GENETIC CHARACTERISATION AND BREEDING OF WINE YEASTS

by

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DECLARATION

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

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SUMMARY

To remain competitive in the market place, the South African wine industry will have to direct well-planned yeast strain-development programmes. However, the winemaker can only benefit from the extensive biochemical and molecular information of the yeast cell and the impressive arsenal of genetic techniques available, if the wine industry defines its requirements in genetic terms. The successful application of these genetic and recombinant deoxyribonucleic acid (DNA) techniques in breeding programmes depends on the availability of rapid and reliable techniques to differentiate between parental and hybrid strains.

Ten strains of *Saccharomyces cerevisiae* used for commercial production of wine in South Africa, were characterised by electrophoretic banding patterns of total soluble cell proteins, DNA restriction fragments and chromosomal DNA. Variations in the protein and DNA profiles of strains N6, N21, N66, N76, N95 and N97 were apparent in the number, position and intensity of the bands. Strains N93 and N181 originated from the same culture and, as expected, displayed the same characteristic protein, DNA restriction fragment and chromosomal banding patterns. Similar protein and DNA profiles were also obtained for killer strain N96 and strain N91. Strain N91 is a derivative of strain N96, cured of the K_2 killer character. Results obtained by electrophoretic fingerprinting and karyotyping corresponded well, indicating that these techniques are valuable in the identification and quality control of industrial wine yeasts.

The value of electrophoretic fingerprinting and karyotyping was also demonstrated in a breeding programme. The aim of this breeding programme was to obtain hybrids that combine the desired oenological characteristics of strains N76 and N96, and of strains N96 and N181. The protein banding patterns of hybrids USM21, USM22 and USM23 were identical and contained a combination of prominent unique bands present in the profiles of parental strains, N76 and $N96^H$ ($N96^H$ is a haploid derived from N96). The DNA restriction fragment profiles of hybrids USM21, USM22 and USM23 contained slight variations, whereas their profiles were quite different from those of their parental strains, N76 and $N96^H$. The contour clamped homogeneous electric field (CHEF) karyotypes of hybrids USM21, USM22 and USM23 were identical but differed from those of their parental strains, N76 and $N96^H$. The protein profiles of hybrid USM30 and its parental strains, $N96^H$ and N181, were similar, whereas their DNA restriction fragment banding patterns and CHEF karyotypes showed discrete differences. In conclusion, protein and DNA fingerprinting

techniques were found to be valuable in selecting four hybrid killer strains after mass spore-cell mating. These four killer hybrids contain desirable oenological properties long sought after by the South African wine industry. Fermentation trials and evaluation of these hybrids were conducted independently by the Deparment of Oenology, University of Stellenbosch and by Stellenbosch Farmers' Winery and they have now been released for commercial wine production.

OPSOMMING

Om mededingend in die handel te bly, sal die Suid-Afrikaanse wynbedryf wel-oorwoë gisras-ontwikkelingsprogramme moet loods. Die wynmaker sal egter slegs voordeel kan trek uit die omvattende biochemiese en molekul—re inligting oor die gissel en die indrukwekkende arsenaal van genetiese tegnieke wat beskikbaar is, indien die wynbedryf sy vereistes in genetiese terme definieer. Die suksesvolle toepassing van hierdie genetiese en rekombinante deoksiribonukleïensuur (DNA) tegnieke in telingsprogramme sal afhang van die beskikbaarheid van vinnige en betroubare tegnieke om tussen ouerlike en hibried-rasse te onderskei.

Tien rasse van *Saccharomyces cerevisiae* wat vir kommersiële wynproduksie in Suid-Afrika gebruik word, is met behulp van elektroforetiese bandpatrone van totale oplosbare selproteïene, DNA-restriksiefragmente en chromosomale DNA gekarakteriseer. Variasies in die proteïen- en DNA-profiele van rasse N6, N21, N66, N76, N95 en N97 het geblyk uit die aantal, posisie en intensiteit van die bande. Rasse N93 en N181 het uit dieselfde kultuur ontstaan en het, soos verwag, dieselfde karakteristieke proteïen-, DNA-restriksiefragment- en chromosomale bandpatrone getoon. Soortgelyke proteïen en DNA profiele is ook vir killerras N96 en ras N91 verkry. Ras N91 is 'n variant van ras N96 wat die K_2 killerkenmerk verloor het. Resultate wat met behulp van elektroforetiese vingermerking en kariotipering verkry is, het goed ooreengestem en dui daarop dat hierdie tegnieke waardevol is vir die identifisering en beheer van industriële giste.

Die waarde van elektroforetiese vingermerking en kariotipering in telingsprogramme is ook gedemonstreer. Die doel van hierdie telingsprogram was om hibriede te kry waarin die gewenste kenmerke van rasse N76 en N96, en van rasse N96 en N181, gekombineer is. Die proteïen-bandpatrone van hibriede USM21, USM22 en USM23 was identies en het 'n kombinasie van prominente unieke bande, teenwoordig in die profiele van hul ourlike rasse, N76 en $N96^H$ ($N96^H$ is 'n haploïde afstammeling van N96), bevat. Die DNA-restriksiefragment-profiele van hibriede USM21, USM22 en USM23 toon geringe onderlinge verskille, maar hul profiele het wesenlik van die van hul ouerlike rasse, N76 en $N96^H$, verskil. Die kontoergeklampde-homogene-elektriese-veld (CHEF) elektroforetiese kariotipes van hibriede USM21, USM22 en USM23 was identies, maar het verskil van die van hul ouerlike rasse, N76 en $N96^H$. Die proteïenprofiele van hibried USM30 en sy ouerlike rasse, $N96^H$ en N181, was soortgelyk, terwyl hul DNA-restriksiefragment-bandpatrone en CHEF-kariotipes

diskrete verskille getoon het. Ten slotte is gevind dat proteïen- en DNA-vingermerkingstegnieke waardevol was in die seleksie van vier hibried-killerrasse na massa spoor-sel paring. Hierdie vier killerhibriede beskik oor gewenste wynkundige eienskappe waarna die Suid-Afrikaanse wynbedryf reeds lank soek. Fermentasie-proewe en evaluering is onafhanklik deur die Departement Wynkunde, Universitiet van Stellenbosch en deur Stellenbosch-Boerewynmakery gedoen en hulle is nou vir kommersiële wynproduksie vrygestel.

BIOGRAPHICAL SKETCH

Theunes Johannes van der Westhuizen was born on the 22nd September, 1962 in Cape Town. 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 B.Sc. Honours degree in Microbiology was awarded to him in 1988. In 1988 he enrolled as a M.Sc. student in Microbiology at the University of Stellenbosch and was appointed as Technical Officer in the Department of Microbiology.

This thesis is dedicated to my parents.

Hierdie tesis is opgedra aan my ouers.

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PREFACE

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the journal to which it was submitted.

Chapter 2 "Impact of yeast genetics and recombinant DNA technology on the wine industry" has been submitted for publication in *South African Journal for Enology and Viticulture*.

Chapter 3 " The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes" has been submitted for publication in *Antonie van Leeuwenhoek*.

CONTENTS

1. INTRODUCTION

Until recently, the major direction of wine improvement has involved the breeding of improved grape varieties, cultivation of grapes, fermentation and winemaking practices (Snow, 1983). Despite the keyrole that wine yeasts play in winemaking through their fermentation activities, the study of wine yeasts and especially their genetic potential has remained neglected (Thornton, 1983). With traditional wine fermentation methods there has been little demand for improved yeast strains. Consequently, the wine industry has failed to define its requirements in genetic terms and this has impeded the identification of realistic targets for strain development. However, new trends in the beverage markets demand genetic modification of traditional wine yeast strains and the development of more cost-effective winemaking practices.

Yeast has become the experimental model for unravelling molecular mechanisms such as gene regulation and recombination. This has lead to generation of extensive biochemical, genetic and molecular information on the yeast cell and the development of an impressive arsenal of genetic and recombinant DNA techniques (Snow, 1983). Many of the oenological characteristics of the various wine yeast strains have a genetic basis and are amenable to genetic manipulation (Thornton, 1983). Not withstanding the complexity of the genetic make-up of commercial wine yeast strains, techniques like clonal selection of variants, mutation and selection, hybridisation, rare-mating, spheroplast fusion, and gene cloning and transformation have great potential in the breeding of wine yeasts with new and improved oenological properties. The successful application of these genetic techniques in strain development depends on the ability to differentiate between parental and hybrid strains.

Traditionally, yeast cultures used in the beverage industry are characterised by cell and colony morphology, physiological and biochemical criteria, and the ability to flocculate or to form a film (Kunkee & Amerine, 1970). These techniques are time consuming, sometimes unreliable and not universally adept at differentiating between strains of the same species. Furthermore, it is apparent that many of the physiological and biochemical characteristics used are encoded by a small portion of the genome. However, genome comparisons through determination of DNA base composition, DNA reassociation and DNA sequencing are impractical. New approaches to identify wine yeast strains include gas-liquid chromatographic analysis of cellular long-chain fatty acids (Augustyn & Kock, 1989; Tredoux *et al.*, 1987) and protein and DNA fingerprinting. Protein fingerprinting is obtained by visual comparison of the electrophoretic protein banding patterns or numerical analysis of

electropherograms. DNA fingerprinting includes the following techniques: (i) direct analysis of DNA restriction fragments, (ii) analysis of specific DNA restriction fragments by probing, (iii) karyotyping by pulsed field gel electrophoresis and (iv) gene amplification with sequencing (Meaden, 1990).

AIMS OF THIS STUDY

There is an urgent need for the South African wine industry to formulate reliable yeast quality control measures and a well-planned strain-development programme. The yeast quality control and breeding programmes must be tailored to fulfil the specific requirements of the local wine industry. The objective of the present study was twofold; firstly to evaluate the electrophoretic fingerprinting and karyotyping techniques in yeast quality control and strain-development programmes, and secondly, to breed killer wine yeast strains with desirable oenological properties.

The specific aims and approaches of this study were the following:

1. Reviewing of the taxonomic and genetic methods that are useful in yeast quality control and breeding programmes, and the identification of potential targets for strain development.
2. Characterisation of ten yeast strains used for commercial production of wine, by electrophoretic banding patterns of total soluble cell proteins, DNA restriction fragments and chromosomal DNA.
3. Hybridisation of a killer yeast strain (N96) with two sensitive strains (N76 and N181), by spore-cell mating and selection of progeny with desired oenological characteristics (strains N76, N96 and N181 are currently the most popular wine yeasts in South Africa).

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2. IMPACT OF YEAST GENETICS AND RECOMBINANT DNA TECHNOLOGY ON THE WINE INDUSTRY

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IMPACT OF YEAST GENETICS AND RECOMBINANT DNA TECHNOLOGY ON THE WINE INDUSTRY

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1. INTRODUCTION

Yeasts provided food and drink for more than 8 000 years before their existence was recognised in 1680 by the Dutch microscopist, Antonie van Leeuwenhoek. Long before Charles Cagniard de la Tour of France and Theodor Schwann and Friedrich Traugott Kützing of Germany proposed that the products of fermentation were created by a microscopic form of life, yeast had been used to leaven bread, brew beer, bubble champagne and sparkle wine (Demain & Solomon, 1981; Angier, 1986). During the second half of the nineteenth century the French biochemist, Louis Pasteur, disproved the mechanistic theory of leading German chemists, von Liebig and Wöhler, by proposing his vitalistic theory and showing that living yeast cells were responsible for the conversion of sugar to mainly ethanol and carbon dioxide (Demain & Solomon, 1981). Originally, yeasts present on grape skins and equipment were responsible for the "spontaneous" fermentation which took place. No deliberate inoculation was made to start the fermentation. It was only with the development of a technique to isolate pure cultures on solid media by Robert Koch of Germany that it became possible to select yeast strains on the basis of their fermentation behaviour and on the characteristics of their product. In 1883 a pure culture derived from a single yeast cell by the method of Emil Christian Hansen was used for the first time on a production scale in the fermentation of wort to beer in the Carlsberg Brewery in Denmark. Because of its origin this yeast strain was named *Saccharomyces carlsbergensis* Hansen 1883 (Stewart & Russell, 1986). Following the lead of Hansen, Müller-Thurgau sent out pure yeast cultures for winemaking as early as 1890 from Geisenheim-am-Rhein (Kunkee & Amerine, 1970). For the last century, the availability of pure yeast culture has improved reproducibility in fermentations and in product quality (Tubb & Hammond, 1987).

Another milestone in the history of fermentation microbiology was achieved in 1935 with the pioneering genetic studies of Ojvind Winge and colleagues at the Carlsberg Laboratories who established the basic life cycle of *Saccharomyces* (Stewart & Russel, 1986). Today Winge is regarded as the "Father of Yeast Genetics". In 1937 Winge and Lausten also demonstrated the first Mendelian segregation of genetic traits in yeast (Von Wettstein, 1983). Genetic studies on *Saccharomyces cerevisiae* were extended by Lindegren and co-workers, who unravelled the details of the yeast life cycle and identified two opposite mating types (Tubb & Hammond, 1987). It was now possible to interbreed yeast strains and produce new hybrids. Improved understanding of these processes initiated the explosive advance in microbial genetics and molecular biology that is still underway today.

Until recently, studies directed toward wine improvement have concentrated upon improvement of grape varieties and their cultivation, and on fermentation and winemaking practices. However, little attention has been paid to genetic improvement of the other major organism involved in wine production, the wine yeast (Snow, 1983). It is sad to admit that there are still far too many winemakers who use the wine yeast as a chemical and do not handle it as a living organism and who do not realise the potential of its powerful genetic system. Yeast has become one of the premier organisms for basic genetic research and the ideal experimental model for molecular biologists probing the intimate details of genes and proteins in eucaryotic cells. The winemaker can thus benefit from both the extensive biochemical and molecular information on the yeast cell and the impressive repertoire of genetic techniques, and also from decades of practical experience in handling this leading industrial microorganism in large-scale fermentations (Snow, 1983; Rank *et al.*, 1988).

2. CLASSIFICATION AND CHARACTERISATION OF WINE YEASTS

2.1 Classification of wine yeasts

The original wine yeast strains were derived from the yeasts that occur naturally on the grape skins, including species of *Saccharomyces*, *Kloeckera* and *Hanseniaspora* (Snow, 1983). The first pure culture to be used to convert grape must into wine on a production scale was designated *Saccharomyces ellipsoideus*. Based on sugar fermentation and assimilation patterns, wine yeasts of the genus *Saccharomyces* have previously been classified into at least 29 different species or varieties, including *S. ellipsoideus, S. vini, S. bayanus, S. fermentati* and *S. oviformis* (Lodder, 1970; Kunkee & Goswell, 1977). Subsequently, these species were reclassified as *S. cerevisiae* (Kreger-van Rij, 1984). However, it should be emphasised that the assignment of all the wine yeast strains to a single species does not imply that all strains of *Saccharomyces* are equally suitable for wine fermentation. Wine yeast strains differ mainly in their ability to contribute to the bouquet of wine and in their fermentation performance. It is, therefore, of cardinal importance to both the winemaker and yeast geneticist to have reliable taxonomic techniques at their disposal to characterise individual strains.

Conventional yeast taxonomy is usually based on phenotypic traits such as morphological characteristics, sexual reproduction and certain physiological and biochemical features. These taxonomic procedures allow for distinction between species, but are time consuming and not always reliable. Yeast cultures in the alcoholic beverage industry are usually characterised by cell and colony morphology,

physiological tests and the ability to form a film, or flocculate (Kunkee & Amerine, 1970; Van Vuuren & Van der Meer, 1987). In the search for additional taxonomic characteristics, more advanced techniques have been proposed. These include comparison of ascospore surfaces by scanning electron microscopy (SEM), serology (Tsuchiya *et al.*, 1965), proton magnetic resonance (PMR), spectra of cell wall mannans (Gorin & Spencer, 1970) and grouping based on the co-enzyme Q-system (Yamada *et al.*, 1980). Recently, gas-liquid chromatographic analysis of the cellular long-chain fatty acid composition of wine yeasts proved to be a useful technique for rapid identifcation of wine yeast strains (Tredoux *et al.*, 1987; Augustyn, 1989; Augustyn & Kock, 1989).

Many of the traditional criteria used for speciation of yeasts were derived from analysis of a small portion of the genome. Phenotypic characteristics serve a purpose in classification since not all of these characteristics are unstable and insignificant. However, phenotypic traits 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 deoxyribonucleic acid (DNA) base composition and DNA sequence homology in different yeasts. Some of the genetic techniques that have been used to characterise yeasts successfully will now be discussed briefly.

2.2 Genetic characterisation of wine yeasts

Verification of species relationships through hybridisation (sexual compatibility) studies is generally regarded as an ideal way to define taxa (Kurtzman *et al.*, 1983). Lack of fertility among yeasts, however, does not preclude conspecificity because only a few genes affect the ability to mate (Hicks & Herskowitz, 1976). Genome comparisons through determination of DNA base composition, DNA reassociation, restriction length fragment polymorphisms (RFLP), fingerprinting by protein profiles and karyotyping by chromosome banding patterns are being used increasingly in the classification of yeasts. Although the ultimate classification scheme would be to determine and compare the entire nucleotide sequence of the genomes from different yeasts, this is as yet impractical. Only time will tell whether this method will ultimately provide the definitive data for taxonomy of yeasts (Campbell, 1987).

2.2.1 DNA base composition and DNA relatedness

DNA base composition

The DNA base composition, expressed as molar percentages of guanine + cytosine (mol% G+C), has been determined for many yeasts (Price *et al.*, 1978; Kreger-van Rij, 1984). The mol% G+C can be determined from thermal denaturation profiles, buoyant density in isopycnic cesium salt gradients, chemical analysis, absorbance ratios, or high-pressure liquid chromatography of nucleotides or free bases (Kurtzman *et al.*, 1983). The mol% G+C thermal denaturation method (Marmur & Doty, 1962) is used most frequently but is greatly affected by sample impurities and/or minor DNA species and has to be interpreted with caution (Kurtzman *et al.*, 1983). Cesium salt buoyant density determinations (Schildkraut *et al.* 1962) are generally the most accurate, since they are unbiased by the presence of contaminating ribonucleic acid (RNA), mitochondrial DNA and other impurities such as carbohydrates and proteins (Kurtzman *et al.*, 1983). Two yeasts with DNA base composition values that differ by more than 1,5 to 2,5 mol% G+C are not regarded as closely related (Price *et al.*, 1978). These methods have the disadvantage of being more complicated to perform than the physiological tests and they all share a lack of specificity. The taxonomic uses of mol% G+C values are mainly exclusionary, because yeast species range in mol% G+C content from approximately 28 to 70 mol% and overlap between unrelated species is inevitable (Kurtzman, 1987). For example, the 40 mol% G+C content of *S. cerevisiae* is a property shared with at least 36 other yeast species, spanning the genera *Ambrosiozyma, Brettanomyces, Candida, Debaryomyces, Hanseniaspora, Kluyveromyces, Lodderomyces, Nematospora, Pichia, Saccharomyces, Schizosaccharomyces* and *Zygosaccharomyces* (Kreger-van Rij, 1984; Campbell 1987). Therefore, although of some value to classification, the application of these methods to distinguish between individual wine yeast strains is limited.

DNA reassociation

The methods for assessing DNA relatedness vary, but short of actual sequencing rely on measuring the extent and stability of renatured DNA strands from two yeasts, i.e., the fidelity of complementary base pairing (Kurtzman *et al.*, 1983). DNA reassociation is possible when the bases are in essentially the same sequence over the DNA molecule. Depending on the method, the DNA may or may not need to be labelled with radioisotopes. *In vivo* labelling of DNA is frequently done using ^{14}C, ^{3}H or ^{32}P, while *in vitro* labelling is done with ^{125}I or by nick translation. Following

labelling, DNA reassociation reactions can be performed using the membrane method, which involves immobilising single strands of one of the DNA species onto nitrocellulose filters and allowing sheared single strands of the second DNA to react with the immobilised DNA. Alternatively, both DNA species can be allowed to react in free solution and the degree of reassociation assessed by percent binding of the resulting duplexes to hydroxylapatite or by resistance to hydrolysis by S_1 nuclease (Price *et al.*, 1978; Johnson, 1981; Kurtzman *et al.*, 1983; Kurtzman, 1987). Renaturation reactions of DNA that has not been labelled with radioisotopes can be monitored spectrophotometrically by measuring the kinetics of duplex formation (Kurtzman *et al.*, 1980). Relatedness can also be estimated from the thermal stability of the renatured heterologous DNA as compared with renatured homologous DNA. This can be done by monitoring denaturation profiles spectrophotometrically or with labelled DNA by thermal elution from hydroxylapatite columns (Kurtzman *et al.*, 1983). A number of factors affect DNA renaturation reactions, including impurities, contaminating RNA and mitochondrial DNA, repetitive DNA sequences, DNA fragment size and ionic strength of the incubation buffer (Kurtzman *et al.*, 1983).

Determination of mol% G+C might be of limited value, but determination of the extent and stability of renatured DNA strands from different yeasts is of fundamental importance in demonstrating genetic relatedness. Although, for example, the mol% G+C values recorded for the DNA of *Brettanomyces anomalus*, *Pichia quercuum* and *S. cerevisiae* is 40, the difference in base sequences prevents any significant re-annealing of separated DNA strands unless both strands were derived from closely-related yeasts (Campbell, 1987). Despite identical mol% G+C values, DNA renaturation of only 80% or higher was accepted by Price *et al.* (1978) as indicative that the pair of test yeasts were of the same species. This figure has in general been accepted by other yeast taxonomists (Campbell, 1987).

Restriction fragment length polymorphism

Genetic relatedness can also be detected with a technique known as restriction fragment length polymorphism (RFLP). This involves Southern blot hybridisation and the use of specific structural genes or other evolutionarily conserved DNA sequences as hybridisation probes. Once the genomic DNA of a yeast has been isolated, the DNA is digested by one of the several restriction endonucleases. Restriction endonucleases like *Eco*RI, *Bam*HI and *Hin*dIII each recognise a six base pair palindrome and cleave the DNA, generating numerous restriction fragments. Recently, restriction endonucleases (e.g. *Not*I and *Sfi*I) that recognise specific eight base pair sequences have also become available commercially and these generate fewer but larger fragments. These restriction fragments are separated according to

size by agarose gel electrophoresis and the fragment patterns visualised in the presence of ultraviolet light after staining with ethidium bromide. Van der Westhuizen & Pretorius (1989) showed that ethidium bromide-stained electropherograms can be used to distinguish among different wine yeast strains. Using the Southern blot technique, the restriction fragments are transferred from the gel on to nitrocellulose or nylon filter membranes and probed with labelled, specific DNA sequences (Southern, 1975). Gene probes will hybridise to homologous DNA sequences that may occur on restriction fragments of different lengths. This variation in fragment length can be visualised by autoradiography and is referred to as RFLP. Secondly, the intensity of hybridisation (as determined by densitometric scanning of autoradiographs) corresponds to the stability of heteroduplexes formed between the chromosomal DNA and the DNA probe. This stability depends on the degree of homology between the DNA species and can be used to determine genetic relatedness among yeasts.

Restriction fragment length polymorphisms have proved useful in the taxonomic evaluation of yeast genera and species, and even to identify different strains of one species. Various gene probes, including genes encoding rRNA (*RDN1*), enzymes of the pyrimidine (*URA3*) and amino acid synthetic (e.g., *HIS4, LEU2, TRP1*) and glycolytic pathways (*PDC1, PFK1, PFK2, PGI1, PGM1, PGK1, PYK1*) as well as transposable elements (*Ty1*) have been used previously to distinguish among yeast genera, species and strains (Pedersen, 1983a, b; Von Wettstein, 1984; Braus *et al.*, 1985; Decock & Iserentant, 1985; Keiding, 1985; Martens *et al.*, 1985; Pedersen, 1985a, b; Seehaus *et al.*, 1985; Pedersen, 1986a, b; Laaser *et al.*, 1989; Sakai *et al.*, 1990). Pedersen (1983a) used the *HIS4* and *RDN1* gene probes to distinguish *S. cerevisiae* strains formerly known as *Saccharomyces uvarum, Saccharomyces pastorianus* and *S. bayanus*. The *RDN1* gene, encoding the cytosolic 25S, 5,8S, 18S and 5S rRNA molecules, is present in over 100 tandemly repeated copies and is generally highly conserved in nucleotide sequence and overall organisation. The *Ty1* elements resemble retroviruses in structure and function, and typically occur at several positions in the *S. cerevisiae* genome. When *RDN1* and *Ty1* were used as probes together with *HIS4* and *LEU2*, it was possible to identify different restriction fragment patterns for lager (bottom fermenting) and ale (top fermenting) brewing yeast strains (Pedersen, 1985a). Seehaus *et al.* (1985) used *PDC1, PFK1, PFK2, PGI1, PGM1, PGK1, PYK1, URA3* and *TRP1* as probes to assess the degree of genetic relatedness between different yeast genera and species, including *S. cerevisiae*, two commercial baking yeasts and a commercial wine yeast. This study concluded that different degrees of conservation were evident in the genes used as hybridisation probes. The most conserved genes were found to be *PDC1* (pyruvate decarboxylase), *PFK1* (β subunit of phosphofructokinase) and *PYK1*

(pyruvate kinase). These findings indicated a strong conservation of genes encoding enzymes of the central metabolic pathways, like the glycolytic pathway.

Restriction endonuclease analysis of mitochondrial DNA (mtDNA) have also been used to distinguish among different yeasts (Lee & Knudsen, 1985; Vezinhet *et al.*, 1990). Because the mtDNA is approximately 200-fold smaller in size than the nuclear DNA, fewer fragments are generated by restriction endonucleases, resulting in less complex ethidium bromide-stained electropherograms. One potential difficulty with restriction pattern analysis is that mtDNA polymorphisms, that arise from insertions and deletions, will give the erroneous appearance of greater sequence divergence than really exists (Kurtzman, 1987). Since mtDNA evolves much more rapidly (up to ten-fold faster in some organisms) than nuclear DNA, the resolution afforded by mtDNA patterns may not be sufficient to recognise the more divergent strains of a species (Kurtzman, 1987). It remains to be proved that fingerprinting of mtDNA will be able to differentiate among wine yeast strains.

2.2.2 Genetic fingerprinting of wine yeasts

Fingerprinting by protein electrophoresis

Protein expression is genetically determined; the set of proteins and their copy numbers from a specific yeast strain are constant when growing under standardised conditions. Electrophoresis of total soluble proteins of a yeast strain yields a complex pattern, with each band usually consisting of a number of structurally different protein species with the same electrophoretic mobility. Identical electrophoretic mobility of different proteins from a series of yeasts does not necessarily imply that these proteins possess identical protein components. However, proteins of genetically related strains display similar or almost identical electropherograms (Kersters & De Ley, 1980). Two basic polyacrylamide gel electrophoretic (PAGE) techniques are used to fingerprint yeasts, namely the cylindrical gel technique (Van Vuuren & Van der Meer, 1987) and the vertical and horizontal slab gel techniques (Raymond & Wang, 1960; Laemmli, 1970). Integral reference proteins are used to normalise and compare the electropherograms. Protein profiles of a few samples can be visually compared. Quantitative comparison and grouping of normalised densitograms of a large number of electropherograms, however, can only be done with the assistance of computer programs. These computer programs take the relative mobility, the sharpness of bands and the relative protein concentrations of the peaks and valleys into account (Kersters & De Ley, 1980).

