Characterization and evaluation of indigenous *Saccharomyces cerevisiae* strains isolated from South African vineyards

by

T.J. van der Westhuizen

Dissertation presented for the Degree of Doctor of Philosophy at the University of Stellenbosch

December 1999

Promoter:
Prof. I.S. Pretorius

Co-promoter:
Dr. O.P.H. Augustyn

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.

TJ van der Westhuizen

22.11.1999

Date

SUMMARY

The bioconversion of grape juice into wine by simply allowing the yeasts, associated with grape berries and winery equipment, to ferment the sugars to ethanol, carbon dioxide and other minor, but important metabolites, is an ancient process. The art and science of winemaking has been extensively studied since the time when Louis Pasteur demonstrated, for the first time, the relationship between yeast and alcoholic fermentation. It is now recognized that the fermentation of grape must and production of premium quality wines is a complex ecological and biochemical process involving the sequential development of microbial species, as affected by a particular environment. This complex heterogeneous microbiological process includes the interaction of many microbial species, represented by fungi, yeasts, lactic acid bacteria and acetic acid bacteria, as well as the mycoviruses and bacteriophages affecting these grape-associated microorganisms. However, of all these different microbes and viruses, yeast represent the heart of the harmonious biochemical interaction with the musts derived from the various varieties of *Vitis* species which, in turn, are largely products of their respective genetic make-ups and the *terroir*. These yeasts are significant in winemaking because they not only conduct the alcoholic fermentation, but can also spoil wine during conservation in the cellar and after packaging, and they affect wine quality through the production of fermentation metabolites and through autolysis. A sound understanding of yeast systematics, biogeography and ecology is therefore essential to endeavours to preserve and exploit the hidden oenological potential of the untapped wealth of yeast biodiversity in our wine-producing regions. One of the main thrusts of this kind of eco-taxonomic survey is to determine the actual contribution of the indigenous strains of the so-called *wine yeast* (*Saccharomyces cerevisiae*) and *wild yeasts* (non-*Saccharomyces* species) to the sensory properties of wines and to eventually develop new starter culture strains for guided fermentations, including mixed starter cultures tailored to reflect the characteristics of a given wine region.

Against this background, a comprehensive, long-term biogeographical survey and strain development programme was launched. This dissertation represents the first phase of this long-overdue research programme aimed to systematically catalogue yeasts in different climatic zones of the 350-year-old wine-producing regions of the Western Cape and to develop new yeast starter cultures that would further increase the quality of South African wine. The specific aims of this dissertation included (i) the evaluation of yeast fingerprinting techniques for their suitability to accurately and rapidly differentiate amongst *S. cerevisiae* strains; (ii) the isolation and characterization of *S. cerevisiae* strains from the coastal regions of the Western Cape; (iii) to determine the natural population dynamics of *S. cerevisiae* strains in selected vineyards over a four-year period; (iv) to make a preliminary determination of the possible effect that these indigenous *S. cerevisiae* isolates may have on wine flavour, and (v) to breed new starter culture strains with improved characteristics.

Eighteen strains of *S. cerevisiae* used for commercial production of wine in South Africa were characterized by means of long-chain fatty acid analysis, randomly amplified polymorphic DNA (RAPD-PCR) and electrophoretic karyotyping (CHEF-DNA analysis). Variations in DNA profiles of the strains were apparent in the number, position and intensity of bands. It was found that electrophoretic karyotyping, as a single technique, seemed to be the most useful method to be used for routine fingerprinting. However, it was proposed that the combined use of these three techniques would provide the most reliable means of differentiating amongst wine yeast strains.

Two of these fingerprinting techniques, CHEF-DNA and RAPD-PCR analysis, were used to determine the geographic distribution of indigenous *S. cerevisiae* strains isolated from local vineyards. Grapes were aseptically harvested from 13 sites in five areas in the coastal regions of the Western Cape during 1995. These sites were Groot Constantia and Buitenverwachting in the Constantia area; Jordan, Lievland, Mont Fleur and Nietvoorbij in the

Stellenbosch area; Vergelegen in Somerset West; De Rust, Oak Valley, White Hall and Wildekrans in the Elgin/Bot River area; and Bouchard Finlayson and Hamilton Russel in the Hermanus area. After fermentation, 30 yeast colonies per sample were isolated and examined for the presence of *S. cerevisiae*. Five sampling sites yielded no *S. cerevisiae* strains. Electrophoretic karyotyping revealed the presence of 46 unique karyotypes in eight of the remaining sites. No dominant strain was identified and each site had its own unique collection of strains. The number of strains per site varied from two to 15. Only in four cases did one strain appear at two sites, while only one instance of a strain occurring at three sites was recorded. All sites contained killer and sensitive strains, however, killer strains did not always dominate. Commercial strains were recovered from three sites. Although commercial yeasts dominated the microflora at two sites, it appears that fears of commercial yeasts ultimately dominating the natural microflora seem to be exaggerated.

As an extension of the 1995 survey samples were taken from the same locations at Groot Constantia, Buitenverwachting, Jordan, Lievland, Mont Fleur, Vergelegen, Bouchard Finlayson and Hamilton Russel during 1996 to 1998. This was done in an effort to assess how the natural population dynamics of *S. cerevisiae* are affected over the long term by abiotic factors. Thirty colonies per site were isolated and the *S. cerevisiae* strains were characterized by electrophoretic karyotyping. The identity of strains appearing at more than one site in the same, or different years, was confirmed by RAPD-PCR analysis. Strain numbers per site varied over the four-year study period. Weather conditions resulting in severe fungal infestations and heavy applications of chemical sprays during 1996 and early 1997 dramatically reduced the numbers of *S. cerevisiae* strains recovered during 1997. A return to normal weather patterns during mid 1997 resulted in a gradual recovery of the indigenous population as noted during the 1998 harvest. Indications are that some of the strains isolated are widespread in the study area and may represent yeasts typical of the area. Again, commercial wine yeast strains were recovered in only a few instances and the likelihood that commercial yeasts will eventually replace the natural yeast microflora in vineyards therefore seems remote.

As a preliminary study to determine the possible effect of these indigenous *S. cerevisiae* strains on wine flavour, 33 of the indigenous yeasts were allowed to ferment Chenin blanc wine in laboratory fermentations. The juice was analyzed. The ability to form esters, fatty acids and higher alcohols was compared to that of two local commercial yeasts. None of the indigenous strains were found to be suitable for fermenting white must at 15°C. Their ability to ferment red musts at much higher temperatures still needs to be assessed. Furthermore, differences noted indicate that some of these strains show potential to be included in our extensive yeast-breeding programme as this would broaden the genetic pool.

In parallel with the search to isolate and identify indigenous *S. cerevisiae* strains with good oenological potential, an extensive selection and breeding programme with cultures from our strain collection was undertaken. The aim of this programme was to generate new strains that are better suited to New World winemaking styles and conditions prevailing in South Africa. As a result, 145 hybrids, differentiated by elecrophoretic karyotyping and long-chain fatty acid analysis, were produced. Fifty-eight of the hybrids were able to ferment juice to dryness at 15°C in less than 21 days during microvinification trials. Five of the strains were released for commercial use after extensive industrial-scale evaluation. Based on the success of these interstrain hybridizations, the breeding programme will now be expanded to include some of the indigenous *S. cerevisiae* strains.

In conclusion, it is only when we have a much better understanding of yeast biodiversity, biogeography, ecology and the interaction within yeast communities that we will be able to optimally harness the genetic pool in our strain development programme, aimed to benefit both the wine producer and the consumer.

OPSOMMING

Die biologiese omskakeling van druiwesap na wyn is 'n oeroue proses waartydens giste wat met druiwekorrels en keldertoerusting geassosieer is, bloot toegelaat word om die suiker na etanol, koolstofdioksied en ander mindere, maar belangrike metaboliete te fermenteer. Die kuns en wetenskap van wynbereiding word al sedert die tyd toe Louis Pasteur die verwantskap tussen gis en alkoholiese fermentasie gedemonstreer het, bestudeer. Daar word nou aanvaar dat die fermentasieproses en die produksie van premiumkwaliteit wyn 'n komplekse ekologiese en biochemiese proses is wat die opeenvolgende ontwikkeling van mikrobiese spesies wat deur die spesifieke omgewing beïnvloed word, behels. Hierdie komplekse heterogene mikrobiologiese proses sluit die interaksie van baie spesies soos swamme, giste, asynsuur- en melksuurbakterieë, asook mikovirusse en bakteriofage, wat dié druifgeassosieerde mikroörganismes beïnvloed, in. Giste verteenwoordig nietemin die kern van die harmonieuse biochemiese interaksie met sap afkomstig van verskillende variëteite van *Vitis*-spesies, wat op hul beurt weer grootliks produkte van hul individuele genetiese samestellings en die *terroir* is. Hierdie giste is belangrik in die wynmaakproses, want hulle speel nie slegs 'n rol in alkoholiese fermentasie nie, maar kan ook wynbederf en wynkwaliteit deur die produksie van metaboliese komponente tydens fermentasie en outolise beïnvloed. 'n Deeglike begrip van gissistimatiek, biogeografie en ekologie is dus belangrik om die wynkundige potensiaal van ons wynproduserende areas se ryke gisbiodiversiteit te bewaar en te benut. Een van die hooffokuspunte van dié eko-taksonomiese ondersoeke is om die werklike bydrae van die inheemse rasse van die sogenaamde wyngis (*Saccharomyces cerevisiae*) en wildegiste (nie-*Saccharomyces cerevisiae*) met betrekking tot die sensoriese gehalte van wyn te bepaal. Dit sal dus tot die ontwikkeling van nuwe kommersiële rasse, wat die eienskappe van die area weerspieël, lei.

Teen hierdie agtergrond is 'n langtermyn biogeografiese opname en gisras-ontwikkelingsprogram begin. Dié proefskrif verteenwoordig die eerste fase van hierdie program wat katagorisering van die inheemse giste in die verskillende klimaatsones van die 350 jaar oue wynproduserende areas van die Wes-Kaap en ontwikkeling van nuwe kommersiële gisrasse, wat die kwaliteit van die Suid-Afrikaanse wyn sal verbeter, behels. Die spesifieke doelwitte van die proefskrif sluit die volgende in: (i) die evaluering van die geskiktheid van giskarakteriseringstegnieke om akkuraat en vinnig tussen verskillende *S. cerevisiae*-rasse te onderskei; (ii) die isolasie en karakterisering van *S. cerevisiae*-rasse uit die kusstreke van die Wes-Kaap; (iii) die bepaling van die populasiedinamika van die inheemse *S. cerevisiae*-rasse in geselekteerde wingerde oor 'n vierjaartermyn; (iv) voorlopige bepaling van die moontlike invloed van inheemse *S. cerevisiae*-isolate op wynaroma; en (v) teling van nuwe kommersiële giskulture met verbeterde eienskappe.

Agtien kommersiële wyngisrasse wat in Suid-Afrika gebruik word, is met behulp van langkettingvetsuuranalises, lukraak geamplifiseerde polimorfiese DNA-tegniek (RAPD-PCR) en elektroforetiese kariotipering (CHEF-DNA-analise) gekarakteriseer. Die verskille in die aantal, posisie en intensiteit van die bande van die DNA-profiele van die verskillende gisrasse was duidelik waarneembaar. As enkeltegniek, was die elektroforetiese kariotipering die beste tegniek wat vir roetine-tipering gebruik kan word. Die gesamentlike gebruik van die drie tegnieke sal egter die akkuraatste resultaat lewer indien daar tussen rasse onderskei moet word.

Twee van die tegnieke, CHEF-DNA- en RAPD-PCR-analises, is gebruik om die geografiese verspreiding van inheemse *S. cerevisiae*-rasse wat uit die wingerde geïsoleer is, te bepaal. Druiwe is in 1995 asepties by 13 plekke in vyf areas van die kusstreke van die Wes-Kaap geoes. Dit was Groot Constantia en Buitenverwachting in die Constantia-area; Jordan, Lievland, Mont Fleur en Nietvoorbij in die Stellenbosch-area; Vergelegen in Somerset-Wes; De Rust, Oak Valley, White Hall en Wildekrans in die Elgin/Botrivier-area; en Bouchard Finlayson en Hamilton Russell in die Hermanus-area. Dertig giskolonies is na fermentasie

per monster geïsoleer en vir die teenwoordigheid van *S. cerevisiae*-rasse ondersoek. Vyf plekke het geen *S. cerevisiae*-giste gehad nie. Elektroforetiese kariotipering het die teenwoordigheid van 46 unieke kariotipes in die agt oorblywende plekke uitgewys. Geen dominante gisras is gevind nie en elke isolasieplek het sy eie unieke versameling rasse gehad. Die aantal gisrasse het van twee tot 15 per isolasieplek gewissel. Slegs in vier gevalle is een ras by twee plekke gevind, terwyl daar slegs een geval was waar een ras by drie plekke voorgekom het. Killer- en sensitiewe gisrasse is by alle plekke gevind, maar die killerrasse was nie altyd dominant nie. Kommersiële rasse is by slegs drie plekke gevind. Alhoewel kommersiële rasse die dominante giste by twee plekke was, blyk dit dat die vrese dat kommersiële gisrasse die inheemse mikroflora sal oorheers, oordrewe is.

Ter aansluiting by die 1995-opname, is daar gedurende 1996 tot 1998 weer op dieselfde plekke te Groot Constantia, Buitenverwachting, Jordan, Lievland, Mont Fleur, Vergelegen, Bouchard Finlayson en Hamilton Russell monsters geneem. Die doel hiermee was om te bepaal hoe die inheemse populasiedinamika van *S. cerevisiae* oor die langtermyn deur abiotiese faktore beïnvloed is. Dertig kolonies is per isolaat geïsoleer en die *S. cerevisiae*-rasse is met behulp van elektroforetiese kariotipering gekarakteriseer. Die identiteit van gisrasse wat in dieselfde, of verskillende, jare by meer as een isolasieplek verskyn het, is deur middel van RAPD-PCR-analise bevestig. Die aantal rasse per plek het oor die vierjaarstudieperiode gewissel. Nat en warm weerstoestande gedurende laat 1996 en vroeg 1997 het tot ernstige swaminfeksies gelei en die verhoogde gebruik van chemiese spuitmiddels het die aantal *S. cerevisiae*-rasse wat tydens 1997 geïsoleer is, drasties verlaag. Die terugkeer van normale weerstoestande teen die middel van 1997 het tot die stadige herstel van die inheemse gispopulasie gelei. Daar was aanduidings dat sekere van die rasse oor groot dele van die studiegebied versprei is en dit kan dus dui op gisrasse wat kenmerkend van die area is. Daar is weereens slegs 'n paar kommersiële giste geïsoleer en dit bevestig dus dat die moontlikheid dat kommersiële rasse die inheemse populasie sal oorheers, baie gering is.

Tydens 'n voorlopige studie waarin die moontlike uitwerking van inheemse *S. cerevisiae*-rasse op wynaroma bestudeer het, is 33 van die inheemse giste in laboratorium-eksperimente toegelaat om Chenin blanc-sap te fermenteer. Hierdie wyne is chemies ontleed. Die giste se vermoë om esters, vetsure en hoër alkohole te produseer, is daarna met dié van twee kommersiële rasse vergelyk. Daar is bevind dat nie een van dié inheemse rasse geskik is vir die fermentasie van witmos by 15°C nie. Hul vermoë om rooimos by veel hoër temperature te gis, moet egter nog bepaal word. Verder is daar tog aanduidings dat hierdie giste oor genoegsame potensiaal beskik om in ons gisteelprogram opgeneem te kan word om sodoende die genepoel te vergroot.

Daar is ook 'n intensiewe seleksie- en teelprogram met giste uit ons kultuurversameling onderneem wat gelyktydig met die isolasie en identifikasie van inheemse *S. cerevisiae*-rasse geloop het. Die doel van dié program is die daarstelling van nuwe wyngisrasse aan die Suid-Afrikaanse wynbedryf, wat meer vir Nuwe Wêreld-wynmaakstyle en Suid-Afrikaanse toestande geskik is. Die resultaat was 145 hibriede, wat deur elektroforetiese en langketting-vetsuuranalises onderskei kan word. Tydens kleinskaalse wynbereidingseksperimente het 58 van dié hibriede daarin geslaag om sap binne 21 dae droog te fermenteer. Vyf van dié giste is na intensiewe kommersiële evaluering kommersieel vrygestel. Die sukses met die hibriede het nou tot gevolg dat die teelprogram uitgebrei sal word deur sekere van die inheemse *S. cerevisiae*-rasse in te sluit.

Die gevolgtrekking is dat ons gisbiodiversiteit, biogeografie, ekologie en die interaksies tussen gisgemeenskappe beter sal moet verstaan indien ons die genetiese poel tot ons beskikking optimaal in die teelprogram wil aanwend om sodoende die wynprodusent en verbruiker te bevoordeel.

This dissertation is dedicated to *Aletta*, *Andro* and *Janke*.

Hierdie proefskrif is opgedra aan *Aletta*, *Andro* en *Janke*.

BIOGRAPHICAL SKETCH

Theunes Johannes van der Westhuizen was born in Cape Town, South Africa, on 22 September 1962. He matriculated at President High School, Vrijzee, Cape Town in 1980.

He entered the University of Stellenbosch and obtained a B.Sc. degree in Microbiology and Botany in 1984. The Hons.B.Sc. degree in Microbiology was awarded to him in 1988 and an M.Sc. degree in Microbiology in 1990 from the same institution. He was appointed as researcher at Infruitec in 1991 and as senior wine yeast researcher at the Wine and Fermentation Technology Division at ARC-Fruit, Vine and Wine Research Institute, Nietvoorbij Centre for Vine and Wine in 1993. His research objective was to select and breed more suitable yeasts for the South African wine industry. In 1995 he enrolled as a Ph.D. student in Microbiology at the University of Stellenbosch. He joined Anchor Yeast in 1997 as manager of the Wine Yeast Section.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- **Prof. I.S. Pretorius**, Institute for Wine Biotechnology, University of Stellenbosch, for his enthusiasm, devotion and guidance throughout this project.
- **Dr. O.P.H. Augustyn**, Wine and Fermentation Technology Division at ARC-Fruit, Vine and Wine Research Institute, Nietvoorbij Centre for Vine and Wine, for his enthusiasm, guidance and invaluable discussions.
- **The staff** of the **Wine and Fermentation Technology Division at ARC-Fruit, Vine and Wine Research Institute**, Nietvoorbij Centre for Vine and Wine, for their assistance.
- The **South African wine industry (Winetech)**, the **National Research Foundation** (NRF) and **Anchor Yeast** for financial support.
- My wife, **Aletta**, for her support and encouragement.
- **The Almighty**, for this opportunity.

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 for Enology and Viticulture* to which Chapters 1, 2, 3, and 4 were submitted for publication.

Chapter 1 **Literature Review and Project Aims**

Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry (*S. Afr. J. Enol. Vitic.*, in press)

Chapter 2 **Research Results I**

The value of long-chain fatty acid analysis, randomly amplified polymorphic DNA and electrophoretic karyotyping for the characterization of wine yeast strains (*S. Afr. J. Enol. Vitic.* 20, 3-10)

Chapter 3 **Research Results II**

Geographic distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the coastal regions of the Western Cape in South Africa (*S. Afr. J. Enol. Vitic.*, in press)

Chapter 4 **Research Results III**

Seasonal variation of indigenous *Saccharomyces cerevisiae* strains isolated from Western Cape vineyards (*S. Afr. J. Enol. Vitic.*, in press)

Chapter 5 **Research Results IV**

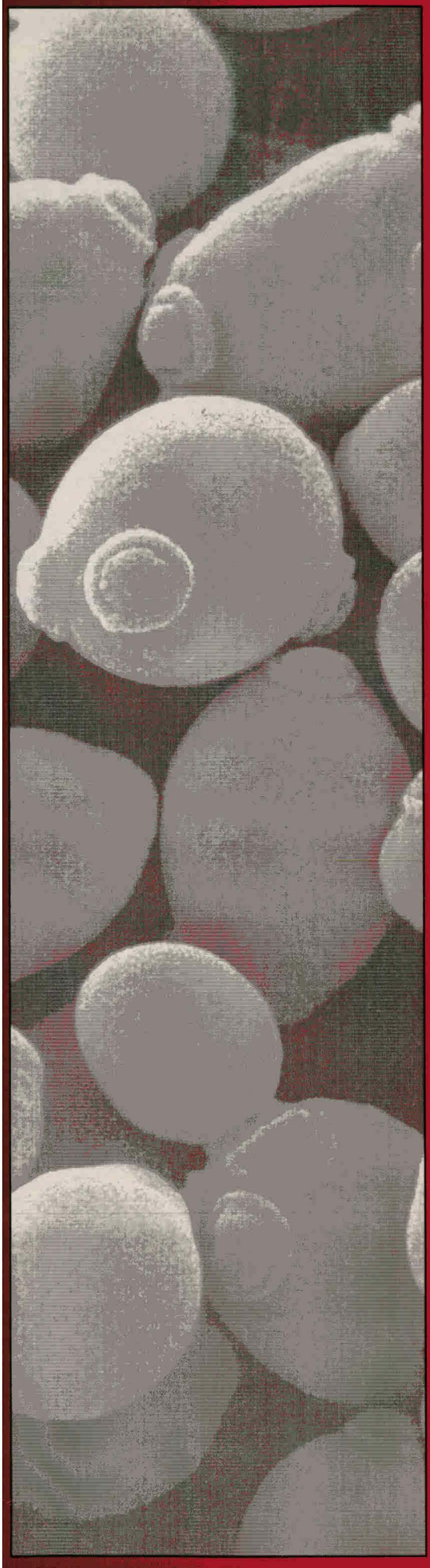
Preliminary study on the ability of indigenous *Saccharomyces cerevisiae* strains to affect the flavour composition of wine

Chapter 6 **Research Results V**

The breeding of new wine yeast strains

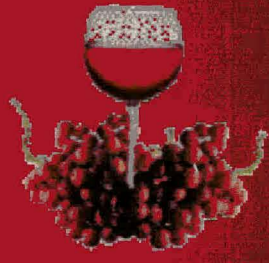
Chapter 7 **General Discussion and Conclusions**

CONTENTS



CHAPTER 1

Literature Review and Project Aims



Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry

(*S. Afr. J. Enol. Vitic.*, in press)

1. LITERATURE REVIEW AND PROJECT AIMS

Yeast Biodiversity in Vineyards and Wineries and its Importance to the South African Wine Industry

Abstract

The art of winemaking is as old as human civilization and the use of yeast in this complex ecological and biochemical process dates back to ancient times. Traditionally, yeasts associated with grape berries were simply allowed to ferment the sugars to ethanol, carbon dioxide and other minor, but important, metabolites. Spontaneous fermentations are still being used in *boutique* wineries that depend more on vintage variability. Various microbes found on the surface of grape skins and the indigenous microbiota associated with winery surfaces participate in these natural wine fermentations. Yeasts of the genera *Kloeckera*, *Hanseniaspora* and *Candida* predominate in the early stages, followed by several species of *Metschnikowia* and *Pichia* (including those species that were previously assigned to the genus *Hansenula)* in the middle stages when the ethanol rises to 3-4%. The latter stages of natural wine fermentations are invariably dominated by the alcohol-tolerant strains of *Saccharomyces cerevisiae.* However, other yeasts, such as species of *Brettanomyces*, *Kluyveromyces, Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* also may be present during the fermentation and can occur in the resultant wine. By contrast, the rule, rather than the exception, for modern wineries depending on reliable fermentation and the production of wines with predictable quality, is the use of specially selected starter cultures of *Saccharomyces.* However, the use of such cultures may not necessarily prevent the growth and metabolic activity of indigenous, winery associated strains of *S. cerevisiae* or other wild yeasts such as *Kloeckera apiculata*, *Hanseniaspora uvarum*, *Candida stellata* and *Torulaspora delbrueckii.* It is therefore clear that both spontaneous and inoculated wine fermentations are affected by the diversity of yeasts associated with the vineyard (natural habitat) and winery (man-made niche). In light of this, focused taxonomic surveys within an ecological framework are essential to preserve and exploit the hidden oenological potential of the untapped wealth of yeast biodiversity in our wine-producing regions. To achieve this, yeast taxonomists need to continue to isolate and characterize new yeast species and strains, while wine microbiologists develop improved identification techniques that differentiate more efficiently among individual strains. At the same time such biological surveys will complement strain development and the current international effort of molecular biologists to assign a biological function to the products of each of the 6000 genes identified by computer analysis of the nucleotide sequence of the 16 chromosomes of a laboratory strain of *S. cerevisiae.* Furthermore, only when we have a much better understanding of yeast biodiversity, biogeography, ecology and the interaction within yeast communities will we be able to optimally harness gene technology that will benefit both the wine producer and the consumer.

1.1 INTRODUCTION

The art of winemaking is far older than recorded history and the development of fermentation technology underpinning this ancient process stretches over a period of nearly 7000 years. The fermentation of grape must and production of premium quality wines is a complex ecological and biochemical process involving the interaction of many microbial species, represented by fungi, yeasts, lactic acid bacteria and acetic acid bacteria, as well as the mycoviruses and bacteriophages affecting these grape-associated microorganisms (Fleet, 1993). Of all these different microbes and viruses, yeasts, being the primary catalysts of the bioconversion of grape juice into wine, represent the heart of the harmonious biochemical interaction with the musts derived from the various varieties of *Vitis* species which, in turn, are largely products of their respective genetic make-ups and the *terroir*. This article, summarizing the most important aspects of yeast biodiversity and ecology, illustrates the importance of thorough biological surveys within the ecological framework of the wine-producing regions of South Africa.

1.1.1 YEAST TAXONOMY

Yeasts can be defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth predominantly results from budding or fission and which do not form their sexual states within or upon a fruiting body (Kurtzman & Fell, 1998b). The world's growing awareness of the importance of biodiversity re-focused the attention on new taxonomic surveys within an ecological framework (Lachance & Stramer, 1998). Based on improved methods of yeast isolation, identification and classification and other taxonomic tools, the number of yeast taxa has increased dramatically over the last three decades.

The first level of yeast classification is based on aspects of yeasts' sexuality (Ascomycotina or Basidiomycotina) or the lack of it (Deuteromycotina), while the lower taxonomic subdivisions (*families*, *subfamilies*, *genera*, *species* and *strains*) are based on various morphological, physiological and genetic characteristics (recently reviewed by Kurtzman & Fell, 1998a). As an example the classification of ascomycetous yeasts and a list of the currently accepted yeast genera are given in **Tables 1.1** and **1.2**, respectively. Rules for taxonomy of the yeasts fall under the authority of the *International Code of Botanical Nomenclature* (Greuter *et al.*, 1994) and publication of a new species must include a description of essential characters as well as a diagnosis that distinguishes the taxon from a previously described species (Kurtzman & Fell, 1998b). The number of yeast genera and species have more than doubled since the release of the second edition of the monographic series, *The Yeasts, A Taxonomic Study* (Lodder, 1970). The fourth and latest edition of the series describes 100 yeast genera representing over 700 species (Kurtzman & Fell, 1998a).

Table 1.1 Classification of the ascomycetous yeasts (Kurtzman & Fell, 1998a).

Class Order Family Genus	Family Genus
Phylum Ascomycota	Metschnikowiaceae
"Archiascomycetes"	*Clavispora*
Schizosaccharomycetales	***Metschnikowia****
Schizosaccharomycetaceae	Saccharomycetaceae
Schizosaccharomyces*	Arxiozyma
Taphrinales	*Citeromyces*
Taphrinaceae	*Cyniclomyces*
Taphrina	***Debaryomyces****
Lalaria (Anamorph of *Taphrina*)	***Dekkera****
Protomycetales	*Issatchenkia*
Protomycetaceae	***Kluyveromyces****
Protomyces	*Lodderomyces*
Saitoella (Anamorphic genus)	*Pachysolen*
Pneumocystidales	***Pichia****
Pneumocystidaceae	***Saccharomyces****
Pneumocystis	*Saturnispora*
Euascomycetes	***Torulaspora****
Endomyces (E. scopularum)	*Williopsis*
Oosporidium	***Zygosaccharomyces****
Hemiascomycetes	Saccharomycodaceae
Saccharomycetales (synonym Endomycetales)	***Hanseniaspora****
Ascoideaceae	*Nadsonia*
Ascoidea	***Saccharomycodes****
Cephaloascaceae	*Wickerhamia*
Cephaloascus	Saccharomycopsidaceae
Dipodascaceae	*Ambrosiozyma*
Dipodascus	*Saccharomycopsis*
Galactomyces	Candidaceae (Anamorphic)
Sporopachydermia	Aciculoconidium
Stephanoascus	*Arxula*
Wickerhamiella	*Blastobotrys*
Yarrowia	*Botryozyma*
Zygoascus	***Brettanomyces****
Endomycetaceae	***Candida****
Endomyces (E. decipiens)	*Geotrichum*
Helicogonium	***Kloeckera****
Myriogonium	*Myxozyma*
Phialoascus	*Schizoblastosporion*
Trichomonascus	*Sympodiomyces*
Eremotheciaceae	*Trigonopsis*
Eremothecium	
Coccidiascus	
Lipomycetaceae	
Babjevia	
Dipodascopsis	
Lipomyces	
Zygozyma	

*Genera that are frequently encountered in vineyards, wineries, grape must and/or wine are typed in bold.

Table 1.2 An overview of yeast genera according to Kurtzman and Fell (1998a).

Teleomorphic ascomycetous genera (Ascomycotina)	Anamorphic ascomycetous genera (Deuteromycotina)	Teleomorphic heterobasidiomycetous genera (Basidiomycotina)	Anamorphic heterobasidiomycetous 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			

*Genera that are frequently encountered in vineyards, wineries, grape must and/or wine are typed in bold.

1.1.2 YEAST BIODIVERSITY AND ECOLOGY

Notwithstanding the spectacular growth in the number of described yeast species between 1970 and 1998, the wealth of yeast biodiversity is still largely untapped. This point is best illustrated by the fact that for ascomycetes in general, the numbers of undescribed genera and species have been calculated at 62 000 and 669 000, respectively (Hawksworth & Mouchacca, 1994). Therefore, in order to discover the hidden oenological potential with respect to the immense untapped yeast biodiversity, it is imperative to continue to develop ways of characterizing and preserving remaining species and strains. According to Lachance & Stramer (1998) these new taxonomic surveys, should, however, not be a meaningless catalogue or inventory of yeast names, devoid of real biological relevance, but rather a study of yeast species in the context of their environment. Several molecular biological taxonomic approaches are now assisting in the fundamental understanding of yeast communities in specific habitats and niches. This will undoubtedly provide improved means to preserve and exploit yeast biodiversity (Roberts & Wildman, 1995) and to track and monitor the spread of genetically modified yeasts when used for the production of fermented foods and beverages in future.

