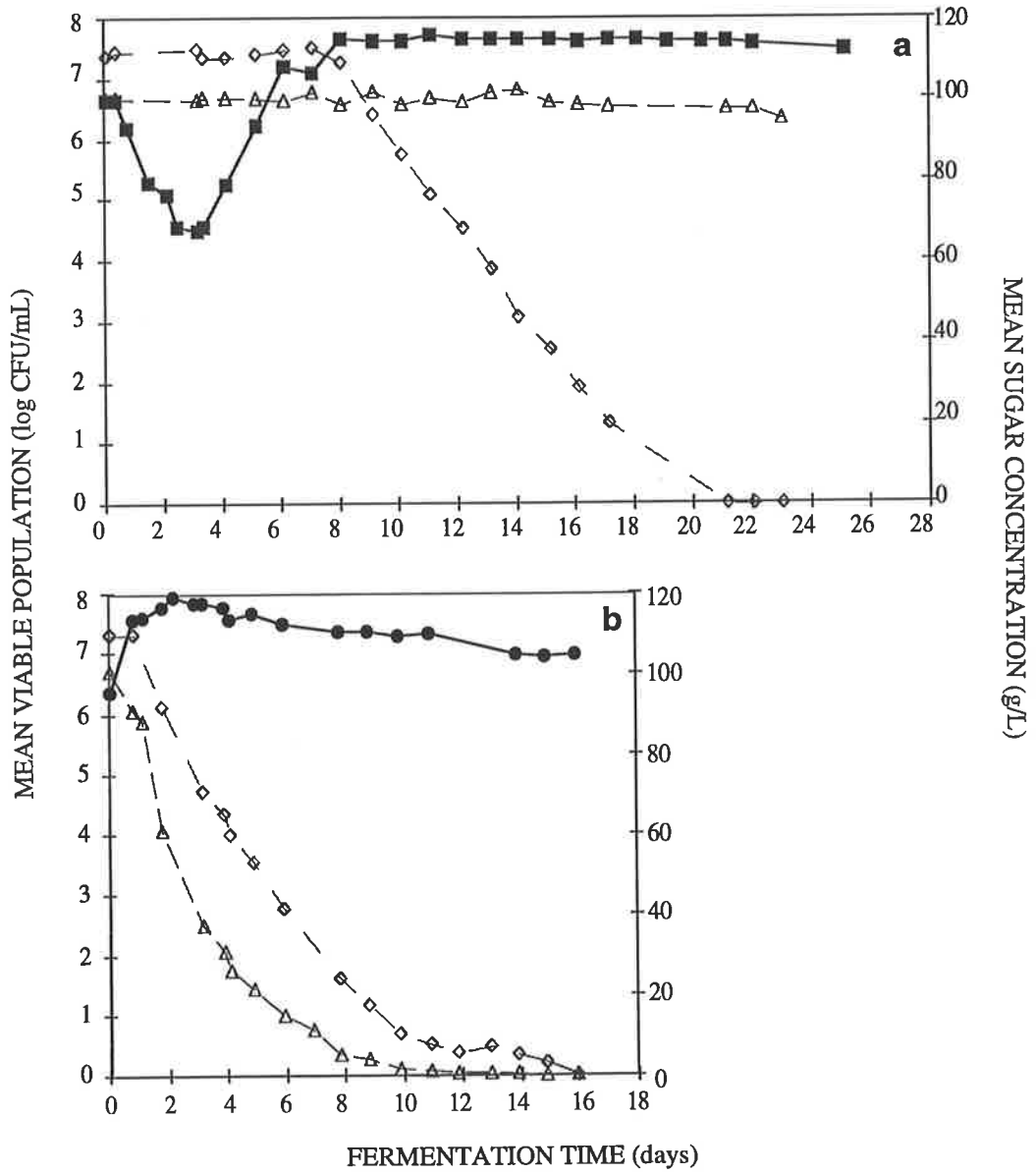
Figure 5.1. Effect of inoculation treatment on yeast population and sugar depletion during fermentation of a Chardonnay juice.

a. Treament A *C. stellata* CBS 2649 monoculture

b. Treatment B *S. cerevisiae* EC1118 monoculture

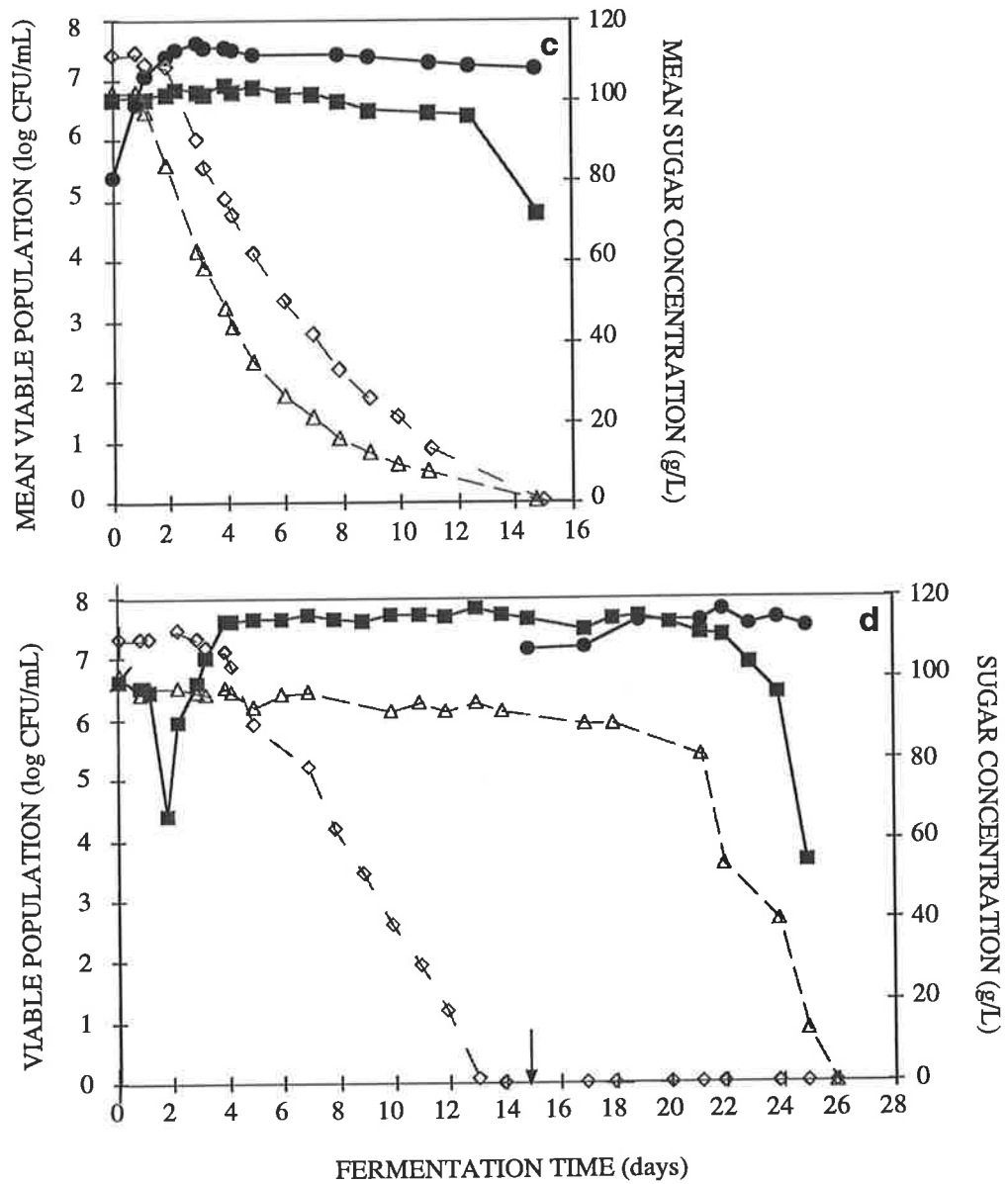—■— CBS 2649   —●— EC1118   —△— glucose   —◇— –fructose

Figure 5.1. Effect of inoculation treatment on yeast population and sugar depletion during fermentation of a Chardonnay juice

c. Treament C, Coinoculation of *C. stellata* CBS 2649 and *S. cerevisiae* EC1118.

d. Treatment D, Sequential inoculation of CBS 2649 and EC1118, arrow indicates time of inoculation of EC1118.

─■─CBS 2649   ─●─EC1118   ─△─glucose   ─◇─ -fructose

unaffected by this superinoculation, although a decline three days before the completion of fermentation on day 23 was noted, occurring 11 days after EC1118 was added.

5.3.2. Chemical composition

The concentration of organic acids, glycerol and sugars is shown in Table 5.2 for unfermented juice and the wines. The concentration of ethyl acetate, acetaldehyde and ethanol, and pH and TA values are shown in Table 5.3.

Table 5.2. Mean concentration (g/L) of organic acids, sugars and glycerol in unfermented juice and the wines

Treatment	citric acid	tartaric acid	malic acid	succinic acid	lactic acid	acetic acid	glycerol	glucose	fructose
unfermented juice	0.9 0	2.8 0	2.2 0	0 0	0 0	0 0	0 0	92.0 0	102.0 0
A	0.6 0	2.8 0.14	2.5 0.14	0.5 0.07	0 0	1.0 0.08	12.9 0.49	91.5 4.67	0 0
B	0.3 0	2.8 0	2.2 0	0.5 0.07	0.1 0	0.3 0	4.8 0.07	0.2 0.07	0 0
C	0.3 0	2.8 0	2.2 0	0.5 0.07	0.1 0	0.4 0.09	5.2 0	0.2 0	0 0
D partial	0.7 0.05	2.8 0	2.6 0.05	0.5 0.05	0 0	1.0 0.18	13.6 0.6	92.1 2.96	0 0
D complete	0.7 0.07	3.0 0.2	2.5 0.14	0.8 0	0.1 0	0.8 0.01	15.7 0.49	0.2 0.07	0 0

mean,
standard deviation
Treatments: A, *C. stellata* CBS 2649 monoculture; B, *S. cerevisiae* EC1118 monoculture; C, coinoculation; D partial, partial fermentation by *C. stellata*; D complete, fermentation of D partial completed by sequential inoculation with EC1118

Table 5.3. pH and TA values and the concentration of acetaldehyde, ethyl acetate and ethanol in unfermented juice and the wines

Treatment	pH	TA (g/L)	Acetaldehyde (mg/L)	Ethyl acetate (mg/L)	Ethanol (% v/v)
unfermented juice	3.27	6.3	–	–	–
A *C. stellata* CBS 2649	3.17 0.01	8.3 0.14	27.7 2.5	57.0 5.4	5.8 0.07
B *S. cerevisiae* EC1118	3.28 0.01	6.2 0.07	37.7 2.1	69.3 2.1	12.5 0.07
C Coinoculation	3.30 0.01	6.2 0.28	24.7 1.5	67.7 5.0	12.4 0.28
D Sequential inoculation	3.19 0.01	8.2 0	20.3 1.5	112.0 9.5	11.8 0.28

mean
standard deviation
– = not determined
Treatments: A, *C. stellata* CBS 2649 monoculture; B, *S. cerevisiae* EC1118 monoculture; C, coinoculation; D sequential fermentation (complete)

For treatment A, 91.5 g glucose/L remained when fermentation had ceased at 5.8% ethanol v/v. The fructose was completely fermented to produce 13 g glycerol/L and 1.0 g acetic acid/L, greater than for EC1118 in monoculture (treatment B). The production of glycerol at high concentration by other strains of *C. stellata* has been found (Ciani and Ferraro 1996). The concentration of acid and hence TA of this wine was the highest of any of the treatments and was in part due to the formation of acetic acid.

