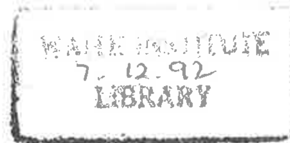
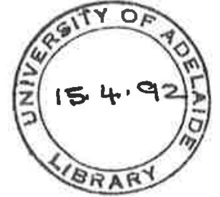

MOLECULAR GENETIC MANIPULATION OF WINE YEASTS

BY

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DECLARATION

The work presented in this thesis is my own unless otherwise acknowledged, and has not previously been submitted to any university for the award of any degree of diploma. This thesis may be made available for loan or photocopying provided that an acknowledgement is made in the instance of any reference to this work.

Jenny E. Petering

December 1991

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Conference proceedings

Petering, J.E., P.A. Henschke and P. Langridge (1989). Genetic engineering of wine yeasts. Proc. 7th Aust. Wine Ind. Tech. Conf. Adelaide, Australia. pp. 176-179.

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Summary

A system has been established for the transformation of wine yeast strains. This system utilizes a mutant *Saccharomyces cerevisiae* acetolactate synthase gene which confers resistance to the herbicide sulfometuron methyl and acts as a dominant selection marker. Satisfactory transformation efficiencies were achieved with both a self-replicating plasmid and an integrating vector in several wine yeast strains. The integrating vector was successfully targeted to the *ILV2* locus on chromosome XIII of the yeast genome, and was stably maintained throughout fermentation. Fermentation trials indicated that the transformation system does not adversely affect the growth kinetics or fermentation rate of the wine yeast strain. Similarly, there were no significant differences in the pH measurements or the alcohol content of wines produced by the parent and transformed strains.

This transformation system was used to develop a procedure for the genetic marking of wine yeast strains. Marking was achieved by the introduction into wine yeasts of the *Escherichia coli* β-glucuronidase (GUS) gene. The GUS gene was adapted for expression in yeast by ligating the *Saccharomyces cerevisiae* alcohol dehydrogenase promoter and terminator sequences to the coding region of the *Escherichia coli* uidA gene. The GUS construct was introduced into the genome of a wine yeast strain by integration into chromosome XIII. The marked strain did not show any significant differences in fermentation performance when compared with the original parent strain. Stability of the marker was confirmed by the observation that the GUS construct was maintained in > 99% of the total yeast population at the end of the fermentation. A simple assay procedure was developed to detect GUS activity in the marked yeast cells or colonies. This method provides a means for genetic marking and subsequent rapid identification of wine yeast strains of choice.

The application of the marked strain to studies in oenology was demonstrated in two separate investigations. First, the efficiency of killer toxin in fermenting grape juice was determined. The marked killer strain was cured of its M-dsRNA genome to enable direct assessment of the efficiency of killer toxin under fermentation conditions. Killer activity was clearly evident in fermenting Riesling grape juice of pH 3.1 at 18°C but depended on the proportion of killer to sensitive cells at the time of inoculation. Killer activity was detected only when the ratio of killer to sensitive cells exceeded 1:2. At the highest ratio of killer to sensitive cells tested (2:1), complete elimination of sensitive cells was not achieved.

Secondly, inoculation efficiency of the marked strain under various fermentation conditions was analysed. Variables studied were time of inoculation, addition to the must of SO_2 (100 mg/L), production of killer toxin and fermentation temperature. Results indicated that dominance of the ferment by the inoculated strain could be ensured by an early inoculation regardless of any other variable. A combination of SO_2 treatment and low fermentation temperature (10°C) was also effective in ensuring inoculation success.

This thesis describes, therefore, the establishment of wine yeast transformation procedure and subsequent use in the development of a yeast genetic marking system; and demonstrates the application of marked yeast strains to the wine industry.