1.1.2.1 Yeast communities, habitats and niches

Yeasts occur widespread in nature although they are not as ubiquitous as bacteria (Phaff, Miller & Mrak, 1978). However, these chemoorganotrophic fungi, requiring fixed, organic forms of carbon for growth, do not occur randomly throughout the biosphere. They form communities of species and each community is defined by its *habitat*, the actual place where an assemblage of yeasts lives, and by the niches of its component species (for a recent review see Lachance & Stramer, 1998). The *niche* consists of the attributes that make a yeast capable of sharing a habitat with other *autochthonous* (essential components of the community) and *allochthonous* (components that are transient or present fortuitously) members of the community and is therefore the sum of all physical, chemical or biotic factors required for successful existence (Lachance & Stramer, 1998). For example, different yeasts are able to utilize different carbon sources and nutritional selectivity determines yeast species diversity in particular niches. Therefore, *generalist* yeasts are endowed with a broad niche and as a consequence occupy many habitats, whereas *specialist* yeasts exhibit great specialization for their habitat and thus occur in very unique habitats (Lachance & Stramer, 1998). The Atlas & Bartha (1993) classification of the various types of community interactions is outlined in **Table 1.3**.

1.1.2.2 Yeast flora of grapes

Being non-motile, yeasts rely on aerosols, animal vectors and human activity for their natural dispersal (Walker, 1998). They can be isolated from terrestrial, aquatic and aerial environments, but preferred habitats are plant tissues. The microflora of grapes are highly variable, with a predominance of the low alcohol-tolerant species of *Kloeckera* and its anamorph *Hanseniaspora* (*e.g.*, *K. apiculata* and *H. uvarum*) that account for about 50-75% of the total yeast population. Furthermore, significant by their presence, but at lesser numbers than the latter species, are species of *Candida* (especially *C. stellata* and *C. pulcherrima*), *Brettanomyces*, *Cryptococcus*,

Kluyveromyces, *Pichia* (including those species that were previously assigned to the genus *Hansenula*) and *Rhodotorula* (for a recent review see Fleet & Heard, 1993). However, fermentative species of *Saccharomyces* (*e.g.*, *S. cerevisiae*) occur at extremely low populations on sound, undamaged grapes and are rarely isolated from intact berries (Du Plessis, 1959; Peynaud & Domercq, 1959; Van Zyl & Du Plessis, 1961; Martini & Vaughan-Martini, 1990; Martini, 1993). In fact, Vaughan-Martini & Martini (1995) concluded that a natural origin for *S. cerevisiae* should be excluded. According to these authors *S. cerevisiae* strains present in spontaneously fermenting grape must originate from various surfaces in the winery. On the other hand, Török *et al.* (1996) supplied data indicating that the vineyard is in fact the primary source of this yeast. They also noted that each plant and grape cluster is different as regards the presence/absence of *S. cerevisiae*. To elude the problems associated with the recovery of such low numbers of yeast, the majority of surveys on the population kinetics and geographic distribution of natural-occurring yeast species invariably included an enrichment procedure after the isolation (Frezier & Dubourdieu, 1992; Fleet, 1993; Querol, Barrio & Ramon, 1994; Schütz & Gafner, 1994; Versavaud *et al.*, 1995; Constanti *et al.*, 1997).

Table 1.3 Classification of the various types of community interactions, the interaction of yeasts and other microbes in specific habitats (Atlas & Bartha, 1993).

Type of interaction	Definition
Microbe-microbe interaction	
Neutralism	Sparse, independent populations sharing the same habitat
Commensalism	A population benefits from the activity of another, *synergism* (cooperative modification of resources leading to close spatial relationships)
Mutualism/symbiosis	Very strong synergistic association
Competition	Utilization of limited common resources
Amensalism	Chemical interference
Predation	Ingestion
Parasitism	Long term destructive contact
Animal-microbe interactions	
Grazing	
Food enrichment	
Epizoic relationships	
Plant-microbe interactions	
Rhizosphere	
Rhizoplane	
Mycorrhizae	Mutualistic interaction
Phyllosphere	
Epiphytes	
Pathogens	

The combined effect of several factors affects the microflora of grapes. These factors, as summarized by Fleet & Heard (1993), include the grape variety; temperature, rainfall and other climatic influences; soil, fertilization and irrigation; development stage at which grapes are examined; physical damage caused by mould, insect or bird attack; and fungicides applied to vineyard. It is also possible that

viticultural practises such as canopy management, which affect the microclimate within the vine canopy, play a role in defining the range of microbes present in a particular vineyard. However, further research needs to be conducted in this regard before definite conclusions can be made.

1.1.2.3 Winery yeast flora

In addition to natural habitats, some yeasts have found niches in man-made environments such as wine cellars. The surfaces of winery equipment that come into contact with grape juice and wine become locations for the development of a so-called *residential* or *winery yeast flora* (Peynaud & Domercq, 1959; Rosini, 1984). The extent of the development of a residential yeast flora (*e.g.*, species of *Saccharomyces*, *Candida* and *Brettanomyces*) will depend upon the nature of the surface and the degree to which the particular surface has been cleaned and sanitized (Fleet & Heard, 1993). Unlike its low occurrence in natural habitats such as grapes, *S. cerevisiae* is prevalent on these surfaces (Rosini, 1984; Martini, Ciani & Scorzetti, 1996). In fact, *S. cerevisiae* is by far the most dominant yeast species colonizing surfaces in wineries demonstrating the selective effects of grape juice and wine as growth substrates (Martini, 1993).

Although the presence, and importance of winery yeasts have been known, or surmised, for many years (Peynaud & Domercq, 1959; Van Zyl & Du Plessis, 1961), their actual contribution to must fermentations has been all but ignored. Rosini (1984) supplied preliminary data indicating that these yeasts actually dominate spontaneous fermentations conducted in well-established wineries. Martini, Ciani & Scorzetti (1996) indicated that fermentation of grape must aseptically prepared in the laboratory is often anomalous. They also speculated that resident winery yeasts will compete with pure *S. cerevisiae* cultures used to inoculate musts. This view is supported by data recently generated by Constanti *et al.* (1997). These authors found that a resident winery yeast completely dominated an inoculated fermentation in a two-year old Spanish winery. Török *et al.* (1996), however, do not support the idea that yeasts resident on winery surfaces play an important, or even dominant, role during spontaneous fermentation of grape must. Clearly the origin (if present at all), composition and actual contribution to fermentation of resident wineries yeast need to be studied much more extensively.

1.1.2.4 Yeast flora in grape must

The microbiota of grape must are affected indirectly by all factors influencing the indigenous grape microflora and the winery flora as mentioned in the previous two sections as well as by the following aspects: Method of grape harvest (handpicked or mechanical, grape temperature), transport from vineyard to cellar (time, initial grape temperature, air temperature, sulfite addition), condition of grapes (time, temperature, sulfite addition) and must pretreatment (cellar hygiene, aeration, enzyme treatment, sulphite addition, clarification method, temperature, inoculation with yeast starter cultures).

Although grape must is considered to be relatively complete in nutrient content, it can only support the growth of a limited number of microbial species. According to Henschke (1997), the low pH and high sugar content of grape must exert strong

selective pressure on the micro-organisms, such that only several yeast and bacterial species can proliferate. Furthermore, the use of restrictive concentrations of sulfur dioxide as an anti-oxidant and anti-microbial preservative, imposes addiditional selection, particularly against undesirable oxidative microbes. The selectivity of fermenting must is further strengthened once anaerobic conditions are established, certain nutrients become depleted and the increasing levels of ethanol start to eliminate alcohol-sensitive microbial species (Henschke, 1997). Therefore, spontaneous fermentation of grape juice into wine can be regarded as a heterogeneous microbiological process involving the sequential development of various yeasts and other microbiological species, affected by the prevailing fermentation conditions in a particular vat or tank. The fermentation is usually started by yeasts of the genera *Kloeckera*, *Hanseniaspora* and *Candida* and, to a lesser extent, *Metschnikowia* and *Pichia*. These yeasts predominate during the early and middle phases of fermentation until ethanol levels rise to around 3 to 4% (Fleet & Heard, 1993; Mortimer *et al.*, 1994). These ethanol-sensitive yeasts are then overtaken by the stronger fermenting and alcohol-tolerant species of *Saccharomyces* which carry out and complete fermentation, reaching ethanol levels in the range of 12 to 14%. It is also amply reported by numerous authors that other yeasts, such as species of *Brettanomyces, Kluyveromyces, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces* may also be present during the fermentation and can occur in the resultant wine, some of which are capable of adversely affecting the sensorial quality of the end product (Fleet & Heard, 1993; Henschke, 1997). From this it is clear that, when must is used as a culture medium, the abovementioned selective pressures always yield biased results in favour of the yeasts with the most efficient fermentative catabolism, particularly strains of *S. cerevisiae* (Martini, 1993) and perhaps strains of closely related species such as *S. bayanus* (Vaughan-Martini & Martini, 1998).

1.1.3 WINE YEAST STARTER CULTURES

1.1.3.1 Wine yeast strain variation

Over the years, strains of *S. cerevisiae* were isolated from vineyards and selected to be used as commercial starter cultures. The genetic variation normally present in all *Saccharomyces* populations also served as a gene pool for the breeding of improved starter strains. The natural genetic heterogeneity in wine yeast strains is mainly due to mitotic recombination and spontaneous mutation.

It is now believed that strains of *Saccharomyces* indigenous to vineyards and wineries, tend to be homozygous for most of the genes by a process known as *genome renewal* (Mortimer *et al.*, 1994). This phenomenon is based on the capability of homothallic haploid *Saccharomyces* cells to switch their mating-type from **a** to α and *vice versa* through the so-called *HO*-controlled cassette model and to conjugate with cells of the same single-spore colony. According to Mortimer *et al.* (1994) continued propagation of yeast cells in their natural (*e.g.*, vineyards) or man-made (*e.g.*, wineries) habitats leads to a situation where strains of *Saccharomyces* accumulate heterozygous recessive mutations and the concomitant heterozygotes can change to completely homozygous diploids by sporulation and homothallic

switching of individual haploid spores. This process would eliminate the recessive lethal or deleterious genes that adversely affect yeast fitness (*e.g.*, slower growth, lower fermentation rate, reduced spore viability, *etc.*). Furthermore, *genome renewal* could also be responsible for the replacement of the parental heterozygous strains by the new homozygous diploids bearing new recessive alleles that increase fitness (**Fig. 1.1**). Perhaps this is the reason why most indigenous strains of *Saccharomyces* isolated from grapes, wineries and musts, are homothallic since homothallism, together with the capability to sporulate, would provide the yeast community with a mechanism by which cells carrying deleterious recessive mutations can be eliminated, thereby enabling them to efficiently adapt to changing environmental conditions.

The practical implications of *genome renewal* and yeast population dynamics in the vineyards and wineries, and even within yeast starter cultures, are far reaching, whether winemakers rely on spontaneous fermentation of grape juice or whether they inoculate grape must with selected or tailor-made wine yeast strains.

1.1.3.2 Spontaneous versus inoculated fermentations

Originally all wine was made by utilizing the natural micoflora in spontaneous fermentations. The practise remained prevalent in *old world* wine-producing areas until the 1980s. Many *boutique* wineries, depending on vintage variability, still utilize this process today. However, the emergence of large-scale wine production where rapid, reliable, trouble free fermentations are essential for consistent wine flavour and predictable quality, necessitated the use of selected pure yeast inocula of known ability (Henschke, 1997). It is these large wineries that will be the main beneficiaries of programmes aimed at yeast strain development. These programmes will continually produce new yeast strains that have even more reliable performance so reducing processing inputs, and consequently facilitate the production of affordable high-quality wines (Henschke, 1997).

Besides the primary role of wine yeast to catalyze the rapid, complete and efficient conversion of grape sugars to alcohol without the development of off-flavours, yeast starter cultures should also possess properties such as high tolerance to sulfite, osmotic stress, ethanol and copper; genetic stability; production of glycerol and β-glucosidase; minimal lag phase on rehydration; complete fermentation of sugar at low temperatures; and limited production of foam, sulfur dioxide, hydrogen sulfide, volatile acidity, acetaldehyde, pyruvate, ethyl carbamate precursors and polyphenol oxidase (for recent reviews see Pretorius & Van der Westhuizen, 1991; Degré, 1993; Henschke, 1997; Pretorius, 1999). The importance of these additional yeast characteristics largely depends on the type and style of wine to be made and the technical requirements of the winery.

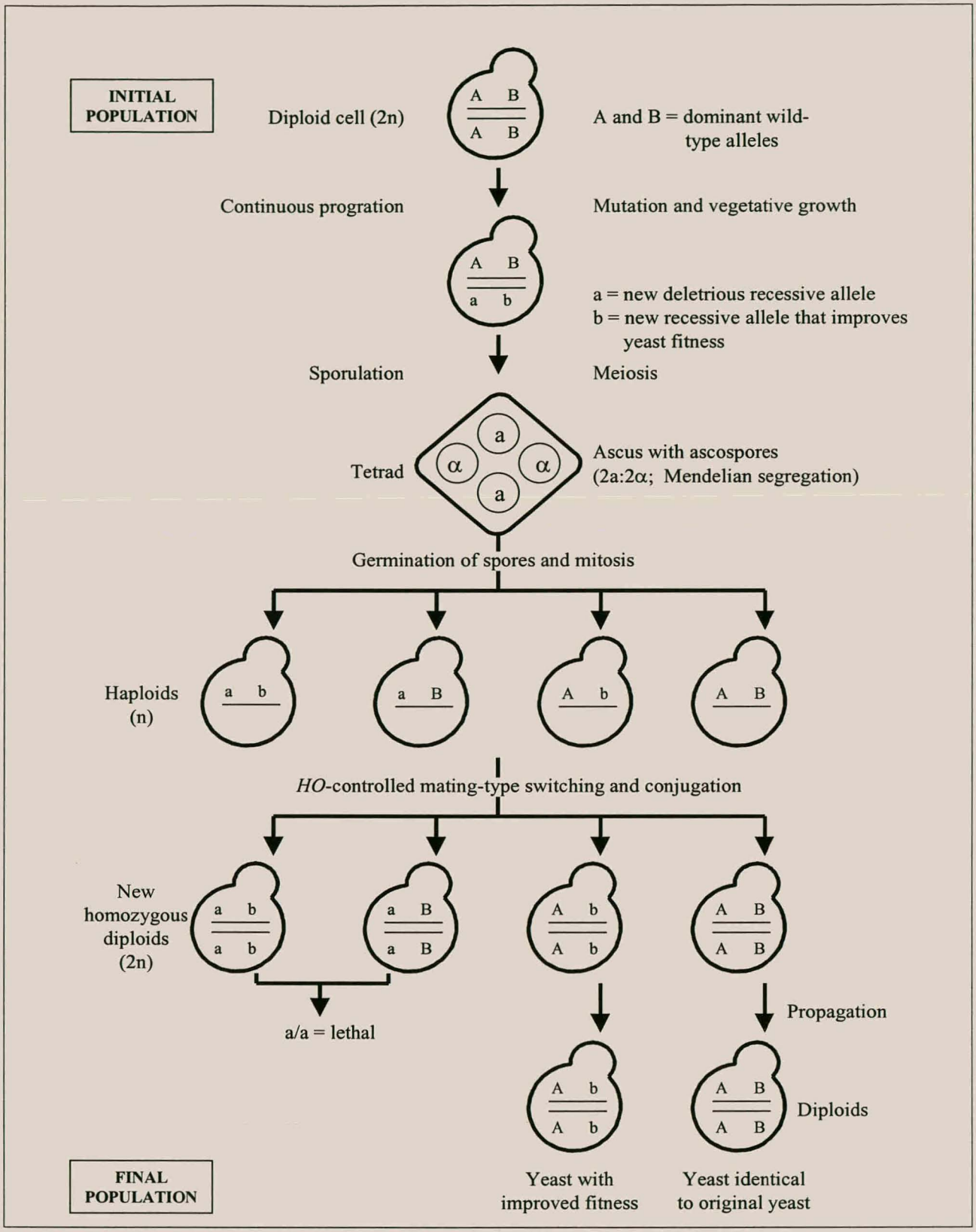


Figure 1.1 A hypothetical scheme to describe a possible succession of events leading to the replacement of one *Saccharomyces* population by another through the process of *genome renewal* (adapted from Mortimer *et al.*, 1994). Most natural wine yeasts are diploid and are homozygous for the *HO* homothalism gene. Homothallic diploids accumulate recessive mutations (spontaneous mutations at a frequency of approximately 10^{-6} per generation at any particular locus) and will also sporulate in their natural environment. The resultant ascospores will diploidize because of the *HO*-controlled mating-type switches. It is therefore possible that some of these new diploids will be more fit than their sibs or parent and will replace the parental strain

1.1.3.3 Industrial-taxonomic relationship for wine yeast strains

Saccharomyces strains with specific characteristics are preferred when making different types of wine, such as dry white, dry red, sparkling, sweet and fortified wine, and flor sherry (Henschke, 1997). This led to a situation where these wine yeast strains of *Saccharomyces* were classified into several different species or varieties, including *S. bayanus*, *S. beticus*, *S. capensis*, *S. chevaleri*, *S. ellipsoideus*, *S. fermentati*, *S. oviformis*, *S. rosei* and *S. vini* (Lodder & Kreger-van Rij, 1952; Lodder, 1970). In fact, the characteristics of some of the yeasts used for the production of specific wine types were so marked that a strong taxonomic linkage was believed to exist (Henschke, 1997). For example, while *S. ellipsoideus* were widely used for the production of dry wine, ethanol-tolerant and flocculent strains with autolytic properties (*e.g.*, *S. bayanus* and *S. oviformis*) were prefered for the production of bottle-fermented sparkling wine, film-forming strains with strong oxidative capabilities (*e.g.*, *S. beticus* and *S. capensis*) for the production of flor sherry and osmotolerant strains forming little or no volatile acids (*e.g.*, *S. rosei*) for sweet wines (Henschke, 1997). Despite this strong industrial-taxonomic relationship that has developed over a number of decades, and the considerable phenotypic differences among these yeasts, most of them are now, based on results obtained using sophisticated genetic taxonomic techniques, considered to be physiological strains of *S. cerevisiae* (Kreger-van Rij, 1984; Barnett, 1992; Vaughan-Martini & Martini, 1995; Kurtzman & Fell, 1998a). Of all these so-called *wine yeasts*, only *S. fermentati* and *S. rosei* were not re-classified as *S. cerevisiae* but rather as *Torulaspora delbrueckii*. The current description of the genus *Saccharomyces* together with the list of the currently accepted species and strains of *S. cerevisiae* and *S. bayanus* are given in **Tables 1.4, 1.5, 1.6** and **1.7**, respectively. The morphology and growth patterns of *Saccharomyces* are depicted in **Fig. 1.2**.

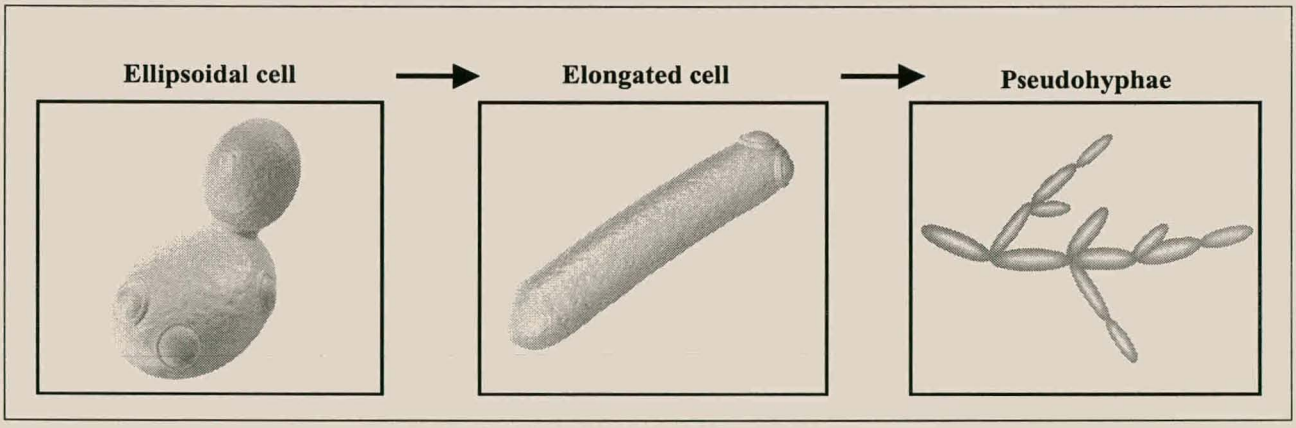


Figure 1.2 Morphology of yeast-type and filament-type *Saccharomyces* cells.

The assignment of most of the traditional wine yeast strains to a single species does, however, not imply that all strains of *S. cerevisiae* are equally suitable for the various wine fermentations. These physiological strains of *S. cerevisiae* differ significantly in their fermentation performance and their ability to contribute to the final bouquet and quality of the various types of wine and distillates. Therefore, to ensure strain authenticity, security and proper strain management, it is of cardinal importance to have reliable taxonomic techniques available to identify and characterize individual

strains of commercial cultures, and to continue to characterize and select new isolates and to genetically improve these wine yeast strains for the production of premium quality wines (Pretorius, 1999).

Table 1.4 Diagnosis of the genus *Saccharomyces* Meyen ex Reess (adapted from Vaughan-Martini & Martini, 1998).

Vegetative reproduction is by multilateral budding.
Cells are globose, ellipsoidal or cylindroidal.
Pseudohyphae may be formed but not septate hyphae.
The vegetative phase is predominantly diploid (or of higher ploidy), conjugation occurs on or soon after germination of the ascospores; diploid ascospores may be formed.
Ascospores are globose to short ellipsoidal, with a smooth wall and usually one to four per ascus. Asci are persistent.
Vigorous fermentation of sugars.
Starch-like compounds are not produced.
Absence of growth with nitrate as a sole source of nitrogen.
Diazonium blue B reaction is negative.

Table 1.5 Key characteristics of species of the genus *Saccharomyces* (adapted from Vaughan & Martini, 1998).

Species	Fermentation			Assimilation									Growth				Fructose transport system
				Carbon source						N Source							
	Sucrose	Raffinose	Trehalose	Sucrose	Maltose	Raffinose	D-Ribose	Ethanol	D-Mannitol	Cadaverine 2HCl	Ethylamine HCl	L-Lysine	Cyclohexamide 1000[a]	30°C	37°C	Vitamin-free medium	
S. barnettii	+	+	s	+	-	+	-	-	-	-	-	-	-	-	-	-	n
S. bayanus	+	+	-	+	+	+	-	+	v	-	-	-	-	+	-	+	+
S. castellii	-	-	-	-	-	-	+/v	-	-	-	-	-	-	+	v	-	n
S. cerevisiae	+	+	-	+	+	+	-	+	-	-	-	-	-	+	v	-	-
S. dairenensis	-	-	-	-	-	-	v	v	-	-	-	-	-	+	v	-	n
S. exiguus	+	s	+	+	-	+	-	s	-	-	-	-	v	+	-	-	n
S. kluyveri	+	+	-	+	+	+	-	+	+	+	+	+	-	+	+	-	n
S. paradoxus	+	+	-	+	+	+	-	+	+	-	-	-	-	+	+	-	-
S. pastorianus	v	+	-	+	+	+	-	+	-	-	-	-	-	+	-	-	+
S. rosinii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	n
S. servazzii	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	n
S. spencerorum	+	-	+	+	-	-	-	-	-	+	+	+	-	+	+	-	n
S. transvaalensis	-	-	-	-	-	-	-	v	-	v	-	v	-	+	+	-	n
S. unisporus	-	-	-	-	-	-	-	+	-	+	+	+	+	+	v	-	n

[a] Indicates resistance to 1000 ppm cycloheximide in the medium.
n, not determined / no data; +, positive; -, negative; v, variable; s, positive but slow.

Table 1.6 Classification and reclassification of *Saccharomyces* species* between 1952 and 1998 as depicted in major taxonomic reference works during this period.

1952 classification (Lodder & Kreger-van Rij, 1952)	1970 classification (Lodder, 1970)	1984 classification (Kreger-van Rij, 1984)	1998 classification (Kurtzman & Fell, 1998)
S. bayanus	***S. bayanus*** (syn. ***S. beticus***, *S. cheriensis*, ***S. oviformis***, *S. pastorianus*)	***S. cerevisiae*** *	***S. bayanus***, *S. pastorianus*, ***S. cerevisiae***, *S. paradoxus*
S. oviformis			
S. pastorianus			
S. uvarum	*S. uvarum*		
S. carlsbergensis			
S. logos			
S. cerevisiae (syn. ***S. vini***)	***S. cerevisiae***		
S.c. var. ***ellipsoideus***			
S. willianus			
S. chevalieri	***S. chevalieri***		
S. fructuum			
S. italicus	*S. italicus*		
S. steineri			
S. heterogenicus	*S. heterogenicus*, *S. aceti*, ***S. capensis***, *S. coreanus*, *S. diastaticus*, *S. globosus*, *S. hienipiensis*, *S. inusitatus*, *S. norbensis*, *S. oleaceus*, *S. oleaginosus*, *S. prostoserdovii*		
S. exiguus	*S. exiguus*	*S. exiguus*	*S. barnettii*, *S. exiguus*, *S. spencerorum*
S. bailii	*S. bailii*	*Zygosaccharomyces bailii*	*Z. bailii*
S. acidifaciens			
S. elegans			
S. bisporus	*S. bisporus* var. *bisporus*	*Zygosaccharomyces bisporus*	*Z. bisporus*
S. mellis	*S. bisporus* var. *mellis*	*Zygosaccharomyces rouxii*	*Z. rouxii*
S. rouxii	*S. rouxii*		
S. rouxii var. *polymorphus*	*S. bailii* var. *osmophilus*		
S. delbrueckii	*S. inconspicuus*, *S. delbrueckii*, ***S. fermentati***, ***S. rosei***, *S. saitoanus*, *S. vafer*, *S. microellipsodes* var. *osmophilus*	***Torulaspora delbrueckii***	***T. delbrueckii***
S. fermentati (syn. ***S. beticus***)			
S. rosei			
S. marxianus	*Kluyveromyces marxianus*	*K. marxianus*	*K. marxianus*, *K. lactis*
S. fragilis	*Kluyveromyces fragilis*		
S. lactis	*Kluyveromyces lactis*		
S. veronae	*Kluyveromyces veronae*	*K. thermotolerans*	*K. thermotolerans*
	S. amurcae, *S. cidri*	*Zygosaccharomyces cidri*	*Z. cidri*
S. microellipsodes	*S. microellipsodes*	*Zygosaccharomyces microellipsoides*, *S. servazzii*	*Z. microellipsoides*, *S. servazzii*
S. pastori	*Pichia pastoris*	*Pichia pastoris*	*Pichia pastoris*
	S. dairenensis	*S. dairenensis*	*S. castellii*, *S. dairenensis*, *S. rosinii*
S. florentinus	*S. florentinus*, *S. eupagycus*	*Zygosaccharomyces florentinus*	*Z. florentinus*
	S. unisporus	*S. unisporus*	*S. unisporis*
	S. kluyveri	*S. kluyveri*	*S. kluyveri*
	S. telluris	*S. telluris*	*Arxiozyma telluris*
	S. kloeckerianus	*Torulaspora globosa*	*T. globosa*
	S. montanus	*Zygosaccharomyces fermentati*	*Z. fermentati*
	S. mrakii	*Zygosaccharomyces mrakii*	*Z. mrakii*
	S. transvaalensis	*Pachytichospora transvaalensis*	*S. transvaalensis*
	S. pretoriensis	*Torulaspora pretoriensis*	*T. pretoriensis*

*Author citations to the species are to be found in Lodder & Kreger-van Rij (1952), Lodder (1970), Kreger-van Rij (1984) and Kurtzman & Fell (1998a). (Meaning of abbreviations: syn. = synonym; var. = variety).

**Saccharomyces sensu stricto* have been separated into four species: *S. bayanus*, *S. cerevisiae*, *S. paradoxus* and *S. pastorianus*.

Table 1.7 Simplified list of synonyms to *Saccharomyces cerevisiae* and *Saccharomyces bayanus* (Kurtzman & Fell, 1998a).

Saccharomyces cerevisiae	
S. abuliensis	*S. hutensis*
S. aceti	*S. ilicis*
S. acidosaccharophillii	*S. intermedius* var. *turicensis*
S. agavica sylvestre	*S. italicus* var. *melibiosa*
S. anamensis	*S. joannae*
S. annulatus	*S. lindneri*
S. awamori	*S. logos*
S. batatae	*S. mandshuricus*
S. beticus*	*S. mangini* var. *miso*
S. boulardii	*S. marchalianus*
S. brasiliensis	*S. multisporus*
S. capensis*	*S. norbensis*
S. carbajali	*S. odessa*
S. carlsbergensis var. *alcoholophila, mandshuricus, monacensis, polymorphus, valdensis*	*S. oleaceus*
	S. oleaginosus
S. cartilaginosus var. *cartilaginosus, italiens*	*S. onubensis*
S. cerasi	***S. oviformis**** var. *bisporus , cheresiensis*
S. cerevisiae* var. *cratericus, ellipsoideus, festinans, fructuum, marchalianus, onychophilus, pelliculosa, pulmonalis, turbidans*	*S. oxidans*
	S. peka
	S. praecisus
S. cheresiensis	*S. prostoserdovii*
S. chevalieri* var. *lindneri*	*S. robustus*
S. chodati	*S. sake*
S. cordubensis	*S. shaoshing*
S. coreanus	*S. steineri*
S. cratericus	*S. thermantitonum*
S. diastaticus	*S. tokyo*
S. ellipsoideus* var. *major, umbra*	*S. turbidans*
S. elongatus	*S. uvarum* var. *carlsbergensis, melibiosus*
S. eryobotryae	*S. valesiacus*
S. festinans	*S. validus*
S. formosensis	***S. vini**** var. *cartilaginosus, cerevisiae*
S. fructuum	***S. vini-muntz****
S. gaditensis	*S. vordermanii*
S. hienipiensis	*S. willianus*
S. hispalensis	*S. yedo*
S. hispanica	
Saccharomyces bayanus	
S. abuliensis	
S. globosus	
S. heterogenicus	
S. intermedius var. *valdensis*	
S. inusitatus	
S. tubiformis	
S. uvarum	

*Author citations to the species and strains (var. = variety) are to be found in & Fell (1998a).
*Strains of *S. cerevisiae* that are frequently associated with winemaking are typed in bold.

1.1.3.4 Strain identification methods

Contrary to yeast taxonomists whose aims are to classify yeasts to species level, identification of individual strains is more the focus of wine microbiologists. Molecular genetic techniques can easily discriminate between wine yeast strains with similar physiological properties (Cavalieri *et al.*, 1998; Van der Westhuizen, Augustyn & Pretorius, 1999). The most frequently used molecular methods for wine yeast strain differentiation are listed in **Table 1.8**. For example, pulse-field electrophoretic karyotype analysis can be used to detect chromosome length polymorphisms (CLPs) in commercial yeast strains because each individual strain possesses its own and

characteristic banding pattern (**Fig. 1.3**). These fingerprinting techniques have also been used with great success in biological surveys of yeast strains isolated from grapes and musts, as well as in wine yeast breeding programmes (Van Vuuren & Van der Meer, 1987; Vézinhet, Blondin & Hallet, 1990; Yamamoto *et al.*, 1991; Van der Westhuizen & Pretorius, 1992; Naumov, Naumova & Gaillardin, 1993; Cavalieri *et al.*, 1998). Nevertheless, although such molecular identification approaches for wine yeasts are considered to be more objective, sensitive and reproducible compared with traditional morphological and biochemical tests, they are still not rapid enough to satisfy the needs of modern wine production operations (Walker, 1998).

