Characterisation, evaluation and use of non-*Saccharomyces* yeast strains isolated from vineyards and must

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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SUMMARY

Wine is the product of a complex biological and biochemical interaction between grapes and different microorganisms (fungi, yeasts, lactic acid bacteria and acetic acid bacteria, as well as the mycoviruses and bacteriophages affecting them) in which yeasts play the most important role regarding the alcoholic (primary) fermentation. These wine-associated yeasts can be divided into *Saccharomyces* and non-*Saccharomyces* yeasts. During fermentation, there is a sequence of dominance by the various non-*Saccharomyces* yeasts, followed by *Saccharomyces cerevisiae*, which then completes the fermentation. This is especially evident in spontaneously fermenting must, which has a low initial *S. cerevisiae* concentration. Some non-*Saccharomyces* yeasts can also be found throughout the fermentation. The non-*Saccharomyces* presence in the fermentation can affect wine quality, either positively or negatively. A positive contribution could be especially useful to improve wines produced from grape varieties with a neutral flavour profile due to non-optimal climatic conditions and/or soil types. As part of a comprehensive South African research programme, the specific objectives of this study were: the isolation of indigenous non-*Saccharomyces* yeasts from vineyards and musts; the identification of these isolates; the characterisation and evaluation of predominant species under winemaking conditions; and the development of a protocol for their use in enhancing wine quality.

Initially, 720 isolates representing 24 different species, were isolated from grape (vineyard) and must samples taken over three vintages from four distinctly different wine producing regions. The isolates were characterised and grouped utilising biochemical profiles and DNA karyotyping, whereupon representative isolates were identified. The yeast species that had the highest incidence of predominance in the vineyard was *Kloeckera apiculata.* However, some vineyard samples were characterised by low numbers or absence of this yeast, which is not according to generally accepted norms. Other species that also predominated in a few of the vineyard samples were *Candida pulcherrima*, *Kluyveromyces thermotolerans*, *Rhodotorula* sp. and *Zygosaccharomyces bailii.* Generally, there was a greater diversity of yeasts in the processed must than from the vineyard samples.

Furthermore, while each sample showed a different yeast population, no pattern linking species to climatic zone was observed.

Four species i.e. *Candida colliculosa*, *Candida pulcherrima*, *Candida stellata* and *Kloeckera apiculata*, were found to predominate in grape must samples. Representative strains consequently received further attention during laboratory and small-scale winemaking trials. A protocol was developed whereby individual species could be used in co-inoculated fermentations with *S. cerevisiae* in the small-scale production of wine. An improvement in wine quality was achieved and it was found that there was a link between specific species and grape cultivar. The ability of *C. pulcherrima* to improve Chenin blanc wine quality was investigated further. Results over three vintages showed that the wine produced by the co-inoculated fermentation was superior to that of a reference wine (produced by *S. cerevisiae* only). The improvement in wine quality was not linked to increased ester content nor were the standard chemical analyses adversely affected. The effects of pH and wine production parameters i.e. SO_2, fermentation temperature and use of di-ammonium phosphate (DAP), on this yeast followed the same pattern as that known for *S. cerevisiae*. This study was successfully completed and the developed protocol can be used for the improvement of Chenin blanc wine where additional aroma and quality is needed.

OPSOMMING

Wyn is die produk van 'n komplekse biologiese en biochemiese interaksie tussen druiwe en mikroörganismes (swamme, giste, melksuurbakterieë, asynsuurbakterieë, asook die mikovirusse en bakteriofage wat hul beïnvloed) waar gis die belangrikste rol speel ten opsigte van die alkoholiese (primêre) fermentasie. Die betrokke giste kan in *Saccharomyces*- en nie-*Saccharomyces*-giste verdeel word. Tydens gisting vind daar 'n opeenvolging van dominansie deur die verskillende nie-*Saccharomyces* giste plaas, gevolg deur *Saccharomyces cerevisiae*, wat dan die gisting voltooi. Dit is veral in spontaan fermenterende mos, waarin aanvanklik lae konsentrasies *S. cerevisiae*-gisselle voorkom, waarneembaar. Sekere nie-*Saccharomyces*-giste kan ook regdeur die verloop van fermentasie gevind word. Die teenwoordigheid van nie-*Saccharomyces*-giste kan 'n bydrae maak tot wynkwaliteit, hetsy positief of negatief. 'n Positiewe bydrae kan veral nuttig wees vir die verbetering van wyn geproduseer van druifsoorte met neutrale geurprofiele as gevolg van nie-optimale klimaatstoestande en/of grondtipes. As deel van 'n uitgebreide Suid-Afrikaanse navorsingsprogram, was die doelwitte van hierdie studie soos volg: die isolasie van inheemse nie-*Saccharomyces*-giste vanuit wingerde en mos; die identifikasie van hierdie isolate; die karakterisering en evaluering van spesies wat tydens wynbereiding oorheers; en die ontwikkeling van 'n protokol waarin geselekteerde nie-*Saccharomyces*-giste gebruik kan word vir die verbetering van wynkwaliteit.

Druif- en mosmonsters is oor drie oestye vanuit vier duidelik onderskeibare wynproduserende gebiede geneem en 720 isolate, verteenwoordigend van 24 verskillende spesies, is hieruit geïsoleer. Hierdie isolate is volgens biochemiese profiele en DNA-kariotipering gekarakteriseer en gegroepeer waarna verteenwoordigende isolate geïdentifiseer is. Die gisspesie wat die meeste in wingerde voorgekom het, was *Kloeckera apiculata.* Sommige wingerde is egter deur lae getalle of afwesigheid van dié gis gekenmerk, 'n feit wat afwyk van die algemeen aanvaarde norm. Ander spesies, nl. *Candida pulcherrima*, *Kluyveromyces thermotolerans*, *Rhodotorula* sp. en *Zygosaccharomyces bailii*, het ook in enkele gevalle in die wingerdmonsters oorheers. Oor die algemeen was daar 'n groter diversiteit van giste in die geprosesseerde mos as in die wingerdmonsters. Verder is

elke monster gekenmerk deur verskillende gispopulasies, maar geen verband tussen gisspesie en klimaatsone is waargeneem nie.

Vier spesies, nl. *Candida colliculosa*, *Candida pulcherrima*, *Candida stellata* en *Kloeckera apiculata*, het in hoë getalle in die druiwemosmonsters oorheers en verteenwoordigende rasse het verdere aandag tydens laboratorium- en kleinskaalse wynmaakproewe geniet. 'n Protokol, waar hierdie rasse individueel gebruik is in gesamentlike geïnokuleerde fermentasies met *S. cerevisiae* vir die kleinskaalse produksie van wyn, is ontwikkel. 'n Verbetering in wynkwaliteit is verkry en daar is 'n verband tussen spesifieke gisspesies en druifvariëteit gevind. Gevolglik is die vermoë van *C. pulcherrima* om die gehalte van Chenin blanc wyn te verbeter, verder ondersoek.

Resultate oor drie oesjare het gewys dat die wyn wat met die *C. pulcherrima* / *S. cerevisiae* kombinasie geproduseer is, beter was as 'n verwysingswyn (deur slegs *S. cerevisiae* geproduseer). Die waargenome verbetering in wynkwaliteit was egter nie aan 'n verhoging in esterinhoud te danke nie en die standaard chemiese analises het geen negatiewe afwyking uitgewys nie. Verder is gevind dat die effek van pH en wynproduksieparameters, nl. die gebruik van SO_2, fermentasietemperatuur en die gebruik van di-ammoniumfosfaat (DAP), dieselfde patroon as die bekend vir *S. cerevisiae* gevolg het. Die ontwikkelde protokol kan nou aangewend word waar verhoogde Chenin blanc wynaroma en kwaliteit verlang word.

This dissertation is dedicated to Juanita.
Hierdie proefskrif is opgedra aan Juanita.

BIOGRAPHICAL SKETCH

Neil Paul Jolly was born in Stellenbosch, South Africa, on 19 September 1963. He matriculated at Paul Roos Gymnasium, Stellenbosch in 1980. He then entered the University of Stellenbosch and obtained a B.Sc. degree in Microbiology and Biochemistry in 1984 and an Hons. B.Sc. degree in Microbiology in 1985. An M.Sc. degree in Microbiology from the same institution was awarded to him in 1992. He was appointed as an assistant agricultural researcher at the Viticultural and Oenological Research Institute (VORI/NIWW) in 1991 and is currently Senior Microbiologist in the Post-Harvest and Wine Technology Division at ARC Infruitec-Nietvoorbij. His current research fields include the occurrence and use of non-*Saccharomyces* yeasts in wine production, the role of yeast lees during wine production and the development and evaluation of *Saccharomyces cerevisiae* yeast strains for wine production in South Africa.

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PREFACE

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the *South African Journal of Enology and Viticulture*. Chapters 3, 5 and 6 have been published as indicated.

Chapter 1 **Introduction and Project Aims**

Chapter 2 **Literature Review**
Characterisation, role and use of non-*Saccharomyces* yeasts found on grapes and in must

Chapter 3 **Research Results I**
The occurrence of non-*Saccharomyces* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa (*South African Journal for Enology and Viticulture* 24, 35-42)

Chapter 4 **Research Results II**
The occurrence of apiculate yeasts in grape and must samples from the Robertson area of South Africa during the 2000 vintage.

Chapter 5 **Research Results III**
The effect of non-*Saccharomyces* yeasts on fermentation and wine quality (South African Journal for Enology and Viticulture 24, 55-62)

Chapter 6 **Research Results IV**
The use of *Candida pulcherrima* in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc (South African Journal of Enology and Viticulture 24, 63-69)

Chapter 7 **General Discussion and Conclusions**

CONTENTS

CHAPTER 1

INTRODUCTION AND PROJECT AIMS

1. INTRODUCTION AND PROJECT AIMS

Characterisation, evaluation and use of non-*Saccharomyces* yeast strains isolated from vineyards and must

1.1 INTRODUCTION

Wine quality is determined by a number of factors, including topography, soils, viticultural practices, and oenological practices (Jackson, 1994, 2000; Lambrechts & Pretorius, 2000; Pretorius, 2000; Fleet, 2003). All these aspects are being researched in an ongoing effort to increase wine quality. However, a large volume of the mass-produced wine lacks aroma and complexity (J. Marais, personal communication, 1994; Lambrechts & Pretorius, 2000). Specific viticultural and oenological practices can be used to increase overall wine quality, but are often not sufficient. The role of yeasts in enhancing wine quality is therefore receiving increasing attention.

The yeasts found during wine fermentation can be divided into *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts. *Saccharomyces cerevisiae,* known as the 'wine yeast', is primarily responsible for the conversion of the grape sugars to alcohol (Amerine *et al.*, 1967; Jackson, 1994). A secondary, but equally important role, is the production of complementary metabolites and the conversion of grape aroma precursors. These yeasts are naturally present in grape must and are derived from the grapes in the vineyard, where they are found in low numbers, as well as from the cellar equipment, where they are usually found in higher numbers (Peynaud & Domercq, 1959; Van Zyl & Du Plessis, 1961; Rankine, 1972; Vaughan-Martini & Martini, 1995; Török *et al.*, 1996). They may also be added as a commercial culture to initiate a fast and efficient fermentation.

The non-*Saccharomyces* yeasts are derived primarily from the grapes (vineyard), where they are generally found in higher numbers than the *S. cerevisiae* yeasts, and secondly from the cellar equipment (Peynaud & Domercq, 1959; Bisson & Kunkee, 1991; Martini *et al.*, 1996; Török *et al.*, 1996). During the harvesting of the grapes and subsequent crushing in the cellar, these yeasts are carried over to the

grape must. Their presence in the grape must has long been known (Amerine *et al.*, 1967). Furthermore, winemakers, in especially the Old World countries, saw indigenous yeasts as an integral part of their method of wine production (Amerine *et al.*, 1972). However, wine scientists believed that the non-*Saccharomyces* yeasts disappeared during the early stages of the fermentation due to the toxicity of the rising ethanol concentration and therefore played no role in the fermentation process. It was also widely accepted that the non-*Saccharomyces* yeasts were a potential source of wine spoilage.

Research during the last 20 years has shown, to the contrary, that some of the indigenous non-*Saccharomyces* yeasts can survive during the winemaking process (Heard & Fleet, 1985; Fleet, 1990; Gafner *et al.*, 1996). It has also been shown that their cell numbers can reach 1×10^7 cells/ml, which is comparable to that obtained by the wine yeast *S. cerevisiae*. This sustained presence of non-*Saccharomyces* yeast during wine fermentation has led to speculation that they do play a role in wine fermentation and affect wine aroma and general quality.

Non-*Saccharomyces* yeasts found in grape must can be divided into fermentative and non-fermentative yeasts. Some of these yeasts are known to form metabolites that are either aroma compounds or precursors to aroma compounds (Amerine *et al.*, 1967; Bisson & Kunkee, 1991; Heard, 1999). They can also form volatile acidity and other compounds that are detrimental to wine quality. However, not all strains within a species form the same levels of beneficial or detrimental metabolites (Romano *et al.*, 1992).

To obtain specific species and strains of potentially useful non-*Saccharomyces* yeasts, a range of these yeasts would have to be isolated, preserved and then screened for beneficial wine production and quality attributes. Thereafter, their behaviour under winemaking conditions will have to be ascertained. Finally, the controlled use of the selected strains could be applied to improve wine aroma and quality.

Currently, a comprehensive South African research programme regarding yeast biodiversity and yeast development is being undertaken. This programme was launched by several microbiologists from the Wine and Fermentation Technology

Division (currently the Post-Harvest and Wine Technology Division) at the ARC Infruitec-Nietvoorbij (the Fruit, Vine and Wine Research Institute of the Agricultural Research Council) and the Institute for Wine Biotechnology at the University of Stellenbosch. The long-term objectives of the programme were detailed by Pretorius *et al.* (1999). They are:

(i) the systematic cataloguing (isolation and characterisation) of yeasts occurring in the wine producing regions of the Western Cape of South Africa and the preservation of the natural yeast biodiversity;

(ii) a survey of the geographic distribution of the various yeast species and strains associated with the Cape's vineyards falling into different climatic zones;

(iii) a study of the origin, composition and actual contribution of the resident winery yeasts and how these yeasts vary between cellars in a particular area;

(iv) an investigation into the occurrence of area-specific indigenous yeasts and winery yeasts that impart a distinctive characteristic to wines from that area;

(v) an investigation of the effect of climatic conditions (and concomitant use of different dosages of pesticides and anti-fungal spray programmes) on yeast ecology and the seasonal variation of the indigenous yeast populations in these vineyards and in spontaneous wine fermentations;

(vi) the tracking and dissemination of wine yeast starter cultures that have been used over the past at annual sales in excess of 50 tonnes;

(vii) the calculation, estimation and prognosis of possible risks involved in the uncontrolled spread of genetically modified wine yeast strains when the use of such yeasts eventually becomes acceptable to wine producers and consumers;

(viii) the evaluation of yeast isolates for continuous wine yeast selection and strain development programmes which have the ultimate aim of providing an appropriate yeast per cultivar, per area, per wine type;

(ix) and the future exploitation of the genetic biodiversity by using these isolates as sources and hosts of cloned genes that are important to the wine industry.

Under the umbrella of the above-mentioned programme, this study partly addresses objectives (i), (ii), (iv) and (viii) and is the first in the programme to address the non-*Saccharomyces* group of yeasts. The specific aims of this study are therefore:

(i) the isolation of indigenous non-*Saccharomyces* yeasts from South African vineyards and musts;

(ii) the identification of these isolates;

(iii) the characterisation and evaluation of predominant species under winemaking conditions;

(iv) and the development of a protocol for their use in enhancing wine quality.

In the following chapter a broad overview is given of non-*Saccharomyces* yeasts associated with vineyards, wineries and wine, their taxonomy, methods of characterisation and their role and potential use in wine production. The isolation and characterisation of South African non-*Saccharomyces* yeasts used in this study are given in Chapters 3 and 4. This is followed in Chapter 5 by the effect non-*Saccharomyces* yeasts have on fermentation and wine quality. In Chapter 6, the use of *Candida pulcherrima* in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc wine is investigated. Finally, Chapter 7 contains a general discussion and guidelines for future non-*Saccharomyces* yeast research.

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CHAPTER 2

LITERATURE REVIEW

Characterisation, role and use of non-*Saccharomyces* yeasts found on grapes and in must

2. LITERATURE REVIEW

Characterisation, role and use of non-*Saccharomyces* yeasts found on grapes and in must

2.1 INTRODUCTION

Wine is the product of a complex biological and biochemical interaction between grapes (grape juice) and different microorganisms (fungi, yeasts, lactic acid bacteria, acetic acid bacteria as well as the mycoviruses and bacteriophages affecting them) (Fleet, 2003). The process starts in the vineyard, continues through fermentation and maturation and concludes at packaging. Amongst the involved microorganisms, yeasts play the most important role i.e. they conduct the alcoholic fermentation (conversion of grape sugar to ethanol and CO_2). Furthermore, although wine flavour is directly determined by grape variety, yeasts also affect wine flavour and quality by production and excretion of metabolites during growth and through autolysis (Fleet, 1993, 2003 and the references therein). In some instances yeasts can also act as spoilage organisms during wine production (including maturation) and after packaging (Loureiro & Malfeito-Ferreira, 2003). Yeasts present during fermentation can be derived from grapes and the vineyard where they were harvested, the equipment used in the cellar, cellar surfaces and external sources such as selected cultures added to facilitate the fermentation process.

Since 1866 when Louis Pasteur first elucidated the bio-conversion of grape juice into wine, this complex process and the role of the yeast therein has been studied extensively, but, more than a century later, many areas are still not fully understood (Pretorius, 2000 and the references therein). It is especially the role of the numerous non-*Saccharomyces* yeasts normally associated with grape must and wine that was neglected in the past. These yeasts, naturally present in all wine fermentations to a greater or lesser extent, are metabolically active and their metabolites can impact on wine quality. While they were originally seen as a source of microbially related problems in wine production, winemakers, in especially the Old World countries, saw indigenous yeasts as integral to the

authenticity of their wines by imparting distinct regional and other desired characteristics (Amerine *et al.*, 1972; Jackson, 1994). Evidence supporting this view has been published (Fleet, 1990; Heard, 1999) and the role of the non-*Saccharomyces* yeasts in wine fermentation is receiving more and more attention by wine microbiologists in Old and New World wine producing countries.

2.2 YEAST CLASSIFICATION

Yeasts can be defined as unicellular fungi, either ascomycetous or basidiomycetous, that have vegetative states that predominantly reproduce by budding or fission and which do not form their sexual states within or on a fruiting body (Kurtzman & Fell, 1998a).

Current taxonomies recognise 100 genera comprising more than 700 species (Kurtzman & Fell, 1998b) of which approximately 20 are relevant to winemaking (Fleet, 1993). An overview of yeast genera, with those non-*Saccharomyces* yeasts relevant to winemaking indicated in bold type, is given in Table 2.1.

Rules for taxonomy of yeasts fall under the authority of the International Code of Botanical Nomenclature (Greuter *et al.*, 1994). Publication of new species must include a description of essential characters as well as a diagnosis that distinguishes the taxon from previously described species. Names of taxa must be given in Latin or modified in such a way that they follow the rules of Latin derivation including appropriate designations.

The first level of yeast classification is based on the lack of a sexual phase during the life cycle (Deuteronmycotina) or aspects of the sexual phase (Ascomycotina and Basidiomycotina). Further taxonomic subdivisions (orders, families, genera and species) are based on morphological, physiological, biochemical and genetic properties (Kreger-van Rij, 1984; Kurtzman & Fell, 1998b) that are elucidated by conducting 55 to 70 tests. Many of these tests can be used individually to characterise a selection of yeasts.

Some yeasts are found in a sexual (teleomorphic) type and produce ascospores. A similar form of the same yeast is the asexual (anamorphic) type that does not

TABLE 2.1
An overview of yeast genera[1] according to Kurtzman & Fell (1998b).

Teleomorphic ascomycetous genera (Ascomycotina)	Anamorphic ascomycetous genera (Deuteromycotina)	Teleomorphic heterobasidio-mycetous genera (Basidiomycotina)	Anamorphic heterobasidio-mycetous genera (Basidiomycotina)
Ambrosiozyma	*Aciculoconidium*	*Agaricostilbum*	*Bensingtonia*
Arxiozyma	*Arxula*	*Bulleromyces*	*Bullera*
Ascoidea	*Blastobotrys*	*Chionosphaera*	***Cryptococcus***
Babjevia	*Botryozyma*	*Cystofilobasidium*	*Fellomyces*
Cephaloascus	***Brettanomyces***	*Erythrobasidium*	*Hyalodendron*
Citeromyces	***Candida***	*Fibulobasidium*	*Itersonilia*
Clavispora	*Geotrichum*	*Filobasidiella*	*Kockovaella*
Coccidiascus	***Kloeckera***	*Filobasidium*	*Kurtzmanomyces*
Cyniclomyces	*Lalaria*	*Holtermannia*	*Malassezia*
Debaryomyces	*Myxozyma*	*Leucosporidium*	*Moniliella*
Dekkera	*Oosporidium*	*Mrakia*	*Phaffia*
Dipodascopsis	*Saitoella*	*Rhodosporidium*	*Pseudozyma*
Dipodascus	*Schizoblastosporion*	*Sirobasidium*	*Reniforma*
Endomyces	*Sympodiomyces*	*Sporidiobolus*	***Rhodotorula***
Eremothecium	*Trigonopsis*	*Sterigmatosporidium*	*Sporobolomyces*
Galactomyces		*Tilletiaria*	*Sterigmatomyces*
Hanseniaspora		*Tremella*	*Sympodiomycopsis*
Issatchenkia		*Trimorphomyces*	*Tilletiopsis*
Kluyveromyces		*Xanthophyllomyces*	*Trichosporon*
Lipomyces			*Trichosporonoides*
Lodderomyces			*Tsuchiyaea*
Metschnikowia			
Nadsonia			
Pachysolen			
Pichia			
Protomyces			
Saccharomyces			
Saccharomycodes			
Saccharomycopsis			
Saturnispora			
Schizosaccharomyces			
Sporopachydermia			
Stephanoascus			
Torulaspora			
Wickerhamia			
Wickerhamiella			
Williopsis			
Yarrowia			
Zygoascus			
Zygosaccharomyces			
Zygozyma			

[1] Non-*Saccharomyces* genera that can be encountered in vineyards, on winery surfaces, in grape musts and/or in wine are indicated in bold type.

form ascospores. The ability to form ascospores can be lost during long-term storage (Yarrow, 1998; M. Th. Smith, personal communication, 2000).

Sporulation is also difficult to induce for some yeasts. Whether the yeast is subsequently identified as the teleomorphic or anamorphic type can therefore

TABLE 2.2
Anamorphic, teleomorphic and synonyms of some of the non-*Saccharomyces* yeasts in the Ascomycetous genera encountered in wine fermentations (Kurtzmann & Fell, 1998b).

Anamorphic yeast	Teleomorphic yeast	Synonyms[1]
Brettanomyces bruxellensis	*Dekkera bruxellensis*	*Saccharomyces bailii*
Candida colliculosa	*Torulaspora delbrueckii*	*Saccharomyces rosei*
Candida famata	*Debaryomyces hansenii*	
Candida globosa	*Citeromyces matritensis*	
Candida guilliermondii	*Pichia guilliermondii*	
Candida hellenica	*Zygoascus hellenicus*	
Candida lambica	*Pichia fermentans*	
Candida pelliculosa	*Pichia anomala*	*Hansenula anomala*
Candida pulcherrima	*Metschnikowia pulcherrima*	*Torulopsis pulcherrima*
Candida reukaufii	*Metschnikowia reukaufii*	
Candida sorbosa	*Issatchenkia occidentalis*	
Candida stellata	-[2]	*Torulopsis stellata*
Candida valida	*Pichia membranifaciens*	
Kloeckera africana	*Hanseniaspora vineae*	
Kloeckera apiculata	*Hanseniaspora uvarum*	
Kloeckera apis	*Hanseniaspora guilliermondii*	
Kloeckera corticis	*Hanseniaspora osmophila*	
Kloeckera javanica	*Hanseniaspora occidentalis*	
-[3]	*Issatchenkia terricola*	*Pichia terricola*
-[3]	*Kluyveromyces thermotolerans*	
-[3]	*Saccharomyces kluyveri*	
-[3]	*Saccharomycodes ludwigii*	
-[3]	*Zygosaccharomyces bailii*	*Saccharomyces bailii*
-[3]	*Pichia farinosa*	

[1]Names sometimes found in older literature.
[2]No teleomorphic form.
[3]No anamorphic form.

largely depend on time lapse between isolation and identification and adherence to methodology. This can lead to confusion when authors report on isolates as essentially the same yeast species may be referred to by different names. If any uncertainty exists in determining sporulation, it is therefore preferable to use the anamorphic name where applicable. Some of the more commonly encountered anamorphic yeasts and their teleomorphic counterparts in must and wine are given in Table 2.2.

2.3 YEAST CHARACTERISATION

When conducting an investigating into any group of yeasts, it is important to be able to distinguish between them so that relevant information regarding their identity and growth parameters can be obtained. Techniques used to characterise yeasts are normally developed and utilised for the *Saccharomyces* genus and

specifically for *Saccharomyces cerevisiae,* the eukaryotic model research microorganism. None the less, usually with necessary adaptations, the techniques can be applied to non-*Saccharomyces* yeasts as well. Characterisation techniques are varied, but can broadly be divided into non-molecular (physiological and biochemical) and molecular (based on DNA composition) methods. Some of the more discriminating characterisation techniques have obtained widespread usage in routine laboratories for diagnostic use.

2.3.1 Non-molecular characterisation techniques

There are numerous physiological and biochemical tests that can be carried out to characterise a yeast. These include colony morphology, growth at specific temperatures and sugar assimilation and fermentation. Traditionally, these tests were used for classification of yeasts (Lodder & Kreger-Van Rij, 1952). They mostly involve making up individual test tubes of appropriate media and/or agar plates. This exercise can become very time consuming when dealing with a large number of yeast isolates.

In order to facilitate rapid yeast identification, several commercial diagnostic kits have been developed for routine use in clinical microbiology for the identification of pathogenic yeasts (De Louvois *et al.*, 1979; Fleet, 1993). Consequently, their databases are limited to 60 or less species of clinical importance and their use in food (and wine) microbiology can be limited. However, they can be used for identification of food-borne yeasts if their databases are extended and additional supplementary tests can be performed (Deák, 1993). These kits are based on the utilisation (assimilation) by the yeast of a range of carbon compounds. Results, indicated by turbidity or colour changes, can be obtained in 24 to 48 hours. Both manual and automated systems are available. Among the different manual systems, the API 20C system is probably most widely used, and is often the reference when evaluating other systems (Deák, 1993).

The API system, based on assimilation tests in small cupules in a plastic strip, is read 48 to 72 hours after inoculation. The growth or lack of growth is used to generate a numerical profile for which a taxonomic identity from a database can be assigned.

The API 20 AUX system with 30 yeasts in its database has been evaluated for its ability to identify 72 wine yeasts (Subden *et al.*, 1980). The response of the API 20 AUX was consistent with those of conventional assimilation tests. However, not all the wine yeasts could be directly identified. Supplementary conventional tests could be used for the unidentified yeasts. However, the authors concluded that the limited database of wine yeast assimilation and fermentation capacities prevented the adequate evaluation of the efficiency of the API 20 AUX for wine yeast identification.

An expanded system, the API ATB 32 C for clinical yeasts with 63 yeasts in its database, has also been evaluated for the identification of food borne yeasts (Rohm *et al.*, 1990). This system also fared poorly in identifying the yeasts, due to the limited number of food related yeasts in its database. The authors had better results when the individual API ATB 32 C results were computed with the Barnett *et al.* (1985) computer program. This program compares entered results with 497 species in its database. This system was also used by Fernández *et al.* (2000) in conjunction with other tests recommended by Barnett *et al.* (1990) for successfully identifying the non-*Saccharomyces* yeasts in their study. A further advantage of the API ATB 32 C system is that the test strips can be read automatically, which reduces subjectivity.

Another non-molecular technique that has been used for wine yeast characterisation is based on the analysis of fatty acids. Fatty acids and fatty acid esters comprise the largest groups of aroma compounds synthesised by yeasts during fermentation (Suomalainen & Lehtonen, 1979). Cellular long-chain fatty acids can be extracted from yeast cells by saponification, analysed as methyl esters by gas-liquid chromatography and then compared to reference standards. Tredoux *et al.* (1987) used this technique to distinguish between strains of *S. cerevisiae* and other yeast strains associated with the wine industry. In subsequent work Augustyn & Kock (1989) and Augustyn (1989) showed that various strains of *S. cerevisiae* are characterised by unique strain specific variations in the mean relative percentages (MRP) of the different fatty acids present in the cellular material. These variations were reproducible and by using the MRP of the C14:0, C14:1(9), C15:0, C15:1(9), C16:0, C16:1(9), C16:1(11), C18:0, C18:1(9) and C18:1(11) fatty acids, they could differentiate between

strains (a 1% difference in MRP was used as a criterion for a different strain). Rigid standardisation of experimental conditions and techniques is, however, necessary when using cellular fatty acids as criterion for strain differentiation (Augustyn & Kock, 1989; Degré *et al.*, 1989). Parameters that require special attention include incomplete extraction of fatty acids from cellular material; auto-oxidation of polyunsaturated fatty acids; incomplete esterification and structural changes during esterification; incomplete separation of fatty acids present in the sample; and failure to elucidate the exact structures of the fatty acids under study. Furthermore, the fatty acid content is influenced by cultural conditions and rigid standardisation of growth media and cultural conditions is required (Augustyn & Kock, 1989; Degré *et al.*, 1989). The different growth rates of the non-*Saccharomyces* yeast makes the use of this technique for diagnostic means problematic.

Numerous other non-molecular methods that can be used for characterisation exist. Some of these include production of secondary metabolites, killer phenotype, spectroscopic and electrometric methods. Secondary products of fermentation (acetaldehyde, ethyl acetate, higher alcohols, acetoin and acetic acid) can also be used to determine the diversity of yeast species and strains within a species. This approach was used to characterise a range of non-*Saccharomyces* yeasts including *Kloeckera apiculata, Candida stellata, Candida valida* and *Zygosaccharomyces fermentati* (Romano *et al.*, 1997b, Romano & Marchese, 1998). Phenotypes within each species could be distinguished by the production of different amounts of these secondary products.

Two spectroscopic approaches to whole-organism fingerprinting were investigated by Timmins *et al.* (1998). Their approach entailed the use of pyrolysis mass spectrometry and Fourier transform-infrared spectroscopy. They were able to utilise these techniques to discriminate between 22 brewery *S. cerevisiae* strains. They also showed that their approach could mirror the known genotype of the yeasts studied. Both these techniques have the advantage of being fast (2 to 10 minutes), so a large number of yeast samples could be done on a day. They concluded, however, that this procedure could not be used in isolation, but rather in tandem with other procedures for yeast strain quality assurance. Furthermore, sophisticated, very expensive equipment is essential for this technology.

Electrometric methods can be used for routine investigations. These methods are based on the metabolic breakdown of substrates during growth of microorganisms that lead to changes in the electrical conductivity of the medium (Deák, 1995). These changes can be measured and the metabolic activity so estimated, but not yeast identity. Sophisticated apparatus is, however, also required. With rigid standardisation, this technology could be used to discriminate between pure cultures of non-*Saccharomyces* with widely differing growth rates.

The previously mentioned techniques all supply phenotypic characteristics of the yeast under investigation, but can be largely influenced by environmental factors impacting on the cell growth, thereby leading to varying results. For these reasons the advent of molecular biology with the accompanying techniques based on the yeast genome offers more reproducible results.