The composition of the treatment B wine was within a range expected for vinification with *S. cerevisiae* (Amerine and Ough 1980). The analyte values for the coinoculated wines in treatment C did not vary greatly from those of treatment B, with the exception of a higher concentration of glycerol.

Treatment D was analysed after the completion of the first stage of fermentation with CBS 2649 (Table 5.2, treatment D partial), and as expected, was very similar to treatment A in composition. After superinoculation with EC1118 and completion of fermentation, the wine analysis had changed (Table 5.2, treatment D complete). The concentration of acetic acid possibly decreased slightly, and that of glycerol increased to 15.7 g/L, the highest for any of the treatments. The final concentration of ethanol, after fermentation of the remaining 92 g glucose/L, was 11.8% v/v which was lower than for the other treatments that completed fermentation (treatments B and C). The pH was lower, and the TA higher than for these treatments also. The concentration of acetaldehyde was also found to be highest for treatment B and lowest for treatment D, but within an expected range for table wines (Amerine and Ough 1980). The concentration of ethyl acetate was lowest for the CBS 2649 monoculture (57.0 mg/L) and unexpectedly high for the sequentially inoculated treatment (112.0 mg/L), both of which exceeded the range of 6–24 mg/L previously reported for this species (see Table 1.5).

5.3.3. Sensory analysis
5.3.3.1. Difference testing of treatment duplicates

To describe and quantify the extent of any differences in aroma due to the inoculation treatments, sensory descriptive analysis was carried out. Duo-trio difference tests were also performed to assess whether there were significant differences in aroma between treatment replicates (Table 5.4). A highly significant difference between duplicates was found for treatment D, and accordingly all eight of the wines were presented for descriptive analysis.

5.3.3.2. Descriptive analysis of treatment differences

Due to a restriction in wine availability, the sample presentation design for descriptive analysis was such that a judge was not necessarily presented with a particular wine more than once, and hence it was not possible to evaluate an individual judge's consistency in scoring. Table 5.5 shows the results of an ANOVA of the descriptive analysis data for the eight aroma attributes, for the effects of sample and session.

Table 5.4. Duo-Trio test for aroma differences between treatment replicates

Test	Comparison	No. of responses	No. of correct responses	Significance
1	A1 vs. A2	23	10	ns
2	B1 vs. B2	23	11	ns
3	C1 vs. C2	23	15	ns
4	D1 vs. D2	23	19	***

ns, not significant, ***, significant at p<0.001

Table 5.5. Analyses of variance of aroma attribute ratings for four yeast inoculation treatments: F-ratio and degrees of freedom (df)

Aroma attribute	Treatment	Session	Sample x session
Tropical fruit	2.62	0.37	0.38
Apricot	7.51 ***	0.99	0.54
Banana	2.66	4.70 *	0.69
Honey	22.69 ***	0.50	0.81
Lime	7.27 ***	1.38	0.69
Rose	6.61 ***	0.76	0.99
Ethyl acetate	6.71 ***	1.03	1.01
Sauerkraut	15.64 ***	0.93	0.98
df	3	2	14

Significant differences denoted as *, p<0.01, ***, p< 0.001

The sample effect was highly significant for each of the attributes, except for 'tropical' and 'banana', and consequently these two attributes were not considered in any further data analysis. The session effect or the interaction term was not significant for any of the attributes except for 'banana'. This indicates that there was no significant difference in the mean ratings for all but one of the attributes, even though not all judges scored the same samples during the three sessions.

In order to assess differences between the eight samples for the six aroma attributes, principal components analysis was performed (Figure 5.2) (Meilgaard 1991). The first two components account for 87% of the variance in the data set. The first principal component (PC) separated the samples on the basis of their relative scores for 'lime' and 'rose', as opposed to the ratings for the 'honey', 'sauerkraut' and to a lesser extent 'apricot' attributes. The second PC differentiated the samples on the basis of the 'ethyl acetate' descriptor. The samples from treatments B and C were each scored relatively highly in winensity of 'rose' and 'lime', and were lowly scored for all other attributes. In contrast the CBS 2649 monoculture wine (treatment A) was low in 'rose' and 'lime' aroma intensity and exhibited more intense 'sauerkraut', 'apricot' and 'honey' aromas. The sequentially inoculated treatment D tended
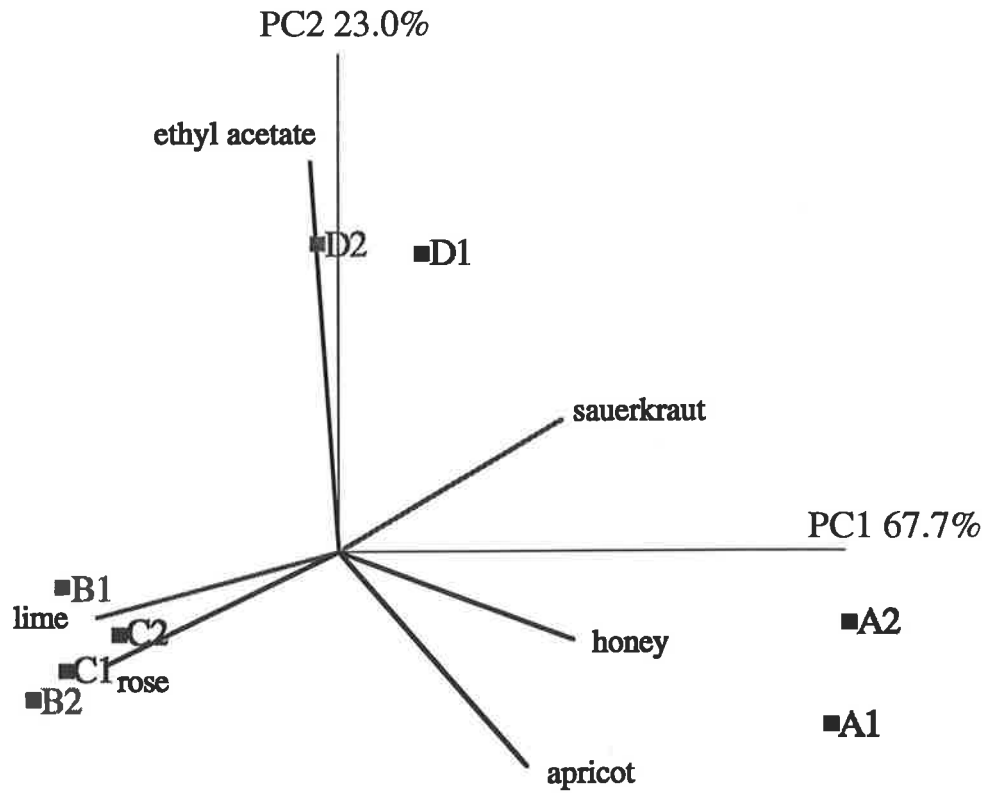
Figure 5.2. Descriptive analysis data projected onto principal components PC1 and PC2. Aroma attribute vectors are shown for samples A1 and A2, *C. stellata* CBS 2649 monocultures; B1 and B2, *S. cerevisiae* EC1118 monocultures; C1 and C2, coinoculation of CBS 2649 and EC1118; D1 and D2, sequential inoculation of CBS 2649 then EC1118.

to be intermediate in intensity for these particular attributes, and in addition, was rated as more intense in 'ethyl acetate', which further separated it from the other treatments.

From the PCA representation it is evident that the two fermentation replicates for each treatment were situated in close proximity to each other, including those of treatment D, which were found to be significantly different by duo-trio difference testing (5.3.3.1). This suggests that the major differences among the three groups of samples seen here resulted from the inoculation treatment rather than being an artefact due to fermentation variation.

To allow an assessment of statistically significant differences between the treatments, two sample t-tests were carried out to find whether it was justifiable to pool the fermentation replicate data. From these tests (data not shown) it was found that there were no significant differences between fermentation replicate rating for any of the treatments.

Figure 5.3 shows the mean aroma attribute scores for the four treatments. The coinoculated wine in treatment C was not significantly different from the EC1118 treatment B for any attribute. There were substantial differences between the CBS 2649 and EC1118 monocultures (treatments A and B respectively), with the former exhibiting significantly more intense 'apricot', 'honey', 'ethyl acetate' and 'sauerkraut', whilst the EC1118 wine showed significantly more intense 'lime' and 'rose' aroma.

The sequential inoculation treatment D — where the first half of the fermentation was conducted by CBS 2649, followed by inoculation with *S. cerevisiae* — was significantly different from the two monocultures for several attributes. Treatment D was rated significantly higher in 'ethyl acetate' than the other three treatments. The attributes dominant in the *S. cerevisiae* treatment B, 'lime' and 'rose', were significantly diminished by the sequential inoculation protocol, also the most intense aromas of the CBS 2649 treatment A, 'honey' and 'apricot', were significantly reduced. The 'sauerkraut' aroma score of treatment D was not significantly different to that of the *C. stellata* monoculture, treatment A.

5.3.3.2. Difference testing between treatments B and C

The descriptive analysis study did not reveal any significant differences between the EC1118 monoculture, treatment B, and the coinoculated treatment, C, for any aroma attribute, despite the presence of CBS 2649 throughout the coinoculated fermentation treatment. Considering the significant effect of this yeast upon wine aroma when sequentially inoculated (treatment D), it was important to confirm the lack of difference between treatments B and C by the more sensitive technique of difference testing. The results of duo-trio analysis are shown in Table 5.6.
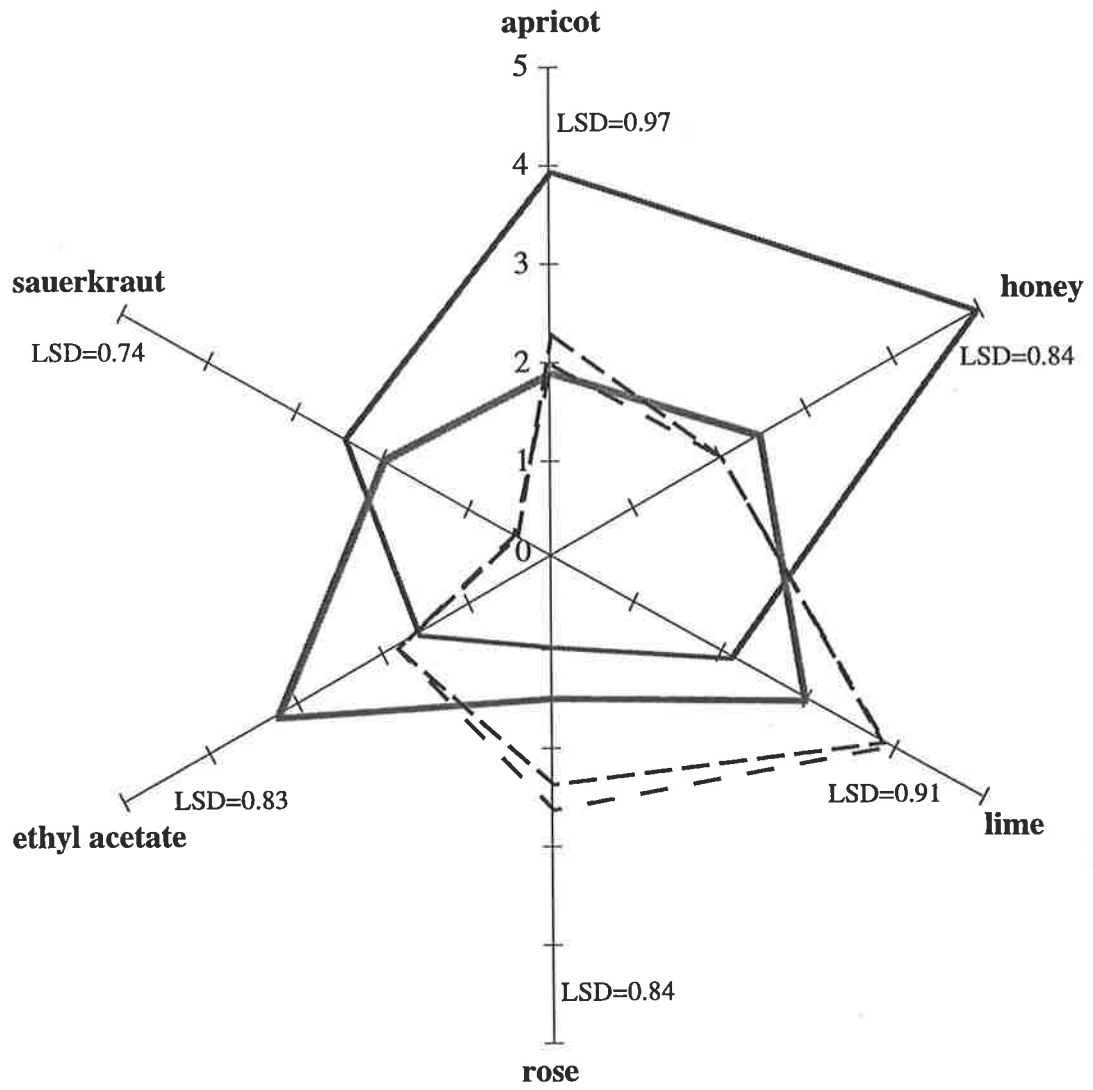
Figure 5.3. Aroma profiles plotted as mean intensity scores for four inoculation treatments. LSD = least significant differences.