Table 1.8 Molecular methods for wine yeast strain differentiation (adapted from Walker, 1998).

Method	Description
Chromatography	Pyrolysis-gas chromatography or gas chromatography (of long-chain fatty acid methyl esters).
Poly**a**crylamide **g**el **e**lectrophoresis (*PAGE*)	Total soluble yeast proteins are electrophoresed and banding patterns analysed by computer.
Restriction enzyme analysis (DNA fingerprinting)	Total, ribosomal or mitochondrial DNA is digested with restriction endonucleases and specific fragments hybridized after electrophoretic separation with multi-locus DNA probes such as the *Ty1* retrotransposon. **R**estriction **f**ragment **l**ength **p**olymorphisms (*RFLP*s) are detected.
Electrophoretic karyotyping (chromosome fingerprinting)	Whole yeast chromosomes are separated electrophoretically using pulse-field techniques; **c**hromosome **l**ength **p**olymorphisms (*CLP*s).
Polymerase **c**hain **r**eaction (*PCR*)	Specific DNA sequences are exponentially propagated *in vitro* and the amplified products analysed after electrophoretic separation. **R**andomly **a**mplified **p**olymorphic **D**NA can also be analysed by PCR (*RAPD-PCR*).
Genetic tagging	Specific genetic sequences, including selectable markers, are introduced into yeasts to facilitate their recognition (*e.g.* replacement of chloramphenicol resistance sequences with a 'tag' which confers sensitivity to the antibiotic).

1.2 IMPORTANCE OF YEAST BIODIVERSITY TO THE WINE INDUSTRY

Until quite recently, winemakers in *old world* wine producing countries relied on spontaneous fermentations because of the long-held belief that superior yeast strains associated with specific vineyards, gave a distinctive quality to wine (Martini, 1993). More recent work has indicated that the contribution of resident winery yeasts to wine aroma is far superior to that of the indigenous microflora present on the grapes (Rosini, 1984; Constanti *et al.*, 1997). This view is not shared by all. Whereas Török *et al.* (1996) dismiss the importance of resident winery yeasts, Heard (1999) notes that it is now recognized that yeasts from both the vineyard and winery environment are important in the fermentation of grape must. On the other hand, a better understanding of wine aroma composition has led to the notion that wine flavour is more closely linked to the accumulation of secondary metabolites by the grape (Abbott, Williams & Coombe, 1993 as quoted by Henschke, 1997). While the adage "*the best wines are made in the vineyard*" is undoubtedly true, it is also true that different yeasts contribute differently to the aroma and quality of the final product. It is

therefore not surprizing that there is an ever-increasing quest for new and improved wine yeast strains (for recent reviews see Pretorius & Van der Westhuizen, 1991; Barre *et al.*, 1993; Henschke, 1997; Pretorius, 1999).

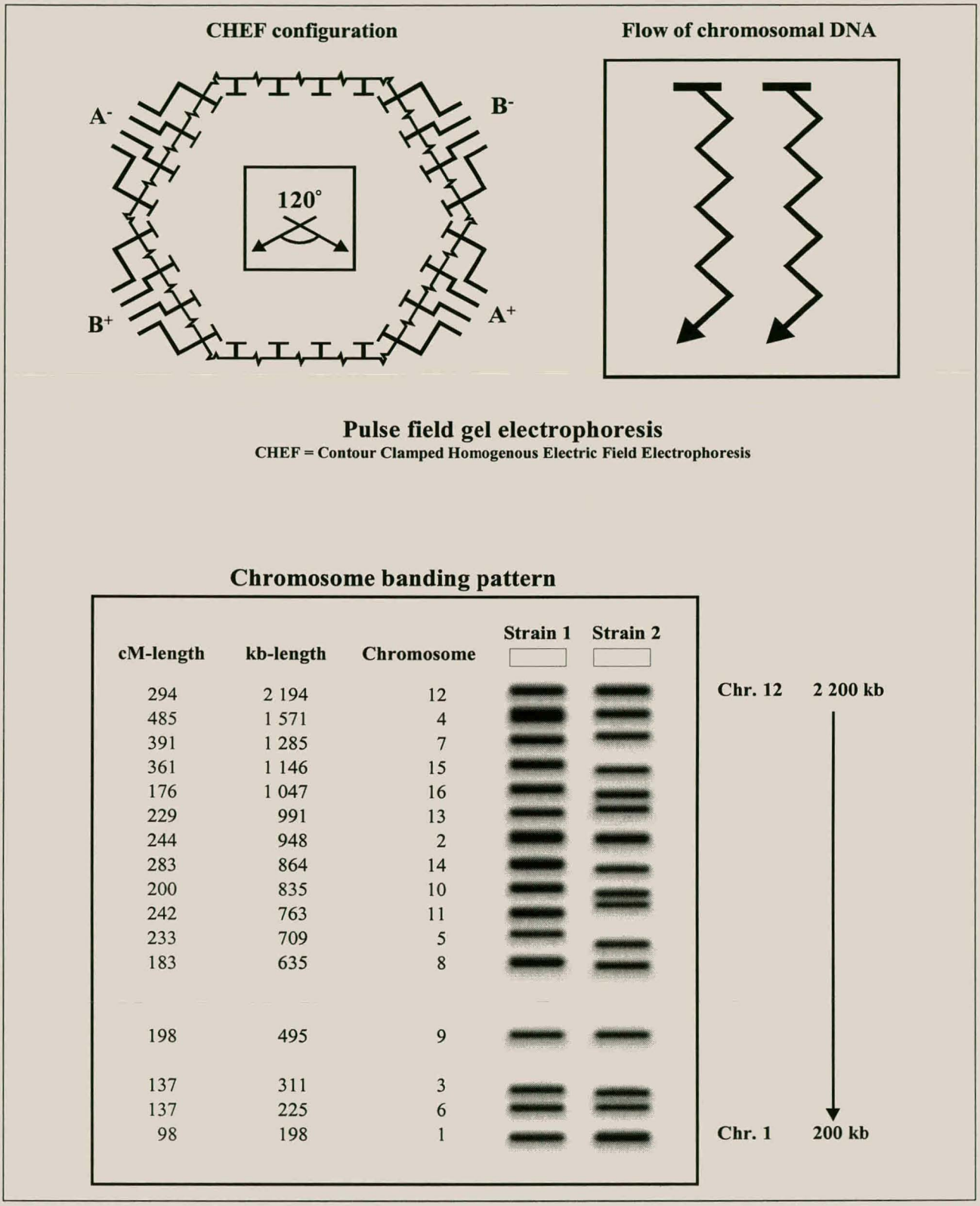


Figure 1.3 Pulse field electrophoretic karyotype analysis for the detection of chromosome length polymorphisms (CLPs) in wine yeast strains. The drawing depicts a schematic representation of the CHEF pulse-field gel electrophoresis system and the separation of the 16 chromosomes of *Saccharomyces* cells according to size. Each individual yeast strain possesses its own, and characteristic banding pattern.

Against this background, a comprehensive, long-term research programme has been launched by several microbiologists from the Wine and Fermentation Technology Division at the ARC-Fruit, Vine and Wine Research Institute, Nietvoorbij Centre for Vine and Wine, and the Institute for Wine Biotechnology at the University of Stellenbosch. The objectives of this research programme include the following:

(i) the systematic cataloging (isolation and characterization) of yeasts occurring in the wine-producing regions of the Western Cape in 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 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 antifungal 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 decade at annual sales in excess of 50 tons;

(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 our 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.

As part of this long-term programme, the specific aims of this dissertation included

(i) the evaluation of yeast fingerprinting techniques for their suitability to accurately and rapidly differentiate amongst *S. cerevisiae* strains;

(ii) the isolation and characterization of *S. cerevisiae* strains from the coastal regions of the Western Cape;

(iii) to determine the natural population dynamics of *S. cerevisiae* strains in selected vineyards over a four-year period;

(iv) to preliminary determine the affect of these indigenous *S. cerevisiae* isolates on wine flavour; and

(v) to breed new starter culture strains with improved characteristics.

This kind of biological survey complements the current international effort to assign a biological function to the products of each of the 6000 genes identified by computer analysis of the nucleotide sequence of the 16 chromosomes of a laboratory strain of *S. cerevisiae* (Goffeau *et al.*, 1996; Oliver, 1996; Mewes *et al.*, 1997). The challenge facing us is therefore a daunting one but not beyond the realms of possibility. This research programme will clearly run over a number of years before one would be

able to draw final conclusions and make recommendations to the wine industry. However, the potential benefits to the wine industry seem to make this tremendous task worthwhile.

1.3 LITERATURE CITED

ABBOTT, N., WILLIAMS, P.J. & COOMBE, B.G., 1993. Measure of potential wine quality by analysis of grape glycisides. In: Stockley, C.S. & Lee, T.H. (eds). *Proceedings of the eigth Australian wine industry technical conference.* Melbourne, Victoria. Winetitles, Adelaide. pp. 72-75.

ATLAS, R.M. & BARTHA, R., 1993. *Microbial ecology, fundamentals and applications.* Addison Wesley, Reading.

BARRE, P., VEZINHET, F., DEQUIN, S. & BLONDIN, B., 1993. Genetic improvement of wine yeast. In: FLEET, G.H. (ed.). *Wine microbiology and biotechnology.* Harwood Academic Publishers, Singapore. pp. 421-447.

BARNETT, J.A., 1992. The taxonomy of the genus *Saccharomyces* Meyen *ex* Reess – A short review for non-taxonomists. *Yeast* **8**, 1-23.

CAVALIERI, D., BARBERIO, C., CASALONE, E., PINZAUTI, F., SEBASTIANI, F., MORTIMER, R. & POLSINELLI, M., 1998. Genetic and molecular diversity in *Saccharomyces cerevisiae* natural populations. *Food Technol. Biotechnol.* **36**, 45-50.

CONSTANTI, M., POBLET, M., AROLA, L., MAS, A. & GUILLAMON, J.M., 1997. Analysis of yeast populations during alcoholic fermentation in a newly established winery. *Am. J. Enol. Vitic.* **48**, 339-344.

DEGRé, R., 1993. Selection and commercial cultivation of wine yeast and bacteria. In: FLEET, G.H. (ed.). *Wine microbiology and biotechnology.* Harwood Academic Publishers, Singapore. pp. 421-447.

DU PLESSIS, L. DE W., 1959. The study of the microorganisms associated with the flowers and ripening berries of a number of grape varieties (in Afrikaans). Thesis, University of Stellenbosch, Stellenbosch, South Africa.

FLEET, G.H., 1993. The microorganisms of winemaking – Isolation, enumeration and identification. In: FLEET, G.H. (ed.). *Wine microbiology and biotechnology.* Harwood Academic Publishers, Singapore. pp. 1-25.

FLEET, G.H. & HEARD, G.M., 1993. Yeasts – Growth during fermentation. In: FLEET, G.H. (ed.). *Wine microbiology and biotechnology.* Harwood Academic Publishers, Singapore. pp. 27-54.

FREZIER, V. & DUBOURDIEU, D., 1992. Ecology of yeast strain *Saccharomyces cerevisiae* during spontaneous fermentation in a Bordeaux winery. *Am. J. Enol. Vitic.* **43**, 375-380.

GOFFEAU, A., BARRELL, B.G., BUSSEY, H., DAVIS, R.W., DUJON, B., FELDMANN, H., GALIBERT, F., HOHEISEL, J.D., JACQ, C., JOHNSTON, M., LOUIS, E.J., MEWES, H.W., MURAKAMI, Y., PHILIPPSEN, P., TETTELIN, H., & OLIVER, S.G., 1996. Life with 6000 genes. *Science* **274**, 546-567.

GREUTER, W., BARRIE, F.R., BURDET, H.M., CHALONER, W.G., DEMOULIN, V., HAWKSWORTH, D.L., JøRGENSON, P.M., NICHOLSON, D.H., SILVA, P.C., TREHANE, P. & McNEIL, J. (eds), 1994. *International Code of Botanical Nomenclature.* Koeltz Scientific Books, Königstein.

HAWKSWORTH, D.L. & MOUCHACCA, J., 1994. Ascomycete systematics in the nineties. In: HAWKSWORTH, D.L. (ed.). *Ascomycete systematics – Problems and perspectives in the nineties.* Plenum Press, New York. pp. 3-11.

HEARD, G., 1999. Novel yeasts in winemaking – looking to the future. *Food Australia* **51**, 347-352.

HENSCHKE, P.A., 1997. Wine yeast. In: ZIMMERMANN, F.K. & ENTIAN, K.-D. (eds). *Yeast sugar metabolism.* Technomic Publishing Co., Lancaster, Pennsylvania. pp. 527-560.

KREGER-VAN RIJ, N.J.W., 1984. *The yeasts, a taxonomic study.* (3rd ed.). Elsevier Science Publishers, Amsterdam.

KURTZMAN, C.P. & FELL, J.W., 1998a. *The yeasts, a taxonomic study.* (4th ed.). Elsevier Science Publishers, Amsterdam.

KURTZMAN, C.P. & FELL, J.W., 1998b. Definition, classification and nomenclature of the yeasts. In: KURTZMAN, C.P. & FELL, J.W. (eds). *The yeasts, a taxonomic study.* (4th ed.). Elsevier Science Publishers, Amsterdam. pp. 3-5.

LACHANCE, M.-A. & STRAMER, W.T., 1998. Ecology and yeasts. In: KURTZMAN, C.P. & FELL, J.W. (eds). *The yeasts, a taxonomic study.* (4th ed.). Elsevier Science Publishers, Amsterdam. pp. 21-30.

LODDER, J.L., 1970. *The yeasts, a taxonomic study.* (2nd ed.). North-Holland Publ. Co., Amsterdam.

LODDER, J.L. & KREGER-VAN RIJ, N.J.W., 1952. *The yeasts, a taxonomic study.* North-Holland Publ. Co., Amsterdam.

MARTINI, A., 1993. The origin and domestication of the wine yeast *Saccharomyces cerevisiae. J. Wine Res.* **4**, 165-176.

MARTINI, A., CIANI, M. & SCORZETTI, G., 1996. Direct enumeration and isolation of wine yeasts from grape surfaces. *Am. J. Enol. Vitic.* **47**, 435-440.

MARTINI, A. & VAUGHAN-MARTINI, A., 1990. Grape must fermentation - Past and present. In: SPENCER, J.F.T. & SPENCER, B.M. (eds.). *Yeast technology.* Springer-Verlag, Berlin. pp. 105-123.

MEWES, H.W., ALBERMANN, K., BäHR, M., FRISHMAN, D., GLEISSNER, A., HANI, J., HEUMANN, K., KLEINE, K., MAIERL, A., OLIVER, S.G., PFEIFFER, F. & ZÖLLNER, A., 1997. Overview of the yeast genome. *Nature* **387**, 7-9.

MORTIMER, R.K., ROMANO, P., SUZZI, G. & POLSINELLI, M., 1994. Genome renewal: A new phenomenon revealed from a genetic study of 43 strains of *Saccharomyces cerevisiae* derived from natural fermentation of grape musts. *Yeast* **10**, 1543-1552.

NAUMOV, G., NAUMOVA, E & GAILLARDIN, C., 1993. Genetic and karyotypic identification of wine *Saccharomyces bayanus* yeasts isolated in France and Italy. *System. Appl. Microbiol.* **16**, 274-279.

OLIVER, S.G., 1996. From DNA sequence to biological function. *Nature* **379**, 597-600.

PHAFF, H.J., MILLER, M.W. & MRAK, E.M., 1978. *The life of yeasts.* (2nd ed.). Harvard University Press, Cambridge.

PEYNAUD, E. & DOMERCQ, 1959. A review on microbiological problems in wine making in France. *Am. J. Enol. Vitic.* **10**, 69-77.

PRETORIUS, I.S., 1999. Engineering designer genes for wine yeasts. *Aust. N.Z. Wine Indust. J.* **14**, 42-47.

PRETORIUS, I.S. & VAN DER WESTHUIZEN, T.J., 1991. The impact of yeast genetics and recombinant DNA technology on the wine industry: a review. *S. Afr. Enol. Vitic.* **12**, 3-31.

QUEROL, A., BORRIO, E. & RAMON, D., 1994. Population dynamics of natural *Saccharomyces* strains during wine fermentation. *Int. J. Food Microbiol.* **21**, 315-323.

ROBERTS, I.N. & WILDMAN, H.G., 1995. The diverse potential of yeast. *Bio/Technol.* **13**, 1246.

ROSINI, G. 1984. Assessment of dominance of added yeast in wine fermentation and origin of *Saccharomyces cerevisiae* in wine-making. *J. Gen. Appl. Microbiol.* **30**, 249-256.

SCHÜTZ, M. & GAFNER, J., 1994. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Lett. Appl. Microbiol.* **19**, 253-257.

TÖRÖK, T., MOTIMER, R.K., ROMANO, P., SUZZI, G. & POLSINELLI, M., 1996. Quest for wine yeasts - an old story revisited. *J. Industrial Microbiol.* **17**, 303-313.

VAN DER WESTHUIZEN, AUGUSTYN, O.P.H. & PRETORIUS, I.S., 1999. The value of long-chain fatty acid analysis, randomly amplified polymorphic DNA and electrophoretic karyotyping for the characterization of wine yeast strains. *S. Afr. J. Enol. Vitic.* **20**, 3-10.

VAN DER WESTHUIZEN, T.J. & PRETORIUS, I.S., 1992. The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. *Ant. v. Leeuwen.* **61**, 249-257.

VAN VUUREN, H.J.J. & VAN DER MEER, L., 1987. Fingerprinting of yeasts by protein electrophoresis. *Am. J. Enol. Vitic.* **38**, 49-53.

VAN ZYL, J.A, & DU PLESSIS, L. DE W., 1961. The microbiology of South African winemaking. Part 1. The yeast occurring in vineyards, musts and wines. *S. Afr. J. Agric. Sci.* **4**, 393-403.

VAUGHAN-MARTINI, A. & MARTINI, A., 1995. Facts, myths and legends on the prime industrial microorganism. *J. Indust. Microbiol.* **14**, 514-522.

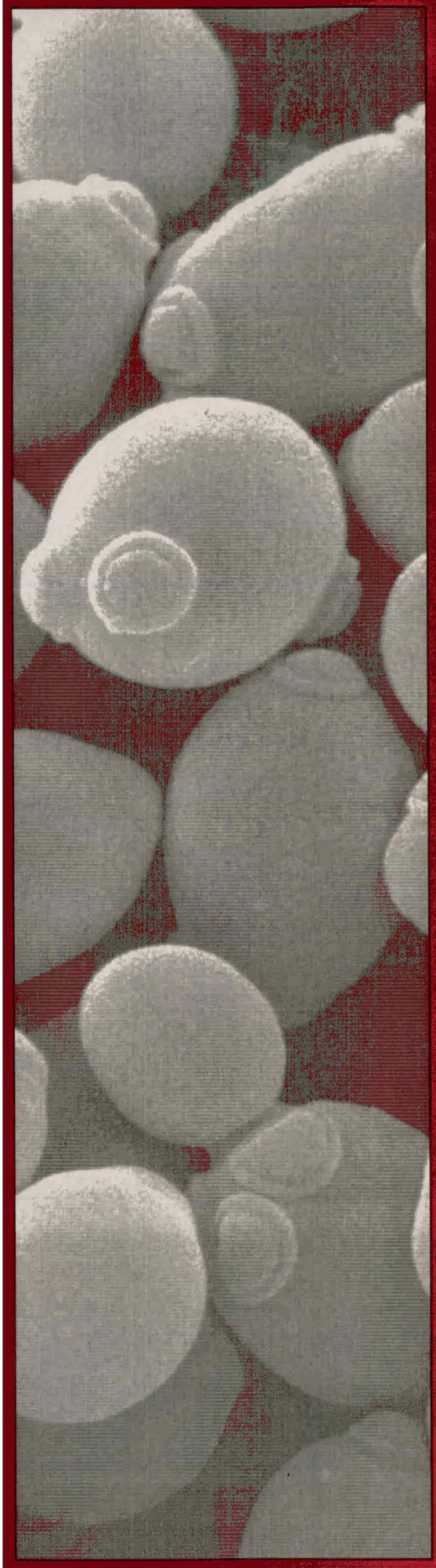
VAUGHAN-MARTINI, A. & MARTINI, A., 1998. *Saccharomyces* Meyen ex Reess. In: KURTZMAN, C.P. & FELL, J.W. (eds). *The yeasts, a taxonomic study.* (4th ed.). Elsevier Science Publishers, Amsterdam. pp. 358-371.

VERSAVAUD, A., COURCOUX, P., ROULLAND, C., DULAU, L. & HALLET, J.-N., 1995. Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strains from wine-producing area of Chanrentes, France. *Appl. Eviron. Microbiol.* **61**, 3521-3529.

VÉZINHET, F., BLONDIN, B. & HALLET, J.-N., 1990. Chromosomal DNA patterns and mitochondrial DNA polymorphism as tools for identification of enological strains of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **32**, 568-571.

WALKER, G.M., 1998. *Yeast physiology and biotechnology.* John Wiley & Sons, New York.

YAMAMOTO, N., YAMAMOTO, N., AMEMIYA, H., YOKOMORI, Y., SHIMIZU, K. & TOTSUKA, A., 1991. Electrophoretic karyotypes of wine yeasts. *Am. J. Enol. Vitic.* **42**, 358-363.



CHAPTER 2

Research Results I

The value of long-chain fatty acid analysis, randomly amplified polymorphic DNA and electrophoretic karyotyping for the characterization of wine yeast strains

(*S. Afr. J. Enol. Vitic.* 20. 3-10)

2. RESEARCH RESULTS I

The Value of Long-Chain Fatty Acid Analysis, Randomly Amplified Polymorphic DNA and Electrophoretic Karyotyping for the Characterization of Wine Yeast Strains

Abstract

Wine yeast strains of *Saccharomyces* had previously been classified into several different species or varieties. This classification system was based mainly on sugar fermentation and assimilation patterns. Subsequently, most of these species were reclassified as *Saccharomyces cerevisiae*. The assignment of the majority of wine yeast strains to a single species does, however, not imply that all strains of *S. cerevisiae* are equally suitable for wine fermentation. These physiological strains of *S. cerevisiae* differ significantly in their fermentation performance and their ability to contribute to the final bouquet and quality of the various types of wine and distillates. Therefore, to ensure strain authenticity, security and proper strain management, it is of cardinal importance to have reliable taxonomic techniques available to identify and characterize individual strains of commercial cultures. In this study, 18 commercial wine yeast strains were characterized in order to evaluate and compare three taxonomic techniques, namely long-chain fatty acid analysis, randomly amplified polymorphic DNA (RAPD) and electrophoretic karyotyping. As a single identification technique, electrophoretic karyotyping seems to be the most useful method for routine fingerprinting of wine yeast strains. However, we propose that the combined use of these three techniques provides the most reliable means of differentiating among commercial wine yeast strains.

2.1 INTRODUCTION

Many of the traditional taxonomic criteria (e.g., sugar fermentation and assimilation patterns) used for the speciation of yeasts were derived from the analysis of a small portion of the genome. These phenotypic characteristics still serve a useful purpose in classification, since not all of them are unstable and insignificant. Phenotypic traits, however, do not necessarily reflect genetic relatedness, since the same phenotype may be a result of convergent evolution. Conversely, the phylogenetic relationships should be reflected in similarities at the level of base composition of deoxyribonucleic acid (DNA) and DNA sequence homology as well as ribosomal RNA/DNA sequence relatedness (ribotypes) in different yeasts (Pretorius & Van der Westhuizen, 1991; Kurtzman & Fell, 1998).

Wine yeast strains of the genus *Saccharomyces* were traditionally classified into several different species or varieties, including *S. bayanus*, *S. beticus*, *S. capensis*

S. ellipsoideus, *S. fermentati*, *S. oviformis* and *S. vini* (Lodder & Kreger-van Rij, 1952; Lodder, 1970). A strong wine-type/-taxonomic relationship for the various wine yeasts was believed to exist. For example, strains of *S. beticus* and *S. capensis* were preferred for producing flor sherry because of their superior film-forming ability and desirable oxidative metabolism. Also, strains of *S. ellipsoideus* were generally preferred for making table (dry, still) wines, whereas strains of *S. bayanus* were chosen for making sparkling wines, because the latter strains were often more tolerant to alcohol and could ferment to a lower residual sugar concentration (Henschke, 1997). Using genetic taxonomic techniques, most of these strains were reclassified as *Saccharomyces cerevisiae* (Kreger-van Rij, 1984; Kurtzman & Fell, 1998). However, the assignment of most of these wine yeast strains to a single species does not imply that all strains of *S. cerevisiae* are equally suitable for wine fermentation. It is well known that these physiological strains of *S. cerevisiae* differ significantly in their fermentation performance and their ability to contribute to the final bouquet and quality of the various types of wine and distillates. Characterization and identification of wine yeasts to the strain level is therefore, of key importance to ensure strain authenticity, security and proper strain management.

Due to the complexity of the vinification process and the widespread practice of seeding must with dry commercial wine yeast cultures with favourable characteristics, it has become increasingly important to use an identifiable yeast strain, so as to ensure consistency of wine type, style and quality. The difficulty of identifying yeasts by standard microbiological methods prompted the development of a large number of different identification techniques. However, these techniques are not universally adept at differentiating amongst strains of the same species. As a result, a variety of additional methods of strain identification are in use i.e. fingerprinting of industrial strains by protein profiles (Van Vuuren & Van der Meer, 1987; Degré *et al.*, 1989; Van der Westhuizen & Pretorius, 1989, 1992), restriction analysis of genomic and mitochondrial DNA (Querol *et al.*, 1992; Van der Westhuizen & Pretorius, 1992; Versavaud *et al.*, 1995), electrophoretic karyotyping (Degré *et al.*, 1989; Yamamoto *et al.*, 1991; Bidenne *et al.*, 1992; Van der Westhuizen & Pretorius, 1992; Naumov, Naumova & Gaillardin, 1993; Grando & Calato, 1994; Kishimoto, Soma & Goto, 1994), randomly amplified polymorphic DNA analysis (Huffman, Molina & Jong, 1992; Ness *et al.*, 1993; Lalvallée *et al.*, 1994; De Barros Lopes *et al.*, 1995; Quesada & Cenis, 1995) and gas-liquid chromatographic analysis of the cellular fatty acids (Tredoux *et al.*, 1987; Augustyn, 1989; Augustyn & Kock, 1989; Rozes *et al.*, 1992). However, despite the variety of strain identification methods in use, there is no single method which is sufficiently reliable to consistently differentiate amongst strains of the same yeast species. It is therefore of key importance to compare and evaluate the various techniques in terms of their usefulness as routine aids for proper management and security of commercial wine yeast cultures.

This paper describes the characterization of 18 commercial wine yeast strains by comparing the three most commonly used differentiation techniques in the wine industry, *viz*. long-chain fatty acid analysis, randomly amplified polymorphic DNA (RAPD) and electrophoretic karyotyping. It is proposed that the use of these three techniques in combination gives a reliable method for yeast strain identification.

2.2 MATERIALS AND METHODS

2.2.1 YEAST STRAINS

The commercial wine yeast strains used in the study are listed in **Table 2.1**. All strains were purified and verified before use. Despite their considerable phenotypic differences, all these strains are considered to be physiological strains of *S. cerevisiae*. According to the yeast manufacturers, strains N96, Maurivin PDM and Zymaflore F10 are referred to as *S. bayanus.* However, it must be kept in mind that, according to the latest classification (Kurtzman & Fell, 1998), some of these strains might in fact be *S. cerevisiae* strains.

Table 2.1 Commercial yeast strains used in this study.

Strain	Yeast manufacturer/ distributor
VIN7	Anchor Yeast (Warren Chem)
VIN13	Anchor Yeast (Warren Chem)
WE14	Anchor Yeast (Warren Chem)
N96	Anchor Yeast (Warren Chem)
228	Anchor Yeast (Warren Chem)
WE372	Anchor Yeast (Warren Chem)
Levuline BRG	Groupe Oeno France
Fermol bouquet	Pascal Biotech (AEB Africa)
Maurivin AWRI 796	Maurivin (CJ Pedro Chemicals)
Fermivin cryo	Gist-brocades (Chemserve)
Actiflore Killer F5	Laffort (Vintec)
Maurivin PDM	Maurivin (CJ Pedro Chemicals)
Blastosel kappa	Perdomini Spa
Zymaflore F10	Laffort (Vintec)
Lalvin 71B	Lallemand (Protea chemicals)
Fermol Killer	Pascal Biotech (AEB Africa)
Fermirouge	Gist-brocades (Chemserve)
Enoferm Bordeaux Red	Lallemand (Protea chemicals)

2.2.2 FATTY ACID ANALYSIS

The yeast strains were cultivated, harvested and lyophilized, their long chain cellular fatty acids recovered, methylated and analyzed according to the techniques described by Augustyn & Kock (1989). The yeast strains were cultivated on a rotary shaker at 30°C in flasks equipped with a side arm to facilitate direct reading of the optical density in a Klett apparatus equipped with a 640-nm filter. Culture medium consisted of 80 g/l glucose and 6,7 g/l yeast nitrogen base (Difco). The preculture consisted of 40 ml culture medium (250-ml flasks) and organisms were cultivated for 16 h (minimum Klett reading of 190-200). Slow-growing yeast strains were left in the preculture until the required Klett reading was reached. For the second stage of the cultivation, 10 ml preculture was added to 300 ml culture medium in a 1-litre flask. Cultivation then proceeded for 48 h to ensure that organisms were harvested in the stationary phase. Growth was continually monitored on the Klett apparatus. Strains that had not entered stationary phase were left until that stage was reached. Cells

were harvested by centrifugation at 8 000 x g for 15 min at 4°C, the sediment washed once with cold saline solution and the lyophilized cells stored in glass bottles in a desiccator at -8 to –10°C. The lyophilized yeast cells (0,12 g) were mixed with 5 ml 2,5% KOH in 50% CH_3OH/H_2O and placed in a screw-capped (Teflon-lined) glass tube. After saturating the contents with N_2, the tube was sealed and heated for 1 h at 100°C with occasional shaking. After cooling to room temperature, nonsaponifiable material was extracted by shaking with two successive 5-ml aliquots of 1:4 $CHCl_3/C_6H_{14}$ and the extracts discarded. The reaction mixture was then acidified by the addition of 1,06 ml 32% HCl and free acids extracted by shaking with two successive 5-ml aliquots of 1:4 $CHCl_3/C_6H_{14}$. Combined extracts were evaporated to dryness in a clean glass tube, 2 ml 20% BF_3/CH_3OH added, the contents of the tube saturated with N_2 and the tube sealed and heated at 100°C for 5 min. After cooling to room temperature, 4 ml saturated NaCl solution was added and methyl esters extracted with three successive 2-ml aliquots of 1:4 $CHCl_3/C_6H_{14}$. Combined extracts were dried over anhydrous $MgSO_4$ (1 h) in a refrigerator and the liquid then decanted into a graduated centrifuge tube containing 1 ml anhydrous $MgSO_4$. After centrifugation at 1260 x g for 10 min, the clear extract was decanted, concentrated to 0,25 ml under a slow stream of N_2 and refrigerated until analyzed. A Varian 3700 gas chromatograph equipped with an FID detector and coupled to a DELTA integrating data system was used throughout. The instrumentation parameters were as follows: injector temperature 220°C, detector temperature 240°C, temperature program 150°C x 2°C/min to 190°C, hold 1 h. A J&W-DB-wax column (30 m x 0,32 mm id, coating 0,25 μm) was used with the carrier gas He at 1 ml/min, make-up gas He at 29 ml/min, split ratio 40:1, injection volume 1,5 μl. The mean relative percentages (MRPs) of nine fatty acids, *viz.* myristic acid (14:0), myristoleic acid (14:1), pentadecanoic acid (15:0), pentadecenoic acid (15:1), palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid [18:1(9)] and vaccenic acid [18:1(11)] were used to differentiate between the strains. Data were statistically analyzed using the SAS program.