Table of Contents

Page

Chapter 1 General introduction and Project aims 1

Chapter 2 Literature Review 5

2.1 ORIGIN AND CLASSIFICATION OF WINE YEASTS 5
2.2 GENETIC FEATURES OF WINE YEASTS 7
2.2.1 Life cycle and sporulation 7
2.2.2 Chromosomes and ploidy 9
2.2.3 Extrachromosomal elements 11
2.3 GENETIC TECHNIQUES FOR YEAST STRAIN IMPROVEMENT 14
2.3.1 Clonal selection 14
2.3.2 Clonal selection after mutagenesis 15
2.3.3 Hybridization 15
2.3.4 Rare mating 16
2.3.5 Spheroplast fusion 17
2.3.6 Recombinant DNA technology 18
2.4 TARGETS FOR WINE YEAST STRAIN DEVELOPMENT 18
2.4.1 Sedimentation and flocculation 19
2.4.2 Non-foaming mutants 20
2.4.3 Sulfite and Sulfide Production 21
2.4.4 Ethanol tolerance 23
2.4.5 Higher alcohols 25
2.4.6 Killer factor 26
2.4.7 Malolactic fermentation 26
2.4.8 Genetic marking 27
2.5 CONCLUSIONS 28

Chapter 3 Materials and Methods 30

3.1 STRAINS AND MEDIA 30
3.2 YEAST TRANSFORMATION METHODS 30
3.2.1 Alkali cation transformation 30
3.2.2 Spheroplast fusion method 31
3.2.3 Electroporation of intact yeast cells 32
3.3 RECOMBINANT DNA TECHNIQUES 33
3.3.1 Restriction digests, fragment isolation and ligation 33
3.3.2 Electrophoresis of DNA 33
3.3.3 Bacterial transformation 34
3.3.4 Plasmid isolation 34
3.3.5 Yeast DNA isolation 34

3.3.6 Southern hybridization 35
3.4 PROTEIN SYNTHESIS ANALYSIS 35
3.4.1 Incorporation of radioactivity into yeast proteins 35
3.4.2 Electrophoresis of proteins 36
3.5 PULSED FIELD GEL ELECTROPHORESIS 36
3.6 β-GLUCURONIDASE (GUS) ASSAYS 37
3.6.1 Enzyme activity assays 37
3.6.2 Agar plate assays 38
3.6.3 Microscopic visualization 38
3.7 KILLER YEAST MANIPULATIONS 38
3.7.1 Curing of killer strain 3AM 38
3.7.2 Assay for cured strain 38
3.7.3 dsRNA isolation 39
3.8 MICROVINIFICATION TRIALS 39
3.8.1 Fermentation procedure 39
3.8.2 Wine analysis 40
3.9 LARGE SCALE LABORATORY FERMENTATIONS 40

Chapter 4 Establishing a system for the genetic manipulation of wine yeasts 42
4.1 INTRODUCTION 42
4.1.1 Selection systems for identifying transformants 42
4.1.2 Gene transfer vectors 45
4.1.3 Tranformation procedures 48
4.1.4 Expression and secretion of foreign proteins 50
4.1.5 Chapter aims 52
4.2 RESULTS 52
4.2.1 Choice of a selectable marker 52
4.2.2 Choice of a transformation procedure 57
4.3.3 Analysis of transformants 60
4.3.4 Fermentation trials 64
4.3.5 Gene regulation during fermentation 68
4.3 DISCUSSION 69

Chapter 5 Development of a system for wine yeast strain marking and identification 72
5.1 INTRODUCTION 72
5.1.1 Yeast chromosomal fingerprints 74
5.1.2 Marked strains 78
5.2 RESULTS 79

5.2.1 Wine yeast chromosome fingerprinting 79
5.2.2 GUS-Vector construct 80
5.2.3 Transformation and Southern analysis 81
5.2.4 Development of a GUS assay for yeasts 83
5.2.5 β-Glucuronidase enzyme activity 87
5.2.6 Fermentation trials 89
5.2.7 Stability of GUS construct 92
5.3 DISCUSSION 93

Chapter 6 Determination of killer toxin activity in fermenting grape juice using a marked *Saccharomyces* strain 99
6.1. INTRODUCTION 99
6.2 RESULTS 101
6.2.1 Curing of Strain 3AM 101
6.2.2 Analysis of killer activity during fermentation 102
6.3 DISCUSSION 107

Chapter 7 Comparison of fermentation conditions by use of a marked strain 109
7.1 INTRODUCTION 109
7.1.1 Killer yeast inoculation 110
7.1.2 Addition of sulfur dioxide to grape must 111
7.1.3 Temperature of fermentation 111
7.2 RESULTS 112
7.2.1 Fermentations conducted at 20°C 114
7.2.2 Fermentations conducted at 10°C 117
7.2.3 Killer activity in the indigenous yeast population 119
7.3 DISCUSSION 119

