The Impact of Yeast Genetics and Recombinant DNA Technology on the Wine Industry - A Review

I.S. Pretorius and T.J. van der Westhuizen

Department of Microbiology and Institute for Biotechnology, University of Stellenbosch, 7600 Stellenbosch, Republic of South Africa

Submitted for publication: September 1990
Accepted for publication: February 1991
Key words: Recombinant DNA, genetic manipulation, wine yeasts, yeast breeding, fingerprinting

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. In this review we highlight the importance of the wine yeast to the wine industry and the necessity for well-planned breeding programmes. First, we summarise reliable taxonomic methods that are useful as diagnostic techniques in such breeding strategies. Second, we emphasise the complexity of the genetic features of commercial wine-yeast strains. Third, we review the genetic techniques available and point out the potential of these techniques (individually and in combination) in strain-development programmes. Finally, we attempt to stimulate interest in the genetic engineering of wine yeasts by discussing a few potential targets of strain development. The impact of yeast genetics and recombinant DNA technology on the wine industry promises to be impressive.

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 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 the 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 that 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 Hansen's lead, Müller-Thurgau sent out pure yeast cultures for wine making 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 Øjvind 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 on the improvement of grape varieties and their cultivation, and on fermentation and wine-making 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 wine makers who use the wine yeast as a chemical and do not handle it as a living organism, nor 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 wine maker 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 micro-organism in large-scale fermentations (Snow, 1983; Rank *et al.*, 1988).

Acknowledgements: We thank Dr P. Sollitti, Department of Biochemistry, Albert Einstein College of Medicine, New York and Mrs Angelika Alberts, Department of English, University of Stellenbosch, for their critical review of the manuscript.

THE CLASSIFICATION AND CHARACTERISATION OF WINE YEASTS

The 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 juice into wine on a production scale was designated *Saccharomyces ellipsoideus*. Based on sugar fermentation and assimilation patterns, wine yeasts of the genus *Saccharomyces* had 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). It should be emphasised, however, 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 both to the wine maker and the 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 the comparison of ascospore surfaces by scanning electron microscopy (SEM), serology (Tsuchiya *et al.*, 1965). Proton magnetic resonance spectroscopy (PMR), spectra of cell-wall mannans (Gorin & Spencer, 1970), and grouping based on the co-enzyme Q-system (Yamada *et al.*, 1980). Recently, the gas-liquid chromatographic analysis of the cellular long-chain fatty-acid composition of wine yeasts has proved to be a useful technique for the rapid identification of wine-yeast strains (Tredoux *et al.*, 1987; Augustyn, 1989; Augustyn & Kock, 1989).

Many of the traditional criteria used for the speciation of yeasts were derived from the 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. Phenotypic traits, however, do not necessarily reflect genetic relatedness, since the same phenotype may be a result of convergent evolution. Conversely, the phylogenetic relationships should be reflected in similarities at the level of the base composition of deoxyribonucleic acid (DNA) 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.

The genetic characterisation of wine yeasts

The verification of species relationships through hybridisation (sexual compatibility) studies is generally regarded as an ideal way to define taxa (Kurtzman *et al.*, 1983). A lack of fertility among yeasts does not, however, preclude conspecificity, because only a few genes affect the ability to mate (Hicks & Herskowitz, 1976). Genome comparisons through the determination of DNA base composition, DNA reassociation, restriction fragment length 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 the taxonomy of yeasts (Campbell, 1987).

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 the 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 all of them lack 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 overlapping 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 radio-isotopes. *In vivo* labelling of DNA is frequently done by feeding the cells ^{14}C, ^{3}H or ^{32}P, whereas *in vitro* labelling is done with ^{125}I or ^{32}P by

nick translation and random priming. 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). The renaturation reactions of DNA that has not been labelled with radio-isotopes 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 elusion 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 the ionic strength of the incubation buffer (Kurtzman *et al.*, 1983).

The determination of mol% G+C might be of limited value, but the 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 recognises 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 the densitometric scanning of autoradiographs) corresponds to the stability of heteroduplexes formed between the chromosomal DNA and the DNA probe. The stability depends on the degree of homology between the DNA species and the stringency of the hybridisation conditions; this 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 the 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) has also been used to distinguish among different yeasts (Lee & Knudsen, 1985; Vezinhet *et al.*, 1990). Because the mtDNA is approximately 200-fold smaller 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, which 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 tenfold 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.

The genetic fingerprinting of wine yeasts

Fingerprinting by protein electrophoresis: Protein expression is genetically determined; the set of proteins and their individual abundance in a specific yeast strain are constant when the strain is grown under standardised conditions. Electrophoresis of the total soluble proteins of a yeast strain yields a complex pattern. Each band usually represents a number of structurally different protein species with the same electrophoretic mobility. The 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. The quantitative comparison and grouping of normalised densitograms of a large number of electropherograms can, however, be done only with the assistance of computer programs which take the relative mobility, the sharpness of bands and the relative protein concentrations of the peaks and valleys into account (Kersters & De Ley, 1980).

A 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 the 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. The 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 the 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 are therefore never visible microscopically. This fact implies that yeasts cannot be karyotyped conventionally as with plants and animals. With the advent of pulsed-field gel electrophoretic systems, however, 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. The first new system to follow was field-inversion gel electrophoresis (FIGE) or reverse-field electrophoresis (RFE), in which 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 than the smaller ones to reorientate and travel in the newly defined direction. 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 using pulsed-field gel electrophoresis. The karyotypes of the various yeasts show great variation in the size 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). These banding patterns are highly reproducible under controlled electrophoretic conditions, however, 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 system 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

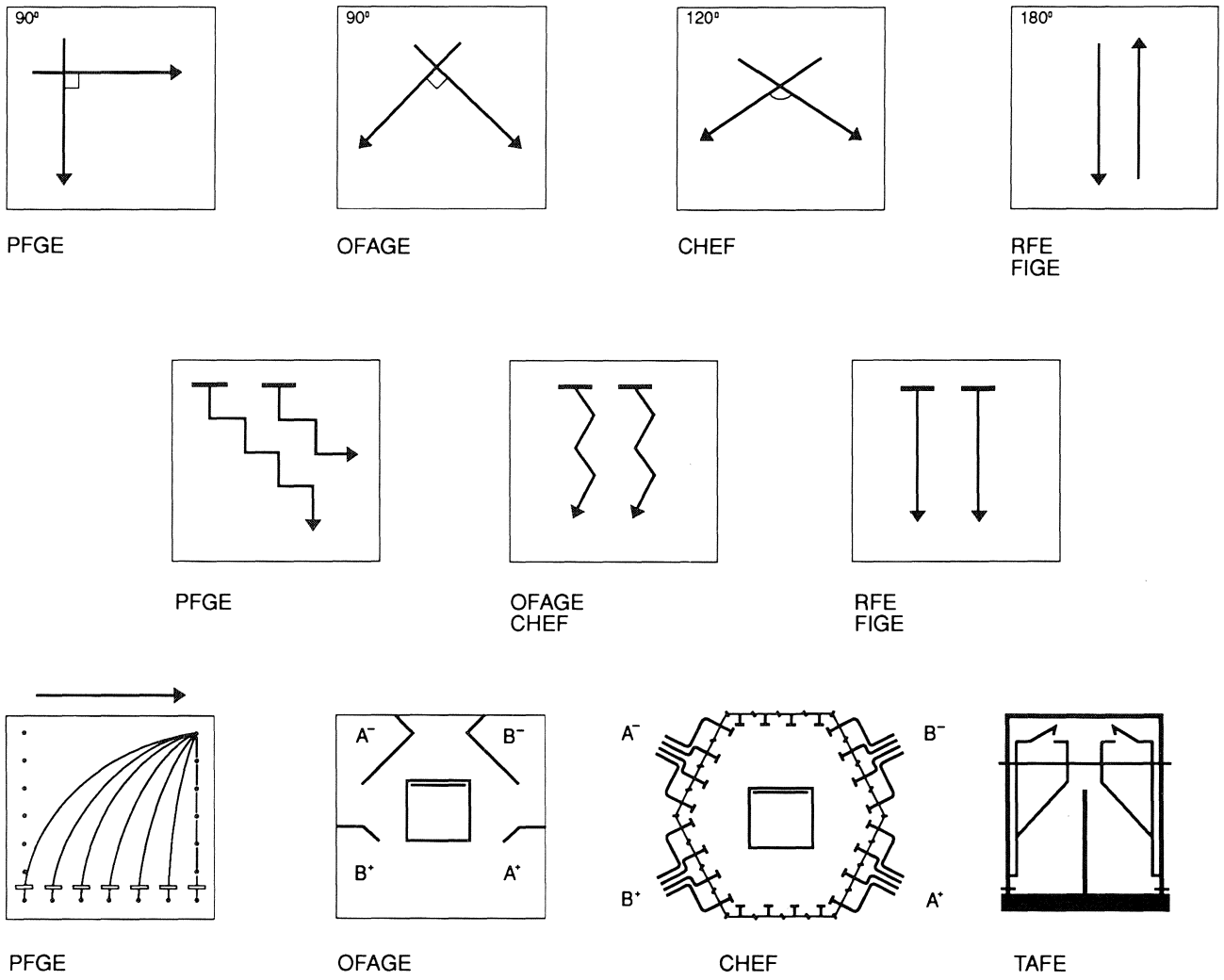

FIGURE 1

The different electrode configurations caused 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.

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 application 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 gluco-amylase gene was probed with the cloned *STA2* gluco-amylase 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. It can be stated that karyotyping using chromosomal banding patterns is a simple and reliable technique to identify individual wine-yeast strains.

THE GENETIC FEATURES OF WINE YEASTS

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 μm in diameter. Fig. 2 is a diagrammatic representation of a *S. cerevisiae* cell. *S. cerevisiae* reproduces 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, 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), whereas 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 cellular 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 maintained stably 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

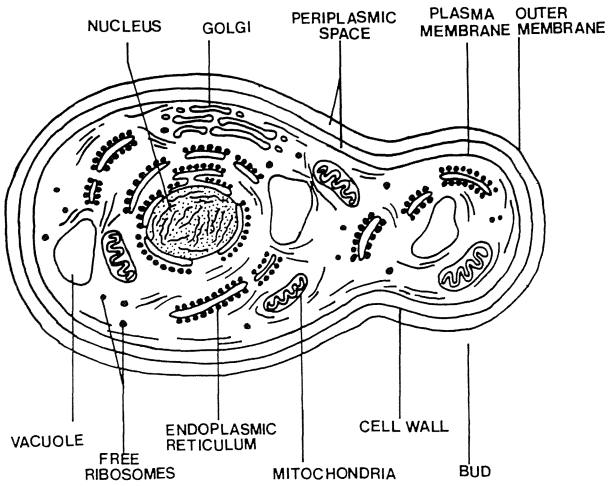

FIGURE 2

Diagram of a *S. cerevisiae* cell.