2.2.3 RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Yeast cells were cultured and the DNA isolation was performed using the method as described by Van der Westhuizen & Pretorius (1992). Cells in the late logarithmic growth phase were harvested, washed in 1 ml 50 mM Tris, 50 mM EDTA, pH 8,0 and incubated with 0,1 ml 3 mg/ml lyticase (Sigma) at 37°C for 30 min. The pellet was resuspended in 0,4 ml 0,5 M EDTA, pH 8,0, and treated with 0,1 ml 20 mg/ml proteinase K (Boehringer Mannheim) and 1% final concentration sodium dodecyl sulphate (SDS) for 2 h at 37°C. One tenth volume of 5 M potassium acetate was added and the solution was left on ice, after mixing, until a white precipitate formed. The supernatant was treated with pancreatic RNase (heat-treated) for 1 h at 37°C. One volume of chloroform/isoamyl alcohol (24:1) was added, mixed and the aqueous layer was spun at 8 000 x g for 10 min. The upper layer was decanted and 2 volumes of cold 100% ethanol were gently added until a visible cocoon of DNA was formed. This was recovered, washed with 70% ethanol and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 7,5). To this, a 0,1 volume of 3 M sodium acetate (pH 7,0) was added and the DNA reprecipitated by adding a 0,54 volume of isopropanol. The purified DNA was dissolved in 50 μl TE (pH 7,5).

Polymerase chain reactions (PCR) were performed in a 50 µl volume and 1 U *Taq* polymerase (Promega Corporation, Madison, USA). PCR conditions were 3 min at 94°C followed by 35 cycles of 40 s at 94°C, 1 min at 37°C and 1 min at 72°C. Finally the mixture was heated at 72°C for 3 min and subsequently cooled to 4°C. Decamer oligonucleotides of random sequence were purchased from Operon Technologies (Alameda, CA). Twenty primers from the OPERON Kit C were used in the preliminary experiments to determine their differentiating ability. PCR products were separated using contour clamped homogeneous electric field (CHEF) electrophoresis. Gels were run for 5,5 h at a constant voltage of 200 V. The pulse duration was 1 s with no ramp. Gels were stained with ethidium bromide (10 mg/ml), viewed on a transilluminator and photographed.

2.2.4 SEPARATION OF INTACT CHROMOSOMAL DNA BY PULSE FIELD GEL ELECTROPHORESIS

Chromosomal DNA samples were prepared according to the embedded-agarose procedure of Carle & Olson (1985). Cells were cultivated to the late logarithmic growth phase in 100 ml YPD medium (1% yeast extract, 2% peptone and 2% glucose), harvested and washed twice with 20 ml 50 mM EDTA, pH 7,5 at 4°C. The final cell pellet was suspended in 3,25 ml 50 mM EDTA, pH 7,5. The cell suspension (3 ml) was mixed with 5 ml of 1% low-gelling temperature agarose (prepared in 0,125 M EDTA, pH 7,5, at 37°C) and 1 ml of solution I [prepared by mixing 10 ml SCE buffer (0,1 M sorbitol, 0,6 M sodium citrate, pH 5,8), 0,5 ml 2-mercaptoethanol and 10 mg of lyticase (Sigma)]. This mixture was poured into a Petri dish and allowed to gel. The gelled agarose was cut into 5 mm square plugs. The agarose plugs were placed in a McCartney bottle containing 5 ml of solution II (0,45 M EDTA, pH 9,0 / 10 mM Tris-HCl, pH 8,0 / 7,5% v/v 2-mercaptoethanol) and incubated overnight at 37°C. This solution was replaced with 5 ml of solution III [0,45 M EDTA, pH 9.0 / 10 mM Tris-HCl, pH 8,0 / 1% sodium N-lauroylsarcosinate / 1 mg of proteinase K (Boehringer Mannheim)] per ml. The bottles were placed in a 50°C waterbath for 48 h. Solution III was finally replaced with 5 ml of 0,5 M EDTA, pH 9,0 and stored at 4°C for further use.

Intact chromosomal DNAs were separated using the CHEF pulse field gel electrophoresis system. Thin sections of the DNA-agarose plugs were loaded into wells and sealed with 1% low melting temperature agarose prior to the run. The apparatus used was the CHEF-DR11 (Bio-Rad Laboratories, Richmond, USA). All separations were carried out in a 20 cm square, 6 mm deep, 1% agarose gel made in 0,5 x TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8,0) according to the electrophoretic conditions of Van der Westhuizen & Pretorius (1992). The average temperature of the 0,5 x TBE electrophoresis buffer was maintained at 14°C. Gels were run for 26 h at a constant voltage of 200 V. The pulse duration was 60 s for the first 15 h and 90 s for the last 11 h. Gels were stained with ethidium bromide (10 mg/ml), viewed on a transilluminator and photographed.

2.3 RESULTS AND DISCUSSION

2.3.1 FATTY ACID ANALYSIS

Discriminant analysis revealed that the MRPs of the nine fatty acids supplied sufficient data to differentiate amongst the 18 commercial wine yeast strains studied (Wills' lambda 0,001) (**Table 2.2**). This result corresponds to results generated by Augustyn (1989) and Augustyn & Kock (1989) who, respectively, differentiated amongst 13 and 46 *S. cerevisiae* strains using the MRPs of ten fatty acids.

Table 2.2 Mean* relative percentages of nine fatty acids in 18 commercial wine yeast strains.

Strain	Fatty acids								
	1 14:0	2 14:1(9)	3 15:0	4 15:1(9)	5 16:0	6 16:1(9)	7 18:0	8 18:1(9)	9 18:1(11)
VIN 7	2,05	0,45	0,45	0,20	16,90	41,10	4,25	31,85	1,85
VIN13	0,50	0,20	0,20	0,20	7,70	43,55	6,65	37,80	1,85
WE14	1,00	0,40	0,20	0,20	7,60	45,70	5,15	37,25	1,35
N96	0,80	0,20	0,45	0,20	12,75	52,45	5,15	34,60	2,15
228	0,65	0,25	0,30	0,25	9,55	44,40	6,95	34,25	1,85
WE372	0,70	0,20	0,20	0,10	10,20	42,30	6,65	36,80	1,75
Levuline BRG	0,50	0,10	0,10	0,10	6,50	30,60	9,40	48,70	1,90
Fermol bouquet	1,05	0,40	0,20	0,25	10,35	43,95	6,20	32,00	1,90
Maurivin AWRI 796	0,80	0,20	0,20	0,10	12,60	42,70	6,70	32,20	2,00
Fermivin cryo	0,80	0,25	0,25	0,15	10,30	40,55	4,50	39,20	1,20
Actiflore Killer F5	0,55	0,30	0,10	0,20	7,45	37,40	5,25	46,25	1,30
Maurivin PDM	0,75	0,45	0,15	0,20	6,95	46,90	5,10	36,40	1,25
Blastosel kappa	0,95	0,60	0,10	0,20	5,70	43,60	4,85	41,50	0,90
Zymaflore F10	1,10	0,30	0,01	0,10	9,15	37,00	4,70	41,20	1,10
Lalvin 71B	1,00	0,80	0,40	0,45	7,30	50,15	4,75	33,00	1,20
Fermol Killer	0,85	0,50	0,01	0,01	6,40	38,05	5,50	43,75	1,25
Fermirouge	0,90	0,50	0,20	0,25	7,45	48,25	5,05	33,35	1,15
Enoferm Bordeaux Red	0,90	0,50	0,20	0,30	7,10	44,60	4,00	40,15	0,95

*Mean of two replicates per strain.

Calculation of the "Pairwise General Squared Distances" between organisms indicated that whilst some strains were widely separated (e.g. VIN7/ Levuline BRG = 11 565,00), others were not (e.g. Maurivin PDM/ Fermirouge = 35,21). A plot of the first and second canonical variables (**Fig. 2.1**) clearly illustrates this close proximity of some strains. As the number of strains to be distinguished increases this close proximity will undoubtedly lead to overlap and therefore a loss of differentiating ability. This scenario is more likely in the case of large numbers of genetically closely related strains.

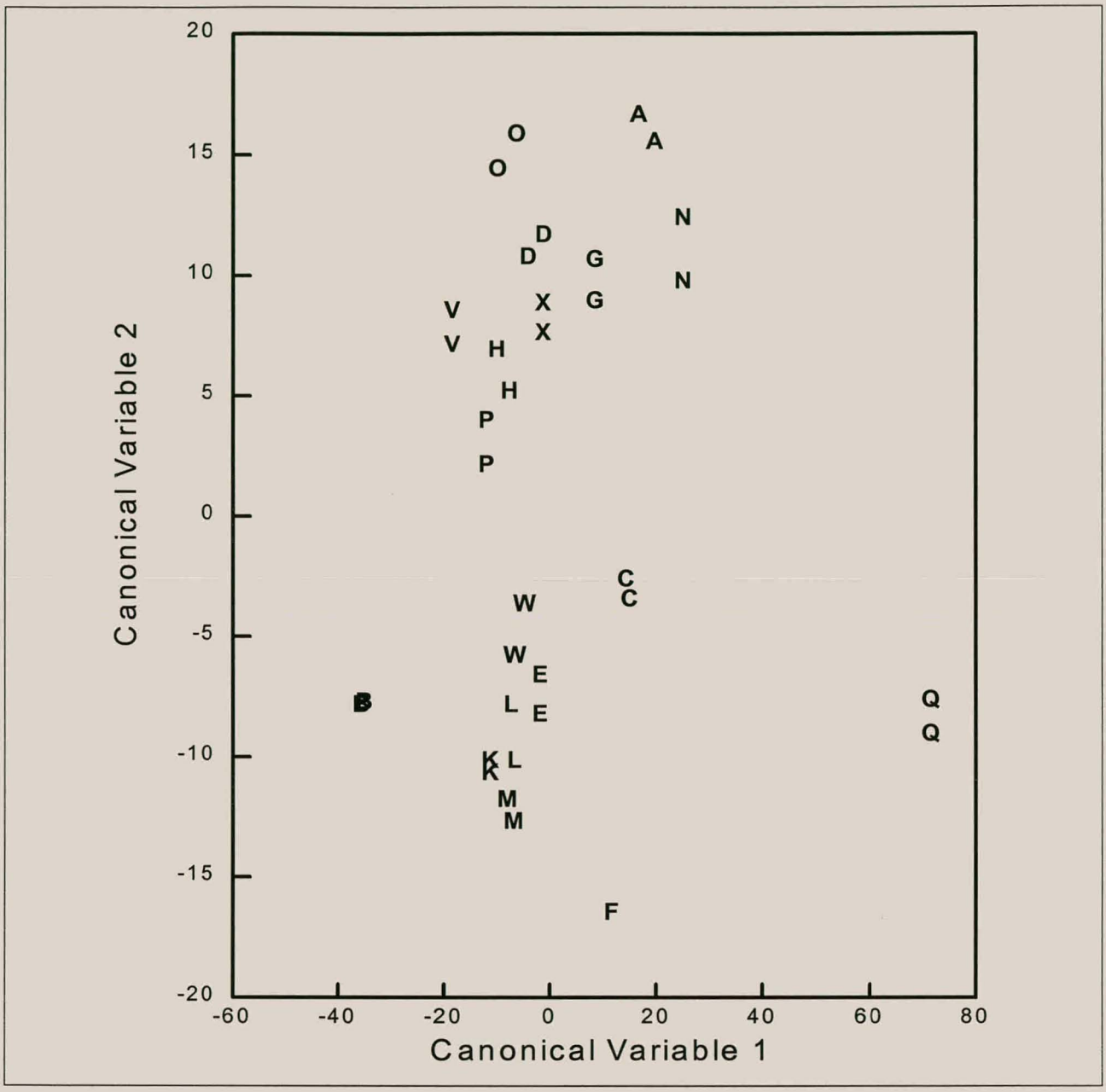


Figure 2.1. Canonical plot. Code for canonical plot: A = Maurivin AWRI 796; B = Levuline BRG; C = Fermivin cryo; D = Lalvin 71B; E = Enoferm Bordeaux Red; F = Zymaflore F10; G = Fermol bouquet; H = Fermirouge; K = Blastocel kappa; L = Actiflore killer F5; M = Fermol killer; N = N 96; O = 228; P = Maurivin PDM; Q = VIN 7; V = VIN 13; W = WE 14; X = WE 372.

Stepwise Discriminant Analysis indicated that in this study fatty acid 9 [18:1(11)] made no contribution to the differentiating ability of the fatty acid analysis technique. This acid was therefore not considered in the rest of this study. Fatty acids 5, 7 and 1 made the greatest contribution to the differentiating ability of the technique. Upon reclassification of the duplicates per strain using "Linear Discriminant Functions" calculated from data for only fatty acids 5, 7 and 1, only one of the duplicates for Lalvin 71B and WE14 exhibited a probability of less than 0,700 for reclassification as itself. In all other instances probability for correct reclassification exceeded 0,920 while for 22 of the 36 duplicates the probability was 1,000.

In general, the fatty acid analysis technique is reliable but time-consuming which makes it unsuitable as a routine characterization technique. However, this technique is still useful to verify ambiguous results obtained with other characterization techniques.

2.3.2 RAPD-PCR BANDING PATTERNS

Only some of the primers used in this study gave satisfactory results in amplification reactions with all the yeast strains studied. The amplification banding patterns obtained when using primer OPC-09 for the different strains are shown in **Fig. 2.2A** and **2.2B**. A considerable number of shared bands were generated for the different strains. It was apparent that one could not distinguish amongst the South African yeast strains, VIN13, WE14, N96, 228 and WE372, using this primer. No differences in their DNA banding patterns were found between Maurivin PDM and Blastosel kappa. Different RAPD-PCR profiles were generated for the rest of the strains. Results obtained with the OPC-13 primer indicated that strain Maurivin PDM and Blastosel kappa had different profiles (**Fig. 2.2C** and **2.2D**). Strains VIN13, WE14, N96, 228 and WE372 also displayed different banding patterns when using the OPC-13 primer. However, where it was possible to differentiate between strains, Fermol bouquet and Maurivin AWRI 796, using OPC-09, this was not possible when using OPC-13. It is interesting to see that both the PCR-profiles obtained for strains Blastosel kappa and Fermol killer were different, but the electrophoretic karyotypes were similar.

RAPD-PCR is a rapid technique that does not require prior knowledge of the genome sequence and reveals more polymorphisms. The main disadvantage is the time and effort needed to select an appropriate primer for differentiating a new strain from a group of previously characterized strains. Another problem of RAPD-PCR frequently cited is the poor reproducibility of some bands. There are many variables in the reaction that may contribute to this problem, including the different time-temperature responses of various types of thermal cyclers, the specific activity of commercial DNA-polymerase preparations, the concentration of DNA and primers, and the composition of the reaction buffer. However, with the correct standardization of the reaction variables, the problem can be minimized. The effect of some of these variables on the reproducibility of the bands was reported by MacPherson *et al.* (1993). It is therefore imperative to standardize the reaction parameters and perform replications of the same reaction on different days. The separation of the PCR-generated DNA fragments by CHEF pulse field gel electrophoresis is another important factor that contributes to clear differences between RAPD banding patterns thereby enhancing the differentiating power of this technique.

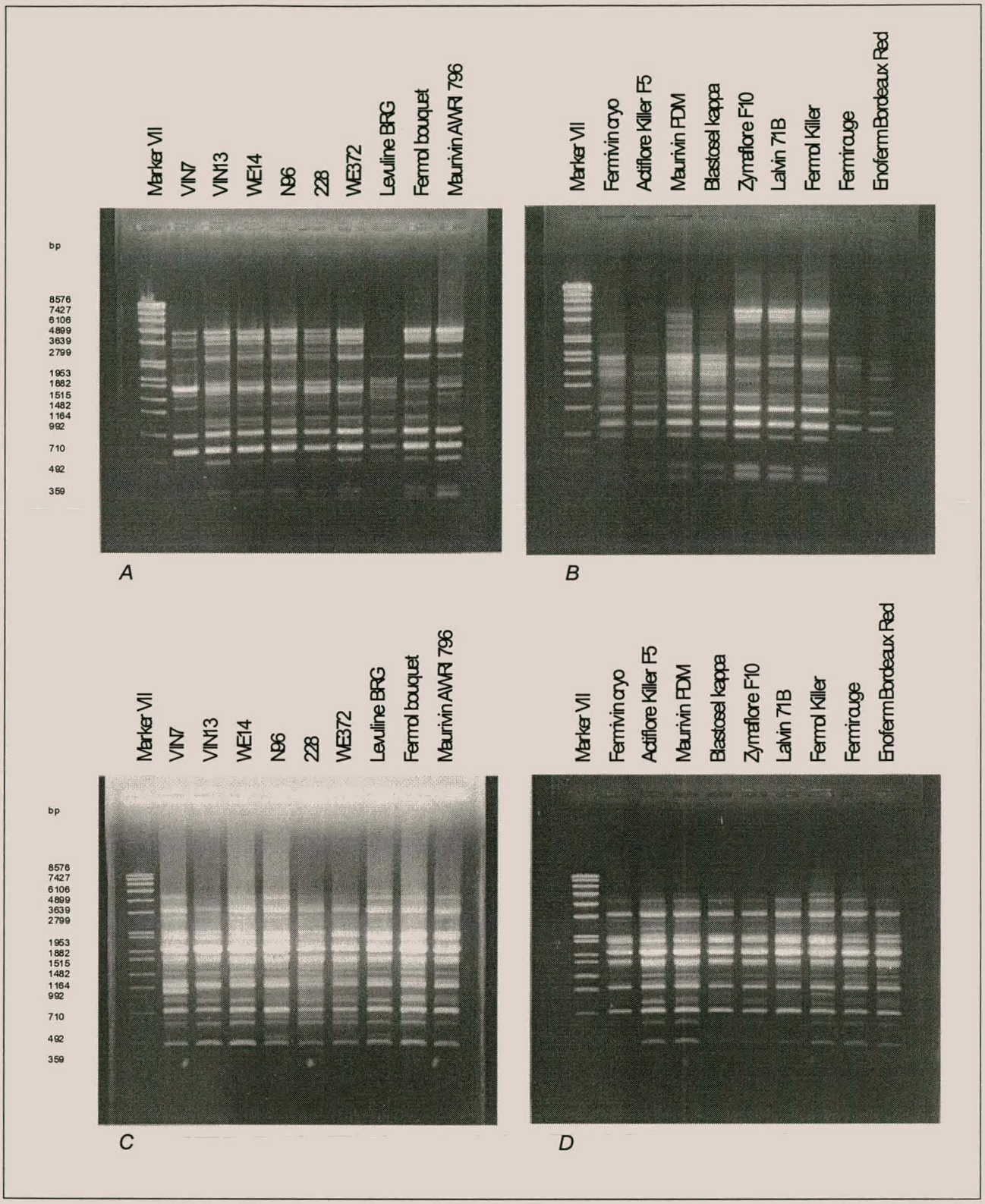


Figure 2.2 Patterns of amplified DNA obtained with different wine yeast strains in PCR. Primer OPC-09 (5'-CTCACCGTCC-3') was used in A and B, and primer OPC-13 (5'-AAGCCTGTCC-3') was used in C and D. Molecular weight marker VII (Boehringer Mannheim) was used as standard

2.3.3 ELECTROPHORETIC KARYOTYPING

The chromosomal banding patterns of the different yeast strains are depicted in **Fig. 2.3**. Almost all the strains had different karyotypes and the variations were apparent in the number, position and intensity of the bands. Identical profiles were obtained for only two of the strains, Blastosel kappa and Fermol killer. It was, however, possible to differentiate between these yeasts by means of RAPD-PCR (both primers) and long-chain cellular fatty acid analysis.

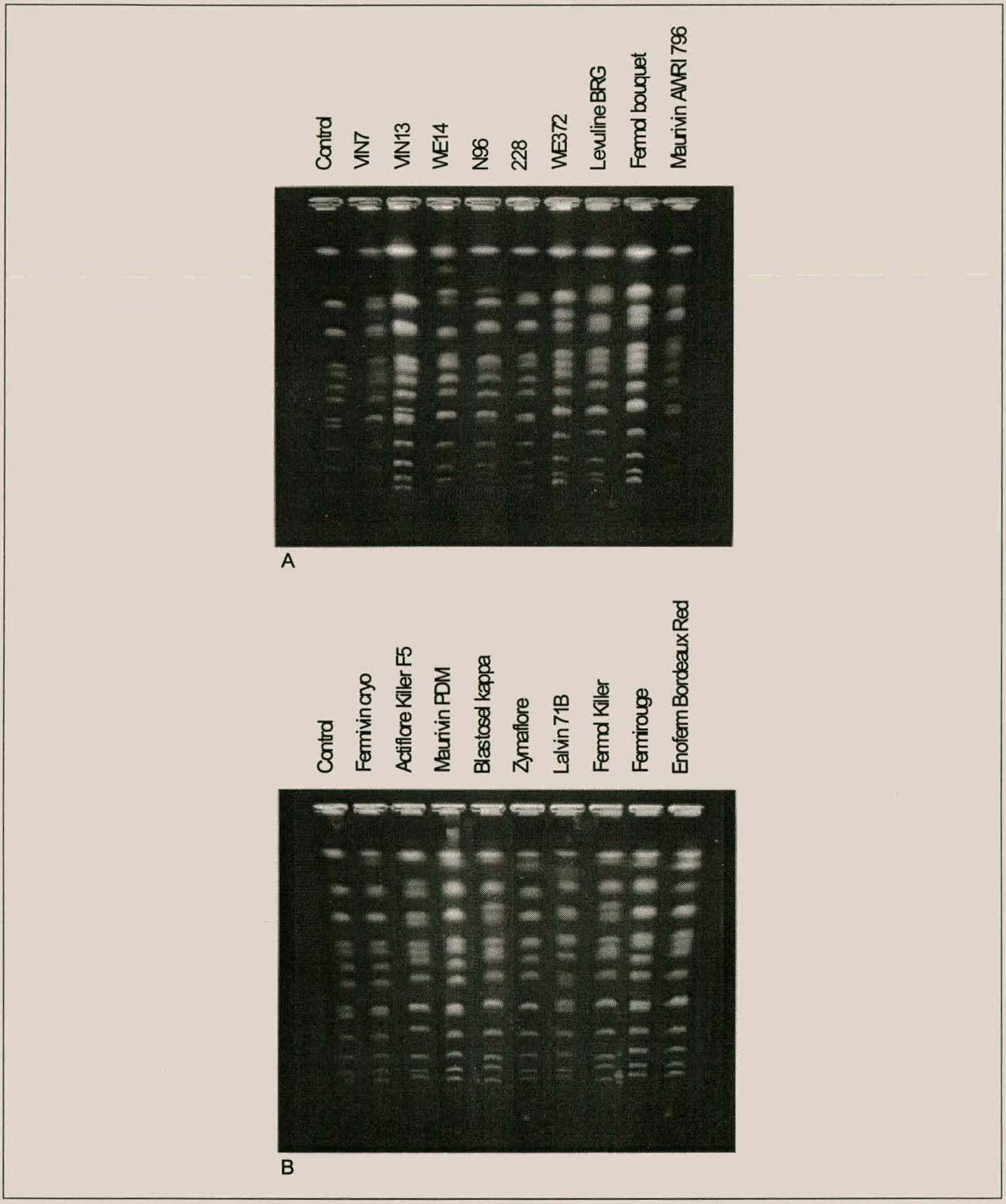


Figure 2.3 Electrophoretic karyotypes of commercial wine yeast strains.

The chromosome numbers of the 18 commercial wine yeast strains varied from 12 to 20. It is known that *Saccharomyces* strains contain the most chromosomes of all the yeast genera. This unique chromosome complexity of *S. cerevisiae* is advantageous from the viewpoint of this species being amenable to identification and fingerprinting by comparing chromosomal banding patterns. Although time-consuming, electrophoretic karyotyping is highly reliable and widely used to differentiate amongst wine yeast strains.

2.4 CONCLUSIONS

From practical experience, when using these three identification techniques and comparing them to one another in terms of accuracy, clarity and ease of interpreting results, reliability and reproducibility, time and cost effectiveness, as well as appropriateness for differentiating between a wide variety of commercial wine yeast strains, the following conclusions can be made:

(i) The combined use of these three techniques provides the most reliable means of differentiating amongst commercial wine yeast strains.

(ii) As a single identification technique, electrophoretic karyotyping seems to be the most useful method for routine fingerprinting of wine yeast strains and should therefore be used as the primary means of differentiating between these yeast strains and confirming their authenticity.

(iii) In cases of uncertainty, RAPD-PCR and/or long-chain fatty acid analysis could be used as back-up methods to verify the results obtained by electrophoretic karyotyping.

2.5 LITERATURE CITED

AUGUSTYN, O.P.H., 1989. Differentiation between yeast species, and strains within a species, by cellular fatty acid analysis. 2. *Saccharomyces cerevisiae. S. Afr. J. Enol. Vitic.* **10**, 8-17.

AUGUSTYN, O.P.H. & KOCK, J.F.L., 1989. Differentiation of yeast species, and strains within a species, by cellular fatty acid analysis. 1. Application of an adapted technique to differentiate between strains of *Saccharomyces cerevisiae*. *J. Microbiol. Meth.* **10**, 9-23.

BIDENNE, C., BLONDIN, B., DEQUIN, S. & VEZINHET, F., 1992. Analysis of the chromosomal DNA polymorphisms of wine yeast strains of *Saccharomyces cerevisiae*. *Curr. Genet.* **22**, 1-7.

CARLE, G.F. & OLSON, M.V., 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci. USA* **82**, 3756-3760.

DE BARROS LOPES, M., SODEN, A., LANGRIDGE, P. & HENSCHKE, P.A., 1995. Identification of wine yeast. *Proc. 7th Int. Confer. Yeast Genet. Mol. Biol.*, Lisbon, Portugal. (*Yeast* **11**, 14-51B Special Issue).

DEGRé, R., THOMAS, D.Y., ASH, J., MAILHIOT, K., MARIN, A. & DUBORD, C., 1989. Wine yeast strain identification. *Am. J. Enol. Vitic.* **40**, 309-315.

GRANDO, M.S. & COLATO, L., 1994. Polimorfismo del cariotipo elettroforetico in lieviti *Saccharomyces cerevisiae* di interesse enologico. *Vignivini* **5**, 57-61.

HENSCHKE, P.A., 1997. Wine yeast. In: ZIMMERMANN, F.K. & ENTIAN, K.-D. (eds). *Yeast sugar metabolism.* Technomic Publishing Co., Lancaster, Pennsylvania. pp. 527-560.

HUFFMAN, J.L., MOLINA, F.I. & JONG, S-C., 1992. Authentication of ATCC strains in the *Saccharomyces cerevisiae* complex by PCR fingerprinting. *Exper. Mycol.* **16**, 316-319.

KISHIMOTO, M., SOMA, E. & GOTO, S., 1994. Classification of cryophilic wine yeasts based on electrophoretic karyotype, G + C content and DNA similarity. *J. Gen. Appl. Microbiol.* **40**, 83-94.

KREGER-VAN RIJ, N.J.W., 1984. *The yeasts, a taxonomic study.* (3rd ed.). Elsevier Science Publishers, Amsterdam.

KURTZMAN, C.P. & FELL, J.W., 1998. *The yeasts, a taxonomic study*. (4th ed.). Elsevier Science Publishers, Amsterdam.

LODDER, J.L. & KREGER-VAN RIJ, N.J.W., 1952. *The yeasts, a taxonomic study.* (1st ed.). North-Holland Publ. Co., Amsterdam.

LODDER, J.L., 1970. *The yeast, a taxonomic study.* (2nd ed.). North-Holland Publ. Co., Amsterdam.

LALVALLÉE, F., SALVAS, Y., LAMY, S., THOMAS, D.Y., DEGRÉ, R. & DULAU, L., 1994. PCR and DNA fingerprinting used as quality control in production of wine yeast strains. *Am J. Enol. Vitic.* **45**, 86-91.

MACPHERSON, J.M., ECKSTEIN, P.E., SCOLES, G.J. & GAJADHAR, A.A., 1993. Variability of the randomly amplified polymorphic DNA assay among thermal cyclers, and effects of primer and DNA concentration. *Mol. Cell. Probes* **7**, 293-299.

NAUMOV, G., NAUMOVA, E. & GAILLARDIN, C., 1993. Genetic and karyotypic identification of wine *Saccharomyces bayanus* yeasts isolated in France and Italy. *System. Appl. Microbiol.* **16**, 274-279.

NESS, F., LALVALLÉE, F., DUBOURDIEU, D., AIGLE, M. & DULAU, L., 1993. Identification of yeast strains using polymerase chain reaction. *J. Sci. Food Agric.* **62**, 89-94.

PRETORIUS, I.S. & VAN DER WESTHUIZEN, T.J., 1991. The impact of yeast genetics and recombinant DNA technology on the wine industry: a review. *S. Afr. J. Enol. Vitic.* **12**, 3-31.

QUEROL, A., BARRIO, E., HUERTA, T. & RAMON, D., 1992. Molecular monitoring of wine fermentations conducted by active dried yeast strains. *Appl. Environ. Microbiol.* **58**, 2948-2953.

QUESADA, M.P. & CENIS, J.L., 1995. Use of randomly amplified polymorphic DNA (RAPD-PCR) in the characterization of wine yeasts. *Am. J. Enol. Vitic.* **46**, 204-208.

ROZES, N., GARCIA-JONES, C., LARUE, F. & LONVAUD-FUNEL, A., 1992. Differentiation between fermenting and spoilage yeasts in wine by total free fatty acid analysis. *J. Sci. Food Agric.* **59**, 351-357.

TREDOUX, H.G., KOCK, J.F.L., LATEGAN, P.M. & MULLER, H.B., 1987. A rapid identification technique to differentiate between *Saccharomyces cerevisiae* strains and other yeast species in the wine industry. *Am. J. Enol. Vitic.* **38**, 161-164.