2.3.2 Molecular characterisation techniques

A number of methods based on the DNA composition of the yeast for the fast detection and identification of yeasts in food exist i.e. pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism analysis (RFLP) and polymerase chain reaction (PCR) based techniques e.g. random amplified polymorphic DNA (RAPD–PCR) (Deák, 1993; 1995; Querol & Ramón, 1996; Pretorius, 2000). However, most of the molecular methods require expensive equipment and require some form of computerised assistance for comparison of the numerous bands generated.

The various yeasts isolated from wine differ strongly in the number and size of their chromosomes. PFGE is a technique whereby the intact chromosomes, previously isolated, are subjected to alternating electric fields in an agarose gel. As the direction of the electric field changes (pulsed), chromosomal DNA is separated according to size. Due to chromosomal length polymorphism each individual strain has a unique banding pattern. The resultant banding pattern, visualised by staining with ethidium bromide and viewed under ultra-violet light, represents the electrophoretic karyotype of the yeast (Carle & Olson, 1985; Van der Westhuizen & Pretorius, 1992). A number of electrophoretic methods are available for the separation of the chromosomes. They include field inversion gel electrophoresis (FIGE) (Carle *et al.,* 1986; Degré *et al.*, 1989), transverse

alternating pulsed field electrophoresis (TAFE) (Bilinski & Casey, 1989; Henschke, 1990; Vezinhet *et al.*, 1990), orthogonal field alternation gel electrophoresis (OFAGE) (De Jonge *et al.*, 1986; Johnston & Mortimer, 1986; Takata *et al.*, 1989; Yokomori *et al.*, 1989) and contour clamped homogeneous electric field electrophoresis (CHEF) (Casey *et al.*, 1988; Van der Westhuizen & Pretorius, 1992).

PFGE, although having the disadvantages of being time consuming and requiring sophisticated equipment (Querol *et al.*, 1992), has proven to be a reliable method for characterising and identifying yeast strains (Petering *et al.*, 1988, Degré *et al.*, 1989; Takata *et al.*, 1989; Vezinhet *et al.*, 1990; Van der Westhuizen & Pretorius, 1992; Jolly *et al.*, 1993; Schütz & Gafner, 1994). Furthermore, this technology was able to differentiate between indigenous and commercial *S. cerevisiae* isolates from vineyards and industrial-scale fermentations (Querol *et al.*, 1992; Vezinhet *et al.*, 1992; Van der Westhuizen *et al.*, 2000b; Van der Westhuizen *et al.*, 2002a). Yeast diversity during wine fermentation could also be monitored (Schütz & Gafner, 1993).

Total, ribosomal or mitochondrial DNA can be cleaved by restriction enzymes at specific nucleotide sequences and the resultant fragments separated electrophoretically on agarose gels. RFLP is detected as different banding patterns that may be viewed under UV-light after ethidium bromide staining. Alternately, the DNA may be transferred from the gel to a suitable membrane (blotting) and hybridised with radioactively labelled known DNA sequences (probes). Regions of hybridisation may then be observed on X-ray film. Mitochondrial DNA restriction analysis has been used to characterise and monitor yeast population during fermentation (Sabate *et al.*, 1998; Gutiérrez *et al.*, 1999).

PCR and RAPD-PCR avoids some of the variability of RFLP and are *in-vitro* procedures whereby specific or random segments of DNA can be exponentially amplified and the products viewed after electrophoretic separation (Williams *et al.*, 1990; Deák, 1993). PCR has been used to characterise and differentiate between indigenous wine and non-*Saccharomyces* yeasts (De Barros Lopes *et al.*, 1998; Fernández *et al.*, 1999; Cocolin *et al.*, 2002; Zahavi *et al.*, 2002; Capece *et al.*, 2003). RAPD-PCR has been evaluated for use in the taxonomic identification of

S. cerevisiae and non-*Saccharomyces* wine yeasts (Quesada & Cenis, 1995; Capece *et al.*, 2003). These included yeasts of the genera *Candida, Pichia, Torulaspora, Hansenula, Rhodotorula* and *Saccharomyces*. The authors could differentiate between all their strains by using the accumulated information of a number of reactions. PCR and RFLP can be combined when PCR fragments produced can be cut by selected enzymes to give more detailed information thus allowing better separation between different yeast strains.

PCR may also be linked to RFLP where the PCR products are analysed by RFLP typing and mapping (Masneuf *et al.*, 1996; Smole-Mozina *et al.*, 1998; Fernández *et al.*, 1999; Yamagishi *et al.*, 1999). This technique was used to study wine yeasts isolated from vineyards (Vezinhet, *et al.*, 1992; Constanti *et al.*, 1998) and was found to be good for the discrimination between wine yeasts. However, much variability can be found in the arrangement of the short fragments. Often, too many small fragments are produced and all the yeast strains look the same.

PCR and PCR-RAPD require less time and effort compared to other molecular techniques and should therefore be more cost effective (Quesada & Cenis, 1995). The difficulty in PCR analyses, however, is the search for characteristic sequences that can be amplified. A further disadvantage is the need for several reactions to distinguish between a large number of strains. This puts limitations on this method. The monitoring of fermentations can also be problematic, as pure cultures are needed without interference of grape must constituents for best results. Poor reproducibility has also been cited (Quesada & Cenis, 1995) and rigid standardisation of reaction parameters is important.

2.3.3 General remarks

All the characterisation techniques discussed deliver information about the yeast. However, the extent to which different yeasts can be distinguished from each other largely depends on the number of strains that are being studied. Generally the greater the number, the more discriminating the technique should be. Many techniques are, however, not sufficiently discriminating to be used on their own. Therefore, most authors suggest using a combination of techniques for the most reliable means of differentiating between yeasts (Augustyn, 1989; Timmins *et al.*, 1998; Van der Westhuizen *et al.*, 1999, Khan *et al.*, 2000). The technique of

choice will usually be the one that gives the best results with the least input regarding time and costs. A further important criterion is the availability of the required instrumentation and the financial constraints presented by increasing costs of equipment and chemicals and decreasing budgets in real terms of research facilities.

Amongst the methods of electrophoretic karyotyping, fatty acid analysis and RAPD-PCR, electrophoretic karyotyping has been suggested as the most useful for routine fingerprinting of wine yeasts and can be used as a primary means for differentiating between yeasts (Van der Westhuizen *et al.*, 1999).

2.4 ECOLOGY OF YEASTS

Yeasts are found throughout nature. However, they do not occur randomly, but are found in specific habitats where the different species form communities (Lachance & Starmer, 1998). The different species found in a habitat can either be autochothonous (those that are essential components of the community) or allochothonous (those that are transient or there by chance). The component species within yeast communities are further defined by niches i.e. the physical, chemical and biotic attributes required by the yeast to survive and grow (Lachance & Starmer, 1998).

Yeasts found in many different habitats are considered generalists (broad niche) while those found in unique habitats are considered specialists (narrow niche) (Lachance & Starmer, 1998). Within the winemaking environment (habitat), the vineyard (grape surfaces) and cellar (equipment surfaces and must) can be considered specialised niches where the wine related yeasts can form communities. These niches differ broadly. The surface of the grape berry before ripeness presents limitations regarding nutrients. These are alleviated as berries ripen and/or are damaged. External factors such as fungicides and pesticides used in the vineyard will have a negative impact on populations. However, it has been reported that some pesticides can stimulate certain yeasts i.e. *K. apiculata* when they were tested in laboratory fermentations (Cabras *et al*, 1999).

The cellar environment presents a more nutrient rich niche. Grape must is a rich nutritive environment, but low pH and high osmotic pressure of the must and the use of SO_2 detracts from this ideal yeast niche. Surfaces of equipment can also harbour numerous microorganisms due to constant contact with grape must. Cellar hygiene will consequently play a big role in this niche.

2.5 YEASTS ASSOCIATED WITH VINEYARDS, WINERY AND WINE

The yeast species associated with the different niches of grape growth (vineyards) and wine production (wineries, grape must, fermentation and wine) can be arbitrarily divided into two groups i.e. the *Saccharomyces* group and the non-*Saccharomyces* group.

The *Saccharomyces* group with its primary representative, *Saccharomyces cerevisiae,* is present on the grape skins in low numbers (Van Zyl & Du Plessis, 1961; Rankine, 1972; Török *et al.*, 1996), as well as on winery equipment in greater numbers (Peynaud & Domercq, 1959; Vaughan-Martini & Martini, 1995). The second group, the non-*Saccharomyces* yeasts, contains numerous species dominated numerically by the apiculate yeasts e.g. *Kloeckera* spp., and *Candida* spp. that are found predominantly on grapes and in freshly processed must. Lesser numbers are also found on winery equipment.

The microflora of grapes are affected by a number of factors. These include vineyard altitude and aspect, climatic conditions (temperature, rainfall, humidity, maritime influences), grape variety (cultivar), thickness of grape skin, viticultural practices (fertilisation, irrigation, canopy management, fungicides, use of elemental sulphur), developmental stage of grapes, health of grapes (physical damage to berries, insect vectors) and winery waste disposal practices (Bisson & Kunkee, 1991; Regueiro *et al.*, 1993; Boulton *et al.*, 1996; Epifanio *et al.*, 1999; Pretorius, 2000). The way that the grapes are sampled (e.g. berries or bunches) and processed (washing of berries vs. crushing) can also determine what yeasts are isolated (Martini *et al.*, 1980; 1996), as the yeast population is higher close to the peduncle than to the centre and lower part of the bunch (Bisson & Kunkee, 1991).

At harvest, grape temperature, method of harvest (hand vs. mechanical), method of transport to the cellar (picking crates/baskets, tipsters), time of transport to cellar, time lapse before crushing, sulphite and enzyme addition can all affect the yeast population (Pretorius, 2000). The yeasts found on the surface of grapes are introduced into the must at crushing (Bisson & Kunkee, 1991; Lonvaud-Funel, 1996). Other strains found on the surface of cellar equipment can also be transferred to the must (Boulton *et al.*, 1996). Populations are further affected by method of crushing i.e. pressing whole bunches vs. berries, sulphite addition, enzyme addition, cellar hygiene, type of equipment, clarification method and temperature control (Regueiro *et al.*, 1993; Epifanio *et al.*, 1999; Pretorius, 2000).

The specific environmental conditions in the must i.e. high osmotic pressure, presence of SO_2, and temperature all play a role in determining what species can survive and grow (Bisson & Kunkee, 1991; Longo *et al.*, 1991). The fermentation rapidly becomes anaerobic and the alcohol levels increase, which further affect the yeast population.

Despite all the variables in grape harvest and wine production, the yeast species generally found on grapes and in wines are similar throughout the world (Amerine *et al.*, 1967). However, the proportion or population profile of yeasts in the different regions shows distinct differences (Amerine *et al.*, 1967; Longo *et al.*, 1991).

2.5.1 The *Saccharomyces* group

The prime representative of this group, *S. cerevisiae,* is the most important yeast for wine production and is responsible for the metabolism of grape sugar to alcohol and CO_2 (Fleet, 1993; Pretorius, 2003). It has an equally important role to play in formation of secondary metabolites of importance to wine (Fleet, 1993; Pretorius, 2003) as well as conversion of grape aroma precursors to varietal aromas in wine (Dubourdieu, 1996; P. Darriet, personal communication, 1996; Ribéreau-Gayon *et al.*, 2000). For these reasons *S. cerevisiae* is often simply referred to as "the wine yeast" (Henschke, 1997; Pretorius, 2003). Much of the knowledge pertaining to *S. cerevisiae* during wine fermentation can be equally applied to non-*Saccharomyces* yeasts under the same conditions and in the same environment.

The *S. cerevisiae* wine yeasts have been renamed taxonomically a number of times giving rise to confusion amongst wine makers. Previously, numerous different species of "wine yeasts" were recognised including *Saccharomyces ellipsoideus*, *Saccharomyces bayanus*, *Saccharomyces oviformis*, *Saccharomyces beticus* and *Saccharomyces capensis* (Lodder & Kreger-Van Rij, 1952). Species such as *S. cerevisiae*, *S. bayanus, S. capensis* and *Saccharomyces chevalieri* considered taxonomically similar were later grouped under *S. cerevisiae* (Kreger-van Rij, 1984). This classification was controversial and the *S. cerevisiae* yeasts were later divided into four species based on DNA homology viz. *S. cerevisiae, S. bayanus, Saccharomyces pastorianus* and *Saccharomyces paradoxus* (Vaughan Martini & Kurtzman, 1985; Vaughan Martini & Martini, 1987). This last reclassification has been accepted in the latest yeast taxonomy volume of Kurtzman & Fell (1998b). However, a recent phylogenetic analyses of 106 widely distributed orthologous genes suggest a different classification of *Saccharomyces* yeasts to the one presently accepted (Rokas *et al.*, 2003). A further reorganisation of the *Saccharomyces* group of yeasts can therefore be expected in future taxonomic volumes.

Whatever the correct classification of the *Saccharomyces* yeasts may be, it is generally, although possibly incorrectly, accepted by wine microbiologists and oenologists that the "wine yeasts" commercially available and those normally isolated from fermenting musts all belong to the species *S. cerevisiae*. The inability of many research facilities to do DNA homology studies and gene analyses further clouds the issue.

The origin of *S. cerevisiae* i.e. from the cellar (man-made environment) or from the grapes in the vineyard has been debated extensively. As cells of *S. cerevisiae* are rarely isolated from natural surfaces, such as grapes and vineyard soil (Bisson & Kunkee, 1991; Vaughan-Martini & Martini, 1995; Martini *et al.*, 1996; Mortimer & Polsinelli, 1999), Vaughan-Martini & Martini (1995) maintain that the cellar environment is the only source of *S. cerevisiae*. They further surmise that *S. paradoxis* is the only *Saccharomyces* species to come from natural environments and could possibly represent the original ancestor of the domesticated fermenting yeast, *S. cerevisiae*. However, recent ribosomal DNA sequence analysis suggests divergence times for these two species at five to 20

million years (Kellis *et al.*, 2003), suggesting that *S. cerevisiae* does have a natural origin.

This is in agreement with other authors who have shown that the vineyard is the primary source for *S. cerevisiae*, where it can be found, albeit in low numbers (less than 0.1% of the naturally occurring yeast) (Török *et al.*, 1996), on the grape surfaces (Rankine, 1972; Boulton *et al.*, 1996; Ross, 1997; Mortimer & Polsinelli, 1999). Therefore, enrichments are usually carried out in order to obtain *S. cerevisiae* isolates (Van der Westhuizen *et al.*, 2000a; 2000b; Khan *et al.*, 2000). However, not every vine or berry in a cluster will harbour *S. cerevisiae* (Török *et al.*, 1996). The yeasts are usually found around the stomata or at cracks in the cuticle where nutrients can seep out. Damaged grape berries therefore carry a higher number of cells than healthy grapes (up to $1 \times 10^5 - 1 \times 10^6$ cells/berry) (Mortimer & Polsinelli, 1999). It has also been shown that *S. cerevisiae* is generally less numerous in cool viticultural regions (Jackson, 1994 and the references therein).

Approximately 80% of yeasts colonising winery surfaces belong to the genus *Saccharomyces* (Loureiro & Malfeito-Ferreira, 2003 and the references therein) and form part of the indigenous flora. However, a selected *Saccharomyces* strain added initially to the must is able to out compete the indigenous flora (Rosini, 1984; Querol & Ramón, 1996). Furthermore, a commercial *Saccharomyces* strain once used in a cellar can become part of the indigenous cellar flora and can reappear in following vintages (Constanti, *et al.*, 1997). This indigenous flora is responsible for conducting spontaneous fermentations. Although a large number of strains can be found in musts, usually only a small number are capable of dominating the fermentation (Frezier & Dubourdieu, 1992; Querol & Ramón, 1996). A sequence of dominance by the different strains may also occur (Schütz & Gafner, 1994; Querol & Ramón, 1996). Dominance by a yeast strain is independent of grape cultivar or time of harvest and the same yeast strain can be dominant over a number of years (Frezier & Dubourdieu, 1992; Constanti, *et al.*, 1997; Torija *et al.*, 2001). Indigenous yeast strains can also compete with inoculated yeasts and dominate if they are stronger (Constanti, *et al.*, 1997). This may be due to indigenous yeasts being better adapted to the chemical and microbiological characteristics of the must from their winery (Lema *et al.*, 1996).

Many different strains of *S. cerevisiae* have been isolated from wine fermentations, each different regarding acetic acid, esters, higher alcohols and volatile sulphur compound formation (Fleet *et al.*, 1984; Lema *et al.*, 1996). Wine quality is therefore significantly influenced by the particular strain of *S. cerevisiae* that conducts the fermentation.

2.5.2 The non-*Saccharomyces* group

Numerous different non-*Saccharomyces* yeasts can be found on grapes and in fermenting must. In areas with high rainfall during harvest numbers of non-*Saccharomyces* yeasts increase (Querol *et al.*, 1990). Pesticides and other chemical sprays used in the vineyard can also affect yeast populations (Cabras *et al.*, 1999; Guerra *et al.*, 1999).

The range of species isolated (Table 2.3) often depends on the time and place during the winemaking process where the samples for yeast isolation were taken. The method of isolation and enumeration that is followed can also impact on the type of yeasts that are isolated as a perusal of Table 2.3 shows. These include shaking grape berries in a broth or crushing whole berries and plating on nutrient agar media. The technique of crushing berries before plating is closer to practical winemaking protocols than shaking in a broth. The objective of the investigation would also influence the method chosen.

The type of agar medium can also play an important role by limiting the growth of specific yeasts. The use of Lysine Medium, for example, does not allow the growth of *S. cerevisiae* due to its inability to utilise lysine as sole carbon source (Fowell, 1965; Heard & Fleet, 1986). However, some non-*Saccharomyces* yeasts may also not be able to utilise lysine and will then not be detected. On general nutrient medium faster growing yeasts can also overgrow slower growers. For yeasts with similar growth rates, only yeasts present in the same numerical order will be detected and more specific techniques and media are needed to isolate slower growing yeasts or yeasts found in lower numbers.

2.5.2.1 Non-*Saccharomyces *yeasts in vineyards and on grapes: Initially on unripe grapes low numbers of yeasts are found (10-10^3 cfu/g), but as the grapes ripen and mature, the population increases to 10^4-10^6 cfu/g (Fleet, 2003).

TABLE 2.3.
Non-*Saccharomyces* yeasts isolated from grapes.

Yeast species Anamorph/Teleomorph [Synonym][1]	Form isolated A/T/S[1]	Region or country	Isolation material	Brief description of method of isolation	Reference
Candida albicans		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985
Candida edax		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985
Candida famata / Debaryomyces hansenii	A	Israel	Muscat d' Alexandrie	20-50 berries shaken in sterile distilled water & plated onto basal yeast agar.	Zahavi *et al.*, 2002
	A	Israel	Cabernet Sauvignon	20-50 berries shaken in sterile distilled water & plated onto basal yeast agar.	Zahavi *et al.*, 2002
Candida glabrata [*Torulopsis glabrata*]	S	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Candida globosa / Citeromyces matritensis	T	Israel	Cabernet Sauvignon, Colombard	20-50 berries shaken in sterile distilled water & plated onto basal yeast agar.	Zahavi *et al.*, 2002
Candida guilliermondii	A	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
	A	Israel	Cabernet Sauvignon	20-50 berries shaken in sterile distilled water & plated onto basal yeast agar.	Zahavi *et al.*, 2002
Candida humicola		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985
Candida inconspicua [*Torulopsis inconspicua*]	S	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Candida krusei		Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium.	Van Zyl & Du Plessis, 1961

TABLE 2.3 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	Form isolated A/T/S[1]	Region or country	Isolation material	Brief description of method of isolation	Reference
Candida melinii		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Candida mycoderma		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Candida pulcherrima / Metschnikowia pulcherrima [*Torulopsis pulcherrima*]	A	Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium.	Van Zyl & Du Plessis, 1961
	A	Israel	Cabernet Sauvignon	20-50 berries shaken in sterile distilled water & plated onto basal yeast agar.	Zahavi *et al.*, 2002
Candida reukaufi / Metschnikowia reukaufi	T	Israel	Muscat de Alexandrie	20-50 berries shaken in sterile distilled water & plated onto basal yeast agar.	Zahavi *et al.*, 2002
Candida sake		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985
Candida stellata [*Torulopsis bacillaris*]		Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium.	Van Zyl & Du Plessis, 1961
		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Candida zeylanoides		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Van Zyl & Du Plessis, 1961

TABLE 2.3 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	Form isolated A/T/S[1]	Region or country	Isolation material	Brief description of method of isolation	Reference
Cryptococcus albidus		Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium.	Van Zyl & Du Plessis, 1961
		Japan	Zenkoji & Koshu	Berries crushed; dilutions plated.	Yanagida *et al.*, 1992
		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Cryptococcus diffluens		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Cryptococcus humicolus		Spain	Traditional grape varieties	Ten berries washed in saline and plated on Sabouraud dextrose agar.	Rementeria *et al.*, 2003
Cryptococcus laurentii		Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium.	Van Zyl & Du Plessis, 1961
		Japan	Chardonnay, Zenkoji & Koshu	Berries crushed; dilutions plated.	Yanagida *et al.*, 1992
		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Cryptococcus neoformans		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Hanseniaspora occidentalis		Japan	Niagra	Berries crushed; dilutions plated.	Yanagida *et al.*, 1992

TABLE 2.3 (continued)

Yeast species Anamorph/Teleomorph [Synonym][1]	Form isolated A/T/S[1]	Region or country	Isolation material	Brief description of method of isolation	Reference
Kloeckera apiculata / Hanseniaspora uvarum	A	Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium.	Van Zyl & Du Plessis, 1961
	A	Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
	A	Japan	Niagra, Zenkoji & Koshu	Berries crushed; dilutions plated.	Yanagida *et al.*, 1992
	T	California	Vineyard	Fermentations at 13°C & 18°C; dilutions plated on WL medium	Pallmann *et al.*, 2001
Kloeckera corticis / Hanseniaspora osmophila [*K. magna*]		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985
Lodderomyces elongisporus		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985
Pichia fermentans		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Pichia membranifaciens		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985
Rhodotorula glutinus		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985

TABLE 2.3 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	Form isolated A/T/S[1]	Region or country	Isolation material	Brief description of method of isolation	Reference
Rhodotorula glutinis (continued)		Western Cape	Grapes	Berries incubated in sterile grape juice until growth observed and then plated on malt extract and grape juice agar medium.	Van Zyl & Du Plessis, 1961
		Japan	Chardonnay & Zenkoji	Berries crushed; dilutions plated.	Yanagida *et al.*, 1992
		Spain	Traditional grape varieties	Ten berries washed in saline and plated on Sabouraud dextrose agar.	Rementeria *et al.*, 2003
		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Rhodotorula aurantiaca		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973
Rhodotorula minuta		North Carolina	Muscadine (*Vitis rotundifolia*)	Ten berries shaken in peptone buffer and plated on potato dextrose agar.	Parish & Carroll, 1985
		Japan	Zenkoji	Berries crushed; dilutions plated.	Yanagida *et al.*, 1992
Rhodotorula mucilaginosa		Western Cape	Grapes	Berries shaken in sterile distilled water with Tween 80 and plated on grape must agar.	Le Roux *et al.*, 1973

[1]Synonym or alternate name used in older literature.
[2]A = anamorph; T = teleomorph; S = synonym.

This is due to sugars that leach or diffuse out from inner tissue to the grape skin surfaces consequently providing nutrition for the yeasts. Therefore, the health of the grapes and/or degree of damage to grape berry will also largely determine the population numbers.

Generally, between nine and 12 yeast species are found on grapes (Van Zyl & Du Plessis, 1961; Parish & Caroll, 1985; Yanagida *et al.*, 1992; Regueiro *et al.*, 1993; Zahavi *et al.*, 2002; Rementeria *et al.*, 2003). They include predominantly *Hanseniaspora/Kloeckera* spp., *Metschnikowia/Candida* spp., *Rhodotorula* spp. and *Cryptococcus* spp. Unfortunately, comparisons between different studies are difficult, as different approaches were used for grape sampling and yeast isolation (Table 2.3). In addition, the state of ripeness and berry health is never given. Further factors that can influence a yeast population include specific meso- and micro-climates in the vineyards. Notwithstanding, there is general agreement that the most frequently occurring species in vineyards are usually the apiculate yeasts, *K. apiculata/Hanseniaspora uvarum* (50-75% of isolates) (Van Zyl & Du Plessis, 1961; Yanagida *et al.*, 1992; Pretorius, 2000 and the references therein). It has been reported that in warm to hot regions the teleomorphic form (*Hanseniaspora uvarum*) tends to replace the anamorphic form (*K. apiculata*) while the anamorphic form is present in greater numbers in cooler regions (Bisson & Kunkee, 1991; Jackson, 1994; Boulton *et al.*, 1996). In moderate climates, both types occur in equal numbers. However, this distribution between the teleomorphic and anamorphic forms may be region dependent. Altitude also appears to play a role as it has been reported that *Kloeckera* spp. are found more frequently at higher altitudes and *Hanseniaspora* spp. more at lower altitudes. This may be linked to temperature. Identification of *Kloeckera* vs. *Hanseniaspora* yeasts can also depend on how long the yeasts were conserved before identification. Teleomorphic yeasts can loose their ability to sporulate the longer they are kept in storage (Yarrow, 1998; M. Th. Smith, personal communication, 2000) and will therefore be identified as the anamorphic form.

According to Van Zyl & Du Plessis (1961) the following yeasts occur in highest frequency in South African vineyards i.e. *K. apiculata, Rhodotorula glutinis, Candida krusei, Candida pulcherrima, Candida laurentii, Cryptococcus albidus* and

Candida stellata (*T. bacillaris*). Further studies showed that grapes infected with the *Botrytis cinerea* fungus influenced the non-*Saccharomyces* population i.e. *C. krusei* and *K. apiculata* increased and *R. glutinis* decreased (Le Roux *et al.*, 1973). *R. glutinis* can also be predominant on grapes (Rementeria *et al.*, 2003). Other non-*Saccharomyces* yeasts found on grapes and in vineyards are shown in Table 2.3.

2.5.2.2 Non-Saccharomyces yeasts associated with cellar equipment: Non-*Saccharomyces* yeasts are present in lower numbers on cellar equipment where *S. cerevisiae* predominates (Peynaud & Domercq, 1959; Rosini, 1984; Lonvaud-Funel, 1996; Pretorius, 2000). The cellar surface population would be expected to be similar to that of the grapes and/or fermenting must with which it had contact. Species that have been isolated include *Pichia anomala, Pichia membranifaciens, Candida* spp., *Cryptococcus* spp. and more rarely *Rhodotorula* spp., *Debaryomyces hansenii*, *K. apiculata* and *Metschnikowia pulcherrima* (Loureiro & Malfeito-Ferreira, 2003).

2.5.2.3 Non-Saccharomyces yeasts associated with fermenting must: During crushing, the non-*Saccharomyces* yeasts on the grapes, cellar equipment and in the cellar environment (air and insects) are carried over to the must (Peynaud & Domercq, 1959; Bisson &-Kunkee, 1991; Boulton *et al.*, 1996; Lonvaud-Funel, 1996; Török *et al.*, 1996; Constanti *et al.*, 1997; Mortimer & Polsinelli, 1999; Fleet, 2003). However, the hygienic procedures of most modern wineries can limit contamination of the juice by resident cellar flora (Jackson, 1994; Pretorius; 2000). It can therefore be expected that the dominant yeasts in must after crushing will be the same as on grapes (Rementeria *et al.*, 2003).

The specific environmental conditions in grape must are limited and hostile to yeasts due to low pH, high sugar (high osmotic pressure), equimolar mixture of glucose and fructose, presence of SO_2 and non-optimal growth temperature during cold settling (Bisson & Kunkee, 1991; Longo *et al.*, 1991; Pretorius, 2000). Furthermore, the environment rapidly becomes anaerobic with increasing levels of ethanol that is toxic to yeasts. Nitrogen levels are usually sufficient at the start of fermentation (Bisson & Kunkee, 1991), but can be limiting towards the end of fermentation unless supplemented. The clarification of white must (centrifugation, enzyme treatments,

cold settling) can also reduce the initial population of yeasts in must (Fleet, 1990; Lonvaud-Funel, 1996; Pretorius, 2000).

Non-*Saccharomyces* yeasts found in grape must and during fermentation (Table 2.4) can be divided into three groups viz.

(i) yeasts with mainly oxidative behaviour e.g. *Pichia* spp., *Debaryomyces* sp., *Rhodotorula* spp., *Candida* spp. (e.g. *C. pulcherrima and C. stellata*), and *C. albidus*;

(ii) apiculate yeasts with low fermentative activity e.g. *K. apiculata (H. uvarum), Kloeckera apis, Kleockera javanica;* and

(iii) yeasts with fermentative metabolism e.g. *Saccharomyces kluyveri, Kluyveromyces marxianus, Torulaspora* spp. (e.g. T. *globosa* and *T. delbrueckii*) and *Zygosaccharomyces* spp. (Fleet *et al.*, 1984; Querol *et al.*, 1990; Bisson & Kunkee, 1991; Longo *et al.*, 1991; Lonvaud-Funel, 1996; Lorenzini, 1999; Torija *et al.*, 2001).

During fermentation and especially apparent in spontaneous fermentations where the initial high inoculum of *S. cerevisiae* cultured wine yeasts is absent, there is a sequential development of yeasts. Initially, species of *Kloeckera* (*Hanseniaspora*), *Rhodotorula, Pichia, Candida* (*C. stellata, C. pulcherrima//M. pulcherrima, Candida sake*) and *Cryptococcus*, are found at low levels in the fresh must (Parish & Caroll, 1985; Bisson & Kunkee, 1991; Frezier & Dubourdieu, 1992; Jackson, 1994; Granchi *et al.*, 1998; Fleet, 2003). Of these species, *K. apiculata* is usually present in the highest numbers, followed by the various *Candida* spp..

In a study of South African musts, however, very few *K. apiculata* yeasts were found (Van Zyl & Du Plessis, 1961). This was attributed to the addition of large quantities of SO_2 to the must to aid settling. In another study on muscadine grapes (*Vitis rotundifolia*) from North Carolina, *Hanseniaspora uvarum* (*Kloeckera apiculata*) was absent, but *Hanseniaspora osmophilia* and *Pichia membranifaciens* predominated during initial stages of the fermentation (Parish & Caroll, 1985). This may be an association for that particular geographic area or grape type. However, viticultural practices could have affected the normal non-*Saccharomyces* population. At the

TABLE 2.4.
Non-*Saccharomyces* yeasts isolated from grape must.