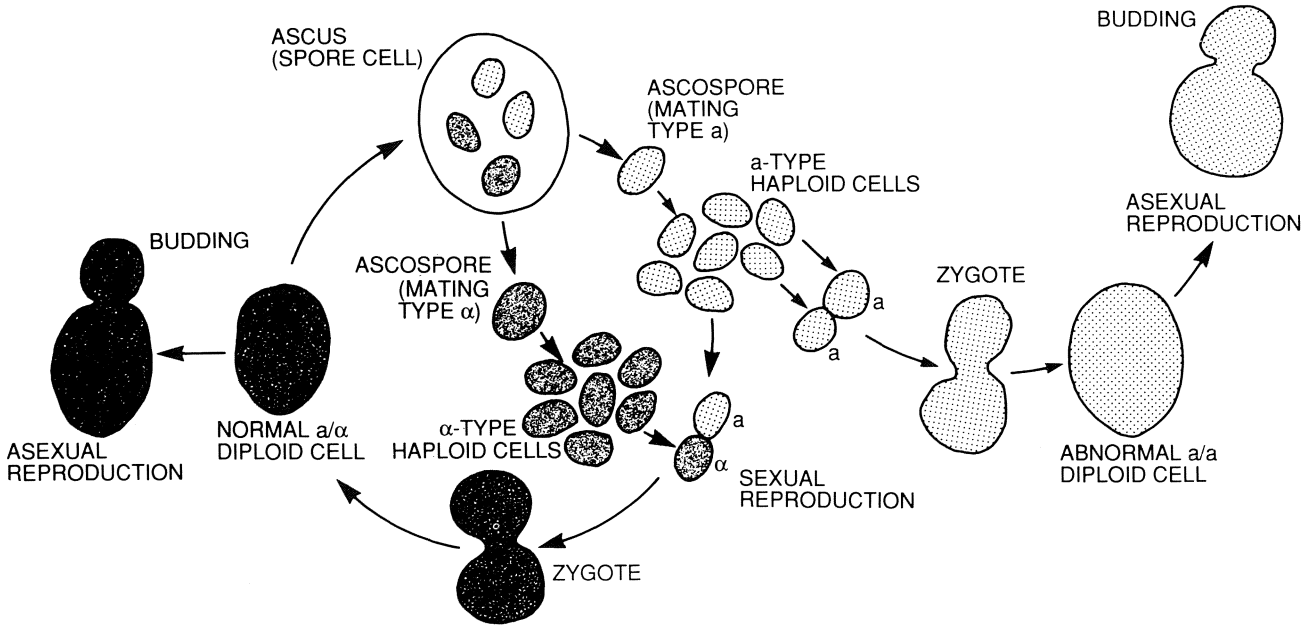

FIGURE 3

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

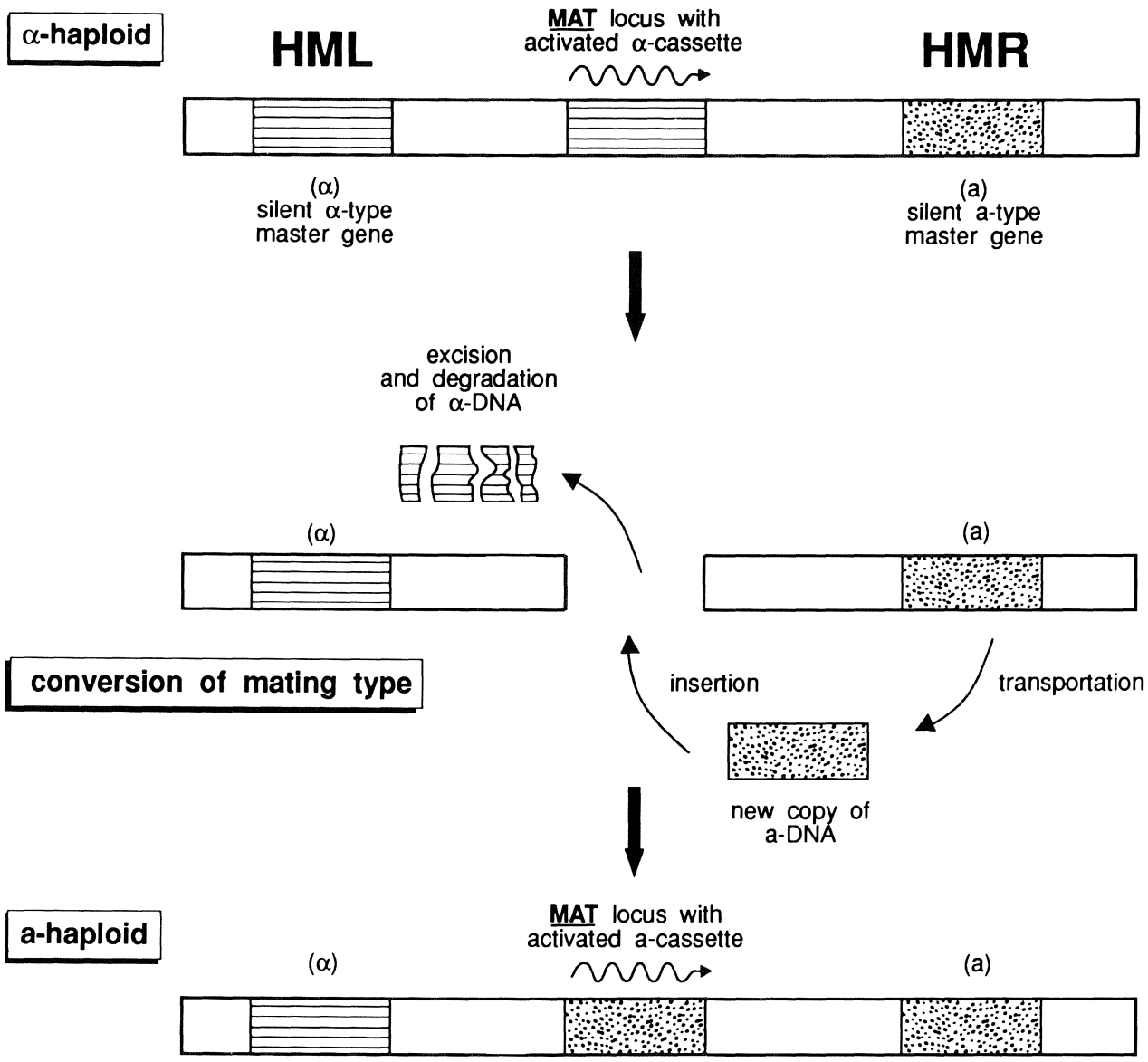

FIGURE 4

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

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 *MAT*α or *vice versa* as often as every cell division (Strathern & Herskowitz, 1979). It was found that chromosome III possesses 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, *HMR*, to the right of *MAT*, contains information equivalent to what is expressed at *MAT*α. 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 & Herskowitz, 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 the 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, whereas 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). Because of the need for constant properties in industrial fermentations there may have been an unintentional selection against sexual reproduction in yeast strains used for wine making.

Chromosomes, ploidy and genetic stability

Since *S. cerevisiae* is a eucaryote, its choromosomes 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, III, IV and IX, which 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 from 198 to 2 194 kb long (Mortimer & Schild, 1985), arranged as chromatin and containing basic histone molecules. The 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). The 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 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. Industrial strains, however, 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 the tetrad-segregation analysis of mating type and morphological characteristics (Emeis, 1961; Gunge, 1966; Fowell, 1969). The determination of the 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. The determination of DNA content is dependent on very specific cell concentrations. Variation in the choromosomal sizes of industrial strains could also affect 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, accurately determined ploidy (including aneuploidy) 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). Consequently, 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. On the basis of these results it was concluded that heterosis rather than ploidy is responsible for improvement in 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 merely 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 problematic. The term *pure culture* signifies that it has been derived from a single cell but not that 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. The heterogeneity of a *pure* culture was pointed out by Zimmermann (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 the sporulation and spore viability of pure yeast cultures are generally poor and that there is a considerable variation in growth-rate between spore clones (Thornton & Eschenbruch, 1976). Some

of this genetic heterozygosity of pure cultures is undoubtedly due to the 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 (Paquin & Adams, 1983). 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 the 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 influence the *Ty* transposable elements and the respective contributions of nuclear and cytoplasmic (particularly mitochondrial) genomes have on the genetic drift in wine yeasts.

Extrachromosomal elements

Mitochondria: Mitochondria are complex organelles specialized in respiration and oxidative phosphorylation (Dujon, 1981). Rapidly growing cells usually contain less than 10 mitochondria, whereas cells from a stationary-phase culture contain up to 50 mitochondria 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 rich in adenine-thymine (A-T), 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, the replication of mtDNA differs from that of nuclear DNA. The 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, so that 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 encodes proteins that perform only a few activities. One explanation for the persistence of this large mitochondrial genome is that in yeast it could play 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 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 the 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 the formation of flavour compounds (Lewis *et al.*, 1976; Hammond & Eckersley, 1984). Thus, although wine yeasts are not required to respire during the fermentation of grape must, some mtDNA-encoded functions are important, and for this reason *petite* strains are not used for wine making.

Killer factors: The killer phenomenon is *S. cerevisiae* is associated with the presence of intracellular virus particles (Wickner, 1981; Tipper & Bostian, 1984; Young, 1987). Virus particles in killer yeasts, which 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 killertype (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 showed killer activity against the K_1 neutral strain that is immune to the toxin. Furthermore, the K_1 toxin is not active below pH 4 whereas 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).

2μm Plasmids: The 2μm 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 although 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*° strains) that lack the 2μm plasmid. There are usually 50 to 100 copies of 2μm DNA per cell and they represent about 5% of the total yeast DNA. These circular DNA molecules consist of two idential 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 (4μm, 6μm) forms of the plasmid also occur. The 2μm DNA is transcribed into three separate poly-adenylated 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 2μm DNA (Cox, 1983). The *REP1* and *REP2* genes are required for the autonomous replication of the 2μm plasmid (Broach, 1982). Other than its own maintenance, the 2μm plasmid appears to confer no advantage on the host cells. The 2μm 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 2μm origin of replication.

GENETIC TECHNIQUES FOR STRAIN DEVELOPMENT

S. cerevisiae can be manipulated genetically in many ways. Whereas some techniques alter limited regions of the genome, others are used to recombine or rearrange the entire genome. Techniques having the greatest potential in the genetic programming of wine-yeast strains are the 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 has dramatically increased the genetic diversity that can be introduced into yeast cells.

The clonal selection of variants: The 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. The 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.

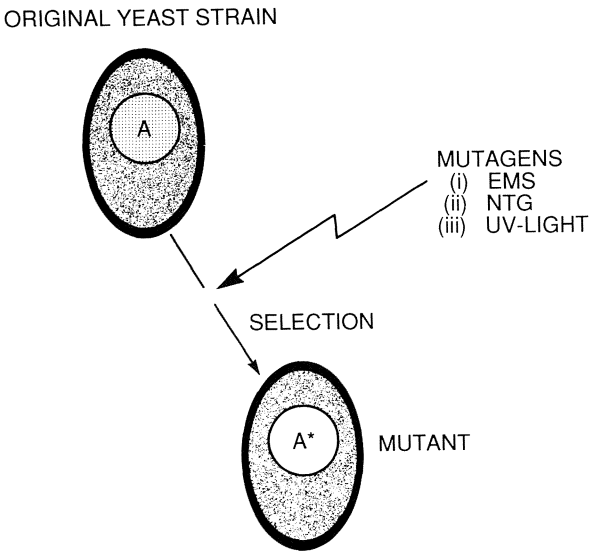

FIGURE 5

The 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.

The value of continuous culture for the isolation of variants without prior mutagenesis was amply demonstrated by Zimmermann (Snow, 1983), who isolated variants of an Epernay yeast with improved fermentation charactistics. 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).

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 (Fig. 5). 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). The mutation of wine yeasts can however, lead to the 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, the presence of non-mutated alleles cause them to be less easily detected in diploid and polyploid cells. 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 genetically stable 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 iso-amyl 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 in wine yeasts. Athough use of mutagens for directed strain development is limited, the method could be applied to isolate new variants of wine-yeast strains prior to further genetic manipulation (Sturley & Young, 1986).

Hybridisation: Intra-species hybridisation involves the mating of haploids of opposite mating-types to yield a heterozygous diploid. The process is illustrated in Fig. 6. Recombinant progeny are recovered by sporulating the diploid, recovering individual haploid ascospores and repeating the mating/sporulation cycle as required (Tubb & Hammond, 1987). Two general methods are used for the 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* can be recovered for analysis. Futhermore, tetrad analysis can also be used (i) to determine whether a gene is inherited chromosomally (i.e. a 2:2 segregation pattern) or cytoplasmically (i.e. a 4:0 segregation pattern); (ii) to assign a gene to a linkage group (chromosome) and map its chromosomal location; or (iii) to 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 requiring no special equipment or skills (Ingolia & Wood, 1986).

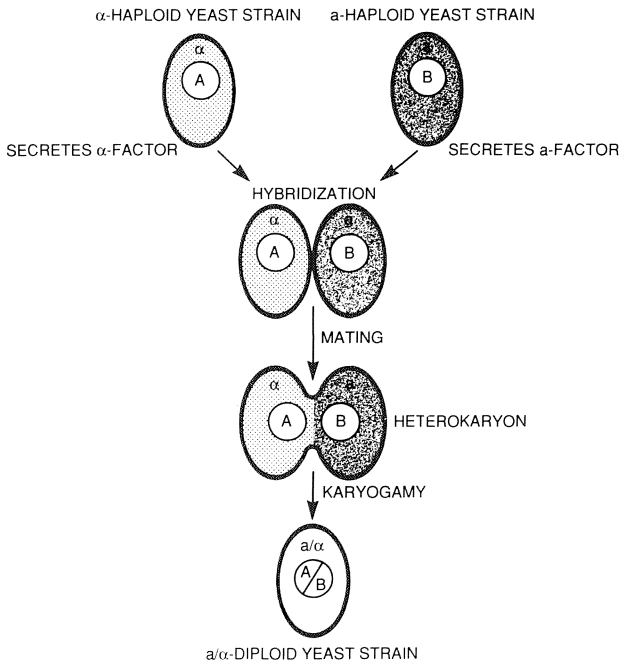

FIGURE 6

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

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 the development of wine-yeast strains has proved difficult (Thornton & Eschenbruch, 1976; Snow, 1979; Van der Westhuizen & Pretorius, 1990). This problem can, however, be circumvented by direct spore-cell mating (Thornton, 1983). As illustrated in Fig. 7 four homothallic

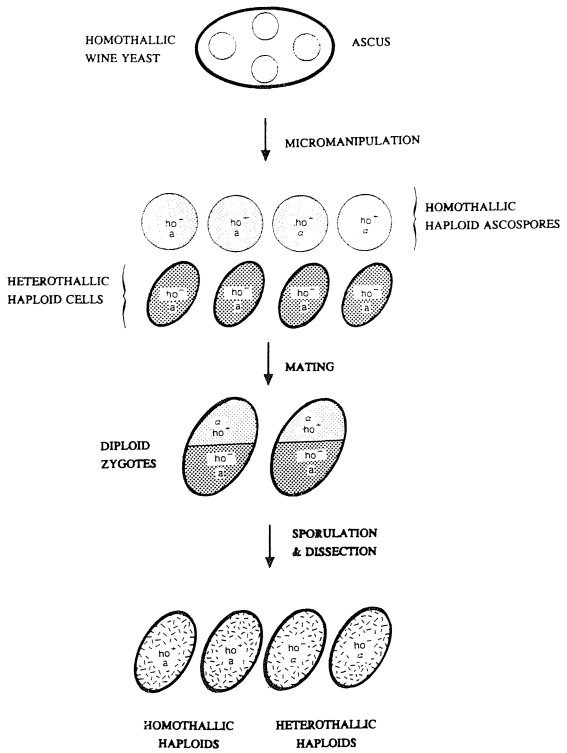

FIGURE 7

The 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).