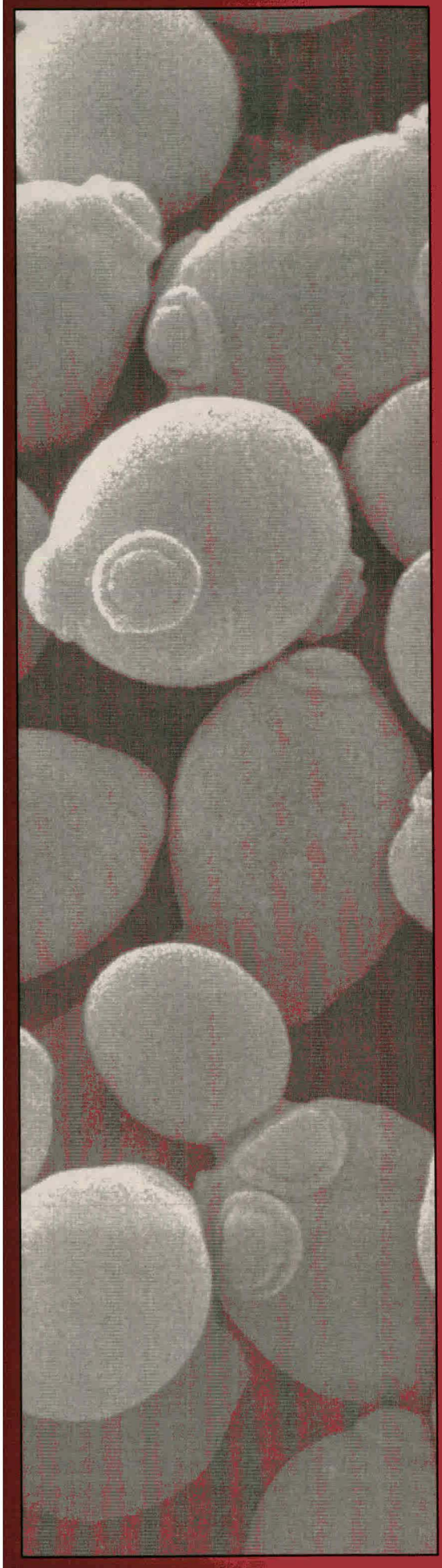
VAN DER WESTHUIZEN, T.J. & PRETORIUS, I.S., 1989. Genetic characterization and breeding of wine yeasts. *S. Afr. Soc. Microbiol. Cong. 6th, Stellenbosch* (pp 15.15).

VAN DER WESTHUIZEN, T.J. & PRETORIUS, I.S., 1992. The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. *Anton. v. Leeuwen.* **61**, 249-257.

VAN VUUREN, H.J.J. & VAN DER MEER, L., 1987. Fingerprinting of yeasts by protein electrophoresis. *Am. J. Enol. Vitic.* **38**, 49-53

VERSAVAUD, A., COURCOUX, P., ROULLAND, C., DULAU, L. & HALLET, J.-N., 1995. Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strains from the wine-producing area of Chanrentes, France. *Appl. Eviron. Microbiol.* **61**, 3521-3529.

YAMAMOTO, N., YAMAMOTO, N., AMEMIYA, H., YOKOMORI, Y., SHIMIZU, K. & TOTSUKA, A., 1991. Electrophoretic karyotypes of wine yeasts. *Am. J. Enol. Vitic.* **42**, 358-363.



CHAPTER 3

Research Results II

Geographic distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the coastal regions of the Western Cape in South Africa

(*S. Afr. J. Enol. Vitic.*, in press)

3. RESEARCH RESULTS II

Geographic Distribution of Indigenous *Saccharomyces cerevisiae* Strains Isolated from Vineyards in the Coastal Regions of the Western Cape in South Africa

Abstract

Notwithstanding numerous studies on the yeast biota of grapes and grape must, the origin of the primary wine yeast *Saccharomyces cerevisiae* has been rather controversial. One school of thought claims that the primary source of *S. cerevisiae* is the vineyard, whereas another believes that ecological evidence points to a strict association with artificial, man-made environments such as wineries and fermentation plants. One of the main thrusts of these kind of investigations is to understand the succession of yeasts during fermentation of wine and to determine the actual contribution of indigenous strains of *S. cerevisiae* and wild yeast species to the overall sensorial quality of the end-product, even in guided fermentations using selected *S. cerevisiae* starter cultures. There is increasing interest in the wine community for the use of indigenous strains of *S. cerevisiae* and mixed starter cultures, tailored to reflect the characteristics of a given region. Against this background, we have launched a comprehensive and long overdue, biogeographical survey systematically cataloging yeasts in different climatic zones of the 350-year-old wine-producing regions of the Western Cape. The present paper represents the first phase of this programme aimed to preserve and exploit the hidden oenological potential of the untapped yeast biodiversity in South Africa's primary grape-growing areas. Grapes were aseptically harvested from 13 sites in five areas in the coastal regions of the Western Cape. After fermentation, 30 yeast colonies per sample were isolated and examined for the presence of *S. cerevisiae*. Five sampling sites yielded no *S. cerevisiae*. CHEF-DNA analysis revealed the presence of 46 unique karyotypes in eight of the remaining sites. No dominant strain was identified and each site had its own unique collection of strains. The number of strains per site varied from two to 15. Only in four cases did one strain appear at two sites, while only one instance of a strain occurring at three sites was recorded. All sites contained killer and sensitive strains, however, killer strains did not always dominate. Commercial strains were recovered from three sites. Although commercial yeasts dominated the micro flora at two sites, it appears that fears of commercial yeasts ultimately dominating the natural microflora seem to be exaggerated.

3.1 INTRODUCTION

According to Jemec *et al.* (1997), the biotransformation of grape juice into wine cannot be viewed as a simple biochemical process since it is a complex heterogenous microbiological process involving the sequential development of various yeasts and other microbial species, as affected by a particular environment.

This statement accurately summarizes the findings of numerous papers as reviewed by Bisson & Kunkee (1991), Fleet & Heard (1993), Henschke (1997) and Pretorius, van der Westhuizen & Augustyn (1999), amongst others.

From the above it has also become clear that strains of *Saccharomyces cerevisiae* are rarely isolated from grapes (Van Zyl & Du Plessis, 1961; Benda, 1964; Parish & Carroll, 1985; Martini, Ciani & Scorzetti, 1996). On the other hand, strains of this species predominate amongst the microflora resident on different surfaces in the winery (Peynaud & Domercq, 1959; Rosini, 1984). These resident yeasts, and particularly the *S. cerevisiae* strains, should therefore play an important role in, or even dominate, spontaneous fermentations. The importance of resident yeasts is well illustrated by the work of Constanti *et al.* (1997). These authors reported the almost complete take-over of an inoculated fermentation by a yeast resident in a two-year old Spanish winery. Clearly, analysis of the yeast present in juice prepared in a winery will not reflect the true composition of the microflora present in the vineyard.

High-risk spontaneous fermentations have largely been replaced by a more controlled process utilizing one, or more, commercially prepared active dried wine yeast preparation(s). This is particularly true in South Africa where spontaneous fermentations are a rarity. However, recurrent fermentation problems experienced during the mid 1970's to late 1980's resulted in the launch of an extensive wine yeast selection and hybridization programme aimed at producing new yeasts better adapted to local fermentation conditions.

Apart from some early work (Du Plessis, 1959; Van Zyl & Du Plessis, 1961), the composition of vineyard microflora in South Africa has received no attention at all. Furthermore, there is growing interest in the wine community for the use of indigenous strains of *S. cerevisiae* and mixed starter cultures, tailored to refelect the characteristics of a given region (Heard, 1999). This fact, coupled to specific needs of the local wine industry and the current emphasis on the preservation of all forms of genetic biodiversity, resulted in the expansion of the natural wine yeast selection programme to encompass the goals set out in Pretorius *et al.* (1999).

The aim of this study, as part of the programme mentioned above, was to determine the natural distribution of *S. cerevisiae* strains in the coastal vineyards of the Western Cape in South Africa.

3.2 MATERIALS AND METHODS

3.2.1 AREAS SAMPLED

Vineyards were sampled in the following areas during the 1995 harvest: Constantia (2 farms), Stellenbosch (4 farms), Somerset West (1 farm), Elgin/Bot River (4 farms) and Hermanus (2 farms) (**Fig. 3.1**). Sampling sites are identified in **Table 3.1**.

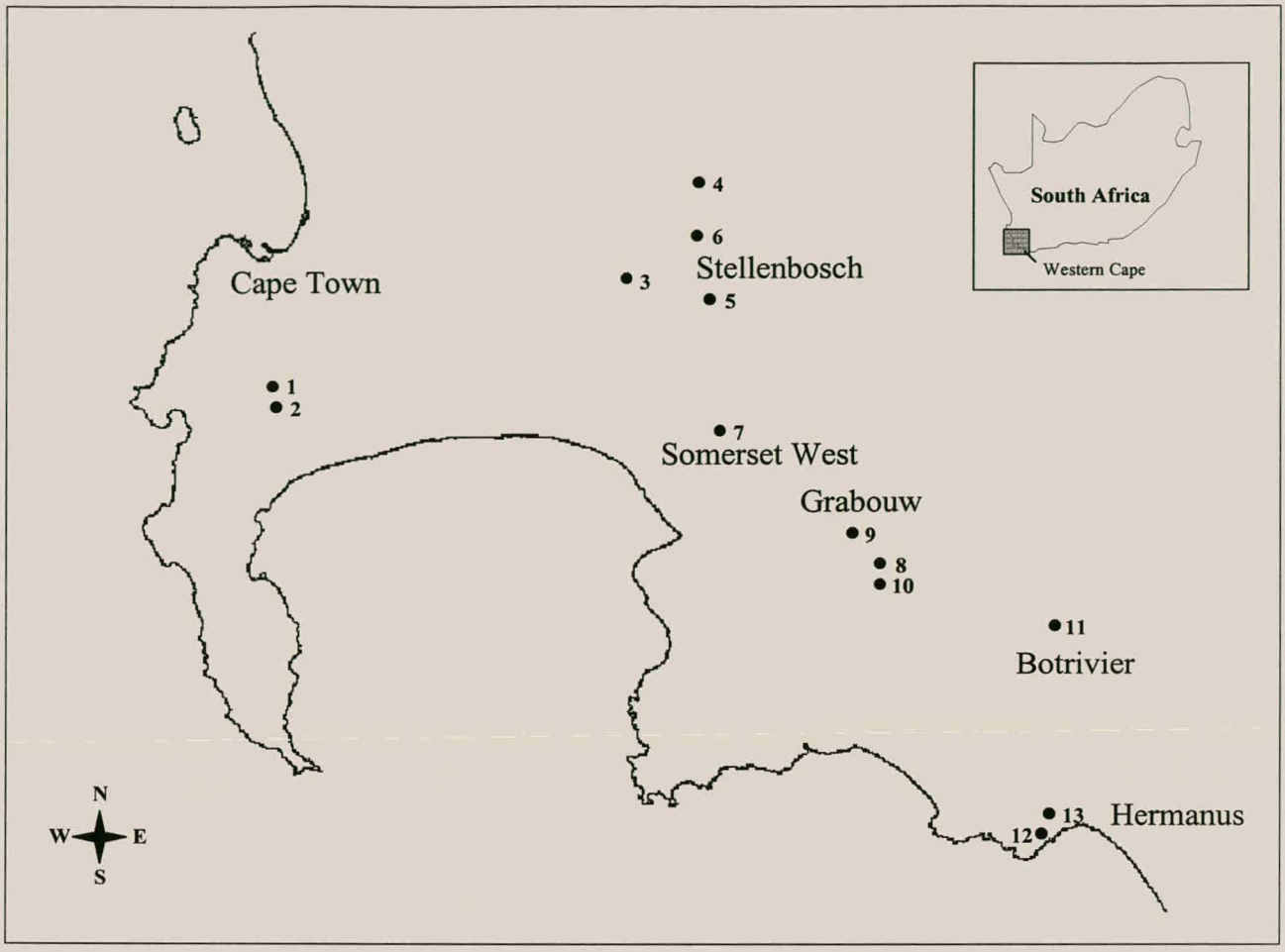


Figure 3.1 Location of sampling sites (listed in **Table 3.1**) in five different areas in the coastal regions of the Western Cape, South Africa.

3.2.2 SAMPLE COLLECTION AND YEAST ISOLATION

Whole clusters [3-4 kg of bunches per sampling site, gathered from 10-15 vines of white varieties (Chardonnay, Chenin blanc, Sauvignon blanc and Riesling) at sugar levels of above 20°Brix] were gathered aseptically and dropped directly into sterile plastic bags. The tightly sealed plastic bags were transported to the laboratory in cool bags. At the laboratory grapes were crushed by hand in the still tightly sealed plastic bags. After thorough shaking, the bags were opened and the juice (500 ml) poured into 750 ml sterile bottles which were immediately sealed by affixing sterile fermentation caps. The bottles were then placed in a dark, temperature-controlled room (15°C). Progress of fermentation was determined by measuring mass loss. Samples were taken from successful fermentations when residual fermentable sugar was less than 4 g/l. Before withdrawing samples, bottles were shaken to thoroughly mix the contents and get all organisms in suspension. Bottles in which fermentation was not so successful (high residual sugar) were sampled after 80 days. Each of these samples was streaked on 10 Petri dishes containing YPD agar medium (1% yeast extract, 2% peptone and 2% glucose) and incubated at 30°C for 3 days to allow colony formation. Thirty colonies were randomly selected (three from each of the 10 Petri dishes) and plated individually. Cultures were stored at 4°C until further analysis. Yeasts (30 isolates per site) were characterized on the basis of killer activity, galactose utilization and pulse field gel electrophoresis.

Table 3.1 Distribution of natural *Saccharomyces cerevisiae* strains in the Western Cape region.

Area	Farm	Fermentation time* (days)	Strain	Number out of 30	Percentage
Constantia	Groot Constantia (1)	63	C5-1	14	47%
			C5-2	8	26%
			C5-3 = B5-6 = F5-3	6	20%
			C5-6		7%
	Buitenverwachting (2)	38	B5-1	1	3%
			B5-2	1	3%
			B5-3	3	10%
			B5-4	1	3%
			B5-5 = HR5-7	2	7%
			B5-6 = C5-3 = F5-3	2	7%
			B5-7	2	7%
			B5-8	1	3%
			B5-9	1	3%
			B5-10	1	3%
			B5-11	1	3%
			B5-12	1	3%
			B5-13	4	13%
			B5-14	8	29%
			B5-15	1	3%
Stellenbosch	Jordan (3)	55	J5-1 (VIN13)	23	77%
			J5-2	1	3%
			J5-3	5	17%
			J5-4	1	3%
	Lievland (4)	31	L5-1	1	3%
			L5-2 (VIN13)	5	17%
			L5-3 (N96)	24	80%
	Mont Fleur (5)	27	M5-1	3	10%
			M5-2	10	34%
			M5-3	2	7%
			M5-4	1	3%
			M5-5	2	7%
			M5-6	7	23%
			M5-7	1	3%
			M5-8	1	3%
			M5-9	3	10%
	Nietvoorbij (6)	80	-	0	0%
Somerset West	Vergelegen (7)	36	V5-1 = HR5-10	28	94%
			V5-2	2	6%
Elgin/Bot River	De Rust (8)	80	-	0	0%
	Oak Valley (9)	80	-	0	0%
	White Hall (10)	80	-	0	0%
	Wildekrans (11)	80	-	0	0%
Hermanus	Bouchard Finlayson (12)	61	F5-1 = HR5-4	2	7%
			F5-2	26	87%
			F5-3 = C5-3 = B5-6	1	3%
			F5-4	1	3%
	Hamilton Russell (13)	49	HR5-1	3	10%
			HR5-2 (VIN7)	2	7%
			HR5-3	5	17%
			HR5-4 = F5-1	3	10%
			HR5-5	2	7%
			HR5-6	1	3%
			HR5-7 = B5-5	4	13%
			HR5-8	2	7%
			HR5-9	2	7%
			HR5-10	5	17%

*Time required to complete natural fermentation prior to yeast isolation. Incomplete fermentations (sites 6, 8, 9, 10 and 11) were sampled after 80 days yielding only non-*Saccharomyces* yeasts.

3.2.3 DETERMINATION OF KILLER ACTIVITY

Methylene blue agar plates, buffered at pH4,5, were used to detect zones of growth inhibition caused by the K_2 toxin (zymocin) secreted by killer yeasts. The strain designated as Geisenheim was used as sensitive lawn, and two South African commercial wine yeast strains (produced by Anchor Yeast in Cape Town), N96 (killer-positive) and VIN7 (killer-negative), as controls. Methylene blue plates were incubated for 48 h at 25°C and then examined to note killer activity.

3.2.4 GALACTOSE UTILIZATION

Galactose-utilizing strains were identified by the presence of yellow halos on YPGB medium containing 1% yeast extract, 2% peptone, 2% galactose and 2% bromthymol blue (4 mg/ml). Results of the galactose test were determined after 24 h incubation at 30°C.

3.2.5 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 DNAs were separated using contour clamped homogenous electric field (CHEF) electrophoresis (CHEF-DR11, Bio-Rad Laboratories, Richmond, USA). All separations were carried out in 1% agarose gels according to the electrophoretic conditions of Van der Westhuizen & Pretorius (1992) as applied by Van der Westhuizen, Augustyn & Pretorius (1999). Gels were stained with ethidium bromide (10 mg/ml), viewed on a transilluminator and then photographed.

A standard reference strain was used on each CHEF gel as three gels were needed to characterize the 30 isolates per site. The banding pattern of each yeast isolate was digitized and compared to all the other patterns using a customized computer program. Computer and visual data were used as primary criteria when comparing strains. Final results were confirmed by running additional gels.

3.2.6 RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Yeast cells were cultured and the DNA isolation was performed using the method as described by Van der Westhuizen & Pretorius (1992). Polymerase chain reactions (PCR) were performed with primer OPC-09 (5'-CTCACCGTCC-3') as applied by Van der Westhuizen *et al.* (1999). RAPD-PCR analysis was only carried out to verify that those yeast isolates that display identical electrophoretic karyotypes are indeed the same.

3.3 RESULTS AND DISCUSSION

3.3.1 SAMPLE PREPARATION AND FERMENTATION

Aseptic harvesting of grapes and preparation of juice avoided the contamination of samples by yeasts, not resident on the grape samples. The simple juice preparation technique followed here was considered adequate in spite of the results generated

by Martini, Frederici & Rosini (1980). These authors indicated that complete recovery of all micro-organisms associated with many natural surfaces required aggressive recovery techniques such as sonication. Bisson & Kunkee (1991), however, pointed out that although Martini *et al.* (1980) did recover greater quantities of micro-organisms through application of these aggressive techniques, they did not identify any novel organisms. Their results, in fact, qualitatively confirm results generated after application of much milder sample preparation techniques.

Fermentation rates in the 13 samples differed dramatically. Whereas the fastest fermentation (juice from site 5, **Table 3.1**) was completed after 27 days, some samples (juice from site 8) still had a residual fermentable sugar content of 180 g/l after 80 days. These differences in fermentation rates clearly indicated the presence of different yeasts.

Using fermentation as an enrichment tool for the elusive strains of *S. cerevisiae* will clearly bias results towards yeasts with a high ethanol tolerance. In addition, killer activity will probably result in the demise of some killer sensitive strains. The final picture of *S. cerevisiae* strains isolated in this study will, therefore, only reflect those strains that could possibly have some oenological use.

3.3.2 YEAST IDENTIFICATION BY MEANS OF KARYOTYPING

Electrophoretic karyotyping was used to determine the identity of each of the 30 isolates per sample (site). Results are presented in **Table 3.1**. No *S. cerevisiae* strains were found in samples (sites 6, 8, 9 and 11) with high residual fermentable sugar. In addition, no sample contained both *Saccharomyces* and non-*Saccharomyces* yeasts. This is a surprising result. Although it is a well known fact that most of the non-*Saccharomyces* yeasts are killed when must alcohol levels reach 3-4% (Bisson & Kunkee, 1991; Fleet & Heard, 1993), other reports have indicated that some of these "wild" yeasts may be present at the end of fermentation (Heard & Fleet, 1988).

Clearly the anaerobic conditions, initial high sugar concentration (20°B) and low fermentation temperature (15°C) inhibited non-*Saccharomyces* yeasts in the spontaneously fermented grape juice sampled at sites 1, 2, 3, 4, 5, 7, 12 and 13. Therefore, these selective pressures and high ethanol levels after completion of fermentation limited the probability to isolate non-*Saccharomyces* yeasts in the latter samples.

3.3.3 GEOGRAPHIC DISTRIBUTION OF *S. CEREVISIAE* STRAINS

Fifty-one different *S. cerevisiae* isolates representing 46 unique karyotypes were recovered (**Table 3.1**). RAPD-PCR analysis was used to confirm that those yeast isolates indicated to be identical by means of electrophoretic karyotyping, were in fact the same (data not shown). The number of *S. cerevisiae* strains recovered per site varied from two (site 7) to 15 (site 2). No *Saccharomyces* strains were recovered from sites 6, 8, 9, 10 and 11. The sites sampled in the Elgin/Bot River area (8, 9, 10, 11) were all young vineyards in an area only recently planted to grapevines, with no wineries in the vicinity. Such conditions have often been associated with the complete absence of fermentation (Martini *et al.*, 1996). However, the situation at Nietvoorbij (site 6) is completely different in that this is a well-established wine farm

with two wineries. No reason, other than that put forward by Martini *et al.* (1996), for this absence of wine yeasts was apparent. These authors coupled the complete absence of *S. cerevisiae* in seven of eight fermentations in aseptically prepared juice to the paucity of this yeast in nature. They concluded that either no cells were present in the 3 to 4 kg of grapes sampled, or the small number actually present could not survive amongst the numerous non-*Saccharomyces* yeast normally found on grapes. If this was the case for the Nietvoorbij sample, there is no reason why it could not also be the case for the Elgin/Bot River samples. From **Table 3.1** it is also apparent that a high/low number of yeast strains per site was not associated with a particular area. It is therefore tempting to speculate that the spraying programme followed at a particular site could have affected the microflora present on the grapes at that site. Therefore, it is quite possible that a high/low number of *S. cerevisiae* strains per site is directly the result of an intense/less intense spray programme. This possibility was not examined in the study because spraying is a general practice and therefore forms part of the habitat in which the sought-after yeasts have to survive.

Generally speaking, all eight sites had a unique spectrum of *S. cerevisiae* strains and very few strains were found at more than one site. A strain appeared at two different sites in four cases (B5-5/HR5-7; J5-1/L5-2; V5-1/HR5-10; F5-1/HR5-4), while only one strain (F5-3/C5-3/B5-6) was recorded at three sites. While strains J5-1/L5-2 and F5-1/HR5-4 were isolated from sites in close proximity to each other, the sites for B5-5/HR5-7 and V5-1/HR5-10 were separated by 140 km and 90 km, respectively. In the case of F5-3/C5-3/B5-6 site 12 (F5-3) was separated from sites 1 and 2 by 140 km. If the presence of these yeasts at the particular sites persists over a number of years they might be considered to be representative of an area or terroir (Vézinhet *et al.*, 1992). If these yeasts are indeed representative of the different areas, more extensive sampling should indicate their presence at many more sites within the different areas.

Strains J5-1/L5-2 are not included in the above reasoning as they represent VIN13, currently the most popular active dried wine yeast used in South Africa. Strains L5-3 and HR5-2 were also considered to represent recovered commercial yeasts, although identification was not positive in the case of L5-3. Given the fact that winemakers in South Africa have been using active dried wine yeasts almost exclusively over the last two decades, the number of recovered commercial yeasts is very small. Commercial yeasts were only recovered at sites 4, 5 and 13. Sites 4 and 5 are very close together and far from site 13. Commercial yeasts dominated the fermentations at sites 3 and 4, but formed a minor component of the yeast spectrum at site 13. Reasons for this difference will have to be elucidated in future studies. One possible reason could be found in the sampling strategy and the location of the sampling sites in relation to the source of commercial yeasts.

Vézinhet *et al.* (1992) also recovered commercial yeast strains from nature (EC-1118 and 8130) in their study of wild *S. cerevisiae* strains found in Champagne between 1980 and 1985. However, as both these strains had originally been isolated from the Champagne area (Vézinhet, Blondin & Hallet, 1990) this does not indicate an invasion of an area by alien organisms. Schütz & Gafner (1994) isolated three yeast strains with clearly related, very similar chromosomal banding patterns from a spontaneous fermentation at Wädenswil. These banding patterns were very similar to the banding pattern of Lallemand W7, a commercial yeast strain originally isolated from the Wädenswil area. Although the possible spread of commercial yeast strains, and particularly that of genetically modified yeast, in nature will have to receive further attention, the results generated here do not point to a major problem.

3.3.4 OCCURRENCE OF GALACTOSE-UTILIZING AND KILLER YEASTS

The ability to utilize galactose as a sole carbon source together with killer activity are often used as important phenotypes to differentiate amongst strains of *S. cerevisiae* in strain development programmes (Van der Westhuizen & Pretorius, 1992). From **Table 3.2** it is clear that only six strains were unable to ferment galactose and killer strains were found in all fermentations that contained *S. cerevisiae* (**Table 3.2**). Abundance of killers varied from 6% to 100%. Vagnoli *et al.* (1993) published similar results based on a study of spontaneous fermentations in 18 Tuscan wineries. In their study, however, four of the 33 fermentations did not contain killer yeasts. The very low percentage of killer yeast (6) present in the sample from site 7 clearly indicates that killers do not automatically dominate all fermentations, thus confirming an earlier report by Tredoux, Tracey & Tromp (1986).

Table 3.2 Distribution and occurrence of natural killer *Saccharomyces cerevisiae* strains in the Western Cape region.

Farm / sampling sites	Strains	Number out of 30	Killer	Galactose utilization	% Killer
Groot Constantia (1)	C5-1	14	+	-	
	C5-2	8	-	+	
	C5-3 = B5-6 = F5-3	6	-	+	
	C5-6	2	+	+	53%
Buitenverwachting (2)	B5-1	1	+	+	
	B5-2	1	+	+	
	B5-3	3	-	+	
	B5-4	1	-	+	
	B5-5 = HR5-7	2	+	+	
	B5-6 = C5-3 = F5-3	2	-	+	
	B5-7	2	-	+	
	B5-8	1	-	+	
	B5-9	1	-	+	
	B5-10	1	+	+	
	B5-11	1	+	+	
	B5-12	1	+	+	
	B5-13	4	+	+	
	B5-14	8	+	+	
	B5-15	1	+	+	63%
Jordan (3)	J5-1 (VIN13)	23	+	+	
	J5-2	1	+	+	
	J5-3	5	-	+	
	J5-4	1	-	+	80%
Lievland (4)	L5-1	1	+	+	
	L5-2 (VIN13)	5	+	+	
	L5-3 (N96)	24	+	-	100%
Montfleur (5)	M5-1	3	+	+	
	M5-2	10	+	+	
	M5-3	2	+	+	
	M5-4	1	-	-	
	M5-5	2	+	+	
	M5-6	7	-	-	
	M5-7	1	+	+	
	M5-8	1	+	+	
	M5-9	3	+	+	74%
Vergelegen (7)	V5-1 = HR5-10	28	-	+	
	V5-2	2	+	+	6%
Bouchard Finlayson (12)	F5-1 = HR5-4	2	+	-	
	F5-2	26	+	+	
	F5-3 = C5-3 = B5-6	1	-	+	
	F5-4	1	+	+	97%

Table 3.2 *(continued)*

Farm / sampling sites	Strains	Number out of 30	Killer	Galactose utilization	% Killer
Hamilton Russell (13)	HR5-1	3	-	+	
	HR5-2 (VIN7)	2	-	+	
	HR5-3	5	-	+	
	HR5-4 = F5-1	3	+	-	
	HR5-5	2	-	+	
	HR5-6	1	-	+	
	HR5-7 = B5-5	4	+	+	
	HR5-8	2	-	+	
	HR5-9	2	+	-	
	HR5-10	5	-	+	33%

3.4 CONCLUSIONS

The absence of *S. cerevisiae* strains on grapes from five of 13 sites sampled confirms that these yeasts are not necessarily present on all wine grape clusters. Characterization of 240 colonies, representing 46 unique karyotypes isolated from the eight remaining sites, indicated the absence of a yeast common to the areas sampled. Many more sites per area need to be sampled in order to confirm, or disprove, this apparent localization of yeast biodiversity. These studies should include consideration of the chemical sprays applied in the different areas, if they in fact differ, to determine their effect on the observed biodiversity. The individual sites sampled originally should also be sampled over a number of years to determine the stability/evolution of the yeast population.

3.5 LITERATURE CITED

BENDA, I., 1964. Die Hefeflora des frankischen Weinbaugebietes. *Weinberg und Keller* **11**, 67-80.

BISSON, L.F. & KUNKEE, R.E., 1991. Microbial interactions during wine production. In: ZEIKUS, J.F. & JOHNSON, E.A. (eds). *Mixed cultures in biotechnology*. McGraw-Hill, New York. pp. 37-68.

CARLE, G.F. & OLSON, M.V., 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci. USA* **82**, 3756-3760.

CONSTANTI, M., POBLET, M., AROLA, L., MAS, A. & GUILLAMON, J.M., 1997. Analysis of yeast populations during alcoholic fermentation in a newly established winery. *Am. J. Enol. Vitic.* **48**, 339-344.

DU PLESSIS, L. DE W., 1959. The study of the microorganisms associated with the flowers and ripening berries of a number of grape varieties (in Afrikaans). Thesis, University of Stellenbosch, Stellenbosch, South Africa.

FLEET, G.H. & HEARD, G.M., 1993. Yeasts – Growth during fermentation. In: FLEET, G.H. (ed.). *Wine microbiology and biotechnology.* Harwood Academic Publishers, Singapore. pp. 27-54.

HEARD, G., 1999. Novel yeasts in winemaking – looking to the future. *Food Australia* **51**, 347-352.

HEARD, G.M. & FLEET, G.H., 1988. The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *J. Appl. Bacteriol.*, **65**, 23-28.

HENSCHKE, P.A., 1997. Wine yeast. In: ZIMMERMANN, F.K. & ENTIAN, K.-D. (eds). *Yeast sugar metabolism.* Technomic Publishing Co., Lancaster, Pennsylvania. pp. 527-560.

JEMEC, K.D., PECJAK, M., DEREANI, V.R., PODJAVORŠEK, J., MOZINA, S.S. & RASPOR, P., 1997. Population kinetics of yeasts during wine fermentations in Carst and coastal regions of Slovenia. In: RASPOR, P., MOZINA, S.S., ZAGORC, T. & BATIC, M. (eds). *Yeast nutrition and natural habitats.* Book of abstracts, 18th International Specialized Symposium on Yeasts, 24 to 29 August 1997, Bled, Slovenia. P4-12.

MARTINI, A., FREDERICI, F. & ROSINI, G., 1980. A new approach to the study of yeast ecology of natural substrates. *Can. J. Microbiol.* **26**, 856-859.