Numerical analysis of total soluble cell protein patterns has been used to

fingerprint and group wine yeasts (Van Vuuren & Van der Meer, 1987) and brewing yeasts (Van Vuuren & Van der Meer, 1988). The protein banding patterns of 27 strains of *Saccharomyces* used for commercial production of wine were compared and used to distinguish different yeast groups. Van Vuuren & Van der Meer (1987) confirmed the reclassification of *S. uvarum*, *S. carlsbergensis* and *S. bayanus* as *S. cerevisiae*. Since a number of reputedly genetically unique yeast strains are being sold commercially, it has become necessary to fingerprint individual yeast strains used in wine fermentations. Visual comparison of total soluble cell protein patterns can be used to fulfil this need in the wine industry (Van Vuuren & Van der Meer, 1987). Protein profiles were also used successfully in breeding experiments of wine yeasts, where it is of utmost importance to be able to distinguish between the parental and hybrid strains (Van der Westhuizen & Pretorius, 1989).

Karyotyping by chromosomal banding patterns

Unlike the chromosomes of higher eucaryotes, yeast chromosomes are never in a mitotically condensed form and therefore never visible microscopically. This fact implies that yeasts cannot be karyotyped conventionally as with plants and animals. However, with the advent of pulsed-field-gel-electrophoretic systems it became possible to separate and identify the different yeast chromosomes. Pulsed field gradient electrophoresis (PFGE) and orthogonal field alternation gel electrophoresis (OFAGE) were first described by Schwartz & Cantor (1984) and Carle & Olson (1984) respectively. The intact yeast chromosomes migrate along diagonal paths, making it impossible to compare large numbers of samples with the PFGE and OFAGE systems. To achieve straight migration of DNA, investigators altered the geometry of the electric fields in various ways and the first new system to follow was field inversion gel electrophoresis (FIGE) or reverse field electrophoresis (RFE), where a computer-aided switch is used to invert the electric field in a time gradient mode (Carle *et al.*, 1986). The contour-clamped homogeneous electric field (CHEF) (Chu *et al.*, 1986), transverse alternating field electrophoresis (TAFE) (Gardiner *et al.*, 1986) and autonomously controlled electrode gel electrophoresis (PACE) (Clark *et al.*, 1988) systems followed. Anand (1986) has compared the geometry, advantages and disadvantages of the different systems. The different electrode configurations and migration patterns are schematically summarised in Fig.1. All of these systems make use of two electric fields; the orientation, the angle of intersection and the distribution of field strengths of the two electric fields vary with the design of the apparatus. The separation of chromosomes by size occurs as the direction of the electric field is changed: the larger chromosomes take longer to reorientate and travel in the newly defined direction than the smaller ones. The smaller

chromosomes migrate more rapidly through the gel because they can reorientate themselves more rapidly and become stuck in the gel matrix less frequently. Size separation thus occurs in an electric field that is continuously changing its orientation (Smith & Cantor, 1987: Casey *et al.*, 1988b). Pulse time (time of directional change of the electric field), electric field strength, agarose concentration, temperature, the angle between the electric fields and field geometry all affect resolution to a great extent (Heller & Pohl, 1989). A given set of these parameters should be optimised and standardised for the size range of the DNA to be separated.

The chromosome banding patterns of *S. cerevisiae* (Carle & Olson, 1985; De Jonge *et al.*, 1986), *Candida albicans* (Snell & Wilkens, 1986), *Schizosaccharomyces pombe* (Smith *et al.*, 1987; Vollrath & Davis, 1987), *Kluyveromyces* (Steensma *et al.*, 1988; Sor & Fakuhara, 1988) and *Cryptococcus neoformans* (Polacheck & Lebens, 1989) have been determined with pulsed field gel electrophoresis. The karyotypes of the various yeasts show great variation in length and number of chromosomes. Such variants are noted even among strains of one species. The bands obtained are not a reliable measure of chromosome number because of the inability of certain large chromosomes to separate in the gel matrix (De Jonge *et al.*, 1986). However, these banding patterns are highly reproducible under controlled electrophoretic conditions and chromosome profiles represent a relatively simple method of fingerprinting and identifying a specific yeast strain. Digital image processing techniques are used to store data obtained from DNA fingerprinting and to resolve minute differences among a large number of electrophoretic karyotypes (Pedersen, 1989).

The FIGE and OFAGE systems were used to show that certain chromosome length polymorphisms segregate in a 2:2 ratio, indicating single structural alterations of the chromosomes (Ono & Ishino-Arao, 1988). Chromosome length polymorphisms, however, can also result from two or more structural alterations per chromosome and are not restricted to specific chromosomes. The TAFE system was used for the analysis of chromosomal segregations and inheritance (Bilinski & Casey, 1989). Viljoen *et al.* (1989) used OFAGE to establish possible anamorph/telomorph relations of yeasts and could differentiate between the assumed perfect species, *Saccharomyces exiguus*, and its imperfect counterpart, *Candida holmii*. Another appliction of pulsed field gel electrophoresis rests with the localisation of specific genes and the distinction of two yeast strains that differ only in the chromosomal location of a specific gene. A Southern blot of an OFAGE ethidium bromide-stained gel containing the resolved chromosomes of four *S. cerevisiae* var. *diastaticus* strains, that differ only in the presence and/or chromosomal position of a glucoamylase gene was probed with the cloned *STA2* glucoamylase gene to map the genes and to distinguish the strains from each other (Pretorius & Marmur, 1988).

The electrophoretic karyotypes of some brewing (Pedersen, 1987; Takata *et*

al., 1989), distilling (Johnston *et al.*, 1989) and baking yeasts (Casey *et al.*, 1988b) have been determined. The first electrophoretic karyotypes of wine yeasts were reported by Van der Westhuizen & Pretorius (1989 & 1990). It was shown that the karyotypes of ten yeast strains used currently in the South African wine industry were unique. These karyotypes, obtained by using the CHEF system, were also used to distinguish parental and hybrid strains from one another in a breeding experiment and to point out genetic drift over a number of years in a particular wine yeast strain (Van der Westhuizen & Pretorius, 1989; Van der Westhuizen & Pretorius, 1990). By comparing the chromosomal banding patterns of 22 oenological strains of *S. cerevisiae*, Vezinhet *et al.* (1990) were able to identify 20 different karyotypes. In conclusion it can be stated that karyotyping using chromosomal banding patterns is a simple and reliable technique to identify individual wine yeast strains.

3. GENETIC FEATURES OF WINE YEASTS

3.1 Life cycle and sporulation

S. cerevisiae is a unicellular fungus and a member of the Ascomycetes. It has oblately spheroid or ovoid shaped cells some 3 *u*m in diameter. Figure 2 is a diagrammatic representation of a *S. cerevisiae* cell. *S. cerevisiae* reproduce asexually (budding) or sexually (formation of ascospores). Budding means that each cell gives rise to a daughter cell made of entirely new cell surface material. During the mitotic division the bud receives a full complement of chromosomes before it is pinched off. The daughter cell is smaller than the mother cell and must increase in size before it initiates chromosome duplication and bud-formation (reviewed by Herskowitz, 1988). Under optimal nutritional and cultural conditions *S. cerevisiae* doubles its mass every 90 minutes. *S. cerevisiae* can exist in either the haploid (one set of chromosomes) or diploid (two sets of chromosomes) state, with the haploid cells being either of two sexes (mating types), designated *MATα* and *MATa*. Cells of the *MATα* mating type produce a peptide of 13 amino acids, the α factor (Duntze *et al.*, 1970; Kurjan & Herskowitz, 1982); while the *a* mating type cells produce a peptide of 12 amino acids, the *a* factor (Wilkinson & Pringle, 1974). When in close proximity, the α arrests the growth of *MATa* cells, permitting the cells to mate. The mating process results in cell and nuclear fusion. The *MATa/MATα* diploid cell formed by mating can neither produce nor respond to mating pheromones and will under satisfactory nutritional and cultural conditions grow and divide, maintaining the diploid state. Upon nutritional starvation, the *MATa/MATα* diploid cell undergoes meiosis, generating four haploid ascospores (two *MATa* and two *MATα* ascospores) that are encapsulated within a sac, the ascus. When released from the ascus, the

ascospores germinate to commence new rounds of haploid existence (Herskowitz, 1988). Strains that can be stably maintained for many generations as haploids are termed heterothallic. Strains in which sex reversals, cell fusion and diploid formation occur are termed homothallic (Jensen *et al.*, 1983). The basic life cycles of heterothallic and homothallic strains of *S. cerevisiae* are shown in Fig. 3. The presence of the haploid-specific gene, *HO*, in homothallic strains brings about a high frequency of switching between mating types during vegetative growth. However, cells of homothallic yeast strains have to bud at least once before they are competent to switch mating type (Herskowitz & Oshima, 1981). In *HO* strains the mating type locus, *MAT*, changes from *MATa* to *MATα* or *vice versa* as often as every cell division (Strathern & Herskowitz, 1979). It was found that chromosome III posesses both an active *MAT* gene and two unexpressed mating-type loci. One unexpressed locus, *HML*, is situated 200 kilobase pairs (kb) to the left of the *MAT* locus and contains a silent copy of the α information; the other silent gene, to the right of *MAT*, *HMR*, contains information equivalent to what is expressed at *MATα*. The change in the *MAT* locus occurs by a programmed genetic rearrangement in which silent genetic information becomes activated by moving from *HML* or *HMR* to *MAT* (Oshima & Takano, 1971; Hicks & Herkowitz, 1977) (Fig. 4). Although meiotic recombination (mating and sporulation) is important for evolutionary change, most *S. cerevisiae* strains found in nature are homothallic, with heterothallic strains usually restricted to laboratory variants that have been selected for this trait. Homothallism leads to the early diploidisation of the descendants of all ascospores, preventing expression of harmful mutations in the haploid progeny. Furthermore, mating-type switching that leads to mating and diploidisation also confers a more rapid sporulation response to unfavourable environmental conditions.

Most industrial yeast strains are homothallic, while sporulation efficiency is strain dependent (Haber & Halvorson, 1975). The majority of brewing yeasts either do not sporulate or sporulate very poorly and have low spore viability (Gjermansen & Sigsgaard, 1981). Distilling strains sporulate more freely than brewing strains, but few of the segregants produced are capable of mating with either *MATa* or *MATα* haploid strains. It was reported that strains used in baking also sporulate more freely than brewing yeasts (Johnston, 1965). It was found that most wine yeast strains are homothallic, have a high sporulation efficiency and a higher spore viability than brewing and distilling yeasts (Thornton & Eschenbruch, 1976; Van der Westhuizen & Pretorius, 1990). It may be that there has been an unintentional selection against efficient sexual reproduction in yeast strains used for winemaking, because of the need for constant properties in industrial fermentations.

3.2 Chromosomes, ploidy and genetic stability

Since *S. cerevisiae* is an eucaryote, its chromosomes are encased in a nuclear membrane. Haploid strains contain 12,54 megabases (mb) of nuclear DNA (non ribosomal DNA; non rDNA), distributed along 17 linear chromosomes. The minimum total genetic length of the genome is 4 295 centi Morgans (cM) with a 0,34 cM/kb ratio (Mortimer *et al.*, 1989). The cM/kb ratios for different chromosomes are close to this value except for the shorter chromosomes I, VI, III and IX that have significantly higher values in crossing over per physical unit (Kaback *et al.*, 1989; Mortimer *et al.*, 1989). Each chromosome is a single DNA molecule between 198 and 2 194 kb long (Mortimer & Schild, 1985), arranged as chromatin, containing basic histone molecules. Chromosomal DNA of *S. cerevisiae* contains relatively few repeated sequences (Fangman & Zakian, 1981) and most genes appear to be present as single copies in the haploid genome. However, each amino acid specific transfer RNA (tRNA) is present in 12 to 15 copies and the ribosomal RNA (rRNA) genes are highly repetitive (80 to 160 copies) and tightly linked (Warner, 1982). The genome of *S. cerevisiae* also contains transposable elements (called *Ty*). Some 35 copies of these mobile elements, consisting of a 6 kb DNA sequence flanked by 334 base pair (bp) repetitive sequences (δ) (Boeke *et al.*, 1985), are present within the genomes of most yeast strains, including those of industrial strains and "wild" isolates (Tubb & Hammond, 1987). Substantial rearrangements of the genome and mutated regulatory elements frequently arise as a consequence of the transposition of *Ty* elements from one chromosomal location to another (Scherer *et al.*, 1982). Random excision and insertion of *Ty* elements into the genomes of wine yeasts can thus inactivate genes encoding desirable proteins and cause genetic instability of selected strains. The reverse can also occur so that improved wine yeast strains evolve. Furthermore, *Ty* sequences can also be used as probes that can be employed to distinguish wine yeast strains from one another, because the pattern of dispersion of these multiple transposable elements on their respective genomes is unique.

Most laboratory-bred strains of *S. cerevisiae* are either haploid or diploid. However, industrial strains are predominantly diploid or polyploid. Ploidy of yeast strains can be estimated by one of several methods. In the early 1960s ploidy was determined by means of tetrad segregation analysis of mating-type and morphological characteristics (Emeis, 1961; Gunge, 1966; Fowell, 1969). Determination of DNA content per cell, measurement of cell volume, and irradiation death rate are also used (Gunge & Nakatomi, 1971; Lewis *et al.*, 1976; Russell & Stewart, 1979; Aigle *et al.*, 1983; Leuch *et al.*, 1985; Takagi *et al.*, 1985). Talbot *et al.* (1988) have used a method where 4,6-diamidine-2-phenylindole (DAPI) intercalates into DNA and fluoresces under ultraviolet light. The DNA

concentration is determined by measuring the fluorescent yield of the samples and comparing the values to that of haploid control strains. Determination of DNA content is dependent on very specific cell concentrations. Variation in chromosomal sizes of industrial strains could also alter the precision of the test. Although critical size is clearly a function of ploidy, most individual strains of the same ploidy have critical sizes significantly different from those of other strains in the same ploidy group. Aneuploidy cannot be determined by this method (Aigle *et al.*, 1983). Casey (1986), however, determined ploidy (including aneuploidy) accurately by visualising yeast chromosomes in a gel and probing with specific gene probes. The majority of attempts to estimate the ploidy of brewing and distilling yeasts have relied on measuring the DNA content per cell and comparing this with the value obtained from defined haploid strains. Results from these studies suggest that many brewing and distilling strains are polyploid, particularly triploid, tetraploid or aneuploid (Tubb & Hammond, 1987). It was also found that baking yeasts are typically polyploid (Gunge, 1966; Fowell, 1969; Gunge & Nakatomi, 1971). As such, a lack of mating ability, a low frequency of sporulation and poor ascospore viability are to be expected (Tubb & Hammond, 1987). Wine yeast strains were found to be mainly diploid (Thornton & Eschenbruch, 1976; Cummings & Fogel, 1987). One widely used commercial German wine yeast strain (Hefix 1000) was reported to be tetraploid and had an *a/a/a/α* mating-type genotype (Takahashi, 1978). It is not yet clear whether polyploidy in industrial yeast strains is advantageous. Emeis (1963) constructed a series of homozygous and heterozygous strains with ploidy from one to eight, and reported that the heterozygous triploids and tetraploids were more efficient in fermentation than the homozygous strains of higher or lower ploidy. Based on these results it was concluded that heterosis rather than ploidy is responsible for improvement of fermentation performance. Other researchers claim that the polyploid state might enable industrial yeasts to harbour a high dosage of genes important for efficient fermentation (Mowshowitz, 1979; Stewart *et al.*, 1981). These reports only emphasise the fact that the relationship between the fermentation ability and the ploidy of a yeast strain is rather complicated (Tubb & Hammond, 1987).

The maintenance of the genetic identity of strains in a pure culture is problematical. The term *pure culture* describes that it has been derived from a single cell, but does not mean the culture is genetically uniform (Snow, 1983). Even under closely controlled conditions of growth a yeast strain reveals slow but distinct changes after many generations. This might be due to a number of different processes, including mutation and more frequently mitotic crossing-over or gene conversion. Heterogeneity of a *pure* culture was pointed out by Zimmermann (1978) (see Snow, 1983), who was able to isolate a strain with considerably improved characteristics

from successive single-cell cultures of an Epernay yeast. It is well known that sporulation and spore viability of pure yeast cultures are generally poor and that there is considerable variation in growth rate between spore clones (Thornton & Eschenbruch, 1976). Some of this genetic heterozygosity of pure cultures is undoubtedly due to segregation of aneuploid chromosome complements from a polyploid or aneuploid parental strain; the remaining variation probably reflects the segregation of lethal genes or genes compromising efficient growth (Snow, 1983). Mating between *MATa* and *MATα* ascospores, generated by sporulation, can also cause genetic instability (Emeis, 1965). Increased homozygosity in polyploid yeasts is expected to confer greater genetic stability (Emeis, 1965). It has also been reported that the rate of genetic drift of yeast strains increases with ploidy. This finding is contrary to a popular belief that the polyploid state protects against mutation and genetic variability. Since wine yeasts most probably harbour recessive mutations (as is the case with brewing yeasts), genetic stability is likely to be a function of the frequency of segregational events leading to expression of mutant genes, rather than the frequency of mutation itself (Tubb & Hammond, 1987). It would seem unwise to assume *a priori* that all wine yeast strains are genetically stable. It is not yet clear what the influence of the *Ty* transposable elements and the respective contributions of nuclear and cytoplasmic (particularly mitochondrial) genomes are to the genetic drift in wine yeasts.

3.3 Extrachromosomal elements

3.3.1 Mitochondria

Mitochondria are complex organelles specialized in respiration and oxidative phosphorylation (Dujon, 1981). Rapidly growing cells contain usually less than 10 mitochondria, whereas cells from a stationary-phase culture contain up to 50 mitonchondria per cell (Stevens, 1981). Actively respiring mitochondria are rounded or elongated and are regularly distributed in the cytoplasm. Individual mitochondria can fuse to create filamentous and branched forms (Stevens, 1981).

Mitochondria possess their own genetic system and their own protein synthetic machinery. *S. cerevisiae* has among the largest mitochondrial DNAs (mtDNAs) of any organism, consisting of 75 kb circles (Hollenberg *et al.*, 1970). However, the mitochondrial genome of *S. cerevisiae* is adenine-thymine (A-T) rich, carrying the genetic information for only a few, essential mitochondrial components and does not even code for the majority of the enzymes involved in the generation of ATP (Fangman & Zakian, 1981). Furthermore, replication of mtDNA differs from that of nuclear DNA. Replication of mtDNA is not limited to the S phase of the cell cycle

and takes place throughout the cell cycle (Newlon & Fangman, 1975). The mtDNA polymerase also lacks proofreading (exonuclease) activity, resulting in a much higher mutation rate within the mtDNA than within nuclear genes and so mtDNA can evolve extremely rapidly (Evans, 1983; Tzagoloff & Dieckmann, 1990). This lack of an error repair mechanism during mtDNA replication is partly compensated for by the abundance of mitochondria in a single cell. With a genome that is much larger than required, the yeast mtDNA carries out only a few activities. One explanation for the persistence of this large mitochondrial genome is that in yeast it plays the additional role of a reservoir of genetic diversity, capable of serving the nuclear genome by contributing evolved sequences. This could be one contributing factor in the observed genetic heterogeneity of pure cultures of wine yeasts.

Unlike other eucaryotic cells, yeasts can survive without its mtDNA. Mitochondrial mutants usually lack vital oxidative enzymes, rendering them unable to generate ATP oxidatively. As a result mitochondrial mutants grow slowly and form smaller (*petite*) colonies on solid agar surfaces. Petite mutants are respiratory-deficient and are unable to utilise non-fermentable substrates. The term cytoplasmic *petite* mutant describes respiratory-defective strains with cytoplasmically inherited mutations, ranging from point mutations (mit^-) through deletion mutations (rho^-) to complete elimination of the mtDNA (rho^0). To distinguish cytoplasmic *petite* mutants from respiratory-deficient strains with genetic lesions in nuclear genes, the latter are referred to as nuclear *petite* or *pet* mutants (Tzagoloff & Dieckmann, 1990). The mitochondrial genome is involved in cell functions other than respiratory metabolism. Since the generation of petite mutants of wine yeasts occurs spontaneously at quite high rates, it is important to note that yeasts with different mtDNAs could differ in their flocculation characteristics, lipid metabolism, higher alcohol production and formation of flavour compounds (Lewis et al., 1976; Hammond & Eckersley, 1984). Thus, although wine yeasts are not required to respire during fermentation of grape must, mtDNA-encoded functions are important and for this reason *petite* strains are not used for winemaking.

3.3.2 Killer factors

The killer phenomenon in *S. cerevisiae* is associated with the presence of intracellular virus particles (Wickner, 1981; Tipper & Bostian, 1984; Young, 1987). Virus particles in killer yeasts, that are cytoplasmically inherited, contain two major linear double-stranded ribonucleic acid (dsRNA) types, the L and M genomes. The L genome encodes a polymerase and the viral coat protein that encapsulates both genomes. The M genome encodes both a proteinaceous toxin and an immunity factor. The toxin is secreted by the killer strains and is lethal to sensitive strains of

the same species. Three types of *S. cerevisiae* killers, K_1, K_2 and K_3, have been described (Young & Yagiu, 1978). The size of the L genome is 4,5 kb and the M dsRNA genomes 1,9, 1,5 and 1,3 kb, respectively. Pfeiffer and Radler (1982) reported a fourth killer type (strain KT28) and a fifth has been described by Extremera *et al.* (1982). Some yeast strains are immune to K_1 toxin but do not produce active toxin. These so-called neutral strains do contain an M dsRNA genome. This genome codes for the production of the immunity factor but not for the production of an active toxin. Wingfield *et al.* (1990a) first characterised a K_2 neutral *S. cerevisiae* strain. The K_2 neutral M dsRNA was found to be larger than the K_2 killer yeast M dsRNA and homoduplex analysis revealed an inverted duplication.

Killer yeasts have been isolated as contaminants in several commercial fermentation processes (Maule & Thomas, 1973; Naumov *et al.*, 1973; Imamura *et al.*, 1974). Van Vuuren & Wingfield (1986) recently showed that stuck or sluggish wine fermentations can be caused by contaminating killer yeasts. The size of viral dsRNA genomes from 11 killer yeast strains, isolated from stuck wine fermentations, have been compared with those of K_2 and K_3 killer strains (Wingfield *et al.*, 1989). It was reported that the size of the L genomes of all these isolates was similar (4,5 kb) but that the M genomes varied in size from 1,3 to 1,5 kb. Since Wingfield *et al.* (1990b) found that M_3 is a deletion of M_2, it can be stated that the killer yeasts isolated from the wineries by Wingfield *et al.* (1989) belong to the K_2 type. This conclusion was supported by the observation that these killer isolates were immune to both the K_2 and K_3 toxins and that they showed killer activity against the K_1 neutral strain that is immune to the K_1 toxin. Furthermore, the K_1 toxin is not active below pH 4 while the K_2 and K_3 toxins are in fact active at the low pH in wines. In addition to the L and M genomes, some of the killer isolates from the wineries also contained other minor dsRNA species (Wingfield *et al.*, 1989).

3.3.3 2um Plasmids

The 2*u*m DNA is the only naturally occurring plasmid thus far found in the nucleoplasm of yeasts. This extrachromosomal element is inherited in a non-Mendelian fashion and while most strains of *S. cerevisiae* contain this circular, 6300-bp plasmid, its biological function has not yet been discovered (Broach, 1981). No consistent difference in properties has been observed in those relatively rare strains (*cir*^{o} strains) that lack the 2*u*m plasmid. There are usually 50 to 100 copies of 2*u*m DNA per cell and they represent about 5% of the total yeast DNA. These circular DNA molecules consist of two identical repeats of 599 bp separated by two unique regions of 2774 bp and 2346 bp (Broach, 1981). Reciprocal recombination between

the repetitive sequences generates a mixture of two forms of the plasmid (Broach, 1981). In addition, multimeric (4 *u*m, 6 *u*m) forms of the plasmid also occur. The 2*u*m DNA is transcribed into three separate polyadenylated messenger RNA (mRNA) molecules that can direct protein synthesis *in vitro*. One of the three genes, *FLP*, produces a protein that is responsible for the reciprocal recombination of 2*u*m DNA (Cox, 1983). The *REP*1 and *REP*2 genes are required for the autonomous replication of the 2*u*m plasmid (Broach, 1982). Other than its own maintenance, the 2*u*m plasmid appears to confer no advantage on the host cells. The 2*u*m DNA, however, serves as an important tool to molecular biologists involved in the genetic manipulation of wine yeasts, as many plasmid vectors are based on the 2*u*m origin of replication.

4. GENETIC TECHNIQUES FOR STRAIN DEVELOPMENT

S. cerevisiae can be manipulated genetically in many ways. Some techniques alter limited regions of the genome, while other techniques are used to recombine or rearrange the entire genome. Techniques having the greatest potential in genetic programming of wine yeast strains are: clonal selection of variants, mutation and selection, hybridisation, rare-mating, spheroplast fusion as well as gene cloning and transformation. The combined use of classical genetic techniques and recombinant DNA methods have dramatically increased the genetic diversity that can be introduced into yeast cells.

4.1 Clonal selection of variants

Selection of variants is a simple direct means of strain development that depends on the genetic variation normally present in all wine yeast strains. Genetic heterogeneity in wine yeast strains is due mainly to mitotic recombination during vegetative growth and spontaneous mutation. Successful isolation of variants depends on the frequency at which they occur and the availability of selection procedures to isolate strains containing the improved characteristic. Dramatic improvements in most characteristics can not be expected; nevertheless intra-strain selection has been used for decades to obtain improved wine yeast strains.

The value of continuous culture for the isolation of variants without prior mutagenesis was amply demonstrated by Zimmermann (see Snow, 1983) who isolated variants of an Epernay yeast with improved fermentation characteristics. Selection in continuous culture has also been used to obtain non-foaming variants (Ouchi & Akiyama, 1971; Eschenbruch & Rassell, 1975), variants with improved

ethanol tolerance (Brown & Oliver, 1982a) as well as variants with reduced H_2S production (Rupela & Taura, 1984). Strain degeneration caused by the accumulation of undesirable mutations or somatic recombinants can be efficiently prevented by using clonal selection coupled with the analysis of variance tests (Azevedo *et al.*, 1978).

4.2 Mutation and selection

The average spontaneous mutation frequency in *S. cerevisiae* at any particular locus is approximately 10^{-6} per generation (Ingolia & Wood, 1986). The use of mutagens greatly increases the frequency of mutations in a wine yeast population. Mutation and selection appear to be a rational approach to strain development when a large number of performance parameters are to be kept constant while only one is to be changed (Kielland-Brandt *et al.*, 1983). However, mutation of wine yeasts can lead to improvement of certain traits with the simultaneous debilitation of other characteristics. Although mutations are probably induced with the same frequency in haploids, diploids or polyploids, they are not as easily detected in diploid and polyploid cells because of the presence of non-mutated alleles. Only if the mutation is dominant, is a phenotypic effect detected without the need for additional alterations (Kielland-Brandt *et al.*, 1983). Therefore, haploid strains of wine yeasts are preferred, though not essential, when inducing mutations. Successful mutation breeding is usually associated with mutations in meiotic segregants, where the two mating parents of a well-behaving hybrid provide a good basis for the introduction of recessive mutants.