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Brettanomyces bruxellensis / Dekkera bruxellensis	T	Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al.*, 1999
[*Brettanomyces vini*]	A	Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
	T	Spain	Ribeiro	Dilution & plating on YPD	Cansado *et al.*, 1989
Candida sp.		Spain	Ribeiro	Dilution & plating on YPD	Cansado *et al.*, 1989
Candida colliculosa / Torulaspora delbrueckii [*T. rosei*]	A	Australia	Hermitage (red)	Spread inoculation on malt extract and Lysine agar	Heard & Fleet, 1985
	A	Catalonia	Macabeo (white) & Grenache (red)	Dilution and plating on YPD	Torija *et al.*, 2001
	T	Bordeaux	White variety	Not given.	Peynaud & Domercq, 1959
	T	Italy	Variety not given	Not given.	Castelli, 1955
	T	Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al.*, 1999
Candida famata / Debaryomyces hansenii	T	Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al.*, 1999
	A	Israel	Muscat de Alexandrie	20-50 berries shaken in sterile distilled water & plated on basal yeast agar.	Zahavi *et al.*, 2002
	T	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
	T	Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
(*Debaryomyces* sp.)	T	Spain	Ribeiro	Dilution & plating on YPD	Cansado *et al.*, 1989

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Candida glabrata		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Candida glucosophila		Spain	White and red (traditional varieties)	Samples plated on Sabouraud dextrose agar.	Rementeria *et al.*, 2003
Candida guilliermondii	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Candida krusei		Bordeaux	Semillon	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
Candida lusitaneae		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Candida pulcherrima / Metschnikowia pulcherrima [*Torulopsis pulcherrima*]	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
	T	Catalonia	Macabeo (white) & Grenache (red)	Dilution and plating on YPD	Torija *et al.*, 2001
	A	Australia	Riesling (white) and Malbec (red)	Spread inoculation on malt extract and Lysine agar	Heard & Fleet, 1985
	A	Bordeaux	Red grape variety	Not given	Peynaud & Domercq, 1959
	T	Bordeaux	Semillon	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Candida pulcherrima / Metschnikowia pulcherrima [*Torulopsis pulcherrima*] (continued)	A	Majorca	Prensal blanc (white)	Dilutions plated on YM and Lysine agar	Mora & Mulet 1991
	T	Italy	Nebbiola (red)	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	T	Germany	Pinot noir (red)	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	S	Switzerland	Pinot noir (red)	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	T	Italy	Variety not given	details not given.	Castelli, 1955
Candida rugosa		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Candida steatolytica		Majorca	Prensal blanc (white)	Dilutions plated onto YM and Lysine agar	Mora & Mulet, 1991
Candida stellata [*Torulopsis stellata*; *Torulopsis bacillaris*]		Australia	Riesling, Semillon (white), Malbec and Hermitage (red)	Spread inoculation on malt extract and Lysine agar	Heard & Fleet, 1985
		Catalonia	Garnatxa	Dilutions plated on malt extract agar	Constanti *et al.*, 1997
		Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al.*, 1990
		Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
		Bordeaux	Semillon	Dilutions plated on malt extract agar	Fleet *et al.*, 1984

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Candida stellata [Torulopsis stellata; Torulopsis bacillaris] (continued)		Catalonia	Macabeo (white) & Grenache (red)	Dilution and plating on YPD	Torija *et al.*, 2001
		Bordeaux	Red & white variety	Not given	Peynaud & Domercq, 1959
		Majorca	Chenin blanc & Prensal blanc	Dilutions plated on YM and Lysine agar	Mora & Mulet, 1991
Candida valida / Pichia membranifaciens	T	Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al.*, 1999
Hanseniaspora occidentalis		North Carolina	Muscadine (*Vitis rotundifolia)*	Direct plating on potato dextrose agar.	Parish & Carroll, 1985
Hansenula anomala		Australia	Hermitage (red)	Spread inoculation on malt extract and Lysine agar	Heard & Fleet, 1985
		Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
		Majorca	Chenin blanc	Dilutions plated on YM and Lysine agar	Mora & Mulet, 1991
Issatchenkia terricola		Majorca	Chenin blanc	Dilutions plated on YM and Lysine agar	Mora & Mulet, 1991
Kloeckera africana / Hanseniaspora vineae		Bordeaux	Red grape variety	Not given	Peynaud & Domercq, 1959
	A	Italy	Variety not given	Not given	Castelli, 1955
	T	Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al.*, 1999

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Kloeckera apis / Hanseniaspora guilliermondii		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Kloeckera apiculata / Hanseniaspora uvarum	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
	A	Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al.*, 1990
	T	Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al.*, 1990
	T	Catalonia	Macabeo (white) & Grenache (red)	Dilution and plating on YPD	Torija *et al.*, 2001
	T	Catalonia	Garnatxa	Dilutions plated on malt extract agar	Torija *et al.*, 2001
	A	Majorca	Chenin blanc & Prensal blanc	Dilutions plated onto YM and Lysine agar	Mora & Mulet, 1991
	A	Australia	Riesling, Semillon (white), Malbec and Hermitage (red)	Spread inoculation on malt extract and Lysine agar	Heard & Fleet, 1985
	T	Bordeaux	Semillon	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
	A	Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
	A	Bordeaux	Semillon	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Kloeckera apiculata / Hanseniaspora uvarum (continued)	A	Bordeaux	Red & white varieties	Not given	Peynaud & Domercq, 1959
	T	Italy	Nebbiola (red)	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	T	Germany	Pinot noir	Dilutions plated on yeast extract agar	Schütz & Gafner, 1993
		Bordeaux	Red varieties	Not given	Peynaud & Domercq, 1959
	T	Switzerland	Pinot noir	Dilutions plated on Phytone yeast extract agar	Schütz & Gafner, 1993
	T	Switzerland	Pinot noir	Details not given	Lorenzini, 1999
	A	Italy	Different varieties	Details not given.	Castelli, 1955
Kloeckera corticis / Hanseniaspora osmophila [*K. magna*]	T	Italy	Variety not given	Details not given.	Castelli, 1955
Kloeckera jensenii		Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
Kloeckera sp.		Spain	Ribeiro	Dilution & plating on YPD	Cansado *et al.*, 1989
Kloeckera javanica / Hanseniaspora occidentalis	A	Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
	A	Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al.*, 1990
	T	Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al.*, 1999

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Kluyveromyces marxianus		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Kluyveromyces thermotolerans		Catalonia	Macabeo (white) & Grenache (red)	Dilution and plating on YPD	Torija *et al.*, 2001
		Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al.*, 1999
Pichia etchelsii		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Pichia farinosa		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Pichia fermentans		Bordeaux	Red & white varieties	Not given	Peynaud & Domercq, 1959
Pichia kluyveri		Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
Pichia kudriavzevii		Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
Pichia membranifaciens		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
		North Carolina	Muscadine (*Vitis rotundifolia*)	Direct plating on potato dextrose agar.	Parish & Carroll, 1985

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Pichia membranifaciens (continued)		Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al.*, 1990
		Majorca	Prensal blanc	Dilutions plated on YM and Lysine agar	Mora & Mulet, 1991
		Bordeaux	Red & white varieties	Not given	Peynaud & Domercq, 1959
Pichia terricola		Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
Rhodotorula glutinus		Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
		Tenerife	White Listan	Random harvesting; further details not given.	De Cos *et al.*, 1999
		Spain	White and red (traditional varieties)	Samples plated on Sabouraud dextrose agar.	Rementeria *et al.*, 2003
Rhodotorula graminis		Bordeaux	Semillon	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
Rhodotorula minuta		Alicante	Monastrell (red)	Dilutions plated on malt extract agar	Querol *et al.*, 1990
Rhodotorula mucilaginosa		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
Rhodotorula sp.		Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1985

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Saccharomyces fermentati		Bordeaux	Merlot	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
Saccharomyces kluyveri		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991
		Tenerife	White Listan	Random harvesting; no further details.	De Cos *et al.*, 1999
Saccharomyces krusei		Bordeaux	Semillon	Dilutions plated on malt extract agar & grape juice agar	Fleet *et al.*, 1984
Saccharomycodes sp.		Spain	Ribeiro	Dilution & plating on YPD	Cansado *et al.*, 1989
Saccharomycodes ludwigii [*Saccharomyces ludwigii*]	S	Tenerife	White Listan	Random harvesting; no further details.	De Cos *et al.*, 1999
Schizosaccharomyces spp.		Catalonia	Macabeo (white) & Grenache (red)	Dilution and plating on YPD	Torija *et al.*, 2001
Torulaspora delbrueckii		Bordeaux	White variety	Not given	Peynaud & Domercq, 1959
Torulaspora globosa		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991

TABLE 2.4 (continued).

Yeast species Anamorph/Teleomorph [Synonym][1]	**Form isolated A/T/S[2]**	**Country or region**	**Isolation material** (must variety)	**Brief description of method of isolation**	**Reference**
Torulaspora sp.		Spain	Ribeiro	Dilution & plating on YPD	Cansado *et al.*, 1989
		Bordeaux	Red & white varieties	Not given	Peynaud & Domercq, 1959
Torulopsis famata		Bordeaux	Red variety	Not given	Peynaud & Domercq, 1959
Zygosaccharomyces bailii		Spain	White & red varieties	Samples of must diluted and plated on agar malt and Sabouraud plates	Regueiro *et al.*, 1993
Zygosaccharomyces florentinus		Spain	Abarino, Godello (white) and Mencia (red)	Dilution plated on juice-agar medium	Longo *et al.*, 1991

[1]Synonym or alternate name used in older literature.
[2]A = anamorph; T = teleomorph; S = synonym.

start of fermentation an initial proliferation of apiculate yeasts (*Kloeckera* and *Hanseniaspora*) normally occurs. This is usually more apparent in red must than white, possibly due to the higher pH of the red must.

In the past it was generally believed that all non-*Saccharomyces* yeasts died soon after the commencement of the alcoholic fermentation due to the rising ethanol concentration and added SO_2. Recently, numerous authors have reported that some non-*Saccharomyces* yeasts can survive to a later stage of fermentation than initially believed (up to 8 days) and others can be present throughout the fermentation reaching cell concentrations of 10^6 to 10^8 cells/mL (Fleet *et al.*, 1984; Heard & Fleet, 1985; Fleet, 1990; Longo *et al.*, 1991; Todd, 1995; Gafner *et al.*, 1996; Granchi *et al.*, 1998; Fleet, 2003). This sustained growth of non-*Saccharomyces* is more evident in spontaneous fermentations where there is no initial large number of *S. cerevisiae* that can suppress their growth. Abnormal vintages e.g. excessive rainfall during the ripening of the grapes, also contribute to a greater presence of non-*Saccharomyces* in the initial stages and later in the fermentation (Querol *et al.*, 1990). Non-*Saccharomyces* yeasts have also been observed to grow to levels of *ca.* 10^4 cells/ml in red wines during malo-lactic fermentations (Fleet *et al.*, 1984).

Despite the sustained presence of certain non-*Saccharomyces* yeasts, the majority do disappear during the early stages of the vigorous fermentation (Fleet *et al.*, 1984; Jackson, 1994; Henick-Kling *et al.*, 1998). This may be due to their slow growth and inhibition by the combined effects of sulphur dioxide, low pH, high ethanol and oxygen deficiency (Jackson, 1994). This is consistent with their oxidative or weak fermentative metabolism. Nutrient limitation and strength of *S. cerevisiae* inoculum would also have a suppressive effect. Granchi *et al.* (1998) reported that the decline of specifically *K. apiculata* started once *S. cerevisiae* became dominant rather than when the fermentation temperature and ethanol concentration reached values known to inhibit apiculate yeast growth. It has also been reported that *T. delbrueckii* and *K. thermotolerans* are less tolerant to low oxygen levels and this rather than ethanol toxicity affects their growth and leads to their death during fermentation (Hansen *et al.*, 2001). The non-*Saccharomyces* that do survive and are present until the end of fermentation may also have a

higher tolerance to ethanol. It has been documented that *C. stellata* (*Torulopsis stellata*) can tolerate 10 to 12% ethanol which would account for its sustained presence during fermentation. Other species reported throughout fermentation are *Pichia* sp. (Bisson & Kunkee, 1991) and *Zygosaccharomyces bailii* (synonym = *Saccharomyces acidifaciens*) (Peynaud & Domercq, 1959).

The extent to which different factors affect the non-*Saccharomyces* yeasts are dependent on the characteristics of the individual species and growth parameters for one species will not necessarily be the same for others. Strains within a species can also vary.

2.5.2.4 Non*-Saccharomyces *yeasts associated with wine: Non-*Saccharomyces* yeasts in wine are usually associated with wines in barrels and post fermentation spoilage (Grbin, 1999; Loureiro & Malfeito-Ferreira, 2003 and the references therein). However, only a small number are able to tolerate the adverse conditions in wine and multiply (Van Kerken, 1963). These include *Brettanomyces* spp. (*Dekkera* spp.), *Z. bailii*, *P. membranifaciens*, *C. krusei* and *C. valida* (Van Kerken, 1963; Fleet *et al.*, 1984; Parish & Caroll, 1985; Bisson & Kunkee, 1991; Grbin, 1999). Some of these species e.g. *Brettanomyces* and *Zygosaccharomyces* are as ethanol tolerant as *S. cerevisiae* and may be found in bottled wine. Their presence is influenced by the degree of filtration that precedes bottling and cellar hygiene during bottling.

2.6 THE ROLE OF NON-*SACCHAROMYCES* YEASTS IN WINE PRODUCTION

The role of non-*Saccharomyces* yeasts in wine production has been debated extensively (Castor, 1954; Van Zyl *et al.*, 1963; Fleet *et al.*, 1984; Heard & Fleet, 1985; Fleet, 1990; Herraiz *et al.*, 1990; Longo *et al.*, 1991; Romano *et al.*, 1992; Todd, 1995; Gafner *et al.*, 1996; Gil *et al.*, 1996; Lema *et al.*, 1996; Granchi *et al.*, 1998; Henick-Kling *et al.,* 1998; Lambrechts & Pretorius, 2000; Fleet, 2003 and the references therein; Rementeria *et al.*, 2003). As already discussed in earlier sections, grape musts contain a mixture of yeast species. Wine fermentation is therefore not a single species fermentation (Fleet, 1990), although the dominance of *S. cerevisiae* (inoculated or indigenous) in the fermentation is expected and desired. However, the indigenous non-*Saccharomyces* yeasts, already present in

the must in greater numbers than *S. cerevisiae*, are adapted to the specific environment and in an active growth state, giving them a competitive edge.

Despite a long held belief among winemakers in the Old World wine regions that spontaneous fermentations (comprising mixed cultures of non-*Saccharomyces* and *Saccharomyces* yeasts) gave superior wines in comparison to pure culture fermentations, earlier authors usually referred to the non-*Saccharomyces* yeasts as spoilage organisms (Amerine & Cruess, 1960; Van Zyl & Du Plessis, 1961; Van Kerken, 1963; Rankine, 1972; Le Roux *et al.*, 1973). This was substantiated by their frequent isolation from stuck fermentations and from spoiled bottles of wine.

Furthermore, although it was known that some non-*Saccharomyces* yeasts could form metabolites e.g. esters, leading to aromas not always detrimental to wine quality (Castor, 1954; Amerine & Cruess, 1960; Van Zyl *et al.*, 1963), this was outweighed by the high levels of volatile acidity and other negative compounds produced (Castor, 1954; Amerine & Cruess, 1960; Van Zyl *et al.*, 1963; Amerine *et al.*, 1967; 1972). Some yeasts e.g. *Candida, Pichia* and *Hansenula* spp. are capable of forming films on the surface of the wines with exposure to oxygen. Off-odours like acetic acid, ethyl acetate and acetaldehyde are also associated with their growth (Grbin, 1999). *Brettanomyces* spp. (*Dekkera* spp.) can produce ethylphenols and tetrahydropyridines that contribute to 'animal/farmyard/mousy' taints in wines (Parish & Caroll, 1985; Grbin, 1999, Grbin & Henschke, 2000; Arvik & Henick-Kling, 2002). Other non-*Saccharomyces* yeasts such as *Saccharomycodes ludwigii*, more commonly a contaminant of sulphated musts due to its high resistance to SO_2, produce large amounts of ethyl acetate and acetaldehyde that will negatively affect wine aroma and quality (Ciani & Maccarelli, 1998).

The earlier authors also considered non-*Saccharomyces* yeasts sensitive to SO_2 in must and added this compound primarily to control their growth and that of spoilage bacteria (Amerine & Cruess, 1960; Van Zyl & Du Plessis, 1963; Amerine *et al.*, 1972). Non-*Saccharomyces* yeasts were also known to be poor fermenters of grape must and intolerant to ethanol (Castor, 1954) especially in the presence of SO_2 (Amerine *et al.*, 1972). It was therefore accepted that those non-*Saccharomyces* yeasts not initially inhibited by the SO_2, died during fermentation

due to the combined toxicity of the SO_2 and alcohol. Consequently, the non-*Saccharomyces* were seen to be of little significance in normal wine production and it was recommended that only proven strains of the wine yeast *S. cerevisiae* be used in commercial fermentations (Amerine & Cruess, 1960; Amerine *et al.*, 1972).

During ongoing wine microbiological research, more recent authors (as already mentioned), showed that non-*Saccharomyces* yeasts survive during fermentation (Fleet *et al.*, 1984; Heard & Fleet, 1985; Longo *et al.*, 1991; Todd, 1995; Gafner *et al.*, 1996). It has also been reported that the non-*Saccharomyces* populations can reach concentrations of 10^6 to 10^8 cells/mL (Fleet *et al.*, 1984), which is similar to concentrations reached by *S. cerevisiae* yeasts. The more recently reported higher numbers of non-*Saccharomyces* yeasts may be a result of improved cellar technology and hygiene in modern cellars that has led to the reduction of SO_2 usage, which will result in the survival of a greater number and diversity of non-*Saccharomyces* yeasts. Coupled to this is the use of modern laboratory techniques making the detection of non-*Saccharomyces* yeasts easier.

These high numbers and sustained presence of non-*Saccharomyces* yeasts in modern wine fermentations resulted in wine microbiologists revisiting the role of these yeasts in wine fermentation. The previously mentioned belief of the Old World winemakers has also been reconsidered. Spontaneous fermented wines, although carrying a higher risk of spoilage, are generally regarded to have improved complexity, mouth-feel (texture) and integration of flavours compared to inoculated wines (Heard & Fleet, 1985; Bisson & Kunkee, 1991; Gil *et al.*, 1996; Lema *et al.*, 1996; Grbin, 1999; Soden *et al.*, 2000). This is due to specific metabolic end products.

Documented cases bearing out the belief of the winemakers include the study on Albariño wine aroma components by Lema *et al.* (1996). These authors concluded that the predominance of the inoculated *S. cerevisiae* strain along with a notable growth rate of the indigenous non-*Saccharomyces* population during the first days of the wine fermentation, contributed significantly to the good aromatic properties of the wines. Herraiz *et al.* (1990) and Gil *et al.*, (1996) also reported that wines produced by pure and mixed cultures of *S. cerevisiae* and apiculate yeasts

(*K. apiculata* and *H. uvarum*) differ regarding their aromatic compounds. The low frequency of *Kloeckera* spp. during fermentation has also been suggested as a reason for the lack of complexity of bouquet of Folle blanche wines in the Basque region in Spain (Rementeria *et al.*, 2003).

The range of flavour compounds produced by different yeasts is well documented (Lambrechts & Pretorius, 2000 and the references therein). These metabolic products resulting from non-*Saccharomyces* growth include glycerol, acetaldehyde, acetic acid, succinic acid, higher alcohols, ester aldehydes, ethyl esters and fatty acid esters (Fleet *et al.*, 1984; Bisson & Kunkee, 1991; Jackson, 1994; Boulton *et al.*, 1996; Lonvaud-Funel, 1996; Heard, 1999).

Glycerol, after ethanol, is the next major yeast metabolite produced during wine fermentation and is important in yeast metabolism for regulating redox potential in the cell (Scanes *et al.*, 1998; Prior *et al.*, 2000). Glycerol has been attributed to add smoothness (mouth-feel) and complexity to wines. Glycerol can also add sweetness (Ciani & Maccarelli, 1998). The contribution that glycerol makes to a wine will depend on the grape variety and style of wine being produced. It generally appears that Chardonnay, Sauvignon blanc and Chenin blanc quality will not be improved by increased glycerol concentrations (Nieuwoudt *et al.*, 2002). However, as all wine sensory profiles are unique, an individual wine may benefit from increased glycerol levels.

According to Henick-Kling *et al.* (1998) spontaneously fermented wines have higher glycerol levels indicating a possible contribution by non-*Saccharomyces* yeasts. It is known that *C. stellata* has elevated glycerol production and it can produce between 10 and 14 g/L (Ciani & Picciotti, 1995; Ciani & Ferraro, 1998) in comparison to the 4 to 7 g/L range of *S. cerevisiae* (Ciani & Maccarelli, 1998; Prior *et al.*, 2000). Unfortunately, increased glycerol production is usually linked to increased acetic acid production (Prior *et al.*, 2000) which can be detrimental to wine quality. This makes the growth of *C. stellata* during fermentation problematic, unless a balance can be achieved between enough glycerol production to add smoothness and/or complexity and limited acetic acid production that will not detract from wine quality.

Apiculate yeasts (*K. apiculata* and *H. guilliermondii*) have also been implicated in glycerol production. These yeasts can generally be divided into two groups i.e. high-glycerol (3 g/L), low ethanol (0.9% $^{v}/_{v}$) producers and low glycerol (1 g/L), high ethanol (2.4% $^{v}/_{v}$) producers (Romano *et al.*, 1997a). Apiculate yeasts are also known as high producers of acetic acid and that makes them undesirable for wine production (Ciani & Picciotti 1995). However, it has been reported that large strain variability exists and that not all strains of *Kloeckera* spp. form high levels of acetic acid (Romano *et al.*, 1992). Some strains form less than 1 g/L and are comparable to *S. cerevisiae*. If these strains also form higher levels of glycerol, their use can benefit a wine where higher glycerol levels are needed.

The primary flavour of wine is derived from the grapes. However, secondary flavours are derived from ester formation by yeasts during wine fermentation (Lambrechts & Pretorius, 2000). Over 160 esters have been distinguished in wine (Jackson, 2000). These esters can have a positive effect on wine quality, especially in wine from varieties with neutral flavours that are consumed shortly after production e.g. some Chenin blanc wines (Lambrechts & Pretorius, 2000). Non-*Saccharomyces* yeasts isolated from South African musts during 1961 could be divided into two groups viz. neutral yeasts (producing little or no flavour compounds) and flavour producing species (both desired and undesired) (Van Zyl *et al.*, 1963). Flavour producing yeasts included *Pichia anomala* (*Hansenula anomala*) and *K. apiculata*. *C. pulcherrima* are also known to be high producers of esters (Bisson & Kunkee, 1991). Unfortunately *C. pulcherrima* has an antagonistic action against *S. cerevisiae* and some other yeasts (Panon, 1997; Nguyen & Panon, 1998). When aerobic growth of *C. pulcherrima* cultures was followed by *S. cerevisiae*, delays in fermentation occurred. This killer effect is not similar to the classical *S. cerevisiae* killer effect, but is linked to the pulcherrimin pigment formed by *C. pulcherrima*.

Other yeasts that can play a role in wine production are those of the genus *Brettanomyces* (*Dekkera*). Different *Brettanomyces* strains can develop flavours ranging from pleasantly fruity and toffee to volatile and unpleasant when grown in sterile grape juice (Eschenbruch & Wong, 1993). *Brettanomyces* spp. have also been linked to the formation of tetrahydropyridines which give wines a 'mousy' taint and 4-ethyl phenol that has a 'medicinal-like' aroma (Grbin *et al.*, 1995; Grbin

& Henschke, 2000; Arvik & Henick-Kling, 2002). The 'mousy/medicinal' character, usually referred to as "Brett", is considered to be an off-flavour. However, anecdotal evidence indicates that the "Brett" character, at low levels in a red wine with an overall complex aroma, can be a positive addition.

Some non-*Saccharomyces* yeasts e.g. *T. delbrueckii* are able to form succinic acid (Ciani & Maccarelli, 1998). This correlates to high ethanol production and ethanol tolerance. Succinic acid production could positively influence the analytical profile of wines by contributing to the total acidity in wines with insufficient acidity. However, succinic acid has a salt-bitter-acid taste (Amerine *at al.*, 1972) and too high levels will negatively influence wine quality.

Different non-*Saccharomyces* yeasts also produce different levels of higher alcohols (n-propanol, isobutanol, isoamyl alcohol, active amyl alcohol) (Romano *et al.*, 1992; Lambrechts & Pretorius, 2000). This can be potentially important during wine production, as high concentrations of higher alcohols are generally not desired, but lower values can add to wine complexity (Romano & Suzzi, 1993b). Non-*Saccharomyces* yeasts often form lower levels of higher alcohols than *S. cerevisiae,* but great strain variability is found (Romano *et al.*, 1992, 1993; Zironi *et al.*, 1993).

Other non-*Saccharomyces* metabolites can act as intermediaries in aroma metabolic pathways. Romano *et al.* (1993) showed that *H. guilliermondii* and *K. apiculata* strains produced 50.3 to 258.1 mg/L and 55.8 to 187.4 mg/L acetoin, respectively, in grape must. Acetoin is considered a relatively odourless compound in wine with a threshold value of approximately 150 mg/l (Romano & Suzzi 1996). However, diacetyl and 2,3-butanediol (both off-flavours in wine) can be derived from acetoin by chemical oxidation and yeast mediated reduction, respectively. This indicates that acetoin can play a role in off-flavour formation in wines. High concentrations of acetoin produced by non-*Saccharomyces* yeasts can also be utilised by *S. cerevisiae.* This was shown by Zironi *et al.* (1993) in their chemical analysis results of wines from pure culture compared to mixed and sequential culture fermentations. They could, however, not confirm what metabolites are formed by *S. cerevisiae* from acetoin.

TABLE 2.5
The presence of extracellular hydrolytic enzymes in non-*Saccharomyces* yeasts.

Species (teleomorph / anamorph)	Extracellular enzyme activity										
	Pectinase	Protease	Glucanase	Lichenase	Glucosidase	Cellulase	Xylanase	Amylase	Sulphite reductase	Lipase	Reference
Zygoascus hellenicus / Candida hellenica			+		+				+		S
Brettanomyces clausenii	+										F
Kluyveromyces thermotolerans	+										F
Pichia fermentans / Candida lambica			+						+		S
-[2] / *Candida oleophila*	+						+		+		S
Issatchenkia occidentalis / Candida sorbosa			+						+		S
-[2] / *Candida stellata*	+	+	+	+		+	+	+	+	+	C, F, S
Pichia membranifaciens / Candida valida	+	+			+				+		F, S
Debaryomyces hansenii / Candida famata		+			+				+		R[4], S
Hanseniaspora uvarum / Kloeckera apiculata	+	+	+	+	+	+	+	+	+		R[4], C, S
Issatchenkia orientalis / Candida krusei										+	C
Metschnikowia pulcherrima / Candida pulcherrima	+	+	+	+	+	+	+	+	+	+	C, F, S
Pichia anomala / Candida pelliculosa		+			+				+		R[4], C, F, S
Torulaspora delbrueckii / Candida colliculosa										+	C
Pichia farinosa / -[3]			+								S
Debaryomyces castellii / -[3]					+						R[4]
Debaryomyces polymorphus / -[3]					+						R[4]
Pichia kluyveri / -[3]			+								S

[1] R = Rosi *et al.*, 1994; C = Charoenchai, *et al.*, 1997; Fernández *et al.*, 2000 and S = Strauss *et al.*, 2001.
[2] No teleomorph.
[3] No anamorph
[4] Only screened for β-glucosidase activity.

These and other compounds not mentioned e.g. volatile fatty acids, carbonyl and sulphur compounds, are all known to play a role in the sensory quality of wine (Lambrechts & Pretorius, 2000 and references therein). However, as quoted by Guth (1997), there are over 680 documented compounds and a large number of these can, depending on concentration, contribute either positively or negatively to wine aroma and flavour. It is also not known how these compounds are affected by, and contribute to, the metabolism of the different yeast species found in fermentation.

Furthermore, certain flavour and aroma compounds are present in grapes as glycosidically bound precursors with no sensory properties (Todd, 1995; Pretorius, 2003). These compounds may be hydrolysed by the enzyme β-glycosidase to form free volatiles that can improve the flavour and aroma of the wine. No *S. cerevisiae* has been found to naturally possess β-glucosidase activity (Ubeda-Iranzo *et al.*, 1998), but certain non-*Saccharomyces* yeasts do have this enzyme. Rosi *et al.* (1994) and Todd (1995) have shown that yeasts belonging to the genera *Debaryomyces, Hansenula* and *Candida* possess various degrees of β-glucosidase activity. These enzymes are capable of releasing monoterpenols from grape glycoside extract. An intracellular β-glucosidase has also been isolated and purified from *Debaryomyces hansenii.* This enzyme, tolerant to glucose and ethanol, was used during fermentation of Muscat grape juice resulting in an increase in concentration of monoterpenols in the wine (Yanai & Sato, 1999).

Other extracellular enzymatic activity (proteolytic, polygalacturonase) can also potentially be of use to winemaking. Some extracellular enzymes found in different non-*Saccharomyces* species are shown in Table 2.5. Species showing the greatest number of extracellular enzymes tested for are *C. stellata*, *H. uvarum/K. apiculata* and *M. pulcherrima/C. pulcherrima*.

2.7 FACTORS AFFECTING GROWTH OF NON-*SACCHAROMYCES* YEASTS DURING WINE FERMENTATION

The contribution by non-*Saccharomyces* yeasts to wine flavour will depend on the concentration of metabolites formed. Therefore, any factors affecting the growth rate of individual non-*Saccharomyces* species will determine the extent of their

contribution to flavour development (Heard, 1999). These include intrinsic factors of the juice (pH, sugar concentration and clarity), processing methods (method of clarification, use of preservatives and/or SO_2), fermentation temperatures, oxygen content (aeration) and effect of *S. cerevisiae* yeast e.g. ethanol formed.

Non-*Saccharomyces* yeasts appear to be less tolerant to low oxygen availability, especially in comparison to *S. cerevisiae* (Fleet, 2003 and the references therein). The removal of oxygen by vigorously fermenting *S. cerevisiae* can contribute to an early death of some non-*Saccharomyces* yeasts.

For some non-*Saccharomyces* yeasts i.e. *C. stellata, C. colliculosa* and *C. pulcherrima,* it has been shown that the pH of the medium or must does not have a large effect on growth rate (Gao & Fleet, 1988; Heard & Fleet, 1988). The effect of pH on other non-*Saccharomyces* yeasts is not well defined. Increase in sugar concentration, however, does have an effect on these yeasts due to their differing osmotolerances (Heard, 1999).

Temperature and SO_2 are possibly the two factors that have the greatest effect on non-*Saccharomyces* yeasts. Some non-*Saccharomyces* yeasts are inhibited at temperatures above 25°C (Fleet, 1990; Boulton *et al.*, 1996). Therefore, lowering of temperatures can lead to greater contribution by non-*Saccharomyces* species (Sharf & Margalith, 1983). *Kloeckera apiculata*, for example, is able to ferment better at 10°C than at 25°C (Heard & Fleet, 1988; Bilbao *et al.*, 1997). The ethanol tolerance of *C. stellata* and *K. apiculata* is also improved at lower temperatures (e.g. 10°C compared to 25°C) (Gao & Fleet, 1988). This ethanol tolerance has no relationship to the maximum ethanol that can be produced by the yeasts and the ethanol tolerance concentrations are significantly higher than the ethanol produced (Heard & Fleet, 1988). Subsequently, the lowering of temperature of fermentation will lead to an increased presence of *K. apiculata* and *C. stellata* yeasts. The same could be expected to be true for some other non-*Saccharomyces* yeasts. This change in the ecology of the fermentation will also result in a change in the concentration of aroma and flavour metabolites formed by the non-*Saccharomyces* yeasts with consequential impact on wine quality.

While it has generally been accepted that SO_2 added to wine suppresses non-*Saccharomyces* yeasts, the work of Henick-Kling *et al.* (1998), Constanti *et al.*

(1998), Egli *et al.* (1998) and Rementeria *et al.* (2003) puts this generalised statement into a clearer perspective. Low sulphur addition (20 mg/l) does not suppress non-*Saccharomyces* yeasts, higher levels (50 mg/l) does suppress some, but others e.g. *C. guilliermondii* and *Zygosaccharomyces* spp. can survive. The effectiveness of SO_2 also depends on the type of must and the number of non-*Saccharomyces* yeasts present in the first place. Results of Fleet (1990) and Granchi *et al.* (1998) supports this statement. They found that SO_2 in the range 50 to 100 mg/L did not prevent growth of non-*Saccharomyces* yeasts in red wine fermentations. These concentrations are normally effective in white wine fermentations. Suppression of sensitive yeasts by SO_2 allows more tolerant yeasts, present in lower numbers, to proliferate. The judicious use of SO_2 can therefore be used to alter the non-*Saccharomyces* population profile in some instances. In others, the addition of SO_2 will decrease the population, but not the species diversity (Rementeria *et al.*, 2003).