MARTINI, A., CIANI, M. & SCORZETTI, G., 1996. Direct enumeration and isolation of wine yeasts from grape surfaces. *Am. J. Enol. Vitic.* **47**, 435-440.

PARISH, M.E. & CARROLL, D.E., 1985. Indigenous yeasts associated with Muscadine (*Vitis rotundifolia*) grapes and musts. *Am. J. Enol. Vitic.* **36**, 165-169.

PEYNAUD, E. & DOMERCQ, S., 1959. A review on microbiological problems in wine making in France. *Am. J. Enol. Vitic.* **10**, 69-77.

PRETORIUS, I.S., VAN DER WESTHUIZEN, T.J. & AUGUSTYN, O.P.H., 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. *S. Afr. J. Enol. Vitic.* (submitted).

ROSINI, G., 1984. Assessment of dominance of added yeast in wine fermentation and origin of *Saccharomyces cerevisiae* in wine-making. *J. Gen. Appl. Microbiol.* **30**, 249-256.

SCHÜTZ, M. & GAFNER, J., 1994. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Lett. Appl. Microbiol.* **19**, 253-257.

TREDOUX, H.G., TRACEY, R.D. & TROMP, A., 1986. Killer factor in wine yeasts and its effect on fermentation. *S. Afr. J. Enol. Vitic.* **7**, 105-112.

VAN DER WESTHUIZEN, T.J., AUGUSTYN, O.P.H. & PRETORIUS, I.S., 1999. The value of long-chain fatty acid analysis, randomly amplified polymorphic DNA and electrophoretic karyotyping for the characterization of wine yeast strains. *S. Afr. J. Enol. Vitic.* **20**, 3-10.

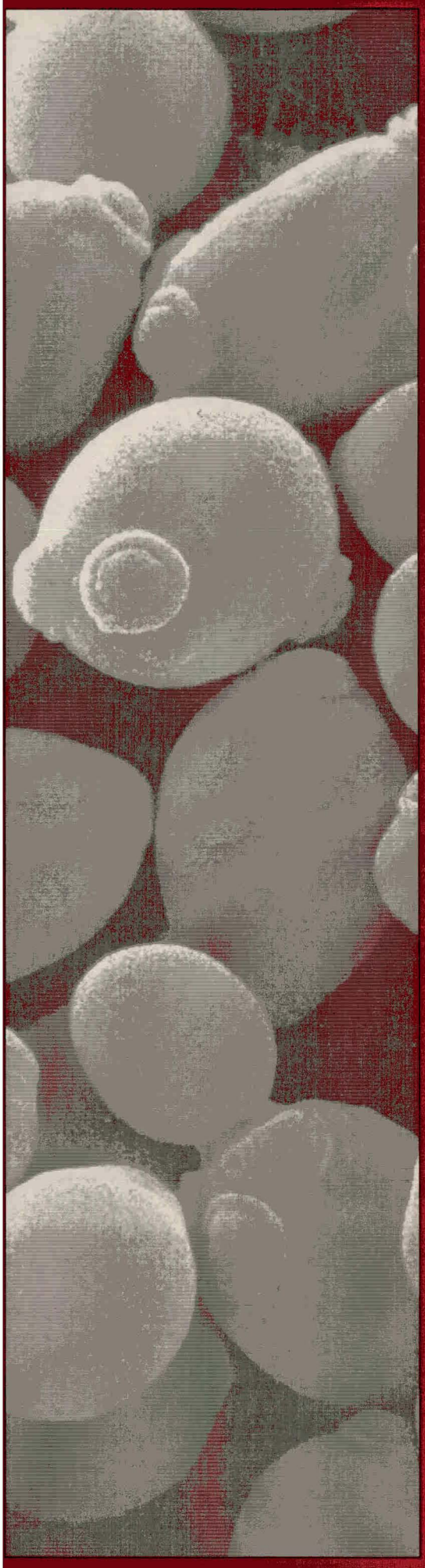
VAN DER WESTHUIZEN, T.J. & PRETORIUS, I.S., 1992. The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. *Ant. v. Leeuwen.* **61**, 249-257.

VAN ZYL, J.A, & DU PLESSIS, L. DE W., 1961. The microbiology of South African winemaking. Part 1. The yeast occurring in vineyards, musts and wines. *S. Afr. J. Agric. Sci.* **4**, 393-403.

VAGNOLI, P., MUSMANNO, R.A., CRESTI, S., MAGGIO, T.D. & CORATZA, G., 1993. Occurrence of killer yeasts in spontaneous wine fermentations from the Tuscany region in Italy. *Appl. Environ. Microbiol.* **59**, 4037-4043.

VÉZINHET, F., BLONDIN, B. & HALLET, J.-N., 1990. Chromosomal DNA patterns and mitochondrial DNA polymorphism as tools for identification of enological strains of *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **32**, 568-571.

VÉZINHET, F., HALLET, J.-N., VALADE, M. & POULARD, A., 1992. Ecological survey of wine yeast strains by molecular methods of identification. *Am. J. Enol. Vitic.* **43**, 83-86.



CHAPTER 4

Research Results III

Seasonal variation of indigenous *Saccharomyces cerevisiae* strains isolated from Western Cape vineyards

(*S. Afr. J. Enol. Vitic.*, in press)

4. RESEARCH RESULTS III

Seasonal Variation of Indigenous *Saccharomyces cerevisiae* Strains Isolated from Western Cape Vineyards

Abstract

There is strong support for the use of naturally-occurring *Saccharomyces cerevisiae* strains that improve the sensory quality of wines and reflect the characteristics of a given region. Contrary to popular belief, *S. cerevisiae* is found at very low numbers on healthy, undamaged grapes and is rarely isolated from intact berries. The majority of studies on the population kinetics and geographic distribution of indigenous *S. cerevisiae* strains have not adequately focused on the variation in their numbers over a longer period of time. This paper discusses the results obtained in the first phase of a comprehensive research programme aimed at assessing how the natural population dynamics of *S. cerevisiae* are affected over the long term by abiotic factors. Indigenous strains of *S. cerevisiae* were aseptically isolated from eight sites in four areas in the coastal regions of the Western Cape, South Africa, during 1995 through to 1998. Thirty colonies per site were isolated and the *S. cerevisiae* strains were characterized by electrophoretic karyotyping. Strain numbers per site varied over the four-year study period. Weather conditions resulting in severe fungal infestations and heavy applications of chemical sprays dramatically reduced the numbers of *S. cerevisiae* strains recovered during 1997. A return to normal weather patterns in 1998 resulted in a gradual recovery of the indigenous population. Indications are that some of the strains isolated are widespread in the study area and may represent yeasts typical of the area. Commercial wine yeast strains were recovered in only a few instances and the likelihood that commercial yeasts will eventually replace the natural yeast microflora in vineyards seems remote.

4.1 INTRODUCTION

In a previous paper, we reported on efforts to isolate strains of *Saccharomyces cerevisiae* from wine grapes at 13 sites in five areas within the coastal region of the Western Cape in South Africa (Van der Westhuizen, Augustyn & Pretorius, 1999b). The sought after yeasts were recovered from eight sites, while grapes from five sites contained no *S. cerevisiae* at all. As the grapes had been harvested and juices prepared under aseptic conditions, the yeasts recovered could only have come from the vineyards sampled.

The presence or absence of *S. cerevisiae* on grapes is the subject of some debate. While some studies, where grapes were harvested under aseptic conditions, indicated that strains of *S. cerevisiae* were present on grapes (Van Zyl & Du Plessis, 1961; Parish & Carroll, 1985; Török *et al.*, 1996; Van der Westhuizen *et al.*, 1999b), others do not (Barnett *et al.*, 1972; Rosini, 1982, 1984). After examining the

evidence at hand, Vaughan-Martini & Martini (1995) concluded that "we must exclude a natural origin for *S. cerevisiae*". However, Török *et al.* (1996) refuted this claim and concluded that the vineyard does, in fact, represent the primary source of these yeasts.

It has become abundantly clear that *S. cerevisiae* is not as plentiful in nature as was thought earlier (Peynaud & Domercq, 1959; Van Zyl & Du Plessis, 1961; Benda, 1964). It has also become clear that *S. cerevisiae* strains resident on surfaces in the winery are much more numerous than those present in the vineyard. These are, in all probability, the yeasts that will dominate spontaneous fermentations (Peynaud & Domercq, 1959; Rosini, 1984) and also play a role in other fermentations inoculated with pure yeast cultures (Rosini, 1984; Constanti *et al.*, 1997).

Some authors have presented evidence that specific strains of *S. cerevisiae* are widely distributed in particular areas (Vézinhet *et al.*, 1992; Versavaud *et al.*, 1995). We did not find such dominance in the coastal regions of the Western Cape (Van der Westhuizen *et al.*, 1999b). Nevertheless, the 46 different karyotypes identified in that study represent a valuable natural resource (Pretorius, Van der Westhuizen & Augustyn, 1999). The aim of this study was to determine the natural population dynamics of *S. cerevisiae* strains in the same vineyards over a four-year period.

4.2 MATERIALS AND METHODS

4.2.1 AREAS SAMPLED

Yeast strains were isolated from grapes sampled in the same vineyards at eight different sites in the Constantia, Stellenbosch, Somerset West and Hermanus areas of the Western Cape, South Africa, during the 1996, 1997 and 1998 seasons. Collection and isolation procedures were done according to the methods described by Van der Westhuizen *et al.* (1999b). In this study yeasts were isolated from the same vineyards at the same sites and areas as noted by Van der Westhuizen *et al.* (1999b). These data were combined with those generated in this study to give a four-year observation period.

4.2.2 METEOROLOGICAL DATA

The monthly rainfall and average monthly maximum temperature (1994 through 1998) for the Constantia, Stellenbosch and Somerset West areas were recorded by automatic weather stations. These data are presented in **Tables 4.1** and **4.2**, respectively.

4.2.3 PREPARATION OF INTACT CHROMOSOMAL DNA AND PULSE FIELD GEL ELECTROPHORESIS

Samples were prepared according to the embedded agarose procedure of Carle & Olson (1985). The methods applied were as in Van der Westhuizen, Augustyn & Pretorius (1999a).

Table 4.1 Monthly rainfall (mm) in the Constantia, Stellenbosch and Somerset West areas (1994 to 1998).

Area/ Year	Month*											
	1	2	3	4	5	6	7	8	9	10	11	12
Constantia												
LTA†	**26,3**	**23,9**	**34,5**	**78,0**	**137,9**	**187,6**	**177,3**	**155,6**	**108,0**	**60,1**	**42,3**	**40,2**
1994	18,8	3,7	2,9	53,7	80,9	513,3	159,2	45,5	74,1	19,5	17,5	8,0
1995	34,2	5,0	8,0	20,6	93,5	120,5	**295,0**	156,1	45,2	**112,8**	20,5	29,8
1996	2,0	**54,0**	39,5	56,0	70,8	225,6	196,8	143,7	**305,7**	**85,4**	**64,7**	**62,7**
1997	18,7	3,3	15,4	62,9	111,3	198,4	51,0	270,6	18,5	24,2	**93,5**	22,4
1998	19,5	0,8	15,5	71,0	250,7	134,6	163,2	78,4	73,3	33,9	65,3	35,9
Stellenbosch												
LTA†	**19,2**	**21,1**	**30,5**	**76,8**	**107,8**	**126,6**	**116,3**	**84,1**	**55,8**	**44,9**	**27,2**	**25,2**
1994	42,3	3,3	15,9	58,6	47,4	278,9	96,0	39,0	45,0	27,5	13,2	16,0
1995	17,1	7,8	12,8	22,5	95,1	136,6	**146,8**	123,6	24,8	**89,3**	12,2	**51,3**
1996	5,3	**56,1**	**41,9**	49,1	57,8	194,9	90,5	128,4	136,7	**106,5**	**60,3**	**55,3**
1997	12,5	1,5	4,6	59,4	91,3	167,9	31,3	91,3	15,2	15,7	**106,3**	11,7
1998	22,4	0,0	27,2	40,6	260,4	79,8	103,6	61,2	35,7	25,6	72,4	36,2
Somerset West												
LTA†	**10,2**	**11,6**	**16,1**	**43,7**	**91,6**	**196,8**	**101,0**	**69,9**	**45,5**	**43,4**	**58,6**	**45,5**
1994	22,8	3,8	8,2	41,0	59,4	296,8	98,2	37,0	67,8	30,4	17,4	27,0
1995	5,6	3,0	21,8	25,9	105,0	147,6	94,4	**116,8**	8,4	25,6	25,8	**91,6**
1996	4,4	**55,8**	33,4	1,2	69,4	194,8	**144,6**	67,0	**98,2**	**120,0**	**74,6**	**53,6**
1997	19,6	3,8	14,2	64,6	94,8	243,0	44,2	37,8	7,4	27,4	**98,6**	28,0
1998	3,6	0,0	18,2	53,2	193,2	101,6	123,8	91,0	45,8	13,4	76,4	55,2

* 1 to 12 : January to December
† LTA : long-term average

4.2.4 RANDOMLY AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Yeast cells were cultured and the DNA isolation was performed using the method as described by Van der Westhuizen & Pretorius (1992). Polymerase chain reactions (PCR) were performed using primer OPC-09 (5'-CTCACCGTCC-3') as applied by Van der Westhuizen *et al.* (1999a).

4.3 RESULTS AND DISCUSSION

4.3.1 YEAST ISOLATION

During 1995 only eight of the original 13 sampling sites contained strains of *S. cerevisiae* (Van der Westhuizen *et al.*, 1999b). These eight sampling sites formed the basis of this study over the next three years. Fermentation rates in the aseptically prepared musts again differed dramatically (data not shown) with some samples not fermenting at all. This not only happened in a particular year when considering all sites sampled, but also over years at a particular site. These observations indicated that while all sites had contained *S. cerevisiae* during 1995, this was not necessarily so over the next three years. This observation may indicate

that external factors (climate, application of different numbers of chemical spray) had affected the natural *S. cerevisiae* population. On the other hand, it may simply be due to the fact that the low numbers of naturally occurring *S. cerevisiae* are normally distributed in a rather haphazard manner (Török *et al.*, 1996).

Table 4.2 Average monthly maximum temperature (°C) in the Constantia, Stellenbosch and Somerset West areas (1994 to 1998).

Area/ Year	Month*											
	1	2	3	4	5	6	7	8	9	10	11	12
Constantia												
LTA†	**24,8**	**25,3**	**24,4**	**22,1**	**19,6**	**17,6**	**16,9**	**17,1**	**18,3**	**20,2**	**22,1**	**23,6**
1994	24,9	26,0	24,9	23,3	18,6	16,6	17,1	17,3	19,2	21,6	22,5	24,5
1995	25,5	26,0	25,2	21,0	20,6	18,0	15,2	17,1	18,7	**19,2**	22,6	24,7
1996	25,3	25,6	23,5	23,6	20,9	18,4	16,0	16,3	16,5	**19,3**	**19,3**	**23,0**
1997	24,5	23,4	23,6	21,3	20,4	15,8	18,0	16,9	21,0	22,7	**20,9**	23,5
1998	24,2	26,6	23,5	22,5	19,4	17,6	16,5	18,0	18,0	20,6	21,7	24,3
Stellenbosch												
LTA†	**27,7**	**28,1**	**26,6**	**22,9**	**20,1**	**17,6**	**17,1**	**18,0**	**20,0**	**22,8**	**25,2**	**26,5**
1994	28,6	30,3	27,6	25,3	19,7	16,6	17,4	18,2	20,6	24,5	24,2	27,7
1995	28,7	30,4	28,1	23,8	22,0	18,1	15,5	17,5	20,0	20,8	24,9	27,4
1996	29,0	**28,5**	**25,8**	25,3	21,8	18,1	16,2	17,0	17,8	**21,2**	**21,3**	**24,8**
1997	27,3	27,0	26,2	22,6	21,8	16,5	18,8	17,6	22,7	25,6	**23,6**	26,7
1998	26,8	29,9	25,9	24,8	19,9	17,7	17,2	18,8	19,7	23,3	24,1	27,4
Somerset West												
LTA†	**28,0**	**29,1**	**27,2**	**24,4**	**21,3**	**18,6**	**17,7**	**18,5**	**20,8**	**23,3**	**23,7**	**26,1**
1994	28,5	29,4	27,4	25,8	20,2	17,6	18,4	18,5	20,8	23,5	23,4	27,0
1995	28,1	30,4	27,8	22,8	22,3	19,1	15,0	17,8	20,2	23,1	24,7	**27,0**
1996	28,5	**29,0**	25,7	27,2	22,4	19,9	16,9	17,9	18,6	**21,4**	**21,0**	**25,7**
1997	27,8	26,9	26,4	23,1	22,1	17,4	19,8	16,9	23,7	25,1	**23,1**	Nd+
1998	26,2	30,0	26,6	25,3	19,9	18,9	18,3	19,7	19,8	22,8	23,2	26,4

* * 1 to 12 : January to December
† LTA: long-term average
+ Not determined

4.3.2 ELECTROPHORETIC KARYOTYPING

CHEF-DNA-analysis was run on 30 isolates per sampling site. Only one sample contained a mixed culture of *S. cerevisiae* and non-*Saccharomyces* yeasts. The remaining samples contained either non-*Saccharomyces* or *S. cerevisiae,* as was also the case in our previous study (Van der Westhuizen *et al.*, 1999b). The *S. cerevisiae* strains recovered during 1996 to 1998 are listed in **Table 4.3**. These data are summarized in **Table 4.4** which lists the number of unique karyotypes per site per year and includes data for 1995 taken from Van der Westhuizen *et al.* (1999b). Data in **Tables 4.3** and **4.4** do not take into account that a particular yeast may be present at more than one site, or occur in more than one year. Comparison of karyotypes between sites and over a period of years indicated that a number of yeasts were present at more than one site or re-occurred over years (**Table 4.5**).

Table 4.3 Distribution and frequency of *S. cerevisiae* strains per sampling site between 1996 and 1998.

Area[1]	Sampling site[2]	*S. cerevisiae* strains per sampling site								
		1996 strain = number[3] (%)			1997 strain = number (%)			1998 strain = number (%)		
A	Groot Constantia (1)	C6-1	30	(100)	None	-	-	C8-1	24	(80)
	Buitenverwachting (2)	B6-1	1	(3)	None	-	-	B8-1	non-*cerevisiae*	
		B6-2	2	(7)				B8-2	1	(3)
		B6-3	9	(30)				B8-3	3	(10)
		B6-4	4	(13)				B8-4	15	(50)
		B6-5	14	(47)				B8-5	5	(17)
								B8-6	3	(10)
								B8-7	1	(3)
B	Jordan (3)	J6-1 (N96)	21	(71)	J7-1 (VIN13)	30	(100)	J8-1	10	(34)
		J6-2	1	(3)				J8-2	1	(3)
		J6-3	1	(3)				J8-3	4	(13)
		J6-4	3	(10)				J8-4	12	(40)
		J6-5	1	(3)				J8-5	1	(3)
		J6-6	3	(10)				J8-6	1	(3)
								J8-7	1	(3)
	Lievland (4)	None	-	-	None	-	-	L8-1	27	(90)
								L8-2	3	(10)
	Mont Fleur (5)	M6-1	16	(54)	None	-	-	M8-1	30	(100)
		M6-2	1	(3)						
		M6-3	1	(3)						
		M6-4	3	(10)						
		M6-5	3	(10)						
		M6-6	3	(10)						
		M6-7	2	(7)						
		M6-8	1	(3)						
C	Vergelegen (7)	V6-1 (VIN13)	17	(57)	V7-1	30	(100)	V8-1	23	(77)
		V6-2	4	(13)				V8-2	7	(23)
		V6-3	5	(17)						
		V6-4	3	(10)						
		V6-5	1	(3)						
D	Bouchard Finlayson (12)	F6-1	30	(100)	F7-1	13	(43)	F8-1	4	(14)
					F7-2 (VIN13)	16	(54)	F8-2	5	(17)
					F7-3	1	(3)	F8-3	5	(17)
								F8-4	7	(23)
								F8-5	6	(20)
								F8-6	1	(3)
								F8-7	1	(3)
								F8-8	1	(3)
	Hamilton Russell 13)	None	-	-	None	-	-	HR8-1	15	(50)
								HR8-2	4	(13)
								HR8-3	6	(20)
								HR8-4	2	(7)
								HR8-5	3	(10)

[1] A = Constantia; B = Stellenbosch; C = Somerset West; D = Hermanus
[2] Sampling site number according to Van der Westhuizen *et al.* (1999b)
[3] Number out of 30

Table 4.4 Summary: Number of *S. cerevisiae* strains per site per year.

Area	Site	1995*	1996	1997	1998
Constantia	1	4	1	0	1
	2	15	5	0	6
Stellenbosch	3	4	6	1	7
	4	3	0	0	2
	5	9	8	0	1
Somerset West	7	2	5	1	2
Hermanus	12	4	1	3	8
	13	10	0	0	5

*Data from Van der Westhuizen *et al.* (1999b).

RAPD-PCR analysis confirmed that yeasts indicated to be the same by means of electrophoretic karyotyping, were in fact the same (data not shown). The number of commercial yeasts recovered was small and none were found during 1998. As noted before (Van der Westhuizen *et al.,* 1999b), the likelihood that commercial yeasts will eventually replace the natural yeast microflora in vineyards seems remote. This finding should appease those people who are concerned about possible detrimental effects of uncontrolled spreading of commercial yeast cultures in the environment.

4.3.3 SEASONAL VARIATION IN THE *S. CEREVISIAE* POPULATION

Data presented in **Table 4.3** clearly indicate that the number of *S. cerevisiae* strains per site varied dramatically between 1996 and 1998. This fact is emphasized when perusing the summarized data (1995 – 1998) presented in **Table 4.4**.

It is well known that grape yeast microflora varies from area to area and from vintage (year) to vintage (Benda, 1964; Frezier & Dubourdieu, 1992; Vézinhet *et al.*, 1992; Schütz & Gafner, 1994). However, authors rarely comment on possible factors that affect yeast populations. Benda (1964), who studied the yeast microflora in Franken during 1959 and 1960, reported July to October 1959 to be a relatively dry and sunny period in contrast to the wet and more overcast 1960. She concluded that these climatic differences had affected the yeast microflora present on the grapes.

Perusal of the data in **Table 4.4** reveals a number of trends, some coupled to area/site, while others are more general. Briefly, from 1995 to 1996, the numbers of *S. cerevisiae* strains recovered at Constantia and Hermanus declined dramatically while numbers actually increased, or stayed approximately the same, at three of the four remaining sites. During 1997, a total of five *S. cerevisiae* strains were detected at only three sites. Strain count increased at all sites during 1998. Consideration of the meteorological data in **Tables 4.1** and **4.2** helps to explain some of these trends. In the Western Cape vines typically flower between August and October depending on factors as grape cultivar, terroir, general growth area (e.g. warmer inland/cooler coastal), specific vintage (e.g. cooler/warmer or wetter/dryer than usual). Harvest takes place from February to April subject to the same factors as mentioned above. **Table 4.1** clearly indicates that late 1995, early 1996 and particularly late 1996 had periods in which unusually high volumes of rain were recorded. This high level of moisture coupled to temperatures (**Table 4.2**) that, although generally lower than the long-term averages, were still condusive to fungal growth resulted in particularly severe outbreaks of disease. Consequently, on average, 2- to 2,5-fold more rounds

of fungicide sprays were applied during these unusually wet periods. Clearly the higher rainfall and concomitant much-increased application of anti-fungal sprays severely affected the naturally occurring yeasts as reflected in the extremely low recovery recorded during 1997. Data in **Table 4.4** represent mean values for an entire area and may differ from that relevant for a particular site (terrain). This fact will help explain the apparent contradiction between the 1995/1996 rainfall patterns (**Table 4.1**) and numbers of *S. cerevisiae* strains present in the Somerset West/Stellenbosch areas (**Table 4.4**). Meteorological data for the Hermanus area were not available, but consultations with winemakers confirmed a situation similar to that recorded for Constantia (**Table 4.1**), a fact reflected in the declining strain counts between 1995 and 1997 (**Table 4.4**). Indigenous yeast populations will clearly always be affected by application of fungicides, even in those "normal" years when producers follow standard spray programmes.

It was pointed out earlier that, except for one exception, must/wine samples prepared from grapes harvested at the various sites contained either *S. cerevisiae* or non-*Saccharomyces* yeasts. Data in **Tables 4.3** and **4.4** clearly indicate that the initially scarce *S. cerevisiae* (Peynaud & Domercq, 1959; Van Zyl & Du Plessis, 1961) were hard hit by the intensified spray programmes mentioned above. By 1997 these yeasts had been almost completely eradicated in the vineyard sections under study. Alternatively, their numbers were reduced to such low levels that they could not maintain themselves in competition with the more numerous non-*Saccharomyces* yeast. It is, therefore, very interesting, though not unexpected, to note that as chemical spray programmes returned to normal (rainfall closer to the long-term average, **Table 4.1**) *S. cerevisiae* strains started reappearing, or increasing in number, at all sampling sites.

4.3.4 DISTRIBUTION OF YEASTS BETWEEN AREAS

Perusal of the data summarized in **Table 4.5** indicates 17 groups of equivalent yeasts. Two additional groups representing recoveries of commercial yeast are presented as a footnote.

No single *S. cerevisiae* strain was present at one single site in all four years (**Table 4.5**). Groups 1, 5, 7, 9, 10, 14 and 15 represent six different yeasts present at the same respective site for two years. Sometimes these yeasts were recorded in successive years (*e.g.,* groups 5, 9, 10 and 15), whilst at other sites the relevant yeast reappeared after one or two years (*e.g.,* groups 1, 7 and 14). This cycle of presence, absence, reappearance was noted earlier during a study of *S. cerevisiae* naturally present on grapes in Champagne and the Loire Valley (Vézinhet *et al.*, 1992). The yeasts represented by groups 3 and 11, respectively, were the only ones to be recorded in three seasons. From **Table 4.5** it is also clear that one of the yeasts was recorded at four different sites (group 3) and another at three sites (group 2). Of the remaining yeasts, seven were found at one site only while eight more were recorded from two sites. The fact that many of these groups represent a yeast recovered from widely separated sites and in different years would seem to indicate the presence of an indigenous microflora typical of the general area under study. That these yeasts were not recovered annually could be ascribed to natural population fluctuations, fluctuations brought about by man-made external factors (chemical sprays), or an inadequate sampling technique that is unable to

compensate for the normal haphazard distribution of *S. cerevisiae* (Török *et al.*, 1996) in the vineyard.

Table 4.5 Yeast strains present at more than one site or at the same site in different years*

Group no.	Equivalent strains			
	1995	1996	1997	1998
1	B5-3:**		-	B8-7
2	B5-5 : HR5-7			F8-7
3	B5-6 : C5-3 : F5-3	F6-1 : M6-1 :		B8-3
4	B5-13 :			B8-4 : V8-2
5	B5-14 :	B6-1		
6		B6-5 : V6-3		
7		C6-1 :		C8-1
8				J8-1 : L8-1
9	M5-2 :	M6-8		
10	M5-4 :	M6-7		
11	M5-6 :	M6-4 :	V7-1	
12	V5-1 : HR5-10			
13	F5-1 : HR5-4			
14	F5-2 :			F8-1
15			F7-3 :	F8-3
16				F8-2 : HR8-2
17	HR5-9 :			B8-1 : HR8-1

* Does not include commercial yeast strains: VIN13 = J5-1 : L5-2 : V6-1 : J7-1 : F7-2; N96 = L5-3 : J6-1.
** Site abbreviations as in **Table 4.3**: B = Buitenverwachting; C = Constantia; J = Jordan; L = Lievland; M = Mont Fleur; V = Vergelegen; F = Bouchard Finlayson; HR = Hamilton Russell.

4.4 CONCLUSIONS

Data generated in this study clearly indicate that *S. cerevisiae* does occur in nature in numbers sufficient to conduct a successful, spontaneous fermentation. Grapes from sites which yielded no *S. cerevisiae* at a particular sampling will more than likely contain sufficient wine yeasts when the sample size is increased, e.g. when harvesting the whole vineyard. Some of the *S. cerevisiae* strains recovered may be widespread in the area studied. It remains to be determined if these yeasts actually contribute to the character of the wines from these areas. Whatever the case, these indigenous wine yeasts represent a valuable natural resource and the best of them will be included in our extensive yeast breeding programme.

4.5 LITERATURE CITED

BARNETT, J.A., DELANY, M.A., JONES, E., MAGSON, B. & WINCH, B., 1972. The number of yeasts associated with winegrapes of Bordeaux. *Arch. Microbiol.* **83**, 52-55.

BENDA, I., 1964. Die Hefeflora des frankischen Weinbaugebietes. *Weinberg und Keller* **11**, 67-80.

CARLE, G.F. & OLSON, M.V., 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci. USA* **82**, 3756-3760.

CONSTANTI, M., POBLET, M., AROLA, L., MAS, A. & GUILLAMON, J.M., 1997. Analysis of yeast populations during alcoholic fermentation in a newly established winery. *Am. J. Enol. Vitic.* **48**, 339-344.

FREZIER, V. & DUBOURDIEU, D., 1992. Ecology of yeast strain *Saccharomyces cerevisiae* during spontaneous fermentation in a Bordeaux winery. *Am. J. Enol. Vitic.* **43**, 375-380.

PARISH, M.E. & CARROLL, D.E., 1985. Indigenous yeasts associated with Muscadine (*Vitis rotundifolia*) grapes and musts. *Am. J. Enol. Vitic.* **36**, 165-169.

PEYNAUD, E. & DOMERCQ, S., 1959. A review on microbiological problems in wine making in France. *Am. J. Enol. Vitic.* **10**, 69-77.

PRETORIUS, I.S., VAN DER WESTHUIZEN, T.J. & AUGUSTYN, O.P.H., 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. *S. Afr. J. Enol. Vitic.* (submitted).

ROSINI, G., 1982. Influenza della microflora saccaromicetica della contina sulla fermentazione delmosto d'uva. *Vigne e Vini* **9**, 43-46 (As quoted in Vaughan-Martini & Martini, 1995)

ROSINI, G., 1984. Assessment of dominance of added yeast in wine fermentation and origin of *Saccharomyces cerevisiae* in wine-making. *J. Gen. Appl. Microbiol.* **30**, 249-256.

SCHÜTZ, M. & GAFNER, J., 1994. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Lett. Appl. Microbiol.* **19**, 253-257.

TÖRÖK, T., MOTIMER, R.K., ROMANO, P., SUZZI, G. & POLSINELLI, M., 1996. Quest for wine yeasts - an old story revisited. *J. Industrial Microbiol.* **17**, 303-313.

VAN DER WESTHUIZEN, T.J., AUGUSTYN, O.P.H. & PRETORIUS, I.S., 1999a. The value of long-chain fatty acid analysis, randomly amplified polymorphic DNA and electrophoretic karyotyping for the characterization of wine yeast strains. *S. Afr. J. Enol. Vitic.* **20**, 3-10.

VAN DER WESTHUIZEN, T.J., AUGUSTYN, O.P.H. & PRETORIUS, I.S., 1999b. Geographic distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the coastal regions of the Western Cape in South Africa. *S. Afr. J. Enol. Vitic.* (submitted).