——————— Treatment A, *C. stellata* CBS 2649 monoculture

— — Treatment B, *S. cerevisiae* EC1118 monoculture

— — — Treatment C, Coinoculation of CBS 2649 and EC1118

▬▬▬▬▬ Treatment D, Sequential inoculation of CBS 2649 then EC1118

Table 5.6. Duo-Trio test for aroma differences between treatments B and C

Test	Comparison	Number of responses	Number of correct responses	Significance
1	B1 vs C1	21	13	ns
2	B2 vs C1	21	12	ns
3	B1 vs C2	21	12	ns
4	B2 vs C2	21	11	ns

ns, not significant.

This analysis showed that there were no significant differences in aroma between treatments B and C. This result supported the descriptive analysis findings, and confirmed that the coinoculation protocol for this treatment did not significantly alter the wine aroma, as compared to EC1118 in monoculture, despite the presence of viable CBS 2649 throughout fermentation.

5.4. Discussion

This study detailed perhaps for the first time quantitative descriptive analysis of a wine made with a yeast other than *S. cerevisiae*. In monoculture *C. stellata* CBS 2649 was a stringently fructophilic yeast that did not complete fermention of the Chardonnay juice, to yield a wine with 5.8% ethanol v/v and 92 g /L residual glucose. The preferential uptake of fructose by this species has also been noted by Minárik *et al.* (1978), but not to the extent that glucose remained unutilised as occurred in treatment A. The CBS 2649 ferment was left for five days after fructose utilisation ceased, but glucose uptake had not occurred during this time and was unlikely to have commenced later.

Glycerol and acetic acid production was greater than by EC1118, ethyl acetate production was lower, and the pH was decreased and the TA increased by fermentation with CBS 2649. The wines made by this yeast were significantly higher in 'apricot', 'honey' and 'sauerkraut' aroma, and were significantly lower in 'lime' and 'rose' when compared to the EC1118 wines.

Discrepancies were noted for yeast growth and the production of ethanol by the *C. stellata* strain CBS 2649 when it was grown in Chardonnay juice, as compared to the synthetic grape juice-like medium (see 4.3.3). This strain was able to complete fermentation and produce a concentration of 11.3% ethanol v/v in the small-scale ferments in synthetic medium, but for large volume ferments of Chardonnay juice, growth was altered and only 6% ethanol v/v was produced. This large discrepancy is presumably due to the lack of glucose uptake by CBS 2649 in the juice, although this strain was able to transport and utilise glucose in the synthetic medium. However, five days after fructose exhaustion glucose uptake had not commenced and the wine was therefore bottled.

Other differences in media composition, and perhaps also the differences in the volume and agitation rate of the ferments are of importance. Sulfur dioxide in the juice may have contributed to the death of CBS 2649 shortly after inoculation as the tolerance of this strain to sulfur dioxide is unknown. This strain tolerated 200 g sugar/L in the synthetic medium without cell death, and as the juice sugar content was slightly less than this, osmotic shock due to sugar would not account for the observed loss of viability. The pH of the juice was 3.57, which was higher than that of the synthetic medium and therefore should have improved the growth and ethanol tolerance of *C. stellata* (Gao and Fleet 1988, Heard and Fleet 1988b). When used to ferment a different Chardonnay juice (pH 3.39) as described in Chapter 3, CBS 2649 was able to produce 8.7% ethanol v/v, but again with no or little depletion of glucose.

As the composition of the synthetic medium appeared to be limiting to the growth of CBS 2649 but not EC1118, the issue of differences in nutritional requirements of the two species is raised. Presumably these factors will also be of importance for the growth of CBS 2649 in grape juices of different composition, and suggest that the effect of different mixed culture protocols will be influenced by different environmental conditions as well as yeast strain and inoculation protocol as determined in this study.

In treatment C, coinoculation of the two yeasts at a relative density of 10:1 favouring CBS 2649 did not support reproduction of this strain, despite the presence of a substantial viable population throughout fermentation. It is important to note however that the post-inoculation cell death of CBS 2649 apparent in monoculture (treatment A) did not occur. Presumably this is due to the buffering effect of the EC1118 cells present, perhaps against the action of sulfur dioxide.

Chemical and sensory differences from the EC1118 wine were not detectable, implying that the isolation of a non-*Saccharomyces* yeast, even from the late stages of fermentation, does not ensure an impact upon the wine composition. Indeed CBS 2649 did not multiply after inoculation, and metabolites characteristic of this yeast, if produced, were not present in sufficient concentration at the end of fermentation to significantly affect the wine aroma. This result showed that addition of a yeast to juice, even at a high density, did not ensure an effect upon fermentation. Again a discrepancy existed between the results for fermentation of grape juice and fermentation of a synthetic grape juice medium. In the synthetic medium, CBS 2649 was still able to grow at this inoculation ratio, although without suppression of EC1118 growth.

In sequential fermentation with EC1118 (treatment D) the growth and metabolic activity of CBS 2649 altered the chemical and sensory character of the wine. This wine had the highest concentration of glycerol and succinic acid and was lower in ethanol than the other two completely fermented wines, although this finding requires confirmation. In aroma intensity, this wine was significantly highest for the attribute 'ethyl acetate'— as confirmed by chemical

analysis — and significantly lower in 'lime' and 'rose' which were high for EC1118, and 'honey' and 'apricot' which were high for CBS 2649.

5.5. Conclusion

There is much dispute over whether the non-*Saccharomyces* yeast contribute to wine aroma and flavour at all, let alone positively. The general belief that each yeast imparts its own flavour and aroma notes upon a wine is difficult to assess for spontaneous fermentations where multiple strains may be involved. By comparing wines made with a *S. cerevisiae* or *C. stellata* strain to the wines made by successful cofermentation with both yeasts, it was found that aroma attributes of both strains were apparent, albeit with significant differences in intensity.

Fleet and Heard (1993) stated that the exploitation of the diversity of biochemical and sensory properties of non-*Saccharomyces* yeasts could be of interest to creative winemakers. This study also suggests, that, with the selection of appropriate strains, and the establishment of inoculation protocols that effectively modulate yeast growth, chemical and sensory enhancement of wine by non-*Saccharomyces* yeasts becomes an interesting, and achievable option for winemaking.

Chapter 6

CONCLUSION

This work has attempted to determine the impact of some selected non-*Saccharomyces* yeasts to the fermentation of grape juice and the flavour of white wine. *S. cerevisiae*, although regarded as the principal wine yeast, is not the only species that contributes to fermentation. Non-*Saccharomyces* yeasts can have a significant numerical presence in both spontaneous and inoculated fermentation, and can therefore introduce ecological and biochemical diversity to winemaking. This study aimed to determine the effect of some non-*Saccharomyces* strains upon fermentation kinetics, wine composition, and wine aroma, with an emphasis on fermentation in the presence of *S. cerevisiae*.

In order to differentiate and identify some unknown isolates collected from spontaneous fermentations in California and Australia a PCR method was developed. The intron based PCR method was rapid, simple and relatively inexpensive to perform, and was applied to both ecological studies and strain verification during mixed culture fermentation experiments. The technique permitted both yeast species identification and strain differentiation, differentiating a number of species from juice and fermentations in California and Australia and showing that multiple strains of a species (especially *Hanseniaspora uvarum*) were present in a single fermentation. It is not however envisaged that this method could replace existing techniques in yeast taxonomy, but it has value as a supplementary tool. The method can assist taxonomists in rapidly detecting unrelated yeasts in a heterogenous species, as was observed with *Candida stellata* and *Pichia* isolates. In this study the method was applied to the indigenous yeasts of grape must and wine, and it may be useful for other yeast ecological studies, and for the preliminary grouping of isolates by amplification pattern commonality — and therefore potential conspecificity. By comparing the PCR amplification pattern of isolates to a data base of type strain fingerprints it may be possible to quickly identify a completely uncharacterised yeast.

As few sensory studies have investigated the effects of individual non-*Saccharomyces* yeasts upon wine flavour, some isolates of the species *C. stellata*, *C. krusei* and *T'spora delbrueckii*. were studied. Ten strains were used to vinify a sterilised Chardonnay juice, and the effect upon chemical composition, aroma profile and aroma preference of the resulting wines was determined.

Fermentation was completed to varying extents by the strains studied. Complete fermentation of sugar was achieved by *S. cerevisiae* EC1118, *C. stellata* CBS 843, and the mixed yeast culture. The non-*Saccharomyces* yeasts are generally thought to produce less ethanol and more secondary metabolites than *S. cerevisiae* (Fleet 1990), although this could

not be confirmed for the strains investigated in this study. The *C. krusei* type strain CBS 573^T, that was not of wine origin, was the least suited for winemaking in terms of fermentative capacity and also wine aroma preference.

From the analysis of some representative compounds of importance to wine aroma, it was evident that production varied with yeast strain, irrespective of the extent of fermentation. In particular, the production of glycerol also varied with strain, with particularly high production by two of the *C. stellata* strains. An increase in sweetness due to glycerol would be perceptible in these wines. Glycerol can also increase wine viscosity, although the concentration in these wines was below the only reported sensory threshold value. The production of glycerol is known to be coupled to a reduction in ethanol yield, which has implications for the production of lower alcohol wines by the involvement of such strains in fermentation, although this was not confirmed for the strains used in this study.