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 wine-making 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. SO_2-tolerant, haploid killer 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).

The 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. Many desirable wine yeast characteristics are, however, specified by several genes or are the result of several gene systems interacting with one another (Thornton, 1983). For instance, the conversion of grape sugar to alcohol by wine yeasts involves at least twelve chemical reactions, each promoted by an enzyme encoded 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 with less than normal activity (Thornton, 1983). Since a hybridisation programme aimed at improving conversion efficiency that focused on individual genes could be time-consuming, a more empirical approach has to be adopted. This can be achieved by isolating haploids from several wine-yeast strains with different sugar-conversion efficiencies. The most efficient haploid strains can be identified after trial fermentations and then mated to generate the first generation of diploid strains. After further trail 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. In this manner Thornton (1980, 1982) employed selective hybridisation over three generations of diploid strains to raise the fermentation efficiency from 84% to 93%.

Rare-mating: Wine-yeast strains that fail to express a mating type can be force-mated with haploid *MATa* and *MATα* strains. This procedure, known as rare mating, is illustrated in Fig. 8. Typically, a large number of cells of the parental strains

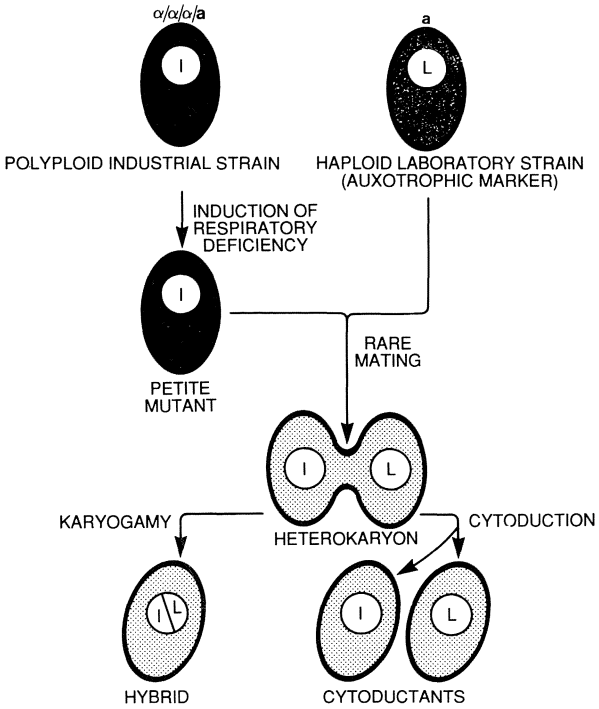

FIGURE 8

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).

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 of or lack mtDNA (respiratory-deficient mutants) can be force-mated with auxotrophic haploid strains having normal respiratory characteristics (Gunge & Nakatomi, 1972; Spencer & Spencer, 1977). Mixing 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 cross-breeding (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, which is 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 that a haploid mating strain carry the *kar1* mutation, 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 *karl* 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 *karl* 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).

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 obviate the need to cross opposite mating types, thereby extending the number of crosses that can be done (e.g. *MATα + MATα; MATa + MATa; MATa/MATα + MATa; MATa/MATα + MATα; MATa/MATa + MATa; MATα/MATα + MATα*). The procedure of spheroplast fusion was described by Van Soligen & Van der Plaat (1977) and is outlined in Fig. 9. 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 are 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. Owing to abortive nuclear fusion this technique rarely yields nuclear hybrids. Similarly, Seki *et al.* (1985) constructed a killer wine yeast and showed that the growth of sensitive cells in grape juice was inhibited by the killer fusant. Yokomori *et al.*

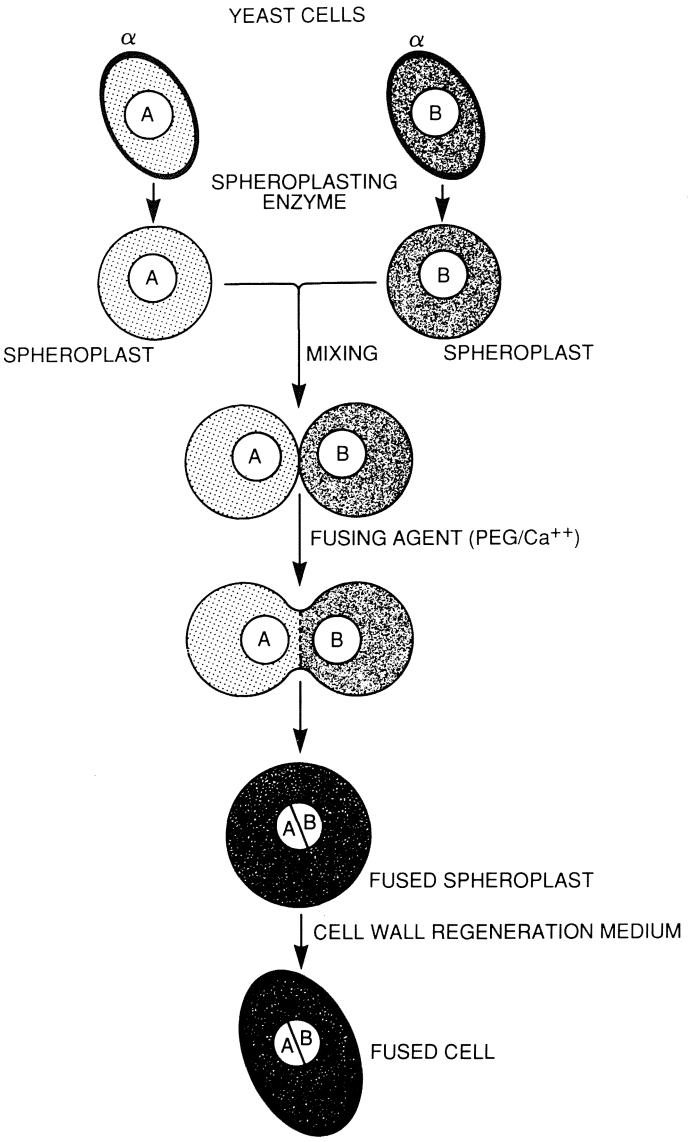

FIGURE 9

Spheroplast fusion between two different yeast cells is a direct asexual technique to produce either hybrids or cytoductants. Spheroplasts are formed by removing 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).

(1989) produced cytoductants of a saké wine yeast by spheroplast fusion that exhibited good fermentation performances and produced quality wine with low volatile acids.

Gene cloning and transformation: Without underrating 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. With 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, 1989). 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 achieving this are now available. Gene cloning and recombinant DNA technology offer exciting prospects for improving wine yeasts (Snow, 1983). Genetic transformation is the changing of the genetic set-up of a yeast cell by the introduction of purified DNA (Fig. 10).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) identifying the target gene and obtaining the DNA fragment to be cloned (passenger DNA) by enzymatic fragmentation of the donor DNA using restriction endonucleases;

(ii) identifying and linearising a suitable vector, whether a plasmid, virus (bacteriophage) or cosmid;

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

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

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

A number of options are available at each of these stages, and the decision to use any particular option will depend on a number of factors, not the least of which will be the extent of information available on 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 a 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

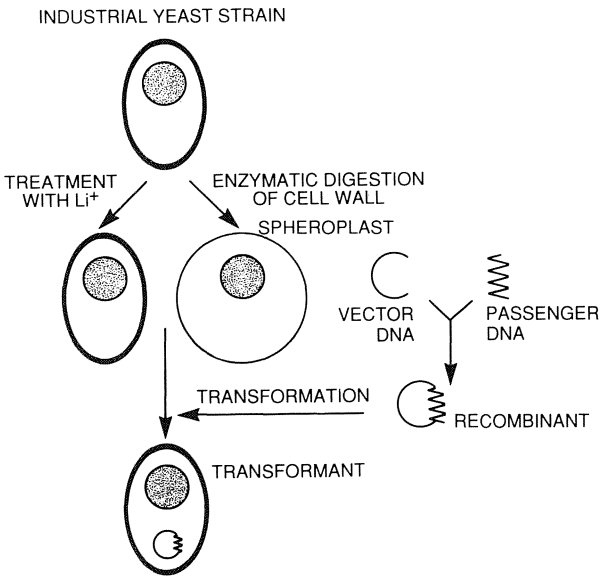

FIGURE 10

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 annealed 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).

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). The methods involving spheroplasts yield high transformation efficiencies. Their disadvantages, however, lie 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, which 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 become a heritable component 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 preferably be stripped of all non-relevant bacterial DNA sequences before transformation into industrial yeast strains.

In addition to the indroduction 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) amplifying gene expression by maintaining a gene on a multi-copy plasmid (Lacroute *et al.,* 1981) integrating 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) the in-frame splicing of a structural gene to a secretion signal to engineer the 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) incorporating genetic information from diverse organisms such as fungi, bacteria, animals and plants.

The 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 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 centred on DNA transformation have revolutionised strategies for strain modification, but it remains difficult to clone unidentified genes. Mutation and selection will, therefore, 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, 1989).

TARGETS FOR STRAIN DEVELOPMENT

Requirements for efficient wine yeasts

Owing 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. With traditional fermentation methods there was moreover, 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 wine-making 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) the rapid initiation of fermentation immediately upon inoculation, without excessive yeast growth;

(ii) growth at 15-18°C;

(iii) fermentation at low temperatures such as 10-14°C;

(iv) the 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, which is normally used in wine-making;

(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 or mercaptan production;

(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 characteristics listed above, and it is well established that wine yeasts vary in their wine-making abilities. While some degree of variation can be achieved by altering the fermentation conditions (e.g. temperature), a major source of variation is the genetic constitution of the wine yeasts (Thornton, 1983).

Specific targets for yeast genetics in wine-making

Improved quality control

Strain maintenance: One of the main objectives for using pure cultures in wine-making 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. The total prevention of heterogeneity in pure cultures is impossible, since homothallism, an inability to sporulate and mate, and polyploidy (multiple gene structure) protect only against genetic drift caused by sexual reproduction and mutation, but not against that caused by gene conversion and transposition. Even stringently controlled conditions for the maintenance of culture collections (i.e. freeze-dried cultures and 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 the 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. A 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 assist 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 the molecular marking of wine yeasts will assist in monitoring yeast strains used in wine fermentations and will also discourage the illegal use of (patented) commercial wine-yeast strains.

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 the improvement of sugar utilisation and an increased tolerance to ethanol, resistance to microbial toxins (e.g. killer toxins) and the production of substances inhibitory to contaminating micro-organisms, resistance to heavy metals, the reduced formation of foam, induced flocculance at the end of fermentation, and the production of extracellular enzymes.

Efficient sugar utilisation and ethanol tolerance: In the wine industry there is an obvious demand 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 are metabolised. *S. cerevisiae* has the ability to take up and ferment a wide range of sugars (e.g. glucose, fructose, mannose, galactose, maltose and maltotriose) and is constitutive to metabolise grape sugar (glucose and fructose) (Stewart & Russell, 1983). 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. 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, the 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 increased efficiency in the conversion of grape sugar to alcohol. Schaaf *et al.* (1989) reported, however, that an 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 disposed 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

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 2μm 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 owing 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 function 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, 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.)

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, particularly the plasma membrane (Ingram & Buttke, 1984), solute transport systems (Van Uden, 1985), the steps of initiation and elongation in the process of protein synthesis, and RNA accumulation (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, for instance phosphofructokinase, 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 (Olivier, 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 process, 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. From a parental strain that could tolerate no more than 14,4% ethanol, 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%. 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 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 yet to be found or constructed. 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. K_2 killer toxin, however, 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. Since commercial killer as well as sensitive wine yeasts are currently being used in the South African wine industry, 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 wine makers 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 programmes 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 strains 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, 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 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 expressing 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, whereas 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 constructing the appropriate plasmids. For example, a gene encoding a yeast gluco-amylase has been incorporated into the yeast expression plasmid and used to transform industrial strains. These transformants now secrete killer toxin and gluco-amylase (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, which 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. It is not clear, however, whether 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 provided an entry into the molecular genetics of industrial yeast strains.

Resistance to metals: Grapes contain small but adequate amounts of the 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 leads 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 occurred even 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 copper-resistant wine yeasts 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.

Foam formation: Excessive foaming during the early stages of a wine fermentation is an undesirable characteristic of some wine-yeast strains. The 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 forth-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, 21 c M 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, recomibnant 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* genes 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. The one-step gene disruption method of Orr-Weaver *et al.* (1981) can be used to replace the *FRO1* and *FRO2* genes in wine yeasts by their disrupted counterparts (Fig. 11).