Mutagens such as ultraviolet light (UV), ethylmethane sulphonate (EMS) and N-methyl-N-nitro-N-nitrosoguanidine (NTG) have each proved surprisingly effective with polyploid strains (Tubb & Hammond, 1987). Ingraham & Guymon (1960) have used ultraviolet light to generate isoleucine- and valine-requiring mutants that produced only traces of isoamyl alcohol and isobutyl alcohol, respectively. From EMS-treated wine yeasts, Rous *et al.* (1983) isolated leucine-auxotrophic recessive mutants that also produced reduced levels of higher alcohols.

Mutagenesis has the potential to disrupt or eliminate undesirable characteristics and to enhance favourable properties of wine yeasts. However, the use of mutagens for directed strain development is limited, but the method could be applied to isolate new variants of wine yeast strains prior to further genetic manipulation (Sturley & Young, 1986).

4.3 Hybridisation

Intra-species hybridisation involves the mating of haploids of opposite mating-types to yield a heterozygous diploid. The process is illustrated in Fig.4. Recombinant progeny are recovered by sporulating the diploid, recovering individual haploid ascospores and repeating the mating/sporulation cycle as required (Tubb & Hammond, 1987). There are two general methods used for isolation of individual ascospores, i.e., tetrad analysis and random spore analysis. Tetrad dissection is done with a micromanipulator and has the advantage that all four products of meiosis in *S. cerevisiae* are recoverable. Futhermore, tetrad analysis can also be used to (i) determine whether a gene is inherited chromosomally (i.e. a 2:2 segregation pattern) or cytoplasmically (i.e. a 4:0 segregation pattern); (ii) assign a gene to a linkage group (chromosome) and map its chromosomal location; or (iii) provide insight into the complexity of the genotype responsible for a particular characteristic of a wine yeast strain (i.e. phenotype) (Tubb & Hammond, 1987). Random spore analysis is used when tetrad analysis is not feasible or necessary, i.e., when a relatively uncomplicated genotype reassortment is desired from meiosis. The advantage of using random spore analysis is that it is a rapid technique that requires no special equipment or skills (Ingolia & Wood, 1986).

Haploid strains from different parental diploids, possessing different genotypes, can be mated to form a diploid strain with properties different from that of either parental strain. Thus, theoretically speaking, crossbreeding can permit the selection of desirable characteristics and the elimination of undesirable characteristics (Thornton, 1983). Unfortunately many wine yeasts are homothallic and the use of hybridisation techniques for development of wine yeast strains has proved difficult (Thornton & Eschenbruch, 1976; Snow, 1979; Van der Westhuizen & Pretorius, 1990). However, this problem can be circumvented by direct spore-cell mating (Thornton, 1983). As illustrated in Fig.5, four homothallic ascospores from the same ascus are placed into direct contact with heterothallic haploid cells by using a micromanipulator. Mating takes place between compatible ascospores and cells. To make wine yeasts more genetically accessible, Bakalinsky & Snow (1990) introduced the *ho* heterothallic allele into three widely used wine strains through spore-cell mating. The resultant hybrids were sporulated and heterothallic segregants were isolated for use in successive back-crosses.

A useful killer saké yeast has been generated by crossing a wild-type killer yeast with an efficient saké yeast. The hybrid was sporulated and back-crossed with the saké yeast six times (Ouchi & Akiyama, 1976). Hybridisation was also used to introduce the killer (Hara *et al.*, 1980), mesophilic (Hara *et al.*, 1981) and cryophilic (Hara *et al.*, 1981) characteristics into wine yeasts. A killer haploid strain derived

from a killer saké yeast was mated with an SO_2-tolerant haploid strain from a yeast with good winemaking qualities. The diploid hybrid that resulted from this cross was an SO_2-tolerant killer. The killer ability was transmitted by the dsRNA in the cell cytoplasm and the SO_2-tolerance by the chromosome in the nucleus. Killer, SO_2-tolerant, haploid strains were isolated from this cross and back-crossed with haploids of the original wine yeast (Thornton, 1983). Selective hybridisation was also used to produce a flocculant, non-foaming wine yeast with a high fermentation rate and high ethanol production (Romano *et al.*, 1985).

Elimination or inclusion of a specific property can thus be achieved relatively quickly by hybridisation, provided that it has a simple genetic basis, for example one or two genes. However, many desirable wine yeast characteristics are specified by several genes or are the result of several gene systems interacting with one another (Thornton, 1983). For instance, conversion of grape sugar to alcohol by wine yeasts involve at least twelve chemical reactions each promoted by an enzyme specified by a different gene. In diploid yeasts, this means that twelve pairs of sister genes are involved and considerable variation in conversion efficiency can arise because some of the genes may be mutant alleles that either fail to produce an enzyme or produce an altered enzyme of less activity than normal (Thornton, 1983). A hybridisation programme aimed at improving conversion efficiency that focused on individual genes could be time consuming and therefore a more empirical approach has to be adopted. This can be achieved by isolating haploids from several wine yeast strains with different conversion efficiencies. The most efficient haploid strains can be identified after trial fermentations and then be mated to generate the first generation of diploid strains. After further trial fermentations, the best diploids can be sporulated and haploid strains can be isolated from them. The most efficient of these haploids can be mated to form the second generation of diploid strains. This mating cycle can be repeated as required. Thornton (1980; 1982) employed selective hybridisation over three generations of diploid strains in this manner to raise the fermentation efficiency from 84 to 93%.

4.4 Rare-mating

Wine yeast strains that fail to express a mating-type can be force-mated with haploid *MATa* and *MATα* strains. The procedure, known as rare-mating, is illustrated in Fig.6. Typically, a large number of cells of the parental strains are mixed together and a strong positive selection procedure is applied to obtain the rare hybrids formed (Tubb & Hammond, 1987). For instance, industrial strains that have a defective form or lack of mtDNA (respiratory-deficient mutants) can be force-mated with auxotrophic haploid strains having normal respiratory characteristics (Gunge &

Nakatomi, 1972; Spencer & Spencer, 1977). Mixing of these non-mating strains at high cell density will generate only a few respiratory-sufficient prototrophs. These true hybrids with fused nuclei can then be induced to sporulate for further genetic analysis and crossbreeding (Spencer & Spencer, 1977). Brewing strains with the ability to ferment wort dextrins have been constructed, using rare-mating (Tubb *et al.*, 1981). Once the *POF1* gene, responsible for the production of phenolic off-flavours, was eliminated by back-crossing, these hybrids produced acceptable low-carbohydrate beers.

Rare-mating is also used to introduce cytoplasmic genetic elements into wine yeasts without the transfer of nuclear genes from the non-wine yeast parent. This method of strain development is termed cytoduction. Cytoductants (or heteroplasmons) receive cytoplasmic contributions from both parents but retain the nuclear integrity of only one (Tubb & Hammond, 1987). Cytoduction requires a haploid mater carrying the *kar1* mutation, that is, a mutation that impedes karyogamy (nuclear fusion) after mating (Conde & Fink, 1976). This more specific form of strain construction can, for example, be used to introduce the dsRNA determinants for the K_2 zymocin and associated immunity into a particular wine yeast. Cytoduction can also be used to substitute the mitochondrial genome of a wine yeast or to introduce a plasmid encoding desirable genetic characteristics into specific wine yeast strains.

Mating between strains, one of which carries the *kar1* allele, occasionally generates progeny that contain the nuclear genotype of one parent together with an additional chromosome from the other parent (Nilsson-Tillgren *et al.*, 1980; Dutcher, 1981). The donation of a single chromosome from an industrial strain to a haploid *kar1* recipient is termed single-chromosome transfer, and is used to examine individual chromosomes of industrial yeast strains in detail (Nilsson-Tillgren *et al.*, 1980; Nilsson-Tillgren *et al.*, 1981; Kielland-Brandt *et al.*, 1983; Casey, 1986; Nilsson-Tillgren *et al.*, 1986; Pedersen 1986b).

4.5 Spheroplast fusion

Spheroplast fusion is a direct, asexual technique that can be used in crossbreeding as a supplement to mating. Like rare-mating, spheroplast fusion can be used to produce either hybrids or cytoductants. Both these procedures overcome the requirement for opposite mating types to be crossed, thereby extending the number of crosses that can be done. The procedure of spheroplast fusion was described by Van Soligen & Van der Plaat (1977) and is outlined in Fig.7. Cell walls of yeasts can be removed by lytic enzymes, viz. Glusulase (isolated from snail gut) or Zymolase (Lyticase, a glucanase isolated from *Arthrobacter luteus*) in the presence of an

osmotic stabiliser (e.g., 1M sorbitol) to prevent osmolysis of the resulting spheroplasts. Spheroplasts from the different parental strains are mixed together in the presence of a fusion agent, polyethylene glycol (PEG) and calcium ions, and then allowed to regenerate their cell walls in an osmotically stabilised selective agar medium. Spheroplast fusion can also be obtained by electroporation (electrofusion) in a weak inhomogeneous alternating electric field. Fusion of the aligned cells can then be induced by applying a higher-intensity electric field (Halfmann *et al.*, 1982; Tubb & Hammond, 1987).

Spheroplast fusion of non-sporulating industrial yeast strains serves to remove the natural barriers to hybridisation. The desirable (and undesirable) characteristics of both parental strains will recombine in the offspring (Sturley & Young, 1986). Cells of different levels of ploidy can be fused. For instance, a diploid wine yeast strain can be fused to a haploid strain to generate triploid strains. Alternatively, two diploid wine yeasts with complementing desirable characteristics can be fused to generate a tetraploid wine yeast strain containing all of the genetic backgrounds of the two parental wine yeasts.

Ouchi *et al.* (1983) described a method for transferring the dsRNA killer genome from UV-killed cells to recipient cells of a saké wine yeast through spheroplast fusion. This technique rarely yields nuclear hybrids due to abortive nuclear fusion. Similarly, Seki *et al.* (1985) constructed a killer wine yeast and showed that growth of sensitive cells in grape juice was inhibited by the killer fusant. Yokomori *et al.* (1989) produced cytoductants of a saké wine yeast by spheroplast fusion that exhibited good fermentation performances and produced quality wine with low volatile acids.

4.6 Gene cloning and transformation

Without underscoring the value of clonal selection, mutagenesis, hybridisation, rare-mating and spheroplast fusion in strain development programmes, one has to keep in mind that these methods lack the specifity required to modify wine yeasts in a well-controlled way. Using these genetic techniques, it may not be possible to define precisely the change required and a new strain may bring an improvement in some aspects, while compromising other desired characteristics (Pretorius, 1989a). Yeast geneticists must, therefore, be able to alter the characteristics of wine yeasts in specific ways: an existing property must be modified, or a new one introduced without adversely affecting other desirable properties. Molecular-genetic techniques capable of this are now available. Gene cloning and recombinant DNA techniques offer exciting prospects for improving wine yeasts (Snow, 1983). Genetic transformation is the change of the genetic set-up of a yeast cell by the introduction

of purified DNA. By using such procedures it should be possible to construct new wine yeast strains that differ from the original strains only in single specific characteristics.

The demonstration of yeast transformation by Hinnen *et al.* (1978) and the development of plasmids, that can be shuttled between *S. cerevisiae* and *E. coli* by Botstein *et al.* (1979), paved the way for genetic engineering in wine yeasts. In principle, there are five major steps in the cloning of a gene. These include:

(i) identification of the target gene and obtaining the DNA fragment to be cloned (passenger DNA) by enzymatic fragmentation of the donor DNA using restriction endonucleases;

(ii) identification and linearisation of a suitable vector; be it a plasmid, virus (bacteriophage) or cosmid;

(iii) joining of the passenger DNA fragments to the linearised vector DNA, thereby generating recombinant DNA molecules, designated a gene library;

(iv) insertion of the recombinant DNA molecules into host cells by transformation (or transduction in the case of viral and cosmid vectors);

(v) screening of transformed cells and the selection of those cells containing the target gene.

A number of options are available at each of these stages (summarised in Fig.8) and the decision to use any particular option will depend on a number of factors, not least of which will be the extent of information available about the target gene product and the gene itself (Gibson, 1987).

Free DNA molecules, however, are not taken up by normal yeast cells; their entry requires the generation of the more permeable spheroplast. DNA is added in the presence of calcium ions and polyethylene glycol that makes the plasma membrane permeable, encouraging the passage of DNA through it (Hinnen *et al.*, 1978). Another method, using *E. coli* protoplasts fused to yeast spheroplasts, yielded up to 10% transformed cells (Gyuris & Duda, 1986). Encapsulating DNA in liposomes that are then fused to spheroplasted yeast cells provides a further approach to yeast transformation and may have applicability for the introduction of large amounts of DNA (e.g., with chromosomes of either natural or recombinant origins) (Tubb & Hammond, 1987). These methods involving spheroplasts yield high transformation efficiency. However, their disadvantage lies in the fact that transformation is somewhat laborious and is associated with a high frequency of cell fusion (Harashima *et al.*, 1984). Also, different strains vary considerably in their transformation competence, that seems to be inherited in a polygenic manner (Johnston et al., 1981). A simpler method has been developed using intact yeast cells and alkali cations, especially lithium acetate (or lithium sulphate) and polyethylene glycol (Ito et al., 1983), or PEG alone (Klebe et al., 1983) to induce DNA uptake.

Currently, the lithium method developed by Ito et al. (1983) seems to be the most commonly used, despite its disadvantage of giving a lower transformation efficiency than the spheroplast method. Further development of this procedure using intact yeast cells increased the transformation efficiency dramatically (Brzobohaty & Kovac, 1986; Bruschi et al., 1987; Gietz & Sugino, 1988; Keszenman-Pereyra & Hieda, 1988; Schiestl & Gietz, 1989). Another method that uses agitation of glass beads (Constanzo & Fox, 1988), is convenient but gives a low frequency. Yeast cells can also be transformed by electroporation (Delorme, 1989).

To be incorporated into the inheritable components of the yeast cell, the transforming DNA normally suffers one of two fates: either it is maintained as a self-replicating plasmid, physically separated from the endogenous yeast chromosomes, or it must integrate into a chromosome and thus be maintained by the functions of the chromosome (Sturley & Young, 1986). A wide range of *E. coli-S. cerevisiae* shuttle vectors, containing bacterial and yeast marker genes and origin of replication sequences, were developed (Parent *et al.*, 1985). These are summarised in Table 1. The introduction of recombinant plasmids into a wine yeast strain requires either that the strain be made auxotrophic before transformation or that the plasmid, used for transformation, carry a marker that is selectable against a wild-type diploid or polyploid background. Positive selectable markers include the kanamycin-resistance gene, the gene encoding resistance to the antibiotic G418 (Jiminez & Davies, 1980; Webster & Dickson, 1983), the copper-resistance (*CUP1*) gene (Fogel *et al.*, 1983; Butt *et al.*, 1984; Henderson *et al.*, 1985), hygromycin B-resistance (Gritz & Davis, 1983; Kaster *et al.*, 1984), resistance to chloramphenicol (Hadfield *et al.*, 1986), methotrexate-resistance (Zhu *et al.*, 1986), resistance to the herbicide sulfometuron methyl (*SMR1* gene) (Casey *et al.*, 1988a), resistance to methylglyoxal (Kimura & Murata, 1989), the L-canavanine-resistance (*CAN1*) gene (Suizu *et al.*, 1989) and the ability to utilise melibiose (Gendre & Guerineau, 1986). Recombinant plasmids with positive selectable markers, containing a particular target gene, are usually either integrated into a chromosome or maintained as a stable minichromosome in industrial yeast strains. Such minichromosomes should be stripped preferably of all non-relevant bacterial DNA sequences before transformation into industrial yeast strains.

In addition to the introduction of specific genes into wine yeasts, recombinant DNA approaches offer wider applicability. Some of the applications provided by recombinant-DNA techniques include (Tubb & Hammond, 1987):

(i) amplification of gene expression by maintaining a gene on a multi-copy plasmid (Lacroute *et al.*, 1981), integration of a gene at multiple sites within chromosomal DNA (Szostak & Wu, 1979) or splicing a structural gene to a highly efficient promoter sequence;

(ii) releasing enzyme synthesis from a particular metabolic control or subjecting it to a new one;

(iii) in-frame splicing of a structural gene to a secretion signal to engineer secretion of a particular gene product into the culture medium;

(iv) developing gene products with modified characteristics by site directed mutagenesis;

(v) eliminating specific undesirable strain characteristics by gene disruption;

(vi) incorporation of genetic information from diverse organisms such as fungi, bacteria, animals and plants.

Successful application of recombinant DNA technology in the wine industry will depend on whether commercial users of genetically manipulated wine yeasts are assured that existing desirable characteristics have not been damaged, that the requirements of beverage legislation are met, that the engineered strain will be stable in practice and that suitable procedures are available for monitoring of new strains (Tubb & Hammond, 1987). The genetic techniques of mutation, hybridisation, cytoduction and transformation discussed in this section will most likely be used in conjunction for commercial wine yeast improvement. Procedures centered around DNA transformation have revolutionised strategies for strain modification, but it remains difficult to clone unidentified genes. Thus, mutation and selection will persist as an integral part of many breeding programmes. Furthermore, although recombinant DNA methods are the most precise way of introducing novel traits encoded by single genes into commercial wine yeast strains, hybridisation remains the most effective method for improving and combining traits under polygenic control (Sturley & Young, 1986; Pretorius, 1989a).

5. TARGETS FOR STRAIN DEVELOPMENT

5.1 Requirements for efficient wine yeasts

Due to technical difficulties and the fact that the requirements of the wine industry have not been defined in genetic terms, no serious strain development has been conducted. Furthermore, with traditional fermentation methods there was little need to change the yeast strain (Tubb & Hammond, 1987). Unlike other yeast-based industries such as baking and brewing, the wine industry has not taken an active interest in yeast genetics and strain-development programmes (Thornton, 1983). New trends in the beverage markets demand the modification of traditional wine yeast strains and the development of more cost-effective winemaking practices. Wine yeast modification must be subject to certain standards and must not impair the flavour and bouquet of the final product. The most desirable characteristics of a

wine yeast include the following (Thornton, 1983; Yap, 1987):

(i) rapid initiation of fermentation immediately upon inoculation without excessive yeast growth;
(ii) growth at 15-18^{o}C;
(iii) fermentation at low temperatures such as 10-14^{o}C;
(iv) efficient conversion of grape sugar to alcohol, with a desirable residual sugar level;
(v) the ability to conduct even fermentation;
(vi) the ability to ferment to dryness, i.e., the yeast has to be ethanol tolerant (at alcohol concentrations up to 14,5% v/v);
(vii) growth and fermentation in musts containing sulphur dioxide, that is normally used in winemaking;
(viii) low foaming ability;
(ix) low volatile acid, acetaldehyde, sulphite and a higher alcohol production;
(x) effective flocculation at the end of fermentation to aid clarification;
(xi) low hydrogen sulphide production or mercaptan fermentation;
(xii) relatively low higher alcohol production to aid "hotness";
(xiii) relatively high glycerol production to contribute to the sensory qualities of the wine;
(xiv) the production of desirable fermentation bouquet and reproducible production of the correct levels of flavour and aroma compounds;
(xv) the retention of viability during storage as well as genetic stability;
(xvi) no production of urea that can result in the formation of ethylcarbamate ;
(xvii) resistance to killer toxins and other zymocidal compounds.

Some of the requirements listed above are complex and difficult to define genetically without a better understanding of the biochemistry involved. A need therefore remains for the careful selection of appropriate strains from yeast culture collections. To date, no wine yeast in commercial use has all the characteritics listed above and it is well established that wine yeasts vary in their winemaking abilities. While some degree of variation can be achieved by altering the fermentation conditions (e.g., temperature), the major source of variation is the genetic constitution of the wine yeasts (Thornton, 1983).

5.2 Specific targets for yeast genetics in winemaking

5.2.1 Improved quality control

Strain maintenance. One of the main objectives for using pure cultures in winemaking is to ensure reproducible fermentation performance and product

quality. It is therefore important to maintain the genetic identity of wine yeasts and to slow down the rate of strain evolution caused by sporulation and mating, mutations, gene conversions and genetic transpositions. Total prevention of heterogeneity in pure cultures is impossible, since homothallism, inability to sporulate and mate, and polyploidy (multiple gene structure) only protect against genetic drift caused by sexual reproduction and mutation, and not against that caused by gene conversion and transposition. Even closely controlled conditions for maintenance of culture collections (i.e., freeze-dried cultures, cultures preserved in liquid nitrogen or in silica gel) will not render full protection against genetic drift in pure yeast cultures. Fermentation trials, continuous strain evaluation and early detection of genetic changes using comparative molecular techniques (Kurtzman, 1987) are the only practical ways to limit possible economic loss. A commercial wine yeast strain, WE500 (widely used in the South African wine industry), was reported to have deteriorated in fermentation performance over a period of several years. Comparison of strain WE500 and its original French parental strain, VIN7, using CHEF chromosome banding patterns, revealed a clear difference in the size of two chromosomes (Van der Westhuizen & Pretorius, 1990). These results indicated the presence of a contaminant or a genetically rearranged strain in the fermentations. The use of fingerprinting will asssist in monitoring the yeast strains used in wine fermentations.

Molecular marking. As an aid to yeast management and trouble-shooting, particularly for wineries using more than one yeast strain, the genomes of commercial wine yeasts can be tagged. Recombinant-DNA techniques can be used to insert specific genetic markers into wine yeasts. This could take the form of synthetic oligonucleotides or foreign genes of known nucleotide sequences. These DNA sequences can then be used as "diagnostic probes" to identify specific wine yeast strains. Labelling could also take the form of a specific labelled compound in the cell wall or the secretion of an unusual or "marker" protein (Tubb & Hammond, 1987). Karyotyping, DNA fingerprinting and molecular marking of wine yeasts will assist in monitoring yeast strains used in wine fermentations and will also discourage illegal use of (patented) commercial wine yeast strains.

5.2.2 Fermentation performance

Many possibilities are available for introducing characteristics into wine yeasts in order to improve their fermentation performance. The efficiency of fermentation would be markedly improved by improvement of sugar utilisation and increased tolerance to ethanol, resistance to microbial toxins (e.g., killer toxins) and the

production of substances inhibitory to contaminating microorganisms, resistance to heavy metals, reduced formation of foam, induced flocculance at the end of fermentation and production of extracellular enzymes.

Efficient sugar utilisation and ethanol tolerance. In the wine industry there is an obvious desire for yeasts that have a high fermentation rate, high ethanol tolerance and high viability. Improved fermentation rates would be brought about by increasing the rate at which the carbohydrates present in grape must, such as sucrose, are metabolised. *S. cerevisiae* has the ability to take up and ferment a wide range of sugars; for example, glucose, fructose, mannose, galactose, maltose and maltotriose. The first step in the utilisation of any sugar by *S. cerevisiae* is usually the passage of the intact sugar across the cell membrane or its initial hydrolysis outside the membrane followed by entry into the cell of some or all of the hydrolysis products (Stewart & Russell, 1983). The dissachariде, sucrose, is first hydrolysed outside the cell membrane by the extracellular enzyme, invertase (β-D-fructofuranoside fructohydrolase, E.C.3.2.1.26) to glucose and fructose. In *S. cerevisiae*, the ability to hydrolyse sucrose is conferred by any one of six (or more) polymeric genes, denoted *SUC1* to *SUC6*. The *SUC2* gene encodes two forms of invertase, intracellular and extracellular, via two differentially regulated mRNA's (Carlson & Botstein, 1982). The non-glycosylated intracellular enzyme is encoded by a 1,8-kb mRNA that is produced constitutively at low levels and has no obvious physiological function. Its substrates are not taken up by the yeast cells to any appreciable extent. The secreted invertase, the physiologically important enzyme that is responsible for sucrose utilisation, is a heavily glycosylated enzyme that is secreted into the periplasmic space under conditions of glucose deprivation. The precursor of this mature form is a polypeptide containing a single peptide at the amino terminus (Perlman *et al.*, 1982). This precursor is encoded by a 1,9-kb mRNA that is regulated by glucose repression (Carlson *et al.*, 1983). The *SUC2* gene has two promoters: a constitutive promoter that is responsible for the synthesis of the 1,8-kb mRNA and a regulated promoter that promotes glucose-repressible transcription of the 1,9-kb mRNA and the concomitant synthesis of the extracellular invertase (Sarokin & Carlson, 1985; Carlson, 1987). An increase in the rate at which the sucrose in grape must is metabolised by wine yeasts can be achieved by cloning the *SUC* genes and increasing the gene dosage through their integration at several sites within the genome. Furthermore, the regulated promoter of the 1,9-kb mRNA of the *SUC2* gene can be substituted by a strong constitutive promoter (e.g., the *ENO1* promoter) that is insensitive to carbon catabolite repression. The amplification of a constitutive *SUC2* gene encoding extracellular invertase should improve sucrose fermentation in wine yeasts.

Unlike some bacteria that ferment a wide range of organic compounds, yeasts (with a few notable exceptions) ferment only those metabolised through the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways (Stewart & Russell, 1983). Furthermore, except for cytochrome-deficient mutants, a yeast that uses sugar anaerobically also uses it aerobically (Barnett, 1981). Reducing the yeast biomass produced during wine fermentation without affecting the fermentation rate will lead to a more efficient fermentation since more sugar will be converted to alcohol. This can be brought about when the twelve glycolytic enzymes of the Embden-Meyerhof-Parnas pathway function efficiently. Gene cloning and transformation can be used specifically to replace mutant alleles of genes encoding glycolytic enzymes. Alternatively, random mating of ascospores derived from several wine yeast strains can be used to yield many new recombinations of these genes, some of which could possibly express improved fermentation characteristics. It was previously suggested that an increase in the dosage of genes encoding the glycolytic enzymes would result in an increase in the efficiency of conversion of grape sugar to alcohol. However, Schaaf *et al.* (1989) reported that overproduction of the different glycolytic enzymes in yeast had no effect on the rate of ethanol formation. Improved conversion efficiency may also be brought about by reducing glycogen accumulation or by reducing the efficacy with which energy regeneration is coupled to biosynthesis reactions (Tubb & Hammond, 1987). The lower biomass would have a secondary benefit in that there would be less surplus yeast to be removed and to dispose of after fermentation.