Unusual sugar fermentation patterns were also noted for the *C. stellata* strains CBS 1713 and CBS 2649, which exhibited a stringent preference for fructose. Sluggish or stuck fermentation can result in, or perhaps be caused by, an excess of fructose, this situation could possibly be prevented by the involvement of such a fructophilic yeast in fermentation. The unusual kinetics of grape sugar utilisation by these strains is worthy of further study to determine the nature of hexose transport.

The differences in chemical composition of the non-*Saccharomyces* monoculture wines were accompanied by aroma differences, as were evident from the judges' descriptions. The role of sensory analysis in the selection of yeast strains in this study was of particular importance considering the unknown impact of these strains to wine aroma and flavour.

Preference ranking of the wines by aroma assessment showed that the EC1118 wine was the most preferred, although statistically the wines ranked first to eighth were not different in preference. The lowest ranked wine was made by the *C. krusei* strain CBS 573^T, that fermented only approximately 20 g sugar/L, to produce objectionable ethyl acetate and sulfidic characters. Therefore it is suggested that the impact of a yeast upon wine flavour can occur early in fermentation, and may not relate only to its overall persistence. This is important especially where yeasts have the potential to spoil wines in the early stages of fermentation, as faults may not necessarily be redeemed by completion of fermentation with another yeast.

This study provides a chemical and sensory profile of wines made by pure cultures of nine non-*Saccharomyces* yeasts — without the involvement of the ubiquitous wine yeast *S. cerevisiae*. Great diversity in winemaking properties exists among the non-*Saccharomyces* yeasts and some potentially useful traits for winemaking such as different sugar utilisation patterns, altered metabolic activity, and the production of novel aromas were identified.
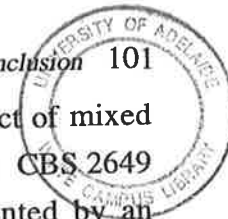
Three yeast strains were selected for fermentation studies to determine the effect of mixed species fermentation. These were *T'spora delbrueckii* AWRI 860, and *C. stellata* CBS 2649 and CBS 843. Although the aroma characteristics of juice partially fermented by an individual strain may not be as evident when another yeast is used to ameliorate or complete fermentation, the assessment of yeasts in pure culture fermentation, as an indication of their winemaking potential as mixed cultures was of value.

The use of non-*Saccharomyces* strains in conjunction with commercial — or at least more strongly fermentative yeasts — offers potential for imparting the novel character of such yeasts in a controlled fermentation of predictable outcome. The effect of different inoculation strategies upon yeast growth in mixed species fermentation was determined. This was necessary to determine how culture in the presence of a commercial strain, as may be necessary to ensure the completion of sugar fermentation, would affect the growth and metabolic activity of non-*Saccharomyces* strains of different fermentative capacity. In these experiments three non-*Saccharomyces* yeasts, *T'spora delbrueckii* AWRI 860, and *C. stellata* CBS 2649, and CBS 843 exhibited different growth and fermentation properties but were all able to yield up to 11.3% ethanol v/v, albeit more slowly than EC1118. The two *C. stellata* strains were characterised by preferential uptake of fructose and production of a high concentration of glycerol, which was useful for inferring the extent of the involvement of these strains in cofermentation.

Coinoculation affected the growth of the participating strains depending upon their fermentative capacity and the relative inoculation density. The growth and metabolism of the non-*Saccharomyces* yeasts could be enhanced, to the detriment of EC1118, to achieve quantitative dominance in fermentation. The effect of a particular coinoculation ratio was strain dependent, relating to the growth and fermentation rate of the participating non-*Saccharomyces* yeast. The slowest fermenter, *C. stellata* CBS 2649, grew best and only suppressed the growth of EC1118 when inoculated at a 50-fold greater density, whereas the more rapid fermenter *T'spora delbrueckii* AWRI 860 was able to dominate fermentation when inoculated at a ten-fold greater density than EC1118. Depending upon the non-*Saccharomyces* strain used and the inoculation treatment, wine composition and fermentation time were also affected.

The effect of coinoculation would appear to depend upon the vigour of the two strains, and decision about what ratios to use in a commercial fermentation would no doubt depend upon the yeast combination and fermentation conditions such as temperature, that would influence the growth of the non-*Saccharomyces* yeasts. Determination of the optimal coinoculation conditions for particular strain combinations however, would lead to improved growth and expression of non-*Saccharomyces* yeast character in fermentation, with effects upon the yield of significant metabolites and the sensory character of the wine.

For the slowest growing yeast *C. stellata* CBS 2649, the effect of inoculating this yeast at different intervals relative to the inoculation of EC1118 was also determined. Such sequential inoculation regimes either greatly suppressed or enhanced the growth of the slow fermenter CBS 2649, depending upon the timing of inoculation. Inoculation of this strain one day before EC1118 improved its growth and suppressed the *S. cerevisiae* strain, whereas preinoculation of CBS 2649 by three days resulted in severe suppression of EC1118 growth. So for both the slow growing non-*Saccharomyces* yeast and the strongly fermentative commercial *S. cerevisiae* strain, preinoculation resulted in significant effects upon growth. The inoculation of faster growing non-*Saccharomyces* yeast strains using this protocol, would surely emphasise the advantage conferred by preinoculation.

In commercial winemaking sequential inoculation would allow uninhibited growth of the novel strain early in fermentation with the option of later inoculation with a selected strain to achieve additional sensory effects and completion of fermentation. It would however be necessary to select strains that are able to effectively initiate fermentation under the required conditions. The timing of the second inoculation would also have to allow potentially for continued growth of the first strain. Sequential inoculation very late in fermentation, when a high concentration of ethanol has been attained, may not be successful if the latter yeast was not first acclimatised to the conditions. Furthermore, nutrient depletion by the first yeast may also limit growth and fermentation by the second yeast.

This study, although somewhat preliminary, has highlighted the effect of inoculation protocol, be it inoculation ratio, or timing of inoculation of two strains, on yeast growth, sugar depletion, fermentation kinetics and the concentration of some wine constituents. Further studies would need to confirm the effects of different combinations of strains, and different fermentation conditions, upon the outcome of particular inoculation protocols. In this way conditions can be further established for the exploitation of the biochemical and sensory diversity of non-*Saccharomyces* yeasts in a controlled fermentation of predictable outcome.

There is much dispute over whether the non-*Saccharomyces* yeast contribute to wine aroma and flavour at all, let alone positively. The general belief that each yeast imparts its own flavour and aroma notes upon a wine is difficult to assess for spontaneous fermentations where multiple strains may be involved. By comparing wines made with either a *S. cerevisiae* or a *C. stellata* strain to the wines made by cofermentation with these yeasts, it was found that aroma attributes of both strains were apparent when the inoculation protocol permitted the growth of both yeasts, and that the wine produced was significantly different in aroma to either reference strain.

This exercise provided, probably for the first time, formal sensory descriptive analysis of wine made — exclusively or partially — by fermentation with a non-*Saccharomyces* yeast.

From this experiment it was also shown that isolation of a yeast, even from the late stages of fermentation, does not necessitate a sensory impact upon that fermentation. It was found that a *C. stellata* strain, coinoculated at a high concentration in the presence of *S. cerevisiae*, did not grow, yet maintained its initial viable population number until almost the end of fermentation. Descriptive analysis and difference testing confirmed that there were no detectable differences in aroma from a wine made by EC1118 alone.

Fleet and Heard (1993) stated that the exploitation of the diversity of biochemical and sensory properties of non-*Saccharomyces* yeasts could be of interest to creative winemakers. This study aimed to contribute to the understanding of the effect of some non-*Saccharomyces* yeasts in fermentation. It is hoped, that with further work on the selection of appropriate yeasts, and the establishment of inoculation protocols that effectively modulate growth, chemical and sensory enhancement of wine made with non-*Saccharomyces* yeasts will become an interesting and feasible winemaking option.

APPENDIX 1. Yeasts associated with winemaking

Only those yeasts isolated from grapes, wine, or the winery environ are listed.

Yeast	Where isolated
Candida apicola	must
C. boidinii	wine
C. cantarellii	must
C. diversa	must
C. incommunis	must
C. intermedia	grapes
C. norvegica	wine cellar
C. sake	must, wine
C. stellata	grapes, juice, wine
C. vanderwaltii	winery equipment
C. veronae	must
C. vini	wine
Cryptococcus albidus	wine
C. laurentii	grapes, wine
Debaryomyces hansenii	juice, wine
Dekkera anomala	wine
D. bruxellensis	must, wine
Dipodascus ingens	wine cellars
Endomycopsella vini	grapes
Filobasidium capsuligenum	wine cellar
Hanseniaspora occidentalis	grapes
H. guilliermondii	juice and must
H. osmophila	grapes
H. uvarum	grapes, must
Hasagawaea japonica var *versatalis*	juice, wine
Issatchenkia orientalis	must
I. terricola	grapes, must, wine
Kluyveromyces thermotolerans	grapes, must, wine
Metschnikowia pulcherrima	grapes
Pichia carsonii	wine
P. guilliermondii	must
P. membranaefaciens	must, wine
Rhodotorula mucilaginosa	wine
Saccharomyces bayanus	fermenting juice
S. cerevisiae	fermenting juice, wine
S. exiguus	must
Saccharomycodes ludwigii	must
Schizosaccharomyces pombe	grapes, juice
Torulaspora delbrueckii	grapes, juice, wine
Wickerhamiella domercqiae	wine vat
Zygoascus hellenicus	must
Zygosaccharomyces bailii	wine
Z. florentinus	must
Z. rouxii	grapes, wine

Adapted from Barnett *et. al.* (1990)

APPENDIX 2. Wine yeasts: sexual and asexual forms

Adapted from Barnett *et. al* (1990). Only those yeasts associated with winemaking (Appendix 1) are included.

Sexual (teleomorph)	Asexual (anamorph)
Debaromyces hansenii	*Candida famata*
Dekkera anomala	*Brettanomyces anomalus*
D. bruxellensis	*B. bruxellensis*
D. custersiana	*B. custersianus*
Dipodascus ingens	*Geotrichum ingens*
Hanseniaspora guilliermondii	*Kloeckera apis*
H'spora occidentalis	*Kl. javanica*
H'spora osmophila	*Kl. corticus*
H'spora uvarum	*Kl. apiculata*
Issatchenkia orientalis	*Candida krusei*
Kluyveromyces thermotolerans	*Candida dattila*
Metschnikowia pulcherrima	*Candida pulcherrima*
Pichia membranaefaciens	*Candida valida*
Saccharomyces cerevisiae	*Candida robusta*
S. exiguus	*C. holmii*
Sporidiobolus pararoseus	*Sporobolomyces shibatanus*
Torulaspora delbrueckii	*Candida colliculosa*
Wickerhamiella domercqiae	*Candida domercqiae*
Yarrowia lipolytica	*Candida lipolytica*
Zygoascus helienicus	*Candida hellenica*

BIBLIOGRAPHY

Amerine, M., Berg, A., Kunkee, R.E., Ough, C.S., Singleton, V.L. and Webb, A.D. (1980) The technology of winemaking. 4th ed. Westport, Connecticut: AVI Publishing Company Inc.

Amerine, M.A. and Joslyn, M.A. (1970) Table wines. The technology of their production. 2nd ed. University of California Press: Berkeley.

Amerine, M.A. and Ough, C.S. (1980) Methods for analysis of musts and wines. John Wiley & Sons: New York.

Amerine, M.A., Pangborn, R.M. and Roessler, E.B. (1965) Principles of sensory evaluation in food. Academic Press: New York.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K.A. (1994) Current protocols in molecular biology. John Wiley and Sons, Inc: New York.