Sedimentation and flocculation: Sedimentation of yeast cells refers to clumpy growth caused by the 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 an irreversible loss of flocculation (Miki *et al.*, 1980), suggesting that proteins on the surface 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). The attachment of yeast cells to one another can apparently also 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

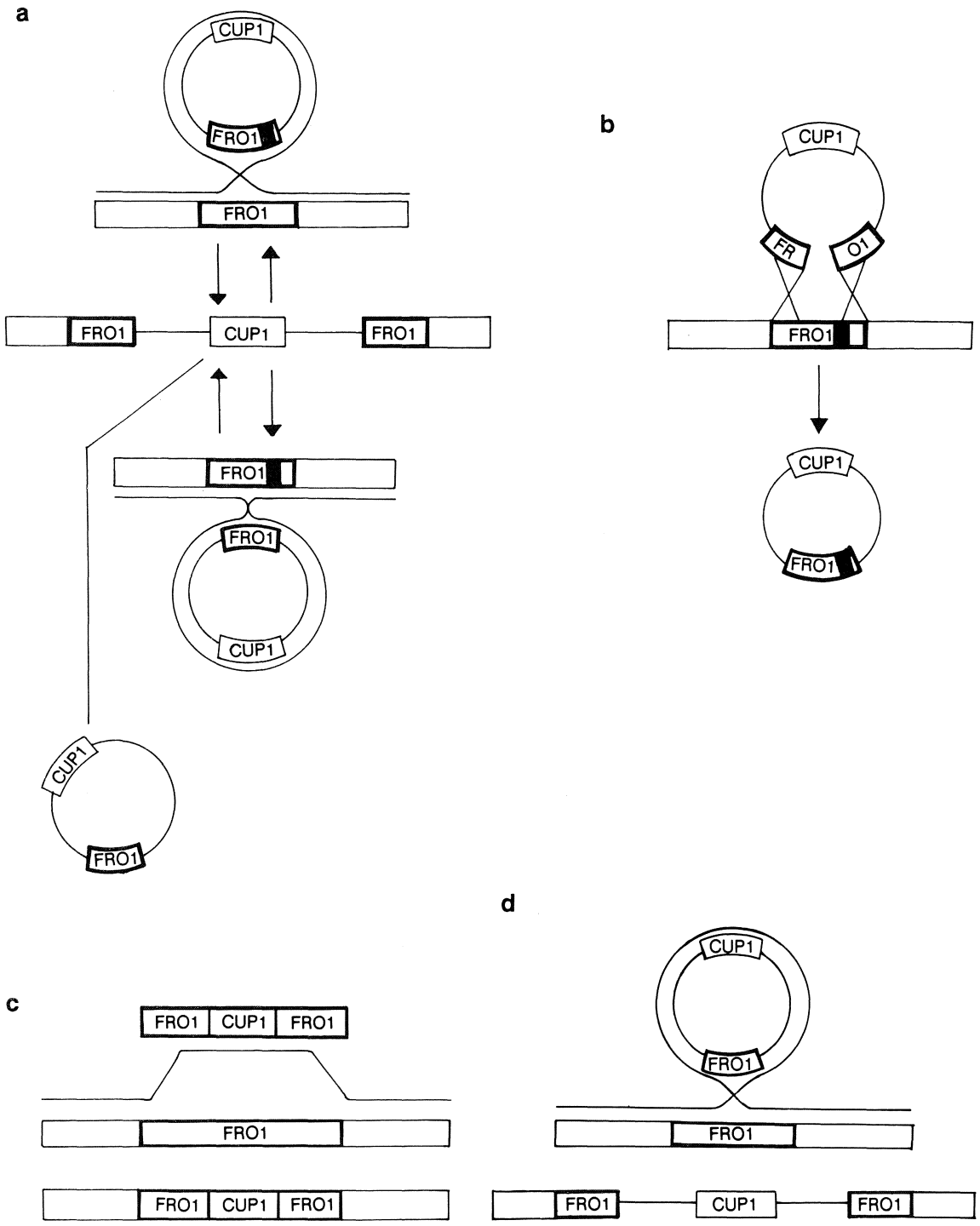

FIGURE 11

Homologous recombination can be used to transfer mutations into and out of the normal locus on a yeast chromosome. (a) The 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 the homologous recombination of a mutant plasmid into a normal locus. Depending on the position of the crossover event, the 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 the 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).

flocculation is under genetic control. At least one dominant gene, *FLO1*, will cause flocculation when expressed in a yeast strain (Russel *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 it would be possible to clone the *FLO1* gene by using reverse genetics. A cloned *FLO1* gene could then be transformed into a wine-yeast strain without adversely affecting other desirable properties through 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 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 wine maker 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 the β-glucans normally present in certain types of grape must to be degraded during fermentation and so prevent the development of glucan hazes and gels. The removal of β-glucans in this way promises to improve the efficiency of the 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 β 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 be 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. hydrolases and lyases). Most wine makers add commercial preparations of fungal pectinases to grape must to clarify it and to increase the juice yield. 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 *MFα1* promoter and secretion signal (leader) sequences and transformed into laboratory-bred strains of *S. cerevisiae*. The recombinant plasmid, containing the *pelE* gene, integrated 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 may 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. 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 contain high levels of malic acid, resulting in wines with a particularly acid taste. Malolactic fermentation (induced secondary fermentation by strains of *Lactobacillus* and *Leuconostoc*) is of considerable concern to wine makers (Edwards & Beelman, 1989). First, it reduces the acidity of some wines (especially the vinous-type Burgundy-styled wines); second, it contributes to microbiological stability following growth of bacteria; and, third, 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, yeast geneticists have been trying for several years to select or genetically construct wine-yeast strains that would utilise the malic acid in high-acidity musts. Toward this end fusions between *S. bailli*, *S. rouxii* or *Schiz. 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 malolactic 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. Owing 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 *Schiz. pombe* into *S. cerevisiae*, and it would seem that the genetic construction of a wine-yeast strain capable of complete malo-ethanolic fermentation is a distinct possibility in the near future (Subden & Osothsilp, 1987).

Contribution to wine bouquet

The single most important factor in wine making 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 determines the bouquet of good wines. Together with the grape variety and wine-making practices, the wine-yeast strain makes an important contribution to the complex character of good quality 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 seen as health hazards (e.g. sulphur dioxide and ethylcarbamate).

The 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 high levels of ethyl caprate, ethyl caproate, ethyl caprylate and iso-amyl 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 appears to be a promising line of future work.

Fusel-oil production: Alcohols with carbon numbers greater than that of ethanol, such as isobutyl, isoamyl and active amyl alcohol, are termed fusel oil. These higher alcohols are produced by wine yeasts during alcoholic fermentation from intermediates in the branched-chain amino-acids pathway leading the 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 in wines, however, are usually present at concentration levels below their threshold values and 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 iso-amyl 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 iso-amyl alcohol during fermentation than the prototrophic parent (Snow, 1983). It will be of great interest to see whether the integrative disruption of specific *ILE, LEU* and *VAL* genes of wine yeasts will result in lower levels of fusel oil in wine.

Sulphite and sulphide production: It is general knowledge that *S. cerevisiae* can use sulphate, sulphite and elemental sulphur as sole sources of sulphur (Rose, 1987) and that the formation of SO_2 and H_2S by wine yeasts greatly affects the quality of wine. Sulphur dioxide is used regularly as an antimicrobial and anti-oxidative additive in the production of white wine. Health concern has led to ever-increasing demands for the restriction of its use as a preservative. Consequently, the production of SO_2 by wine yeasts has in itself become a point of debate. Whereas SO_2, when properly used, has beneficial effects, the reverse is true for H_2S, one of the most undesirable of yeast metabolites, since it affects the taste and smell of wines (Snow, 1983).

Sulphur is essential for yeast growth and is naturally available as sulphate in grape juice. Dusting vines with elemental sulphur provides another source of sulphur. Sulphite is formed only from sulphate, whereas sulphide is formed from sulphate, from 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 affect sulphite formation. The formation of sulphide was shown to be influenced indirectly by the amount of yeast growth, pantothenate or pyridoxine deficiencies, or by excess levels of certain amino acids (which 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 retain most of the H_2S produced intracellularly (Rupela & Tauro, 1984). Snow (1983) suggested that in addition to the 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, especially effective as an adjuvant to sterile filtration in wine making 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 reduce the production of ethylcarbamate: (i) restrict 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 high-aldehyde, low-sugar flor sherry (Snow, 1983). It has been reported that the film-forming characteristic segregated 2:2 is asci of these sherry yeasts, indicating that the flor formation is controlled by a single dominant gene (Santa Maria & Vidal, 1973). The cloning and sequencing of the gene responsible for flor formation and the 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.

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 incorporating 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 heterologous genes encoding 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, co-enzymes 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 highlighted several advantages of industrial yeasts as hosts for the expression of foreign genes, namely that (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) the 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 wine makers have vast experience of the yeast-based fermentations, they are well placed to explore new opportunities offered by the golden age of biotechnology.

LITERATURE CITED

AIGLE, M., ERBS, D. & MOLL, M., 1983. Determination of brewing yeast ploidy by DNA measurement. *J. Inst. Brew.* **89**, 72-74.

ALIKHANYAN, S.I. & NALBANDYAN, G.M., 1971. Selection of wine yeasts using mutagens. *Sov. Genet.*, **2**, 1200.

ANAND, R., 1986. Pulsed field gel electrophoresis: a technique for fractionating large DNA molecules. *Trends Genet.* **2**, 278-283.

ANGIER, N., 1986. A stupid cell with all the answers. *Discover* **7**, 71-83.

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

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

AZEVEDO, J.L., TAVARES, F.C.A. & CRUZ, M.R.M., 1978. Genetic improvement of yeasts. In: BACILA, M., HORECKER, B.L. & ANDRES, O.M. (eds.). Biochemistry and genetics of yeasts: pure culture and applied aspects. Academic Press, New York. pp. 563-575.

BAKALINSKY, A.T. & SNOW, R., 1990. Conversion of wine strains of *Saccharomyces cerevisiae* to heterothallism. *Appl. Environ. Microbiol.* **56**, 849-857.

BARNETT, J.A., 1981. The utilization of disaccharides and some other

sugars by yeasts. *Adv. Carbohydr. Chem. Biochem.* **39,** 347-404.

BEAVEN, M.J., CHARPENTIER, C. & ROSE, A.H., 1982. Production and tolerance of ethanol in relation to phospholipid fatty-acyl composition in *Saccharomyces cerevisiae* NCYC431. *J. Gen. Microbiol.* **128,** 1447-1455.

BILINSKI, C.A. & CASEY, G.P., 1989. Developments in sporulation and breeding of brewer's yeast. *Yeast* **5,** 429-438.

BOEKE, J.D., GARFINKEL, D.J., STYLES, C.A. & FINK, G.R., 1985. *Ty* elements transpose through an RNA intermediate. *Cell* **40,** 491-500.

BOSTIAN, K.A., ELLIOT, Q., BUSSEY, H., BURN, V., SMITH, A. & TIPPER, D.J., 1984. Sequence of the preprotoxin dsRNA gene of type 1 killer yeast: multiple processing events produce a two-component toxin. *Cell* **36,** 741-751.

BOTSTEIN, D., FALCO, S.C., STEWART, S.E., BRENNAN, M., SCHERER, S., STINCHCOMB, D.T., STRUHL, K. & DAVIS, R.W., 1979. Sterile host yeasts (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. *Gene* **8,** 17-24.

BRAUS, G., FURTER, R., PRANTL, F., NIEDERBERGER, P. & HÜTTER, R., 1985. Arrangement of genes *TRP1* and *TRP3* of *Saccharomyces cerevisiae* strains. *Arch. Microbiol.* **142,** 383-388.

BROACH, J.R., 1981. The yeast plasmid 2µm circle. In: STRATHERN, J.N., JONES, E.W. & BROACH, J.R. (eds.). The molecular biology of the yeast *Saccharomyces:* life cycle and inheritance. Cold Spring Harbor, New York. pp. 445-470.

BROACH, J.R., 1982. The role of site-specific recombination in expression of the yeast plasmid 2 µm circle. *Rec. Adv. Yeast Mol. Biol.* **1,** 93-112.

BROWN, A.P.J., GOODEY, A.R. & TUBB, R.S., 1981. Interstrain transfer of the 2µm DNA plasmid of *Saccharomyces* by cytoduction. *J. Inst. Brew.* **87,** 234-238.

BROWN, S.W. & OLIVER, S.G., 1982a. The effect of temperature on the ethanol tolerance of the yeast, *Saccharomyces uvarum. Biotechnol. Lett.* **4,** 269-274.

BROWN, S.W. & OLIVER, S.G., 1982b. Isolation of ethanol-tolerant mutants of yeast by continuous selection. *Eur. J. Appl. Microbiol. Biotechnol.* **16,** 119-122.

BRUSCHI, C.V., COMER, A.R. & HOWE, G.A., 1987. Specificity of DNA uptake during whole cell transformation of *S. cerevisiae. Yeast* **3,** 131-137.

BRZOBOHATY, B. & KOVAC, C.L., 1986. Factors enhancing genetic transformation of intact yeast cells modify cell wall porosity. *J. Gen. Microbiol.* **132,** 3089-3093.

BUSSEY, H. & MEADEN, P., 1985. Selection and stability of yeast transformants expressing cDNA of an M1 killer toxin-immunity gene. *Curr. Genet.* **9,** 258-291.

BUTT, T.R., STERNBERG, E., HERD, J. & CROOKE, S.T., 1984. Cloning and expression of a copper metallothionein gene. *Gene* **27,** 23-33.

CALLEJA, G.B., 1987. Cell aggregation. In: ROSE, A.H. & HARRISON, J.S. (eds.). The yeasts, Vol. 2. Academic Press Inc., London, pp. 165-237.

CAMPBELL, I., 1987. Systematics of yeasts. In: PRIEST, F.G. & CAMPBELL, I. (eds.). Brewing microbiology. Elsevier Applied Science Publishers, Essex, England. pp. 1-14.

CANTWELL, B.A., BRAZIL, B., MURPHY, N. & McCONNELL, D.J., 1986. Comparison of expression of the endo-β-1,3-1,4-glucanase gene from *Bacillus subtilis* in *Saccharomyces cerevisiae* from the *CYC1* and *ADH1* promoters. *Curr. Genet.* **11,** 65-70.