Efficient sugar utilisation by wine yeasts cannot be discussed without referring to their tolerance to ethanol. The yield of ethanol is independent of sugar concentration above a critical value of the sugar. It has long been recognised that yeasts are sensitive to ethanol and a number of methods of defining ethanol tolerance have been proposed. Fermentation rate, glucose consumption, biomass yield, growth rate and cell viability have all been used as indicators of the relative sensitivity or tolerance of various yeast strains to the alcohol (Oliver, 1987). Considering the complexity of ethanol toxicity, it is no surprise that ethanol has different and separable effects on the growth rate, fermentation rate and viability of wine yeasts (Brown *et al.*, 1981). This suggests that there are many target sites within the yeast cell for the toxic action of ethanol. These targets include membranes and in particular the plasma membrane (Ingram & Buttke, 1984), solute transport systems (Van Uden, 1985), the steps of initiation and elongation in the process of protein synthesis (Swedes *et al.*, see Oliver, 1987) and RNA accumulation (Stephens & Oliver, see Oliver, 1987). The physiological basis for ethanol tolerance in yeasts remains obscure. Intracellular enzymes are bathed in ethanol concentrations of over 2 M at the end of a fermentation (Rose, 1987) and the V_{max} of a number of

glycolytic enzymes have been shown to be reduced (Nagodawithana *et al.*, 1977). However, the twelve glycolytic enzymes were still found to be able to function fairly efficiently at the ethanol concentrations that they encounter and some, phosphofructokinase, for instance, are noticeably resistant (Millar *et al.*, 1982). The molecular basis for the inhibitory action remains unknown, as does the reason why some yeasts are more tolerant of ethanol than others. Given the pleiotropic nature of the effect of ethanol on wine yeast strains, it is most unlikely that any single gene is solely responsible for the sensitivity or tolerance of the yeast to ethanol (Oliver, 1987). In fact, the response of yeast cells to ethanol has been shown to be strain dependent and affected by many nuclear and mitochondrial genes (Christensen, 1987). In addition to the genome, environmental factors such as osmotic pressure (Panchall & Stewart, 1980), the presence of unsaturated fatty acids and sterols in the medium (Thomas & Rose, 1979; Beaven *et al.*, 1982; Casey *et al.*, 1984) and temperature (Nagodawithana *et al.*, 1974; Nagodawithana & Steinkraus, 1976; Hacking *et al.*, 1984) play a major role in the response of yeast cells to high ethanol concentrations. This situation, in which only small quantitative increases are likely to be obtained as a result of multiple (rather than single) mutations, the use of mutagenesis, hybridisation and continuous (chemostat) selection, has proved to be the most efficient approach in breeding ethanol-tolerant wine yeasts. Alikhanyan & Nalbandyan (1971) used mutagenesis to select mutant strains of the film-forming yeast, *S. oviformis* (now known as *S. cerevisiae*), that could grow well at an ethanol concentration of 17,5%, from a parental strain that could tolerate no more than 14,4% ethanol. Brown & Oliver (1982b) and Christensen (1987) used continuous selection to isolate variant strains possessing high ethanol-tolerance, improved fermentation ability and enhanced viability.

Resistance to microbial toxins. Commercial fermentations that employ *S. cerevisiae* are subject to contamination by "wild-yeasts", that is, yeasts other than those used in fermentation. The ideal yeast strain would be one that is resistant to any microbial toxin (zymocides or zymocins) and one that would itself produce a compound lethal to wild-yeasts as well as contaminating bacteria and fungi. Such a resistant yeast or wide-spectrum antimicrobial compound has not been found or constructed yet. However, K_2 killer yeasts have been isolated from wine (Naumova & Naumov, 1973; Naumov *et al.*, 1973) and beer (Maule & Thomas, 1973; Rogers & Bevan, 1978). Since the optimum pH for the production and stability of the K_1 toxin lies between pH 4,6-4,8 (Woods & Bevan, 1968), K_1 killers are not important in fermenting grape must. However, K_2 killer toxin is stable at pH 2,8-4,8 (Shimizu *et al.*, 1985). Van Vuuren & Wingfield (1986) reported that contaminating K_2 killer yeasts can cause stuck wine fermentations. Jacobs *et al.* (1988) confirmed this result and showed that

a relatively low inoculation with K_2 killer yeasts early in grape juice fermentation can eliminate a sensitive wine yeast strain and eventually dominate the yeast population. Furthermore, commercial killer as well as sensitive wine yeasts are currently being used in the South African wine industry. Thus, fermentations carried out by sensitive wine yeast strains can be overtaken by killer wine yeast strains or spoiled by contaminating killer yeasts from grape skins. An unfortunate consequence of ignorance regarding the role of killer yeasts in wine fermentations was that some winemakers used co-cultures to inoculate fermentations; one strain being a killer and the other a sensitive strain! The advantage of using killer or neutral wine yeasts should not be underestimated. For this reason the aim of many breeding programmmes is to incorporate the mycoviruses from killer yeasts into commercial brewing, saké and wine strains.

Mycoviruses are readily transmitted by cytoplasmic fusion (Conde & Fink, 1976) and have been used to transfer the killer character into commercial yeasts. In most cases, however, the mixing of the genomes of commercial strains and donor strain containing the killer character would prove undesirable even though repeated back-crossing could be used to minimise the unwanted effects (Ouchi & Akiyama, 1976). Thus, Ouchi *et al.* (1979) employed a donor of killer character that was deficient in nuclear fusion and mated this with a haploid (derived from a saké yeast) and selected for saké strains containing cytoplasmic elements of both strains. Van der Westhuizen & Pretorius (1989 & 1990) crossed a haploid (derived from a killer wine yeast) with ascospores from a homothallic, sensitive wine yeast. In this case mixing of the two genomes did not prove to be undesirable as both parental strains are efficient wine yeasts. The result of this cross was an efficient killer wine yeast containing desirable characteristics of both parental wine yeasts. An alternative to the use of hybridisation and cytoduction to introduce the killer character into wine yeasts would be to clone the toxin and immunity genes into wine yeasts. Since both killing and immunity reside on the same MdsRNA molecule, reverse transcription has been used to produce a DNA copy (cDNA) of these two genes (Skipper *et al.*, 1984; Bostian *et al.*, 1984). The cDNA clone contained a region of the M_1dsRNA molecule coding for the preprotoxin and conferred both immunity and the ability to produce toxin. Site-directed mutagenesis of the cDNA clone not only made the killer system genetically accessible, but also paved the way to express cDNA clones of the M_2dsRNA and other toxin-immunity genes in wine yeasts.

Furthermore, the killer cDNA expression plasmids can also be used as a dominant selection system for yeast transformants. Killer expression plasmids can be used to transform yeast hosts lacking selectable markers (Bussey & Meaden, 1985; Thomas *et al.*, 1987). This is of particular interest in the tranformation of wine yeasts and other industrial strains that are diploid or polyploid. Since the killer expression

vector contains both the toxin and immunity genes, non-transformed cells will be eliminated by toxin treatment and killer toxin secreted by transformed cells, while the transformants will be immune to the toxin. This autoselective screening system proved to be sensitive in detecting rare transformants in a variety of yeasts used in different industrial processes (Thomas *et al.*, 1987). This system can now be used to introduce other important genes in wine yeasts by construction of the appropriate plasmids. For example, a gene encoding a yeast glucoamylase has been incorporated into the yeast expression plasmid and used to transform industrial strains. These transformants now secrete killer toxin and glucoamylase (Thomas *et al.*, 1987). Further modifications of this killer expression vector to remove the *E. coli* plasmid segments will make the constructions entirely of *S. cerevisiae* origin, that may be required for some applications in the food industry, including the wine industry. Utilisation of the killer-toxin leader sequence to secrete heterologous proteins from yeast is another obvious use of the killer system. However, it is not clear if the killer-toxin leader has all the advantages of the leader sequences of other secreted yeast proteins in this application. Thomas *et al.* (1987) stated that the killer-toxin-based transformation (and secretion) system provides an entry into the molecular genetics of industrial yeast strains.

Resistance to heavy metals. Grapes contain small but adequate amounts of the heavy metals (calcium, cobalt, copper, iron, magnesium, potassium and zinc) needed for yeast growth and alcoholic fermentation. They also contain sufficient phosphorus, sulphur and iodine. Much more common than an inadequate amount of metal ions is the inhibiting effect of excessive amounts.

Copperoxychloride is widely used in South African vineyards for the control of downy mildew (*Plasmopara viticola*) and to a lesser extent against dead arm (*Phomopsis viticola*) and anthracnose (*Gloeosporium ampelophagum*). The use of copper-containing fungicides leades to copper residues in musts that may cause lagging fermentation and affect wine quality detrimentally (Tromp & De Klerk, 1988). The copper concentration of a settled must obtained from grapes that had received five sprays of copperoxychloride with the last spray applied one week before harvest was found to be 3,91 mg/*l* (Eschenbruch & Kleynhans, 1974). From the results of Tromp & De Klerk (1988) it is evident that where vines were sprayed in a comprehensive programme of six sprays, with the last one applied three days before harvest, the settled musts had a copper content of 43,6 mg/*l*. Tromp & De Klerk (1988) also reported that a copper concentration in excess of 40 mg/*l* caused serious lagging of fermentation. In fact, lagging fermentation even occurred where spraying was terminated 14 days before harvest (leading to a copper content of 13,1 mg/*l* in musts).

The *CUP1* gene encodes a copper-binding protein, copper-chelatin, and it was shown that the copper resistance level of a given yeast strain correlates directly with the *CUP1* copy number (Fogel *et al.*, 1983). One way in which wine yeasts resistant to copper can be engineered would be to clone and integrate the *CUP1* gene at multiple sites into their genomes (Henderson *et al.*, 1985). This will enable the wine yeast to tolerate higher concentrations of copper residues in musts.

Non-foaming. Excessive foaming during the early stages of a wine fermentation is an undesirable characteristic of some wine yeast strains. Formation of a froth-head can result in the loss of grape juice (Thornton, 1983) or reduce the capacity of plant equipment as part of the fermentation vessel may have to be reserved to prevent the froth from spilling over (Snow, 1983; Thornton, 1983). Certain wine yeast strains produce proteins that interact with the grape juice, causing foaming (Molan *et al.*, 1982). The genetic basis for foaming has been investigated in saké yeasts (Kasahara *et al.*, 1974) and wine yeasts (Thornton, 1978a, b). It was found that when non-foaming haploid strains were crossed with foaming haploids the diploid progeny retained the foaming character (Thornton, 1978a). Tetrad analyses of the sporulated diploids showed 4:0, 3:1 and 2:2 segregation patterns for the foaming characteristic. These results indicated that the ability to produce a froth-head was under the control of at least two dominant genes. These genes are denoted as *FRO1* and *FRO2* and are linked on chromosome VII, 21cM from one another and near *ade3* (Thornton, 1978b). In the past, hybridisation was used to breed out the genes that were responsible for foaming. Eschenbruch & Rassell (1975) were able to select non-foaming mutants from two strains of New Zealand wine yeasts. Likewise, Vezinhet (1989) has modified yeast strains by intra-genomic recombination techniques, producing non-foaming hybrids.

In a more specific fashion, recombinant DNA techniques can be used to eliminate the foaming characteristic of wine yeast strains without changing the remainder of their genetic backgrounds. First the *FRO1* and *FRO2* would have to be cloned from yeast strains expressing the foaming character. Following the restriction mapping of the cloned *FRO1* and *FRO2* genes, these genes can be disrupted by integrating a marker gene (e.g., *CUP1*) into their coding regions. Exogenous DNA can be made to integrate into the yeast genome by homologous recombination if sufficient homology exists between the donor and genomic DNA. As such the one-step gene disruption method of Orr-Weaver *et al.* (1981) can be used to replace the *FRO1* and *FRO2* in wine yeasts by their disrupted counterparts.

Sedimentation and flocculation. Sedimentation of yeast cells refers to clumpy growth caused by delayed separation of mother and daughter cells (Snow, 1983). Yeast

flocculation is the phenomenon where cells adhere to one another, forming microscopic clumps of continuous, multicellular associations that settle rapidly from suspension in liquid cultures (Calleja, 1987; Zaworski & Heimsch, 1987). These flocs may encompass many thousands of cells. The mechanism of yeast flocculation remains controversial. Protease treatment leads to irreversible loss of flocculation (Miki *et al.*, 1980), suggesting that proteins on the surfaces of flocculant cells play a role in flocculation. Flocculation receptor sites, present on both flocculant and non-flocculant cells, are insensitive to protease action (Miki *et al.*, 1982). Attachment of yeast cells to one another can also apparently be mediated by ionic-binding, brought about by divalent ions, especially calcium (Mill, 1964). According to the "lectin-like" hypothesis, proteins bind to the mannose residues in neighbouring yeast cell-walls using calcium ions to maintain correct configurations of the lectins (Stratford & Brundish, 1990). It is known that yeast flocculation is under genetic control. At least one dominant gene, *FLO1*, will cause flocculation when expressed in a yeast strain (Russell *et al.*, 1980) unless modified by the presence of suppressor genes (Holmberg & Kielland-Brandt, 1987). Using hybridisation techniques coupled with a back breeding-programme, Thornton (1983) was able to introduce the *FLO1* gene from a laboratory strain into a wine yeast strain. Miki *et al.* (1981 & 1982) attempted to identify the gene product of *FLO1*. The protein patterns from flocculant cells and those from non-flocculant mutants showed insignificant differences, with the exception of one polypeptide of about 13 kDa. It remains to be demonstrated that this 13-kDa protein plays a role in mediating cell-cell interactions. If the FLO1 protein can be positively identified, then it would be possible to clone the *FLO1* gene using reverse genetics. A cloned *FLO1* gene could then be transformed into a wine yeast strain without adversely affecting other desirable properties due to non-specific hybridisations.

Since wine fermentation is a function of the concentration of active wine yeast cells in contact with the grape must substrate, an efficient wine yeast should remain dispersed during fermentation. When its role is over, it should then separate out of suspension, clarifying the wine. To avoid premature flocculation a cloned *FLO1* gene can be linked to an inducible promoter element. This will enable the winemaker to turn on the *FLO1* gene and induce flocculation at the appropriate time, for example, by altering the temperature.

Extracellular enzymes. Potentially, a wide spectrum of extracellular enzyme activities could be introduced into wine yeasts. Introducing the genes encoding endo-β-glucanase and exo-glucanases into wine yeasts would enable β-glucans normally present in grape must to be degraded during fermentation and so prevent the development of glucan hazes and gels. Removal of β-glucans in this way promises to

improve the efficiency of filtration of wine containing high levels of β-glucans. The endo-β-glucanase genes from *Bacillus subtilis* (Hinchliffe & Box, 1984; Cantwell *et al.*, 1986) and from *Trichoderma reesei* (Arsdell *et al.*, 1987; Penttila *et al.*, 1987) have already been cloned and expressed in *S. cerevisiae*. Cantwell *et al.* (1986) used the *CYC1* and *ADH1* promoters to obtain more efficient expression of the endo-β-glucanase gene from *B. subtilis* in *S. cerevisiae*. These cloned glucanase genes can also be spliced in frame to the signal sequence of the *MFα1* gene and the promoter of the *ENO1* gene and then introduced into wine yeasts.

Pectic substances are structural polysaccharides, occurring mainly in the middle lamellae and primary cell walls of higher plants. The α-1,4-glycosidic linkages in the pectic polymers of grapes can be split by extracellular pectinases. These include pectin esterases and pectin depolymerases (i.e. hydrolyses and lyases). Some winemakers add commercial preparations of fungal pectinases to grape must to clarify it. Commercially produced pectinases are also used in the fruit juice industry to liquefy the fruit to increase the juice yield. *S. cerevisiae* produces pectin esterases but no pectin depolymerases. The genes encoding polygalacturonases and pectate lyases can be cloned from other organisms and linked to yeast secretion signal and regulatory sequences. The expression of these genes and the secretion of their encoded pectinases in wine yeasts would be useful in a number of ways. Laing & Pretorius (1990) have cloned the pectate lyase (*pelE*) gene from the bacterium *Erwinia chrysanthemi* into an integrative yeast plasmid. The *pelE* gene was fused to the *MFα* promoter and secretion signal (leader) sequences and transformed into laboratory-bred strains of *S. cerevisiae*. The recombinant plasmid, containing the *pelE* gene, intergrated into the chromosomal DNA of the recipient yeast strain by homologous recombination. The yeast transformant stably expressed the bacterial *pelE* gene under the control of regulatory elements and secreted pectate lyase (PLe) into the culture medium. Pectolytic wine yeasts would contribute to the clarification of wine and would replace or reduce the levels of commercial pectinases needed to clarify the wine. Furthermore, pectinases secreted by wine yeasts would improve liquefaction of the grapes, increasing the juice yield. Since much of the flavour compounds are trapped in the grape skins, pectolysis would also release more of these aromatic compounds and make a positive contribution to the wine bouquet.

Malolactic fermentation. The decarboxylation of malic acid to lactic acid by a number of bacterial species is termed malolactic fermentation. Malic and tartaric acid are the principle acids of grape musts and the level of these acids depends on the climate, the grape variety and the cropping level (Subden & Osothsilp, 1987). Musts from cooler grape-growing regions or over-cropped vineyards contain excess malic acid, resulting in wines with a particularly sour taste. Malolactic fermentation

(induced secondary fermentation by strains of *Lactobacillus* and *Leuconostoc*) is of considerable concern to winemakers (Edwards & Beelman, 1989). Firstly, it reduces the acidity of some wines (especially the vinous-type Burgundy-styled wines); secondly, it contributes to microbiological stability following growth of bacteria and thirdly, it causes changes in the wine flavour, caused by products of the bacterial fermentation (Snow, 1983). Since it would be much more convenient if the wine yeast were able to carry out the malolactic fermentation concurrent with the alcoholic fermentation, winemakers have been trying for several years to select or genetically construct wine yeast strains that will utilise the malic acid in high acidity musts. Toward this end fusions between *S. bailli, S. rouxii* or *S. pombe* and *S. cerevisiae* wine strains were made, but the fusant hybrids had less malate fermenting ability than the parental strains (Subden & Osothsilp, 1987). In other efforts to construct a yeast capable of performing a normal ethanolic and a malolatic fermentation, the gene encoding the malolactic enzyme (known as L-malate: NAD carboxy lyase) was cloned from *Leuconostoc oenos* (Lautensach & Subden, 1984) and from *Lactobacillus delbruekii* (Williams *et al.*, 1984) into *E. coli* and transferred to yeast. Due to expression problems or the limited malate uptake ability of the yeast host, the transformed yeast failed to carry out malolactic fermentation. Attempts are currently underway to introduce the genes encoding malate permease and the malic enzyme from *S. pombe* into *S. cerevisiae* and it would seem that genetic construction of a wine yeast strain capable of complete malo-ethanolic fermentation is a distinct possibility in the near future (Subden & Osothsilp, 1987).

5.2.3 Contribution to wine bouquet

The single most important factor in winemaking is obviously the organoleptic quality of the final product. The presence of desirable flavour compounds and metabolites in a well-balanced ratio as well as the absence of undesirable components eventually determine the bouquet of good wines. Together with the grape variety and winemaking practices, the wine yeast strain makes an important contribution to the complex character of prize-winning wines. Wine yeasts can be bred for the production of metabolites associated with pleasant organoleptic responses (e.g., desirable levels of volatile esters). The breeding strategy can also be directed toward the elimination of compounds that are seen as health hazards (e.g., sulphur dioxide and ethyl carbamate).

Production of volatile esters. Wine yeasts produce a wide variety of esters that are important flavour compounds. The composition of the ester fraction depends on the growth of the yeast, the yeast strain used and the fermentation conditions (Kunkee &

Amerine, 1970). It was found that wine yeasts in general produce more ethyl caprate, ethyl caproate, ethyl caprylate and isoamyl acetate and that there is considerable variation in the production of various esters by different wine yeast strains (Nykänen & Nykänen, 1977). In another study, four esterase (*EST*) loci were identified in wine yeasts sampled from 40 localities in Europe (Wöhrmann & Lange, 1980). This investigation revealed that all the wine strains had at least two active loci, *EST1* and *EST2*, and that some strains carried all four *EST* loci. Snow (1983) speculated that beneficial flavour changes might be obtained by using wine yeast strains carrying esterase mutations that reduce, increase and alter the balance of various esters. Without underestimating the complexity of wine bouquet, we agree with Snow (1983) that this seems like a promising line of future work.

Fusel oil production. The higher alcohols such as isobutyl, isoamyl and active amyl alcohol are termed fusel oil. These alcohols are produced by wine yeasts during alcoholic fermentation from intermediates in the branched chain amino acids pathway leading to production of isoleucine, leucine and valine by decarboxylation, transamination and reduction (Webb & Ingraham, 1963). At high concentrations these higher alcohols have undesirable flavour and odour characteristics (Snow, 1983). Higher alcohols, however, are usually present in wines at concentration levels that do not affect the taste of wine unfavourably. In some cases, they may even contribute to wine quality (Kunkee & Amerine, 1970). Since higher alcohols are concentrated by the distilling process, their reduction in wines that are to be distilled for brandy production is of great importance (Snow, 1983).

Initial attempts to use Ile^-, Leu^- and Val^- auxotrophic mutants succeeded in lowering the levels of isobutanol, active amyl alcohol and isoamyl alcohol production in fermentations, but these mutants were of no commercial use as their growth rate and fermentation rate were compromised (Ingraham & Guymon, 1960; Ingraham *et al.*, 1961). A Leu^- mutant derived from the widely used Montrachet wine yeast (UCD, Enology 522) was reported to produce more than 50% less isoamyl alcohol during fermentation than the prototrophic parent (Rous *et al.*, see Snow, 1983). It will be of great interest to see whether integrative disruption of specific *ILE*, *LEU* and *VAL* genes of wine yeasts will result in lower levels of fusel oil in wine and whether that will improve the quality of the wine.

Sulphite and sulphide production. It is general knowledge that *S. cerevisiae* can use sulphate, sulphite and elemental suphur as sole sources of sulphur (Rose, 1987) and that the formation of SO_2 and H_2S by wine yeasts greatly affect the quality of wine. Sulphur dioxide is used regularly as an antimicrobial and antioxidative additive in white wine fermentations. Health concern has led to ever increasing demands for

restriction of its use as a disinfectant additive. Consequently, the production of SO_2 by wine yeasts itself has become a point of debate. While SO_2, when properly used, has beneficial affects, the reverse is true for H_2S, one of the most undesirable of yeast metabolites, since it effects the taste and smell of wines (Snow, 1983).

Sulphur is essential for yeast growth and is naturally available as sulphate in grape juice. Dusting of vines with elemental sulphur provide another source of sulphur. Sulphite is only formed from sulphate, while sulphide is formed from sulphate, sulphite, from elemental sulphur applied as a fungicide, or from cysteine (Eschenbruch, 1974a, b; Eschenbruch & Bonish, 1976a, b). The formation of both sulphite and sulphide is affected by many factors, including the composition of the fermentation medium. The concentration of sulphate (Eschenbruch, 1974a) and the initial pH (Eschenbruch & Bonish, 1976a) have been reported to effect sulphite formation. The formation of sulphide was shown to be influenced indirectly by the amount of yeast growth, pantothenate or pyridoxine deficiencies or excess levels of certain amino acids (that cause a methionine deficiency, resulting in higher levels of H_2S), metal ions (copper, manganese, zinc) and yeast autolysis (Snow, 1983).

During investigations into the regulation of sulphur metabolism in high and low sulphite-producing wine yeast strains, considerable differences in the levels of activity of sulphate permease (Dott *et al.*, 1977), ATP-sulphurylase (Heinzel & Trüper, 1978) and sulphite reductase (Dott & Trüper, 1978) were reported. Sulphate permease, mediating the uptake of sulphate by the yeasts, was shown not to be repressed by methionine in high sulphite-producing strains (Dott *et al.*, 1977). Heinzel & Trüper (1978) reported that ATP-sulphurylase and ADP-sulphurylase were not regulated by sulphur intermediates in high or low sulphite-producing strains. Unlike the high sulphite-producing strains, the low sulphite-producing strains showed an increased biosynthesis of NADPH-dependent sulphite reductase, O-acetylserine sulphydrylase and O-acetylhomoserine sulphydrylase during the exponentional growth phase in the presence of sulphate, sulphite and djencolic-acid (Dott & Trüper, 1978 & 1979). Methionine and cysteine prevented an increase in the levels of sulphite reductase, O-acetylserine sulphydrylase and O-acetylhomoserine sulphydrylase (Dott & Trüper, 1978 & 1979).

Of importance to the yeast geneticist is that yeast strains differ drastically in their ability to produce sulphite and sulphide (Rankine, 1968; Eschenbruch, 1974a; Eschenbruch & Bonish, 1976b; Eschenbruch *et al.*, 1978; Thornton & Bunker, 1989). One way to take advantage of this fact is to select or develop a wine yeast strain that will either produce less H_2S or that will retain most of the H_2S produced intracellularly (Rupela & Tauro, 1984). Snow (1983) suggested that in addition to exploitation of the genetic heterogeneity in sulphite and sulphide formation, the deliberate introduction of mutations in certain enzymes of the sulphur, sulphur

amino acids, pantothenate and pyridoxine pathways might well enable a stepwise elimination of these characteristics in wine yeasts. The *MET3* gene encoding ATP sulphurylase (the first enzyme in the conversion of intracellular sulphate to sulphite) has been cloned and shown to be regulated at the transcriptional level (Cherest *et al.*, 1985). This may lead to the elucidation of sulphite and sulphide formation by wine yeasts.

Ethylcarbamate. Diethyl dicarbonate (DEDC), also called diethyl pyrocarbonate (DEPC), is an excellent fungicide, and is especially effective as an adjuvant to sterile filtration in winemaking and brewing. The Food and Drug Administration banned its use after Lofroth & Gejvall (1971) indicated that wine treated with DEDC formed ethylcarbamate (carcinogenic when present in high concentrations) in large amounts. The ban continued, although it was shown by Fischer that Lofroth and Gejvall were in error (Ough, 1976a). Ough (1976a & b) indicated that ethylcarbamate is a natural component in wine and probably in other fermented foods, but not in unfermented food. The concentrations present in these products are very low and do not present any health hazard. It is known that diethyl dicarbonate reacts with ammonia to form ethylcarbamate. It appears that most N-carbamyl compounds, (i.e., urea, citrulline, N-carbamyl α-amino acids, N-carbamyl β-amino acid, allantoin and carbamyl phosphate) react with ethanol at acid pH levels to form ethylcarbamate non-enzymatically (Ough *et al.*, 1988b).

It was shown in fermentations that arginine was metabolised to provide precursors for ethylcarbamate formation and that the amount of precursors formed seems to depend not only on arginine, but also on the balance of other amino acids and ammonia (Ough *et al.*, 1988a). Ough *et al.* (1988a) pointed out the following possible solutions to stop or lessen the amount of ethylcarbamate produced: (i) limit vineyard fertilisation to a minimum and add arginine-free yeast nutrients to the juice before fermentation; (ii) develop a yeast that will not metabolise arginine; or (iii) use grape varieties low in arginine (e.g., White Riesling).

Flor formation. The process for the production of flor sherry was developed in the south of Spain over a century ago (Kunkee & Amerine, 1970). The formation of a yeast film on the surface of wine containing about 15% ethanol is a characteristic of this high-aldehyde, low-sugar flor sherry (Snow, 1983). It has been reported that the film-forming characteristic segregated 2:2 in asci of these sherry yeasts, indicating that the flor formation is controlled by a single dominant gene (Santa Maria & Vidal, 1973). Cloning and sequencing of the gene responsible for flor formation and purification of the encoded protein would elucidate the mechanism of this very interesting phenomenon of film formation in flor sherry. Novel applications of film

formation by yeasts may arise from such information.

5.2.4 New products

Genetically modified wine yeasts could be used for the development of new products. Yeasts with a restricted pattern of fermentation could be engineered to produce low-alcohol wines. Another worthwhile target for genetic engineering is to incorporate into yeast cells the ability to utilise carbohydrates other than those normally metabolised, leading to a more complete utilisation of a range of raw materials. The inclusion of enzymes for the degradation of cellobiose, cellulose, raffinose, starch and pentose would result in a more complex fermentation of conventional raw materials. Furthermore, totally new substrates could be used as adjuncts in the production of completely new types of wine. For example, lactose in whey could be used as an adjunct if wine yeasts were provided with the enzymes for lactose uptake and hydrolysis. Alternative wine derivatives or products with unusual flavours could be produced by the introduction of new flavour compounds into wine yeasts or by the genetic modification of other yeast species (e.g., *Candida, Kluyveromyces* and *Schizosaccharomyces* strains). Another area that may be exploited is the use of wine yeasts to produce valuable by-products such as flavourings, vitamins, enzymes, coenzymes and lipids, or even completely new high-price, low-volume materials. This would, however, involve diversification away from the alcoholic beverage industry. Tubb & Hammond (1987) have listed a wide range of possible products that could be profitably manufactured using genetically-tailored brewing yeasts (and for that matter wine yeasts). These products include the following: (i) industrially or therapeutically important enzymes (e.g., amylolytic and pectolytic enzymes, glucose isomerase, proteases, cellulases, rennin, papain, lipases, glucose oxidases, invertases, superoxide dismutase, tissue plasminogen activator, urokinase); (ii) hormones and other pharmaceutical products (e.g., calcitonin, growth hormones, insulin, somatostatin, interferons, interleukin-2, serum albumin, colony stimulating factor, epidermal growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, skeletal growth factor, wound angiogenesis factor, tumor angiogenesis factor); (iii) viral antigens used in the production of vaccines (e.g., hepatitis B, herpes, foot-and-mouth disease viruses); and (iv) products valuable to the food or chemical industries (e.g., thaumatin, solvents from vegetables, sweeteners, thickeners).