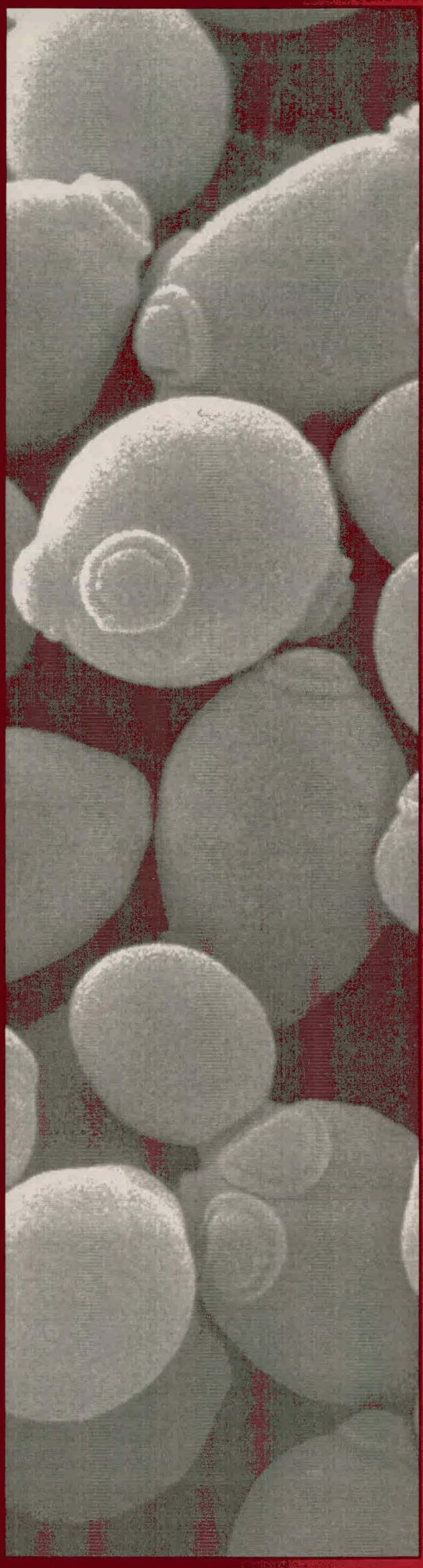
VAN DER WESTHUIZEN, T.J. & PRETORIUS, I.S., 1992. The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. *Ant. v. Leeuwen.* **61**, 249-257.

VAN ZYL, J.A, & DU PLESSIS, L. DE W., 1961. The microbiology of South African winemaking. Part 1. The yeast occurring in vineyards, musts and wines. *S. Afr. J. Agric. Sci.* **4**, 393-403.

VAUGHAN-MARTINI, A. & MARTINI, A., 1995. Facts, myths and legends on the prime industrial microorganism. *J. Indust. Microbiol.* **14**, 514-522.

VÉZINHET , F., HALLET, J.-N., VALADE, M. & POULARD, A., 1992. Ecological survey of wine yeast strains by molecular methods of identification. *Am. J. Enol. Vitic.* **43**, 83-86.

VERSAVAUD, A., COURCOUX, P., ROULLAND, C., DULAU, L. & HALLET, J.-N., 1995. Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strains from wine-producing area of Chanrentes, France. *Appl. Eviron. Microbiol.* **61**, 3521-3529.



CHAPTER 5

Research Results IV

Preliminary study on the ability of indigenous *Saccharomyces cerevisiae* strains to affect the flavour composition of wine

5. RESEARCH RESULTS IV

Preliminary study on the ability of indigenous *Saccharomyces cerevisiae* strains to affect the flavour composition of wine

Abstract

Wine yeasts can affect the aroma component profile and, therefore, the quality of wine. In this preliminary study, the ester, higher alcohol and fatty acid formation ability of 33 indigenous *Saccharomyces cerevisiae* strains was compared to that of two local commercial yeasts. Differences noted indicate that some of these yeasts may find a place in our extensive yeast breeding programme.

5.1 INTRODUCTION

Formation of wine aroma during the fermentation of must is a complex process and is affected by a number of factors. In particular, the final wine aroma depends on the nature and concentration of the chemical species originally present in the must (proportions vary from cultivar to cultivar and between clones of a particular cultivar), the yeast's capacity to utilize these components and techniques applied /conditions prevailing during winemaking (Margalith & Schwartz, 1970; Daudt & Ough, 1973; Houtman & Du Plessis, 1986; Nykänen, 1986; Rapp & Mandery, 1986; Mateo *et al.*, 1992, Gomez, Laencina & Martinez, 1994). The complex mixture of volatile compounds responsible for wine aroma represents a number of different chemical classes (Schreier, 1979). Amongst these aroma contributors the higher alcohols, acids and esters dominate quantitatively (Stashenko, Macku & Shibamato, 1992). The influence of different yeasts (both *Saccharomyces cerevisiae* and non-*Saccharomyces*) on the volatile aroma component profile of wine has been the subject of many studies including Van Zyl, De Vries & Zeeman, 1963; Soles, Ough & Kunkee, 1982; Houtman & Du Plessis, 1986; Nykänen, 1986; Carbrera *et al*, 1988; Giudici, Romano & Zambonelli, 1990; Herraiz *et al.*, 1990; Delteil & Jarry, 1992; Ciani & Picciotti, 1995 and Lema *et al.*, 1996, amongst others. Selection of the right yeast(s) to produce a secondary aroma component profile that complements or enhances the primary varietal aroma will help winemakers produce optimum quality wines. In a recent ecological survey we isolated 42 unique strains of *S. cerevisiae* indigenous to the coastal areas of the Western Cape, South Africa (Van der Westhuizen, Augustyn & Pretorius, 1999). The purpose of this preliminary study was to determine how a number of these yeasts can affect the ester, higher alcohol and fatty acid profiles of wine.

5.2 MATERIALS AND METHODS

5.2.1 YEAST STRAINS AND FERMENTATION

Thirty three *S. cerevisiae* (**Table 5.1**) isolated in the coastal regions of the Western Cape during 1995 (Van der Westhuizen *et al.*, 1999) were used. Local commercial strains WE372 and VIN13 were included for purposes of comparison.

Laboratory scale fermentations (750-ml bottles, in duplicate) of Chenin blanc must, previously sterilized by addition of 100 µl/l dimethyl dicarbonate (Valcorin, Bayer), were conducted at 15°C in a dark room. No SO_2 was added and progress of fermentation was monitored by measuring mass loss. Wine samples were withdrawn after 28 days and stored at 4°C (for not more than 14 days) prior to chemical analyses.

5.2.3 EXTRACTION OF VOLATILES

Wine (50 ml) was placed in a round ball flask and 4 ml internal standard (232 mg/l 4-methyl-2-pentanol [Fluka] in 10% ethanol/water) and 30 ml diethyl ether (Riedel de Haen) added. After rotating the flask (60 rpm) for 30 min at 20°C the upper phase was removed and a 3 ml aliquot injected directly into the gas chromatograph.

5.2.3 GAS CHROMATOGRAPHY

Analyses were done on an HP5890 Series II instrument equipped with an FID detector and a Lab alliance bonded phase Carbowax capillary column (60 mm x 0.32 mm I.D., layer 0.5 µm). Hydrogen was used as carrier gas. Split ratio 20:1, detector temperature 250°C; injector temperature 200°C. Programme 35°C (10 min) x 30°C/min to 230°C. Chromatographic peakes were recorded, integrated and quantified on an HP3396-A integrator.

This extraction / GC-analysis technique is used on a daily basis in the laboratory which performed these analyses. Experience over years has indicated a maximum variation of 5% on some of the components analyzed.

5.3 RESULTS AND DISCUSSION

Data recorded for esters, higher alcohols and fatty acids are presented in **Tables 5.1, 5.2** and **5.3**, respectively. Maximum and minimum values per compound as well as totals per group of compounds are presented in bold type. Data for the indigenous yeasts will be compared to that for the commercial control strains. Yeast strain VIN13, currently the most widely used wine yeast strain in South Africa, is a general purpose strain that does well at low and high fermentation temperatures. Strain WE372, on the other hand, is generally used for red wine production and does not ferment well below 18°C. Perusal of the data for esters (**Table 5.1**) reveals many entries indicated as 0.00 mg/l. In most cases these entries represent trace amounts having peaks so small that they could not be handled by the integration system.

Table 5.1. Ester production (mg/l) by indigenous yeasts in Chenin blanc must.

Strain	EA*	EB	IAM	ECO	HA	ECY	ECA	PEA	ΣESTERS	Σ-EA
C5-1	**1,60**+	**0,00**	**0,00**	**0,00**	**0,00**	0,77	**0,00**	0,18	**2,55**	0,95
C5-2	38,00	0,46	3,38	0,78	0,15	2,08	0,85	0,27	45,99	7,79
C5-3	47,90	0,59	3,68	0,76	0,19	1,42	0,31	0,30	55,15	7,25
C5-4	46,60	0,60	3,38	0,75	0,18	1,36	0,72	0,27	53,86	7,26
B5-1	24,00	0,16	0,86	0,28	0,00	0,36	0,00	0,11	25,77	1,77
B5-2	25,50	0,17	1,85	0,38	0,12	0,59	0,00	0,24	28,85	3,35
B5-4	40,60	0,45	2,92	1,06	0,17	2,64	0,89	0,16	48,89	8,29
B5-5	2,90	0,00	0,00	0,00	0,00	0,35	0,00	0,13	3,38	**0,48**
B5-7	43,10	0,54	4,91	**1,35**	0,20	**3,13**	**1,53**	0,31	55,07	11,97
B5-8	5,10	0,00	0,00	0,00	0,00	0,55	0,73	0,13	6,51	1,41
B5-11	55,30	0,46	5,15	1,04	**0,28**	2,04	1,03	0,40	65,70	10,40
B5-13	46,30	0,42	3,14	0,75	0,23	1,70	0,76	0,28	53,58	7,28
B5-14	56,10	0,44	2,80	0,76	0,22	1,66	1,06	0,32	63,35	7,25
B5-15	25,50	0,00	1,13	0,34	0,10	1,32	0,51	0,37	29,27	3,77
J5-2	7,90	0,00	0,23	0,13	0,00	1,36	0,64	0,35	10,61	2,71
J5-3	8,10	0,00	0,00	0,04	0,04	0,49	0,42	0,09	9,18	1,08
J5-4	61,40	0,67	3,63	0,88	0,20	2,30	1,18	0,24	20,50	9,10
L5-1	33,60	0,18	1,30	0,13	0,00	0,22	0,00	0,18	35,61	2,01
M5-1	49,70	0,59	3,73	0,82	0,22	2,00	0,80	0,32	58,18	8,48
M5-2	36,00	0,24	1,28	0,18	0,00	0,23	0,00	0,10	38,03	2,03
M5-3	41,90	0,21	1,52	0,11	0,00	**0,00**	0,49	**0,06**	44,29	2,39
M5-5	40,90	0,25	1,48	0,11	0,00	0,19	0,00	0,09	43,02	2,12
M5-6	27,60	0,19	0,79	0,00	0,00	0,00	0,00	0,07	28,65	1,05
M5-9	37,50	0,24	1,30	0,08	0,00	0,11	0,00	0,07	39,30	1,80
V5-1	48,10	0,25	1,30	0,12	0,00	0,21	0,00	0,28	50,26	2,16
V5-2	31,50	0,19	0,83	0,00	0,00	0,00	0,00	0,07	32,59	1,09
F5-1	49,80	0,22	2,69	0,00	0,00	0,00	0,00	0,29	53,00	3,20
F5-2	47,90	**0,69**	3,70	0,88	0,20	2,16	0,47	0,32	56,32	8,42
HR5-1	15,30	0,00	0,43	0,23	0,00	1,19	0,71	0,11	17 97	2,67
HR5-3	**76,80**	0,60	**7,63**	0,83	0,27	1,66	0,60	**0,50**	**88,89**	**12,09**
HR5-5	36,30	0,43	2,35	0,93	0,15	1,79	0,91	0,26	43,12	6,82
HR5-8	31,20	0,24	1,42	0,56	0,12	0,71	0,68	0,32	35,25	4,05
HR5-9	23,50	0,25	1,72	0,61	0,14	1,14	0,32	0,21	27,89	4,39
VIN13	39,00	0,15	1,23	0,08	0,03	0,12	0,00	0,09	40,70	1,70
WE372	46,50	0,39	1,76	0,10	0,00	0,20	0,00	0,14	49,09	2,59

* EA = ethylacetate; EB = ethylbutyrate; IAM = iso + active amylacetate; ECO = ethyl caproate; HA = hexyl acetate; ECY = ethyl caprylate; ECA = ethyl caprate; PEA = 2-phenylethyl acetate.

+ All values represent the mean of two analyses. Numbers in bold indicate maximum/minimum values for each compound.

Volatile esters mostly contribute fruity / floral notes to wines aroma (Soles *et al.*, 1982) and play a very important role in the product's fermentation or secondary aroma profile. Clearly then, high levels of ester production would be unwanted in wines which should have a delicate cultivar character (Houtman, Marais & Du Plessis, 1980). On the other hand the opposite would be true for neutral, bland musts ultimately resulting in wines that at best can only be called vinuous. From **Table 5.1** it is clear that ester production by some of the indigenous yeasts differs rather dramatically from that of the two commercial control yeasts. Six of the yeasts (C5-1; B5-5; B5-8; J5-3; M5-6 and V5-2) produce much lower amounts of esters than either of the control strains (**Table 5.1**). This is true for total esters determined as well as total esters minus the ethyl acetate fraction (**Table 5.1**). The winemaking ability of these yeasts should be evaluated in a number of cultivars and under a variety of conditions (Soles *et al.*, 1982) to confirm consistently low ester production. If this ability is confirmed some of these yeasts might make a valuable contribution to the yeast breeding programme mentioned by Pretorius, Van der Westhuizen & Augustyn (1999). Strains such as HR5-3 and J5-4, amongst others (**Table 5.1**), with

a high ester production might be useful when producing yeasts for use in neutral musts. However, ester production is not the only criterion of importance. Excessive production of higher alcohols or fatty acids, especially acetic acid, will clearly also be detrimental to wine quality. Perusal of the higher alcohol data presented in **Table 5.2** indicates that indigenous yeasts once again have concentration levels above and below those produced by the commercial standards. None of the yeasts should be excluded from further evaluation based on the data in **Table 5.2**.

Table 5.2 Higher alcohol production (mg/l) by indigenous yeasts in Chenin blanc must.

Strain	PA*	i-BA	n-BA	IAA	HX	PE	Σ Alcohols
C5-1	**11,50**+	12,90	0,39	96	0,68	13,10	134,57
C5-2	16,20	19,70	0,50	130	0,85	12,50	179,75
C5-3	22,50	21,40	0,35	124	0,85	11,40	180,50
C5-4	23,00	21,80	0,37	116	0,94	10,30	172,41
B5-1	12,30	15,40	**0,13**	78	0,96	7,31	114,10
B5-2	17,20	14,20	0,23	74	**0,40**	9,21	115,24
B5-4	20,40	21,00	0,51	126	0,96	9,57	178,44
B5-5	10,90	25,40	0,23	80	0,43	8,16	125,12
B5-7	17,00	19,40	0,29	130	0,80	14,40	181,89
B5-8	18,10	17,20	0,29	83	1,01	10,40	130,00
B5-11	28,70	17,70	0,48	127	0,84	13,30	188,02
B5-13	23,90	18,50	0,45	97	1,01	0,28	141,14
B5-14	34,30	17,90	0,56	113	1,01	13,50	180,27
B5-15	28,20	15,70	0,50	108	0,91	14,20	167,51
J5-2	**45,40**	12,70	**1,15**	112	0,69	12,20	184,14
J5-3	23,60	16,90	0,25	79	0,87	8,67	129,29
J5-4	40,50	14,80	0,40	100	0,92	9,26	165,88
L5-1	38,60	13,70	0,88	96	0,63	0,18	149,99
M5-1	23,20	20,30	0,48	126	1,05	13,30	184,33
M5-2	25,20	20,00	0,50	124	**1,20**	0,10	171,00
M5-3	23,80	28,90	0,68	135	1,06	0,06	189,50
M5-5	27,40	22,40	0,57	135	1,14	12,60	199,11
M5-6	19,20	17,80	0,35	91	0,81	**0,07**	129,23
M5-9	18,50	18,20	0,36	104	0,67	**0,07**	141,80
V5-1	24,50	**30,20**	0,62	127	0,89	0,28	183,49
V5-2	17,40	**12,50**	0,39	80	0,97	11,50	122,76
F5-1	47,20	16,80	0,82	**168**	0,81	**26,00**	**259,63**
F5-2	20,50	19,20	0,55	125	0,91	12,00	178,16
HR5-1	29,90	23,40	0,56	114	0,92	4,36	173,14
HR5-3	36,20	25,60	0,51	157	0,73	13,20	233,24
HR5-5	20,00	17,40	0,33	105	0,80	10,40	153,93
HR5-8	12,20	19,00	0,25	**69**	0,45	12,20	**113,10**
HR5-9	14,00	19,70	0,49	99	0,68	10,70	144,57
VIN13	40,20	23,20	0,92	133	0,79	0,09	198,20
WE372	36,40	14,50	0,41	107	0,83	10,70	169,84

* PA = propyl alcohol; i-BA = i-butyl alcohol; n-BA = n-butyl alcohol; IAA = iso + active amyl alcohol; HX = hexyl alcohol; PE = 2-phenylethanol.

+ All values represent the mean of two analyses. Numbers in bold indicate maximum/minimum values for each compound.

Excessive volatile acidity is highly undesirable in a wine as it has a drastic negative effect on wine quality. From the fatty acid data in **Table 5.3** it is clear that yeasts B5-14, B5-15, M5-2, M5-3, M5-6 and V5-1, all produce very high concentrations of acetic acid. This characteristic, if confirmed, will exclude these yeasts from direct use in the wine cellar or inclusion into the breeding programme.

This preliminary study has indicated that volatile ester, higher alcohol and fatty acid production of the indigenous yeasts isolated from the coastal areas of the Western Cape varies appreciably. Subsequent small-scale winemaking trials indicated that

none of these yeasts were suitable for fermenting white grape must at 15°C. However, this does not exclude a potential for the fermentation of red musts, which is conducted at much higher temperatures. Furthermore, some of these strains could also be useful sources of desirable and specific characteristics in our strain development programme.

Table 5.3 Fatty acid production (mg/l) by indigenous yeasts in Chenin blanc must.

Strain	AAC*	PAC	i-BAC	n-BAC	i-VAC	n-VAC	HAC	OAC	DAC	Σ Acids	Σ-AAC
C5-1	691+	1,46	2,29	1,60	0,85	0,18	4,50	5,49	2,98	710,35	19,35
C5-2	756	1,47	2,15	1,44	1,46	0,17	4,56	7,36	3,62	778,23	22,23
C5-3	804	1,32	2,49	2,36	1,29	0,40	4,45	7,68	6,82	830,81	26,81
C5-4	923	1,37	2,61	2,39	1,20	0,40	4,18	6,57	6,24	947,96	24,96
B5-1	294	1,18	1,30	1,10	0,93	**0,10**	**2,71**	4,26	1,04	306,62	**12,62**
B5-2	224	1,15	1,54	1,24	1,14	0,24	3,70	6,48	2,36	241,85	17,85
B5-4	830	1,62	2,87	1,82	1,04	0,25	6,10	9,24	5,56	858,50	28,50
B5-5	**221**	0,84	2,02	**1,00**	1,06	0,16	2,99	4,92	2,73	**236,72**	15,72
B5-7	532	1,35	2,03	2,33	1,38	0,21	**7,68**	**11,70**	**9,27**	567,95	**35,95**
B5-8	1033	1,61	1,84	1,79	1,07	0,28	4,61	6,85	6,19	1057,24	24,24
B5-11	774	1,59	2,07	2,00	1,21	0,37	5,30	9,55	8,67	804,76	30,76
B5-13	981	1,65	2,13	1,91	1,18	0,29	4,34	6,98	6,72	1006,20	25,20
B5-14	1291	2,00	1,73	1,67	0,84	0,33	4,48	7,78	5,19	1315,02	24,02
B5-15	1159	1,74	1,75	2,07	0,52	0,35	4,93	7,88	7,61	1185,85	26,85
J5-2	432	1,74	1,19	2,06	1,02	0,66	4,64	7,41	5,55	456,27	24,27
J5-3	730	1,21	2,19	1,78	1,06	0,27	2,71	3,44	2,91	745,57	15,57
J5-4	988	**2,01**	1,42	2,02	0,77	0,65	4,74	7,69	5,17	1012,47	24,47
L5-1	248	1,48	**1,11**	1,90	0,99	0,58	5,24	6,07	1,79	267,16	19,16
M5-1	1050	1,49	2,23	2,24	0,53	0,34	4,70	6,62	5,06	1073,21	23,21
M5-2	**1499**	1,75	4,22	2,11	1,13	0,46	4,02	2,82	2,00	**1517,51**	18,51
M5-3	1355	1,72	3,32	1,95	1,20	0,35	4,03	4,32	2,11	1374,00	19,00
M5-5	1294	1,68	**4,64**	2,33	0,57	0,46	3,99	3,62	1,79	1313,08	19,08
M5-6	523	1,16	1,55	1,60	0,86	0,17	4,51	6,70	2,52	542,07	19,07
M5-9	366	0,98	2,08	1,55	0,47	0,19	3,40	3,95	**0,67**	379,29	13,29
V5-1	1149	1,36	2,40	2,09	0,90	0,50	6,19	7,72	2,55	1172,71	23,71
V5-2	1095	1,50	1,77	1,70	0,46	0,34	4,18	4,90	1,46	1111,31	16,31
F5-1	448	1,78	3,53	2,47	1,19	**0,78**	5,66	7,57	1,97	472,95	24,95
F5-2	917	1,67	2,49	**2,69**	**1,48**	0,30	5,51	8,55	6,30	945,99	28,99
HR5-1	952	1,60	2,91	2,20	1,43	0,32	4,79	6,85	6,21	978,31	26,31
HR5-3	631	1,74	1,75	1,77	0,54	0,30	4,30	4,36	3,09	648,85	17,05
HR5-5	1014	1,40	2,24	1,77	1,22	0,30	5,45	9,59	8,17	1044,14	30,14
HR5-8	310	**0,78**	1,41	1,43	0,62	0,27	5,38	8,29	7,77	335,95	25,95
HR5-9	486	1,19	1,61	1,31	**0,45**	0,14	4,33	7,76	4,30	507,09	21,09
VIN13	724	1,19	3,61	1,72	0,79	0,46	2,81	**2,86**	0,78	738,22	14,22
WE372	796	1,87	1,58	2,57	1,04	0,63	4,80	5,30	1,65	815,44	19,44

* AAC = acetic acid; PAC = propionic acid; i-BAC = i-butyric acid; n-BAC = n-butyric acid, i-VAC = i-valeric acid; n-VAC = n-valeric acid, HAC = hexanoic acid; OAC = octanoic acid; DAC = decanoic acid.

+ All values represent the mean of two analyses. Numbers in bold indicate maximum/minimum values for each compound.

5.4 LITERATURE CITED

CARBRERA, M.J., MORENO, J., ORTEGA, J.M. & MADINA, M., 1988. Formation of ethanol, higher alcohols, esters and terpenes by five yeast strains in musts from Pedro Zimenez grapes in various degrees of ripeness. *Am. J. Enol. Vitic.* **39**, 283-287.

CIANI, M. & PICCIOTTI, G., 1995. The growth kinetics and fermentation behaviour of some non-*Saccharomyces* yeasts associated with wine-making. *Biotechnol. Lett.* **17**, 1247-1250.

DAUDT, C.E. & OUGH, C.S., 1973. Variations is some volatile acetate esters formed during grape juice fermentation. Effects of fermentation temperature, SO_2, yeast strain and grape variety. *Am. J. Enol. Vitic.* **24**, 130-135.

DELTEIL, D. & JARRY, J.M., 1992. Characteristic effects of two commercial yeast strains on Chardonnay wine volatiles and polysaccharide composition. *Wine Indus. J.*, February issue, 29-33.

GIUDICI, P., ROMANO, P. & ZAMBONELLI, C., 1990. A biometric study of higher alcohol production in *Saccharomyces cerevisiae*. *Can. J. Microbiol.* **36**, 61-64.

GOMEZ, E., LAENCINA, J. & MARTINEZ, A., 1994. Vinification effects on changes in volatile compounds of wine. *J. Food Sci.* **59**, 406-409.

HERRAIZ, T., REGLERO, G., HERRAIZ, M., MARTIN-ALVAREZ, P.J. & CABEZUDO, M.D., 1990. The influence of the yeast strain and type of culture on the volatile composition of wine fermented without sulfur dioxide. *Am. J. Enol. Vitic.* **41**, 313-318.

HOUTMAN, A.C. & DU PLESSIS, C.S., 1986. The effect of grape cultivar and yeast strain on fermentation rate and concentration of volatile components in wine. *S. Afr. J. Enol. Vitic.* **7**, 14-20.

HOUTMAN, A.C., MARAIS, J. & DU PLESSIS, C.S., 1980. The possibility of applying present day knowledge of wine aroma components: Influence of several juice factors on fermentation rate and ester production during fermentation. *S. Afr. J. Enol. Vitic.* **1**, 27-33.

LEMA, C.. GARCIA-JARES, C., ORRIOLS, I. & ANGULO, L., 1996. Contribution of *Saccharomyces* and non- *Saccharomyces* populations to the production of some components of Albarino wine aroma. *Am. J. Enol. Vitic.* **47**, 206-216.

MARGALITH, P. & SCHWARTZ, Y., 1970. Flavor and micro-organisms. In: Advances in Applied Microbiology. Perlman, D. (ed.). Acedemic Press, New York. pp. 13-35.

MATEO, J.J., JIMENEZ, M., HUERTA, T. & PASTOR, A., 1992. Comparison of volatiles produced by four *Saccharomyces cerevisiae* strains isolated from Monastrell musts. *Am. J. Enol. Vitic.* **43**, 206-209.

NYKäNEN, L., 1986. Formation and occurrence of flavor compounds in wine and distilled alcoholic beverages. *Am. J. Enol. Vitic.* **37**, 84-96.

PRETORIUS, I.S., VAN DER WESTHUIZEN, T.J. & AUGUSTYN, O.P.H., 1999. Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. *S. Afr. J. Enol. Vitic.* (submitted).

RAPP, A. & MANDERY, H., 1986. Wine aroma. *Experienta* **42**, 873-884.

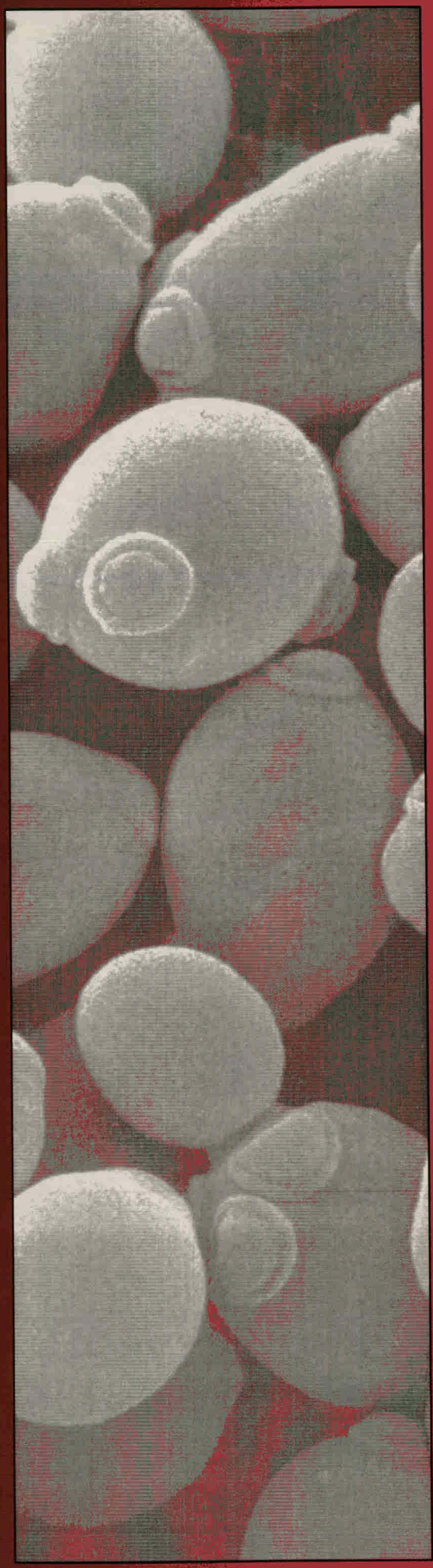
SCHREIER, P., 1979. Flavour composition of wines: A review. *CRC. Crit. Rev. Food Sci. Nutr.* **12**, 59-111.

SOLES, R.M., OUGH, C.S. & KUNKEE, R.E., 1982. Ester concentration differences in wine fermented by various species and strains of yeasts. *Am. J. Enol. Vitic.* **33**, 94-98.

STASHENKO, H., MACKU, C. & SHIBAMATO, T., 1992. Monitoring volatile chemicals formed from must during yeast fermentation. *J. Agric. Food Chem.* **40**, 2257-2259.

VAN DER WESTHUIZEN, T.J., AUGUSTYN, O.P.H. & PRETORIUS, I.S., 1999. Geographic distribution of indigenous *Sacchromyces cerevisiae* strains isolated from Western Cape vineyards. *S. Afr. J. Enol. Vitic.* (submitted)

VAN ZYL, J.A., DE VRIES, M.J. & ZEEMAN, A.S., 1963. The microbology of South African winemaking. III. The effect of different yeasts on the composition of fermented musts. *S. Afr. J. Agric. Sci.* **6**, 165-180.



CHAPTER 6

Research Results V

The breeding of new wine yeast strains

6. RESEARCH RESULTS V

The Breeding of New Wine Yeast Strains

Abstract
The role of yeast, especially strains of *Saccharomyces cerevisiae*, in the wine-making process has been known for many years, but it was only in the last three decades that pure yeast cultures were made available in the dried form. Most of these strains were specifically selected for the European wine styles and did not prove to be very useful in the New World countries where fermentations are controlled at low temperatures and grapes harvested at higher sugar concentrations. This selection and breeding programme was undertaken to select new strains that are better suited to New World winemaking styles and conditions prevailing in South Africa. The breeding programme resulted in 145 hybrids. Extensive microvinification trials were used to select the best of these hybrids. Subsequent commercial evaluation of these selections in sixty wineries resulted in the commercial release of three white and two red wine strains. The white wine strains, NT7, NT45 and NT116, consistently produced wines with tropical fruity, citric and grassy aromas, respectively. The red wine strains, NT50 and NT112, promoted fruity and varietal aromas, respectively.