CARLE, G.F., FRANK, M. & OLSON, M.V., 1986. Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. *Science* **232,** 65-68.

CARLE, G.F. & OLSON, M.V., 1984. Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis. *Nuc. Acids Res.* **12,** 5647-5664.

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

CASEY, G.P., 1986. Molecular and genetic analysis of chromosomes X in *Saccharomyces carlsbergensis. Carlsberg Res. Commun.* **51,** 343-362.

CASEY, G.P., MAGNUS, C.A. & INGLEDEW, W.M., 1984. High-gravity brewing: effects of nutrition on yeast composition, fermentation ability, and alcohol production. *Appl. Eviron. Microbiol.* **48,** 639-646.

CASEY, G.P., XIAO, W. & RANK, G.H., 1988a. A convenient dominant selection marker for gene transfer in industrial strains of *Saccharomyces* yeast: *SMR1* encoded resistance to the herbicide sulfometuron methyl. *J. Inst. Brew.* **94,** 93-97.

CASEY, G.P., XIAO, W. & RANK, G.H., 1988b. Application of pulsed field chromosome electrophoresis in the study of chromosome XIII and the electrophoretic karyotype of industrial strains of *Saccharomyces* yeasts. *J. Inst. Brew.* **94,** 239-243.

CHEREST, H., THAO, N.N. & SURDIN-KERJAN, Y., 1985. Transcriptional regulation of the *MET3* gene of *Saccharomyces cerevisiae. Gene* **34,** 269-281.

CHRISTENSEN, B.E., 1987. Breeding for ethanol-tolerant distillers yeast. In: STEWART, G.G., RUSSELL, I., KLEIN, R.D. & HIEBSCH, R.R. (eds.). Biological research on industrial yeasts, Vol. I. CRC Press Inc., Boca Raton, Florida. pp. 111-120.

CHU, G., VOLLRATH, D., & DAVIS, R.W., 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. *Science* **234,** 1582-1585.

CLARK, S.M., LAI, E., BIRREN, B.W. & HOOD, L., 1988. A novel instrument for separating large DNA molecules with pulsed homogeneous electric fields. *Science* **241,** 1203-1205.

CONDE, J. & FINK, G.R., 1976. A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. *Proc. Natl. Acad. Sci., USA* **73,** 3651-3655.

CONSTANZO, M.C. & FOX, T.D., 1988. Transformation of yeast by agitation with glass beads. *Genetics* **120,** 667-670.

COX, M.M., 1983. The FLP protein of the yeast 2-µm plasmid: expression of a eukaryotic genetic recombination system in *Escherichia coli* (gene expression/site-specific recombination/gene cloning). *Proc. Natl. Acad. Sci., USA* **80,** 4223-4227.

CUMMINGS, J. & FOGEL, S., 1987. Genetic homology of wine yeasts with *Saccharomyces cerevisiae. J. Inst. Brew.* **84,** 267-270.

DECOCK, J.P. & INSERENTANT, D., 1985. Ty hydribisation patterns of yeast and their use for strain identification. *Eur. Brew. Conv. Proc. Cong. 20th, Helsinki.* pp. 195-202.

DE JONGE, P., DE JONGH, F.C.M., MEIJERS, R., STEENSMA, H.Y. & SCHEFFERS, W.A., 1986. Orthogonal-field-alternation gel electrophoresis banding patterns of DNA from yeasts. *Yeast* **2,** 193-204.

DELORME, E., 1989. Transformation of *Saccharomyces cerevisiae* by electroporation. *Appl. Eviron. Microbiol.* **55,** 2242-2246.

DEMAIN, A.L. & SOLOMON, N.A., 1981. Industrial microbiology: introducing an issue on how products useful to man are manufactured by micro-organisms. *Sci. Am.* **245,** 43-51.

DOTT, W., HEINZEL, M. & TRÜPER, H.G., 1977. Sulfite formation by wine yeasts IV. Active uptake of sulfate by "low" and "high" sulfite producing wine yeasts. *Arch. Microbiol.* **112,** 283-285.

DOTT, W. & TRÜPER, H.G., 1978. Sulfite formation by wine yeasts VI. Regulation of biosynthesis of NADPH- and BV-dependent sulfite reductases. *Arch. Microbiol.* **118,** 249-251.

DOTT, W. & TRÜPER, H.G., 1979. Sulfite formation by wine yeasts VII. Regulation of the enzymatic steps between sulfide and sulfur-containing amino acids. *Arch. Microbiol.* **121,** 251-253.

DUJON, B., 1981. Mitochondrial genetics and functions. In: STRATHERN, J.N., JONES, E.W. & BROACH, J.R. (eds.). The molecular biology of the yeast *Saccaromyces:* life cycle and inheritance. Cold Spring Harbor Laboratory. New York. pp. 505-635.

DUNTZE, W., MacKAY, V.L. & MANNEY, T.R., 1970. *Saccharomyces cerevisiae:* a diffusible sex factor. *Science* **168,** 1472-1473.

DUTCHER, S.K., 1981. Internuclear transfer of genetic information in *karl-1/KAR1* heterokaryons in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **1,** 245-253.

EDWARDS, C.G. & BEELMAN, R.B., 1989. Inducing malolactic fermentation in wines. *Biotechnol. Adv.* **7,** 333-360.

EMEIS, C.C., 1961. Polyploide kulturhefer. *Eur. Brew. Conv. Proc. Cong., 8th Vienna.* pp. 205-215.

EMEIS, C.C., 1963. Kulturversuche mit polyploiden hefer. *Eur. Brew. Conv. Proc. Cong. 9th, Brussels.* pp. 362-369.

EMEIS, C.C., 1965. Stabilität diploider und polyploider kulturhefe-stämme. *Eur. Brew. Proc. Conv. Cong. 10th, Stockholm.* pp. 156-163.

ESCHENBRUCH, R.E., 1974a. On the hydrogen sulphide formation of wine yeasts. *Wynboer* **508,** 8-12,46.

ESCHENBRUCH, R., 1974b. Sulfite and sulfide formation during winemaking- a review. *Am. J. Enol. Vitic.* **25,** 157-161.

ESCHENBRUCH, R. & BONISH, P., 1976a. The influence of pH on sulphite formation by yeasts. *Arch. Microbiol.* **107,** 229-231.

ESCHENBRUCH, R. & BONISH, P., 1976b. Production of sulphite and sulphide by low- and high-sulphite forming wine yeasts. *Arch. Microbiol.* **107,** 299-302.

ESCHENBRUCH, R., BONISH, P., & FISHER, B.M., 1978. The production of H_2S by pure culture wine yeasts. *Vitis* **17,** 67-74.

ESCHENBRUCH, R. & KLEYNHANS, P.H., 1974. The influence of copper-containing fungicides on the copper content of grape juice and on

hydrogen sulphide formation. *Vitis* **12,** 320-324.

ESCHENBRUCH, R. & RASSELL, J.M., 1975. The development of non-foaming yeast strains for wine-making. *Vitis* **14,** 43-47.

EVANS, I.H. 1983. Molecular genetic aspects of yeast mitochondria. In: SPENCER, J.F.T., SPENCER, D.M. & SMITH, A.R.W. (eds.). Yeast genetics fundamental and applied aspects. Springer-Verlag, New-York. pp. 269-370.

EXTREMERA, A.L., MARTIN, I. & MONTOYA, E., 1982. A new killer toxin produced by *Saccharomyces cerevisiae*. *Curr. Genet.* **5,** 17-19.

FANGMAN, W.L. & ZAKIAN, V.A., 1981. Genome structure and replication. In: STRATHERN, J.N., JONES, E.W. & BROACH, J.R. (eds.). The molecular biology of the yeast *Saccharomyces:* life cycle and inheritance. Cold Spring Harbor Laboratory, New York. pp. 27-58.

FOGEL, S., WELCH. J.W., CATHALA, G. & KARIN, M., 1983. Gene amplification in yeast: *CUP1* copy number regulates copper resistance. *Curr. Genet.* **7,** 347-355.

FOWELL, R.R., 1969. Sporulation and hybridization of yeasts. In: ROSE, A.H. & HARRISON, J.S. (eds.). The yeast. Academic Press, London and New York. pp. 303-383.

GARDINER, K., LAAS, W. & PATTERSON, D., 1986. Fractionation of large mammalian DNA restriction fragments using vertical pulsed-field gradient gel electrophoresis. *Som. Cell Mol. Genet.* **12,** 185-195.

GENDRE, F. & GUERINEAU, M., 1986. Construction of an *E. coli* yeast shuttle vector with a double positive selection in order to transform industrial strains of *Saccharomyces cerevisiae*. *Yeast* **2,** S127.

GIBSON, R.K., 1987. The technology of genetic manipulation. *J. Appl. Bacteriol. Symp. Suppl.* pp. 7S-19S.

GIETZ, R.D. & SUGINO, A., 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74,** 527-534.

GJERMANSEN, C. & SIGSGAARD, P., 1981. Construction of a hybrid brewing strain of *Saccharomyces carlsbergensis* by mating of meiotic segregants. *Carlsberg Res. Commun.* **46,** 1-11.

GORIN, P.A.J. & SPENCER, J.F.T., 1970. Proton magnetic resonance spectroscopy- an aid in identification and chemotaxonomy of yeasts. *Adv. Appl. Microbiol.* **13,** 25-89.

GRITZ, L. & DAVIES, J., 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25,** 179-188.

GUNGE, N., 1966. Breeding of baker's yeast- determination of the ploidy and an attempt to improve practical properties. *Japan. J. Genet.* **41,** 203-214.

GUNGE, N. & NAKATOMI, Y., 1971. Genetic mechanisms of rare matings of the yeast *Saccharomyces cerevisiae* heterozygous for mating type. *Genetics* **70,** 41-58.

GYURIS, J. & DUDA, E.G., 1986. High-efficiency transformation of *Saccharomyces cerevisiae* cells by bacterial minicell protoplast fusion. *Mol. Cell. Biol.* **6,** 3295-3297.

HABER, J.E. & HALVORSON, H.O., 1975. Methods in sporulation and germination of yeasts. In: PRESCOTT, D.M. (ed.). Methods in cell biology, Vol. XI. Academic Press, New York. pp. 45-69.

HACKING, A.J., TAYLOR, I.W.F. & HANAS, C.M., 1984. Selection of yeast able to produce ethanol from glucose at 40°C. *Appl. Microbiol. Biotechnol.* **19,** 361-363.

HADFIELD, C., CASHMORE, A.M. & MEACOCK, P.A., 1986. An efficient chloramphenicol-resistance marker for *Saccharomyces cerevisiae* and *Escherichia coli*. *Gene* **45,** 149-158.

HALFMANN, H.J., ROCKEN, W., EMEIS, C.C. & ZIMMERMANN, U., 1982. Transfer of mitochondrial function into a cytoplasmic respiratory-deficient mutant of *Saccharomyces* yeast by electro-fusion. *Curr. Genet.* **6,** 25-28.

HAMMOND, J.R.M. & ECKERSLEY, K.W., 1984. Fermentation properties of brewing yeast with killer character. *J. Inst. Brew.* **90,** 167-177.

HARA, S., IIMURA, Y. & OTSUKA, K., 1980. Breeding of useful killer wine yeasts. *Am. J. Enol. Vitic.* **31,** 28-33.

HARA, S., IIMURA, Y., OYAMA, H., KOZEKI, T., KITONA, K. & OTSUKA, K.-I., 1981. The breeding of cryophilic killer wine yeasts. *Agric. Biol. Chem.* **46,** 1327-1334.

HARASHIMA, S., TAKAGI, A. & OSHIMA, Y., 1984. Transformation of protoplasted yeast cells is directly associated with cell fusion. *Mol. Cell. Biol.* **4,** 771-778.

HEINZEL, M.A. & TRÜPER, H.G., 1978. Sulfite formation by wine yeasts V. Regulation of biosynthesis of ATP- and ADP-sulfurylase by sulfur- and selenium-compounds. *Arch. Microbiol.* **118,** 243-247.

HELLER, C. & POHL, F.M., 1989. A systematic study of field inversion gel electrophoresis. *Nuc. Acids Res.* **17,** 5989-6003.

HENDERSON, R.C.A., COX, B.S. & TUBB, R., 1985. The transformation of brewing yeasts with a plasmid containing the gene for copper resistance. *Curr. Genet.* **9,** 133-138.

HERSKOWITZ, I., 1988. Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52,** 536-553.

HERSKOWITZ, I. & OSHIMA, Y., 1981. Control of cell type in *Saccharomyces cerevisiae:* mating type and mating type interconvertion. In: STRATHERN, J.N., JONES, E.W. & BROACH, J.R. (eds.). The molecular biology of the yeast *Saccharomyces:* life cycle and inheritance. Cold Spring Harbor, New York. pp. 181-209.

HICKS, J.B. & HERSKOWITZ, I., 1976. Interconversion of yeast mating types. I. Direct observations of the action of the homothallism (*HO*) gene. *Genetics* **83,** 245-258.

HICKS, J.B. & HERSKOWITZ, I., 1977. Interconversion of yeast mating types. II. Restoration of mating ability to sterile mutants in homothallic and heterothallic strains. *Genetics* **85,** 373-393.