Tubb & Hammond (1987) have also highlighted several advantages of industrial yeasts as hosts for the expression of foreign genes, namely (i) *S. cerevisiae* is of GRAS (Generally Regarded As Safe) status and has a long association with man and his food; (ii) there is a plethora of literature describing the biochemistry and

genetics of *S. cerevisiae* and a multitude of genetic techniques are available for strain modification; (iii) the large-scale fermentation and separation technology already exists and any excess yeast biomass is a commercially exploitable by-product; and (iv) secretion of foreign gene products into the culture medium will reduce recovery costs and will also take advantage of post-translational events such as glycosylation and endoproteolysis that will allow for the formation of correct secondary structures during the secretory process. Since winemakers have vast experience of the yeast-based fermentations, they are well placed to explore new opportunities offered by the golden age of biotechnology.

6. PERSPECTIVES

In this review we have highligted the importance of the wine yeast to the wine industry and the necessity for well-planned breeding programmes. Firstly, we summarised reliable taxonomic methods that are useful as diagnostic techniques in such breeding strategies. Secondly, we emphasised the complexity of the genetic features of commercial wine yeast strains. In the third place, we reviewed the genetic techniques available and pointed out the potential of these techniques (individually and in combination) in strain development programmes. Finally, we attempted to stimulate interest in the genetic engineering of wine yeasts by discussing a few potential targets of strain development.

Despite our limited knowledge of the genetic make-up of commercial wine yeasts and the fact that the advantages of genetic manipulation of wine yeasts have not yet been demonstrated in practice, the wine industry has to realise that the name of the game is recombinant DNA and that the pace of progress is fast. The technology is so powerful that it now enables manipulation of the genome in ways hard to imagine only a decade ago. The impact of yeast genetics and recombinant DNA technology on the wine industry promises to be impressive!

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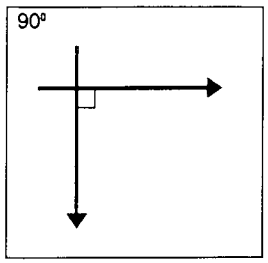
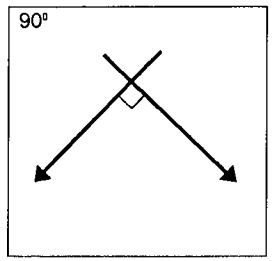
FIG. 1

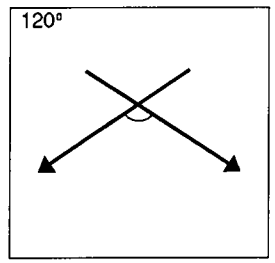
The different electrode configurations used in pulsed field gel electrophoresis (PFGE = pulse field gradient electrophoresis; OFAGE = orthogonal field alternation gel electrophoresis; RFE = reversed field electrophoresis; CHEF = contour clamped homogeneous electric field electrophoresis; TAFE = transverse alternating field electrophoresis) and DNA migration patterns (Pretorius, 1989b).



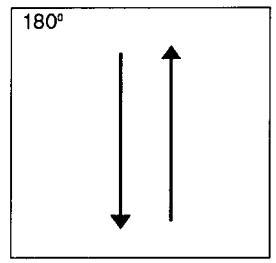
PFGE



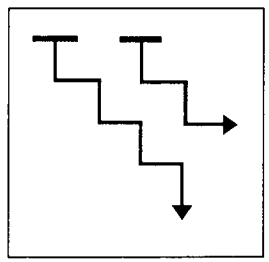
OFAGE



CHEF



RFE
FIGE



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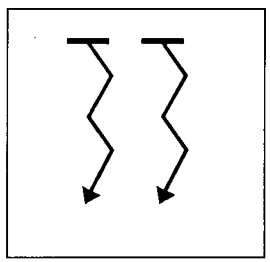
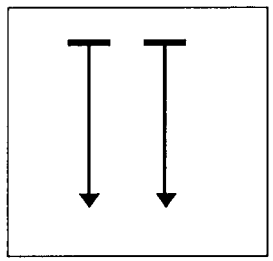


OFAGE
CHEF



RFE
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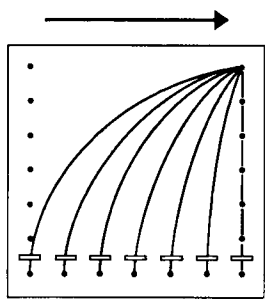

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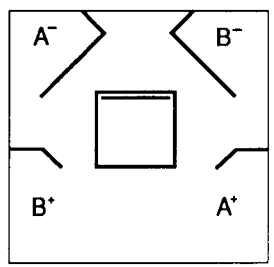

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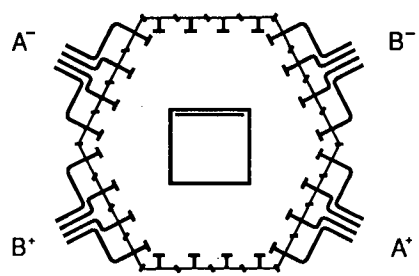

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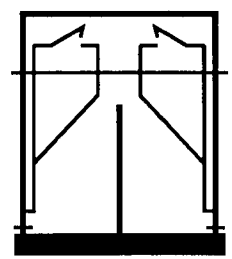


TAFE

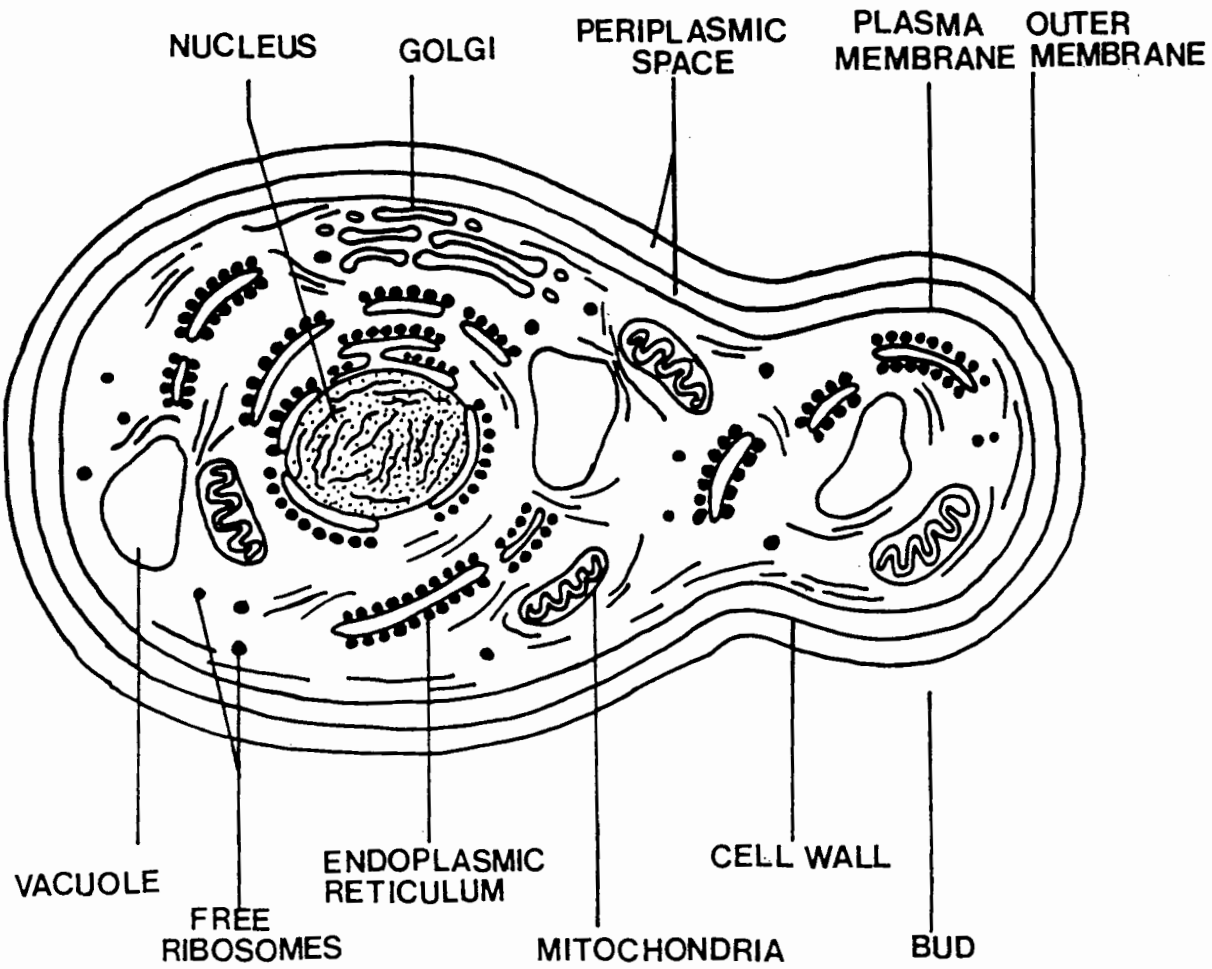
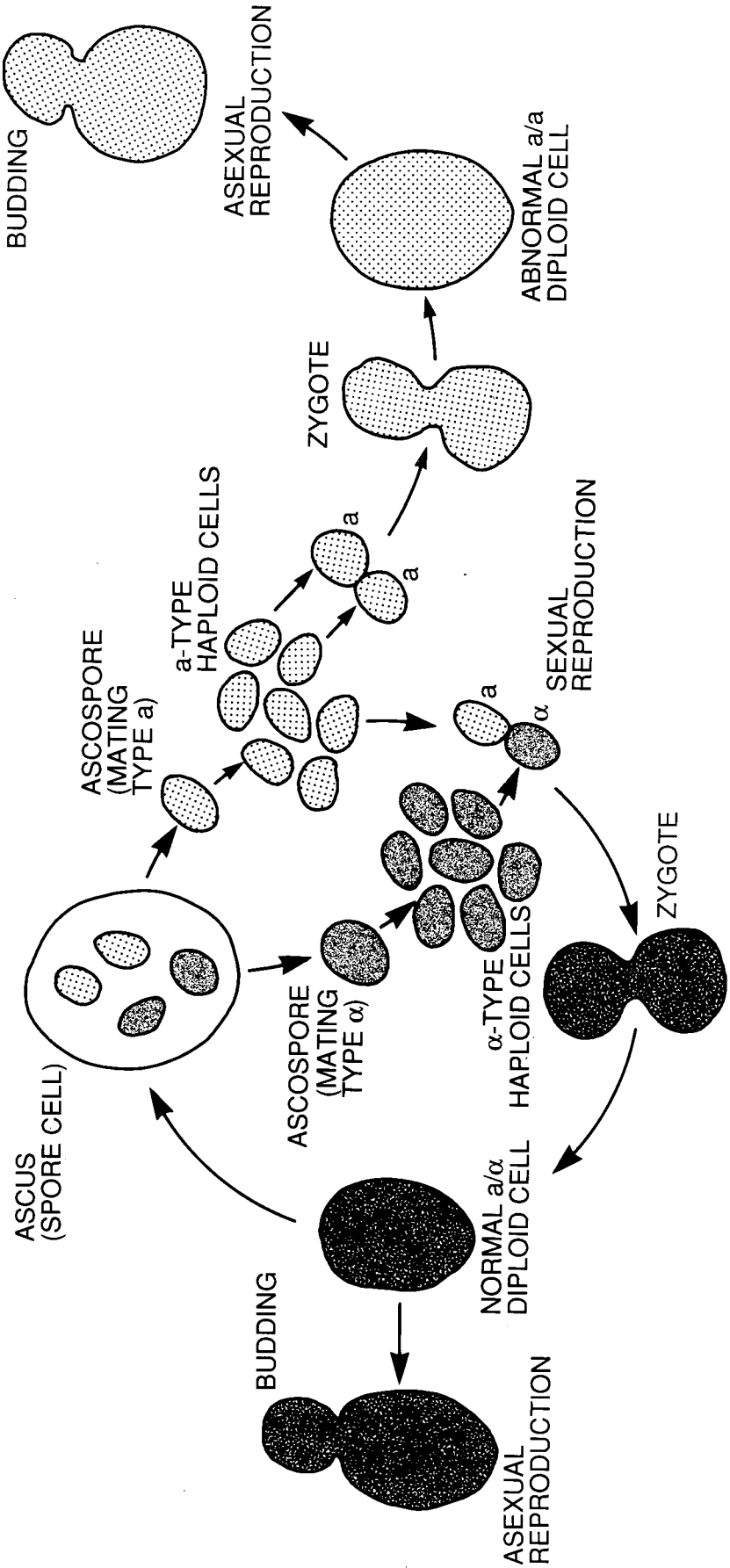


FIG. 2

Diagram of a *S. cerevisiae* cell (Pretorius, 1989b).

FIG. 3.

The basic life cycles of homothallic and heterothallic strains of *S. cerevisiae* (Phaff, 1981).



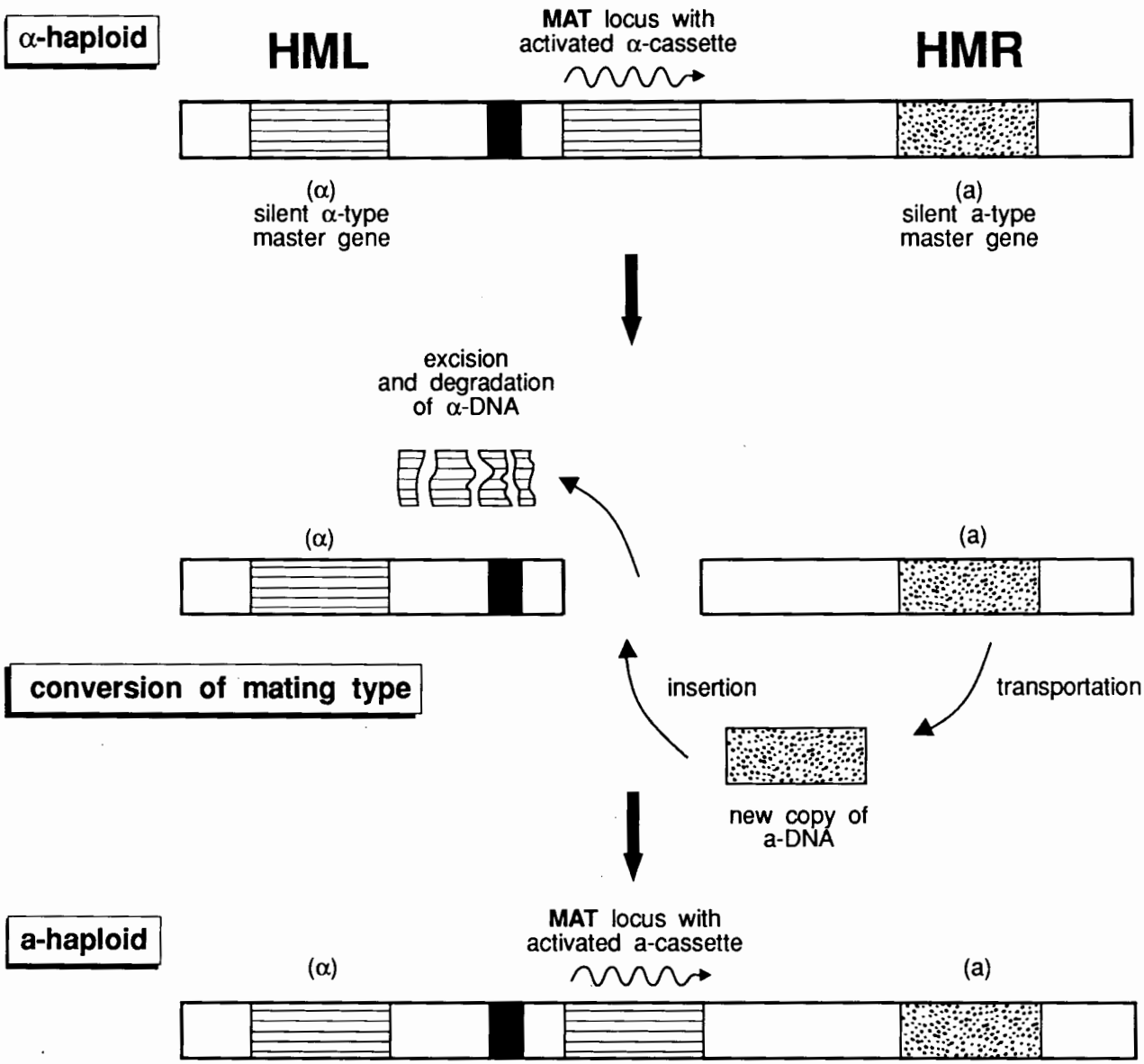


FIG. 4

The cassette model of mating-type switching in *S. cerevisiae*. Here the *a* cassette replaces the *α* cassette in the mating-type locus that is expressed (Herskowitz & Oshima, 1981).

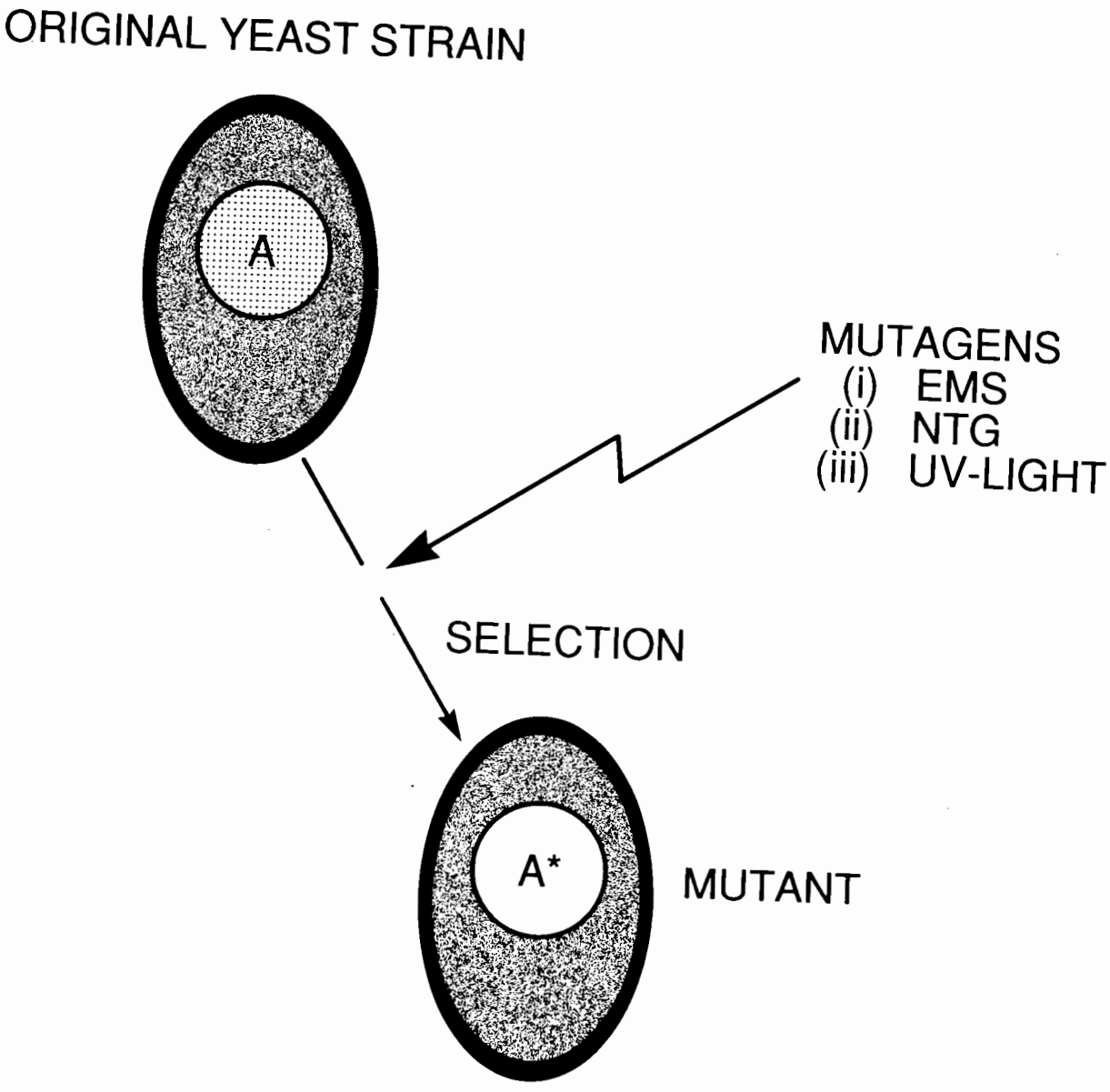


FIG. 5

Induction of mutation in *S. cerevisiae*. The use of mutagens, such as, ultraviolet light (UV-light), ethylmethane sulphonate (EMS) and N-nitro-N-nitrosoguanidine (NTG) increases the proportion of mutants within a given yeast population (Pretorius, 1989b).

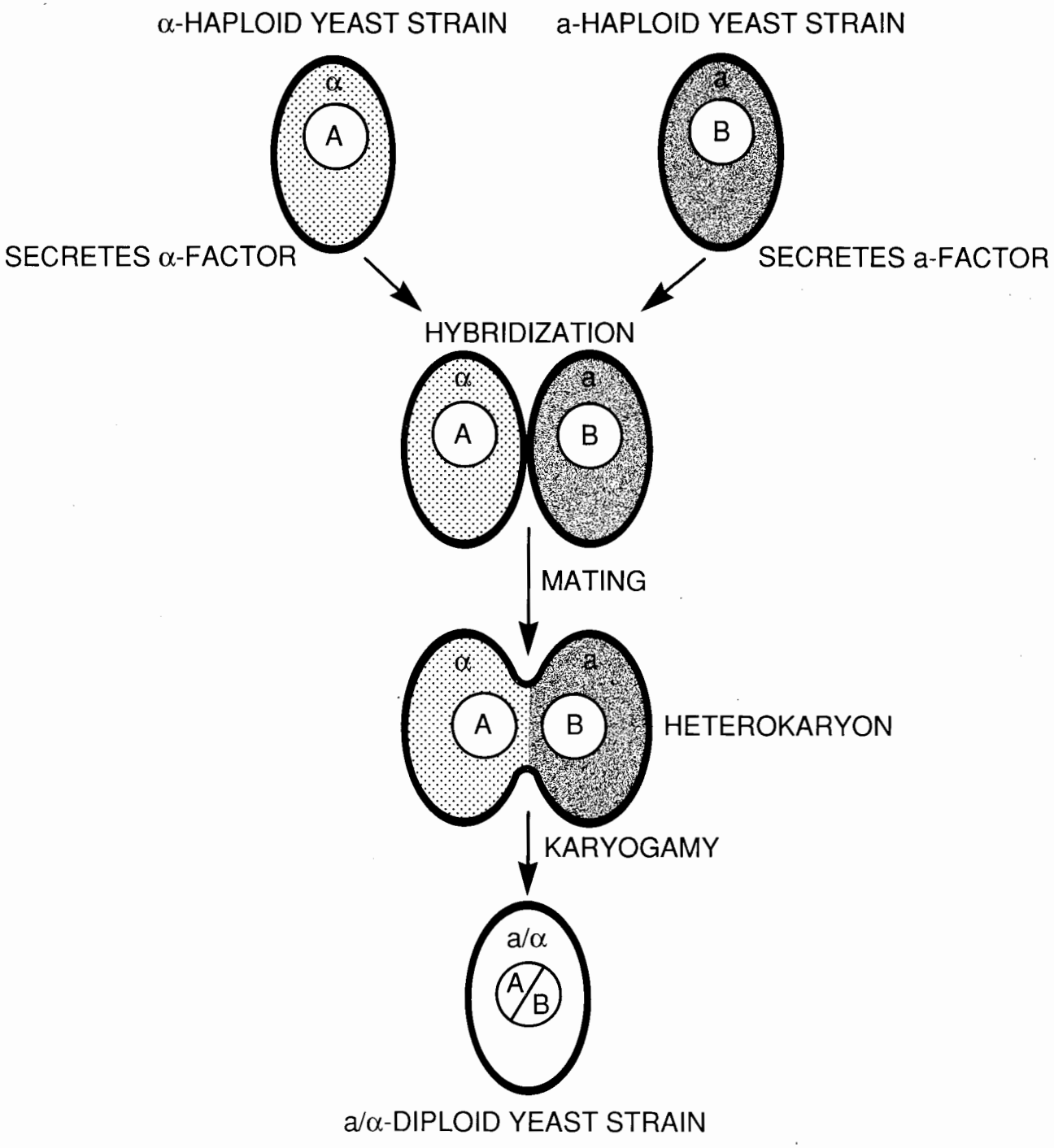


FIG. 6

Hybridisation (mating) between haploids of two opposite mating-types in *S. cerevisiae* (Pretorius, 1989b).

FIG. 7

Rare-mating between industrial and laboratory strains of *S. cerevisiae*. Industrial strains that fail to show a mating-type are force-mated with haploid strains, exibiting a or α mating-type. A large number of cells of the parental strains are mixed and the rare hybrids are selected as respiratory-sufficient prototrophs from crosses between a respiratory-deficient mutant of the industrial strain and an auxotrophic haploid laboratory strain (Tubb & Hammond, 1987).