Chapter 8 General Conclusions 125

References 127

Appendix 1 Publications 156

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Chapter 1 General introduction and Project aims

Yeasts were employed in the production of wine for several thousand years before their existence was recognised by Antonie van Leeuwenhoek in 1680. During the second half of the nineteenth century, Louis Pasteur demonstrated that living yeast cells were responsible for fermentation, or the conversion of sugar to ethanol and carbon dioxide (Demain and Solomon, 1981). Originally, yeasts present on grape skins and equipment were responsible for the "spontaneous" fermentation involved in wine-making. For the last century, however, the availability of pure yeast cultures has improved reproducibility in fermentations and the quality of the product (Tubb and Hammond, 1987). It is now possible to select specific yeast strains on the basis of their fermentation performance and the characteristics of their product.

Although the wine industry rapidly adopted pure culture inoculation technology, it has taken little active interest in yeast genetics and strain development programs (Thornton, 1983). Recent trends in the wine market, however, demand the modification of traditional wine yeast strains in the development of more cost-effective wine making practices. Toward this end, it is important to define the requirements of the wine industry in genetic terms - to select specific targets for yeast breeding programs. In order to identify these targets, it is necessary to consider the most desirable characteristics of a wine yeast (Thornton,1983):

- the efficient conversion of grape sugar to alcohol;
- the rapid initiation of fermentation immediately upon inoculation;
- the ability to ferment at low temperatures such as 10-14°C;
- tolerance to sulfur dioxide (used in wine making as a sterilizing agent and as an anti-oxidant);
- tolerance to ethanol (in order to ferment to dryness);

- low foaming activity;
- low production of volatile acids, acetaldehyde and sulphite;
- low hydrogen sulfide or mercaptan production;
- relatively low higher alcohol production;
- effective flocculation at the end of fermentation to aid clarification;
- relatively high glycerol production to contribute to the sensory qualities of the wine;
- the production of desirable fermentation bouquet;
- resistance to killer toxins and other zymocidal compounds.

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. The major source of this variation is the genetic constitution of the wine yeasts (Thornton, 1983). Although some of the requirements listed above are complex and difficult to define genetically without a better understanding of the biochemistry involved, they comprise specific targets for wine yeast modification programs.

Other possible targets for strain modification are processes involved in yeast management and in the synthesis of new products. For example, genetic manipulation can be used to insert specific markers into wine yeast strains as an aid to strain identification. This would be particularly useful for wineries using more than one strain, and in the optimization of must processing strategies. An area which has not yet been exploited is the use of wine yeasts to produce valuable by-products. Spent yeast from industrial processes is already used to produce yeast extracts and as an ingredient in foods and flavourings. Possibilities exist to produce higher value materials such as vitamins, enzymes, carbohydrates and lipids from wine yeasts. Genetic manipulation could facilitate maximum formation of the required product.

These and other potential applications of genetic manipulation to industrial yeast strains have been recognised for some time (Spencer and Spencer, 1983). Since the pioneering genetic studies of Winge (1935), in which the basic life-cycle of *Saccharomyces* was established, yeast strains have been interbred to produce new hybids. Classical genetic methods which have been involved in yeast manipulation programs to date include mutation and selection, hybridization, rare mating and spheroplast fusion (Tubb and Hammond, 1987). These techniques have enjoyed only limited success in the past, essentially for two reasons. First, methods such as cross breeding involve mating which requires spore formation; industrial yeasts, however, tend to be polyploid, which means they have a reduced ability to form spores. Secondly, although other techniques do not require spore formation on the part of yeast strains, they do involve gross and non-specific exchanges of genetic material. Therefore, while it is possible to introduce favourable characteristics into yeast strains using these techniques, it is quite probable that deleterious properties will be introduced simultaneously.

The relatively new methods of recombinant DNA technology overcome some of the problems inherent in the more traditional techniques. Recombinant DNA technology does not require spore formation by the parental yeast strains. It is, by nature, highly specific in its action as it allows the transfer of single genes from one yeast strain to another. Another advantage of this technology is that it offers the potential to introduce genes from any organism (plant, animal, bacterial or fungal) into yeast strains.