HINCHLIFFE, E. & BOX, W.G., 1984. Expression of the cloned endo-1,3-1,4-ß-glucanase gene of *Bacillus subtilis* in *Saccharomyces cerevisiae*. *Curr. Genet.* **8,** 471-475.

HINNEN, A., HICKS, J.B. & FINK, G.R. 1978. Transformation of yeast. *Proc. Natl. Acad. Sci. USA* **75,** 1929-1933.

HOLLENBERG, C.P., BORST, P. & VAN BRUGGEN, E.F.J., 1970. A 25-μ closed circular duplex DNA molecule in wild-type yeast mitochondria. Structure and genetic complexity. *Biochem. Biophys. Acta* **209,** 1-15.

HOLMBERG, S. & KIELLAND-BRANDT, M. C., 1987. A mutant of *Saccharomyces cerevisiae* temperature sensitive for flocculation. Influence of oxygen and respiratory deficiency on flocculation. *Carlsberg Res. Commun.* **43,** 37-47.

IMAMURA, T., KAWAMOTO, M. & TAKAOKA, Y., 1974. Characteristics of main mash infected by killer yeast in saké brewing and nature of its killer factor. *J. Ferment. Technol.* **52,** 293-299.

INGOLIA, T.D. & WOOD, J.S., 1986. Genetic manipulation of *Saccharomyces cerevisiae*. In: DEMAIN, A.L. & SOLOMON, N.A. (eds.). Manual of industrial microbiology and biotechnology. American Society for Microbiology, Washington, D.C. pp. 204-213.

INGRAM, L.O. & BUTTKE, M., 1984. Effects of alcohols on micro-organisms. *Adv. Microbiol. Physiol.* **25,** 253-300.

INGRAHAM, J.L. & GUYMON, J.F., 1960. The formation of higher aliphatic alcohols by mutant strains of *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **88,** 157-166.

INGRAHAM, J.L., GUYMON, J.F. & CROWELL, E.A., 1961. The pathway of formation of n-butyl and n-amyl alcohols by a mutant strain of *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **95,** 169-175.

ITO, H., FAKUDA, Y., MURATA, K. & KIMURA, A., 1983. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153,** 163-168.

JACOBS, C.J., FOURIE, I. & VAN VUUREN, H.J.J., 1988. Occurrence and detection of killer yeasts on chenin blanc grapes and grape skins. *S. Afr. J. Enol. Vitic.* **9,** (2), 28-31.

JENSEN, R., SPRAGUE, G.F. & HERSKOWITZ, I., 1983. Regulation of yeast mating-type interconversion: feedback control of *HO* gene expression by the yeast mating type locus. *Proc. Natl. Acad. Sci. USA* **80,** 3035-3039.

JIMENEZ, A. & DAVIS, J., 1980. Expression of a transposable antibiotic resistance element in *Saccharomyces*. *Nature* **287,** 869-871.

JOHNSON, J.L., 1981. Genetic characterization. In: GERHARDT, P. (ed.). Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C. p. 450.

JOHNSTON, J.R., 1965. Breeding yeasts for brewing I. Isolation of breeding strains. *J. Inst. Brew.* **71,** 130-135.

JOHNSTON, J.R., CURRAN, L., CONTOPOULOU, R.C. & MORTIMER, R. K., 1989. Electrophoretic karyotyping of commercial brewing and distilling strains of *Saccharomyces* and of other yeasts. *Yeast (Spec. Iss.)* **5,** S255-S259.

JOHNSTON, J., HILGER, F. & MORTIMER, R., 1981. Variation in frequency of transformation by plasmid YRp7 in *Saccharomyces cerevisiae*. *Gene* **16,** 325-329.

KABACK, D.B., ANGERER, L.M. & DAVIDSON, N., 1979. Improved methods for the formation and stabilization of R-loops. *Nuc. Acids Res.* **6,** 2499-2517.

KASAHARA, H., OUCHI, K., KURATA, K., ISHIDO, T. & NUNOKAWA, T., 1974. Genetic characters of non-foaming mutants of saké yeast. *J. Ferment. Technol.* **52,** 348-351.

KASTER, K.R., BURGETT, S.G. & INGOLIA, T.D., 1984. Hygromycin B resistance as dominant selectable marker in yeast. *Curr. Genet.* **8,** 353-358.

KEIDING, A.K., 1985. Genetic and molecular characterization of a distiller's yeast. *Carlsberg Res. Commun.* **50,** 95-125.

KERSTERS, K. & DE LEY, J., 1980. Classification and identification of bacteria by electrophoresis of their proteins. In: GOODFELLOW, M. & BOARD, R.G. (eds.). Microbial classification and identification. Academic Press, London. pp. 273-297.

KESZENMAN-PEREYRA, D. & HIEDA, K., 1988. A colony procedure for transformation of *Saccharomyces cerevisiae*. *Curr. Genet.* **13,** 21-23.

KIELLAND-BRANDT, M.C., NILSSON-TILLGREN, T., PETERSEN, J.G.L., HOLMBERG, S. & GJERMANSEN, C., 1983. Approaches to the genetic analysis and breeding of brewer's yeast. In: SPENCER, J.F.T., SPENCER, D.M. & SMITH, A.R.W. (eds.). Yeast genetics- fundamental and applied aspects. Springer-Verlag. New-York. pp. 421-437.

KIMURA, A. & MURATA, K., 1989. Breeding of a jumbo yeast and its application to production of useful compounds. *Yeast (Spec. Iss.)* **5,** S89-S92.

KLEBE, R.J., HARRISS, J.V., SHARP, Z.D. & DOUGLAS, M.G., 1983. A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. *Gene* **25,** 333-341.

KREGER-VAN RIJ, N.J.W., 1984. The yeasts, a taxonomic study. Elsevier Science Publishers, Amsterdam, The Netherlands.

KUNKEE, R.E. & AMERINE, M.A., 1970. Yeast in wine-making. In: HARRISON, J.S. & ROSE, A.H. (eds.). The yeast, Vol. III. Academic Press, New York. pp. 5-57.

KUNKEE, R.E., & GOSWELL, R.W., 1977. Table wines. In: ROSE, A.H. (ed.). Economic microbiology. Academic Press, New York. pp. 315-386.

KURJAN, J. & HERSKOWITZ, I., 1982. Structure of a yeast pheromone gene (*MF*α): a putative α-factor precursor contains four tandem copies of mature α-factor. *Cell* **30,** 933-943.

KURTZMAN, C.P., 1987. Molecular taxonomy of industrial yeasts. In: STEWART, G.G., RUSSELL, I., KLEIN, R.D. & HIEBSCH, R.R. (eds.). Biological research on industrial yeasts, Vol. I. CRC Press Inc., Boca Raton, Florida. pp. 27-45.

KURTZMAN, C.P., PHAFF, H.J. & MEYER, S.A., 1983. Nucleic acid relatedness among yeasts. In: SPENCER, J.F.T., SPENCER, D.M. & SMITH, A. R.W. (eds.). Yeast genetics: fundamental and applied aspects. Springer-Verlag, New York. pp. 139-159.

KURTZMAN, C.P., SMILEY, M.J., JOHNSTON, C.J., WICKERHAM, L.J. & FUSON, G.B., 1980. Two new and closely related heterothallic species, *Pichia amylophila* and *Pichia mississippiensis:* characterization by hybridization and deoxyribonucleic acid reassociation. *Int. J. Syst. Bacteriol.* **30,** 208-216.

LAASER, G., MÖLLER, E., JAHNKE, K.-D., BAHNWEG, G., PRILLINGER, H. & PRELL, H.H., 1989. Ribosomal DNA restriction fragment analysis as a taxonomic tool in separating physiologically similar basidiomycetous yeasts. *System. Appl. Microbiol.* **11,** 170-175.

LACROUTE, F., BACH, M.L., CHAVELLIER, M.R., HUBERT, J.C., LOSSON, R., BOTSTEIN, D. & LOISON, G., 1981. In: VON WETTSTEIN, D., STENDERUP, A., KIELLANDT-BRANDT, M. & FRISS, J. (eds.). Molecular genetics in yeast, Alfred Benzon Symposium, Vol. 16. Munksgaard, Copenhagen. p. 383.

LAEMMLI, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680-685.

LAING, E. & PRETORIUS, I.S., 1990. Cloning of an *Erwinia chrysanthemi* pectate lyase gene into *Saccharomyces cerevisiae*. *S. Afr. Soc. Microbiol. Cong. 6th, Stellenbosch.* p. 4.6.

LAUTENSACH, A. & SUBDEN, R.E., 1984. Cloning of malic acid assimilating activity from *Leuconostoc oenos* in *E. coli*. *Microbios* **39,** 29-39.

LEE, S.Y. & KNUDSEN, F.B., 1985. Differentiation of brewery yeast strains by restriction endonuclease analysis of their mitochondrial DNA. *J. Inst. Brew.* **91,** 169-173.

LEUSCH, H.G., HOFFMANN, M. & EMEIS, C.C., 1985. Fluorometric determination of the total DNA content of different yeast species using 4',6-diamidino-2 phenyl-indol-dihydrochloride. *Can. J. Microbiol.* **31,** 1164-1166.

LEWIS, C.W., JOHNSTON, J.R. & MARTIN, P.A., 1976. The genetics of yeast flocculation. *J. Inst. Brew.* **82,** 158-160.

LODDER, J., 1970. The yeasts (second edition). North-Holland Publ. Co., Amsterdam, The Netherlands.

LÖFROTH, G. & GEJVALL, T., 1971. Diethyl pyrocarbonate: formation of urethan in treated beverages. *Science* **174,** 1248-1250.

MALIK, V.S., 1989. Biotechnology- The golden age. *Adv. Appl. Microbiol.* **34,** 263-306.

MARTENS, F.B., VAN DEN BERG, R. & HARTEVELD, P.A., 1985. DNA fingerprinting of yeast strains. *Eur. Brew. Conv. Proc. Cong. 20th Helsinki.* pp. 211-217.

MARMUR, J. & DOTY, P., 1962. Determination of the base composition of DNA from its thermal denaturation temperature. *J. Mol. Biol.* **3,** 208-218.

MAULE, A.P. & THOMAS, P.D., 1973. Strains of yeast lethal to brewery yeasts. *J. Inst. Brew.* **79,** 137-141.

MIKI, B.L.A., POON, N.H., JAMES, A.P. & SELIGY, V.L., 1980. Flocculation in *Saccharomyces cerevisiae:* mechanism of cell-cell interactions. In: STEWART, G.G. & RUSSELL, I. (eds.). Current developments in yeast research. Pergamon Press, Canada Ltd., Toronto. pp. 165-170.

MIKI, B.C.A., POON, N.H., JAMES, A.P. & SELIGY, V.L., 1981. Flocculation in *Saccharomyces cerevisiae:* mechanism of cell-cell interactions. In: STEWART, G.G. & RUSSELL, I. (eds.). Advances in biotechnology: current developments in yeast research. Pergamon Press, Canada Ltd., Toronto. pp. 193-197.

MIKI, B.L.A., POON, N.H., JAMES, A.P. & SELIGY, V.L., 1982. Possible mechanism for flocculation interactions governed by gene *FLO2* in *Saccharomyces cerevisiae*. *J. Bacteriol.* **150,** 878-889.

MILL, P.J., 1964. The nature of the interactions between flocculent cells in the flocculation of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **35,** 61-68.

MILLAR, D.G., GRIFFITHS-SMITH, K., ALGAR, E. & SCOPES, R.K., 1982. Activity and stability of glycolytic enzymes in the presence of ethanol. *Biotechnol. Lett.* **4,** 601-606.

MOLAN, P.S., EDWARDS, M. & ESCHENBRUCH, R., 1982. Foaming in wine making: II. Separation and partial characterization of foam inducing proteins excreted by pure culture wine yeasts. *Eur. J. Appl. Microbiol. Biotechnol.* **16,** 110-113.

MORTIMER, R.K., SCHILD, D., CONTOPOULOU, C.R. & KANS, J.A., 1989. Genetic map of *Saccharomyces cerevisiae,* Edition 10. *Yeast* **5,** 321-403.

MOWSHOWITZ, D.B., 1979. Gene dosage effects on the synthesis of maltose in yeast. *J. Bacteriol.* **137,** 1200-1207.

NAGODAWITHANA, T.W., CASTELLANO, C. & STEINKRAUS, K.H., 1974. Effect of dissolved oxygen, temperature, initial cell count, and sugar concentration on the viability of *Saccharomyces cerevisiae* in rapid fermentations. *Appl. Microbiol.* **28,** 383-391.

NAGODAWITHANA, T.W. & STEINKRAUS, K.H., 1976. Influence of the rate of ethanol production and accumulation of the viability of *Saccharomyces cerevisiae* in "rapid fermentation". *Appl. Environ. Microbiol.* **31,** 158-162.

NAGODAWITHANA, T.W., WHITT, J.T. & CUTAIA, A.J., 1977. Study of the feedback effect of ethanol on selected enzymes of the glycolytic pathway. *J. Am. Soc. Brew. Chem.* **35,** 179-183.

NAUMOV, G.I., TYURINA, L.V., BUR'YAN, N.I. & NAUMOVA, T.I., 1973. Wine-making an ecological niche of type K_2 killer *Saccharomyces cerevisiae*. *Biologicgeskie Nauki* **16,** 103-107.

NAUMOVA, T.I. & NAUMOV, G.I., 1973. Comparative genetics of yeast XIII. Comparative study of killer strains of *Saccharomyces* from different collections. *Genetika* **6,** 140-145.