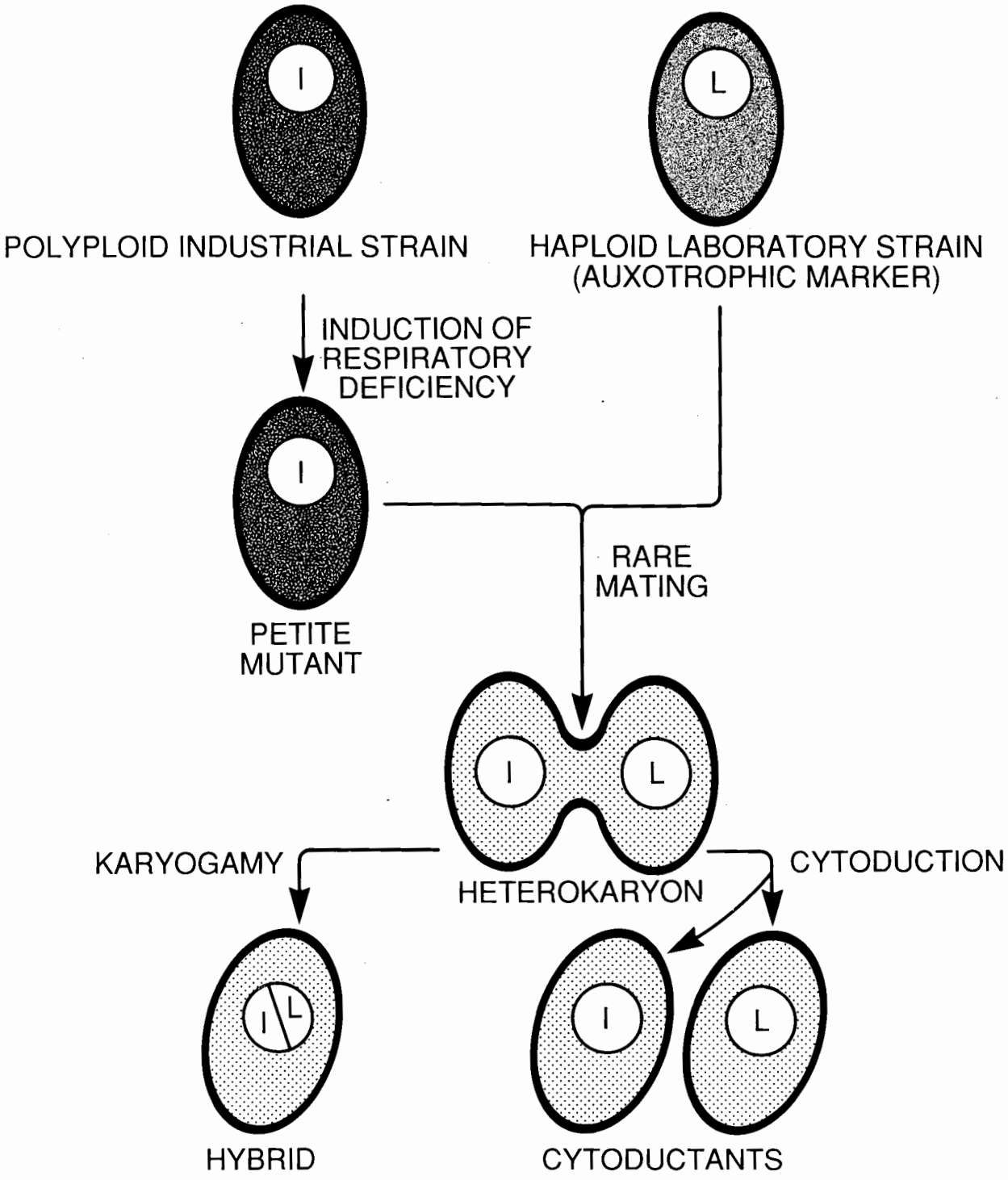
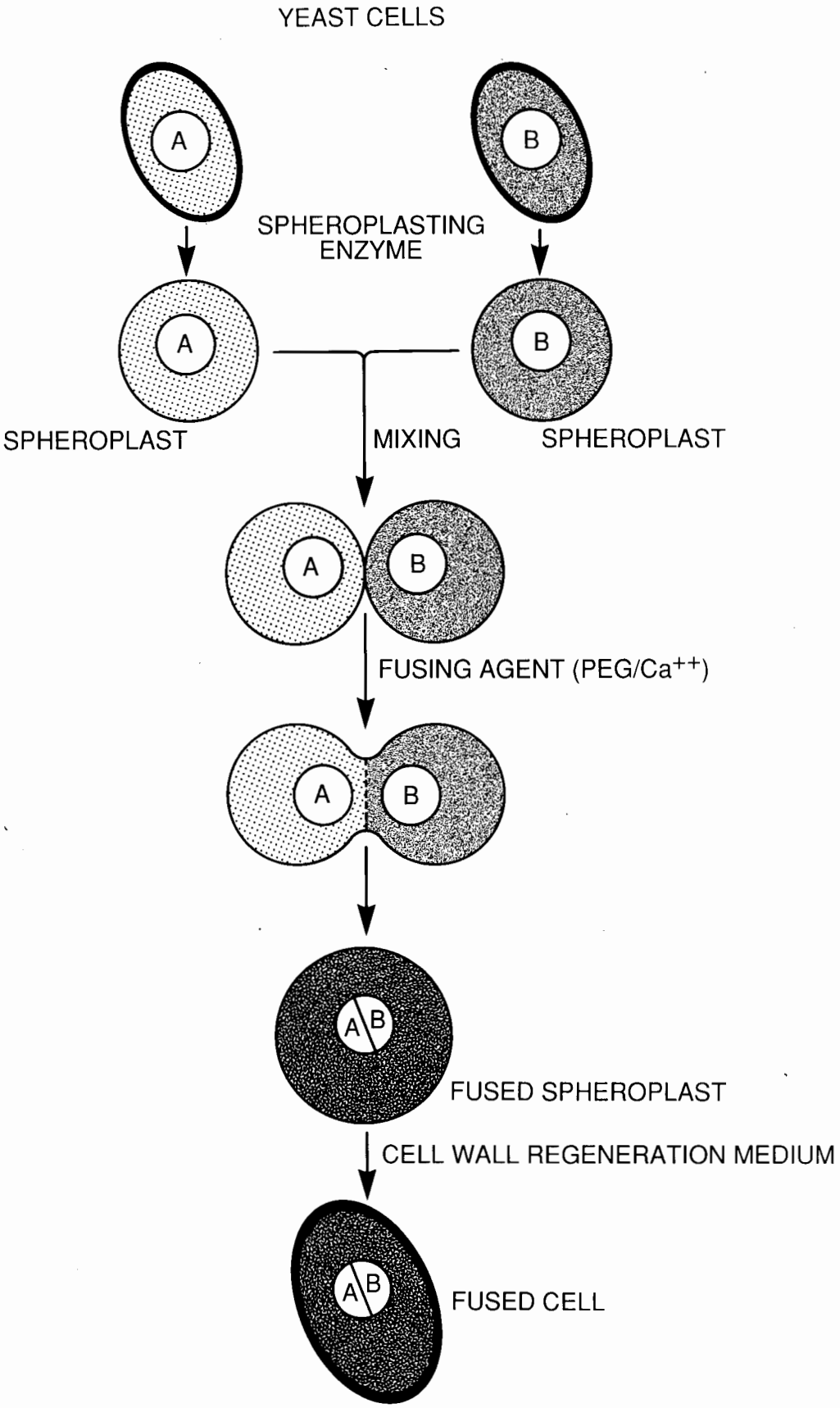


FIG. 8

Spheroplast fusion between two different yeast cell is a direct asexual technique to produce either hybrids or cytoductants. Spheroplasts are formed by removal of the cell wall with an appropriate lytic enzyme preparation such as Glusulase or Zymolase in an osmotic stabilised medium to prevent lysis. Spheroplasts from two different strains are mixed together in the presence of polyethylene glycol and calcium ions to fuse. The fused cells are allowed to regenerate their cell walls in an osmotically stabilised agar medium (Tubb & Hammond, 1987).



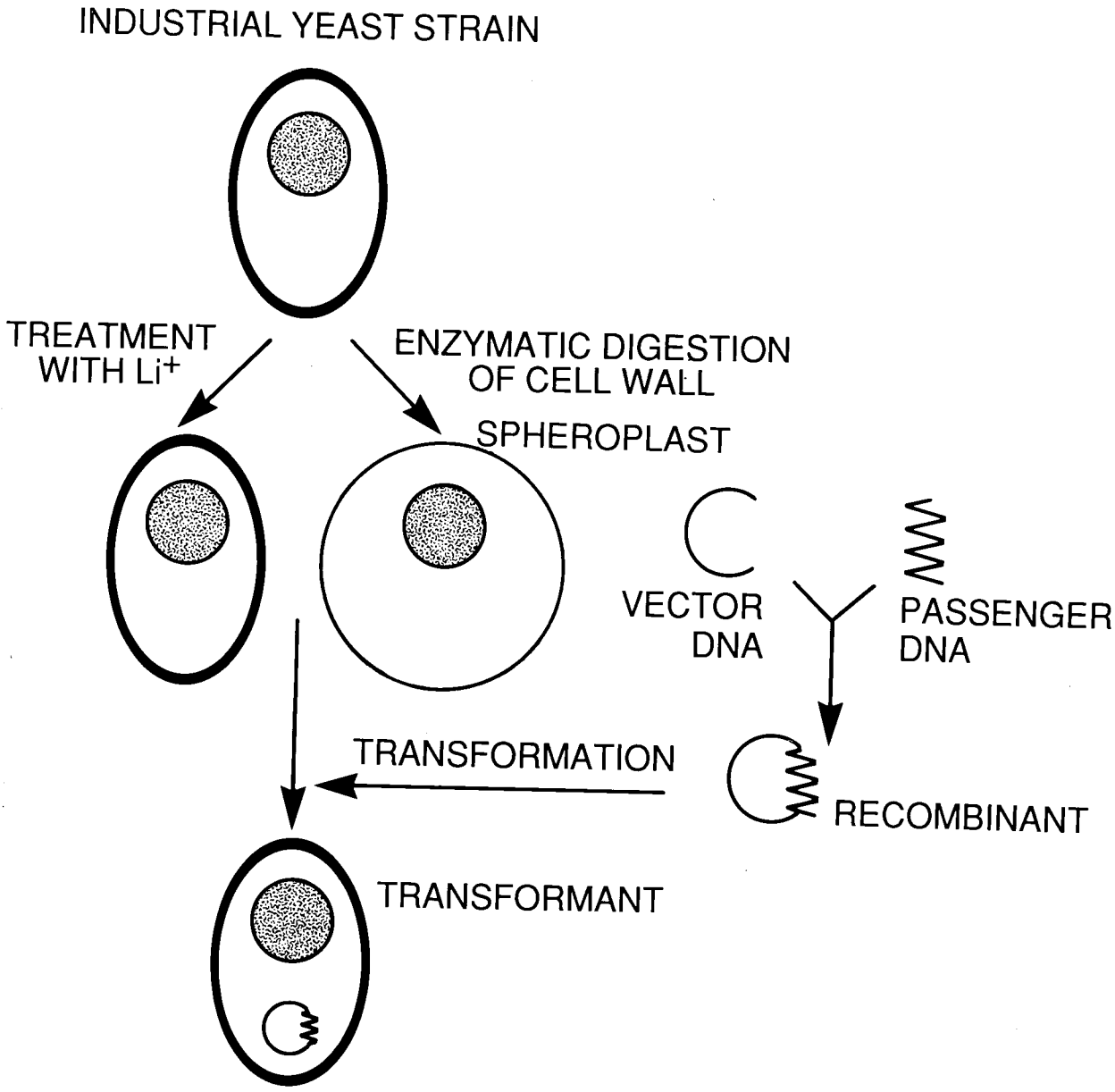


FIG. 9

Yeast transformation is used to introduce recombinant DNA molecules (e.g., possessing a useful gene) into yeasts. Yeast cells are made competent to take up naked DNA by enzymatic removal of the cell wall or by treatment with monovalent cations such as Li^+. The passenger DNA is annealled and ligated to the vector DNA to form recombinant DNA molecules. The recombinant DNA plasmids are introduced into competent yeast cells in the presence of PEG (Tubb & Hammond, 1987).

FIG. 10

Homologous recombination can be used to transfer mutations into and out of the normal locus on a yeast chromosome. (a) Integration of a cloned gene (*FRO1*) by homologous recombination into the mutant locus results in a heterogenic duplication. The same duplication can be produced by homologous recombination of a mutant plasmid into a normal locus. Depending on the position of the cross-over event, excision of the plasmid by homologous recombination from the duplication can result in either a mutant or a wild-type gene at the locus. If one digests the DNA containing the duplication with a suitable restriction endonuclease and ligates the fragments, one can obtain the mutation by selection for vector markers in *E. coli*. (b) A mutation on a yeast chromosome can be recovered by recombination repair after transformation with a suitable gapped plasmid carrying the wild-type gene. (c) Gene disruption can be accomplished by integration of a linear fragment containing an insertion or deletion containing a selectable marker. (d) Integrative gene disruption occurs when an integral fragment of a gene integrates by homologous recombination into the intact locus, splitting the gene into partially duplicated but incomplete parts, one missing the amino-terminal coding region and the other missing the carboxyl terminal (Malik, 1989).

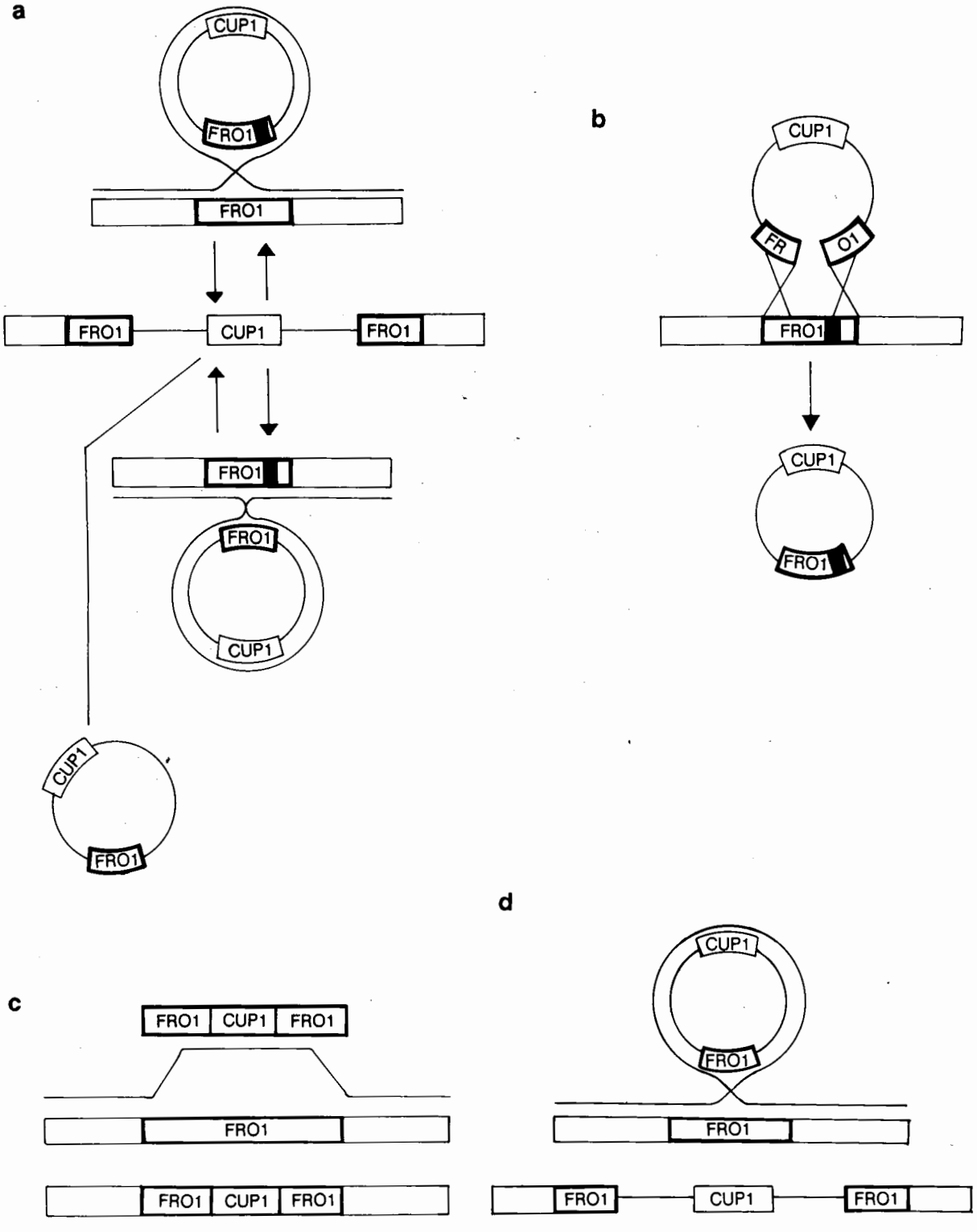


FIG. 11

Isolation of haploid strains from a homothallic yeast by spore-cell mating. Four ascospores from the same ascus are micromanipulated into direct contact with heterothallic haploid yeast cells. Mating takes place between compatible spores and cells. The resulting diploid is sporulated. Since two spores in each ascus are homothallic and two spores are heterothallic, stable haploids can be isolated from the sporulated diploids (Thornton, 1983).

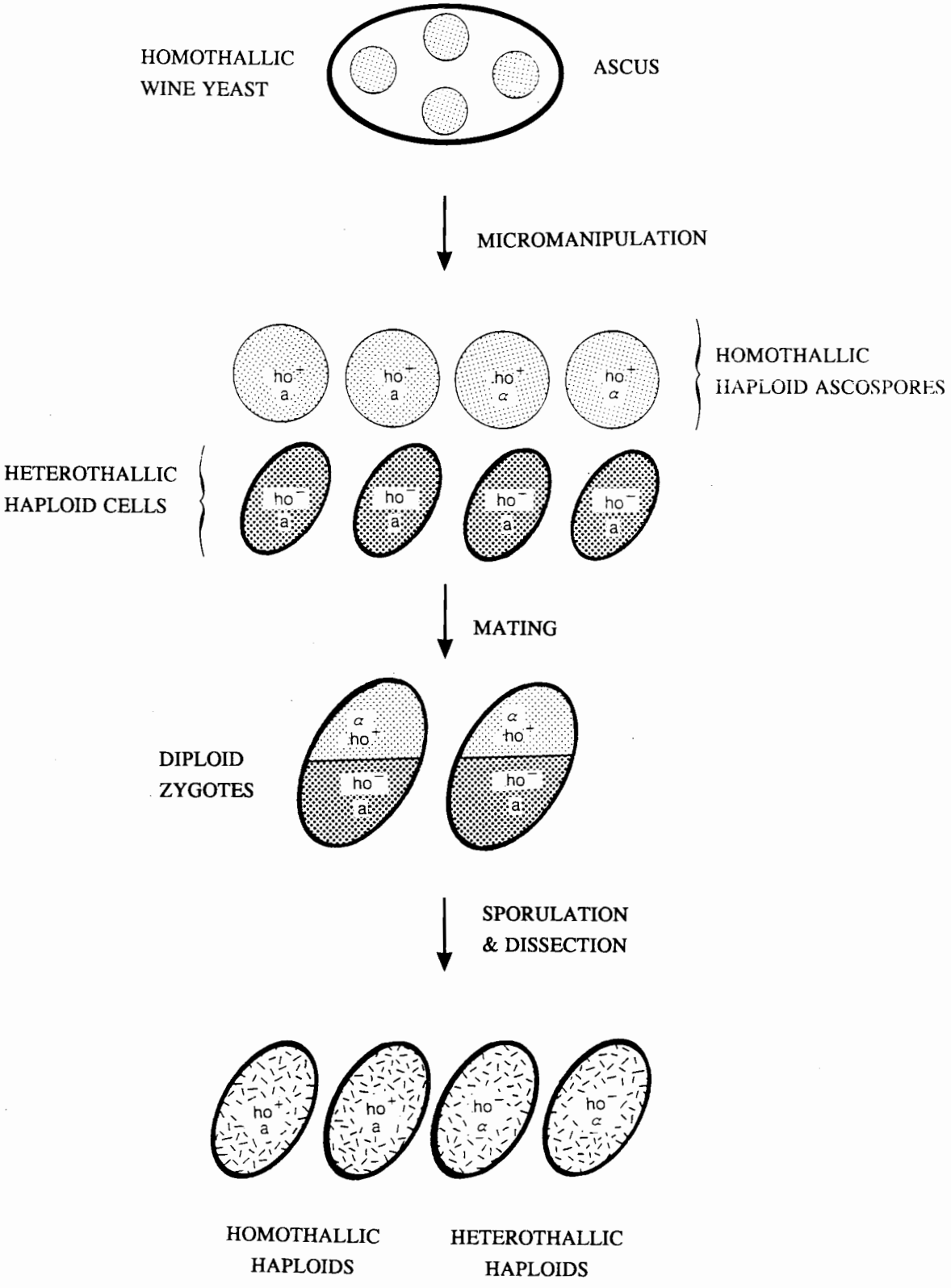


Table 1. Plasmid vector systems for gene cloning in yeast (Parent *et al.*, 1985).

Plasmid vector	Description and comments
YEp (yeast episomal plasmids)	These multi copy, autonomously replicating plasmids contain a region of the naturally occurring 2 *u*m plasmid responsible for its replicative properties. They transform yeast at high frequencies, exist as extrachromosomal elements in the cell and are often very unstable.
YRp (yeast replicating plasmids)	These multi copy, autonomously replicating plasmids contain an *ARS* element and transform yeast at high frequencies due to homologous sequences that presumably act as origins of replication in yeast. They are generally very unstable.
YIp (yeast integration plasmids)	These non-replicating plasmids contain yeast DNA and transform yeast at a low frequency by integration of plasmid DNA into the genome of the transformed cell by homologous recombination.
YCp (yeast centromeric plasmids)	These autonomously replicating plasmids contain a centromere (*CEN*). They are extrachromosomal but are unusually stable mitotically and meiotically, and are present at very low copy number (approximately one per cell).
YTp (yeast telomeric plasmids)	These autonomously replicating plasmids contain *TEL* sequences (telomeric sequences). They are extrachromosomal but stable at low copy number (one per cell).
YLp (yeast linear plasmids)	Linear plasmids contain homologous or heterologous sequences that functions as telomeres in yeast and may also contain centromeric sequences. The ends of these plasmids acquire additional sequences $(C_{1\text{-}3}A)_n$ upon replication in yeast. Some contain functional centromeres and behave as eucaryotic minichromosomes and are termed yeast artificial chromosomes (YAC).
YXp (yeast expression plasmids)	Expression vectors contain a transcriptional promoter, and in many instances, transcriptional terminator sequences, to which homologous or heterologous gene sequences may be fused for expression in yeast. Some of these plasmids possess coding sequences that direct post translational processing and protein secretion.
YPp (yeast promoter plasmids)	These plasmids possess an easily assayed protein coding sequence to which promoter containing transcriptional and/or translational signals can be fused, enabling investigation of promoter structure and function.
YHp (yeast hybrid plasmids)	These are complex vectors, usually consisting of hybrid gene sequences. They provide interesting models for studying particular aspects of genotypic expression in yeast (i.e., nuclear or extracellular protein localisation, RNA processing, etc.).

3. THE VALUE OF ELECTROPHORETIC FINGERPRINTING AND KARYOTYPING IN WINE YEAST BREEDING PROGRAMMES

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The value of electrophoretic fingerprinting and karyotyping in wine yeast breeding programmes

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Key words: wine yeasts, fingerprinting, CHEF, karyotyping, spore-cell mating

Abstract

Electrophoretic banding patterns of total soluble cell proteins, DNA restriction fragments and chromosomal DNA were used to characterise ten strains of *Saccharomyces cerevisiae* used for commercial production of wine. These fingerprinting procedures provided unique profiles for all the different yeast strains and can therefore be used to identify and control industrial strains. Furthermore, the protein profiles, restriction fragment banding patterns and electrophoretic karyotyping by contour clamped homogeneous electric field electrophoresis (CHEF), were valuable to differentiate hybrid and parental strains in yeast breeding programmes. Hybrid strains, with desirable oenological properties, were obtained by mass spore-cell mating between a heterothallic killer yeast and two homothallic sensitive strains and all were shown to have unique DNA fingerprints and electrophoretic karyotypes.

Introduction

Unlike other yeast-based industries such as baking and brewing, the wine industry has not taken an active interest in yeast genetics and strain-development programmes (Thornton 1983). With traditional wine fermentation methods there was little need to manipulate the yeast strain. However, new trends in beverage markets demand the modification of traditional wine yeast strains and the development of more cost-effective winemaking practices. The fact that the requirements of the wine industry have not been defined in genetic terms, has impeded the identification of realistic targets for strain development. Furthermore, genetic programming of homothallic wine yeasts by inter-strain hybridisation was problematical. This obstacle was, however, overcome by breeding techniques like

spheroplast fusion, rare mating and mass spore-cell mating (for a review see Van der Westhuizen & Pretorius 1990b). The successful application of these genetic techniques in strain development depends on the ability to differentiate between parental and hybrid strains.

Yeast cultures used in the alcoholic beverage industries are usually characterised by cell and colony morphology, physiological tests and the ability to flocculate or to form a pellicle (Kunkee & Amerine 1970). However, these techniques are not universally adept at differentiating between strains of the same species. Furthermore, it is apparent that many of the physiological and biochemical characteristics used for identification are encoded by a small portion of the genome. This resulted in the fingerprinting of industrial yeast strains by protein profiles (Van der Westhuizen & Pretorius 1989, 1990a; Van Vuuren & Van der Meer 1987), restriction fragment length polymorphisms of genomic or mitochondrial DNA (Keiding 1985; Lee & Knudsen 1985; Panchal et al. 1987; Pedersen 1985b, 1986a), electrophoretic karyotyping (chromosomal banding patterns) (Casey & Pringle 1990; Petering et al. 1990; Van der Westhuizen & Pretorius 1989, 1990a; Vezinhet et al. 1990) and gas-liquid chromotographic analysis of the cellular long-chain fatty acids (Augustyn & Kock 1989; Tredoux et al. 1987).

The present report describes the characterisation of ten wine yeast strains by visual comparison of total soluble cell protein patterns, restriction fragment banding patterns and electrophoretic karyotyping. We also describe the hybridisation of a heterothallic, killer yeast with two homothallic, sensitive strains by mass spore-cell mating. This report highlights the value of electrophoretic fingerprinting and karyotyping in breeding programmes.

Materials and methods

Yeast strains and genetic methods

The following strains of *Saccharomyces cerevisiae*, obtained from the Viticulture and Oenological Research Institute (VORI; Stellenbosch, South Africa), were used in this study: N6 (WE14), N21 (Geisenheim), N66 (WE372), N76 (228), N91 (WE466), N93 (WE500), N95, N96, N97 and N181 (VIN7). Strain N96 was previously classified as *Saccharomyces bayanus* (Kreger-van Rij 1984). Strains N93 and N181 originated from the same culture. Standard yeast genetic methods of sporulation, purifying and selecting haploids were carried out according to Sherman et al. (1986). Hybridisation between haploid cells isolated from heterothallic strains, and ascospores isolated from homothallic strains, was performed according to the mass

spore-cell mating method described by Salmon et al. (1989).

Media and screening procedures

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. Galactose utilising strains 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). 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.

Isolation and electrophoresis of proteins

Preparation of cell-free extracts and isolation of total soluble cell proteins from yeasts were carried out according to the methods described by Van Vuuren & Van der Meer (1987). Protein extracts were stored at -18°C. The protein concentration was determined by the Folin-Lowry method (Plummer 1971) and samples were adjusted to a concentration of 2 mg/ml with 6.4 mM Tris (hydroxymethyl) aminomethane buffer (pH 8.4). Slab gel electrophoresis (SE 600 Cooled Vertical Slab Unit; Hoefer Scientific Instruments, San Francisco, USA) was used to obtain protein profiles. The lower electrode buffer [63 mM Tris (hydroxymethyl) aminomethane, 50 mM HCl, pH 7.5] was kept at 8°C. The upper electrode buffer contained 37.7 mM Tris (hydroxymethyl) aminomethane and 40 mM glycine (pH 8.9). The gel was 1.5 mm thick and consisted of a 5% (w/v) acrylamide stacking gel and a 7% (w/v) acrylamide resolving gel. Samples of 50 *u*l were loaded into the wells. Bromophenol blue (0.1%, w/v) in 50% (w/v) sucrose served as loading buffer. Electrophoresis was performed at a constant current of 35 mA for 5 h. The gels were fixed with 12.5% (v/v) trichloric acid, stained with 0.25% (w/v) Coomassie blue R-250 and destained with a 7% (v/v) acetic acid-5% (v/v) methanol solution.