Barnett, J.A., Delaney, M.A., Jones, E., Magson, M.A. and Winch, B. (1972) The numbers of yeasts associated with wine grapes of Bordeaux. *Archives of Microbiology* 83, 52-55.

Barnett, J.A., Payne, R.W. and Yarrow, D. (1990) Yeasts: Characteristics and identification. 2nd ed. Cambridge University Press: Cambridge.

Belin, J.M. (1972) Recherches sur la répartition des levures à la surface de la grappe de raisin. *Vitis* 11, 135-145.

Benda, I. (1970) Natural and controlled microbial processes in grape must and in young wine. *Bayerisches Landwirtschchaftliches Jahrbuch* 47, 19-29.

Benda, I. (1982) Wine and brandy. In Prescott and Dunn's industrial microbiology. Reed, G. ed. pp. 293-402. AVI Publishing: Westport, Connecticut.

Benda, I. (1989) Occurence of killer toxin producing strains of film forming yeasts in grape must and their influence on the alcoholic fermentation. *Viticultural and Enological Science* 44, 56-60.

Bertrand, A. (1968) Utilisation de la chromatographie en phase gazeuse pour le dosage des constituants volatils du vin. *Connaisance de la Vigne et du Vin* 2, 175-270.

Bevan, E.A. and Makower, M. (1963) The physiological basis of the killer character in yeast. In Proceedings of the eleventh International Congress of Genetics. Hague 1963, Goerts, S.J. Ed. pp. 202-203.

Bisson, L.F. (1993) Yeasts — Metabolism of sugars. In Wine microbiology and biotechnology. Fleet, G.H. ed. pp. 55-75. Harwood Academic Publishers: Chur.

Bisson, L.F. and Kunkee, R.E. (1991) Microbial interactions during wine production. In Mixed cultures in biotechnology. Zeikus, J.G. and Johnson, E.A. eds. pp. 37-68. McGraw-Hill: New York.

Blondin, B. and Vezinhet, F. (1988) Identification de souches de levures oenologiques par leurs caryotypes obtenus en électrophorèse en champ pulsé. *Revue Française d'Oenologie* 28, 7-19.

Boekhout, T., Kurtzman, C.P., O'Donnell, K. and Smith, M.T. (1994) Phylogeny of the yeast genera *Hanseniaspora* (anamorph *Kloeckera apiculata*), *Dekkera* (anamorph *Brettanomyces*) and *Eeniella* as inferred from partial 26S ribosomal DNA nucleotide sequences. *International Journal of Systematic Biology* 44, (4), 781-786.

Bullard, R. (1994) In the home of scientific winemaking, more Californian vintners use wild yeast strains. *Wine Spectator* Sept 15, 10-11.

Bureau, G., Brun, O., Vigues, A. Maujean, A. Vesselle, G. and Feuillat, M. (1982) Étude d'une microflore levurienne champenoise. *Connaissance de la vigne et du vin*, 16, (1), 15-32.

Cabrera, M.J., Moreno, J., Ortega, J.M. and Medina, M. (1988) Formation of ethanol, higher alcohols, esters and terpenes by five yeast strains in musts from Pedro Ximénez grapes in various degrees of ripeness. *American Journal of Enology and Viticulture* 39,(4), 283-286.

Cairncross, S.E. and Sjöström, L.B. (1950) Flavor profiles—a new approach to flavor problems. *Food Technology* 4, 308-311.

Cantarelli, C. (1957) On the activation of alcoholic fermentation in wine making. Part 1. *American Journal of Enology and Viticulture*, 8, (3), 113-120.

Cardinali, G. and Martini, A. (1994) Electrophoretic karyotypes of authentic strains of the *sensu stricto* group of the genus *Saccharomyces*. *International Journal of Systematic Bacteriology* 44, 791-797.

Caridi, A., Tini, V., Benevelli, M. and Zambonelli, C. (1991) Caratteristiche enologiche di *Hanseniaspora guilliermondii*. *Vini d'Italia* 33, (2), 51-57.

Cartwright, C.P., Rose, A.H., Calderbank, J. and Keenan, M.H.J. (1989) Solute transport. In The yeasts. Vol. 2. 2nd. ed. Rose, A.H. and Harrison, J.S. eds. pp. 5-56. Academic Press: London.

Casey, G.P. and Ingledew, W.M. (1985) Re-evaluation of alcohol synthesis and tolerance in brewer's yeast. *MBAA Technical Quarterly* 22, 133-141.

Castelli, T. (1957) Climate and agents of wine fermentation. *American Journal of Enology and Viticulture* 8, (4), 149-156.

Charpentier, C. and Feuillat, M. (1993) Yeast Autolysis. In Wine microbiology and biotechnology. Fleet, G.H. ed. pp. 225-242. Harwood Academic Publishers: Chur.

Chatonnet, P., Dubourdieu, D., Boidron, J-N. and Pons, M. (1992) The origin of ethylphenols in wines. *Journal of the Science of Food and Agriculture* 60, 165-178.

Ciani, M. and Ferraro, L. (1996) Enhanced glycerol content in wines made with immobilized *Candida stellata* cells. *Applied and Environmental Microbiology* 62, (1), 128-132.

Ciani, M. and Maccarelli, F. (1998) Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology* 14, 199-203.

Ciani, M. and Picciotti, G. (1995) The growth kinetics and behaviour of some non-*Saccharomyces* yeasts associated with wine-making. *Biotechnology Letters* 17, (11), 1247-1250.

Cochran, W.G. and Cox, G.M. (1957) Experimental designs. 2nd ed. John Wiley & Sons, Inc.: New York.

Cottrell, T.H.E. and McLellan, M.R. (1986) The effect of fermentation temperature on chemical and sensory characteristics of wines from seven white grape cultivars grown in New York state. *American Journal of Enology and Viticulture* 37, (3), 190-194.

Curschmann, K., Köhler, H.J., Miltenberger, R., Schindler, E. and Geßner, M. (1994) Die Hefe prägt den Wein. *Rebe und Wein* 47, (6), 186-188.

D'Amore, T. and Stewart, G. (1987) Ethanol tolerance of yeast. *Enzyme and Microbial Technology* 9, 322-330.

Darriet, P., Boidron, J.N. and Dubordieu, D. (1988). L'hydrolyse des hétérosides terpéniques du muscat à petits grains par les enzymes périplasmiques de *Saccharomyces cerevisiae*. *Connaissance de la vigne et du vin* 22, (3), 189-195.

Davenport, R.R. (1974) Microecology of yeasts and yeast-like organisms associated with an English vineyard. *Vitis* 13, 123-130.

de Barros Lopes, M. (1998). Personal communication.

de Barros Lopes, M., Soden, A., Henschke, P.A., and Langridge, P. (1996) PCR differentiation of commercial yeast strains using intron splice site primers. *Applied and Environmental Microbiology* 62, (12), 4514-4520.

Deak, T., and Beuchat, L.R. (1993) Yeasts associated with fruit juice concentrates. *Journal of Food Protection* 56, 777-782.

Degré, R., Thomas, D.Y., Ash, J., Mailhiot, K., Morin, A., & Dubord, C. (1989) Wine yeasts strain identification. *American Journal of Enology and Viticulture* 40, (4), 309-315.

Delteil, D., and Aizac, T. (1988) Comparaison de différentes techniques de levurage par suivi de l'implantation d'une souche de levure oenologique marquée. *Revue Française d'Oenologie* 113, 11-18.

Di Stefano, R., and Ciolfi, G. (1985) Volatile compounds produced by *Torulopsis stellata*. *Vini d'Italia* 27, (4), 31-36.

Dittrich, H.H. (1977) Mikrobiologie des Weines. Handbuch der Getränkemikrobiologie. Ulmer: Stuttgart.

Dittrich, H H. (1978) Analytical differences between wines fermented with spontaneous yeasts and wines fermented with pure yeast cultures. In Lemperle, E. and Frank, J. eds. 5th International Oenological Symposium. pp. 257-263. Auckland, New Zealand.

Dittrich, H.H., and Wenzel, K. (1976) Die Abhängigkeit der Schaumbildung bei der Gärung von der Hefe und von der Most-Behandlung. *Weinwissenschaften* 31, 263

Dubordieu, D., Sokol, A., Zucca, P., Thalouarn, P., Dattee, A. and Aigle, M. (1987) Identification des souches de levures isolées de vins par l'analyse de leur ADN mitochondrial. *Connaissance Vigne Vin* 21, (4), 267-78.

Edinger, W.D. and Henick-Kling, T. (1994) Yeast population in spontaneous fermentation and inoculated wine fermentation with different must sulfite treatments. Cornell University, Wine Research Group, Department of Food Science and Technology, New York State Agricultural Experiment Station, Geneva NY.

Emmerich, W. and Radler, F. (1983). The anaerobic metabolism of glucose and fructose by *Saccharomyces bailii*. *Journal of General Microbiology* 129, 3311-3318.

Etiévant, P.X. (1991) Wine. In Volatile compounds in foods and beverages. Maarse, H. ed. pp. 483-546. Marcel Dekker, Inc: New York.

Farkas, J. (1988) Technology and Biochemistry of Wine. Vol. 1. Gordon and Breach Science Publishers: New York.

Fell, J.W. (1993) Rapid identification of yeast species using three primers in a polymerase chain reaction. *Molecular Marine Biology and Biotechnology* 2, (3), 174-180.

Ferreira, J.D. (1959) The growth and fermentation characteristics of six yeast strains at several temperatures. *American Journal of Enology and Viticulture* 10, 1-7.

Ferreira, V., Fernández, P., Peña, C., Escudero, A. and Cacho, J.F. (1995) Investigation on the role played by fermentation esters in the aroma of young Spanish wines by multivariate analysis. *Journal of the Science of Food and Agriculture* 67, 381-392.

Feuillat, M. (1984) Mise en évidence de souches de levures à caractère protéolytic: au cours de la fermentation alcoolique du moût de raisin. *Bulletin de L'Office International de la vigne et du vin* 57, 878-890.

Feuillat, M. and Charpentier, C. (1982) Autolysis of yeast in champagne production. *American Journal of Enology and Viticulture* 33, (1), 6-13.

Fink, G.R. (1987) Pseudogenes in yeast? *Cell* 49, 5-6.

Fleet, G.H. (1990) Growth of yeasts during wine fermentations. *Journal of Wine Research* 1, (3), 211-223.

Fleet, G.H. (1992) Spoilage yeasts. *Critical Reviews in Biotechnology* 12, (1/2), 1-44.

Fleet, G.H. and Heard, G.M. (1993) Yeasts- Growth during fermentation. In Wine microbiology and biotechnology. Fleet, G.H. ed. pp. 27-54. Harwood Academic Publishers: Chur.

Fleet, G.H., Heard, G.M. and Gao, C. (1989) The effect of temperature on the growth and ethanol tolerance of yeasts during wine fermentation. *Yeast* S5, S43-S46.

Fleet, G.H., Lafon-Lafourcade, S. and Ribereau-Gayon, P. (1984) Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology* 48, (5), 1034-1038.

Frayne, R.F. (1986) Direct analysis of the major organic components in grape must and wine using High Performance Liquid Chromatography. *American Journal of Enology and Viticulture* 37, (4), 281-287.

Fuck, E. and Radler, F. (1972) Äpfelsäurestoffwechsel bei *Saccharomyces* 1. Der anaerobe Äpfelsäurabbau bei *Saccharomyces cerevisiae*. *Archives of Microbiology* 87, 149-164.