NEWLON, C.S. & FANGMAN, W.L., 1975. Mitochondrial DNA synthesis in cell cycle mutants of *Saccharomyces cerevisiae*. *Cell.* **5,** 423-428.

NILSSON-TILLGREN, T., GJERMANSEN, C., HOLMBERG, S., PETERSEN, J.G.L. & KIELLAND-BRANDT, M.C., 1986. Analysis of chromosome V and the *ILV1* gene from *Saccharomyces carlsbergensis*. *Carlsberg Res. Commun.* **51,** 309-326.

NILSSON-TILLGREN, T., GJERMANSEN, C., KIELLAND-BRANDT, M.C., PETERSEN, J.G.L. & HOLMBERG, S., 1981. Genetic differences between *Saccharomyces carlsbergensis* and *S. cerevisiae*. Analysis of chromosome III by single chromosome transfer. *Carslberg Res. Commun.* **46,** 65-76.

NILLSON-TILLGREN, T., PETERSEN, J.G.L., HOLMBERG, S. & KIELLAND-BRANDT, M.C., 1980. Transfer of chromosome III during *kar* mediated cytoduction in yeast. *Carslberg Res. Commun.* **45,** 113-117.

NYKÄNEN, L. & NYKÄNEN, I., 1977. Production of esters by different yeast strains in sugar fermentations. *J. Inst. Brew.* **83,** 30-31.

OLIVER, S.G., 1987. Physiology and genetics of ethanol tolerance in yeast. In: STEWART, G.G., RUSSELL, I., KLEIN, R.D. & HIEBSCH, R.R. (eds.). Biological research on industrial yeasts, Vol. I. CRC Press Inc. Boca Raton, Florida, pp. 81-98.

ONO, B-I, & ISHINO-ARAO, Y., 1988. Inheritance of chromosome length

polymorphisms in *Saccharomyces cerevisiae*. *Curr. Genet.* **14,** 413-418.

ORR-WEAVER, T.L., SZOSTAK, J.W. & ROTHSTEIN, R.J., 1981. Yeast transformation: a model system for the study of recombination. *Proc. Natl. Acad. Sci. USA.* **78,** 6354-6358.

OSHIMA, Y. & TAKANO. I., 1970. Mating types in *Saccharomyces:* their convertibility and homothalism. *Genetics* **67,** 327-335.

OUCHI, K. & AKIYAMA, H., 1971. Non-foaming mutants of saké yeasts. Selection by cell agglutination method and by froth flotation method. *Agric. Biol. Chem.* **35,** 1024-1032.

OUCHI, K. & AKIYAMA, H., 1976. Breeding of useful killer saké yeasts by repeated back-crossing. *J. Ferment. Technol.* **54,** 615-623.

OUCHI, K., NISHIYA, T. & AKIYAMA, H., 1983. UV-Killed protoplast fusion as a method for breeding killer yeasts. *J. Ferment. Technol.* **61,** 631-635.

OUCHI, K., WICKNER, R.B., TOH-E, A. & AKIYAMA, H., 1979. Breeding of killer yeast for saké brewing by cytoduction. *J. Ferment. Technol.* **57,** 483-487.

OUGH, C.S., 1976a. Ethylcarbamate in fermented beverages and foods. I. Naturally occurring ethylcarbamate. *J. Agric. Food Chem.* **24,** 323-328.

OUGH, C.S., 1976b. Ethylcarbamate in fermented beverages and foods. II. Possible formation of ethylcarbamate from diethyl dicarbonate addition to wine. *J. Agric. Food Chem.* **24,** 328-331.

OUGH, C.S., CROWELL, E.A. & GUTLOVE, B.R., 1988a. Carbamyl compound reactions with ethanol. *Am. J. Enol. Vitic.* **39,** 239-242.

OUGH, C.S., CROWELL, E.A. & MOONEY, L.A., 1988b. Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acids, urea, and ammonia: effects of fortification on intracellular and extracellular precursors. *Am. J. Enol. Vitic.* **39,** 243-249.

PANCHALL, G.J. & STEWART, G.G., 1980. The effect of osmotic pressure on the production and excretion of ethanol and glycerol by a brewing yeast strain. *J. Inst. Brew.* **86,** 207-210.

PAQUIN, C. & ADAMS, J., 1983. Frequency of fixation of adaptive mutations is higher in evolving diploid than haploid yeast populations. *Nature* **302,** 495-500.

PARENT, S.A., FENIMORE, C.M. & BOSTIAN, K.A., 1985. Vector systems for the expression, analysis and cloning of DNA sequences in *S. cerevisiae*. *Yeast* **1,** 83-138.

PEDERSEN, M. B., 1983a. DNA sequence polymorphisms in the genus *Saccharomyces*. *I. Comparison of the HIS4* and ribosomal RNA genes in lager strains, ale strains and various species. *Carlsberg Res. Commun.* **48,** 485-503.

PEDERSEN, M.B., 1983b. Fingerprinting of DNA in the identification of yeast. *Eur. Brew. Conc. Proc. Cong. 19th, London.* pp. 457-463.

PEDERSEN, M.B., 1985a. Brewing yeast identification by DNA fingerprinting. *Eur. Brew. Conv. Proc. Cong. 20th, Helsinki.* pp. 202-210.

PEDERSEN, M.B., 1985b. DNA sequence polymorphisms in the genus *Saccharomyces*. II. Analysis of the genes *RDN1, HIS4, LEU2* and TY transposable elements in Carslberg, Tuborg and 22 Bavarian brewing strains. *Carlsberg Res. Commun.* **50,** 263-272.

PEDERSEN, M.B., 1986a. DNA sequence polymorphisms in the genus *Saccharomyces*. III. Restriction endonuclease fragment patterns of chromosomal regions in brewing and other yeast strains. *Carlsberg Res. Commun.* **51,** 163-183.

PEDERSEN, M.B., 1986b. DNA sequence polymorphisms in the genus *Saccharomyces*. IV. Homologous chromosomes III of *Saccharomyces bayanus, S. carlsbergensis,* and *S. uvarum*. *Carlsberg Res. Commun.* **51,** 185-202.

PEDERSEN, M.B., 1987. Practical use of electro-karyotypes for brewing yeast identification. *Eur. Brew. Conv. Proc. Cong. 21st, Madrid.* pp. 489-496.

PEDERSEN, M.B., 1989. The use of digital image processing for brewing yeast DNA analysis. *Eur. Brew. Conv. Proc. Cong. 22nd, Zurich.* pp. 521-528.

PENTTILÄ, M.E., ANDRE, L., SALOHEIMO, M., LEHTOVAARA, P. & KNOWLES, J.K.C., 1987. Expression of two *Trichoderma reesei* endoglucanases in the yeast *Saccharomyces cerevisiae*. *Yeast* **3,** 175-185.

PHAFF, H.J. 1981. Industrial micro-organisms. *Sci. Am.* **245,** 76-90.

PFEIFFER, P. & RADLER, F., 1982. Purification and characterization of extracellular and intracellular killer toxin of *Saccharomyces cerevisiae* strain 28. *J. Gen. Microbiol.* **128,** 2699-2706.

POLACHECK, I. & LEBENS, G.A., 1989. Electrophoretic karyotype of the pathogenic yeast *Cryptococcus neoformans*. *J. Gen. Microbiol.* **135,** 65-71.

PRETORIUS, I.S., 1989. Genetic improvement of brewer's yeast. *Inst. Brew. Proc. Sci. Tech. Conv. 2nd, Johannesburg.* pp. 170-186.

PRETORIUS, I.S. & MARMUR, J., 1988. Localization of yeast glucoamylase genes by PFGE and OFAGE. *Curr. Genet.* **14,** 9-13.

PRICE, C.W., FUSON, G.B. & PHAFF, H.J., 1978. Genome comparison in yeast systematics: delimination of species within the genera *Schwanniomyces, Saccharomyces, Debaryomyces* and *Pichia*. *Microbiol. Rev.* **42,** 161-193.

RANK, G.H., CASEY, G. & XIAO, W., 1988. Gene transfer in industrial *Saccharomyces* yeasts. *Food Biotechnol* **2,** 1-41.

RANKINE, B.C., 1968. The importance of yeasts in determining the composition and quality of wine. *Vitis* **7,** 22-49.

RAYMOND, S. & WANG, Y.-J., 1960. Preparation and properties of acrylamide gel for use in electrophoresis. *Anal. Biochem* **1,** 391-396.

ROGERS, D. & BEVAN, E.A., 1978. Group classification of killer yeasts based on cross-reactions between strains of different species and origin. *J. Gen. Microbiol.* 105, 199-202.

ROMANO, P., SOLI, M.G., SUZZI, G., GRAZIA, L. & ZAMBONELLI, C., 1985. Improvement of a wine *Saccharomyces cerevisiae* strain by a breeding program. *Appl. Environ. Microbiol.* **50,** 1064-1067.

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

ROUS, C.V., SNOW, R. & KUNKEE, R.E., 1983. Reduction of higher alcohols by fermentation with a leucine-auxotrophic mutant of wine yeast. *J. Inst. Brew.* **89,** 274-278.

RUPELA, O.P. & TAURO, P., 1984. Isolation and characterization of low hydrogen sulphide producing wine yeast. *Enz. Microb. Technol.* **6,** 419-421.

RUSSELL, I. & STEWART, G.G., 1979. Spheroplast fusion of brewer's yeast strains. *J. Inst. Brew.* **85,** 95-98.

RUSSELL, I., STEWART, G.G., READER, H.P., JOHNSTON, J.R. & MARTIN, P.A., 1980. Revised nomenclature of genes that control yeast flocculation. *J. Inst. Brew.* **86,** 120-121.

SAKAI, A., SHIMIZU, Y. & HISHINUMA, F., 1990. Integration of heterologous genes into the chromosome of *Saccharomyces cerevisiae* using a delta sequence of yeast retrotransposon Ty. *Appl. Microbiol. Biotechnol.* **33,** 302-306.

SANTA MARIA, J. & VIDAL, D., 1973. Genetic control of "flor" formation by *Saccharomyces*. *J. Bacteriol.* **113,** 1078-1080.

SCHAAF, I., HEINISCH, J. & ZIMMERMANN, F.K., 1989. Overproduction of glycolytic enzymes in yeast. *Yeast,* **5,** 285-290.

SCHIESL, R.H. & GIETZ, R.D., 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16,** 339-346.

SCHILDKRAUT, C.L., MARMUR, J. & DOTY, P., 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. *J. Mol. Biol.* **4,** 430-433.

SCHWARTZ, D.C. & CANTOR, C.R., 1984. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37,** 67-75.

SEEHAUS, T., RODICIO, R., HEINISCH, J., AGUILERA, A., SCHMITT, H. D. & ZIMMERMANN, F.K., 1985. Specific gene probes as tools in yeast taxonomy. *Curr. Genet.* **10,** 103-110.

SEKI, T., CHOI, E.-H. & RYU, D., 1985. Construction of killer wine yeast strain. *Appl. Environ. Microbiol.* **49,** 1211-1215.

SCHERER, S., MANN, C. & DAVIS, R.W., 1982. Reversion of a promoter delection in yeast. *Nature* **289,** 815-819.

SHIMIZU, K., ADACHI, T., KITANO, K., SHIMAZAKI, T., TOTSUKA, A., HARA, S. & DITTRICH, H.H., 1985. Killler properties of wine yeasts and characterization of killer wine yeasts. *J. Ferment. Technol.* **63,** 421-429.

SKIPPER, N., THOMAS, D.Y. & LAU, P.C.K., 1984. Cloning and sequencing of the preprotoxin-coding region of the yeast M1 double-stranded RNA. *EMBO J.* **3,** 107-111.

SMITH, C.L. & CANTOR, C.R., 1987. Purification, specific fragmentation, and separation of large DNA molecules. *Meth. Enzymol.* **155,** 449-467.

SMITH, C.L., MATSUMOTO, T., NIWA, O., KLCO, S., FAN, J.-B., YANAGIDA, M. & CANTOR, C.R., 1987. An electrophoretic karyotype for *Schizosaccharomyces pombe* by pulsed field gel electrophoresis. *Nuc. Acids Res.* **15,** 4481-4489.

SNELL, R.G. & WILKINS, R.J., 1986. Separation of chromosomal DNA molecules from *C. albicans* by pulsed field gel electrophoresis. *Nuc. Acids Res.* **14,** 4401-4406.

SNOW, R., 1979. Toward genetic improvement of wine yeast. *Am. J. Enol. Vitic.* **30,** 33-37.

SNOW, R. 1983. Genetic improvement of wine yeast. In: SPENCER, J.F.T., SPENCER, D.M. & SMITH, A.R.W. (eds.). Yeast genetics- fundamental and applied aspects. Springer-Verlag, New York. pp. 439-459.

SOR, F. & FAKUHARA, H., 1989. Analysis of chromosomal DNA patterns of the genus *Kluyveromyces. Yeast* **5,** 1-10.

SOUTHERN, E.M., 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503-517.

SPENCER, J.F.T. & SPENCER, D.M., 1977. Hybridization of non-sporulating and weakly sporulating strains of brewer's and distiller's yeasts. *J. Inst. Brew.* **83,** 287-289.

STEENSMA, H.Y., DE JONGH, F.C.M. & LINNEKAMP, M., 1988. The use of electrophoretic karyotypes in the classification of yeasts: *Kluyveromyces marxianus* and *K. lactis. Curr. Genet.* **14,** 311-317.