Isolation of genomic DNA and electrophoresis of restriction fragments

Genomic DNA was isolated from the parental and hybrid strains according to a method reported by Gupta & Jones (1987). The DNA was digested with the *Hae*III restriction endonuclease according to the specifications of the supplier (Boehringer Mannheim Biochemicals, Mannheim, FRG). Electrophoresis of DNA samples was

performed in a 0.8% (w/v) agarose gel in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) at 100 V for 2 h. Gels were stained with ethidium bromide (10 mg/ml) and viewed on a transilluminator.

Preparation of intact chromosomal DNA and pulsed field gel electrophoresis

Chromosomal DNA samples were prepared according to the embedded-agarose procedure of Carle & Olson (1985). Intact chromosomal DNAs were separated using contour clamped homogeneous electric field (CHEF) electrophoresis. The apparatus used was the CHEF-DR II (Bio-Rad Laboratories, Richmond, USA). All CHEF separations were carried out in a 20 cm square, 6 mm deep, 1.2% agarose gel made in 0.5 x TBE buffer. Thin sections of the DNA-agarose plugs were loaded into the wells and sealed in with 1% low melting temperature agarose just prior to the run. The average running temperature of the 0.5 x TBE electrophoresis buffer was maintained at 14°C by a recirculating water bath set at 4°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) and viewed on a transilluminator.

Results

Fingerprinting of wine yeast strains

The electrophoretic banding patterns of total soluble cell proteins (Fig. 1), DNA restriction fragments (Fig. 2) and chromosomal DNA (Fig. 3) were used to characterise ten strains of *S. cerevisiae* used for commercial production of wine. Variation in the profiles of strains N6, N21, N66, N76, N95 and N97 were apparent in the number, position and intensity of the bands. Strains N93 and N181 originated from the same culture and, as expected, displayed similar characteristic protein profiles, DNA restriction fragment and chromosomal banding patterns. Similar protein and DNA profiles were obtained for strains N93' (a strain once thought to be synonomous with strain N93) and N95, supporting the hypothesis that they originated from the same culture. Identical profiles were also obtained for killer strain N96 and strain N91. Strain N91 is a derivative of strain N96, cured of the K_2 killer character. Although, similar protein profiles were obtained for strains N6 and N76, their DNA restriction fragment and chromosomal banding patterns were different.

Hybridisation of wine yeast strains

The aim of this breeding programme (Fig. 4) was to obtain hybrids that contain a combination of the oenological characteristics of strains N96 and N181, and of strains N76 and N96, respectively. Strain N96 is a K_2 killer (Kil^+) and is unable to utilize galactose as carbon source (Gal^-), whereas strains N76 and N181 are sensitive for the K_2 killer toxin (Kil^-) and capable of galactose assimilation (Gal^+). A haploid was isolated from the heterothallic strain N96 and was designated $N96^H$. No stable haploids could be isolated from the homothallic strains N76 and N181. Ascospores of strains N76 and N181 were therefore mixed with cells of haploid strains $N96^H$ using the mass spore-cell mating procedure. This resulted in the formation of diploids, albeit at low frequency. The diploids were isolated by screening for both killer activity and galactose utilisation (Figs. 5 & 6). One hybrid strain, USM30, resulted from the genetic cross between strains $N96^H$ and N181. Three hybrids, USM21, USM22 and USM23, were obtained from the mass spore-cell mating between strains N76 and $N96^H$. The hybrids (Kil^+ Gal^+) were compared to their parental strains (Kil^+ Gal^- and Kil^+ Gal^+) by using protein profiles (Fig. 7), DNA restriction banding patterns (Fig. 8) and electrophoretic karyotypes (Fig. 9).

Discussion

Traditional methods for distinguishing wine yeast strains have depended on morphological, physiological and biochemical criteria (Kunkee & Amerine 1970). These taxonomic procedures allow for distinction between species, but are time consuming and not always reliable. New approaches attempt to identify yeast strains by an analysis of their protein and DNA content (Meaden 1990).

Numerical analysis of total soluble cell proteins has been used to fingerprint and group wine yeasts (Van Vuuren & Van der Meer 1987) and brewing yeasts (Van Vuuren & Van der Meer 1988). Since a number of reputedly genetically unique yeast strains are being sold commercially, it has become necessary to fingerprint individual yeast strains used in wine fermentations. Van Vuuren & Van der Meer (1987) concluded that visual comparison of total soluble cell protein patterns can be used to fulfil this need in the wine industry. Our results confirmed this statement. Unique protein profiles were obtained for strains N6, N21, N66, N76, N95 and N97 (Fig. 1). Furthermore, we found that protein profiles could also be used to differentiate hybrid and parental strains in a breeding programme. The protein profiles of hybrid USM30 and its parental strains, $N96^H$ and N181, were similar (Fig. 7). This was also evident in the cross between strains N76 and $N96^H$. The protein

banding patterns of hybrids USM21, USM22 and USM23 were similar and contained a combination of the prominent unique bands present in the profiles of parental strains N76 and $N96^{H}$ (Fig. 7).

Direct analysis of restriction fragments of mitochondrial and genomic DNA, using a number of restriction endonucleases, has been applied to differentiate brewing strains, but with mixed success. Aigle et al. (1984) found that restriction fragment banding patterns obtained from the mitochondrial DNA (mtDNA) of different lager strains were identical. Martens et al. (1985) could distinguish between the mtDNA of two ale strains, using double digestion with *Hind*II and *Hind*III. Lee & Knudsen (1985) reported slight (but nevertheless discrete) differences in the pattern of *Ava*I or *Hae*II mtDNA restriction fragments of two lager strains. The preparation of genomic DNA is much more rapid and technically less demanding than the isolation of mtDNA. However, the interpretation of electrophoretic banding patterns of genomic DNA restriction fragments is complicated because discrete fragments are generally not apparent unless they are derived from repeated sequences such as ribosomal DNA (Meaden 1990). Pedersen (1985b) found that the *Eco*RI digested fragments of genomic DNA isolated from 22 Bavarian lager and ale strains generated identical electrophoretic banding patterns. Minor differences in the electrophoretic banding patterns of *Hpa*I digested DNA restriction fragments of ale and lager strains were reported by Panchal et al. (1987). From these results Meaden (1990) concluded that direct analysis of DNA restriction fragments was limited in the information it can provide and that it was therefore unlikely to be a useful method for fingerprinting large numbers of different brewing strains. By contrast our results indicated that direct analysis of DNA restriction fragments was a valuable tool to fingerprint wine yeast strains and to differentiate hybrid and parental strains in a breeding programme. The electrophoretic banding patterns of *Hae*III digested DNA restriction fragments of strains N6, N21, N66, N76, N95 and N97 were unique (Fig. 2). The DNA restriction banding patterns of hybrid USM30 and its parental strains, $N96^{H}$ and N181, were different (Fig. 8). The DNA restriction fragment profiles of hybrids USM21, USM22 and USM23 contained only slight variations, whereas their profiles were quite different from those of their parental strains, N76 and $N96^{H}$. In fact, this rapid fingerprinting method was found to be efficient, rendering analysis of specific DNA restriction fragments by probing unnecessary. However, a substantial amount of DNA fingerprinting has been attempted using labelled DNA probes, including genes encoding rRNA (*RDN1*), enzymes of the pyrimidine (*URA3*) and amino acid synthetic (e.g., *HIS4*, *LEU2*, *TRP1*) and glycolytic pathways (*PDC1*, *PFK1*, *PFK2*, *PGI1*, *PGM1*, *PGK1*, *PYK1*) as well as transposable elements (*Ty*1) (Braus et al. 1985; Decock & Iserentant 1985; Keiding 1985; Laaser et al. 1989; Martens et al. 1985; Pedersen 1983a,b; Pedersen

1985a,b; Pedersen 1986a,b; Sakai et al. 1990; Seehaus et al. 1985) Probing for specific DNA fragments, however, has not taken full advantage of the wide range of cloned yeast genes that are available. Meaden (1990) concluded that any attempt to fingerprint yeast strains by DNA probing was best approached using a variety of probes and restriction endonucleases, until a combination that suits the investigator's needs was found. It can also be expected that molecular marking by integrating unique DNA oligonucleotides into the genomes of wine yeasts will eventually enable "designer" fingerprinting. Gene amplification by the polymerase chain reaction (PCR) with subsequent sequencing, can also be anticipated to filter through to fingerprinting of wine yeast strains and genetic hybrids.

Since the first karyotyping of yeasts by pulsed field gradient electrophoresis (PFGE) (Schwartz & Cantor 1984) and orthogonal field alteration gel electrophoresis (OFAGE) (Carle & Olson 1985) researchers have applied pulsed field electrophoresis to fingerprint a diverse range of yeast genera and species (Casey et al. 1988; De Jonge et al. 1986; Johnston & Mortimer 1986; Johnston et al. 1988; Sor & Fukuhara 1989; Takata et al. 1989). However, rather little has been published on the use of electrophoretic karyotyping to specifically differentiate wine yeast strains. Petering et al. (1988) differentiated several wine yeast strains by transverse alternating field electrophoresis (TAFE). Vezinhet et al. (1990) have reported 20 different TAFE karyotypes for 22 wine yeast strains. Only three strains originating from the same vinyard could not be differentiated by TAFE karyotyping. Our results showed eight different CHEF karyotypes for ten wine yeast strains (Fig. 3). Two strains (N96 and N181) originated from the same culture and strain N91 is a derivative from N96, cured of the K_2 killer MdsRNA. We have also shown that CHEF karyotyping was valuable in the analysis of genetic hybrids in breeding programmes (Fig. 9). The electrophoretic karyotype of hybrid USM30 differed from those of its parental strains, $N96^H$ and N181. The chromosomal banding patterns of hybrids USM21, USM22 and USM23 were identical but differed from those of their parental strains, N76 and $N96^H$. Casey & Pringle (1990) reported that chromosome profiles could also be used in selecting variants with enhanced fermentation performance. In addition, gels with chromosomal banding patterns can also be blotted onto filters and probed with specific DNA fragments to differentiate between various yeasts (Hansen et al 1990; Pretorius & Marmur 1988; Takata et al. 1989). In this study, CHEF karyotyping without chromoblotting was sufficient to differentiate between the parental and hybrid strains.

In conclusion, results obtained by electrophoretic protein and DNA fingerprinting and karyotyping corresponded well, indicating that these techniques are valuable in the identification and control of industrial wine yeasts. Furthermore, these techniques enabled us to select four hybrid strains after mass spore-cell mating

of wine yeasts. These hybrids fermented efficiently and produced wines with desirable oenological characteristics (Van Wyk & Pretorius 1990).

Acknowledgements

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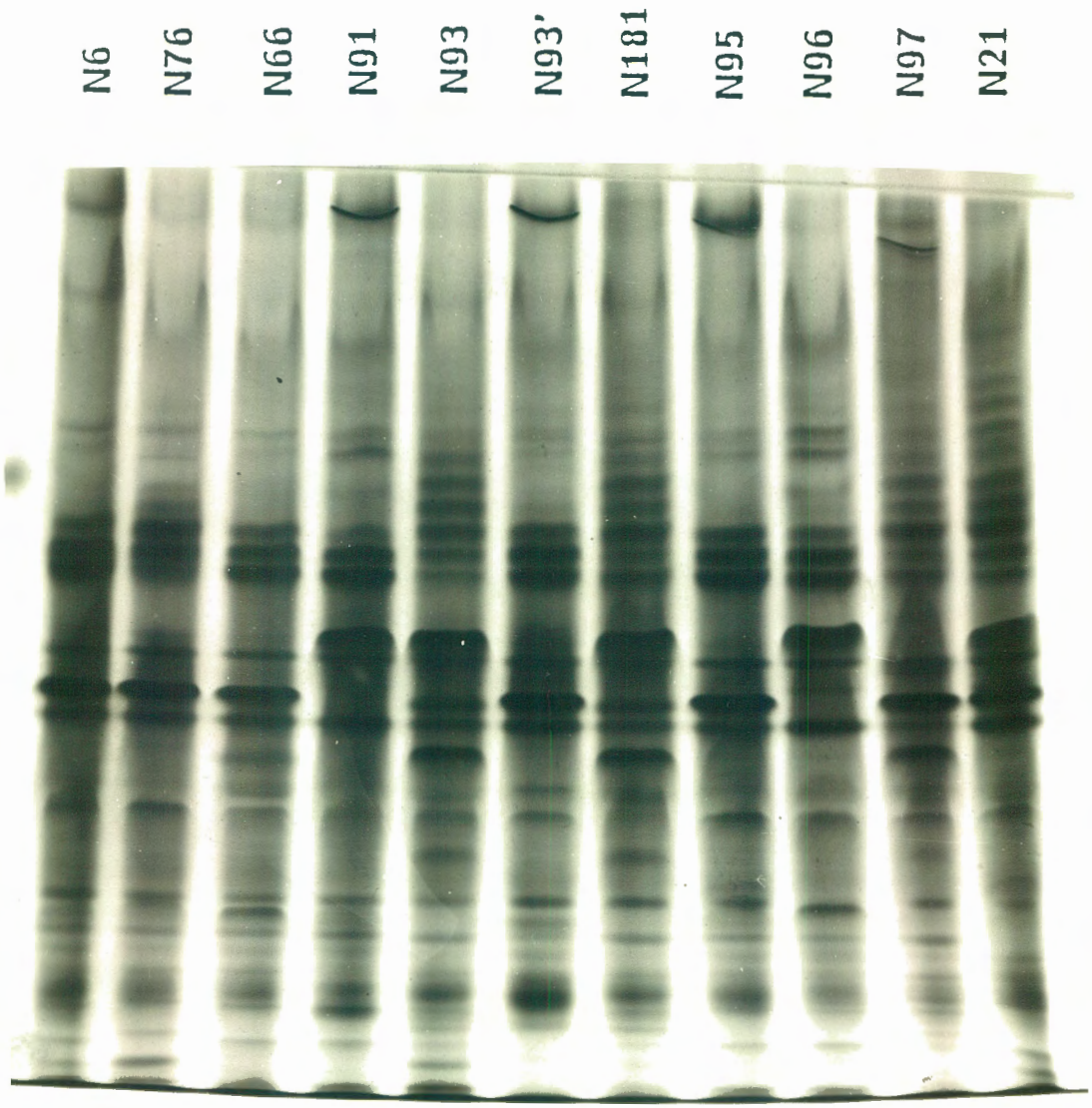


Fig. 1. Total soluble cell protein patterns of wine yeasts strains N6, N76, N66, N91, N93, N93', N181, N95, N96, N97 and N21. Strains N93 and N181 originated from the same culture. Strain N91, a derivative of N96, is cured of the killer character. Strain N93' was previously mistakingly distributed as N93, but was also later shown to be N95. Electrophoresis was performed in a 5% (w/v) acrylamide stacking gel and a 7% (w/v) acrylamide resolving gel.

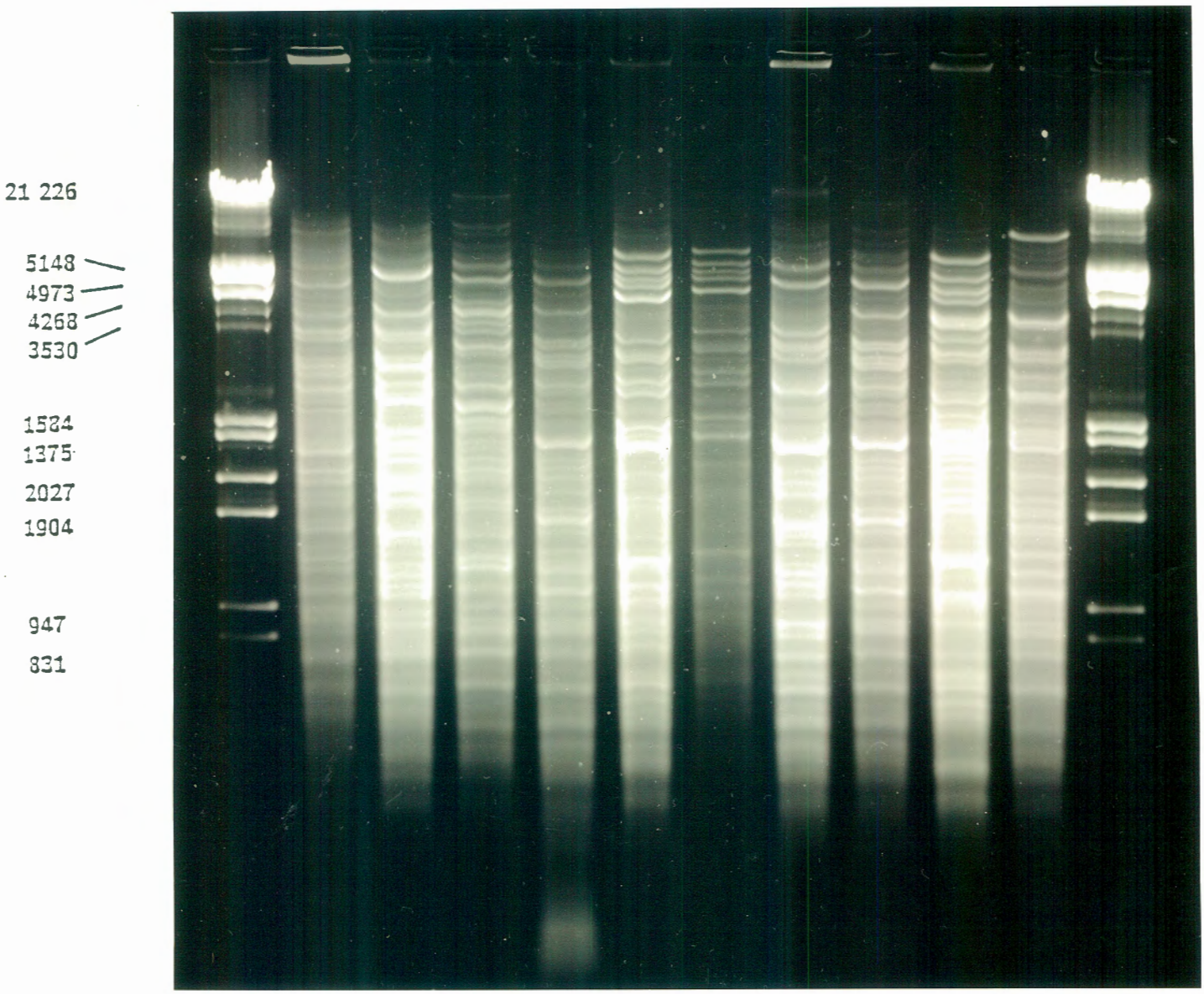


Fig. 2. DNA restriction fragment banding patterns of wine yeast strains N6, N76, N66, N91, N93, N181, N95, N96, N97 and N21. Total genomic DNA of these strains was cleaved with *Hae*III and separated in a 0.8% agarose gel, stained with ethidium bromide.

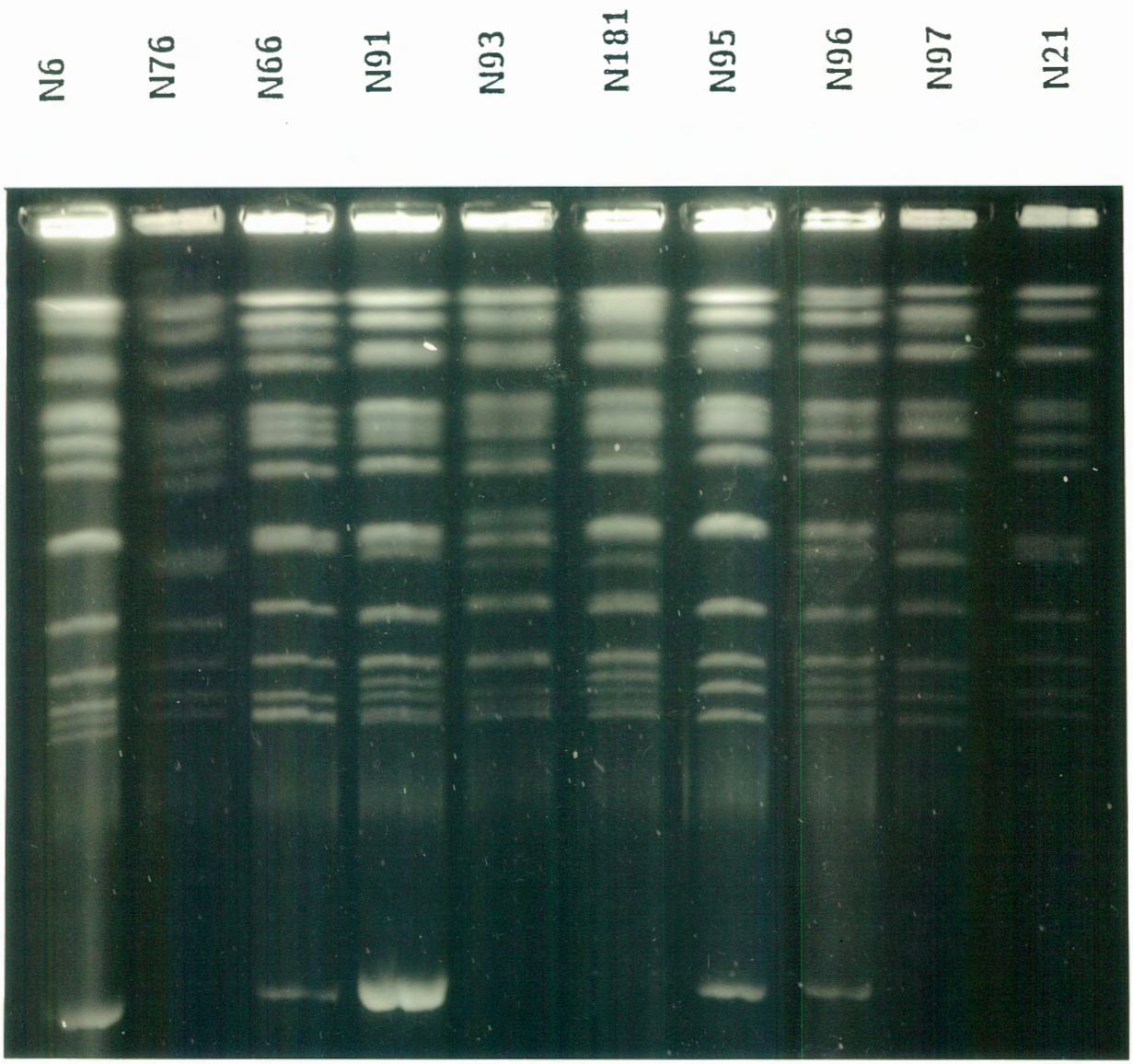


Fig. 3. Contour clamped homogeneous electric field (CHEF) banding patterns of chromosomal DNA of wine yeast strains N6, N76, N66, N91, N93, N181, N95, N96, N97 and N21. Intact chromosomal DNAs were separated in a 1.2% agarose gel stained with ethidium bromide.

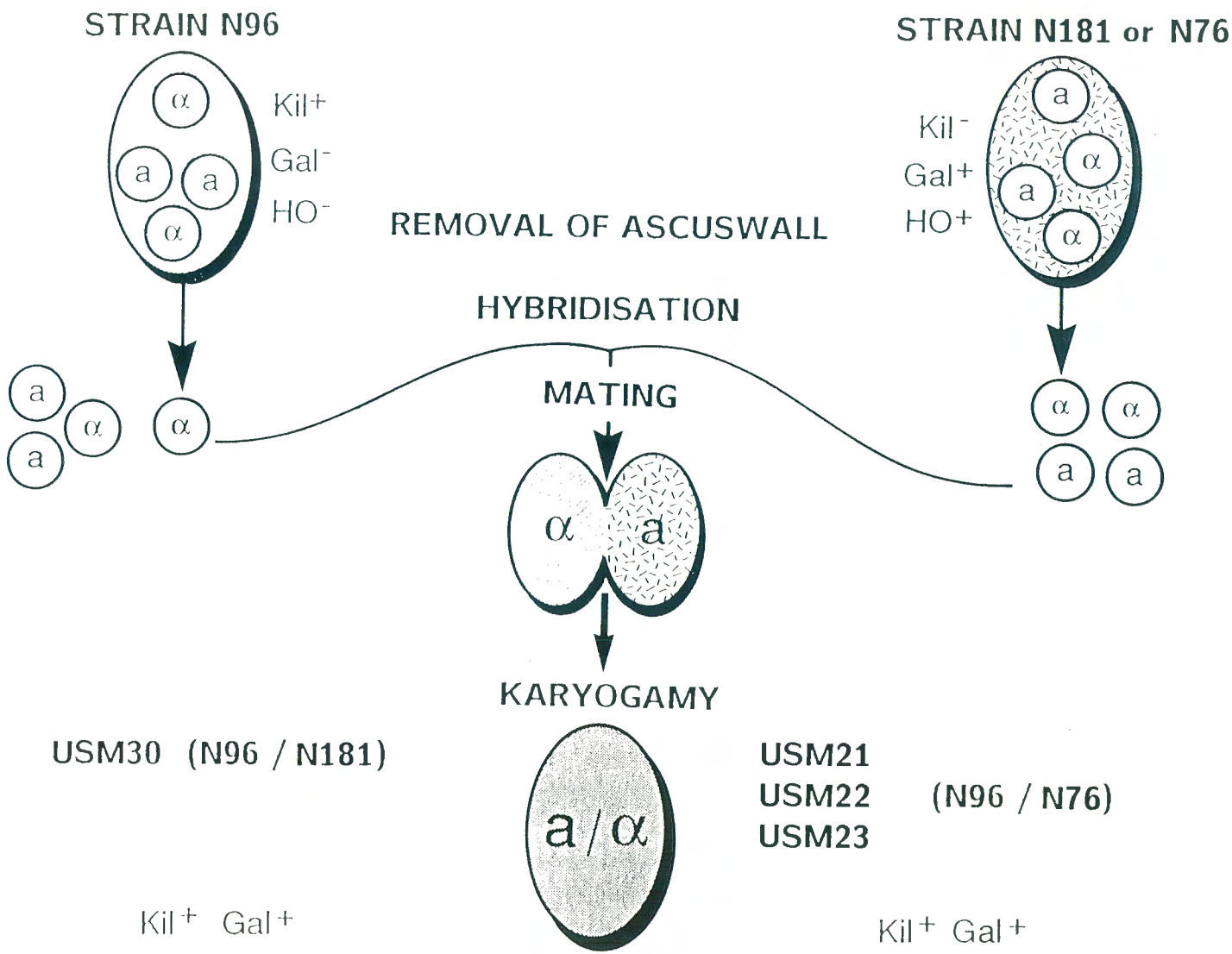
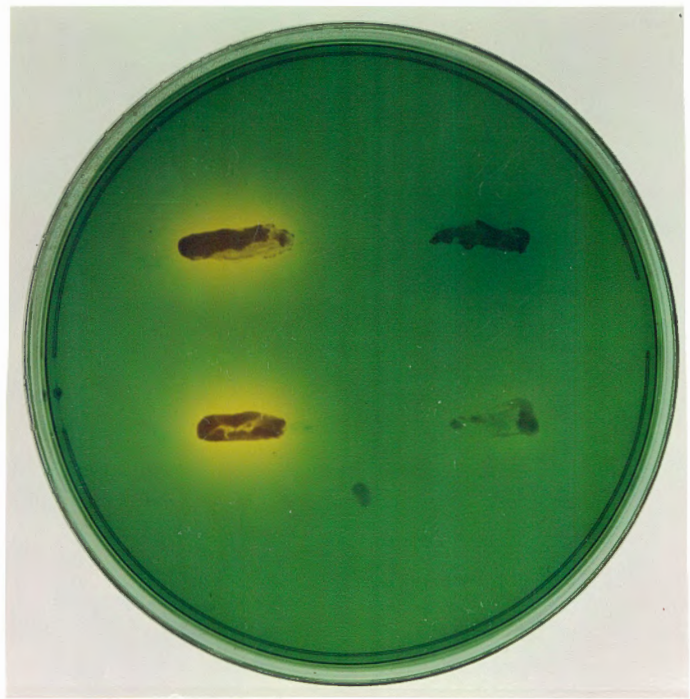


Fig. 4. The breeding strategy scheme. A haploid, $N96^{H}$, isolated from a heterothallic strain, N96, was hybridised with two homothallic strains, N76 and N181, by mass spore-cell mating. Strain N96 is a killer yeast (Kil^{+}) and is unable to utilise galactose as carbon source (Gal^{-}), whereas strains N76 and N181 are sensitive for the K_2 killer toxin (Kil^{-}) and capable of galactose assimilation. Hybrid USM30 (Kil^{+} Gal^{+}) was obtained from the genetic cross between $N96^{H}$ and N181, and three hybrid strains USM21, USM22 and USM23 (Kil^{+} Gal^{+}) were obtained from the cross between N76 and N96.