Other aspects that can impact on the presence and growth of non-*Saccharomyces* yeasts are different types of yeast-yeast and yeast-bacteria interactions e.g. neutralism, commensalism, mutualism/synergism, amensalism or antagonism and competition (Boulton *et al.*, 1996; Henick-Kling *et al.*, 1998; Fleet, 2003 and the references therein). These include the utilisation of specific nutrients e.g. amino acids and vitamins, by one species making the environment less favourable to others. Metabolites produced such as ethanol, inhibitory peptides, proteins and glyco-proteins can inhibit or destroy other species by lysis of their cell walls. Specifically, zymocidal strains of *S. cerevisiae* (killer yeasts) can also affect other species. Other metabolites in turn can lead to enhanced growth. Autolysis with the subsequent release of cellular material can also encourage yeast growth (Fleet, 2003 and the references therein).

Growth of non-*Saccharomyces* yeasts can also be limited by the *S. cerevisiae* starter culture (Constanti *et al.*, 1998; Henick-Kling *et al*, 1998). Different starter cultures will also have different effects on the non-*Saccharomyces* population. Therefore, by controlling the parameters of inoculum i.e. *S. cerevisiae* strain and inoculum concentration, the non-*Saccharomyces* contribution can also be controlled. This is illustrated by the experiments of Egli *et al.* (1998). They compared three types of fermentations i.e. fermentations with indigenous

microflora (spontaneous), a vigorous yeast starter (*S. cerevisiae* strain EC1118) and a slowly fermenting yeast starter (*S. cerevisiae* strain Assmanshausen). Although the yeast related sensory profiles of the wines did not exceed the varietal character, the wines differed, dependent on the type of fermentation followed. The faster growing EC1118 strain limited the growth of the non-*Saccharomyces* component more strongly than the slow growing Assmanshausen. Growth could be further suppressed by the addition of SO_2.

The use of fungicides and pesticides has also been suggested to result in a reduced yeast presence on grapes (Parish & Caroll, 1985; Guerra *et al.*, 1999). Cabras *et al.* (1999), however, showed that certain pesticides could stimulate yeasts (especially *K. apiculata*) to produce more ethanol. This increased vigour would by implication also extend to other metabolites. As pesticides are used to a greater or lesser extent in all vineyards (with a few exceptions where organic farming is followed) there may be instances where certain pesticides can stimulate non-*Saccharomyces* growth on the grapes. The resultant higher levels of yeast may have a knock-on effect in the subsequent wine fermentation. Pesticides may therefore indirectly increase the non-*Saccharomyces* contribution to wine fermentation.

Non-*Saccharomyces* populations may also be affected by "quorum sensing", the mechanism whereby microbial cells can communicate with each other and cause a population to follow a specific growth pattern (Fleet, 2003 and the references therein). For yeast, bicarbonate, ammonia and farnesol have been suggested as cell communication molecules. The effect of "quorum sensing" and its significance to wine production is not known.

2.8 THE USE OF NON-*SACCHAROMYCES* YEASTS IN WINE PRODUCTION

A number of authors have reported on the use of selected and cultured non-*Saccharomyces* yeasts for benefit to wine production. These include *C. stellata, K. apiculata*, *Brettanomyces* and *Zygosaccharomyces* species. As these non-*Saccharomyces* yeasts are all poor fermenters, *S. cerevisiae* yeasts (either indigenous or inoculated) are always needed to complete wine fermentations. Practically, under current winemaking conditions, it would also be impossible to

eliminate *S. cerevisiae* from wine fermentations and therefore all strategies for using non-*Saccharomyces* yeasts will have to incorporate *S. cerevisiae*. Typically, the non-*Saccharomyces* yeasts have been used in sequential fermentations where these yeasts are allowed to grow or ferment between three and fifteen days before inoculation with *S. cerevisiae* (Herraiz *et al.*, 1990; Zironi *et al.*, 1993; Ciani & Ferraro, 1998; Ferraro *et al.*, 2000).

2.8.1 *Candida stellata*

C. stellata is known as a high glycerol producer. Values of up to 11.76 g/L have been reported, which is higher than the sensory threshold level for glycerol sweetness i.e. 5.2 g/L (Ciani & Maccarelli, 1998). Glycerol is also thought to contribute to the mouth-feel and complexity of wine flavour at lower levels (Scanes *et al.*, 1998; Prior *et al.*, 2000). This yeast is therefore a prime candidate for investigating a positive non-*Saccharomyces* contribution to wine quality. Ciani & Picciotti (1995) suggested that it be used as a starter culture to increase glycerol levels in wine.

Ciani & Ferraro (1998) subsequently used a *C. stellata* strain (strain 3827, Industrial Yeast Collection, University of Perugia) to improve the chemical analyses (analytical profile) of small-scale (500 mL) Pinot grigio wines during batch fermentations. The Pinot grigio grapes were harvested at 18.5°B and sweetened to 27°B with sucrose. Immobilised *C. stellata* cells at concentrations of 1 $x10^9$ cells/mL were used in three fermentation types, viz. a simultaneous inoculation with *S. cerevisiae*; a sequential fermentation where *S. cerevisiae* was added three days after *C. stellata*; and a substituted fermentation where *C. stellata* was replaced with *S. cerevisiae* after three days. The *S. cerevisiae* inoculum was 1 x 10^6 cells/mL for all treatments. Results showed that in comparison to the *S. cerevisiae* control fermentations, the combined fermentations had a reduced time of fermentation with an increased glycerol content. This was accompanied by a reduction of acetic acid and higher alcohols and an increase in succinic acid. Other by-products measured were similar to the *S. cerevisiae* control fermentation. Despite the high initial sugar content, all the sugar was consumed due to the complimentary consumption of the glucose and fructose by the *C. stellata* (fructophylic yeast) and *S. cerevisiae* (glucophyllic yeast). It was concluded that of

all three treatments, the sequential fermentation was the best combination for improving the analytical profile of the wines. It was further noted that an important interaction took place between *C. stellata* and *S. cerevisiae.* Acetoin produced by *C. stellata* was utilised by *S. cerevisiae* to form 2,3-butanediol. As already mentioned earlier, 2,3-butanediol can contribute to off-flavour of wine. As no sensory analyses was done it is not clear if the wines would have passed a sensory evaluation.

In a subsequent study, Ferraro *et al.* (2000) confirmed their sequential fermentation laboratory trials during production of pilot-scale (100 L) wine. They used Trebbiano Toscano grape juice at 19°B, pH 2.94 and a free SO_2 of 11.52 mg/L. The inoculum of immobilised *C. stellata* was 5 x 10^8 cells/mL, followed by 5 x 10^6 cells/mL of *S. cerevisiae* after three days. High glycerol production was evident on the fourth day of fermentation while the alcohol was still lower than 5%. Thereafter, the glycerol production was slower and ethanol concentration increased due to the metabolism of mainly *S. cerevisiae*. The final wine had a 70% higher glycerol concentration than their control. Ethanol concentration was 0.6% and acetic acid 0.05 g/L lower than the control fermentation.

Apart from the increase in glycerol, the reduction in alcohol as reported above could be beneficial for the production of wines from grapes with high sugar content (as often found in grapes from hot regions). Reduction in acetic acid concentration will always be beneficial. However, while the analytical profile of the wine was improved, a shortcoming of the work done by Ferraro *et al.* (2000) is that they do not report on the sensory profile of the wine. During the three-day lag before inoculation with *S. cerevisiae*, oxidation of the must may have occurred. However, spontaneous fermentation would have started which would have offset the oxidation risk.

In another investigation Soden *et al.* (1998; 2000) also used *C. stellata* in combination with *S. cerevisiae,* but for production of Chardonnay wines. They used two different inoculation protocols viz. co-inoculation and sequential inoculation starting with the *C. stellata* to compare to *S. cerevisiae* and *C. stellata* monoculture fermentations. All the yeasts were inoculated at a concentration of 5 x 10^6 cells/mL. The exception was the co-inoculated fermentation where the

S. cerevisiae was inoculated at a slightly lower cell count (5 x 10^5 cells/mL). In the sequential fermentation, the *C. stellata* was inoculated first, followed by *S. cerevisiae* 15 days later. At this point the *C. stellata* had depleted all the fructose, but none of the glucose in the must and had stopped fermenting actively. All the wines except the *C. stellata* monoculture fermented dry.

The wines underwent a descriptive sensory analyses and the reference *S. cerevisiae* wine was judged to have tropical 'fruit', 'floral', 'lime' and 'banana' aromas of a typical Chardonnay. The monoculture *C. stellata* wine had significantly more 'apricot', 'honey' and 'sauerkraut' aromas and significantly less 'lime', 'tropical fruit', 'banana' and 'floral' aromas. The co-inoculated wine had aromas similar to the *S. cerevisiae* monoculture, but was scored lower for 'floral' and 'banana'. The sequential fermentations produced a wine significantly different from the *S. cerevisiae* reference wine regarding 'banana', 'floral' and 'lime' aromas, but similar in 'honey', 'apricot' and 'sauerkraut' aromas attributed to the *C. stellata* yeast. Unfortunately, the wine was judged to have a high 'ethyl acetate' aroma, a judgement supported by chemical analysis. This wine also had the highest concentration of glycerol and succinic acid and a lower concentration of ethanol, which as previously discussed can add to wine quality. On the negative side, 'sauerkraut' and 'ethyl acetate' nuances could be considered to detract from wine quality as they are listed under 'microbiological' and 'oxidised' according to wine evaluation terminology (Noble *et al.*, 1987). The long time lapse of 15 days before the inoculation with *S. cerevisiae* in the sequential fermentation would have contributed to the 'oxidised' aroma of the wine. A shorter delay before inoculation with *S. cerevisiae* could have given a better wine. Soden *et al.* (2000) concluded that with selection of the appropriate strains and the establishment of effective inoculation protocols, greater flavour diversity and complexity could be obtained in wine during commercial winemaking.

2.8.2 *Kloeckera* and *Hanseniaspora* spp.

As mentioned in a previous section, the apiculate yeasts *K. apiculata* and *H. uvarum* are generally the non-*Saccharomyces* yeasts found in the highest numbers in grape must and therefore in the best position to make a contribution to wine quality. These yeasts, with low fermentative power, are important in the production of volatile substances in wine and the chemical composition of wines

made with *Kloeckera* spp./*S. cerevisiae* combinations differ from reference wines (Herraiz *et al.*, 1990; Mateo *et al.*, 1991; Zironi *et al.*, 1993; Gil *et al.* 1996).

Caridi & Ramondino (1999) evaluated a range of 20 *Hanseniaspora* spp. (*Kloeckera* spp.) of oenological origin for their ability to ferment a must (17.9°B; pH 3.81). They found that the ethanol produced ranged from 5.02 to 8.72% in comparison to the 11.17% of the control (*S. cerevisiae*), but volatile acidity was higher (0.75g/l to 2.25g/L) than the control (0.65g/L). These results indicate that although some strains of *Hanseniaspora* are able to produce higher levels of ethanol than other strains, the high levels of volatile acidity would be detrimental to the sensory characteristics of the wine. However, Romano *et al.* (1992) and Ciani & Maccarelli (1998) showed that not all *Kloeckera* strains formed high levels of volatile acidity and some were similar to *S. cerevisiae* in this regard. The production of other secondary metabolites i.e. glycerol, acetaldehyde, ethyl acetate and hydrogen sulphide, also differed between strains (Romano *et al.*, 1997a). Selected strains of apiculate yeasts might therefore favour flavour and aroma enhancement in wines.

Most authors utilised *Kloeckera* spp. in small-scale laboratory trials and subsequently only did chemical analyses on the wines. Their approaches in the use of *Kloeckera* differed from each other. Zironi *et al.* (1993) in their sequential fermentations allowed the *Kloeckera/Hanseniaspora* to ferment for six days before inoculating with *S. cerevisiae,* while Herraiz *et al.* (1990) waited eight days. Differences in chemical analyses were noted in the wines. Of more concern was that the initial growth of *Kloeckera* had a retarding effect on the subsequent growth of *S. cerevisiae*. This phenomenon could have further implications as a cause for lagging or stuck fermentations. Therefore, a cautionary approach would have to be taken when considering using *Kloeckera* spp. for wine production.

Sensory evaluations on wines produced by *Kloeckera* spp. were done by Owuama & Saunders (1990). They used *K. apiculata/S. cerevisiae* combinations for the fermentation of cashew apple juice (25°B). Inoculation of the two species was simultaneous and 9.3% ethanol was formed with 4.2% residual sugar. Sensory evaluation (colour, odour, taste and aroma) of the wines showed that although the

Saccharomyces reference wine was the best, the product of the combined fermentation was also acceptable for consumption.

The production of 2-phenyl-ethyl acetate by another apiculate yeast i.e. *H. guilliermondii,* has been investigated in laboratory fermentations (Rojas *et al.*, 2003). This acetate ester contributes to 'rose', 'honey', 'fruity' and 'flowery' aroma nuances (Lambrechts & Pretorius, 2000 and the references therein) and is formed to a greater or lesser extent by most yeasts and, as part of the 'fermentation bouquet', can contribute to the overall flavour of a young wine. However, the high level of ethyl acetate produced by the strain investigated by Rojas *et al.* (2003) was a serious handicap.

Another role that has been suggested for *Kloeckera* and *Hanseniaspora* spp. is that of production of wine destined for vinegar manufacture (Ciani & Picciotti, 1995). The high production of acetoin and ethyl acetate will favourably influence the quality of vinegar.

2.8.3 *Zygosaccharomyces* spp.

Zygosaccharomyces spp. are considered to be winery contaminants and are especially a problem in wineries producing sweet and sparkling wines (Loureiro & Malfeito-Ferreira, 2003 and the references therein). Notwithstanding, *Zygosaccharomyces* spp. have been investigated by Romano & Suzzi (1993a) as contributors to wine fermentation. The species studied included a *Z. fermentati* strain (strain F42) with low acetic acid, H_2S and SO_2 production and high fermentation vigour. A strain of another species, *Z. bailii* (strain F37), showed malic acid degradation and generally low H_2S production. In addition, both species showed flocculation. The authors suggested that these characteristics could benefit wine production e.g. during re-fermentation of wine. Their *Z. fermentati* strain, in particular, had the combined advantages of low acetic acid, H_2S and SO_2 production, and high fermentation vigour.

As most literature recognises *Zygosaccharomyces* yeasts as spoilage organisms producing high quantities of acetic acid, Romano & Suzzi (1993a) further suggested that this could be due to yeasts bearing a close resemblance to

Zygosaccharomyces that in older literature were wrongly regarded as synonyms of *Zygosaccharomyces* species.

Selected strains of *Zygosaccharomyces* spp. may be useful especially for production of alternate beverages. *Z. bailii* is also, in contrast to *S. cerevisiae* and many other non-*Saccharomyces* yeasts, fructophyllic i.e. it uses fructose preferentially to glucose. This could be beneficial in grape musts from riper grapes (high Balling) where the fructose concentration can exceed that of glucose at the start of fermentation (Margalith, 1981).

2.8.4 *Schizosaccharomyces* spp.

Schizosaccharomyces spp. have been used for the production of mango (*Magnifera indida* L.) wine without addition of *S. cerevisiae* (Obisanya *et al.*, 1987). Two *Schizosaccharomyces* strains isolated from palm wine were found to be suitable for the production of sweet mango table wine with between 8 and 9% alcohol. Reference wines produced by *S. cerevisiae* were, however, found to be superior in flavour and taste, while fermenting dry (approximately 4 g/l sugar).

Another characteristic of *Schizosaccharomyces* spp. is their ability to degrade malic acid. This could be put to use for enhancing wine stability in stead of malolactic bacteria. It has been shown that high-density cell suspensions of *Schizosaccharomyces* yeasts could degrade 95-99% of malic acid in a buffered assay system (Gao & Fleet, 1995). However, none of the yeasts could metabolise malic acid fast enough to be of use in a reactor system for the treatment of grape juice. Increasing the cell density had no improved effect.

2.8.5 *Torulaspora delbrueckii*

T. delbrueckii (teleomorph of *C. colliculosa*), formerly classified as *Saccharomyces rosei,* was previously suggested for vinification of musts low in sugar and acid (Castelli, 1955). It has also been used for the commercial production of red and rosé wines in Italy. In more recent experiments carried out by Moreno *et al.* (1991) it was shown that *T. delbrueckii* in pure culture produced lower levels of volatile acidity than the *S. cerevisiae* strains tested. Furthermore, in mixed fermentations (indigenous population plus pure cultures of *T. delbrueckii* or *S. cerevisiae*) the volatile acidity increased with ripeness of grapes fermented, however, these

increases were generally smaller for the *T. delbrueckii* than the *S. cerevisiae* strains.

2.8.6 *Brettanomyces* spp.

As previously mentioned, anecdotal evidence has suggested that the character derived from some *Brettanomyces* metabolites, usually referred to as "Brett", can be a positive addition at low levels in a red wine with an overall complex aroma (Arvik & Henick-Kling, 2002). The contribution of *Brettanomyces* to wine aroma has also been likened to a 'Bordeaux-like' character. Apart from the negative aroma nuances imparted by these yeasts, positive aromas such as 'smoky', 'spicy' and 'toffee' are also cited (Eschenbruch & Wong, 1993; Arvik & Henick-Kling, 2002). *Brettanomyces* has also been implicated in Belgian acidic ale production where it is found in the final 20 to 24 month stage in the fermentation casks (Martens *et al.*, 1997). While the deliberate inoculation of *Brettanomyces* in must or wine for commercial production has not been reported, some winemakers are working with the indigenous populations of *Brettanomyces* found in their cellars to make more complex wines, some of which are highly regarded by virtue of their specific aromas and flavours (Arvik & Henick-Kling, 2002). This topic is being investigated by different research groups (Grbin & Henschke, 2000; Arvik & Henick-Kling, 2002) and a favourable *Brettanomyces* strain or protocol for the beneficial use of *Brettanomyces* spp. for wine production may still be found. However, the formation of 'mousy' off-flavour compounds (2-acetyl- and 2-ethyl-tetrahydropyridine) by *Brettanomyces* spp. will have to be controlled.

2.8.7 *Saccharomycodes ludwigii*

S. ludwigii is a lesser known, lemon-shaped yeast, typically with a large cell size, frequently isolated from wine at the end of fermentation or from wine in storage (Romano *et al.*, 1999). Production of secondary metabolites by this yeast is characterised by high levels of isobutyl alcohol, acetoin and ethyl acetate. It is also known to be highly resistant to SO_2 and tolerant to ethanol. A selected strain of *S. ludwigii* was used to ferment feijoa or pineapple guava (*Feijoa sellowiana*). The resultant beverage was evaluated for aroma, flavour and taste by a consumer panel. Despite the high levels of acetic acid, the beverage was described as "fresh" with a "fruity flavour" akin to apple and kiwi and similar to apple juice in

taste but with more acid. Romano *et al.* (1999) concluded that this beverage had potential as a refreshing summer drink.

2.8.8 Other non-*Saccharomyces* spp.

Other non-*Saccharomyces* yeasts that have also been investigated for their potential contribution to wine include *Pichia* and *Williopsis* spp.

The production of 2-phenylethanol by *Pichia fermentans* has been investigated under optimised culture conditions by Huang *et al.* (2001). This alcohol contributing to an aroma of rose petals is formed to a greater or lesser extent by most yeasts and as part of the 'fermentation bouquet' can contribute to the overall flavour of a young wine. It was found that the production of this compound increased with increase in biomass during the initial stage of fermentation. A maximum concentration was reached after 16 hours.

Other *Pichia* spp. i.e. *P. anomala, P. membranifaciens* and *P. subpelliculosa*, together with *Williopsis saturnus* have also been suggested for use for the production of low-alcohol wines (*ca.* 3%$^{v}/_{v}$) in an aerated vessel (Erten & Camphill, 2001). In this method stirring and agitiation resulted in more yeast biomass being formed and decreased ethanol production from fermentable sugars. The wines, made by *P. subpelliculosa* and *W. saturnus* in particular, were acceptable to a small tasting panel. This method of low alcohol wine production eliminates the need for costly and complex post-production removal of alcohol from wines produced during normal fermentations. The higher levels of flavour compounds, especially esters, produced by these aerobic yeasts appeared to counteract the loss of flavour enhancing properties of alcohol. The "body" of the wine normally attributed to ethanol also appeared to have been replaced by the higher concentration of esters.

The demand by the beverage industry for new and interesting products and the well-developed flavour produced by aerobic non-*Saccharomyces* yeasts makes this technology attractive. However, problems envisioned are the need for non-standard fermentation equipment and the reluctance of wine and beverage producers to deliberately cultivate organisms normally considered contaminants.

The early death of some non-*Saccharomyces* yeasts during fermentation can also be a source of specific nutrients for *S. cerevisiae* enabling it to ferment optimally. These nutrients include cellular constituents such as cell wall polysaccharides (mannoproteins). For this method of nutrient supply to be effective, any killer or other inhibitory effects by the non-*Saccharomyces* yeasts against *S. cerevisiae* should be known (Herraiz *et al.*, 1990; Panon, 1997; Nguyen & Panon, 1998; Fleet, 2003) so that the subsequent *S. cerevisiae* fermentation is not adversely affected.

2.8.9 Combinations of non-*Saccharomyces* yeasts

Eschenbruch & Wong (1993) inoculated a red grape variety (Blauburger) with a combination of non-*Saccharomyces* yeasts i.e. *Kloeckera* sp. and *Brettanomyces* sp. cultures. This was followed after 24 hours with *S. cerevisiae* pure culture wine yeast. They reported that the wine developed an overall flavour and complexity that they termed a "Brettanomyces" character. This character they maintained was a direct result of the contribution of the *Kloeckera* and *Brettanomyces* spp. They reported further that a lower cell concentration was more appropriate as higher cell concentrations (10^6 cells/ml) resulted in off-characters. Their control fermentations showed an indigenous population of *Kloeckera* and *Candida* yeasts, but their numbers declined and disappeared as the fermentation proceeded. No *Brettanomyces* yeasts were detected. After inoculation of the control, the *S. cerevisiae* yeast quickly became the predominant species. This was also noted in the non-*Saccharomyces* inoculated fermentations. The numbers of *Candida* sp. once again decreased as the fermentation proceeded. However, the cell concentrations of the *Brettanomyces* and *Kloeckera* (represented by the added yeasts and indigenous yeasts already present) decreased only slightly during the fermentation. These yeasts therefore could contribute towards the fermentation metabolism resulting in a contribution to taste and flavour differences.

In another investigation with combinations of non-*Saccharomyces* yeasts, *T. delbrueckii* was used in sequential fermentations with *K. apiculata.* The *K. apiculata* was inoculated first followed three days later by *T. delbrueckii* and finally *S. cerevisiae* was inoculated eight days after. The wines produced had volatile compositions different from the *S. cerevisiae* wines, but the wines were not evaluated sensorially (Herraiz *et al.*, 1990).

2.9 RESEARCH TRENDS IN RELATION TO NON-*SACCHAROMYCES* YEASTS

The utilisation of non-*Saccharomyces* yeasts such as *C. stellata*, *T. delbrueckii* and others to improve the analytical profile and flavour of wines has already been discussed. Concurrently with the envisioned use of these new "wine yeasts", new fermentation techniques could be implemented and alternative alcoholic beverages be produced (Ciani & Maccarelli, 1998). It has also been shown that *S. cerevisiae* can initiate biofilm formation (Reynolds & Fink, 2001), a characteristic previously thought to be restricted to bacteria. This has implications for wine fermentation and storage of wines. The ability of wine related non-*Saccharomyces* to form biofilms is not well researched, but it is already known that *C. albicans*, previously isolated from grapes (Parish & Caroll, 1985), forms biofilms which contribute to its pathogeneses (Douglas, 2003). Other non-*Saccharomyces* yeasts, even if unable to initiate biofilms themselves, may become a constituent of a *S. cerevisiae* or *C. albicans* biofilm and in so doing contribute positively or negatively to wine production.

Apart from the investigations and experimentation with non-*Saccharomyces* yeasts in wine production, other uses for non-*Saccharomyces* yeasts and their metabolites are being sought. These include the use of *Pichia pastoris* for the biomodification of citrus aroma oil to improve and add value to the essence (Goodrich *et al.*, 1998). Another application for non-*Saccharomyces* yeasts is the use of Zygocin, a protein toxin produced and secreted by killer strains of *Z. bailii* (Weiler & Schmitt, 2003). Purified forms of the toxin have potential as an antimycotic for a variety of human and phytopathogenic fungi (Schmitt & Breinig, 2002). The use of non-*Saccharomyces* as bio-control alternatives to chemicals has also been investigated. A "sister" species to *M. pulcherrima* (teleomorph of *C. pulcherrima*) i.e. *M. fructicola* isolated from grapes, shows activity against botrytis rot on stored grapes (Kurtzman & Droby, 2001). Table grape storage and post harvest damage due to botrytis is a major problem in grape producing regions that lie far from their respective domestic and international markets.

The possible use of *P. guilliermondii* for copper uptake in bio-remediation of sewage sludge has also been suggested (De Silóniz *et al.*, 2002). Considering the wide bio-diversity found on grapes and in must, the potential for finding yeasts benefiting mankind outside the parameters of wine production is large.

2.10 FINAL COMMENTS

It is generally accepted that the wealth of yeast bio-diversity with hidden potential, especially for oenology, is largely untapped (Pretorius, 2000 and the references therein). However, in order to exploit the potential benefits of non-*Saccharomyces* yeasts in wine production and to minimise potential spoilage, the yeast populations on grapes and in must, as well as the effect of wine making practices on these yeasts, should be known. Furthermore, the specific metabolic characteristics of the non-*Saccharomyces* yeasts should be known (Romano *et al.*, 2003). This knowledge will help realise the predictions of Heard (1999) concerning the use of mixed starter cultures. His vision includes the use of mixed yeast starter cultures tailored to reflect the characteristics of a given wine region and the use of indigenous yeast species with modern technology to produce novel wine based beverages.

The composition of vineyard and wine microflora in South Africa received attention in earlier work done by Du Plessis (1959), Van Zyl & Du Plessis (1961) and Van Kerken (1963). Recently, the distribution of indigenous *S. cerevisiae* yeasts in vineyards in the cooler coastal and warmer, inland regions of South Africa has been covered extensively by Van der Westhuizen *et al.* (2000a, 2000b) and Khan *et al.* (2000), respectively. These studies formed part of a comprehensive and ongoing long-term research programme by role players in the South African wine industry. The programme's nine objectives were detailed by Pretorius *et al.* (1999) and are listed in Chapter 1.

Whatever the outcome of the search for non-*Saccharomyces* yeasts for use in wine production, the accepted list of desirable characteristics as pertaining to the wine yeast *S. cerevisiae* (Pretorius, 2000), will not necessarily all apply to non-*Saccharomyces* yeasts. High fermentation efficiency, high sulphite tolerance and zymocidal (killer) properties, for example, may not be needed in the new

technology of wine production. The new non-*Saccharomyces* wine yeasts will necessarily have their own list of desired characteristics. Furthermore, the already mentioned problems envisioned by Erten & Campbell (2001) regarding the need for non-standard fermentation equipment and the reluctance of wine producers to cultivate and use on a large-scale microorganisms generally considered as spoilage organisms (Loureiro & Malfeito-Ferreira, 2003 and references therein), should be noted. Intensive education will have to accompany any new non-*Saccharomyces* technology in wine production. However, the goals as set out by Pretorius (2000; 2003) regarding efficient sugar utilisation, enhanced production of desirable volatile esters, enhanced liberation of grape terpenoids and production of glycerol to improve wine flavour and other sensory properties can be met by selected non-*Saccharomyces* wine yeasts. This path will bypass current controversies regarding the genetic modification of the workhorse of wine production i.e. the "wine yeast" *S. cerevisiae*. After the acceptance of genetically modified organisms (GMO) by wine consumers and industries, genetic modification of selected non-*Saccharomyces* yeasts can further enhance their performance and role in wine production.

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CHAPTER 3

RESEARCH RESULTS I

The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa

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3. RESEARCH RESULTS I

The occurrence of non-*Saccharomyces cerevisiae* yeast species over three vintages in four vineyards and grape musts from four production regions of the Western Cape, South Africa

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Abstract

The role of non-*Saccharomyces* yeasts in wine production has been extensively debated and there is growing evidence that non-*Saccharomyces* yeasts play an important role in wine quality. It has been suggested that metabolites formed by some non-*Saccharomyces* species may contribute to wine quality. Recently, a comprehensive, long-term research programme was launched by role players in the South African wine industry, whose aims include the isolation, characterisation and preservation of the natural yeast biodiversity of the Western Cape. As part of the programme, this paper investigates the presence of non-*Saccharomyces* yeast species over three vintages in four vineyards and musts in four distinct areas of the Western Cape. Samples were taken and the non-*Saccharomyces* yeast isolates were characterised by biochemical profiling and pulse field gel electrophoresis. In total 720 yeasts representing 24 species were isolated. Predominant species found in the must samples i.e. *Candida stellata, Kloeckera apiculata, Candida pulcherrima* and *Candida colliculosa* should have the most impact on subsequent fermentation.

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3.1 INTRODUCTION

The role of non-*Saccharomyces* yeasts in wine production has been debated extensively. Earlier schools of thought referred to non-*Saccharomyces* yeasts as primarily spoilage organisms (Amerine & Cruess, 1960; Van Zyl & Du Plessis, 1961; Rankine, 1972; Le Roux *et al.*, 1973). It was further accepted that they died during the initial stages of fermentation due to the toxicity of the rising alcohol concentration from the metabolism of *Saccharomyces cerevisiae*. Consequently, they were seen to be of little significance in wine production. Recently, the work of Fleet *et al.* (1984), Heard & Fleet 1985), Longo *et al.* (1991), Todd (1995) and Gafner *et al.* (1996) showed that non-*Saccharomyces* yeasts survived during fermentation and could reach cell concentrations of 10^6 to 10^8 cells/ml. These numbers are similar to those reached by *S. cerevisiae*.

It has been suggested that metabolites formed by some non-*Saccharomyces* species may contribute to wine quality (Fleet *et al.*, 1984; Gil *et al.*, 1996; Lema *et al.*, 1996; Soden *et al.*, 2000). An example is glycerol production by *Candida stellata* (Ferraro *et al.*, 2000) and ester production by *Candida pulcherrima* (Bisson & Kunkee, 1991) that in some wines can have a positive influence on wine quality. Other species, such as *Kloeckera apiculata,* are associated with acetic acid production that can be detrimental to wine quality. However, large strain variability can be found among non-*Saccharomyces* species and not all strains within a particular species form high levels of oenologically-negative compounds (Romano *et al.*, 1992; Romano & Suzzi, 1993). Some non-*Saccharomyces* species also possess β-glycosidase activity that can hydrolyse glycosically-bound aroma precursors (Todd, 1995). This supports the belief of winemakers, in especially the Old World wine regions, that indigenous yeasts impart a distinct regional and desired characteristic to their wines (Jackson, 1994).

The main sources of indigenous yeast flora in the must are the grapes and the equipment used to process the grapes (Peynaud & Domercq, 1959; Rosini, 1984; Lonvaud-Funel, 1996). Non-*Saccharomyces* yeasts are the dominant species on the grapes, but are found in lesser numbers in the cellar and on the cellar

equipment (Vaughan-Martini & Martini, 1995; Boulton *et al.*, 1996; Constanti *et al.*, 1997). At crushing, these yeasts can be introduced into the must.