Gafner, J., Hoffmann, P., Iselin, F., Schütz, M. and Viviani-Nauer, A. (1996) Die Hefeflora im Rebberg und während der Weinbereitung. *Agrarforschung* 3, (8), 373-376

Gancedo, C. and Serrano, R. (1989) Energy-yielding metabolism. In The Yeasts. Vol 3. Rose, A.H. and Harrison, J.S. eds. pp. 205-259. Academic Press: London.

Gao, C. and Fleet, G.H. (1988) The effects of temperature and pH on the ethanol tolerance of the wine yeasts *Saccharomyces cerevisiae, Candida stellata* and *Kloeckera apiculata*. *Journal of Applied Bacteriology* 65, 405-409.

Goldfarb, A. (1994) Wild or natural, native yeasts have a role in modern winemaking. *Wines and Vines* 75, (6), 27-30.

Goto, S. and Yokotsuka, I. (1977) Wild yeast populations in fresh grape musts of different harvest times. *Journal of Fermentation Technology* 55, 417-422.

Goto, S. and Oguri, H. (1983) Identification of the yeasts isolated from wild grapes. *Journal of the Institute of Enology and Viticulture, Yamanashi University* 18, 27-32.

Goto, S., Oguri, H. and Yamazaki, M. (1984) Yeast populations in botrytised grapes. *Journal of the Institute of Enology and Viticulture, Yamanashi University* 19, 1-5.

Goto, S., Yamazaki, M., Yamakawa, Y.and Yokotsuka, I. (1978) Decomposition of malic acid in grape must by wine and wild yeasts. *Hakkokogaku* 56, 133-135.

Großmann, M., Rapp, A. and Reith, W. (1987). Enzymatisch Freisetzung gebundener Aromastoffe in Wein. *Deutsche Lebensmittel Rundschau* 84, 35-37.

Guillamón, J.M., Barrio, E., Huerta, T. and Querol, A. (1994) Rapid characterization of four species of the *Saccharomyces sensu stricto* complex according to mitochondrial DNA patterns. *International Journal of Systematic Bacteriology* 44, 708-714.

Gunata, Y.Z., Bayonove, C.L., Cordonnier, R.E., Arnaud, A. and Galzy, P. (1990) Hydrolysis of grape monoterpenyl glycosides by *Candida molischiana* and *Candida wickerhamii* ß-glucosidases. *Journal of the Science of Food and Agriculture* 50, 499-506.

Guymon, J.F., Ingraham, J.L. and Crowell, E.A. (1961) Influence of aeration upon the formation of higher alcohols by yeasts. *American Journal of Enology and Viticulture* 12, 60-66.

Hall, J.F. (1971) Detection of wild yeasts in the brewery. *Journal of the Institute of Brewing* 77, 513-517.

Hallet, J.N., Craneguy, B., Daniel, P. and Poulard, A. (1990) Caractérisation des souches levuriennes des moûts et des lies par le polymorphisme de restriction de leur ADN mitochondrial. In Proceedings of Actualities Œnologiques 89. Bordeaux, France. Ribereau-Gayon, P. and Longvaud, E. eds. pp. 227-32. Dunod: France.

Hawkins, J.D. (1988) A survey on intron and exon lengths. *Nucleic Acids Research* 16, 9893-9908.

Hawthorne, D. and Philippsen, P. (1994) Genetic and molecular analysis of hybrids in the genus *Saccharomyces* involving *S. cerevisiae*, *S. uvarum* and a new species *S. douglasii*. *Yeast* 10, 1285-1296.

Heard, G.M. (1988) Occurrence and growth of yeasts during the fermentation of Australian wines. Ph.D. thesis. University of New South Wales. Sydney.

Heard, G. M. and Fleet, G. H. (1985) Growth of natural yeast flora during the fermentation of inoculated wines. *Applied and Environmental Microbiology* 50, (3), 727-728.

Heard, G. M. and Fleet, G. H. (1986) Occurrence and growth of yeast species during the fermentation of some Australian wines. *Food Technology in Australia* 38, (1), 22-25.

Heard, G.M. and Fleet, G.H. (1988a) The effect of sulphur dioxide on yeast growth during natural and inoculated wine fermentations. *Wine Industry Journal* Nov 88, 57-60.

Heard, G.M. and Fleet, G.H. (1988b) The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *Journal of Applied Bacteriology* 65, 23-28.

Henschke, P.A. and Jiranek, V. (1993) Yeasts- Metabolism of nitrogen compounds. In Wine microbiology and biotechnology. Fleet, G.H. ed. pp. 77-164. Harwood Academic Publishers: Chur.

Heresztyn, T. (1986) Metabolism of volatile phenolic compounds from hydroxycinnamic acids by *Brettanomyces* yeast. *Archives of Microbiology* 146, 96-98.

Herraiz, T., Reglero, G., Herraiz, M., Martin-Alvarez, P.J. and Cabezudo, M.D. (1990) The influence of the yeast and type of culture on the volatile composition of wines fermented without sulphur dioxide. *American Journal of Enology and Viticulture* 41, (4), 313-318.

Hoeben, P. and Clark-Walker, G.D. (1986) An approach to yeast classification by mapping mitochondrial DNA from *Dekkera/ Brettanomyces* and *Eeniella* genera. *Current Genetics* 10, 371-379.

Hoeben, P., Weiller, G. and Clark-Walker, G.D. (1993) Larger rearranged mitochondrial genomes in *Dekkera/Brettanomyces* yeasts are more closely related than smaller genomes with a conserved gene order. *Journal of Molecular Biology and Evolution* 36, 263-269.

Holloway, P. and Subden, R.E. (1991) Volatile metabolites produced in a Riesling must by wild yeast isolates. *Canadian Institute of Science and Technology Journal* 24, (1/2), 57-59.

Holloway, P., Subden, R.E. and Lachance, M.A. (1990) The yeasts in a Riesling must from the Niagara grape growing region of Ontario. *Canadian Institute of Food Science and Technology Journal* 23, 212-216.

Holloway, P., van Twest, R.A., Subden, R.E. and Lachance, M.A. (1992) A strain of *Candida stellata* of special interest to oenologists. *Food Research International* 25, 147-149.

Jackson, R.S. (1994) Wine Science: Principles and applications. Academic Press: San Diego.

Jacob, M. and Gallinaro, H. (1989) The 5' splice site: phylogenetic evolution and variable geometry of association with U1RNA. *Nucleic Acids Research* 17, 2159-2180.

Johnston, J.R. and Mortimer, R.K. (1986) Electrophoretic karyotyping of laboratory and commercial strains of *Saccharomyces* and other yeasts. *International Journal of Systematic Bacteriology* 36, 569-572.

Joslyn, M.A. (1955) Yeast autolysis. Chemical and cytological changes involved in autolysis. *Wallerstein Laboratories Communications* 18, 191-201.

Killian, E. and Ough, C.S. (1979) Fermentation esters - Formation and retention as affected by fermentation temperature. *American Journal of Enology and Viticulture* 30, (4), 301-305.

Kreger-van Rij, N.J.W. (1984) The yeasts: A taxonomic study. 3rd ed. Amsterdam: Elsevier Science Publishers.

Kuczynski, J.T. and Radler, F. (1982) The anaerobic metabolism of malate of *Saccharomyces bailii* and the partial purification and characterization of malic enzyme. *Archives of Microbiology* 131, 266-270.

Kunkee, R.A. and Amerine, M.A. (1970) Yeasts in wine-making. In Yeast technology. Vol 3. Rose, A.H. and Harrison, J.H. eds. pp. 5-71. Academic Press: London.

Kunkee, R.E. and Bisson, L.F. (1993) Wine-making yeasts. In The yeasts. Vol 5. 2nd ed. Rose, A.H. and Harrison, J.H. eds. pp. 69-127. Academic Press: London

Kunkee, R.E. and Goswell, R.W. (1977). Table wines. In Economic microbiology. Rose, A.H. ed. pp. 315-385. Academic Press: London.

Kurtzman, C.P. (1988) Identification and taxonomy. In Living resources for biotechnology: Yeasts. Kirsop, B. and Kurtzman, C.P. eds. pp. 99-140. Cambridge University Press:New York.

Kurtzman, C. P. (1992) rRNA sequence comparisons for assessing phylogenetic relationships among yeasts. *International Journal of Systematic Bacteriology* 42, 1-6.

Kurtzman, C. P. (1994) Molecular taxonomy of yeasts. Yeast, 10, 1727-1740.

Kurtzman, C.P. and Fell, J.W. (1998) The yeasts- A taxonomic study. 4th ed. Elsevier Sciences: Amsterdam.

Kurtzman, C.P., Smiley, M.J. and Johnson, C.J. (1980) Emendation of the genus *Issatchenkia* Kudriavzev and comparison of species by Deoxyribonucleic Acid reassociation, mating reaction, and ascospore ultrastructure. *International Journal of Systematic Bacteriology* 30, (2), 503-513.

Lafon-Lafourcade, S. (1983) Wine and brandy. In Biotechnology: A comprehensive treatise in 8 volumes Vol. 5. Reed, G.H. ed. pp. 81-163. Verlag chemie: Weinheim.

Lafon-Lafourcade, S., Geneix, C. and Ribéreau-Gayon, P. (1984) Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination by yeast ghosts. *Applied and Environmental Microbiology* 47, 1246-1249.

Lagace, L.S. and Bisson, L.F. (1990) Survey of yeast acid proteases for effectiveness of wine haze reduction. *American Journal of Enology and Viticulture* 41, (2), 147-155.

Lavallée, F., Salvas, Y., Lamy, S., Thomas, D.Y., Degré, R. and Dulau, L. (1994) PCR and DNA fingerprinting used as quality control in the production of wine yeast strains. *American Journal of Enology and Viticulture* 45, (1), 86-91.

Lawless, H.T. and Heymann, H. (1998) Sensory evaluation of food. Principles and practices. Chapman & Hall: New York.

Lewicka, K., Mallie, M. and Bastide, J.-M. (1995) Genetic variability in the *Saccharomyces sensu stricto* complex revealed by multilocus enzyme electrophoresis. *International Journal of Systematic Bacteriology* 45, 538-543.

Longo, E., Cansado, J., Agrelo, D. and Villa, T.G. (1991) Effect of climatic conditions on yeast diversity in grape musts from northwest Spain. *American Journal of Enology and Viticulture* 42, (2), 141-144.

Mager, W.H. and Varela, J.C.S. (1993) Osmostress response of the yeast *Saccharomyces*. *Molecular Microbiology* 10, (2), 253-258.

Magyar, I. and Panyik, I. (1989) Biological deacidification of wine with *Schizosaccharomyces pombe* entrapped in Ca-alginate gel. *American Journal of Enology and Viticulture* 40, (4), 233-240.

Malan, C. (1956) Taste properties of sterile grape musts fermented by pure yeasts. *Rivista di Viticoltura e di Enologi Conegliano* 9, 11-22.

Margalith, P. and Schwartz, Y. (1970) Flavor and microorganisms. *Advances in Applied Microbiology* 12, 35-88.

Margalith, P.Z. (1981) Flavor microbiology. Charles C Thomas: Springfield, Illinois.

Martens, A.L. (1996) Identification and characterization of yeast flora in long term cold stored grape juice. M of Oenology thesis. University of Adelaide: Adelaide.