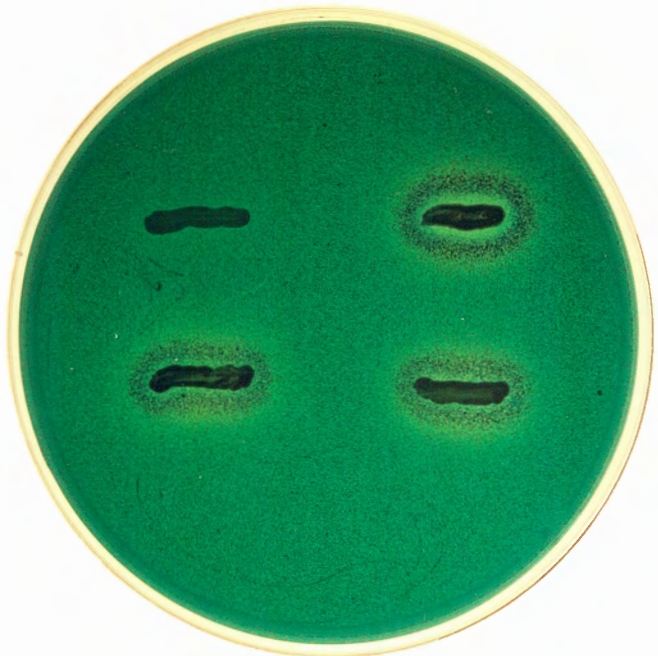


Fig. 5. (A) The formation of yellow zones by strains N181 and USM30 on YPGB agar plates, indicating galactose assimilation. Strain $N96^{H}$ is unable to utilise galactose as carbon source. (B) Killer strains $N96^{H}$ and USM30 streaked out onto methylene blue agar plates previously spread with sensitive strain N21. Killer activity is exhibited by a clear zone of inhibition and a faint halo of dark blue cells. Strain N181 shows no zone of inhibition.

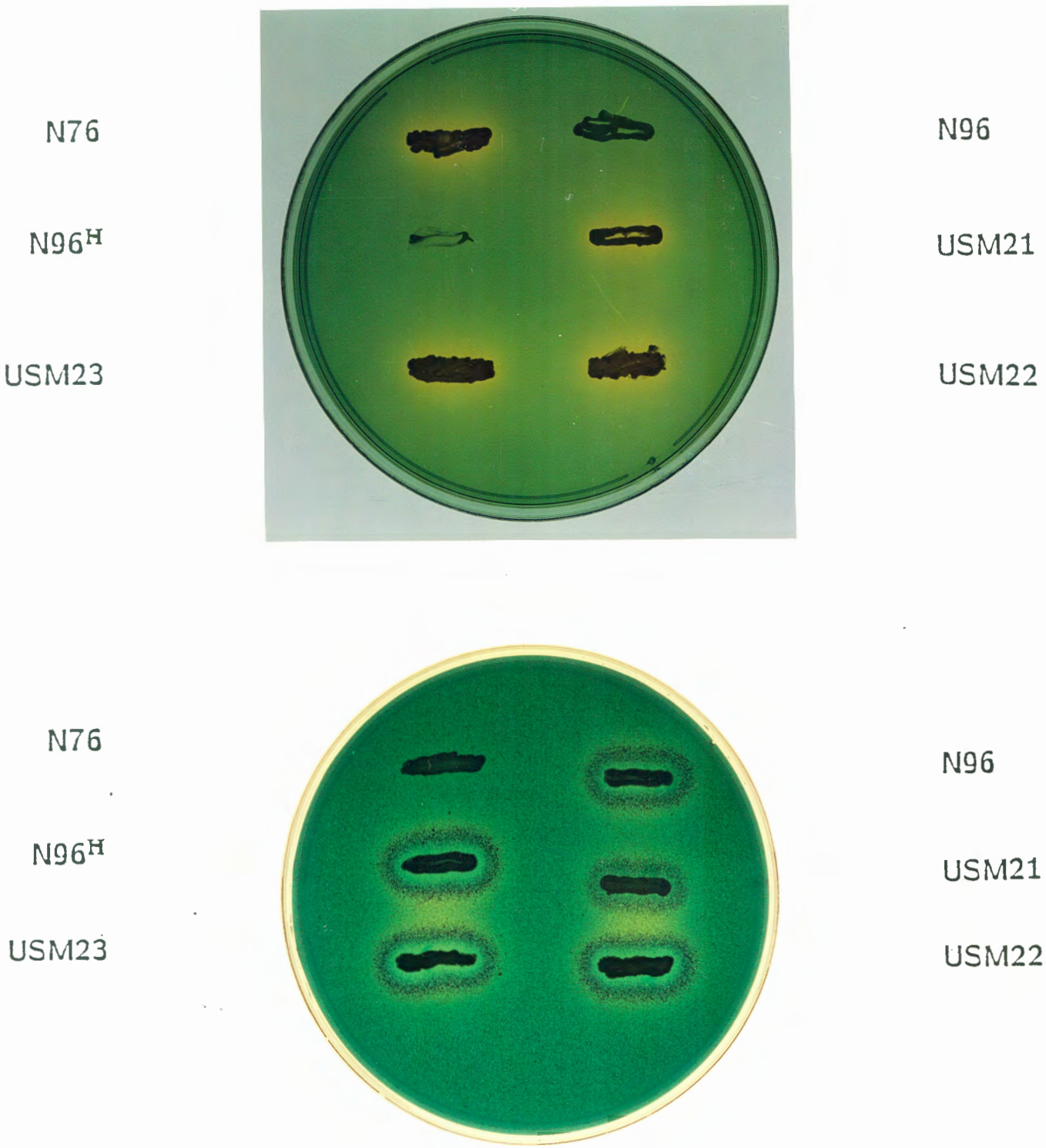


Fig. 6. (A) The formation of yellow zones by strains N76, USM21, USM22 and USM23 on YPGB agar plates, indicating galactose assimilation. Strain $N96^{H}$ is unable to utilise galactose as carbon source. (B) Killer strains $N96^{H}$, USM21, USM22 and USM23 streaked out onto methylene blue agar plates previously spread with sensitive strain N21. Killer activity is exhibited by a clear zone of inhibition and a faint halo of dark blue cells. Strain N76 shows no zone of inhibition.

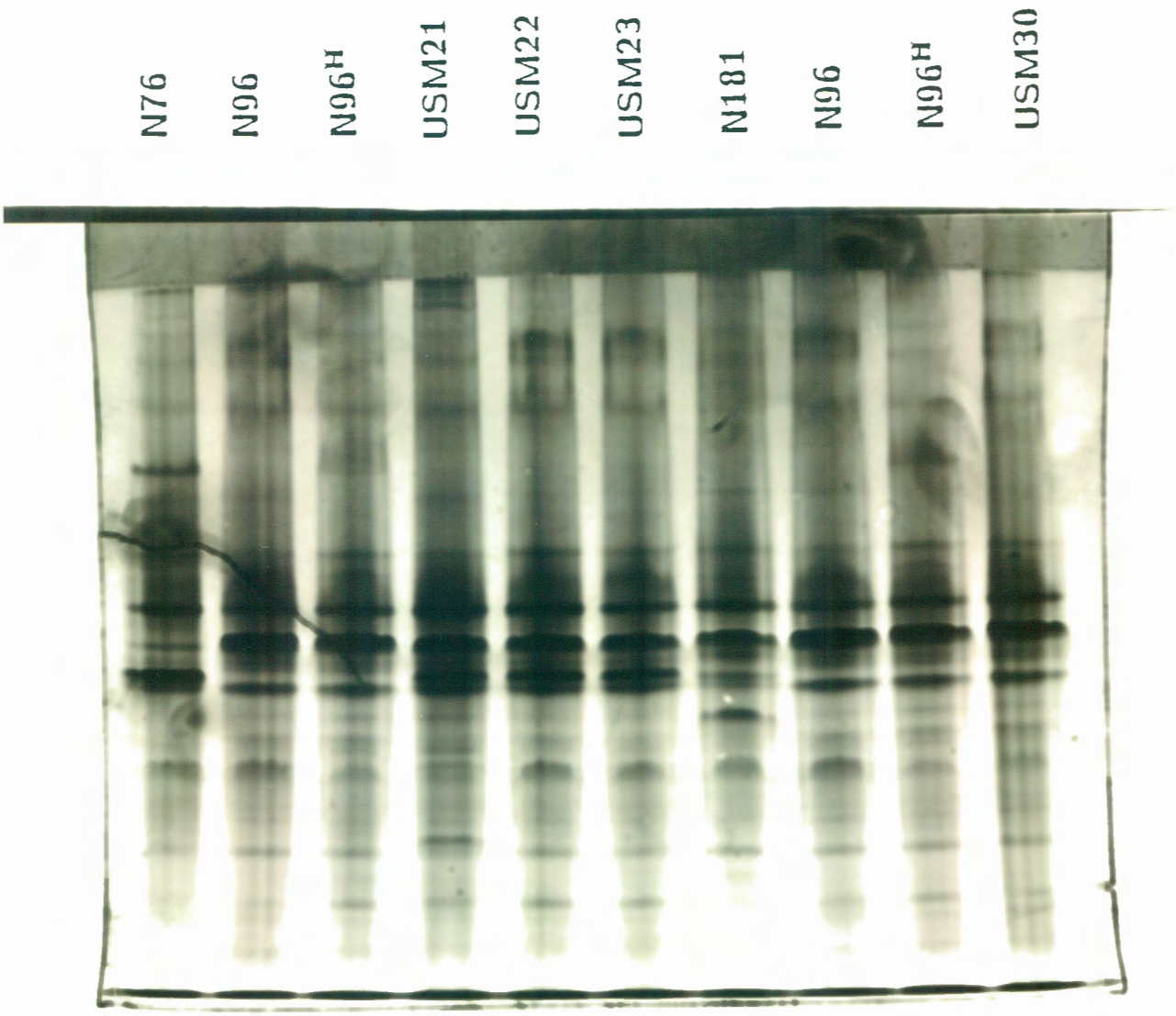


Fig. 7. Total soluble cell protein profiles of parental strains N76, $N96^{H}$ and N181 and hybrid strains USM21, USM22, USM23 and USM30. Electrophoresis was performed in a 5% (w/v) acrylamide stacking gel and a 7% (w/v) acrylamide resolving gel.

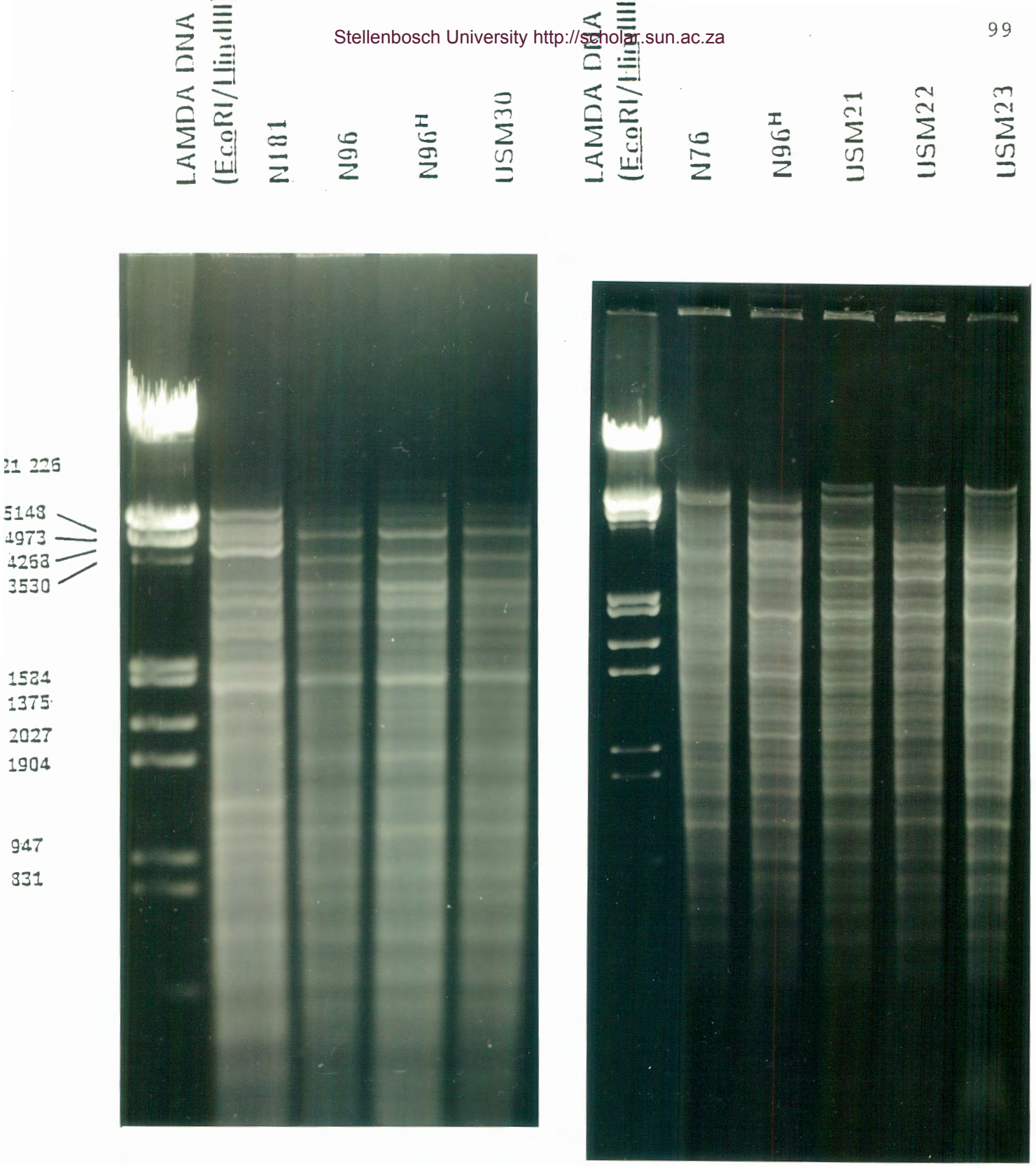


Fig. 8. DNA restriction fragment banding patterns of parental strains N76, $N96^{H}$ and N181 and hybrid strains USM21, USM22, USM23 and USM30. Total genomic DNA of these strains was cleaved with *Hae*III and separated in a 0.8% agarose gel, stained with ethidium bromide.

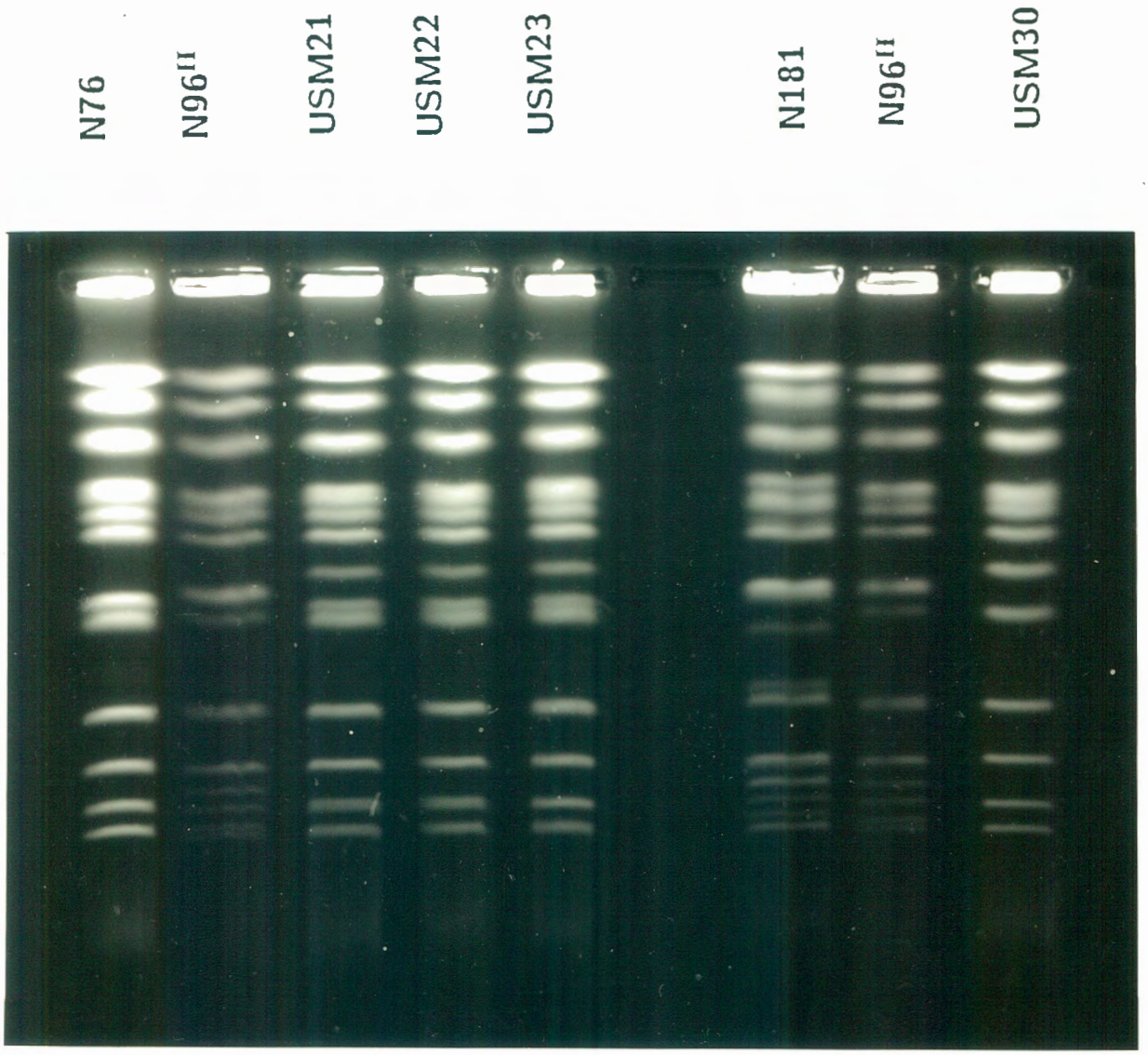


Fig. 9. Contour clamped homogeneous electric field (CHEF) banding patterns of chromosomal DNA of parental strains N76, $N96^{H}$ and N181 and hybrid strains USM21, USM22, USM23 and USM30. Intact chromosomal DNAs were separated in a 1.2% agarose gel stained with ethidium bromide.

4. GENERAL DISCUSSION AND CONCLUSIONS

The first chapter of this thesis emphasises the urgent need for quality control and strain development programmes in the South African wine industry. In this regard, specific aims and approaches of this study is outlined.

Chapter 2 summarizes the impact of yeast genetics and recombinant DNA technology on the wine industry. Previous studies directed towards wine improvement have concentrated mainly upon the improvement of grape varieties and their cultivation, and on fermentation and winemaking practices. Despite the fact that yeast has become one of the premier organisms for basic research and the ideal experimental model for unravelling molecular mechanism, the wine industry remains hesitant to take further advantage of the extensive biochemical and molecular information on the yeast cell. Unlike other yeast-based industries such as baking and brewing, the wine industry has not taken an active interest in the impressive arsenal of yeast genetic techniques and strain-development programmes (Snow, 1983; Thornton, 1983). However, new trends in the beverage markets demand genetic modification of traditional wine yeast strains. Therefore, this chapter highlights the importance of the wine yeast to the wine industry and the necessity for well-planned breeding programmes. The value of reliable taxonomic methods that are useful as diagnostic techniques in such breeding strategies is pointed out. The complexity of the genetic features of commercial wine yeast strains is discussed. The potential of genetic techniques, including clonal selection of variants, mutation and selection, hybridisation, rare-mating, spheroplast fusion and gene cloning and transformation, in strain development programmes is evaluated. Chapter 2 also attempts to stimulate interest in the genetic programming of wine yeasts by focussing on potential targets of strain development such as improved quality control, fermentation performance, enhanced contribution to wine bouquet and production of new products. Since genetic engineering technology has advanced to a point where it is now possible to routinely construct strains of commercial wine yeast carrying recombinant DNA, increasingly such genetically manipulated yeasts will be suggested for commercial application. It is therefore of the utmost importance that the wine industry is aware of the relevant requirements of beverage legislation. To remain competitive in the market place, the South African wine industry must take serious note that the impact of yeast genetic and recombinant DNA technology on the wine industry in general, promises to be impressive.

The third chapter focuses firstly on the availability of reliable techniques to differentiate parental and hybrid strains as a prerequisite for successful control of commercial strains and secondly on the application of genetic techniques in strain

development programmes. Traditional methods for distinguishing wine yeast strains have depended on morphological, physiological and biochemical criteria (Kunkee & Amerine 1970). These technique are time consuming, not always reliable and not universally adept at differentiating between strains of the same species. Furthermore, it is apparant that many criteria used for identification are derived from the analysis of a small portion of the genome. New approaches attempt to identify yeast strains by an analysis of their protein and DNA content.

Numerical analysis of total soluble cell proteins has been used to fingerprint and group wine yeasts (Van Vuuren & Van der Meer, 1987). These authors concluded that visual comparison of total soluble cell protein patterns can be used to fingerprint individual yeast strains. Van der Westhuizen & Pretorius (1989, 1990) have reported that, in addition to protein profiles, electrophoretic banding patterns of DNA restriction fragments and chromosomal DNA, could be used as rapid and reliable methods to fingerprint wine yeast strains. Since 1987 the South African wine industry asked the Department of Microbiology at the University of Stellenbosch to annually fingerprint by protein profiles all wine yeast strains commercially distributed by Anchor Yeast (Cape Town). In 1989 electrophoretic karyotyping was also cross-checked by gas-liquid chromatographic analysis of the cellular, long chain fatty acids (Tredoux *et al.*, 1987; Augustyn & Kock, 1989), as carried out by the Viticulture and Oenological Research Institiute (VORI, Stellenbosch). These quality control measures were responsible for the finding in 1988 that one batch of dried yeast labelled as strain N93 was in fact N95. Again in 1989, it was found prior to the 1990 pressing season that the first batch of dried yeast labelled as strain N93 was indeed strain N95. In future, molecular marking by integrating unique DNA oligonucleotides into the genomes of wine yeasts and concomitant "designer" fingerprinting may also become an integral part of this quality control programme. It can also be expected that gene amplification by the polymerase chain reaction (PCR) with subsequent sequencing, will be applied to fingerprint wine yeast strains.

In Chapter 3, the value of electrophoretic fingerprinting and karyotyping in wine yeast control and breeding programmes is demonstrated. The electrophoretic banding patterns of the total soluble cell proteins, DNA restriction fragments and chromosomal DNA were used to characterise ten strains of *S. cerevisiae*, used for commercial production of wine in South Africa. These techniques were also used to differentiate between parental and hybrid strains in a breeding programme. By way of mass spore-cell mating between strain $N96^{H}$ and strains N76 and N181, four killer hybrids, USM21, USM22, USM23 and USM30 were selected.

Unique protein profiles, electrophoretic DNA restriction fragment banding patterns and CHEF karyotypes were obtained for strains N6, N21, N66, N76, N95 and N97. Variation in these profiles were apparent in the number, position and

intensity of the bands. Strains N93 and N181 originated from the same culture and, as expected, displayed the same characteristic protein, DNA restriction fragment and chromosomal banding patterns. Identical profiles were also obtained for killer strain N96 and strain N91, confirming that strain N91 is a derivative of strain N96, cured of the K_2 killer character. The results obtained by these three fingerprinting techniques corresponded well, indicating that they are valuable in the identification and control of industrial wine yeasts.

The protein profiles of hybrid USM30 and its parental strains, $N96^H$ and N181, were similar, whereas their restriction fragment banding patterns and CHEF karyotypes showed discrete differences. The protein banding patterns of hybrids USM21, USM22 and USM23 were identical and contained a combination of prominent unique bands present in the profiles of parental strains N76 and $N96^H$. The DNA restriction fragment profiles of hybrids USM21, USM22 and USM23 contained only slight variations, whereas their profiles were quite different from those of their parental strains, N76 and $N96^H$. The CHEF karyotypes of hybrids USM21, USM22 and USM23 were identical but differed from those of their parental strains, N76 and $N96^H$. In conclusion, protein and DNA fingerprinting techniques were found to be valuable in selecting four hybrid killer strains after mass spore-cell mating.

The present study has practical implications for the South African wine industry. Apart from the current quality control programme that is based on the report of Van Vuuren & Van der Meer (1987) and this study, the present breeding programme has resulted in killer hybrid strains with desirable properties long sought after by the South African wine industry. Currently, the most popular wine yeast strains in South Africa are the killer strain N96 and the two sensitive strains N76 and N181. These three strains occupy more than 75% of the local wine yeast market. However, the killer activity of strain N96 prevented winemakers using strain N96 as a co-culture with either strain N76 or N181. The complementing characteristics of strains $N96^H$ and N76, and $N96^H$ and N181 were combined in hybrids USM21, USM22 and USM23, and USM30, respectively. These four hybrid killer strains and the three parental strains were subjected to independent fermentation studies on musts of different grape cultivars, by Prof. C. J. van Wyk, Deparment of Oenology, University of Stellenbosch and by Mr. C. J. Jacobs, Stellenbosch Farmers' Winery. Van Wyk & Pretorius (1990) reported that the hybrids fermented at the same rate as strain N96 and N181 but much faster than strain N76. Furthermore, a taste panel of 15 people indicated that the wines produced by these four killer hybrids did not compromise any of the desired oenological characteristics of those produced by the parental strains. The University of Stellenbosch has taken out provisional patents on all four hybrid strains. Two of these hybrids, USM21 (industrial designation VIN13)

designation VIN12) will be released during the forthcoming pressing season on an experimental basis, as dried preparations. Hybrids USM22 and USM23 will be released as wet preparations.

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