The specific environmental conditions in the must, i.e. high osmotic pressure, the presence of SO_2, the temperature and cellar hygiene all play a role in determining which species can survive and grow in the must (Longo *et al.*, 1991). However, in order to exploit the potential benefits and to minimise potential spoilage by undesired non-*Saccharomyces* yeasts, the oenologically important yeast populations on grapes and in must, as well as the effect of winemaking practices on these yeasts, should be known. This knowledge will help realise the predictions of Heard (1999) concerning the use of mixed yeast starter cultures tailored to reflect the characteristics of a given wine region.

The composition of vineyard and wine microflora in South Africa received attention in earlier work done by Du Plessis (1959), Van Zyl & Du Plessis (1961) and Van Kerken (1963), however, it is unclear if any of these isolates have survived to the present. Recently, the distribution of indigenous *S. cerevisiae* yeasts in vineyards in the cooler, coastal and warmer, inland regions of South Africa have been covered extensively by Van der Westhuizen *et al.* (2000a, 2000b) and Khan *et al.* (2000), respectively. These studies formed part of a comprehensive and ongoing long-term, collaborative research programme by ARC Infruitec-Nietvoorbij and Stellenbosch University. The programme's nine objectives were detailed by Pretorius *et al.* (1999). They include the isolation, characterisation and preservation of natural yeast biodiversity from the 350 year old wine-producing regions of the Western Cape of South Africa; an investigation into the occurrence of area-specific indigenous yeasts and winery yeasts that impart a distinctive characteristic to wines from that area; and the evaluation of yeast isolates for continuous wine yeast selection and strain development programmes which have the ultimate aim of providing an appropriate yeast per cultivar, per area, per wine type. While the aforementioned is a daunting task, this study is the first to concentrate on the non-*Saccharomyces* group of yeasts. The aim of this study, as part of the above-mentioned programme, was to obtain an oenologically biased collection of non-*Saccharomyces* yeast that could be used in future selection programmes for use in wine production. Concurrently, a broad overview of some

of the non-*Saccharomyces* yeast species predominantly found in commercial vineyards and grape musts of the Western Cape, South Africa, could be obtained.

3.2 MATERIALS AND METHODS

3.2.1 Areas sampled

One Chardonnay vineyard and accompanying commercial cellar in each of four production areas was selected for sampling in three vintages i.e. 1997, 1998 and 2000. The four vineyards and their respective cellars were in Constantia (cooler temperature zone), Stellenbosch and Slanghoek (intermediate temperature zone) and Robertson (warmer zone) (Fig. 3.1). These four areas were selected according to the climate classification of Le Roux (1974), as adapted by De Villiers (1997).

3.2.2 Sample collection and yeast isolation

Whole bunches of healthy Chardonnay grapes (1-2 kg per sampling point) were aseptically gathered over the whole vineyard from 10-15 vines (both the shaded and unshaded sides) the day before commercial harvesting, dropped directly into sterile plastic bags and transported to the laboratory in cool bags. At the laboratory, the grapes were crushed by hand in the sealed bags. After thorough shaking, the bags were opened and the juice (500 mL) was poured into a sterile closed beaker and mixed (full speed on magnetic stirrer for one min). One mL of juice was taken and a dilution series made in sterile NaCl (0.85%) solution and plated onto YPD agar (1% yeast extract, 2% peptone, 2% glucose) with chloramphenicol (0.1 mg/L). Incubation was at 30°C for 5 days to allow colony formation. From count plates presenting between 30 and 300 colonies, 30 colonies were randomly selected. Selective lysine medium (Biolab, Merck), promoting the growth of yeasts other than *S. cerevisiae,* was used to ensure that no *S. cerevisiae* had been selected (Heard & Fleet, 1986). The selected colonies were purified and stored at 4°C until further analysis. Stock cultures were kept in glycerol at –80°C.

After commercial harvesting and processing of the grapes by the respective cellars, 500 mL samples of sedimented must were collected in sterile bottles.

Isolation of the yeast then followed the same protocol as already described for the vineyard samples.

3.2.3 Characterisation of yeast isolates

The yeast isolates were characterised on the basis of biochemical profiles and pulse field gel electrophoresis.

3.2.3.1 Biochemical profiles: Biochemical profiles of each of the isolates were generated using the ID 32 C AUX system (BioMérieux, France). Profiles were read after 48 h and the identity supplied by the database was used as a preliminary identity. If no identity could be assigned, a code number linked to the biochemical profile was given to the isolate.

3.2.3.2 Preparation of intact chromosomal DNA and pulse field gel electrophoresis: Samples were prepared according to the embedded agarose procedure of Carle & Olson (1985). Intact chromosomal DNA was separated using contour clamped homogenous electric field (CHEF) electrophoresis (CHEF-DR II, Bio-Rad Laboratories, Richmond, USA). All separations were carried out in 1% agarose gels (90 sec pulse for 15 h; 120 sec pulse for 20 h, in TBE buffer at 10°C). Gels were stained with ethidium bromide (10 mg/L), viewed on a trans-illuminator and then photographed.

A standard reference yeast (*S. cerevisiae* strain Vin 13, Anchor Bio-Technologies, South Africa) was used on each CHEF gel, as three gels were needed to characterise the 30 isolates per sampling point. The banding pattern of each yeast isolate was digitised and compared to the others using a customised computer program (CHEF vl.4 © T. Potgieter, ARC Infruitec-Nietvoorbij). This program allows DNA banding patterns to be digitised and stored in a database. Bands can then be compared to a specific pattern or all the patterns in the database.

3.2.4 Grouping and identification of isolates

The computer program, visual data (chromosomal banding patterns) and the biochemical profiles (identity or code number) were used to group yeasts. Identities supplied by the ID 32 C system were noted. Subsequently, one representative yeast from each group was sent to a commercial laboratory (CBS,

Delft, The Netherlands) for identification. That identification was then used as the identification for the other yeasts in the group.

3.2.5 Meteorological data

The monthly rainfall and average monthly maximum temperature (1996 to 2000) for the Constantia, Stellenbosch, Slanghoek and Robertson areas were obtained from Agromet (R. Wentzel, personal communication, 2001).

3.3 RESULTS AND DISCUSSION

3.3.1 Sample areas and climatic conditions

The four areas chosen for sampling represent four different climatic zones (Fig. 3.1). The Constantia area, the coolest, has a mean February temperature (MFT) (February being the harvest month) of 19 to 21°C and is close to the Atlantic Ocean with its cooling sea breezes. The next area, Stellenbosch, is slightly warmer, with a MFT of 21 to 23°C, but also has an open aspect towards the sea. The Slanghoek area has a similar MFT to Stellenbosch, but lies behind a range of mountains that blocks most of the maritime influence. The last area, Robertson, is the furthest inland and is the warmest area, with a MFT of 23 to 25°C.

From heat summation data and rainfall figures, it is evident that the growing and pre-1997 harvest period (December 1996, January-February 1997) had more rain than usual, resulting in a cooler season (V. Bonnardot, personal communication, 2001). The pre-harvest period for 1998 showed less rainfall and was therefore warmer than normal. The pre-harvest period for the 2000 was also warm and dry.

3.3.2 Sample collection and yeast isolation

Aseptic harvesting of the grapes and laboratory preparation of the must avoided contamination of samples by yeasts not present on the grapes. The must preparation technique used for the subsequent isolation of indigenous non-*Saccharomyces* yeasts from the vineyard samples was considered adequate for the same reasons as laid out by Van der Westhuizen *et al.* (2000a) and Bisson & Kunkee (1991). They argued that the more aggressive yeast recovery techniques

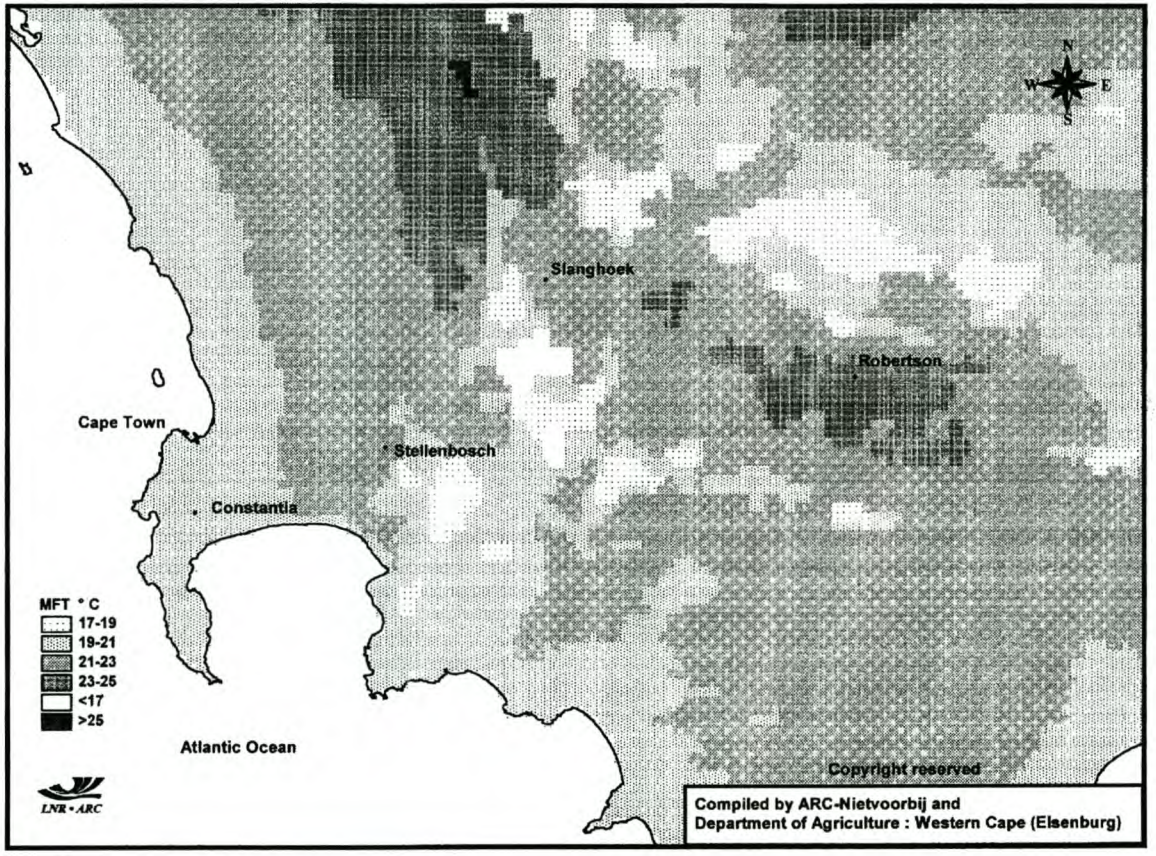

FIGURE 3.1
Mean February temperature (MFT) for the production areas sampled in the Western Cape, South Africa.

i.e. sonification of grapes as followed by Martini *et al.* (1980, 1996), while resulting in larger quantities of yeasts, did not lead to identification of any novel organisms, compared to milder sample preparation techniques. Our approach would furthermore clearly bias results towards yeast that would potentially have some oenological use.

The cellar sample was taken after sedimentation to obtain a representation of vineyard yeasts carried over to the must, as well as yeasts resident on the cellar equipment and surfaces, but excluding any yeasts that normally would be removed during must clarification. The yeast population can change during this initial wine production phase (Mora & Mulet, 1991). However, the remaining yeasts would be more tolerant to sulphur and osmotic pressure and would be the yeasts that play the biggest role in the subsequent fermentation phase.

The use of YPD agar plates incubated for five days ensured that slow growing non-*Saccharomyces* yeasts could also grow, while the added antibiotic prevented any bacterial growth. The use of lysine media at this early stage could have suppressed slow growing non-*Saccharomyces* yeasts, as well as *S. cerevisiae.* However, once the isolates had been selected, the non-*Saccharomyces* status was confirmed by the ability to grow on lysine medium and from electrophoretic karyotyping. Random selection of 30 colonies from the count plates (between 30 and 300 colonies) represents from 100% to only 10% of the possible isolates. While it is obvious that some minor species may have been overlooked on the plates with higher cell counts, the random selection ensured that predominant species were selected.

3.3.3 Yeast characterisation and identification

The use of the biochemical profiles and chromosomal banding patterns proved adequate for the grouping of similar species. However, the preliminary identification supplied by the ID 32 C database was often incorrect. This was to be expected, as the system was designed for the identification of yeasts of clinical importance. However, correct identifications were obtained for *K. apiculata, Candida colliculosa*, *Candida pelliculosa*, *Candida guilliermondii*, *Candida albicans* and *Rhodotorula* spp. While not in the database, *C. stellata* gave a consistent profile, which was correlated to the identification supplied by CBS.

The yeasts identified by CBS were, where applicable, found to be the anamorphic form. Although the teleomorphic form may have been isolated originally, the yeasts had been kept in storage for some time before identification. This can result in a loss of sporulation (Yarrow, 1998; M. Th. Smith, personal communication, 2000).

3.3.4 Non-*Saccharomyces* yeasts in vineyard samples

The four vineyards that were investigated over three vintages represent 12 sampling points (samples). While this study was not an exhaustive survey, and one vineyard cannot be extrapolated to the whole area, it does give us some indication of the type of population dynamics that occurred. Grape microflora are affected by factors such as temperature, rainfall, humidity, vineyard altitude, insect vectors, vineyard spray regimes, nitrogen fertilisation practices, winery waste practices and the health of the grapes (Boulton *et al.*, 1996). It therefore should be expected that different regions will show differences in the proportion of yeasts (Amerine *et al.*, 1967; Longo *et al.*, 1991). Varietal factors, e.g. thickness of grape skins, can also play a role (Bisson & Kunkee, 1991). However, as only one cultivar was investigated, grape skin thickness can be disregarded in this investigation.

The total cell counts (Table 3.1) range from 1.6×10^3 to 1.2×10^6 cells/ml and, apart from a slight reduction in average cell counts in 1998 (by a factor of 10), showed no apparent correlation to weather patterns or climate. However, prior to the 1997 harvest, there was a severe outbreak of fungal diseases in the vineyards due to the cooler weather and rainfall. To combat this, heavy applications of chemical sprays were applied during the growing season. In the 1998 vintage, sprays were used again due to the high fungal spore load from the previous season. This has been suggested as a possible cause for reduced yeast presence on grapes (Parish & Carroll, 1985; Van der Westhuizen *et al.*, 2000a). However, preliminary work done by Sturm *et al.* (2002) showed that yeast species biodiversity was not influenced by pest and disease management practices (integrated pest management, organic farming, application of antagonistic bacteria and copper applications). In contrast, Cabras *et al.* (1999) showed that certain pesticides can stimulate yeasts such as *K. apiculata*.

TABLE 3.1
Total cell counts of non-*Saccharomyces* yeasts over three vintages in four Chardonnay vineyards in the Western Cape, South Africa.

Vineyard	**Total cell count (cells/ml)**			
	Vintage			
	1997	**1998**	**2000**	**Average**
Constantia	1.2×10^5	8.0×10^4	8.9×10^5	3.6×10^5
Stellenbosch	1.2×10^6	5.8×10^4	1.6×10^3	4.2×10^5
Slanghoek	1.4×10^5	8.6×10^4	1.4×10^5	1.2×10^5
Robertson	8.7×10^4	1.1×10^5	1.7×10^4	7.1×10^4
Average total cell count (cells/ml) for each vintage	3.9×10^5	8.4×10^4	2.6×10^5	-

The number of non-*Saccharomyces* species isolated in each sample ranged from one to four, with the exception of Stellenbosch, where eight species were isolated in 1997 (Table 3.2). There appears to be no pattern relating to weather and climatic conditions. In total, 15 species were isolated in this study. In a comparative study, regarding method of yeast isolation, Yanigida *et al.* (1992) isolated 12 species. Direct comparison with other studies is difficult as different approaches for yeast isolation were followed. In the previous South African investigation of 1961 (Van Zyl & Du Plessis, 1961), 15 species of non-*Saccharomyces* were also isolated. However, their approach entailed incubating berries in sterile grape juice until growth was observed. Studies in other parts of the world found between nine and 12 species (Parish & Carroll, 1985; Yanagida *et al.*, 1992).

Single species predominance (>50% of isolates) occurred in 11 of the samples. No single species predominated in the twelfth sample. The species that had the highest incidence of predominance was *K. apiculata,* although in only five of the 12 samples. This scenario was also reported by Yanagida *et al.* (1992), who found *K. apiculata* predominance in only six of 11 sites. In the earlier study of South African vineyards (Van Zyl & Du Plessis, 1961), *K. apiculata* was listed as the most frequent, however, as already mentioned, their methodology would have played a role. Smaller numbers of *K. apiculata* were isolated in a further three samples in this study. However, it was absent in all three Robertson samples. In Constantia there appeared to be a tendency to declining numbers of *K. apiculata* over the three vintages studied, while in Stellenbosch and Slanghoek there was an initial decline followed by an increase in cell numbers.

In three of the remaining six samples, *C. pulcherrima* was the most frequently found yeast. This yeast was reported by Van Zyl & Du Plessis (1961) to be the fourth most frequent in their study.

Other yeasts that dominated in single samples only were *Kluyveromyces thermotolerans*, a *Rhodotorula* sp. and *Zygosaccharomyces bailii*. *K. thermotolerans* has been isolated from grape must sampled in the cellar (Torija *et al.*, 2001), but not from vineyards. *Rhodotorula* spp. were also reported by Van Zyl & Du Plessis (1961) and Parish & Carroll (1985) in vineyards and on grape berry surfaces, but not in predominant numbers. *Z. bailii* has been reported (Peynaud & Domercq, 1959), but it is usually associated with wine spoilage (Sponholz, 1993). However, as far as the authors can ascertain, this yeast has not been reported as predominant in vineyards.

The emergence and disappearance of some of the species over the four years may be coupled to the colonisation of bunches by specific yeasts (Peynaud & Domercq, 1959; Török *et al.*, 1996). During randomised sampling colonised bunches might have been missed. Vineyard practices with resultant physical and micro-climatic changes could also have played a role.

In the Robertson sample of 2000, a large percentage (47%) of the pathogenic yeast *C. albicans* was isolated. This is not the first time that *C. albicans* has been isolated from grapes (Parish & Carroll, 1985). However, due to taxonomic changes, yeasts such as *Candida stellatoidea*, previously reported in vineyards grape vine flowers) have been reclassified to the *C. albicans* species (Meyer *et al.*, 1998).

3.3.5 Non-*Saccharomyces* yeasts in must

Non-*Saccharomyces* yeasts found on grapes and cellar equipment are carried over to the must during crushing (Peynaud & Domercq, 1959; Bisson & Kunkee, 1991; Lonvaud-Funel, 1996). The specific must environment, i.e. low pH, high(sugar content (high osmotic pressure), the presence of SO_2 added at crushing and temperature plays a role in determining which species of yeasts can survive and grow (Bisson & Kunkee, 1991; Longo *et. al.*, 1991). In addition, the

TABLE 3.2
Seasonal variation in non-*Saccharomyces* yeasts over three vintages in four Chardonnay vineyards in the Western Cape, South Africa.

Yeast strain[1]	**Percentage isolates (%)**											
	Constantia			**Stellenbosch**			**Slanghoek**			**Robertson**		
	1997	**1998**	**2000**	**1997**	**1998**	**2000**	**1997**	**1998**	**2000**	**1997**	**1998**	**2000**
Candida albicans												47
Candida guilliermondii										7		
Candida hellenica				7				20				
Candida oleophila				10			7					
Candida pelliculosa				7								
Candida pulcherrima		47	**57**	3			30		**54**	**93**	10	47
Candida stellata	13		23	3								
Candida valida								13				
Cryptococcus albidus											27	
Kloeckera apiculata	**87**	**53**	13	**60**		**73**	**63**	10	40			
Kluyveromyces thermotolerans											**63**	6
Pichia farinosa				7								
***Rhodotorula* sp.**				3	**100**	27			3			
Zygosaccharomyces bailii								**57**	3			
Zygosaccharomyces sp.			7									
Number of species	2	2	4	8	1	2	3	4	4	2	3	3

[1] Dominant yeast (>50%) indicated in bold type.

method of harvesting (hand vs. mechanical), grape temperature, method and time of transport to cellar, time lapse before crushing, method of processing (whole bunches vs. crushing) and methods of clarification also plays an important role in determining what yeast will be present in the clarified must (Fleet, 1990; Mora & Mulet, 1991; Epifanio *et al.*, 1999; Pretorius 2000). From a winemaking point of view, it is important to know what non-*Saccharomyces* yeasts can survive and grow in must, as they are the ones that will have the most influence on wine quality.

In this study, the vineyards were all commercial blocks with differing methods of harvest beyond the control of the authors. The Constantia, Stellenbosch and Robertson vineyards were harvested by hand, while the Slanghoek vineyard was machine harvested. These differences and the time taken to process the grapes can allow the yeast population to increase (Peynaud & Domercq, 1959). This makes actual comparisons between musts from the different cellars difficult.

The total number of cells in each must sample varied from 8.6×10^3 to 5.2×10^6 cells/ml (Table 3.3). On average, the cell counts were higher in the cellar samples than in the vineyard samples. This may be due to the longer time that the yeasts were exposed to the nutrients in the must. It is also an indication that some of the non-*Saccharomyces* yeasts are not as sensitive to SO_2 as is generally assumed. The diversity of yeasts was very similar to the vineyard samples (three to eight species per sample) (Table 3.4). The exception was Robertson (1998) where only one group was isolated. This is comparable to other studies where the total number of species isolated varied from two to 27 (Peynaud & Domercq, 1959; Fleet *et al.*, 1984; Parish & Carroll, 1985; Mora & Mulet, 1991; Longo *et al.*, 1991; Schütz & Gafner, 1993; Constanti *et al.*, 1997).

Predominance by four yeast species was found in eight samples. They were *C. stellata* (four samples), *K. apiculata* (two samples), *C. colliculosa* (one sample) and *C. pulcherrima* (one sample). Another species found in high numbers was *C. guilliermondii* (43%) in Robertson 2000.

C. stellata (Constanti *et al.*, 1997; Torija *et al.*, 2001) and *K. apiculata* (Constanti *et al.*, 1997) have previously been reported as predominant at the start of wine fermentation. However, Van Zyl & Du Plessis (1961) found low numbers of

Kloeckera sp. during their investigation. They attributed this to the high levels of SO_2 used to aid settling. Modern cellars tend to use less SO_2, enabling a higher proportion of non-*Saccharomyces* yeasts to survive. *C. colliculosa* has also previously been reported as predominant in grape must (Heard & Fleet, 1985; Torija *et al.*, 2001) but not *C. pulcherrima* (Bisson & Kunkee, 1991; Longo *et al.*, 1991; Torija *et al.*, 2001).

The isolation of *Rhodotorula* sp. (7%) in Stellenbosch (2000) is unusual and is not in accordance to its non-fermentative metabolism (Longo *et al.*, 1991). However, *Rhodotorula spp.* grow very slowly on normal YPD agar and its presence may easily be overlooked due to the rapid growth of other yeasts. It would be expected

TABLE 3.3
Total cell counts of non-*Saccharomyces* yeasts over three vintages in four Chardonnay musts in the Western Cape, South Africa.

Vineyard	**Total cell count (cells/ml)**			
	Vintage			
	1997	**1998**	**2000**	**Average**
Constantia	2.6×10^6	7.8×10^5	5.2×10^6	2.9×10^6
Stellenbosch	1.6×10^5	4.2×10^4	8.6×10^3	7.0×10^4
Slanghoek	1.5×10^5	8.3×10^5	3.0×10^5	4.3×10^5
Robertson	9.5×10^3	3.3×10^6	2.1×10^4	1.1×10^6
Average total cell count (cells/ml) for each vintage	7.3×10^5	1.2×10^6	1.4×10^6	-

that its numbers would decline very quickly once fermentation commenced as this yeast is very sensitive to ethanol.

3.3.6 General comparison of vineyard and cellar isolates

Geographic location and weather patterns, while obviously impacting on non-*Saccharomyces* populations, are difficult to correlate with specific patterns in yeast population. Furthermore, the cellar processes and environment play an important role in determining the non-*Saccharomyces* population in must. Variations did occur in species diversity and numbers and a small number of non-*Saccharomyces* yeast tended to dominate in vineyards and must, but not in all the samples studied. Generally there was a greater diversity of yeasts in the processed must than from the vineyard samples (Tables 2 and 4). While in some

TABLE 3.4

Distribution of non-*Saccharomyces* yeasts in four Chardonnay musts over three vintages from four cellars in the Western Cape, South Africa

Yeast strain[1]	**Percentage (%) isolates (out of 30)**											
	Constantia			**Stellenbosch**			**Slanghoek**			**Robertson**		
	1997	**1998**	**2000**	**1997**	**1998**	**2000**	**1997**	**1998**	**2000**	**1997**	**1998**	**2000**
Candida albicans										3		
Candida boidinii		3										
Candida colliculosa	7	3					3			3	**100**	7
Candida guilliermondii			3									43
Candida hellenica				13			3			10		
Candida lambica			3							3		
Candida oleophila				3		10						
Candida pulcherrima	7		**67**				47	7	23			17
Candida sorbosa		3			3					20		
Candida stellata	13	**67**	18	30	**63**			**67**	**67**	50		20
Candida valida			3	3	13	7	7					
Debaryomyces hansenii					3					7		
Debaryomyces vanrijiae					3							
Kloeckera apiculata	**73**	24		42	13	**77**	40	27	7			10
Kloeckera apis										3		
Kluyveromyces thermotolerans			3						3			
Pichia kluyveri				7								
Rhodotorula sp.						7						
Zygosaccharomyces bailii			3									3
Number of species	4	5	7	6	6	4	5	3	4	8	1	6

[1] Dominant yeast (>50%) indicated in bold type.

instances the must population contained yeasts also found in the vineyard, this was not always the case (e.g. Robertson in 1997 and 1998). The broader diversity of yeasts in the must may be due to yeasts obtained from the production equipment during grape processing. The yeasts in the must may also have been on the surface of the grapes in very low numbers and therefore were not isolated. During the subsequent crushing and settling, they could have multiplied to such an extent that they could be detected. Other species found in the vineyard, but not in the must, may have been unable to survive in the higher osmotic pressure of the must or may have been sensitive to SO_2 added to the must.

As can be seen from the data presented, it would be unwise to predict the indigenous non-*Saccharomyces* yeast must population purely from a limited study of the grapes in the vineyard. Obvious factors, such as the method of harvest, transportation to the cellar and subsequent processing techniques, will all impact on the non-*Saccharomyces* population. Added to this is the indigenous cellar population and enrichment of yeasts during the sedimentation and pre-alcoholic fermentation phase. The non-*Saccharomyces* yeasts that are finally represented in the clarified must will therefore be those most tolerant to the existing conditions in the must and will be in the best position to continue their growth and have an oenological impact on wine fermentation. The predominant yeasts may be expected to have the most effect on the subsequent wine fermentation and resultant wine quality. *K. apiculata* can form high levels of volatile acidity, however, not all strains carry this trait and other metabolites can have a positive contribution (Romano *et al.*, 1992; Romano & Suzzi, 1993). Glycerol production by *C. stellata* has already been employed for the improvement of the analytical quality of wine (Ciani & Ferraro, 1998; Ferraro *et al.*, 2000), while *C. pulcherrima* is known to produce high amounts of esters (Bisson & Kunkee, 1991). Esters can make a positive contribution to young wines with little cultivar characteristics.

3.4 CONCLUSIONS

As part of the broader programme previously mentioned, this study was successful in isolating and preserving an oenologically biased range of non-*Saccharomyces* yeasts found in South African vineyards and musts. In total this

collection represents 24 different species comprising 720 yeast isolates. Of these, nine species predominated in the various samples investigated. Furthermore, each sampling point had a different yeast population, but no pattern linking species to climatic zone was observed. Four of the predominant species, i.e. *C. colliculosa, C. pulcherrima, C. stellata* and *K. apiculata* were found in the grape must. These species could therefore be expected to have the biggest impact on wine quality and their contribution should receive further attention. In addition, the affect of different winemaking parameters on these yeasts should also be investigated so that conditions favouring their growth can be maintained.

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CHAPTER 4

RESEARCH RESULTS II

The occurrence of apiculate yeasts in grape and must samples from the Robertson area of South Africa during the 2000 vintage

4. RESEARCH RESULTS II

The occurrence of apiculate yeasts in grape and must samples from the Robertson area of South Africa during the 2000 vintage

Abstract

During a previous study low numbers and in some instances no apiculate yeasts i.e. *Kloeckera apiculata* were isolated from the grape and must samples in the Robertson region of South Africa. This is in contrast to the generally accepted norm where apiculate yeasts dominate on grapes and in unfermented grape must. In this study, the investigation into the Robertson region was expanded. In comparison to the previous single site, a further four vineyards and their respective cellars were investigated. Results confirmed initial findings that apiculate yeasts can be found in Robertson, but in low numbers. This can be a contributing factor in the success of spontaneously fermented wines produced in this region

4.1 INTRODUCTION

It is generally accepted that the predominant non-*Saccharomyces* yeasts on grapes and in must at the start of fermentation are the apiculate yeasts (*Kloeckera apiculata/Hanseniaspora uvarum*) (Parish & Caroll, 1985; Bisson & Kunkee, 1991; Frezier & Dubourdieu, 1992; Jackson, 1994; Granchi *et al.*, 1998; Fleet, 2003). These yeasts with their distinctive apiculate or lemon-shaped cell morphology are associated with the production of volatile acidity. However, according to the data of Jolly *et al.* (2003) (Chapter 3) no apiculate yeasts were isolated from the vineyard and from the corresponding cellar samples of the 1997 and 1998 vintages from the Robertson region. During the 2000 vintage apiculate yeasts were isolated, but only 10% of the isolates in the cellar sample belonged to this group. To elucidate whether

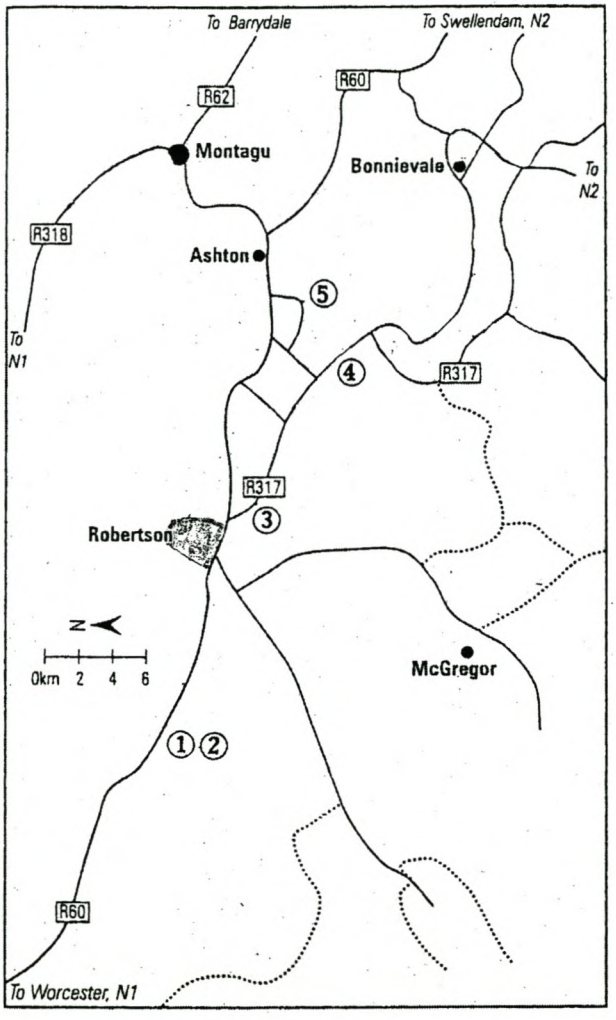

FIGURE 4.1
Map of Robertson sampling area indicating position of sampling sites (numbered 1-5).

the absence and/or low presence of apiculate yeasts in the Robertson samples was specific to the location investigated or was a phenomena of the wider Robertson area, further sites (vineyards) and their corresponding cellars needed to be investigated. The aim of this study was therefore to investigate a further four vineyard and cellar samples for the presence of apiculate yeasts.