STEVENS, B., 1981. Mitochondrial structure. In: STRATHERN, J.N., JONES, E.W. & BROACH, J.R. (eds.). The molecular biology of the yeast *Saccharomyces:* life cycle and inheritance. Cold Spring Harbor, New York. pp. 471-504.

STEWART, G.G. & RUSSELL, I., 1983. Aspects of the biochemistry and genetics of sugar and carbohydrate uptake by yeasts. In: SPENCER, J.F.T., SPENCER, D. M. & SMITH, A.R.W. (eds.). Yeast genetics: fundamental and applied aspects. Springer-Verlag, New York. pp. 461-481.

STEWART, G.G. & RUSSELL, I., 1986. Centenary review. One hundred years of yeast research and development in the brewing industry. *J. Inst. Brew.* **92,** 537-558.

STEWART, G.G., RUSSELL, I. & PANCHAL, C., 1981. The genetic manipulation of industrial yeast strains. In: STEWART, G.G, & RUSSELL, I., (eds.). Current developments in yeast research. Pergamon Press, Toronto. pp. 14-24.

STRATHERN, J.N. & HERSKOWITZ, I., 1979. Asymmetry and directionality in production of new cell types during clonal growth: the switching pattern of homothallic yeast. *Cell* **17,** 371-381.

STRATFORD, M. & BRUNDISH, H.M., 1990. Yeast flocculation: cationic inhibition. *Yeast* **6,** 77-86.

STURLEY, S.L. & YOUNG, T.W., 1986. Genetic manipulation of commercial yeast strains. *Biotechnol. Genet. Engin. Rev.* **4,** 1-38.

SUBDEN, R.E. & OSOTHSILP, C., 1987. Malic acid metabolism in wine yeasts. In: STEWART, G.G., RUSSELL, I., KLEIN, R.D. & HIEBSCH, R.R. (eds.). Biological research on industrial yeasts, Vol. 2. CRC Press Inc., Boca Raton, Florida. pp. 67-76.

SUIZU, T., IIMURA, Y., GOMI, K., TAKAHASHI, K., HARA, S. & YOSHIZAWA, K., 1989. L-Canavanine resistance as a positive selectable marker in diploid yeast transformation through integral disruption of the *CAN1* gene. *Agric. Biol. Chem.* **53,** 431-436.

SZOSTAK, J.W. & WU, R., 1979. Insertion of a genetic marker into the ribosomal DNA of yeast. *Plasmid* **2,** 536-554.

TAKAGI, A., HARASHIMA, S. & OSHIMA, Y., 1985. Hybridization and polyploidization of *Saccharomyces cerevisiae* strains by transformation-associated cell fusion. *Appl. Environ. Microbiol.* **49,** 244-246.

TAKAHASHI, T., 1978. Genetic analysis of a German wine yeast. *Bull. Brew. Sci.* **24,** 39-47.

TAKATA, Y., WATARI, J., NISHIKAWA, N. & KAMADA, K., 1989. Electrophoretic banding patterns of chromosomal DNA from yeasts. *Am. Soc. Brew. Chem.* **47,** 109-113.

TALBOT, N.J., RAWLINS, D. & CODDINGTON, A., 1988. A rapid method for ploidy determination in fungal cells. *Curr. Genet.* **14,** 51-52.

THOMAS, D.S. & ROSE, A.H., 1979. Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as effected by plasmamembrane lipid composition. *Arch. Microbiol.* **122,** 49-55.

THOMAS, D. Y., VERNET, T., BOONE, C., GREENE, D., LOLLE, S.J. & BUSSEY, H., 1987. The yeast killer toxin expression system: functional analysis and biotechnological applications. In: STEWART, G.G., RUSSELL, I., KLEIN, R.D. & HIEBSCH, R.R. (eds.). Biological research on industrial yeasts, Vol. I. CRC Press Inc., Boca Raton, Florida. pp. 155-163.

THORNTON, R.J., 1978a. Investigations on the genetics of foaming in wine yeasts. *Eur. J. Microbiol. Biotechnol.* **5,** 103-107.

THORNTON, R.J., 1978b. The mapping of two dominant genes for foaming in wine yeasts. *FEMS Microbiol. Lett.* **4,** 207-209.

THORNTON, R.J., 1980. Genetic investigation and modification of wine yeast characteristics. Grape and Wine Centennial Symposium Proceedings. University of California Press, Davis. pp. 97-102.

THORNTON, R.J., 1982. Selective hybridisation of pure culture wine yeasts. II. Improvement of fermentation efficiency and inheritance of SO_2 tolerance. *Eur. J. Appl. Microbiol. Biotechnol.* **14,** 159-164.

THORNTON, R.J., 1983. New yeast strains from old- the application of genetics to wine yeasts. *Food Technol. Australia* **35,** 46-50.

THORNTON, R.J. & BUNKER, A., 1989. Characterization of wine yeasts for genetically modifiable properties. *J. Inst. Brew.* **95,** 181-184.

THORNTON, R.J. & ESCHENBRUCH, R., 1976. Homothallism in wine yeasts. *Antonie van Leeuwenhoek* **42,** 503-509.

TIPPER, D.J. & BOSTIAN, K.A., 1984. Double-stranded ribonucleic acid killer systems in yeasts. *Microbiol. Rev.* **48,** 125-156.

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

TROMP, A. & DE KLERK, C.A., 1988. Effect of copperoxychloride on the fermentation of must and on wine quality. *S. Afr. J. Enol. Vitic.* **9**(1), 31-36.

TSUCHIYA, T., FUKAZAWA, Y. & KAWAKITA, S., 1965. Significance of serological studies on yeast. *Mycopathol. Mycol. Appl.* **26,** 1-15.

TUBB, R.S. & HAMMOND, J.R.M., 1987. Yeast genetics. In: PRIEST, F.G. & CAMPBELL, I. (eds.). Brewing microbiology. Elsevier Applied Science Publishers, Essex, England. pp. 47-82.

TUBB, R.S., SEARLE, B.A., GOODEY, A.R. & BROWN, A.J.P., 1981. Rare mating and transformation for construction of novel brewing yeasts. *Eur. Brew. Conv. Proc. Cong. 18th, Copenhagen.* pp. 487-496.

TZAGOLOFF, A. & DIECKMANN, C.L., 1990. *PET* genes of *Saccharomyces cerevisiae. Microbiol. Rev.* **54.** 211-225.

VAN ARSDELL, J.N., KWOK, S., SCHWEICKART, V.L., LADNER, M.B., GELFAND, D.H. & INNIS, M.A., 1987. Cloning, characterization, and expression in *Saccharomyces cerevisiae* of endoglucanase I from *Trichoderma reesei. Bio/Technology* **5,** 60-64.

VAN DER WESTHUIZEN, T.J. & PRETORIUS, I.S., 1989. Genetic characterization and breeding of wine yeasts. *S. Afr. Soc. Enol. Vitic. Cong. 13th, Cape Town.*

VAN DER WESTHUIZEN, T.J. & PRETORIUS, I.S., 1990. Genetic characterization and breeding of wine yeasts. *S. Afr. Soc. Microbiol. Cong. 6th., Stellenbosch.* pp. 15.15.

VAN SOLIGEN, P. & VAN DER PLAAT, J.B., 1977. Fusion of yeast spheroplasts. *J. Bacteriol.* **130,** 946-947.

VAN UDEN, N., 1985. Ethanol toxicity and ethanol tolerance in yeasts. *Ann. Rep. Ferment. Proc.* **8,** 11-58.

VAN VUUREN, H.J.J. & WINGFIELD, B.D., 1986. Killer yeasts - cause of stuck fermentations in a wine cellar. *S. Afr. J. Enol. Vitic.* **7,** 113-118.

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

VAN VUUREN, H.J.J. & VAN DER MEER, L.J., 1988. Characterization of brewing yeast strains by numerical analysis of total soluble cell protein patterns. *J. Inst. Brew.* **94,** 245-248.

VEZINHET, F., 1989. Obtention by intragenomic recombination of non-foaming clones from an enological strain of yeast *Saccharomyces cerevisiae. Sciences Des Aliments* **9,** 253-265.

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

VILJOEN, B.C., KOCK, J.L.F., MILLER, M. & COETZEE, D.J., 1989. The value of orthoganal-field-alternation gel electrophoresis and other criteria in the delimination of anomorphic/telomorphic relations. *System. Appl. Microbiol.* **11,** 305-311.

VOLLRATH, D. & DAVIS, R.W., 1987. Resolution of DNA molecules greater than 5 megabases by contour-clamped homogeneous electric fields. *Nuc. Acids Res.* **15,** 7865-7876.

VON WETTSTEIN, D., 1983. Emil Christian Hansen centennial lecture: from pure yeast culture to genetic engineering in brewers yeast. *Eur. Brew. Conv. Proc., Cong. 19th, London.* pp. 97-119.

VON WETTSTEIN, D., GJERMANSEN, C., HOLMBERG, S., KIELLAND-BRANDT, M.C., NILSSON-TILLGREN, T., PEDERSEN, M.B., PETERSEN, J.G.L. & SIGSGAARD, P., 1984. Genetic engineering in the improvement of brewers yeast. *MBAA Tech. Quart.* **21,** 45-61.

WARNER, J.R., 1982. The yeast ribosome: structure, function, and synthesis. In: STRATHERN, J.N., JONES, E.W. & BROACH, J.R. (eds.). The molecular biology of the yeast *Saccharomyces* - metabolism and gene expression. Cold Spring Harbor Laboratory, New York. pp. 529-560.

WEBB, A.D. & INGRAHAM, J.L., 1963. Fusel oil: a review. *Adv. Appl. Microbiol.* **5**, 317-353.

WEBSTER, T.D. & DICKSON, R.C., 1983. Direct selection of *Saccharomyces cerevisiae* resistant to the antibiotic G418 following transformation with a DNA vector carrying the kanamycin-resistance gene of Tn903. *Gene* **26,** 243-252.

WICKNER, R.B., 1981. Killer systems in *Saccharomyces cerevisiae*. In: STRATHERN, J.N., JONES, E.W. & BROACH, J.R. (eds.). The molecular biology of the yeast *Saccharomyces cerevisiae*. Cold Spring Harbor, New York. pp. 415-444.

WILKINSON, L.E. & PRINGLE, J.R., 1974. Transient G1 arrest of *Saccharomyces cerevisiae* of mating type α by a factor produced by cells of mating type *a*. *Exp. Cell. Res.* **89,** 175-187.

WILLIAMS, S.A., HODGES, R.A., STRIKE, T.L., SNOW, R. & KUNKEE, R.E., 1984. Cloning the gene for the malolactic fermentation of wine from *Lactobacillus delbrueikii* in *Eschereichia coli* and yeasts. *Appl. Environ. Microbiol.* **47,** 288-293.

WINGFIELD, B.D., VAN VUUREN, H.J.J. & PRETORIUS, I.S., 1989. Size differentiation of M_2 genomes among K_2 killer yeasts. *Mycol. Res.* **92,** 364-367.

WINGFIELD, B.D., SOUTHGATE, V.J., PRETORIUS, I.S. & VAN VUUREN, H.J.J., 1990a. A K_2 neutral *Saccharomyces cerevisiae* strain contains a variant K_2M genome. *Yeast* **6,** 159-169.

WINGFIELD, B.D., VAN DER MEER, L.J., PRETORIUS, I.S. & VAN VUUREN H.J.J., 1990b. K_3 killer yeast is a mutant K_2 killer yeast. *Mycol. Res. 94, 901-906.*

WOODS, D.R. & BEVAN, E.A., 1968. Studies on the nature of the killer factor produced by *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **51,** 115-126.

WÖHRMANN, H. & LANGE, P., 1980. The polymorphism of esterases in yeast (*Saccharomyces cerevisiae*). *J. Inst. Brew.* **86,** 174.

YAMADA, Y., TIKINAMI-NAKAMURA, H., TAHARA, Y. & SMITH, M.T., 1980. The coenzyme Q system in the classification of the ascosporogenous yeast genus *Dekkera* and the sporogenous yeast genus *Brettanomyces*. *Antonie van Leeuwenhoek* **46**, 595-599.

YAP, A.S.J., 1987. Development of new yeast strains for wine-making. *The Australian Grapegrower and Winemaker* **287,** 19-21.

YOKOMORI, Y., AKIYAMA, H. & SHIMIZU, K., 1989. Breeding of wine yeasts through protoplast fusion. *Yeast (Spec. Iss.)* pp. S145-S150.

YOUNG, T.W., 1987. Killer yeasts. In: ROSE, A.H. & HARRISON, J.S. (eds.). The yeasts, Vol. II. Academic Press Inc., London. pp. 131-164.

YOUNG, T.W. & YAGIU, M., 1978. A comparison of the killer character in different yeasts and its classification. *Antonie van Leeuwenhoek* **44,** 59-77.

ZAWORSKI, P.G. & HEIMSCH, R.C., 1987. The isolation and characterization of flocculent yeasts. In: STEWART, G.G., RUSSELL, I., KLEIN, R.D. & HIEBSCH R.R. (eds.). Biological research on industrial yeasts, Vol. III. CRC Press Inc., Boca Raton, Florida. pp. 185-196.

ZHU, J., CONTRERAS, R. & FIERS, W., 1986. Construction of stable laboratory and industrial yeast strains expressing a foreign gene by integrative transformation using a dominant selection system. *Gene* **50,** 225-237.