Martínez, J., Millán, C. and Ortega, J.M. (1989) Growth of natural flora during the fermentation of inoculated musts from "Pedro Ximinez" grapes. *South African Journal for Enology and Viticulture* 10, (2), 31-35.

Martini, A. (1993) Origin and domestication of the wine yeast *Saccharomyces cerevisiae*. *Journal of Wine Research* 4, (3), 165-176.

Martini, A. and Vaughan Martini, A. (1990) Grape must fermentation: past and present. In Yeast technology Spencer, J.F.T. and Spencer, D.M. eds. pp. 105-123. Springer-Verlag: Berlin.

Mateo, J.J., Jiminez, M., Huerta, T. and Pastor, A. (1991) Contribution of different yeasts isolated from musts of monastrell grapes to the aroma of wine. *International Journal of Food Microbiology* 14, 153-160.

Mattick, J.S. (1994) Introns: evolution and function. *Current Opinion in Genetics and Development* 4, 823-831.

Meilgaard, M., Civille, G.V. and Carr, B.T. (1991) Sensory evaluation techniques. 2nd ed. CRC Press Inc.: Boca Raton.

Meyer, S.A. and Phaff, H.J. (1972) DNA base composition and DNA-DNA homology studies as tools in yeast systematics. In Proceedings of the First Specialized International Symposium on Yeasts. Smolenice.1971. Kocková-Kratochvilová, A. and Minárik, E. eds. pp. 375-386. Publishing House of the Slovak Academy of Sciences: Smolenice.

Michinik, S., J-L, R., Remize, F., Barre, P. and Dequin, S. (1997) Modulation of glycerol and ethanol yields during alcoholic fermentation in *Saccharomyces cerevisiae* strains overexpressed or disrupted for *GPD1* encoding glycerol 3-phosphate dehydrogenase. *Yeast* 13, 783-793.

Millán, C. and Ortega, J.M. (1988) Production of ethanol, acetaldehyde and acetic acid in wine by various yeast races: role of alcohol and aldehyde dehydrogenase. *American Journal of Enology and Viticulture* 39, 107-112.

Minárik, E., Jungová, O. and Emeriaud, M. (1978) Fruktophile Hefen und deren Einfluß auf süße Natureweine. *Wein-Wissenschaft* 33, (1), 42-47.

Monk, P.R. and Cowley, P.J. (1984) Effect of nicotinic acid and sugar concentration of grape juice and temperature on accumulation of acetic acid during yeast fermentation. *Journal of Fermentation Technology* 62, (6), 515-521.

Moore, K.J., Johnson, M.G. and Morris, J.R. (1988) Indigenous yeast microflora on Arkansas white riesling (*Vitis vinifera*) grapes and in model must systems. *Journal of Food Science* 53, (6), 1725-1728.

Mora, J., Barbas, J.I. and Mulet, A. (1990) Growth of yeast species during the fermentation of musts associated with *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae*. *American Journal of Enology and Viticulture* 41, (2), 156-159.

Mora, J. and Mulet, A. (1991) Effects of some treatments of grape juice on the population and growth of yeast species during fermentation. *American Journal of Enology and Viticulture* 42, (2), 133-136.

Mora, J. and Rossello, C. (1992) The growth and survival of *Pichia membranaefaciens* during fermentation of grape juice. *American Journal of Enology and Viticulture* 43, (4), 329-332.

Naumov, G.I., Naumova, E.S., Lantto, R.A., Louis, E.J. and Korhola, M. (1992) Genetic homology between *Saccharomyces cerevisiae* and its sibling species *S. paradoxus* and *S. bayanus*: electrophoretic karyotypes. *Yeast* 8, 599-612.

Nellen, W., Donath, C., Moos, M. and Gallwitz, D. (1981) The nucleotide sequences of the actin genes from *Saccharomyces calbergensis* and *Saccharomyces cerevisiae* are identical except for their introns. *Journal of Molecular and Applied Genetics* 1, 239-244.

Noble, A.C. (1994) Wine flavour. In Understanding natural flavours. Piggott, J.R. and Paterson, A. eds. pp. 228-242. Blackie Academic and Professional: London.

Noble, A.C. and Bursick, G.F. (1984) The contribution of glycerol to perceived viscosity and sweetness in white wine. *American Journal of Enology and Viticulture* 35, 110-112.

Nykänen, L. (1986) Formation and occurrence of flavour compounds in wine and distilled alcoholic beverages. *American Journal of Enology and Viticulture* 37, 84-96.

Nykänen, L. and Suomalainen (1983) Aroma of beer, wine and distilled alcoholic beverages. D. Reidel Publishing Company: Dordrecht.

O'Mahoney, M. (1986) Sensory evaluation of food. Marcel Dekker, Inc.: New York.

Oosthuizen, A., Koch, J.L.F., Viljoen, B.C., Muller, H.B. and Lategan, P.M. (1987) The value of long-chain fatty acid composition in the identification of some brewery yeasts. *Journal of the Institute of Brewing* 93, 174-176.

Ough, C.S., Fong, D. and Amerine, M.A. (1972) Glycerol in wine: Determination and some factors affecting. *American Journal of Enology and Viticulture* 23, 1-5.

Palumbi, S.R. and Baker, C.S. (1996) Contrasting population structure from nuclear intron sequences and mtDNA of humpback whales. *Molecular Biology and Evolution* 11, 426-435.

Pardo, I., García, M.J., Zúñiga, M. and Uruburu, F. (1989) Dynamics of microbial populations during fermentation of wines from the Utiel-Requena region of Spain. *Applied and Environmental Microbiology* 55, (2), 539-541.

Parish, M.E. and Carroll, D.E. (1985) Indigenous yeasts associated with Muscadine (*Vitis rotundifolia*) grapes and musts. *American Journal of Enology and Viticulture* 36, (2), 165-169.

Pearson, B.M., Carter, A.T., Furze, J.M. and Roberts, I.M. (1995) A novel approach for discovering retrotransposons: characterization of a long terminal repeat element in the spoilage yeast *Pichia membranaefaciens* and its use in strain identification. *International Journal of Systematic Bacteriology* 45, 386-389.

Petering, J.E., Langridge, P. and Henschke, P.A. (1988) Fingerprinting wine yeasts: the application of chromosome electrophoresis. *Australia and New Zealand Wine Industry Journal* 3, (3), 48-52.

Petering, J.E., Langridge, P. and Henschke, P.A. (1990) Identification of yeast strains by molecular biology techniques. In Proceedings of the Ninth International Oenological Symposium. Cascais Portugal. 1990. pp. 178-199. International Association for Modern Winery Technology and Management: Breisach, West Germany.

Peynaud, E. (1984) Knowing and making wine. 2nd ed. John Wiley and Sons: New York.

Price, E. (1996) Natural yeast fermentations- Amberley Estate. In Proceedings of the ASVO Oenology seminar. Advances in juice clarification and yeast inoculation. Melbourne. 1996. Allen, M., Leske, P. and Baldwin, G. eds. pp. 47-48. Australian Society of Viticulture and Oenology: Australia.

Querol, A., Barrio, E. and Ramón, D. (1992a) A comparative study of different methods of yeast strain characterization. *Systematic Applied Microbiology* 15, 439-446.

Querol, A., Barro, E., Huerta, T. and Ramon, D. (1992b) Molecular monitoring of wine fermentations conducted by active dried yeast strains. *Applied and Environmental Microbiology* 58, (9), 2948-2953.

Querol, A., Jiménez, M. and Huerta, T. (1990) Microbiological and enological parameters during fermentation of musts from poor and normal grape-harvests in the region of Alicante (Spain). *Journal of Food Science* 55, (6), 1603-1606.

Radler, F. (1993) Yeasts- Metabolism of organic acids. In Wine microbiology and biotechnology. Fleet, G.H. ed pp. 165-182. Chur: Harwood Academic Publishers.

Radler, F., Dietrich, K. and Schönig, I. (1985) Mikrobiologische Prüfung von Troekenhefepräparaten für die Weinbereitung. *Deutsche Lebensmittel Rundschau* 81, 73-77.

Ramey, D. (1995) Low input winemaking-let nature do the work. In Proceedings of the Ninth Australian Wine Industry Technical Conference. Adelaide, Australia. 1994. Stockley, C.S., Sas, A.N., Johnstone, R.S. and Lee, T.H. eds. pp. 26-29. Winetitles: Adelaide, Australia.

Rankine, B.C. (1967) Formation of higher alcohols by wine yeasts, and relationship to taste thresholds. *Journal of the Science of Food and Agriculture* 18, 583-589.

Rankine, B. C. (1968) The importance of yeasts in determining the composition and quality of wines. *Vitis* 7, 22-49.

Rankine, B.C. and Bridson, D.A. (1971) Glycerol in Australian wines and factors influencing its formation. *American Journal of Enology and Viticulture* 22, 6-12.

Rankine, B.C. and Lloyd, B. (1963) Quantitative assessment of dominance of added yeast in wine fermentations. *Journal of the Science of Food and Agriculture* 14, 793-798.

Rankine, B.C. and Pocock, K.F. (1969) Influence of yeast strain on binding of sulphur dioxide in wines, and on its formation during fermentation. *Journal of the Science of Food and Agriculture* 20, 104-109.

Rankine, B.C. and Pocock, K.P. (1970) Alkalimetric determination of sulphur dioxide in wine. *Australian Wine, Brewing and Spirit Review* 88, (8), 40-44.

Rapp, A. and Versini, G. (1991) Influence of nitrogen compounds in grapes on aroma compounds of wine. Proceedings of the International Symposium on grapes and wine. Seattle, Washington, USA. 1991. Ed. Rantz, J.M. pp. 156-164. American Society for Enology and Viticulture: Davis, California.

Reed, G. and Nagodawithana, T.W. (1988) Technology of yeast usage in winemaking. *American Journal of Enology and Viticulture* 39, (1), 83-90.

Reed, G. and Peppler, H.J. (1973) Yeast technology. AVI publishing company: Westport.

Ribéreau-Gayon, J., Peynaud, E., Ribéreau-Gayon, P. and Sudraud, P. (1975) Traité de Œnologie. Sciences et techniques du vin. Dunod: Paris.

Romano, P. and Suzzi, G. (1992) Preliminary characterization of *Zygosaccharomyces* for oenological purpose. *Biologia Oggi* 1, (1-2), 257-262.

Romano, P. and Suzzi, G. (1993a) Higher alcohol and acetoin production by *Zygosaccharomyces* wine yeasts. *Journal of Applied Bacteriology* 75, 541-545.

Romano, P. and Suzzi, G. (1993b) Potential use for *Zygosaccharomyces* species in winemaking. *Journal of Wine Research* 4, (2), 87-94.

Romano, P., Suzzi, G., Comi, G. and Zironi, R. (1992) Higher alcohol and acetic acid production by apiculate wine yeasts. *Journal of Applied Bacteriology* 73, 126-130.

Romano, P., Suzzi, G., Domizio, P. and Fatichenti, F. (1997) Secondary products formation as a tool for discriminating non-*Saccharomyces* wine strains. Strain diversity in non-*Saccharomyces* wine yeasts. *Antonie van Leeuwenhoek* 71, 239-242.

Romano, P., Suzzi, G., Zironi, G. and Comi, G. (1993) Biometric study of acetoin production in *Hanseniaspora guillermondii* and *Kloeckera apiculata*. *Applied and Environmental Microbiology* 59, (6), 1838-1841.

Rose, A.H. (1987) Responses to the chemical environment. In The Yeasts. Vol 2. Rose, A.H. and Harrison, J.S. eds. pp. 5-40. Academic Press: London.