4.2 MATERIALS AND METHODS

4.2.1 Areas sampled

Four Chardonnay vineyards (Sites 2 to 5) and their accompanying commercial cellars in the wider Robertson area were selected for sampling in 2000. The first vineyard (Site 2) was close (1 km) to Site 1 i.e. the Chardonnay vineyard sampled by Jolly *et al.* (2003) (Chapter 3). The other vineyards viz. Sites 3, 4 and 5 were 14, 24 and 28 km from Site 1, respectively (Fig. 4.1).

4.2.2 Sample collection and yeast isolation

Vineyard and must sample collection was the same as that given in Chapter 3.

4.2.3 Characterisation of yeast isolates

Apiculate yeasts were identified microscopically (400X magnification) and the remaining yeasts were morphologically characterised from colony appearance. The yeasts were subsequently classified as either apiculate, pigmented (orange/red) and other yeasts (non-pigmented).

4.3 RESULTS AND DISCUSSION

4.3.1 Sampling sites, yeast isolation and identification

The four sites sampled during the 2000 vintage together with the Chardonnay vineyard data from Jolly *et al.* (2003) represents ten sampling points (i.e. five vineyards and five cellars). While this still does not represent an exhaustive study of the Robertson area, it does carry more weight than the limited study broad obtained in Chapter 3.

The method of sample collection and yeast isolation has already been discussed by Jolly *et al.* (2003) and the reader is referred to Chapter 3 for further details. As apiculate yeasts have a distinctive cell morphology it was easy to distinguish them from the other yeast isolates microscopically. Their colony morphology is also

somewhat distinctive (smooth, greyish) and for the purpose of this investigation it was not necessary to do any further biochemical and/or karyotypic characterisation.

4.3.2 Apiculate yeasts in vineyard and must samples

The apiculate yeasts generally did not predominate in the vineyard samples and were mostly present in low numbers (Table 4.1). Site 4, the exception, showed in contrast to the other sites, a high percentage (83%) of apiculate yeasts. However, none of the cellar samples had apiculate yeast predominance. This went against expectations, as there should have been a carry over of yeasts to the must during processing. The reduction in numbers of a specific group of yeasts originally present in the vineyard sample has been noted before (Jolly *et al.*, 2003) and can be due to the influences of must clarification techniques and other cellar practices detrimental to yeast growth e.g. addition of SO_2. The reduced presence of apiculate yeasts in must samples has also been noted by Yanagida *et al.* (1992) who used a similar method of yeast isolation as in this study.

TABLE 4.1
Occurrence of apiculate yeasts in grape samples from the Robertson area during the 2000 vintage[1].

Yeast type	% Isolates at sampling points					
	Site 1[1, 2]		Site 2[2]		Site 3	
	Vineyard sample	Cellar sample	Vineyard sampled	Cellar sample	Vineyard sample	Cellar sample
Apiculate yeasts[3]	0	10	13	0	7	0
Pigmented yeasts[4]	47	17	37	23	20	20
Other yeasts	53	73	50	77	73	74

	Site 4		Site 5	
	Vineyard sample	Cellar sample	Vineyard sample	Cellar sample
Apiculate yeasts[3]	83	7	30	7
Pigmented yeasts[4]	0	3	3	0
Other yeasts	17	90	67	93

[1] Data for Site 1 obtained from Jolly *et al.*, 2003.
[2] Sites 1 and 2 share the same cellar.
[3] Apiculate yeasts e.g. *Kloeckera apiculata* and *Hanseniaspora uvarum*.
[4] Pigmented yeasts e.g. *Candida pulcherrima* and *Rhodotorula* spp.

The relative close proximity of the vineyards to each other means that they shared similar macro- and meso-climatic conditions so any climatic influences on the yeast microflora between the sites can be discounted. However, individual viticultural

practices (affecting the micro-climate) and methods of harvesting can vary. This together with cellar practices can impact on the yeast microflora, but these factors were beyond the control of the author.

It was also evident that with the reduced apiculate yeast presence, the pigmented yeasts (mostly *Candida pulcherrima*) were generally present in higher numbers. This may be due to a number of factors such as pigmented yeasts suppressing the growth of the apiculate or they may be purely opportunistic and filling the "gap" left by the absence of the apiculate yeasts. It has been reported that the pigmented yeast *Metschnikowia pulcherrima*/*Candida pulcherrima* has a killer effect against *S. cerevisiae* and some non-*Saccharomyces* yeasts (Nguyen & Panon, 1998). They did not test the effect against apiculate yeasts.

Apiculate yeasts are generally implicated in the production of volatile acidity to the detriment of wine quality (Romano *et al.*, 1992). The absence of apiculate yeasts should therefore imply a smaller risk for winemakers who allow spontaneous fermentation to take place. During this type of fermentation the growth and activity of the indigenous yeasts is not controlled or suppressed by a large inoculum of cultured *Saccharomyces* yeasts. Subsequently, the apiculate yeasts, if present, can proliferate, with the concomitant production of volatile acidity. This strengthens the necessity of knowing the non-*Saccharomyces* species profile and population numbers in a given vineyard and cellar and also the effect these species will have on wine fermentation. Utilisation of this knowledge will enable the wine producer to maximise the desired regional characteristics of his/her wines and minimise any potential spoilage to his/her wine.

One of the cellars investigated in this study (Site 3) makes exclusive use of spontaneous fermentation for their Chardonnay wine, despite the high risk linked to this practice. The results are mostly successful and these wines are sold at premium prices indicating consumer acceptance. Together with their winning combination of viticultural and oenological practices, the low levels of apiculate yeasts in the Robertson area can be contributing to this cellar's success. The belief of the old world wine producers concerning indigenous yeast contribution to the regional

characteristics and quality of wines (Amerine *et al.*, 1972; Jackson, 1994) is therefore substantiated in this case.

4.4 CONCLUSIONS

This study achieved its purpose of elucidating the occurrence of apiculate yeasts in the broader Robertson region. These yeasts are generally not predominant in the Robertson area and this will have a positive impact on the quality and regional characteristics of the wine produced there, especially if spontaneous fermentation practices are followed.

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CHAPTER 5

RESEARCH RESULTS III

The effect of non-*Saccharomyces* yeasts on fermentation and wine quality

(*South African Journal of Enology and Viticulture 24, 56-62*)

5. RESEARCH RESULTS III

The effect of non-*Saccharomyces* yeasts on fermentation and wine quality

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Abstract

Research has shown that non-*Saccharomyces* yeast strains can be detected throughout wine fermentation. Non-*Saccharomyces* yeasts can therefore influence the course of fermentation and also the character of the resultant wine. Previously, it was shown that four non-*Saccharomyces* species, i.e. *Kloeckera apiculata, Candida stellata, Candida pulcherrima* and *Candida colliculosa,* predominated in grape must at the start of fermentation. In this study, these four yeasts were used singularly and in combination with an industrial wine yeast (*Saccharomyces cerevisiae* strain VIN 13) to ferment must on laboratory-scale. The resultant wine was analysed for ethanol, volatile acidity, total SO_2 and glycerol. Results show that, in comparison to the industrial wine yeast, the non-*Saccharomyces* yeast strains could not ferment all the sugar. Furthermore, while the individual non-*Saccharomyces*-fermented wines had a different chemical composition, the wines fermented by the combinations were similar to the wine produced by the industrial yeast only. In subsequent, small-scale winemaking trials, some of the wines produced by combined fermentations were judged to be of better quality than those produced

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by the *S. cerevisiae* only. However, this quality increase could not be linked to increased ester levels.

5.1 INTRODUCTION

The yeasts present in grape must at the onset of wine fermentation can be divided broadly into two groups, i.e. the wine yeast *Saccharomyces cerevisiae* and the non-*Saccharomyces* yeasts. The *Saccharomyces* yeasts are derived primarily from the cellar equipment (Vaughan-Martini & Martini, 1995; Boulton *et al.*, 1996; Martini *et al.*, 1996), but are also present on the grapes, albeit in very low numbers. They are carried over to the must during crushing (Peynaud & Domercq, 1959; Lonvaud-Funel, 1996; Török *et al.*, 1996; Mortimer & Polsinelli, 1999). A third source of *Saccharomyces* yeasts may be the industrial culture added by the winemaker.

The second group, the non-*Saccharomyces* yeasts, is found predominantly on the grapes (Martini *et al.*, 1996), but also in lesser numbers on the cellar equipment. Before inoculation with an industrial *S. cerevisiae*, they are the yeasts present in the highest numbers in the grape must. During the fermentation, there is a sequence of dominance by the various non-*Saccharomyces* yeasts, followed by *S. cerevisiae,* which then completes the fermentation (Fleet *et al.*, 1984; Fleet 1990; Jackson, 1994; Henick-Kling *et al.*, 1998). This is especially evident in spontaneously fermenting grape must, which has a low initial *S. cerevisiae* concentration.

Research has shown that non-*Saccharomyces* yeast strains can be detected throughout wine fermentation (Jolly *et al.*, 2003 and the references therein) and their dominance during the early part of fermentation can leave an imprint on the final composition of the wine (Romano *et al.*, 1997). In a previous investigation (Jolly *et al.*, 2003) we found four different yeast species, i.e. *Kloeckera apiculata, Candida stellata, Candida pulcherrima* and *Candida colliculosa,* predominant (>50%) before the start of fermentation in eight of 12 Chardonnay musts studied.

The aim of this study, which forms part of the comprehensive and ongoing research programme documented by Pretorius *et al.* (1999), was to evaluate a

representative strain of each of the aforementioned species for their effect on wine fermentation and wine quality during laboratory and small-scale winemaking trials. This knowledge will help realise the predictions of Heard (1999) relating to the use of indigenous yeast species to improve the sensory quality of wine. His vision includes the use of mixed yeast starter cultures tailored to reflect the characteristics of a given wine region and the use of indigenous species with modern technology to produce novel wine based beverages.

5.2 MATERIALS AND METHODS

5.2.1 Yeast strains

The five yeast strains used in this study are listed in Table 5.1. Stock cultures of the strains were kept in glycerol at –80°C. The non-*Saccharomyces* strains were selected randomly from a collection of natural isolates (Jolly *et al.*, 2003) and their identities were confirmed by a commercial laboratory (CBS, Delft, The Netherlands). An industrial *S. cerevisiae* wine yeast strain (strain VIN 13, Anchor Bio-Technologies, Cape Town, South Africa) was used as a reference strain and for co-fermentation.

TABLE 5.1
Yeast strains used.

Species	Strain number	Origin
Saccharomyces cerevisiae	VIN 13	Industrial yeast from Anchor Bio-Technologies, South Africa
Candida colliculosa	M2/1	Natural isolate from Chardonnay (1998 vintage)
Candida stellata	770	Natural isolate from Chardonnay (1997 vintage)
Kloeckera apiculata	752	Natural isolate from Chardonnay (1997 vintage)
Candida pulcherrima	825	Natural isolate from Chardonnay (1997 vintage)

5.2.2 Laboratory-scale fermentations

5.2.2.1 Grape must: Fresh Chardonnay grape must, clarified by pectinases (0.5g/hL Ultrazym, Novazymes, Denmark) at 14°C, was stored at –20°C until needed. The thawed juice was thoroughly mixed and 500 mL aliquots were placed in 750 mL glass bottles. After sterilisation (121°C for 15 min), the bottles were closed tightly by plastic fermentation caps filled with sterile distilled water. Three musts (A, B, C) with different sugar concentrations were used. The chemical

analyses were: must A - 21.0°B sugar, 0.50 g/L volatile acidity, 1 mg/L total SO_2; must B - 21.7°B sugar, 0.50 g/L volatile acidity, 1 mg/L total SO_2; and must C - 24.5°B sugar, 0.50 g/L volatile acidity, 0 mg/L total SO_2.

5.2.2.2 Yeast inoculum and fermentation procedure: Yeast starter cultures were grown for 24 h in YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose). Total cell counts were carried out in a Neubauer improved bright-lined counting chamber (1 mm depth) and all inoculations were done at 1 x 10^6 cells/mL per yeast strain.

The four non-*Saccharomyces* yeasts were inoculated individually and in combination with the *S. cerevisiae* yeast strain. In the combined fermentations, the *Saccharomyces* yeast was inoculated one hour after the non-*Saccharomyces* yeast. A reference fermentation was inoculated with *S. cerevisiae* only. All fermentations were conducted in triplicate and the fermentation vessels were placed on an orbital shaker at an ambient temperature of 20°C.

The fermentations were monitored by CO_2 weight loss and were allowed to proceed until the reference fermentation was dry (14 days). Completion of fermentation (no further weight loss) was confirmed by use of glucose test strips (Clinistix, Bayer). The progression of CO_2 weight loss was used to plot a fermentation curve.

During the course of the combined fermentations, 200 µL aliquots were removed from the relevant bottles (must A only) and streaked onto lysine medium (Biolab, Merck) to check for the presence of the non-*Saccharomyces* yeast component.

5.2.3 Small-scale wine production

The four non-*Saccharomyces* yeasts (Table 5.1) were each investigated in combination with a *S. cerevisiae* (strain VIN 13) yeast for small-scale production of wine in 18 L of freshly prepared must from the 2000 vintage. The yeast cultures were propagated and inoculated in the same way as the laboratory-scale fermentations, with the exception of the *S. cerevisiae*, where an active dried VIN 13 culture was used (0.04 g/L). The fermentations were done in duplicate.

Three grape musts were used i.e. Chardonnay (22.2°B, 7.8 g/L total acidity, pH 3.37), Sauvignon blanc (22.9°B, 6.4 g/L total acidity, pH 3.52) and Chenin blanc

(21.7°B, 6.7 g/L total acidity, pH 3.71). Di-ammonium phosphate (0.50 g/L) and SO_2 (50 mg/L) were added and the fermentation was conducted at an ambient temperature of 15°C in 20 L stainless steel containers fitted with fermentation caps. After fermentation, the wines were racked off the yeast lees and the free SO_2 adjusted to 35 mg/L. Bentonite (0.75 g/L) was added and the wine was cold stabilised (0°C) for one week, filtered and transferred to ten bottles according to standard practices for white wine production. The wines were stored at 15°C until evaluated.

5.2.4 Chemical analyses

The wines (laboratory and small-scale) were analysed for alcohol (infralyser technique – SGS Wine & Spirit Laboratory, Stellenbosch) and for residual sugar (Rebelein), volatile acidity and SO_2 as described by Iland *et al.* (2000). Testing for glycerol (must B only) was done with enzymatic test kits (Boehringer Mannheim, Roche, Germany). Analyses for esters (volatile component analyses – Research Chemistry, Distell, Stellenbosch) were carried out at the time of the five-month sensory evaluations. The ester values were analysed by the ANOVA method.

5.2.5 Sensory evaluation of small-scale wines

The duplicate wines were evaluated individually, five and 18 months after production, according to the multi-wine preference tasting method (McCloskey *et al.*, 1995) by two different panels of seven trained wine tasters. The wines were given code numbers, chilled to 15°C and presented in international wine-tasting glasses. The individual scores of the duplicate wines were averaged.

5.3 RESULTS AND DISCUSSION

5.3.1 Yeast strains used

In a previous investigation four yeast species were found to predominate in samples of clarified Chardonnay grape must before the onset of fermentation (Jolly *et al.*, 2003). As predominant species would be in the best position to influence wine fermentation, four representative strains were randomly selected from the aforementioned isolates and used in this study. Although the teleomorphic forms may originally have been isolated, storage before identification

could have led to the loss of the ability to sporulate. For this reason they were identified as the anamorphic forms (M. Th. Smith, personal communication, 2000).

5.3.2 Laboratory-scale fermentations

The laboratory-scale fermentations were done to mimic commercial-scale fermentations as much as possible. However, the must had to be sterilised so that no *S. cerevisiae* inherent to the must could overgrow the slow growing inoculated non-*Saccharomyces* yeasts. The placement of the fermentation vessels on an orbital shaker copied the natural turbulence found in large fermentations as a result of the generation of CO_2 (Henschke, 1990). The tightly sealed fermentation caps ensured that no oxygen entered the fermentation vessels. The one hour lapse between inoculation of the non-*Saccharomyces* yeasts and the *S. cerevisiae* yeast was chosen for practical reasons. Normal winemaking practices do not call for inoculating a wine tank twice due to time constraints on the winemaker during a busy harvest. In addition, a delayed start in alcoholic fermentation can lead to oxidation of the wine and subsequent drop in quality. In order to maximise the effect of the non-*Saccharomyces* yeasts and to minimise any delay in the start of alcoholic fermentation by *S. cerevisiae*, a high inoculum of non-*Saccharomyces* and the short time delay before the inoculation of the *S. cerevisiae* wine yeast, was chosen.

5.3.2.1 Individual yeast fermentations: The non-*Saccharomyces* yeasts were slower fermenters than the reference yeast (*S. cerevisiae* strain VIN 13) in each of the three musts investigated (data for must B only is presented in Fig. 5.1). Furthermore, none of them could complete the fermentation within 14 days, as also indicated by high values of residual sugar (Table 5.2). In decreasing order of fermentation ability, the yeasts were *C. colliculosa, C. stellata, K. apiculata* and *C. pulcherrima. C. colliculosa* can tolerate 10 to 12% (v/v) ethanol (Fleet *et al.*, 1984) and, as the most fermentative species in this investigation, it was able to produce between 9.7 and 12.6% (v/v) alcohol (Table 5.2). It has been reported that the teleomorphic form of *C. colliculosa* (*Torulaspora delbrueckii*) can produce high levels of acetic acid (Fleet *et al.*, 1984), but the strain used in this study had a volatile acidity production comparable to that of *S. cerevisiae*.

TABLE 5.2
Standard wine chemical analyses of single and combined yeast laboratory-scale fermentations in three different Chardonnay musts.

Yeast strain	Chemical analyses[1]						
	Residual sugar (g/L)			Ethanol (% v/v)			Glycerol[2] (g/L)
	Must A	Must B	Must C	Must A	Must B	Must C	Must B
S. cerevisiae (reference)	1.9 ± 0.2	2.5 ± 1.4	1.9 ± 0	12.5 ± 0.1	12.6 ± 0.1	14.5 ± 0.1	8.53 ± 0.76
C. colliculosa	24.8 ± 2.8	47.6 ± 5.0	39.8 ± 7.5	11.2 ± 0.1	9.7 ± 0.9	12.6 ± 0.4	7.76 ± 0.99
C. stellata	85.5 ± 0	108.8 ± 4.0	107.6 ± 2.2	7.7 ± 0	5.9 ± 0.2	8.4 ± 0.1	11.11 ± 1.11
K. apiculata	135.0 ± 9.3	134.9 ± 4.1	141.0 ± 1.0	5.4 ± 0.5	4.3 ± 0.1	6.5 ± 0.2	8.33 ± 1.24
C. pulcherrima	158.9 ± 4.4	159.0 ± 1.4	166.7 ± 1.7	3.5 ± 0.1	2.7 ± 0.1	4.6 ± 0.1	5.79 ± 2.04
C. colliculosa / S. cerevisiae	1.9 ± 0.1	2.5 ± 1.1	2.2 ± 0.3	12.4 ± 0.1	12.6 ± 0	14.6 ± 0	6.89 ± 0.08
C. stellata / S. cerevisiae	1.7 ± 0.1	3.1 ± 0.9	2.1 ± 0.2	12.4 ± 0.1	12.6 ± 0.1	14.6 ± 0.1	7.52 ± 0.53
K. apiculata / S. cerevisiae	1.9 ± 0.2	4.4 ± 0.6	2.1± 0	12.5 ± 0.1	12.7 ± 0.1	14.6 ± 0	7.94 ± 0.10
C. pulcherrima / S. cerevisiae	1.9 ± 0.1	3.1 ± 0.9	2.6 ± 0.2	12.4 ± 0	12.7 ± 0	14.6 ± 0.1	7.47 ± 1.20

Yeast strain	Volatile acidity (g/L)			Total SO_2 (mg/L)		
	Must A	Must B	Must C	Must A	Must B	Must C
S. cerevisiae (reference)	0.22 ± 0.05	0.12 ± 0.04	0.36 ± 0.03	26 ± 1	35 ± 6	34 ± 2
C. colliculosa	0.24 ± 0.01	0.10 ± 0.02	0.30 ± 0.02	50 ± 2	60 ± 1	47 ± 1
C. stellata	0.80 ± 0	0.61 ± 0.04	1.10 ± 0.03	20 ± 0	25 ± 3	23 ± 1
K. apiculata	0.89 ± 0.04	0.71 ± 0.02	1.06 ± 0.04	23 ± 1	24 ± 1	22 ± 2
C. pulcherrima	0.24 ± 0.01	0.20 ± 0.03	0.27 ± 0.02	21 ± 1	21 ± 1	21 ± 3
C. colliculosa / S. cerevisiae	0.14 ± 0.01	0.13 ± 0.03	0.22 ±0.03	28 ± 2	38 ± 2	32 ± 1
C. stellata / S. cerevisiae	0.19 ± 0.03	0.17 ± 0.05	0.35 ± 0.03	27 ± 1	39 ± 2	34 ± 1
K. apiculata / S. cerevisiae	0.18 ± 0.02	0.10 ± 0.04	0.31 ± 0.02	27 ± 1	39 ± 3	33 ± 1
C. pulcherrima / S. cerevisiae	0.21 ± 0.01	0.33 ± 0.17	0.34 ± 0.02	28 ± 2	37 ± 0	37 ± 3

[1] Average value of three fermentations ± standard deviation. Original sugar: must A=21.0°B; must B=21.7°B; and must C=24.5°B.
[2] Glycerol analyses done on must B only.

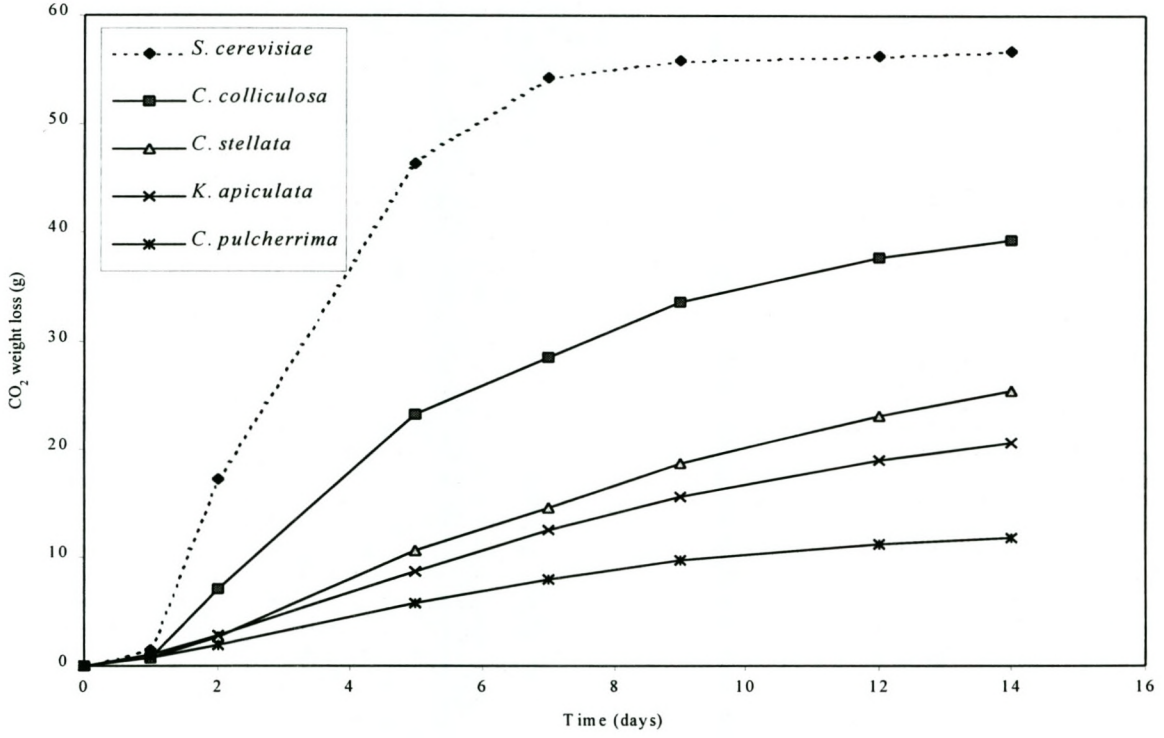

FIGURE 5.1
Fermentation curves of non-*Saccharomyces* yeasts in a Chardonnay must (must B) compared to a reference yeast (*S. cerevisiae* strain VIN 13).

Elevated levels of SO_2 (47 to 60 mg/L) were formed by the *C. colliculosa* strain. They were nearly double that of the reference and other yeasts investigated. This could be detrimental to wine fermentation and quality by inhibiting sensitive wine yeasts and/or malolactic bacteria. The final SO_2 levels in the wine may also be close to or exceed the legal limits, especially as SO_2 is normally added during the winemaking process. The glycerol levels were very similar to that of *S. cerevisiae* (Table 5.2).

In contrast, *C. stellata* did not form excessive SO_2 while producing between 5.9 and 8.4% (v/v) alcohol (Table 5.2). This is lower than the 10% (v/v) alcohol that is accepted as the maximum produced by *C. stellata* (Gao & Fleet, 1998; Jackson, 1994). High levels of glycerol (11.11 g/L) were produced by this strain of *C. stellata*, compared to *S. cerevisiae* (8.53 g/L) (Table 5.2). This could positively benefit the mouth-feel (smoothness) and complexity of a wine (Scanes *et al.*, 1998; Prior *et al.*, 2000). However, increased glycerol production is usually paired with higher acetic acid production due to the cell having to maintain its redox balances (Prior *et al.*, 2000). Production of volatile acidity by *C. stellata* is well known (Jackson, 1994). In this study volatile acidity levels were close to and, for must C, exceeded the sensory threshold (approximately 0.8 g/L) and would obviously have a negative effect on the sensory character of a wine.

K. apiculata is generally known as a high producer of volatile acidity (Van Zyl *et al.*, 1963) and can have a significant negative effect on the chemical composition and therefore the quality of wine (Gil *et al.*, 1996). However, this is strain dependent and Romano *et al.* (1992) showed that some strains produced less than 1 g/l volatile acidity, which is comparable to the production by *S. cerevisiae*. As was expected, the strain used in this study produced high levels of volatile acidity (0.71 to 1.06 g/L) (Table 5.2). Ethanol production was between 4.3 and 6.5% (v/v). This is similar to that documented by Jackson (1994). It has also been reported that *K. apiculata* differs from *S. cerevisiae* in the levels of higher alcohols (n-propanol, iso-butanol, iso-amyl alcohol, and active amyl alcohol) produced (Romano *et al.*, 1992). *K. apiculata* can also produce high concentrations of esters (Bisson & Kunkee, 1991). These all have potential importance for imparting flavour to the wine.

C. pulcherrima was the yeast that was the least able to ferment the grape must (Fig. 5.1 and Table 5.2). This was to be expected from a yeast known to have an oxidative metabolism (Longo *et al.*, 1991). During its limited growth, only low levels of ethanol were produced, while high levels of volatile acidity or SO_2 were not noted. This species can form relatively high concentrations of esters and some fusel oils (Bisson & Kunkee, 1991), which may make a positive contribution to wine flavour.

5.3.2.2 Combined yeast fermentations: The combined yeast fermentation curves (data for must B only is shown in Fig. 5.2) were very similar to that of the *S. cerevisiae* reference and all the fermentations proceeded to dryness (< 4 g/L residual sugar) (Table 5.2). The other chemical analyses, i.e. alcohol and volatile acidity (Table 5.2) for the combined fermentations are similar to the reference fermentation, with the exception of the glycerol concentrations that were lower. However, the characteristic chemical parameters due to the growth of the individual non-*Saccharomyces* yeasts were absent. This scenario was also reported by Moreno *et al.* (1991) when *S. cerevisiae* and *T. delbrueckii* (teleomorph of *C. colliculosa*) yeasts were utilised in single and mixed culture fermentations. This can be due to the suppression of the non-*Saccharomyces* yeasts by the *Saccharomyces* yeasts during the fermentation, or the utilisation of the non-*Saccharomyces* metabolites by *S. cerevisiae*. In the combined fermentations in this study, the non-*Saccharomyces* yeasts could be detected until the fifth day of fermentation (data not shown). The exception was *C. pulcherrima*, the least fermentative of the four species investigated, which could be detected until day 9. This yeast, with preferred aerobic growth, would have been expected to die off earlier in the fermentation.

In contrast, Ciani & Ferraro (1998) and Ferraro *et al.* (2000) reported an improvement in the analytical chemical profile of small-scale Pinot grigio and pilot-scale Trebiano Toscano wines, respectively. They used immobilised *C. stellata* cells in conjunction with *S. cerevisiae* in a sequential fermentation and found that the interaction of the *C. stellata* and *S. cerevisiae* metabolisms led to wines having not only higher glycerol levels, but also lower acetic acid levels. Their inoculation and fermentation strategy, however, differed in that a higher inoculum of *C. stellata* (5×10^8 to 1×10^9 cells/mL) was allowed to grow for three days before

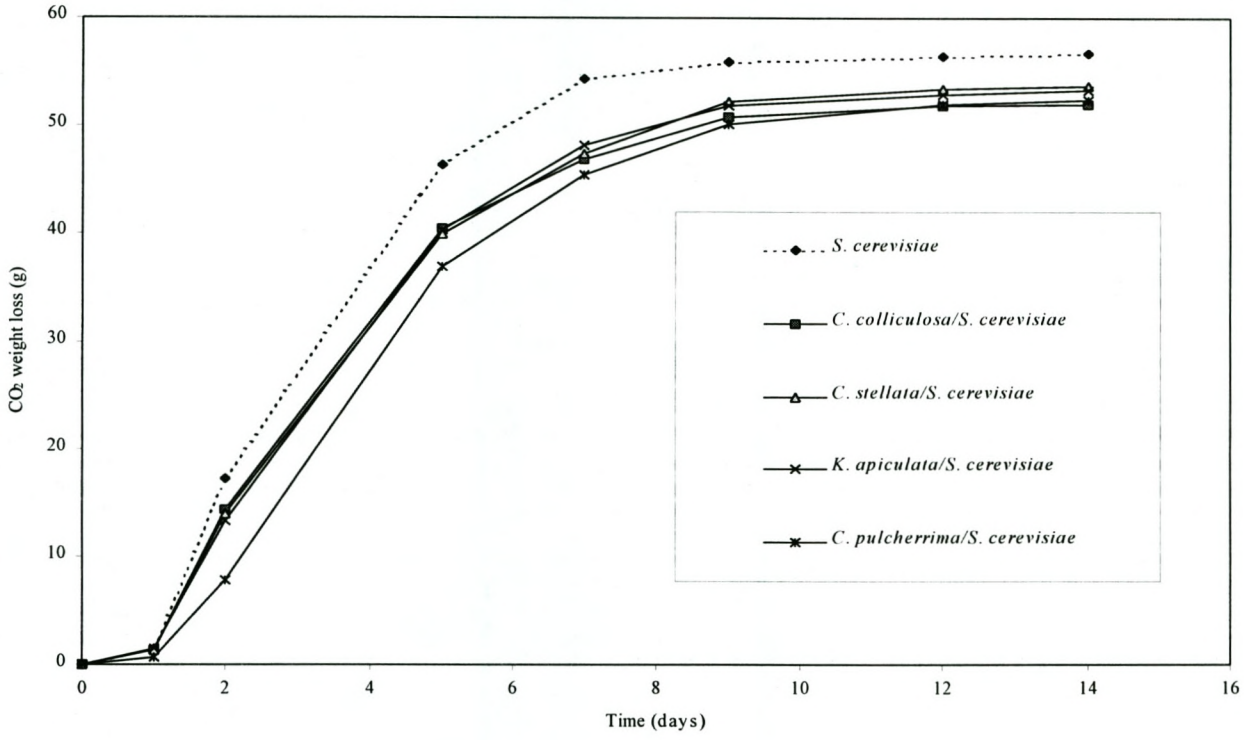

FIGURE 5.2

Fermentation curves of combined non-*Saccharomyces/S. cerevisiae* yeasts in a Chardonnay must (must B) compared to a reference yeast (*S. cerevisiae* strain VIN 13 only).

inoculation with *S. cerevisiae* at 1 to 5 x 10^6 cells/mL. The growth of indigenous non-*Saccharomyces* yeasts, but not indigenous *S. cerevisiae* were controlled by the use of SO_2 during the three-day period. The authors did not report on the sensory characteristics of the wines.