The application of recombinant DNA technology to industrial yeast strains is still a relatively young area of research. Although the brewing industry has invested some effort in this field, the technology is still not yet well enough established for commercial or industrial use. There have been no reports to date of the successful application of recombinant DNA technology to wine yeast strain improvement. The aim of this project is to establish a system for genetic engineering of wine yeast strains which will be suitable for industrial application. The following criteria are considered most important in achieving

this aim: an efficient transformation method for the introduction of foreign DNA into wine yeasts; demonstration that the introduction of foreign DNA does not adversely affect the fermentation performance of the yeast strain; stability of the foreign DNA in the yeast population throughout the fermentation; and appropriate expression of the foreign gene in the yeast cell.

A further objective is to utilise this system in the development of a strain which will have commercial application. The targeted area for development will be the genetic marking of wine yeast strains. The aims here are twofold. First, to introduce a novel property into wine yeasts to enable rapid and unequivocal strain identification. Secondly, an attempt will be made to demonstrate the potential application of such a marked strain in oenological studies.

Chapter 2 Literature Review

2.1 ORIGIN AND CLASSIFICATION OF WINE YEASTS

The original wine yeasts strains were derived from the natural population of yeasts that occur on the skins of grapes. Reports of the isolation of yeast species from grapes, wines and winery equipment from the different wine regions around the world have been reviewed by Kunkee and Amerine (1970), Kunkee and Goswell (1977), Benda (1982), Lafon-Lafourcade (1983) and Farkas (1988). Approximately 200 wine yeast species are listed in the table by Kunkee and Goswell (1977), however, many of these species are only occasionally associated with grapes and wines. According to Lafon-Lafourcade (1983), there are only about 14 yeast species that are frequently isolated from musts and wines and a further 40 species which are sometimes present.

The general conclusions that have been drawn from these reports are that a progression of yeast species are involved in natural fermentation. Grapes and freshly extracted grape juice have a dominant flora of apiculate yeasts such as *Kloeckera apiculata* and *Hanseniaspora uvarum,* as well as species of *Candida*, *Hansenula*, *Metschnikowia* and *Pichia*. These species exhibit limited growth during the early stages of alcoholic fermentation but are inhibited as the ethanol concentration rises to 3-4% (vol/vol). The more alcohol tolerant species of *Saccharomyces*, often initially present in smaller numbers, then proliferate and dominate the remainder of the fermentation. Fermented wines may be spoiled by the growth of alcohol tolerant species such as *Zygosaccharomyces bailii*, species of *Saccharomyces* and film-forming yeasts such as *Pichia membranaefaciens*.

Wine yeasts of the genus *Saccharomyces* have been catagorized taxonomically to at least 29 different species or varieties (Lodder, 1970; Kunkee and Goswell, 1977). Most of these species are separated primarily on the basis of their sugar fermentation and

assimilation patterns. In many cases, however, a mutation in a single gene can result in the loss of capacity to ferment a sugar - few would consider such a mutant strain as a new species. A more fundamental means of classification is based on DNA sequence homology. Vaughan and Martini (1980) have reported that the G+C content of the DNA of *S. cerevisiae*, *S. bayanus*, *S. chevalieri*, *S. italicus*, and *S. uvarum* ranges from 38.5 to 39.5%. Furthermore the DNA sequence homology is greater than 90%. They concluded that it is best to consider all these strains as belonging to the one species; *S. cerevisiae* having taxonomic priority. Because of its dominance in alcoholic fermentation, *S. cerevisiae* has emerged, almost universally, as the single most important species associated with the winemaking process. As a consequence, this species is now widely recognised as 'the wine yeast'.

In some established vineyards of Europe, grape juice is still allowed to ferment naturally with the yeasts originating from the grapes and the flora established on winery equipment. However, in countries such as Australia, USA and South Africa, many winemakers use pure yeast starter cultures (usually a strain of *S. cerevisiae*) to inoculate the must. Pure culture inoculation was first described by Hansen (1886, 1888), who introduced its use into brewery practice. The advantages of inoculating with a pure culture of *S. cerevisiae* are that it promotes a rapid and even onset of fermentation; allows the conduct of a controlled fermentation; and inhibits the growth of indigenous yeasts which may detract from wine quality and even lead to wine spoilage (Rankine and Lloyd, 1963; Rankine, 1977; Kunkee, 1984).