Rosi, I., Costamagna, L. and Bertuccioli, M. (1987) Screening for extracellular acid protease(s) production by wine yeasts. *Journal of the Institute of Brewing* 93, 322-324.

Rosi, I., Vinella, M. and Domizio, P. (1994) Characterization of ß-glucosidase activity in yeasts of oenological origin. *Journal of Applied Bacteriology* 77, 519-527.

Rosini, G. (1984) Assessment of dominance of added yeast in wine fermentation and origin of *Saccharomyces cerevisiae* in wine-making. *Journal of General and Applied Microbiology* 30, 249-256.

Rosini, G., Federici, F. and Martini, A. (1982) Yeast flora of grape berries during ripening. *Microbial Ecology* 8, 83-89.

Ross, J.P. (1997) Going wild: Wild yeast in winemaking. *Wines and Vines* Sept, 16-21.

Saiki, R.K., Skarf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, M.A. and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anemia. *Science* 230, 1350-1354.

Scazzochio, C. (1989) The natural history of fungal mitochondrial genomes. In Proceedings of the Symposium of the British Mycological Society. Bristol. 1989. Rayner, A.D.M., Brasier, C.M. and Moore, D. eds. pp. 55-73. Cambridge University Press: UK.

Scheda, R. and Yarrow, D. (1966) The instability of physiological properties used as criteria in the taxonomy of yeasts. *Archives of Microbiology* 55, 209-225.

Schutte, L. (1975) Precursors of sulfur-containing flavor compounds. In Fenaroli's Handbook of Flavour Ingredients. Furia, T. and Bellanca, N. eds. pp. 184-227. CRC Press: Cleveland.

Schütz, M., Barbic, I., Marugg, D., Hoffman, P. and Gafner, J. (1995) Mikrobiologische Aspekte der Weinqualität: eine Pilotstudie. *Schweizerische Zeitschrift für Obst- und Weinbau, Wadenswil* 131, (5), 119-123.

Schütz, M. and Gafner, J. (1993a) Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. *Journal of Applied Bacteriology* 75, 551-558.

Schütz, M. and Gafner, J. (1993b) Sluggish alcoholic fermentation in relation to alterations of the glucose-fructose ratio. *Chemie Mikrobiologie Technologie der Lebensmittel* 15, ((3/4)), 73-78.

Schütz, M. and Gafner, J. (1994a) Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Letters in Applied Microbiology* 19, (4), 253-257.

Schütz, M. and Gafner, J. (1994b) Population dynamics of a mixed strain culture during fermentation. *ECB6: Proceedings of the 6th European Congress on Biotechnology* 1107-1110.

Sharf, R. and Margalith, P. (1983) The effect of temperature on spontaneous wine fermentation. *European Journal of Applied Microbiology and Biotechnology* 17, 311-313.

Shimazu, Y. and Watanabe, M. (1981) Effect of yeast strains and environmental conditions on formation of organic acids in must during fermentation. *Journal of Fermentation Technology* 59, (1), 27-32.

Shimizu, K. (1993) Killer yeasts. In Wine microbiology and biotechnology. Fleet, G.H. ed. pp. 243-264. Harwood Academic Publishers: Chur.

Shinohara, T. (1984) L'importance des substances volatiles du vin. Formation et effets sur la qualité. *Bulletin de L'Office International de la vigne et du vin* 57, 606-618.

Soles, R.M., Ough, C.S. and Kunkee, R.E. (1982) Ester concentration differences in wine fermented by various species and strains of yeasts *American Journal of Enology and Viticulture* 33, (2), 94-98.

Sols, A. (1956) Selective fermentation and phosphorylation of sugars by Sauternes yeast. *Biochimica et Biophysica Acta* 20, 62-68.

Soufleros, E. and Bertrand, A. (1979). Role de la souche de levures dans la production des substances volatiles au cours de la fermentation du jus de raisin. *Connaisance Vigne Vin* 13, (3), 181-198.

Sponholz, W.R. (1993) Wine spoilage by microorganisms. In Wine microbiology and biotechnology. Fleet, G.H. ed. pp. 243-264. Harwood Academic Publishers: Chur.

Sponholz, W.R. and Dittrich, H.H. (1974) Die Bildung von SO_2-bindenden Gärungs-Nebenprodukten, höheren Alkoholen und Estern bei einigen Reinzuchthefestämmen und bei einigen für die Weinbereitung wichtigen "wilden" Hefen. *Wein-Wissenschaft* 29, (6), 301-314.

Sponholz, W.R., Dittrich, H.H. and Han, K. (1990) The influence on fermentation and acetic acid ethylester formation by *Hanseniaspora uvarum*. *Viticultural and Enological Sciences* 45, 65-72.

Stone, H., Sidel, J.L., Oliver, S., Woolsey, A. and Singleton, R. (1974) Sensory evaluation by quantitative descriptive analysis. *Food Technology* 28, 24-34.

Strauss, C., Wilson, B., Gooley, P.R. and Williams, P.J. (1986) Role of monoterpenes in grape and wine flavour. In 190th meeting of the American Chemical Society. Chicago 1986 American Chemical Society. Parliment, T.H. and Croteau, R. eds. pp. 222-224. American Chemical Society: Chicago.

Stumm, C., Hermans, J.M.H., Middlebeek, E.J., Croes, A.F. and de Vries, G.J.M.L. (1977) Killer-sensitive relationships in yeasts from natural habitats. *Antonie van Leeuwenhoek* 43, 125-128.

Sturley, S.L. and Young, T.W. (1988) Extracellular protease activity in a strain of *Saccharomyces cerevisiae*. *Journal of the Institute of Brewing* 94, 23-27.

Tarlow, J.K., Blakemore, A.I.F., Lennard, A., Solari, R., Hughes, H.N., Steinkasserer, A. and Duff, G.W. (1993) Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-bp tandem repeat. *Human Genetics* 91, 403-404.

van der Merwe, C.A. and van Wyk, C.J. (1981) The contribution of some fermentation products to the odor of dry white wines. *American Journal of Enology and Viticulture* 41, (1), 41-46.

van der Walt, J.P. (1987) The typological yeast species and its delimitation. In Rose, A.H. and Harrison, J.S. eds. The Yeasts. Vol. 1. pp. 95-121. Academic Press: London.

van der Westhuizen, T.J. and Pretorius, I.S. (1992) The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. *Antonie van Leeuwenhoek* 61, 249-257.

van Rooyen, T.J. and Tracey, R.P. (1987) Biological deacidification of musts induced by yeasts or malolactic bacteria and the effect on wine quality. *South African Journal of Enology and Viticulture* 8, (2), 60-69.

Van Zyl, J.A., De Vries, M.J. and Zeeman, A.S. (1963) The microbiology of South African wine-making. III The effect of different yeasts on the composition of fermented musts. *South African Journal of Agricultural Science* 6, 165-180.

Vasserot, Y., Christiaens, P., Chemardin, P., Arnaud, A. and Galzy, P. (1989) Purification and properties of a ß-glucosidase of *Hanseniaspora vineae* Van der Walt and Tscheuschner with the view to its utilization in fruit aroma liberation. *Journal of Applied Bacteriology* 66, 271-279.

Vaughan Martini, A. (1989) *Saccharomyces paradoxus* comb. nov., a newly separated species of the *Saccharomyces sensu stricto* complex based upon nDNA/nDNA homologies. *Systematic and Applied Microbiology* 12, 179-182.

Vaughan Martini, A. and Kurtzman, C.P. (1985) Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces sensu stricto*. *International Journal of Systematic Bacteriology* 35, 508-511.

Vezinhet, F., Dulau, L. and Hallet, J.N. (1994) Comparaison de différentes méthodes d'identification moléculaire de levures d'intérêst Œnologique. *Revue Française d'oenologie* 149, 13-16.

Vezinhet, F., Hallet, .J-N., Valade, M. and Poulard, A. (1992) Ecological survey of wine yeast strains by molecular methods of identification. *American Journal of Enology and Viticulture* 43, (1), 83-86.

Villa, T.G., Notario, V. and Villanueva, J.R. (1979) ß-glucosidases in the yeast *Pichia polymorpha*. *FEMS Microbiology Letters* 6, 91-94.

Webb, A.D. (1967) Some aroma compounds produced by vinous fermentation. *Biotechnology and Bioengineering* 9, 305-319.

Weining, S. and Langridge, P. (1991) Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction. *Theoretical and Applied Genetics* 82, 209-216.

Whiting, G.C. (1976) Organic acid metabolism of yeasts during fermentation of alcoholic beverages: A review. *Journal of the Institute of Brewing* 82, 84-92.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. and Lee, T.H. (1985) Occurrence and growth of lactic acid bacteria in wine: A review. *American Journal of Enology and Viticulture* 36, 302-313.

Williams, A.A. (1974) Flavour research and the cider industry. *Journal of the Institute of Brewing* 80, 455-470.

Winge, Ö and Roberts, C. (1949) Inheritance of enzymatic characters in yeasts, and the phenomenon of long-term adaptation. *Comptes Rendus des Travaux du Laboratoire Carlsberg, Série Physiologique* 24, 263-315.

Woolford, J.L. (1989) Nuclear pre-mRNA splicing in yeast. *Yeast* 5, 439-457.

Yamada, Y., Higashi, T., & Mikata, K. (1996). Should *Petasospora* Boidon et Abadie (Saccharomycetaceae) be retained?- The phylogeny based on the partial sequences of 18S and 26S ribosomal RNAs. *Bioscience, Biotechnology and Biochemistry*, 60, (5), 818-822.

Yokotsuka, K., Otaki, A., Naitoh, A. and Tanaka, H. (1993) Controlled simultaneous deacidification and alcohol fermentation of a high-acid grape must using two immobilized yeasts, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *American Journal of Enology and Viticulture* 44, (4), 371-377.

Young, T. W. (1987). Killer yeasts. In The yeasts. Vol 2. 2nd ed. Rose, A.H. and. Harrison, J. S. eds. pp. 131-164. Academic Press: London.

Zambonelli, C., Romano, P. and Suzzi, G. (1989) Microorganisms of wine. In Biotechnology applications in beverage production. Cantarelli, C. and Lanzarini, G. eds. pp. 17-30. Elsevier Applied Science: London.

Zironi, R., Romano, P., Suzzi, G., Battistutta, F. and Comi, G. (1993) Volatile metabolites produced in wine by mixed and sequential cultures of *Hanseniaspora guilliermondii* or *Kloeckera apiculata* and *Saccharomyces cerevisiae*. *Biotechnology Letters* 15, (3), 235-238.

Zoecklein, W., Fugelsang, K.C., Gump, B.H. and Nury, F.S. (1995) Wine analysis and production. Chapman and Hall: New York.

ERRATA

p. 97. The presence of 15 mg/L free sulfur dioxide in the juice may have caused the shock and death of CBS 2649 shortly after inoculation. The tolerance of this strain to sulfur dioxide could be expected to be lower than that of commercial strains of *S. cerevisiae*, and hence the concentration of molecular SO_2, at approximately 0.5 mg/L may have been inhibitory, as it is generally assumed that concentrations that prevent oxidation are sufficient to reduce the population of indigenous yeast, or at least inhibit their growth (Boulton et al. 1996).

p. 107. Boulton, R.B., Singleton, V.L., Bisson, L.F. and Kunkee, R.E. (1996) Principles and practices of winemaking. Chapman and Hall: New York.