5.3.3 Small-scale wine production

5.3.3.1 Chemical analyses: Similar to the laboratory-scale fermentations, the chemical analyses of the wines (Table 5.3) showed basically no difference between the reference fermentation and the non-*Saccharomyces* combinations. The higher total SO_2 of the wines is a reflection of the SO_2 added during the winemaking process. All the wine fermentations proceeded to dryness.

As some non-*Saccharomyces* yeasts are reported to be high ester producers, the wines were all analysed for total esters at the time of the five-month sensory evaluation. In the Chardonnay wines (Table 5.4), the combined fermentations showed a higher concentration of total esters than the reference wine, however, it was only significant for the *K. apiculata* and *C. stellata* combinations. The values remain higher even after the ethyl acetate and ethyl lactate values are subtracted. In contrast, the total esters of the Sauvignon blanc wines (Table 5.5) were generally lower for the combined fermentations but only of significance for the *K. apiculata* combination. The *C. colliculosa* combination produced a similar amount of total esters than the reference wine. The total esters in the Chenin blanc wine showed no significant differences, however, the *K. apiculata* combination was higher due to the ethyl acetate component (Table 5.6).

5.3.3.2 Sensory analyses: Very little is reported in literature about sensory results of wines produced with the use of non-*Saccharomyces* yeasts. However, in a report by Soden *et al.* (2000), *C. stellata* was used sequentially with *S. cerevisiae* to produce Chardonnay wines with an aroma profile derived from the metabolism of both yeasts. Their strategy involved fermentation by *C. stellata* for fifteen days before inoculation with *S. cerevisiae*. The resultant wine was not judged for overall quality, but a descriptive sensory analyses shows that in comparison to a *S. cerevisiae* reference wine, the intensities for "floral", "banana", "lime" and "tropical fruit" aroma nuances were lower and "honey", "sauerkraut" and "ethyl acetate" were higher. The last two aroma nuances mentioned are grouped under "microbiological" and

TABLE 5.3
Chemical analyses of small-scale wines produced by non-*Saccharomyces/S. cerevisiae* combinations.

Yeast species combination	Analyses[1]				
	Chardonnay				
	Ethanol (% v/v)	Volatile acidity (g/L)	Total SO_2 (mg/L)	Residual sugar (g/L)	Glycerol (g/L)
S. cerevisiae (reference)	13.3 (13.2-13.3)	0.26 (0.26-0.26)	61 (57-65)	1.6 (1.4-1.8)	5.55 (5.45-5.65)
C. colliculosa/S. cerevisiae	13.3 (13.2-13.3)	0.26 (0.25-0.27)	61 (59-62)	1.9 (1.8-1.9)	5.65 (5.48-5.82)
C. stellata/S. cerevisiae	13.6 (13.5-13.6)	0.25 (0.24-0.26)	61 (53-69)	1.7 (1.5-1.8)	5.88 (5.73-6.03)
K. apiculata/S. cerevisiae	13.4 (13.4-13.4)	0.28 (0.28-0.28)	57 (55-58)	1.8 (1.8-1.8)	5.65 (5.57-5.73)
C. pulcherrima/S. cerevisiae	13.4 (13.4-13.4)	0.26 (0.25-0.26)	64 (61-66)	1.8 (1.4-2.1)	5.89 (5.65-6.14)
Yeast species combination	**Sauvignon blanc**				
	Ethanol (% v/v)	Volatile acidity (g/L)	Total SO_2 (mg/L)	Residual sugar g/L)	Glycerol (g/L)
S. cerevisiae (reference)	13.7 (13.7-13.7)	0.23 (0.23-0.23)	82 (80-83)	0.5 (0.4-0.6)	6.06 (6.06-6.06)
C. colliculosa/S. cerevisiae	13.7 (13.7-13.7)	0.21 (0.20-0.22)	74 (72-75)	1.2 (0.2-2.2)	6.08 (6.02-6.14)
C. stellata/S. cerevisiae	13.3 (13.0-13.6)	0.25 0.23-0.26)	82 (75-88)	1.0 (0.9-1.1)	6.10 (5.94-6.25)
K. apiculata/S. cerevisiae	13.7 (13.7-13.7)	0.26 (0.25-0.26)	79 (70-88)	1.5 (1.5-1.5)	5.95 (5.86-6.04)
C. pulcherrima/S. cerevisiae	13.7 (13.6-13.7)	0.24 (0.23-0.24)	78 (77-78)	1.3 (1.2-1.3)	6.15 (6.07-6.23)
Yeast species combination	**Chenin blanc**				
	Ethanol (% v/v)	Volatile acidity (g/L)	Total SO_2 (mg/L)	Residual sugar (g/L)	Glycerol (g/L)
S. cerevisiae (reference)	13.2 (13.1-13.2)	0.19 (0.18-0.19)	104 (104-104)	1.2 (1.0-1.4)	5.55 (5.49-5.61)
C. colliculosa/S. cerevisiae	13.0 (13.0-13.1)	0.18 (0.18-0.18)	98 (96-99)	1.3 (1.2-1.4)	5.51 (5.42-5.59)
C. stellata/S. cerevisiae	13.0 (13.0-13.)	0.20 (0.19-0.20)	95 (91-99)	1.3 (1.1-1.5)	5.82 (5.75-5.88)
K. apiculata/S. cerevisiae	13.1 (13.1-13.1)	0.21 (0.20-0.21)	95 (94-96)	1.6 (1.5-1.6)	6.10 (6.10-6.10)
C. pulcherrima/S. cerevisiae	13.0 (12.9-13.0)	0.19 (0.18-0.19)	98 (94-101)	0.9 (0.2-1.6)	5.53 (5.50-5.56)

[1] Average value of two fermentations. Range indicated in brackets.

TABLE 5.4
GC analyses of small-scale Chardonnay wines of the 2000 vintage produced by non-*Saccharomyces*/*S. cerevisiae* combinations.

Yeast combination	Total esters (mg/L)	Ethyl acetate (mg/L)	Total esters - ethyl acetate (mg/L)	Ethyl lactate (mg/L)	Total esters - ethyl acetate - ethyl lactate (mg/L)
S. cerevisiae (reference)	346.69b[1]	299.14b	47.56b	12.99a	34.57b
C. colliculosa/S. cerevisiae	376.40ab	324.04ab	52.36ab	13.77a	38.59ab
C. stellata/ S. cerevisiae	402.42a	346.02a	56.41a	14.83a	41.58a
K. apiculata/ S. cerevisiae	398.70a	341.08a	57.62a	14.00a	43.62a
C. pulcherrima/ S. cerevisiae	382.67ab	324.45ab	58.22a	14.86a	43.37a

[1] Values within columns followed by the same letter do not differ significantly ($p < 0.05$).

TABLE 5.5
GC analyses of small-scale Sauvignon blanc wines of the 2000 vintage produced by non-*Saccharomyces*/*S. cerevisiae* combinations.

Yeast combination	Total esters (mg/L)	Ethyl acetate (mg/L)	Total esters - ethyl acetate (mg/L)	Ethyl lactate (mg/L)	Total esters - ethyl acetate - ethyl lactate (mg/L)
S. cerevisiae (reference)	384.27a[1]	331.60a	52.67a	14.10a	38.56a
C. colliculosa/S. cerevisiae	384.00a	331.82a	52.18ab	14.06a	38.12a
C. stellata/ S. cerevisiae	371.42ab	320.95a	50.47b	13.51a	36.97b
K. apiculata/ S. cerevisiae	349.92b	299.08a	50.85ab	12.71a	38.13a
C. pulcherrima/ S. cerevisiae	373.26ab	321.24a	52.02ab	14.73a	37.30b

[1] Values within columns followed by the same letter do not differ significantly ($p<0.05$).

TABLE 5.6
GC analyses of small-scale Chenin blanc wines of the 2000 vintage produced by non-*Saccharomyces*/*S. cerevisiae* combinations.

Yeast combination	Total esters (mg/L)	Ethyl acetate (mg/L)	Total esters - ethyl acetate (mg/L)	Ethyl lactate (mg/L)	Total esters - ethyl acetate - ethyl lactate (mg/L)
S. cerevisiae (reference)	216.06a[1]	176.35a	39.71a	11.41a	28.30a
C. colliculosa/S. cerevisiae	213.17a	174.27a	38.90a	10.32a	28.58a
C. stellata/ S. cerevisiae	205.65a	168.65a	36.99a	9.66a	27.33a
K. apiculata/ S. cerevisiae	250.74a	209.42a	41.32a	13.23a	28.09a
C. pulcherrima/ S. cerevisiae	231.62a	188.32a	43.31a	13.97a	29.64a

[1] Values within columns followed by the same letter do not differ significantly ($p<0.05$)

TABLE 5.7
Relative score of small-scale wines produced by non-*Saccharomyces*/*S. cerevisiae* combinations and evaluated by the multi-wine preference method[1].

Yeast combination	Cultivar / Time of evaluation / Relative score[2]					
	Chardonnay		Sauvignon blanc		Chenin blanc	
	5 months	18 months	5 months	18 months	5 months	18 months
S. cerevisiae (reference)	**3** (3, 3)	**2** (1, 3)	-1 (-1, -1)	-3 (-3, -3)	-2 (-5, 1)	-1 (-5, 3)
C. colliculosa/S. cerevisiae	2 (1, 3)	0 (-1, 1)	**3** (1, 5)	1 (-1, 3)	-1 (-1, -1)	3 (3, 3)
C. stellata/ S. cerevisiae	-1 (-2, 0)	-1 (-5, 3)	-3 (-5, -1)	-1 (-1, -1)	1 (-1, 3)	-4 (-5, -3)
K. apiculata/ S. cerevisiae	1 (0, 2)	0 (-3, 3)	0 (-3, 3)	**3** (3, 3)	0 (-3, 3)	-3 (-5, -1)
C. pulcherrima/ S. cerevisiae	0 (-1, 1)	0 (-1, 1)	1 (-1, 3)	0 (-1, 1)	**2** (1, 3)	**5** (5, 5)

[1] McCloskey *et al.*, 1995.
[2] Average values of two wines (seven judges). Range indicated in brackets. Only values within a column are related to each other. Highest score within a column is indicated in bold type.

"oxidised" according to the Wine Aroma Wheel (Noble *at al.*, 1987) and as negative aroma nuances, would detract from an increased quality rating. A co-inoculated (5 x 10^6 cells/mL) wine produced with the same two yeasts appeared to produce a wine with less of the negative aroma nuances. Based on these results, Soden *et al.* (2000) suggested that the controlled use of non-*Saccharomyces* yeasts, such as *C. stellata,* could lead to wines of greater complexity and flavour diversity. Strain selection obviously would be very important. No sensory data was given on the wines produced by Ferraro *et al.* (2000).

In this study the wines were judged for quality according to the method of McCloskey *et al.* (1995). Overall, the different combinations of yeasts produced wines with different average relative qualities (Table 5.7). For the Chardonnay, all the non-*Saccharomyces* combinations produced wines with a lesser quality than the reference wine when judged at five and 18 months, even though the wines differed from each other. The high levels of ethyl acetate formed by the non-*Saccharomyces* yeasts (Table 5.4) would have played a role in this regard.

For the Sauvignon blanc wines (Table 5.7) judged at five months, the *K. apiculata*, *C. pulcherrima* and *C. colliculosa* combinations were all judged to be of better quality than the reference wine. At 18 months, all four of the non-*Saccharomyces* combination Sauvignon blanc wines were judged better, showing that the wine's flavour was still developing.

In the Chenin blanc wine (Table 5.7), all the non-*Saccharomyces* combinations were judged to be better at five months. However, this could not be supported by the ester analyses (Table 5.6). At 18 months, only the *C. pulcherrima* and *C. colliculosa* combinations were judged to be of better quality than the reference.

It thus would appear that specific non-*Saccharomyces/S. cerevisiae* combinations produce wines with increased quality from different grape varieties. Obviously, a large number of winemaking factors, including temperature, SO_2 levels, *S. cerevisiae* strain, time of inoculation and inoculum concentration, can all play a role in the contribution made by non-*Saccharomyces* yeasts. Lower fermentation temperatures (below 25°C) can enhance the ethanol tolerance of yeasts such as *K. apiculata* and *C. stellata* (Fleet, 1990; Bisson & Kunkee, 1991), and their sustained growth may

allow sensorially positive metabolites to become evident. The opposite can also be true and sensorially negative metabolites can detract from wine quality as can be seen in the sensory results of Soden *at al.* (2000). This underlines the importance of a thorough screening and selection programme to choose the most appropriate non-*Saccharomyces* strains.

The random selection of four isolates in this study did result in some incidences of increased wine quality. While ester levels could not be linked to increased quality, numerous other compounds also play a role in wine flavour. More in-depth analyses of the wines could in future elucidate the compounds that may have a subtle impact on wine flavour. Subsequently, the desired indigenous yeast species could be screened for the ability to produce the compounds linked to increased wine quality.

The growth rate of individual species will also determine the extent of their contribution to flavour development (Heard, 1999). Slower growing yeasts would need a longer period of unhindered growth before inoculation with *S. cerevisiae.* The nutrient composition of individual musts, which can vary over vintages and geographic areas and can be influenced by viticultural practices, can also impact on the ability of non-*Saccharomyces* yeasts to contribute to the fermentation. Furthermore, the interactions of the metabolism of the different yeasts with each other should also not be overlooked.

5.4 CONCLUSIONS

An improvement in wine quality was achieved by the use of four non-*Saccharomyces* yeasts even though they were not specifically selected. These improvements were coupled to grape cultivar. Furthermore, the increase in quality was achieved despite the inoculation protocol that primarily addressed cellar practices, and not optimal contribution by the non-*Saccharomyces* yeasts. Improved quality could not be linked to standard chemical analyses of the wine. Further research is now necessary to identify marker components that can be used in turn for selection of non-*Saccharomyces* yeasts. This selection, comprising grape cultivar specific non-*Saccharomyces*/*S. cerevisiae*-combinations, coupled to a specific inoculation protocol (i.e. time between inoculation of the non-*Saccharomyces* and *S. cerevisiae*),

could be successfully used to develop new wine styles, improve aroma of wines with a history of mediocrity or to enhance specific 'terroir'-related characteristics.

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CHAPTER 6

RESEARCH RESULTS IV

The use of *Candida pulcherrima* in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc wine

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6. RESEARCH RESULTS IV

The use of *Candida pulcherrima* in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc wine

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Abstract

Wine fermentations are conducted by naturally occurring or selected industrial wine yeast strains of *Saccharomyces cerevisiae*. However, non-*Saccharomyces* yeasts also occur naturally in fermenting grape musts, especially in the initial stages of the fermentation. It has been speculated that these yeasts can contribute to the overall characteristics of the wine. Generally, it is accepted that *Kloeckera apiculata* is the predominant non-*Saccharomyces* yeast species in grape must. However, it was shown previously that *Candida pulcherrima* was the predominant non-*Saccharomyces* yeast species in a grape must after sedimentation and prior to inoculation with commercial wine yeast. Subsequently, this yeast was investigated in laboratory and small-scale wine fermentations of Chenin blanc wine. As it could not ferment grape juice to dryness on its own, it was used in combination with a *S. cerevisiae* wine yeast strain. The affect of SO_2, di-ammonium phosphate (DAP), pH and temperature on the growth of *C. pulcherrima* was also investigated. In combined fermentations, no change in overall fermentation rate or standard wine chemical analyses could be observed in comparison to a control *S. cerevisiae* fermentation. However, wine production in three consecutive years

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showed that the wine produced by the combined fermentation was of an higher quality than that produced by the *S. cerevisiae* only.

6.1 INTRODUCTION

In South Africa, the most planted white grape variety is Chenin blanc. However, wines produced from this cultivar tend to be neutral in aroma and taste. A number of viticultural (pruning, ripeness at harvest) and winemaking practices (skin contact, post-fermentation lees contact, maturation practices) are currently being investigated to improve wine quality. A further aspect receiving attention is the role of yeast.

Chenin blanc fermentations are normally conducted by selected *Saccharomyces cerevisiae* industrial wine yeast strains. However, non-*Saccharomyces* yeasts also occur naturally in fermenting grape musts, especially in the initial stages of the fermentation (Heard & Fleet, 1985). It has been shown that some non-*Saccharomyces* yeasts can contribute to the overall characteristics of the wine (Romano *et al.,* 1997; Soden *et al.*, 2000). These facts were confirmed by Jolly *et al.* (2003a; 2003b). Furthermore, species present in higher numbers could be expected to have a greater affect on the fermentation and resultant wine quality than species present in lower numbers.

Generally, it is accepted that *Kloeckera apiculata* is the predominant non-*Saccharomyces* yeast species found in grape must (Fleet *et al*, 1984; Querol *et al.*, 1990; Longo *et al.*, 1991), but, as this yeast is usually associated with volatile acidity production, the potential for a positive contribution to wine quality is low (Romano *et al.*, 1992; Gil *et al.*, 1996). Previously, it was shown that *Candida pulcherrima* can also occur in high numbers in must (Schütz & Gafner, 1993; Jolly *et al.*, 2003a). This non-*Saccharomyces* yeast is not normally associated with volatile acidity production, but can form relatively high concentrations of esters (Bisson & Kunkee, 1991). These esters, as well as other metabolites, could have a positive benefit for a Chenin blanc wine with neutral cultivar characteristics. While *C. pulcherrima* has been used to improve wine quality (Jolly *et al.*, 2003b), it is not known how must pH and different winemaking practices, i.e. fermentation temperature, addition of di-ammonium phosphate (DAP) and SO_2, will affect this

yeast. Therefore, the aim of this study, which forms part of the ongoing research programme documented by Pretorius *et al.* (1999), was to investigate the effect of pH, fermentation temperature, DAP and SO_2 addition on the growth of *C. pulcherrima*. Subsequently, two *C. pulcherrima* isolates were investigated in small-scale wine fermentations over three vintages during the production of Chenin blanc wines.

6.2 MATERIALS AND METHODS

6.2.1 Yeast strains

Three yeast strains were used in this investigation, viz. *C. pulcherrima* (strains 825 & C1-15), previously isolated from vineyards and grape must from the Western Cape, South Africa (Jolly *et al.*, 2003a) and an industrial *S. cerevisiae* yeast strain (strain VIN 13, Anchor Bio-Technologies, South Africa). The first *C. pulcherrima* strain (strain 825) was selected randomly for wine production (Jolly *et al.*, 2003b) and the second (strain C1-15) was selected after screening 71 *C. pulcherrima* isolates from South African grape musts. The screening criteria were highest growth tempo, equal or lower formation of volatile acidity (in comparison to *S. cerevisiae* reference) and highest ethanol production in a grape must at 15°C. Strain 825 was used for the laboratory-scale fermentation and wine production for all three vintages, while strain C1-15 was used for wine production in the second and third vintages only.

6.2.2 Laboratory-scale fermentations

Four sets of laboratory-scale fermentations were conducted to determine the individual effects of pH, temperature, DAP and SO_2, respectively. A further two sets of fermentations were done to determine combined effects of aforementioned (Table 6.1).

6.2.2.1 Grape must: A previously frozen white grape must (21.5°B sugar, 5.6 g/L total acidity, pH 3.4, 0.50 g/L volatile acidity and 1 mg/L total SO_2) was used for all laboratory-scale fermentations. After thorough mixing, 500 mL aliquots were placed in 750 mL glass bottles and sterilised (121°C for 15 min). The bottles were closed tightly by plastic fermentation caps filled with sterile distilled water. For the relevant experiments, the pH was adjusted (3.2 & 3.5), DAP (0.5 g/L) and a 10%

solution of sodium meta-bisulphite were added to give 30 and 60 mg/L total SO_2. In the other instances no changes or additions were made to the must.

6.2.2.2 Yeast inoculum and fermentation procedures: Yeast starter cultures were grown for 24 h in YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose). Total cell counts were carried out in a Neubauer improved bright-lined counting chamber (1 mm depth) and all inoculations were done at 1 x 10^6 cells/mL per yeast strain. The two *C. pulcherrima* strains were inoculated individually and all fermentations were conducted in triplicate. Reference fermentations were conducted by *S. cerevisiae* (strain VIN 13) only. The fermentation vessels were placed on an orbital shaker at 20°C, except for the temperature experiment where fermentations were conducted at 15, 20 and 28°C. Fermentations were monitored by CO_2 weight loss and allowed to proceed until the reference fermentation was dry (14 days). Completion of fermentation (no further weight loss) was confirmed by use of glucose test strips (Clinistix, Bayer). The progression of CO_2 weight loss was used to plot a fermentation curve. The slope of the logarithmic phase of fermentation curve was calculated and used for comparison within an experiment as an indication of the yeast' ability to ferment.

6.2.3 Small-scale wine production

The *C. pulcherrima* yeast strains were investigated in combination with the *S. cerevisiae* for small-scale production of Chenin blanc wine.

6.2.3.1 Grape must: The Chenin blanc grapes were commercially harvested from the vineyards of the ARC Infruitec-Nietvoorbij Research Institute, Stellenbosch. After crushing and sedimentation (0.5 g/hL Ultrazym, Novazymes, Denmark), the clear juice was racked off the lees and divided into the fermentation containers. The chemical analyses for the musts were: 21.7°B sugar, 6.9 g/L total acidity, pH 3.71; 23.0°B sugar, 7.8 g/L total acidity, pH 3.33; and 24.3°B sugar, 6.8 g/L total acidity and pH 3.55 for the 2000, 2001 and 2002 vintages, respectively. Di-ammonium phosphate (0.5 g/L) and 10% sodium meta-bisulphite solution (50 mg/L total SO_2) were added before fermentation.

6.2.3.2 Fermentation procedure: The *C. pulcherrima* yeasts were propagated in the same way as for the laboratory-scale fermentations and inoculated at a concentration of 1 x 10^6 cells/mL into 18 L of the freshly prepared grape musts.

This first inoculation was followed one hour later by an active dried *S. cerevisiae* (strain VIN 13) inoculation at a concentration of 0.04 g/L. A reference grape must was inoculated with the *S. cerevisiae* only. Wine production was done in duplicate and continued as described by Jolly *et al.* (2003b).

6.2.4 Sensory evaluation of small-scale wines

The wines (small-scale only) were subjected to a sensory evaluation by different panels of seven trained wine tasters. Descriptive sensory analyses were done on all the wines five months after production. A ten centimetre unstructured line scale was used and the judges were asked to rate 'fruity' and 'guava' aroma intensity (undetectable to prominent) and general quality (unacceptable to excellent). In addition, wines from the first two vintages were evaluated at five and 18 months, while wines from the third vintage were evaluated at five months only according to the multi-wine preferences method (McCloskey *et al.*, 1995). Further wine evaluation protocols were according to Jolly *et al.* (2003b).

6.2.5 Chemical and statistical analyses

The wines (laboratory and small-scale) were analysed for alcohol (infralyser technique – Cape Wine Laboratory, Stellenbosch), glycerol (enzymatic test kits, Boehringer Mannheim, Roche, Germany; and Winescan, Institute for Wine Biotechnology, Stellenbosch University), and for residual sugar (Rebelein), volatile acidity and SO_2 as described by Iland *et al.* (2000). Analyses for esters (volatile component analyses – Research Chemistry, Distell, Stellenbosch) were carried out at the time of the five-month sensory evaluations on the small-scale wines only. Analysis of variance was performed on the ester values and the descriptive sensory analyses values using SAS version 8.2 (SAS, 1999). Shapiro-Wilk test was done to test for non-normality (Shapiro & Wilk, 1965) and Student's t-Least Significant Difference was calculated at the 5% confidence level to compare treatment means (Ott, 1998).

6.3 RESULTS AND DISCUSSION

As our *C. pulcherrima* isolates could not ferment a grape must to dryness on their own (Fig. 6.1), they needed to be co-inoculated with a *S. cerevisiae* wine yeast. The *S. cerevisiae* strain chosen for this, viz. VIN 13, is recommended by the

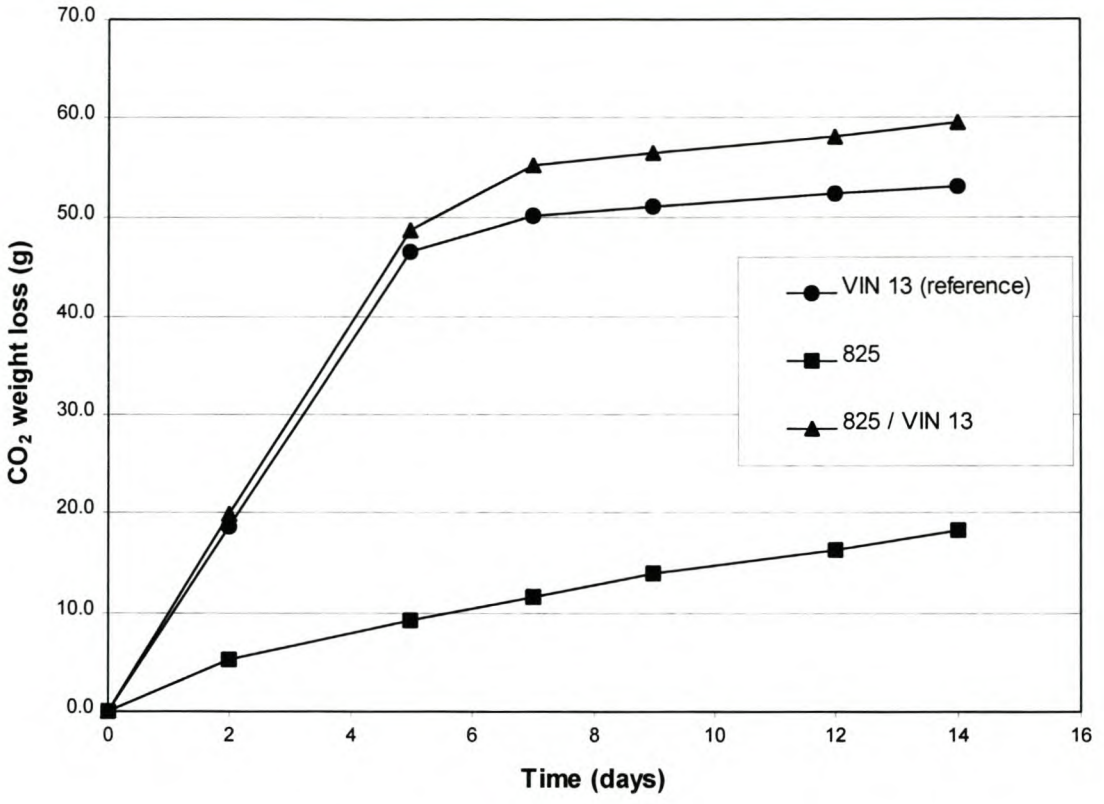

FIGURE 6.1
Laboratory-scale fermentation curves of *C. pulcherrima* (strain 825) and *S. cerevisiae* (strain VIN 13) compared to a combined *S. cerevisiae/C. pulcherrima* fermentation.

manufacturers for the production of aromatic white wines at low temperatures. This made it an ideal choice for Chenin blanc production. It is also a strong fermentor and is not generally implicated in stuck fermentations. Although it has previously been reported that some strains of *C. pulcherrima* have an inhibitory effect against *S. cerevisiae* (Nguyen & Panon, 1998), the two strains used in this investigation did not show this effect against *S. cerevisiae* strain VIN 13 (data not shown). It was, however, expected that the *S. cerevisiae* would be competing for the same nutrients as the *C. pulcherrima,* but there was no reduction in fermentation rate in comparison to the VIN 13 reference fermentation (Fig. 6.1). Typically, *C. pulcherrima* could be detected until the ninth day of a co-inoculated 14 day fermentation by streaking 200µL aliquots onto lysine medium and checking for colonies producing the red-brown pigment pulcherrimin (Heard & Fleet, 1986;

Miller & Phaff, 1998; Jolly *et al.*, 2003b). The one-hour time lapse between inoculating the *C. pulcherrima* and the *S. cerevisiae* yeast was chosen to allow the *C. pulcherrima* to adapt to the must and start its growth, before being dominated by the *S. cerevisiae*. In addition, the short time lapse before the start of fermentation will minimise any detrimental oxidation of the must. Placement of the fermentation vessels on the orbital shaker copied the natural turbulence found in large fermentations due to the generation of CO_2 (Henschke, 1990). The tightly sealed fermentation caps ensured that no oxygen entered the fermentation vessel.

TABLE 6.1
The effect of four different winemaking practices on the slope of the logarithmic-phase of the fermentation curve of laboratory-scale fermentations with *Candida pulcherrima* (strain 825) and *S. cerevisiae* (strain VIN13).

Grape must parameter and winemaking practice		**Slope**[1]	
		***C. pulcherrima* (strain 825)**	***S. cerevisiae* (strain VIN 13) (reference)**
Individual effects:			
pH 3.2 (low)		1.08 ± 0.14	9.37 ± 0.57
pH 3.5 (high)		1.29 ± 0.19	9.04 ± 0.45
DAP (none)		1.04 ± 0.09	9.86 ± 0.23
DAP (0.5g/L added)		1.35 ± 0.07	10.31 ± 0.59
Low temperature	15°C	1.06 ± 0.66	5.17 ± 0.06
Intermediate temperature	20°C	1.64 ± 0.21	14.92 ± 0.23
High temperature	28°C	1.82 ± 0.21	23.88 ± 0.56
SO_2 (0 mg/L)		0.78 ± 0.08	8.38 ± 0.35
SO_2 (30 mg/L)		0.80 ± 0.10	8.53 ± 0.18
SO_2 (60 mg/L)		0.63 ± 0.09	8.40 ± 0.12
Combined effects:			
28°C; pH 3.5; 0.5g/L DAP; No SO_2		2.62 ± 0.16	22.62 ± 0.55
15°C; pH 3.2; no DAP; 60mg/L SO_2		0.54 ± 0.13	5.56 ± 0.99

[1] Slope of the logarithmic phase of the fermentation curve (m = y/x-c/x where m is the slope of the logarithmic growth phase; x and y are the standard co-ordinates; and c is the x-axis intercept). Average value of fermentations in triplicate ± standard deviation.

TABLE 6.2
The effect of four different winemaking practices on chemical analyses of wines produced by laboratory-scale fermentations with *Candida pulcherrima* (strain 825).