Although the term pure culture is still in use, is should be noted that this does not necessarily mean that the culture is genetically uniform. The cultures are pure in the sense that they were derived from a single cell. However, after years of mass propagation of these cultures, mutations are likely to occur and may be expressed (even though they are recessive) through mitotic crossing-over or gene conversion. An example of the heterogeneity of a 'pure' culture is given by Zimmerman (1978) who isolated a strain with

considerably improved characteristics from successive single-cell cultures of an Epernay yeast.

2.2 GENETIC FEATURES OF WINE YEASTS

Genetic studies with *S. cerevisiae* were pioneered at the Carlsberg Laboratories in the 1930's by Winge who first observed haploid and diploid phases in the life cycle of *Saccharomyces* (Winge, 1935). The early literature on yeast genetics has been reviewed by Lindegren, 1949; Winge and Roberts, 1958; and Mortimer and Hawthorne, 1969. A brief review of the genetics of *S. cerevisiae* will be described here, particularly as it relates to wine yeast strains and methods for strain modification.

2.2.1 Life cycle and sporulation

S. cerevisiae can exist in either the haploid or diploid state. Strains in which the haploid form is stable and can be maintained for many generations, are termed heterothallic. The haploids from such strains exist as one of the two mating types, MATa or MATα, and mate to form diploids when a cell of one mating type comes into contact with a cell of the other mating type.

Strains in which cell fusion and diploid formation occur among cells derived from a single spore are termed homothallic. This behaviour is caused by the allele HO (Harashima *et al.*, 1974; Hicks and Herskowitz, 1976), such strains being genotypically HO/HO. The presence of the HO gene brings about a high frequency of switching between mating types during vegetative growth. Under the influence of this gene, the mating type locus, MAT, readily changes from MATa to MATα or vice versa. The MAT gene is found on chromosome III of the yeast genome together with two silent genes HMLα and HMRa which provide the information to allow the switch of mating type at the MAT locus. Cells of homothallic yeasts have to bud at least once before they are competent to switch mating

type, but thereafter a high frequency of switching occurs at each budding for many generations (Herskowitz and Oshima, 1981). The surveys that have been made of wine yeasts indicate that they are typically homothallic (Thornton and Eschenbruch, 1976; Snow, 1979; Kusewicz and Johnston, 1980). Because of poor spore viability it has in many cases been impossible to isolate complete tetrads, and therefore it has not been possible to determine whether the original strain is genotypically HO/HO or HO/ho. The overall information available however, indicates that the majority of wine yeasts are genotypically HO/HO.

In both homothallic and heterothallic strains, mating takes place when the cells of opposite mating type come into close proximity. Cells of α-mating type produce an oligopeptide (12 or 13 amino acid residues) called α-factor which arrests a-mating type cells in the G1 phase and causes a-cells and α-cells to adhere to each other. Cells of a-mating type produce a-factor which has similar effects on α–cells. In the presence of these factors the cells adhere and cytoplasmic fusion takes place to form a heterokaryon. Nuclear fusion follows rapidly to give a zygote (Lindegren and Lindegren, 1943). By subsequent cell division this forms the diploid phase of the yeast life cycle which can be stably maintained for many generations.

Meiosis and sporulation of diploid cells is triggered by nitrogen deprivation in the presence of a non-fermentable carbon source, and will only occur if MATa and MATα genes are both present. Following entry into meiosis the chromosomes in the yeast nucleus undergo premeiotic DNA synthesis, pairing, recombination and segregation. Spore walls grow and envelope four haploid genomes (two each of a and *a* mating types), forming the characteristic four-spored ascus. When placed in suitable nutrient media, the spores germinate to form haploids and begin the cycle once more.

However, wine yeasts behave very differently from laboratory strains: they generally sporulate inefficiently, they produce few viable spores of which most are unable

to mate, their chromosomal constitutions are unknown, they show a great deal of genetic heterogeniety and generally lack selectable genetic markers (Snow, 1983; Spencer and Spencer, 1983; Beckerich *et al.*, 1984; Subden, 1987; Rank *et al.*, 1988).