6.1 INTRODUCTION

The use of spontaneous fermentation, whereby the natural microflora (present on the grapes and winery equipment) is used for fermentation, remained prevalent in Old World wine-producing countries until the 1980s. It became known that the microbiota of grape must is indirectly affected by all the factors influencing the indigenous flora of the grape and winery. The methods of grape harvest, transport of grapes, condition of grapes and must treatment all affect the type and distribution of yeast present in the must. Boutique wineries, relying completely on vintage variability, still utilize this process to produce their unique wines. However, the emergence of large-scale wine production, where reliable, trouble-free fermentations are essential for consistent wine flavour and predictable quality, necessitated the use of selected pure yeast inocula of known ability. It is the large wineries that will be the main beneficiaries of programmes aimed at yeast strain development.

The quality of wine depends not only on the quality and composition of grape musts, but also on the fermentative qualities and performance of the commercial wine yeast used. To produce good wines with reproducible and consistent quality, winemakers need well-characterized yeasts with favourable, stable fermentation abilities. The primary role of wine yeast is to catalyze the rapid and complete conversion of grape sugars to alcohol without the development of any off-flavours. Apart from genetic stability, a minimal lag phase on rehydration and being able to completely ferment sugar at low temperatures, yeast starter cultures should also be tolerant to high levels of sulphite, osmotic stress, ethanol and copper. The production of glycerol and β-glucosidase is required, but the production of foam, sulphur dioxide, hydrogen

sulphide, volatile acidity, acetaldehyde, pyruvate, ethyl carbamate precursors and polyphenol oxidase should be limited (for recent reviews see Pretorius & Van der Westhuizen, 1991; Degré, 1993; Henschke, 1997; Pretorius, 1999). The importance of these additional yeast characteristics largely depends on the type and style of wine to be made and the technical requirements of the winery.

Many researchers have been searching for new yeast strains that possess as many as possible of these desirable characteristics. The emergence of the New World winemaking styles where white wines are produced at low, controlled fermentation temperatures and red wine at higher temperatures with high sugar concentrations, together with the formation of large-scale wineries, has placed new demands on the yeast strains used. Many of the available commercial wine yeast strains are not suitable for these winemaking techniques. The reason being that they were selected for the European wine-producing countries where they generally do not have high sugar containing grape juice and do not produce their white wines at low controlled temperatures. The use of these strains led to numerous lagging and stuck fermentations in South Africa (a typical New World producer).

The aim of this study was to breed and/or select new wine yeast strains with improved performance under the conditions prevalent in the South African wine industry to help local winemakers improve the quality of wines.

6.2 MATERIALS AND METHODS

6.2.1 YEAST STRAINS AND GENETIC METHODS

The yeast strains used in the study are listed in **Table 6.1** and were obtained from ARC-Fruit, Vine and Wine Research Institute, Nietvoorbij Centre for Vine and Wine, Stellenbosch, South Africa. Standard yeast genetic methods of sporulation, purifying and selecting haploids were carried out according to Sherman, Fink & Hicks (1989). Hybridization between haploid cells isolated from heterothallic strains was performed according to the mass-spore-cell mating method described by Salmon, Pinon & Gancedo (1989).

Table 6.1 Parental strains used in the breeding programme.

Strain	Thallism	Killer	Galactose utilization
N96	Heterothallic	Positive	Negative
N642	Heterothallic	Negative	Negative
N650	Heterothallic	Negative	Positive
VIN13 (N96x228)	Homo/heterothallic	Positive	Positive
P35 (N96xN642)	Heterothallic	Positive	Negative
NT1 (VIN7xN96)	Homo/heterothallic	Positive	Positive
N181 (VIN7)	Homothallic	Negative	Positive

6.2.2 MEDIA AND SCREENING PROCEDURE

Yeast strains were grown in a complex medium (YPD) consisting of 1% yeast extract, 2% peptone and 2% glucose. Sporulation of diploid cells was induced in SP medium containing 1% potassium acetate, 0,1% yeast extract and 0,05% glucose. Methylene

blue agar plates, buffered at pH 4,5, were used to detect zones of growth inhibition caused by the K_2 killer toxin secreted by killer yeasts. The strain Geisenheim was used as the sensitive lawn, and N96 (killer positive) and VIN7 (killer negative) as controls. Strains utilizing galactose were identified by the presence of yellow halos on YPGB medium containing 1% yeast extract, 2% peptone, 2% galactose and 2% bromothymol blue (4 mg/ml).

The methylene blue plates were incubated for 48 h at 25°C, while the results of the galactose test were determined after 24 h incubation at 30°C.

6.2.3 MICROVINIFICATION

Bottle fermentations were used to evaluate the fermentation potential of all the new strains. Each 750-ml bottle contained 500 ml Chenin blanc juice and the fermentation was conducted in a 15°C cold room. The bottles were weighed every second day and mass loss was determined over the fermentation time. The fermentations were stopped as soon as the sugar was less than approximately 4 g/l (as measured with Clinistix). The presence of off-flavours in the resulting wines was evaluated by sensory testing.

The winemaking potential of the yeast strains was evaluated in duplicate in 20-litre stainless steel containers containing a homogenous Chenin blanc juice. The chemical analysis of the juice was, sugar 19,8°B, acid 5,6 g/l, pH 3,25. The sulphur was adjusted to 24 ppm free SO_2 and a total SO_2 of 60 ppm. The juice was inoculated, the container sealed with a fermentation cap and fermented at 15°C. All wines that fermented to dryness were bottled for further chemical analysis and sensory evaluation.

6.2.4 FATTY ACID ANALYSIS

The yeast strains were cultivated, harvested and lyophilized, their long-chain cellular fatty acids recovered, methylated and analyzed according to the techniques described by Augustyn & Kock (1989).

6.2.5 PREPARATION OF INTACT CHROMOSOMAL DNA

Samples were prepared according to the embedded agarose procedure of Carle & Olson (1985).

6.2.6 PULSE FIELD GEL ELECTROPHORESIS

Intact chromosomal DNA was separated using contour clamped homogenous electric field (CHEF) electrophoresis (CHEF-DR11, Bio-Rad Laboratories, Richmond, USA). All separations were carried out in 1% agarose gels according to the electrophoretic conditions of Van der Westhuizen & Pretorius (1992) as applied by Van der Westhuizen *et al.* (1999). Gels were stained with ethidium bromide (10 mg/ml), viewed on a transilluminator and photographed.

6.3 RESULTS AND DISCUSSION

6.3.1 HYBRIDIZATION

Many researchers have investigated the possibility of breeding new wine yeast strains, but they were all faced with a typical problem found in *S. cerevisiae*. Wine yeasts are usually diploid, polyploid, or even aneuploid. This phenomenon was well demonstrated by Mortimer *et al.* (1994) who found that the 43 strains they had selected from natural fermenting musts in Italian wineries were all diploid and homothallic, though genetically different. Bakalinsky & Snow (1990) have shown that a single strain can be diploid for some chromosomes and triploid (trisome) or quadruploid (tetrasome) for others. In addition, alloploids (hybrids between different *Saccharomyces* species) can also occur among wine yeasts and other strains of practical use (Vaughan-Martini & Kurtzman, 1985; Kishimoto, Soma & Goto, 1994). Kishimoto (1994) described stable hybrids of cryophillic *Saccharomyces bayanus* and mesophillic *S. cerevisiae* stains. These hybrids did not produce viable spores.

Homothallism and the shortage of suitable selective markers hamper wine yeast breeding programmes. In the past, researchers have been successful in breeding hybrid *Saccharomyces* yeast strains (Miklos & Sipiczki, 1991; Kishimito, 1994, Van der Westhuizen & Pretorius, 1992; Rainieri *et al.*, 1998). Although it might be possible to breed hybrids, the shortage of selective markers makes it difficult to distinguish hybrids from non-hybrid cells. This problem was overcome in that we had some strains that were heterothallic. The ability of some yeast strains to utilize galactose, together with the presence or absence of the killer phenotype, makes it possible to breed hybrids. Hybridization was performed by the spore-cell mating technique, as previously described by Van der Westhuizen & Pretorius (1992). The resulting hybrids were homo/heterothallic strains. This meant that two ascospores per ascus were HO^- and the other two HO^+. It was thus possible to generate stable haploid ascospores from these strains that were used to expand the gene pool. This strategy led to the creation of many genetic recombinations and resulted in the breeding and selection of 145 hybrids.

No recombinant DNA techniques were used to insert genetic markers (or any other foreign DNA) due to current consumer preferences prohibiting the use of genetically altered yeasts in winemaking.

6.3.2 BOTTLE FERMENTATIONS

The 145 hybrids contained a combination of the oenological characteristics of the parental strains used. It was not possible to evaluate the winemaking potential of all the strains. Bottle fermentations at 15°C were used to select the strains most suited to cold fermentations. Seventy two of the hybrids with good fermentation performance potential were subsequently selected for further evaluation (data not shown).

6.3.3 MICROVINIFICATION

Fifty eight of the 72 hybrids were able to ferment the juice to dryness in 21 days or less. It was evident that most of the hybrids had very good fermentation abilities. This is understandable, since most of the parental strains used had good fermentation characteristics. The wine was chemically analyzed (data not shown). All the wines showed normal chemical analyses, with the exception of some wines that had very high total SO_2 values. Some of the results are shown in **Table 6.2**. A few strains had values of 115-150 mg/l while the rest of the strains had values of 19–50 mg/l. It is evident that one should monitor the presence of SO_2 when evaluating hybrid strains since an additive effect has been reported. The production of SO_2 is gene regulated and was most probably overexpressed in these hybrids.

Table 6.2 Fermentation capabilities and SO_2 analysis of some of the hybrids evaluated in Chenin blanc juice at 15°C (free SO_2 - 24 ppm; total SO_2 - 60 ppm).

Strain	Total of fermenting days	Free SO_2	Total SO_2
Positive control	14	9	33
Negative control	23	7	19
NT8	16	7	25
NT35	14	8	51
NT43	14	11	31
NT45	14	14	39
NT66	14	7	70
NT84	16	10	154
NT114	10	15	49
NT123	16	6	117
NT130	14	13	87
NT140	16	5	114

The wine yeast strains selected in the programmes described in Chapters 3 and 4 were also exposed to the same pre-screening and microvinification evaluations. None of these strains passed this microvinification evaluation stage, as they were not able to ferment to dryness in a reasonable time at low temperature (15°C). This does not completely exclude them from direct use in the winery as it is quite possible that evaluation at higher fermentation temperatures, as used for red winemaking, could see some of them pass the prefermentation trials.

6.3.4 SELECTION, CHARACTERIZATION AND COMMERCIALIZATION OF STRAINS

All wines that fermented to dryness were sensorially evaluated by tasting panels after they had been bottled for six months. This was done for both replications of each strain and 14 people were used per tasting. All wines were compared to a reference wine produced by VIN13, the most successful commercial wine yeast strain in South Africa. The tasting panels were also instructed to select the strains with the most interesting aromatic profiles. The evaluation

Table 6.3 Hybrid strains selected for commercial evaluation.

Strain	Parental strain	Parental strain
NT7	NT1 haploid 2	P35 haploid 7
NT45	NT1 haploid 3	P35 haploid 9
NT50	NT1 haploid 5	P35 haploid 6
NT111	N96 haploid 7	P35 haploid 7
NT112	N96 haploid 7	VIN13 haploid 1
NT116	N96 haploid 7	VIN13 haploid 6
NT130	N96 haploid 10	P35 haploid 4
NT143	N650 haploid 1	P35 haploid 2

was repeated at six-weekly intervals for a total of 10 months. The wines with the best fermentation, chemical and aromatic potential were then evaluated by a panel from the wine industry. Based on their recommendation a final selection of yeast strains for commercialization was made.

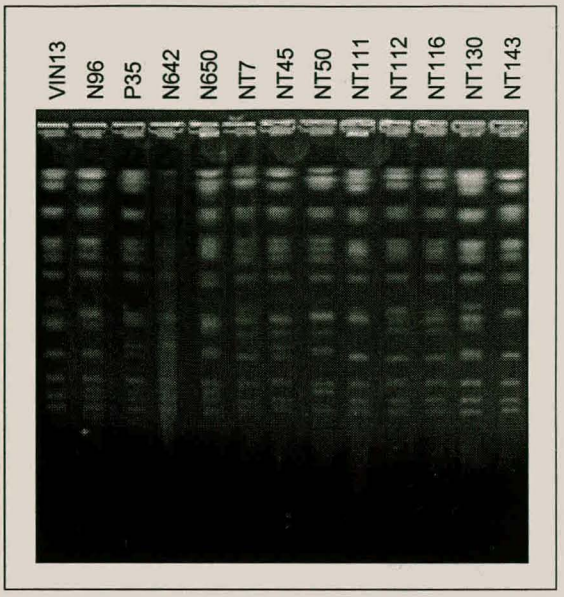


Figure 6.1 Chromosomal DNA banding patterns of the parental and hybrid strains.

All strains selected for commercial evaluation were genetically characterized and patented before release. The parental and hybrid strains were fingerprinted by means of long-chain fatty acid analysis (**Table 6.4**) and chromosomal banding patterns (**Fig. 6.1**) before release to the industry. All hybrids exhibited unique banding patterns that differed from that of the parental strains. This result was confirmed by the long-chain fatty acid analysis. These data were also used to patent the hybrid wine yeast strains.

Selected yeast strains were cultivated on an industrial scale, dried and subsequently released for commercial fermentation trials. Based on *feed-back* from 60 wineries, two white wine strains, NT7 and NT116 were released for commercial sales in 1997, and one white wine strain, NT45, and two red wine strains, NT50 and NT112, were released in 1998. The final decision to commercialize these five yeast strains was based on their appropriate fermentation performance and the interesting aromas conferred to wine. The white wine strains, NT7, NT45 and NT116, consistently produced wines with tropical fruity, citric and grassy aromas, respectively. The red wine strains, NT50 and NT112, promoted fruity and varietal aromas, respectively. The genetic recombinations of the parents of these hybrids are shown in **Table 6.3**.

Table 6.4 Mean relative percentages of nine fatty acids in parental and hybrid wine yeast strains.

Strain	Fatty acids							
	1 14:0	2 14:1(9)	3 15:0	4 15:1(9)	5 16:0	6 16:1(9)	7 18:0	8 18:1(9)
N96	0,8	0,2	0,45	0,2	12,75	52,45	5,15	34,60
N96	0,7	0,2	0,4	0,2	9,9	35,8	4,2	35,5
N642	3,0	1,0	0,5	0,2	14,5	39,7	4,2	34,2
N650	1,2	0,2	0,5	0,1	14,7	36,4	5,0	39,4
VIN13	0,5	0,1	0,1	0,4	9,0	42,0	5,4	41,5
P35	1,1	0,2	0,2	0,1	13,3	31,1	5,2	36,9
NT7	1,8	0,4	0,3	0,1	18,3	36,5	3,7	36,4
NT45	1,2	0,1	0,3	0,1	21,8	31,1	5,0	39,2
NT50	0,8	0,1	0,3	0,0	25,9	21,9	5,8	43,0
NT111	0,8	0,2	0,2	0,1	10,2	41,0	4,4	40,7
NT112	0,3	0,1	0,3	0,2	7,9	42,8	6,4	39,7
NT116	0,5	0,1	0,3	0,2	7,9	40,1	6,3	43,4
NT130	0,6	0,1	0,2	0,1	12,7	33,7	5,6	44,8
NT143	1,1	0,4	0,3	0,2	11,7	44,0	5,5	32,8

6.4 CONCLUSIONS

This selection and breeding programme has proved that it is possible to breed specific wine yeast strains that are better adapted to local winemaking conditions. It was possible to breed many hybrids that fermented very well at low controlled temperatures. The selection programme from vineyards could not identify a single strain over a long period of time that was suitable for such fermentations. Notwithstanding the problem pertaining to homothallism and the lack of selective markers in the naturally-occurring isolates, our current breeding strategy is to include some of the indigenous strains. This will broaden the genetic pool which will hopefully result in even better hybrids.

6.5 LITERATURE CITED

AUGUSTYN, O.P.H. & KOCK, J.F.L., 1989. Differentiation of yeast species, and strains within a species, by cellular fatty acid analysis. 1. Application of an adopted technique to differentiate between strains of *Saccharomyces cerevisiae*. *J. Microbiol. Meth.* **10**, 9-23.

BAKALINSKY, A.T. & SNOW, R., 1990. The chromosomal constitution of wine strains of *Saccharomyces cerevisiae*. *Yeast* **6,** 367-382.

CARLE, G.F. & OLSON, M.V., 1985. An electrophoretic karyotype for yeast. *Proc. Natl. Acad. Sci. USA* **82**, 3756-3760.

DEGRé, R., 1993. Selection and commercial cultivation of wine yeast and bacteria. In: FLEET, G.H. (ed.). *Wine microbiology and biotechnology.* Harwood Academic Publishers, Singapore. pp. 421-447.

HENSCHKE, P.A., 1997. Wine yeast. In: ZIMMERMANN, F.K. & ENTIAN, K.-D. (eds). Yeast sugar metabolism. Technomic Publishing Co., Lancaster, Pennsylvania. pp. 527-560.

KISHIMOTO, M., 1994. Fermentation characteristics of hybrids between the cryophilic wine yeast *Saccharomyces bayanus* and the mesophilic wine yeast *Saccharomyces cerevisiae*. *J. Ferment. Bioeng.* **77**, 432-435.

KISHIMOTO, M., SOMA, E. & GOTO, S., 1994. Classification of cryophilic wine yeasts based on electrophoretic karyotype, G+C content and DNA similarity. *J. Gen. Appl. Microbiol.* **40**, 83-93.

MIKLOS, I. & SIPICZKI, M., 1991. Breeding of a distiller's yeast by hybridisation with a wine yeast. *Appl. Microbiol. Biotechnol.* **35**, 638-642.

MORTIMER, R.K., ROMANO, P., SUZZI, G. & POLSINELLI, M., 1994. Genome renewal: A new phenomenon revealed from a genetic study of 43 strains of *Saccharomyces cerevisiae* derived from natural fermentation of grape musts. *Yeast* **10,** 1543-1552.

PRETORIUS, I.S. & VAN DER WESTHUIZEN, T.J., 1991. The impact of yeast genetics and recombinant DNA technology on the wine industry – A review. *S. Afr. J. Enol. Vitic.* **12**, 3-31.

PRETORIUS, I.S., 1999. Engineering designer genes for wine yeasts. *Aust. N.Z. Wine Indust. J.* **14**, 42-47.

RAINIERI, S., ZAMBONELLI, C., GIUDICI, P. & CASTELLARI, L., 1998. Characterisation of thermotolerant *Saccharomyces cerevisiae* hybrids. *Biotechnol. Lett.* **20**, 543-547.

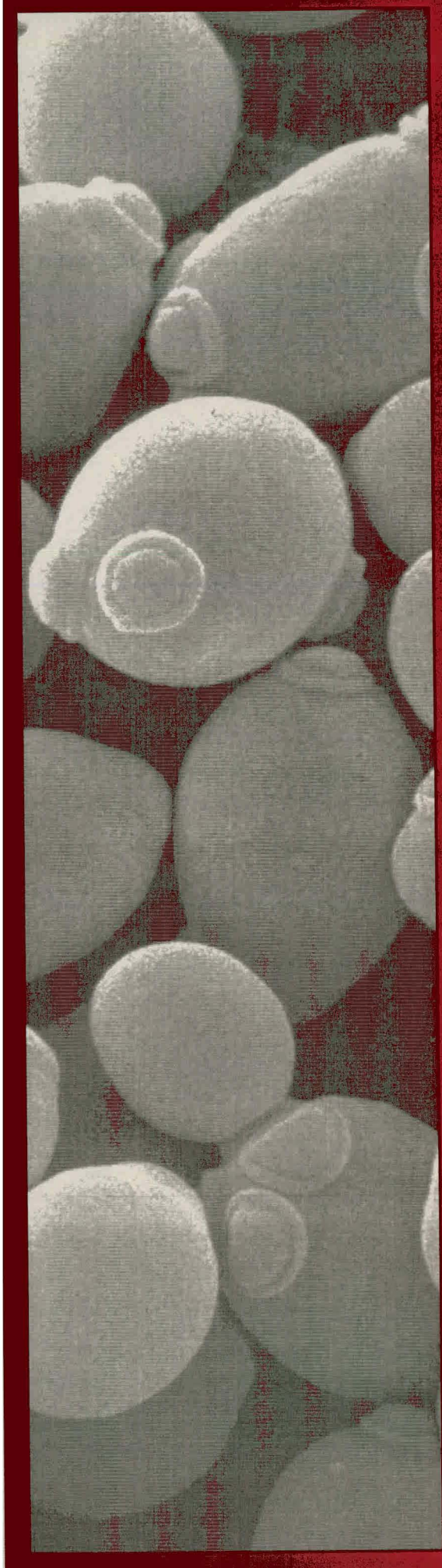
SALMON, J.M., PINON, R. & GANCEDO, C., 1989. Isolation and characterization of mutants of *Saccharomyces cerevisiae* able to sporulate in the presence of glucose. *J. Gen. Microbiol.* **135**, 203-209.

SHERMAN, F., FINK, G.R. & HICKS, J.B., 1989. Laboratory Course Manual of Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

VAN DER WESTHUIZEN, T.J., AUGUSTYN, O.P.H. & PRETORIUS, I.S., 1999. The value of long-chain fatty acid analysis, randomly amplified polymorphic DNA and electrophoretic karyotyping for the characterization of wine yeast strains. *S. Afr. J. Enol. Vitic.* **20**, 3-10.

VAN DER WESTHUIZEN, T.J. & PRETORIUS, I.S., 1992. The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes. *Ant. v. Leeuwen.* **61**, 249-257.

VAUGHAN-MARTINI, A. & KURTZMAN, C.P., 1985. Deoxyribonucleic acid relatedness among species of the genus *Saccharomyces* sensu stricto. *Int. J. Syst. Bacteriol.* **35**, 508-511.



CHAPTER 7

General Discussion and Conclusions

7. GENERAL DISCUSSION AND CONCLUSIONS

7.1 CONCLUDING REMARKS AND PERSPECTIVES

Wine fermentation is a complex microbial process involving the transformation of grape must by the action of different species of yeasts and lactic acid bacteria present on the grapes and winery equipment. Yeast of the genera *Candida*, *Hanseniaspora*, *Kloeckera* and *Pichia* grow during the initial stages of fermentation, but with increasing ethanol concentration, *Saccharomyces cerevisiae* becomes the dominant yeast species until the end of the fermentation (Heard & Fleet, 1985; Frezier & Dubourdieu, 1992; Fleet & Heard, 1993; Querol, Barrio & Ramon, 1994). The microbial fermentation of must has been the subject of numerous studies in which the variability and diversity of wine yeast populations has been well established (for a review see Fleet & Heard, 1993). These studies demonstrated that the microorganisms principally responsible for the alcoholic fermentations are strains of the species *S. cerevisiae*. These strains are well adapted to must conditions and can grow under these conditions to complete the alcoholic fermentation (Martini & Vaughan-Martini, 1990).

The production and use of dry wine yeasts began in the United States of America several decades ago. The addition of selected yeasts to grape must can ensure not only the proper start of the alcoholic fermentation, but also its normal conclusion. For these reasons, when yeast strains are selected, it is essential to establish their oenological properties (Degré, 1993; Henschke, 1997). These oenological properties are classified into three types (Cuinier & Leveau, 1979): favourable (e.g., tolerance to, and good yield of, ethanol; ability to grow under conditions of high sugar concentration and high fermentation activity); unfavourable (*e.g.*, production of H_2S, foam or volatile acidity); neutral (*e.g.*, killer character and malic acid degradation).

Although wine yeast strains have been used for centuries, their unequivocal characterization has only recently been possible, when molecular techniques permitted their identification and the monitoring of their evolution in inoculated and spontaneous fermentations (Querol *et al.*, 1992a, b; Schütz & Gafner, 1993, 1994; Querol & Ramon, 1996; De Barros Lopes *et al.*, 1999). Different methods of yeast characterization have revealed quite extensive variability between *S. cerevisiae* strains. Using mitochondrial DNA (mtDNA) restriction analysis and electrophoretic chromosomal patterns, Vézinhet *et al.* (1992) found evidence for the occurrence of specific native strains. Similar results were obtained by Versavaud *et al.* (1995) using PCR amplification of δ elements, karyotyping and RFLP analysis of mtDNA to analyze the variability, phylogenetic affinities and biogeographic distributions of native *S. cerevisiae* oenological yeasts. The existence of *S. cerevisiae* strains specific to particular wine regions indicates that the species exhibit at least some degree of geographic population structure, perhaps reflecting an adaptation to specific micro-environments (Guillamón *et al.*, 1996; Nadal, Colomer & Piña, 1996). It has been shown that yeast strains are fully adapted to specific climatic environments and substrates, and some oenologists admit that good results can only be obtained with selected yeast starters originating from the micro-area where the wine is produced (Martini & Vaughan-Martini, 1990). It is, therefore, not surprizing

that there is increasing support in the wine community, especially in the Old World, for the use of indigenous yeast species to improve the sensory quality of wines (Heard, 1999).

It is against this backdrop that a comprehensive, long-term research programme has been launched by the Wine and Fermentation Technology Division at the ARC-Fruit, Vine and Wine Research Institute, Nietvoorbij Centre for Vine and Wine, and the Institute for Wine Biotechnology at the University of Stellenbosch. As detailed in Chapter 1 this collaborative research programme is aimed at the preservation and exploitation of the hidden oenological potential of the untapped wealth of yeast biodiversity in our wine-producing regions. Chapter 2 represents the starting point of this daunting task, namely the evaluation of three yeast fingerprinting techniques as means to efficiently differentiate amongst newly-isolated *S. cerevisiae* strains. It was found that electrophoretic karyotyping (CHEF-DNA analysis), as a single technique, seemed to be the most useful method for routine fingerprinting. However, it was proposed that the combined use of CHEF-DNA analysis, randomly amplified polymorphic DNA (RAPD-PCR) and long-chain fatty acid analysis would provide the most reliable means of differentiating amongst *S. cerevisiae* strains. Chapters 3 and 4 descibe the first survey of the geographical distribution of indigenous *S. cerevisiae* strains in the coastal regions of the Western Cape and how this natural yeast population varied over a four-year period. Chapter 5 describes the first preliminary data as to how these indigenous *S. cerevisiae* strains influenced the flavour composition of Chenin blanc wine. None of the indigenous strains were found to be suitable for fermenting white must at 15°C. Their ability to ferment red musts at much higher temperatures still needs to be assessed. Furthermore, there were indications that some of these strains did in fact show sufficient potential to be included in our extensive strain-development programme as this would broaden the genetic pool. Chapter 6 reports that, in parallel with the search to isolate and identify indigenous *S. cerevisiae* strains with good oenological potential, breeding was conducted with cultures from our strain collection. This strain-development programme was highly successful as several of these hybrids were patented and commercialized.

This dissertation lays the foundation for, what seems to be, a very interesting and exciting biogeographical research programme. I firmly believe that only when we have a much better understanding of yeast systematics, biodiversity, biogeography and ecology will we be able to develop further improved starter culture strains. I also share the viewpoint of Heard (1999), who speculated that these starter strains will include mixed culture starter cultures specifically tailored to reflect the characteristics of a given wine region. Sound yeast selection and strain development programmes, together with the effect of the inexhaustible range of *terroir* on the various different grape varieties, will help viticulturalists and oenolgists to improve the sensorial quality of wine while maintaining the most enthralling aspect of wine — its endless variety.

7.2 LITERATURE CITED

CUINIER, C. & LEVEAU, J.Y., 1979. L'identification des levures des vignoble et des vins, méthode rapide a l'aide de la galerie API 20 C. *Vignes et vins* **283**, 44-49.

DE BARROS LOPES, M., RAINIERI, S., HENSCHKE, P.A. & LANGRIDGE, P., 1999. AFLP fingerprinting for analysis of yeast genetic variation. *Int. J. Syst. Bacteriol.* **49**, 915-924.

DEGRé, R., 1993. Selection and commercial cultivation of wine yeast and bacteria. In: FLEET, G.H. (ed.). *Wine microbiology and biotechnology.* Harwood Academic Publishers, Singapore. pp. 421-447.

GUILLAMÓN, J.M., BARRIO, E. & QUEROL, A., 1996. Characterisation of wine yeasts strains of the *Saccharomyces* genus on the basis of molecular markers: relationships between genetic distance and geographical or ecological origin. *Syst. Appl. Microbiol.* **19**, 122-132.

FLEET, G.H. & HEARD, G.M., 1993. Yeasts – Growth during fermentation. In: FLEET, G.H. (ed.). *Wine microbiology and biotechnology.* Harwood Academic Publishers, Singapore. pp. 27-54.

FREZIER, V. & DUBOURDIEU, D., 1992. Ecology of yeast strain *Saccharomyces cerevisiae* during spontaneous fermentation in a Bordeaux winery. *Am. J. Enol. Vitic.* **36**, 375-380.

HEARD, G.M. 1999. Novel yeasts in winemaking – looking to the future. *Food Australia* **51**, 347-352.

HEARD, G.M. & FLEET, G.H., 1985. Growth of natural yeast flora during the fermentation of inoculated wines. *Appl. Environ. Microbiol.* **50**, 727-728.

HENSCHKE, P.A., 1997. Wine yeast. In: ZIMMERMANN, F.K. & ENTIAN, K.-D. (eds). Yeast sugar metabolism. Technomic Publishing Co., Lancaster, Pennsylvania. pp. 527-560.

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

NADAL, D., COLOMER, B. & PIÑA, B., 1996. Molecular polymorphism distribution in phenotypically distinct populations of wine yeast strains. *Appl. Environ. Microbiol.* **62**, 1994-1950.

QUEROL, A., BARRIO, E., HUERTA, T. & RAMÓN, D., 1992a. Molecular monitoring of wine fermentations conducted by active dry yeasts strains. *Appl. Environ. Microbiol.* **58**, 2948-2593.

QUEROL, A., BARRIO, E. & RAMÓN, D., 1992b. A comparative study of different methods of yeast strain characterisation. *Syst. Appl. Microbiol.* **15**, 439-446.

QUEROL, A., BARRIO, E. & RAMÓN, D., 1994. Population dynamics of natural *Saccharomyces* strains during wine fermentation. *Int. J. Food Microbiol.* **21**, 315-323.

QUEROL, A. & RAMÓN, D., 1996. The application of molecular techniques in wine microbiology. *Trends Food Sci. Tech.* **7**, 73-78.

SCHüTZ, M. & GAFNER, J., 1993. Analysis of yeast diversity during spontaneous and induced alcoholic fermentations. *J. Appl. Bacteriol.* **75**, 551-558.

SCHüTZ, M. & GAFNER, J., 1994. Dynamics of the yeast strain population during spontaneous alcoholic fermentation determined by CHEF gel electrophoresis. *Lett. Appl. Microbiol.* **19**, 253-257.

VERSAVAUD, A., COURCOUX, P., ROULLAND, C., DULAU, L. & HALLET, J.-N., 1995. Genetic diversity and geographic distribution of wild *Saccharomyces cerevisiae* strains from the wine-producing area of Charentes, France. *Appl. Environ. Microbiol.* **61**, 3521-3529.

VéZINHET, F., HALLET, J.-N., VALADE, M. & POULARD, A., 1992. Ecological survey of wine yeast strains by molecular methods of identification. *Am. J. Enol. Vitic.* **43**, 83-86.