Grape must parameter and winemaking practice	Chemical analyses[1]							
	Alcohol (% v/v)		Volatile acidity (g/L)		Total SO_2 (mg/L)		Glycerol (g/L)	
	C. pulcherrima (strain 825)	*S. cerevisiae* (strain VIN 13) (reference)	*C. pulcherrima* (strain 825)	*S. cerevisiae* (strain VIN 13) (reference)	*C. pulcherrima* (strain 825)	*S. cerevisiae* (strain VIN 13) (reference)	*C. pulcherrima* (strain 825)	*S. cerevisiae* (strain VIN 13) (reference)
Individual effects:								
pH 3.2 (low)	3.2 ± 0.1	12.8 ± 0.1	0.22 ± 0.02	0.20 ± 0.01	14 ± 1	21 ± 2	5.17 ± 0.96	6.15 ± 0.16
pH 3.5 (high)	3.6 ± 0.1	12.9 ± 0.1	0.24 ± 0.01	0.18 ± 0.02	14 ± 1	20 ± 1	5.51 ± 0.67	6.60 ± 0.10
DAP (none)	3.2 ± 0.2	12.8 ± 0.1	0.23 ± 0.01	0.23 ± 0.03	18 ± 2	27 ± 1	6.14 ± 0.52	6.48 ± 0.48
DAP (0.5g/L added)	3.5 ± 0.2	12.8 ± 0.1	0.25 ± 0.02	0.19 ± 0.04	18 ± 1	30 ± 1	6.38 ± 0.14	6.16 ± 0.43
Low temperature: 15°C	3.9 ± 0.5	12.5 ± 0.1	0.14 ± 0.04	0.18 ± 0.04	12 ± 1	24 ± 1	6.88 ± 0.96	5.76 ± 0.35
Intermediate temperature: 20°C	4.4 ± 0.2	12.5 ± 0.1	0.14 ± 0.02	0.14 ± 0.02	12 ± 2	17 ± 1	6.75 ± 1.22	5.92 ± 0.11
High temperature: 28°C	3.7 ± 0.2	12.5 ± 0.1	0.14 ± 0.01	0.32 ± 0.01	15 ± 1	17 ± 1	5.36 ± 1.51	6.21 ± 0.10
SO_2 (0 mg/L)	2.7 ± 0.2	12.8 ± 0	0.25 ± 0.01	0.35 ± 0.01	17 ± 2	22 ± 1	7.14 ± 0.07	5.05 ± 0.37
SO_2 (30 mg/L)	2.5 ± 0.1	12.8 ± 0	0.23 ± 0.01	0.33 ± 0.02	25± 2	37± 3	6.06 ± 0.38	5.78 ± 0.57
SO_2 (60 mg/L)	2.2 ± 0.1	12.8 ± 0.1	0.19 ± 0.03	0.35 ± 0.02	61± 1	59 ± 4	4.81± 0.63	5.40 ± 0.12
Combined effects:								
28°C; pH 3.83; 0.5g/L DAP; No SO_2	1.2 ± 0.1	13.5 ± 0.1	0.11 ± 0.02	0.27 ± 0	7 ± 2	11 ± 1	12.17 ± 0.57	7.20 ± 0.10
15°C; pH 3.2; no DAP; 60mg/L SO_2	0.7 ± 0.5	13.7 ± 0.4	0.15 ± 0	0.21 ± 0.01	38 ± 2	46 ± 1	6.87 ± 1.31	7.43 ± 0.06

[1] Average of triplicate fermentations ± standard deviation.

6.3.1 Laboratory-scale fermentations

The manipulation of winemaking practices (use of DAP and SO_2), fermentation temperature and must pH generally had similar effects on the fermentation ability of *C. pulcherrima* and the *S. cerevisiae* reference strain (Table 6.1). Di-ammonium phosphate addition, higher pH values and increased temperatures all resulted in a slight increase in the fermentation ability of strain 825 as is shown by the increased slope of the logarithmic phase of the fermentation curve. However, the increase in fermentation ability due to increased fermentation temperature was not as dramatic as for the *S. cerevisiae* reference. Higher levels of ethanol were also formed (Table 6.2), reflecting a more efficient fermentation by strain 825.

Sulphur dioxide in the concentration range normally used in wine fermentation, i.e. 0-30 mg/L, did not affect the fermentation ability of 825. This contrasts with the accepted opinion that all non-*Saccharomyces* yeasts are sensitive to wine-related SO_2 levels. Under normal winemaking conditions, the growth of *C. pulcherrima* strain 825 therefore will not be hampered by SO_2. This insensitivity to SO_2 was also noted by Granchi et al. (1998) who found that SO_2 in the range of 50-100 mg/L did not succeed in preventing the growth of non-*Saccharomyces* yeasts in Sangiovese wine fermentations. However, at higher SO_2 concentrations, i.e. 60 mg/L, the growth of 825 was slightly retarded, as also reflected in the lower alcohol, volatile acidity and glycerol levels. In comparison, the VIN 13 fermentation ability remained unaffected. Excessive volatile acidity, which is often formed when yeasts grow under stress conditions (lower temperature, pH and nitrogen limitation), was not formed by strain 825 during the laboratory-scale fermentations.

The combined effects of lower temperature, low pH, no DAP and high SO_2 had a limiting effect while, as expected, the combined conditions of higher temperature, higher pH with the addition of DAP and no SO_2 enhanced *C. pulcherrima* growth (Table 6.1). Furthermore, the chemical analyses (Table 6.2) show a marked increase in the glycerol concentration. This is supported by a reduction in alcohol of 2% when compared to the previous fermentations (individual effects). This phenomenon was not observed in the previous investigations (Jolly *et al.*, 2003b) and may be due to the high pH and fermentation temperature.

In a warm climate, like that of South Africa, where must pH tends to be high and red wine fermentation temperatures can exceed 25°C, these yeasts, which are present naturally (Jolly *et al.*, 2003a), may already be playing a role in wine quality. Their role would be smaller at lower fermentation temperatures in low pH musts.

6.3.2 Screening of *C. pulcherrima* isolates for wine production

As *C. pulcherrima* strain 825 has a low fermentation ability, a stronger fermentor was desired so that the yeast could make a greater impact on the fermentation. Therefore, fermentation ability and alcohol production were chosen as selection criteria. Production of volatile acidity, as potentially detrimental to wine quality, was also checked. A fermentation temperature of 15°C was used, as it is most representative of the production of South African Chenin blanc in a fruity, non-wooded style.

The screening results (data not shown) did not show much variation from strain 825, although strain C1-15 showed an improvement in fermentation ability, which was also reflected in a marginal improvement in alcohol production (Table 6.3). The volatile acidity was lower than that of strain 825 and comparable to that of the *S. cerevisiae* reference.

TABLE 6.3
Comparison of two *C. pulcherrima* strains and one *S. cerevisiae* strain for three selection criteria during fermentations at 15°C.

Yeast strain	Selection criteria[1]		
	Slope[2]	Alcohol (% v/v)	Volatile acidity (g/L)
S. cerevisiae (reference 1)	5.26 (5.13-5.39)	12.6 (12.5–12.6)	0.33 (0.33-0.33)
C. pulcherrima (strain 825) (reference 2)	0.67 (0.65-0.68)	3.3 (3.1-3.4)	0.39 (0.37-0.40)
C. pulcherrima (strain C1-15)	1.16 (0.77-1.55)	4.6 (3.4-5.7)	0.35 (0.30-0.40)

[1] Average of duplicate fermentation at 15°C. Range indicated in brackets.
[2] Slope of the logarithmic growth phase of the fermentation curve (m= y/x-c/x where m is the slope of the logarithmic growth phase; y is the y co-ordinate; x is the x co-ordinate; and c is the x-axis intercept).

6.3.3 Small-scale wine production

6.3.3.1 Chemical analyses: The fermentations for all three vintages were completed and there were no marked differences between the standard wine chemical analyses of the *S. cerevisiae* reference wine and the wines produced by the *C. pulcherrima*/*S. cerevisiae* combinations (Table 6.4). No yeast counts were

TABLE 6.4
Chemical analyses of Chenin blanc wines fermented by *C. pulcherrima / S. cerevisiae* combinations during small-scale wine production at 15°C.

Yeast strain combination	Vintage	Chemical analyses[1]				
		Residual sugar (g/l)	Ethanol (% v/v)	Volatile acidity (g/l)	Total SO_2 (mg/l)	Glycerol (g/l)
S. cerevisiae (reference)	2000[2]	1.2 (1.0-1.4)	13.2 (13.1-13.2)	0.19 (0.18-0.19)	104 (104-104)	5.6 (5.5-5.6)
C. pulcherrima (strain 825) / *S. cerevisiae*	2000[2]	0.9 (0.2-1.6)	13.0 (12.9-13.0)	0.19 (0.18-0.19)	98 (94-101)	5.6 (5.5-5.6)
S. cerevisiae (reference)	2001	1.8 (1.6-2.0)	14.6 (14.6-14.6)	0.29 (0.28-0.29)	95 (90-100)	7.7 (7.5-7.8)
C. pulcherrima (strain 825) / *S. cerevisiae*	2001	1.4 (1.2-1.5)	14.6 (14.5-14.6)	0.28 (0.27-0.28)	82 (81-82)	7.9 (7.8-7.9)
C. pulcherrima (strain C1-15) / *S. cerevisiae*	2001	1.2 (1.2-1.2)	14.6 (14.6-14.6)	0.29 (0.28-0.30)	88 (82-93)	7.8 (7.8-7.8)
S. cerevisiae (reference)	2002	1.95 (1.90-2.00)	14.9 (14.7-15.1)	0.25 (0.24-0.26)	115 (115-115)	7.9 (7.7-8.0)
C. pulcherrima (strain 825) / *S. cerevisiae*	2002	1.90 (1.90-1.90)	15.0 (14.9-15.0)	0.26 (0.25-0.27)	113 (111-115)	7.7 (7.7-7.7)
C. pulcherrima (strain C1-15) / *S. cerevisiae*	2002	1.90 (1.90-1.90)	15.0 (15.0-15.0)	0.26 (0.25-0.27)	117 (115-118)	8.0 (8.0-8.0)

[1] Average values of duplicate fermentations. Range indicated in brackets.
[2] Data for the 2000 vintage obtained from Jolly *et al.* (2003b).

done during the fermentations, however, it was previously shown that *C. pulcherrima* could be detected for approximately two thirds of a combined fermentation (Jolly *et al.*, 2003b) and the same scenario was expected to have occurred here. The combined fermentations did show a tendency towards lower residual sugars than the *S. cerevisiae* monoculture. This shows a more efficient must sugar utilisation by the dual culture and was also noted by Ciani & Ferraro (1998) in their combined fermentations with *C. stellata* and *S. cerevisiae.* No increased glycerol concentration, as was the case in the laboratory-scale fermentation, was noted. However, this may have been due to the fermentation taking place at 15°C and not at 28°C.

It has been reported previously that *C. pulcherrima* yeasts are high ester producers (Bisson & Kunkee, 1991). As mentioned in the introduction, esters can make a positive contribution to a Chenin blanc wine with neutral cultivar characteristics, even though this may be of short duration. Gas chromatographic analyses of total esters, total higher alcohols and total acids showed no significant differences between the *S. cerevisiae* reference wines and the *C. pulcherrima/S. cerevisiae* combinations (Table 6.5). This may be due to there not being enough *C. pulcherrima* yeast or that a longer period is needed for ester formation to become apparent. The results of the 2001 and 2002 vintages therefore confirmed the findings of Jolly *et al.* (2003b) that *C. pulcherrima* strains 825 and C1-5 did not make a contribution to total esters in the wine under the conditions used.

6.3.3.2 Sensory evaluation: Sensory evaluation can be subjective. However, the human nose is capable of detecting aroma, flavour and other sensory nuances that are not measurable by current instrumentation. Furthermore, in the wine industry most decisions regarding wine quality with the subsequent economic implications often rely more on sensory evaluation and less on chemical analyses. Sensory evaluation is also the final criteria for judging any winemaking manipulation.

In this investigation the sensory evaluation of the two wine types by descriptive analyses (Table 6.6) showed that, for the 2000 vintage wines, there was no significant difference in aroma profile and quality of the five-month old wines.

TABLE 6.5
Gas chromatographic analysis of Chenin blanc wines of the 2000, 2001 and 2002 vintages fermented by *C. pulcherrima / S. cerevisiae* combinations during small-scale wine production.

Yeast Combination	Vintage[1]	Total esters[2] (mg/L)	Total higher alcohols[2] (mg/L)	Total acids[2] (mg/L)	Ethyl-acetate[2] (mg/L)	Total esters -ethyl acetate[2] (mg/L)	Ethyl-lactate[2] (mg/L)	Total esters -ethyl acetate -ethyl lactate[2] (mg/L)
S. cerevisiae (reference)	2000	216.06a	321.45a	233.64a	176.35a	39.71a	11.41a	28.30a
C. pulcherrima (strain 825) / *S. cerevisiae*	2000	231.62a	311.33a	195.60a	188.32a	43.31a	13.97a	29.34a
S. cerevisiae (reference)	2001	109.04a	194.40a	21.54a	57.13a	51.91a	15.96a	35.95a
C. pulcherrima (strain 825) / *S. cerevisiae*	2001	109.19a	198.84a	20.90b	56.25a	52.94a	16.78a	36.17a
C. pulcherrima (strain C1-15) / *S. cerevisiae*	2001	110.50a	195.84a	21.65a	56.76a	53.74a	15.21a	38.53a
S. cerevisiae (reference)	2002	127.18a	208.52a	32.63a	98.68a	28.51a	5.21a	23.3a
C. pulcherrima (strain 825) / *S. cerevisiae*	2002	122.40a	202.90a	30.75a	94.92a	27.48a	4.87a	22.62a
C. pulcherrima (strain C1-15) / *S. cerevisiae*	2002	117.30a	203.70a	29.95a	90.97a	26.34a	4.71a	21.62a

[1] Data for the 2000 vintage obtained from Jolly *et al.* (2003b).
[2] Average value of two wines. Values within columns, for the same vintage, followed by the same letter do not differ significantly ($p<0.05$).

TABLE 6.6
Descriptive sensory analyses of five-month old Chenin blanc wines produced by combinations of *C. pulcherrima* and *S. cerevisiae* yeasts for the 2000, 2001 and 2002 vintages.

Yeast strain	Wine characteristic (%)[1]								
	2000 vintage			2001 vintage			2002 vintage		
	Fruity aroma intensity	Guava aroma intensity	General quality	Fruity aroma intensity	Guava aroma intensity	General quality	Fruity aroma intensity	Guava aroma intensity	General quality
S. cerevisiae (reference)	44.79a	16.57a	48.86a	52.92a	25.17b	52.50a	64.00ab	25.14a	49.43b
C. pulcherrima (strain 825) / *S. cerevisiae*	43.57a	21a	52.36a	59.50a	28.83ab	50.33a	57.86b	23.57a	53.43ab
C. pulcherrima (strain C1-15) / *S. cerevisiae*	-[2]	-[2]	-[2]	49.25a	42.83a	48.00a	67.86a	30.00a	60.57a

[1]Average value of duplicate wines judged by panels of seven judges. Values within columns for the same vintage, followed by the same letter do not differ significantly ($p<0.05$).
[2]Not produced during the 2000 vintage.

TABLE 6.7
Relative score of Chenin blanc wines fermented by *C. pulcherrima* / *S. cerevisiae* combinations during small-scale wine production.

Yeast combination	Vintage / Time of evaluation / Relative score[1]				
	2000 vintage[2]		2001 vintage		2002 vintage
	5 months	18 months	5 months	18 months	5 months
S. cerevisiae (reference)	-2 (-5, 1)	-1 (-5, 3)	-2 (-3, -1)	-2 (-3, -1)	-1 (-3, 1)
C. pulcherrima (strain 825) / *S. cerevisiae*	**2** (1, 3)	**5** (5, 5)	1 (-1, 3)	-1 (-5, 3)	0 (-1, 1)
C. pulcherrima (strain C1-15) / *S. cerevisiae*	-[3]	-[3]	**4** (1, 7)	**3** (1, 5)	**1** (1, 1)

[1] Average score of two wines evaluated by the multi-wine preference method (McCloskey *et al.*, 1995). Only values within a column are related to each other. Highest score in bold type. Range indicated in brackets.
[2] Data for the 2000 vintage obtained from Jolly *et al.*, 2003b.
[3] Not produced during the 2000 vintage.

However, the *C. pulcherrima* (strain 825) wine did have a higher aroma note of 'guava'. Wines from the second vintage (2001) were judged to differ more from each other, with the *C. pulcherrima* wines having the highest scores for 'guava'. For the wines produced with strain C1-15, this was significantly higher than the reference wine (*S. cerevisiae* only). The 'fruity' aroma note and 'general quality' were judged similar (no significant difference). Wines from the third vintage (2002) were again judged similar for 'fruity', but the *C. pulcherrima* strain C1-15 wine again scored the highest for the 'guava' aroma note (although not statistically significant). The quality of the C1-15 wine was also significantly better than the reference wine.

The wines were also tasted according to the multi-wine preference method (McCloskey *et al.*, 1995) to obtain a relative score. During this evaluation, the judges were asked to judge overall wine quality (inclusive of wine colour, aroma, flavour and body/mouthfeel). From Table 6.7 can be seen that the combined *C. pulcherrima*/*S. cerevisiae* wines always scored higher than the reference wine produced by the *S. cerevisiae* only over the three vintages investigated. This, despite the possible subjectivity of the measuring instrument, should be considered significant. Furthermore, in the two vintages for which the selected *C. pulcherrima* strain C1-15 was used, that wine received the highest rating. This supports the choice of selection criteria used for selecting C1-15. It was further noted in the 2002 vintage that the wines judged better, had lower total ester values than the reference wine (Tables 5 & 7), indicating that other metabolites were playing a role in wine quality.

From the data presented it appears that the effect of the *C. pulcherrima* strains in wine fermentation was more complex than could be measured by the chemical techniques used. At this stage of the investigation it is also not yet clear by how much wine quality can be improved. Strain selection criteria could also be sharpened, while combinations with other strains of *S. cerevisiae* can also be investigated, bearing in mind the possible inhibitory effect that *C. pulcherrima* may have on some *S. cerevisiae* strains. Scaling up to pilot and commercial scale should also be carried out, while the extent to which the *C. pulcherrima* yeasts survive during the fermentation can also be ascertained, enabling wine making conditions to be adjusted so that the survival and growth of the *C. pulcherrima* yeast is optimised.

6.4 CONCLUSIONS

The effects of pH, SO_2, DAP addition and temperature on *C. pulcherrima* follows the same pattern as that on *S. cerevisiae*. However, elevated levels of SO_2 can suppress the growth of *C. pulcherrima,* but these levels are much higher than normally found in practice. The use of a selected strain of *C. pulcherrima* had a positive influence on wine quality not linked to ester levels, which also did not detrimentally affect standard wine chemical analyses. Further isolation from grape musts and strain selection for more vigorous strains will make it possible to optimise the improved quality contribution. Further chemical analyses and methodology development could identify the metabolites responsible for this quality improvement. In the interim, the selected strains could be employed for the improvement of South African Chenin blanc wines.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7. GENERAL DISCUSSION AND CONCLUSIONS

7.1 CONCLUDING REMARKS

Wine is the product of a complex biological and biochemical interaction between grapes and different microorganisms, amongst which yeasts play the most important role during the alcoholic (primary) fermentation. For the purpose of this investigation the yeasts were arbitrarily divided into two groups i.e. *Saccharomyces* and non-*Saccharomyces*. This division was convenient for this study, however, a much larger number of non-*Saccharomyces* species are found in grape juice and wine than the *Saccharomyces* species. The non-*Saccharomyces* group could therefore, for future studies, be further subdivided into smaller groups containing fewer representative species. This further grouping does not necessarily have to be based on taxonomic norms, but rather on the basis of potential oenological usefulness. Predominance versus presence in lower numbers could also be used as a criterium for grouping. Smaller groupings would ensure a more focussed approach in research projects.

The role of the "wine yeast" i.e. *Saccharomyces* yeasts, in wine fermentation has long been recognised. They are primarily responsible for the conversion of grape sugar to ethanol and CO_2 (Fleet, 1993; Pretorius, 2003). Their secondary role is that of contributing to wine aroma and flavour (Ribéreau-Gayon *et al.*, 2000). The non-*Saccharomyces* yeasts, on the other hand, were traditionally viewed as spoilage organisms (Loureiro & Malfeito-Ferreira, 2003 and references therein). Their occurrence and subsequent isolation from lagging and stuck fermentations and spoiled bottles of wine contributed to this viewpoint. It was also believed that the non-*Saccharomyces* yeasts were sensitive to the SO_2 used, and disappeared from the fermentation due to the toxicity of the ethanol (Amerine & Cruess, 1960; Van Zyl & Du Plessis, 1961). The belief of, especially the Old World winemakers (Amerine *et al.*, 1972), concerning the importance of indigenous yeasts (which includes the non-

Saccharomyces yeasts) to wine quality was largely discounted by several wine microbiologists and oenologists.

It was not until the watershed publication of Fleet *et al.* (1984) that the interest in the non-*Saccharomyces* yeasts in wine was revived. Since then, the role of the non-*Saccharomyces* yeasts in wine fermentation has received increasing attention (Heard & Fleet, 1985; Fleet, 1990; Gafner *et al.*, 1996; Longo *et al.*, 1991; Todd, 1995; Granchi *et al.*, 1998; Fleet, 2003). The work of these more recent authors showed that some non-*Saccharomyces* yeasts are present throughout fermentation and that some them have the potential of making a positive contribution to wine quality.

The reason for the sustained presence of non-*Saccharomyces* yeasts in modern wine fermentations has never been clearly explained. Although there have been references to the "art of winemaking" being older than history (Pretorius *et al.*, 1999; Pretorius, 2000) and it is true that the basic process has not changed over the years, enormous improvements in technology have occurred. Winemaking styles are also constantly changing to accommodate this technology and to satisfy market trends. For example, improved technology and cellar hygiene, together with consumer resistance to the use of SO_2, has resulted in lower levels of SO_2 being used in many modern wineries. Automatically, non-*Saccharomyces* yeasts previously suppressed can grow. It has further been shown that some non-*Saccharomyces* yeasts are not as sensitive to SO_2 as previously surmised (Fleet, 1990; Constanti *et al.*, 1998; Egli *et al.*, 1998; Granchi *et al.*, 1998; Henick-Kling, 1998; Rementeria *et al.*, 2003). Changes in fermentation temperatures will also have a direct effect on what yeasts can grow during fermentation. Non-*Saccharomyces* yeasts are more tolerant to ethanol below 25°C (Gao & Fleet, 1998), so cold fermentations will also have a greater presence of these yeasts. The advancement in microbiological methods has also contributed to easier detection of yeasts. These factors may have all contributed to the new view of the role of non-*Saccharomyces* yeasts in wine fermentation.

As previously mentioned, non-*Saccharomyces* yeasts can make a positive contribution to wine quality, substantiating winemaker's belief in the role of indigenous

yeasts in wine quality. The use of specific non-*Saccharomyces* yeasts can be especially beneficial to the production of wines from grape varieties showing little varietal characteristics due to non-optimal climatic conditions, soils and/or clonal selection e.g. some Chenin blanc wines in South Africa. These wines often have a neutral flavour profile despite the application of appropriate viticultural and oenological practices. However, prior to this investigation an indigenous non-*Saccharomyces* yeast collection did not exist. The comprehensive research programme described by Pretorius *et al.* (1999) highlighted this void. The specific objectives of this study, as part of the above-mentioned programme, were therefore the isolation of indigenous non-*Saccharomyces* yeasts from vineyards and musts; the identification of these isolates; the characterisation and evaluation of predominant species under winemaking conditions; and the development of a protocol for their use in enhancing wine quality.

These tasks were successfully completed and an oenologically biased range of non-*Saccharomyces* yeasts was isolated from selected South African vineyards and grape must. This collection, containing 720 isolates, representing eight genera and 24 different species, was isolated over three vintages from four distinctly different wine producing regions i.e. Constantia, Stellenbosch, Slanghoek and Robertson (Jolly *et al.*, 2003a). The oenological bias was achieved by crushing grape samples prior to yeast isolation as well as isolations from sedimented must. Although some species normally present on grapes and in must would certainly not have been recovered, this was justifiable for this investigation. Only yeasts robust enough to handle the harsh conditions of grape must were sought, as they not only had to grow, but also compete with *S. cerevisiae* during wine fermentation.

The four regions chosen for the study represented the major climatic condition of the Western Cape and were geographically far enough apart to ensure that a broad diversity of yeasts could be isolated. The three vintages, spread over four years, further ensured that differing weather patterns with subsequent impact on yeast diversity could also be accommodated. Although the objective of this study was the isolation of non-*Saccharomyces* yeasts, it was never intended to be a comparative

regional yeast biodiversity study. For that, many more vineyards in differing locations would have had to be sampled. Nonetheless, a broad overview on yeast diversity and seasonal dynamics was obtained (Jolly *et al.*, 2003a).

The isolates were all successfully characterised by the ID 32 C system and fingerprinted by DNA karyotyping (Jolly *et al.*, 2003a). Although many other techniques for yeast characterisation are available (De Louvois, 1979, Deák, 1993; 1995, Pretorius, 2000), these two served their purpose well. The ID 32 C system is relatively fast (two days) and although too slow for routine diagnostic use, was suitable for this type of ecological study where sufficient time was available. As this technique was only used primarily for determining biochemical profiles, the limited number of yeast species in the database did not matter. However, identifications obtained were useful, even if only used as preliminary identifications.

Characterisation by DNA karyotyping, although more time consuming (approximately six days), gave accurate results and made grouping of similar yeasts possibly. This confirms the conclusions of Van der Westhuizen *et al.* (1999) regarding the use of this technique as a primary means of differentiating between yeasts.

Linking the karyotype groups to the biochemical profiles of the ID 32 C made it possible to send only single representative yeasts of each group to CBS (The Netherlands) for final identification. This contributed to large financial savings. However, final identities of the yeasts conferred by CBS resulted in grouping of yeasts by species and not strains within species. A further study could concentrate on distinguishing the individual strains within the species. The database that now exists containing the biochemical profiles and DNA karyotypes makes it easier for future unknown isolates to be identified. This is a valuable resource in the ongoing research programme.

The species that had the highest incidence of predominance in the vineyard samples was *K. apiculata*. This is in accordance with the generally accepted norm and was also noted by Van Zyl & Du Plessis (1961). Notable exceptions were the Robertson samples that were characterised by low numbers or absence of this yeast (Jolly *et al.*,

2003a; Chapter 4). An absence of *Kloeckera* spp. is not unique and was also noted by Yanagida *et al.* (1992). Although it has been reported that *Kloeckera* spp. can make a positive contribution to the quality of wine due to increased production of volatile compounds (Gil *et al.*, 1996), they are generally seen as detrimental to wine quality when producing too much volatile acidity. The absence of *Kloeckera* spp. or low levels can therefore contribute to wine quality as already discussed in Chapter 4. Other species predominance in the vineyard samples was in decreasing order *C. pulcherrima*, *K. thermotolerans*, *Rhodotorula* sp. and *Z. bailii* (Jolly *et al.*, 2003a). These yeasts can potentially be carried over to the must and can play a role in wine fermentation. *Z. bailii* and *C. pulcherrima* have already been investigated for their contribution to wine quality by Romano & Suzzi (1993) and Jolly *et al.* (2003c), respectively, but further research is still needed. The role of *K. thermotolerans* and *Rhodotorula* sp. should also receive attention in future investigations. The oxidative nature of yeasts should not be used as a reason to discount a potential contribution in wine fermentation.

Other species which predominated in clarified grape must were *C. colliculosa, C. pulcherrima, C. stellata* and *K. apiculata* (Jolly *et al.*, 2003a). Predominant species in must would be expected to have the biggest impact on subsequent wine quality. It has already been shown that *K. apiculata*, *C. pulcherrima* and *C. colliculosa* can be used to improve Sauvignon blanc wine quality (Jolly *et al.*, 2003b), while *C. pulcherrima* could improve Chenin blanc wine quality (Jolly *et al.*, 2003b; 2003c). Future research into other non-*Saccharomyces* yeast / grape variety combinations for wine quality improvement should also be continued. These combinations may result in their own specific optimum inoculation protocol and more of the objectives of the research programme of Pretorius *et al.* (1999) can consequently be reached.

Species present in lower numbers should, however, not be entirely discounted. It is possible that some minor species produce aroma and/or flavour compounds with low threshold concentrations. These minor non-*Saccharomyces* yeasts can also be the topic for future investigations.

The initial work done by Jolly *et al.* (2003b; 2003c) was done on randomly selected isolates. A prior screening of yeasts for desired oenological properties may have resulted in the selection of a more suitable strain. This was evident in the successful selection of the isolate C1-15 from the 127 available *C. pulcherrima* isolates in the collection (Jolly *et al.*, 2003a). Considering the limited sampling and the potential number of different strains that can be isolated, the scope for finding a strain with better characteristics in future ecological studies is large.

The ability of *C. pulcherrima* to improve Chenin blanc wine quality was shown over three vintages. This took place despite the expected variability of must composition over vintages and subjectivity of sensory panels. The similarity in standard chemical analyses between the control wines and the co-inoculated fermentations was, however, not expected, especially for the ester concentrations as *C. pulcherrima* is known as a producer of high levels of esters. Another compound or group of compounds is therefore responsible for the increased quality of the wines. Considering that more than 680 aroma compounds have been identified in wine (as quoted by Guth, 1997), many potential compounds could be involved. The "guava" aroma note, detected as being stronger in the *C. pulcherrima* wines, indicate that a sulphur containing compound may be involved in the perceived aroma (Ribéreau-Gayon *et al.*, 2000). Analyses of the wines for this group of compounds should therefore receive further attention in future studies. Unfortunately, present limitation in availability of expertise and instrumentation to carry out these specialised analyses could hamper further progress.

Subsequent to this study, the best *C. pulcherrima* strain was used during another vintage for slightly larger-scale (180 L fermentations). This work is ongoing and pilot-scale fermentations of 500 L are envisioned for the next vintage. The following hurdle will be the cultivation of the yeasts in sufficient quantities coupled to the drying of the yeasts in an active dried state for semi-commercial trials. However, drying of the yeast may prove to be difficult. Drying non-*Saccharomyces* yeast is not the norm and new drying technology may have to be developed with the accompanying potential financial risks. The interest and application of these yeasts to industry will have to be

proven to commercial yeast manufacturers before they embark on such a venture. The concerns of Erten & Campbell (2001) regarding the possible reluctance of wine producers to cultivate and use on a large-scale microorganisms generally considered as spoilage organisms will also have to be addressed. This can be achieved by setting an example and producing superior quality wines in a small-scale production cellar e.g. that attached to a research facility such as ARC Infruitec-Nietvoorbij. As already mentioned, further isolations of *C. pulcherrima* strains that may be superior to the ones investigated here should also be carried out. The selection criteria used by Jolly *et al.* (2003c) could be used and linked to analyses of aroma impact compounds once identified.

A more in depth investigation into the time that *C. pulcherrima* yeasts are able to survive during fermentation should also be carried out. This latter information will make it possible to adjust the inoculation protocol to address the optimal contribution by *C. pulcherrima*. In the interim, the developed protocol can be used where the improvement of Chenin blanc wine aroma and quality is desired.

The non-*Saccharomyces* isolates, held in the culture collection at the microbiological laboratory of the ARC Infruitec-Nietvoorbij research institute, represent a valuable source of yeast bio-diversity for screening for specific traits in future research projects. A selection has already been screened for the production of extracellular enzymes of importance to wine production (Strauss *et al.*, 2001). The monitoring of non-*Saccharomyces* yeast population during ripening of grapes is also receiving attention and certain populations may still be linked to optimal ripeness of grapes. The search for alternative alcoholic beverages utilising non-*Saccharomyces* yeasts (Obisanya *et al.*, 1987; Ciani & Maccarelli, 1998) will become more important as the market for new and improved beverages expands. These studies as well as other investigations into the use of non-*Saccharomyces* yeasts outside the constrains of wine production (Kurtzman & Droby, 2001; De Silóniz *et al.*, 2002; Weiler & Schmitt, 2003) makes the field of non-*Saccharomyces* research exciting and profitable.

7.2 LITERATURE CITED

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