2.2.2 Chromosomes and ploidy

The chromosomes of *S. cerevisiae* are located in the cell nucleus and account for 80-85% of the total yeast DNA (Petes, 1980). In haploid strains, chromosomal DNA has a molecular weight of 10^{10} Da, which is equivalent to 17000 kilobase pairs (kbp). To date, approximately 750 loci have been mapped to 17 chromosomes (Mortimer *et al.*, 1989). Each chromosome is a single DNA molecule of between 150 and 2500 kbp. As is the case with higher eukaryotes, yeast chromosomes also contain basic histone molecules. In contrast with higher organisms, however, *S. cerevisiae* DNA contains a relatively small fraction of repeated sequences (Fangman and Zakian, 1981).

Mobile genetic elements are found in *Saccharomyces* strains. These elements (called Ty) consist of a 5.1 kbp DNA sequence flanked by a 250 bp repeated sequence (Boeke *et al.*, 1985). As many as 35 copies of Ty can be present per haploid genome and their ability to transpose from one chromosomal location to another can result in substantial rearrangements of the genome (Scherer *et al.*, 1982). The random excision and insertion of Ty elements into the genomes of wine yeasts can therefore inactivate genes encoding desirable proteins and cause genetic instability of selected strains. The reverse can also occur, so that improved wine yeast strains evolve.

In most laboratory studies, the strains of *S. cerevisiae* used are either haploid or diploid. Industrial strains, however, are predominantly diploid or polyploid. The precise determination of the chromosome number of yeast strains is difficult, since they are too small for direct chromosome counts. Methods including the determination of DNA-content per cell, measurement of cell volume, and irradiation and death rate have been used to

estimate the ploidy of yeast strains (Gunge and Nakatomi, 1971; Lewis *et al.*, 1976; Aigle *et al.*, 1983; Takagi *et al.*, 1985). These procedures, however, are problematic. The determination of DNA content is dependent on very specific cell concentrations. Variation in the chromosomal sizes of industrial strains could also affect the precision of the test. Although the cell size is clearly a function of ploidy, most individual strains of the same ploidy have sizes significantly different from other strains in the same ploidy group. Furthermore, aneuploidy cannot be determined by these methods. 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 yeasts are polyploid, particularly triploid, tetraploid or aneuploid (Tubb and Hammond, 1987)

Where strains have been crossed to laboratory haploids, segregations of genetic markers can provide insight into the ploidy of industrial yeast strains. Using this approach, Cummings and Fogel (1978) were able to show that two wine yeasts were almost certainly normal diploids since matings of ascospores with cells of laboratory strains gave regular 2:2 segregations for markers on 13 of the 16 known yeast chromosomes. One of the wine yeast strains studied by Thornton and Eschenbruch (1976) was also probably diploid, as it showed 2:2 segregations for most markers on six different chromosomes. On the other hand, Takahashi (1978), in a study of a widely used commercial German wine yeast (Hefix 1000), concluded that it was about, if not exactly, tetraploid and had an a/a/a/α mating locus genotype.

Circumstantial evidence supporting the wide-spread occurrence of aneuploidy and/or polyploidy among wine yeast strains includes observations of poor spore viability, great variability in growth rates among spore progeny, and a very low frequency of mating-competent meiotic segregants. It is not yet clear whether polyploidy on wine yeasts is advantageous. Some 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). It is known, however, that polyploid/anueploid state makes analyses of important oenological traits more difficult and complicates the genetic improvement of wine yeast strains.

2.2.3 Extrachromosomal elements

A number of extrachromosomal genetic elements have been described in yeast, and are discussed below.

2 µm DNA

Most laboratory strains of *S.cerevisiae* contain a class of small extrachromosomal DNA molecules that are about 2µm in length (Sinclair *et al.*, 1967). The molecules are generally referred to as the 2µm plasmids. There are usually 50 to 100 copies of 2µm DNA per cell and they represent approximately 5% of the total yeast DNA. These circular DNA molecules consist of two identical repeats of 599 bp separated by two unique regions of 2774 and 2346 bp (Broach, 1981). Portions of the DNA are transcribed into three different polyadenylated mRNA molecules which can direct protein synthesis *in vitro*. One of these genes (FLP) produces a protein which is actively involved in 2µm recombination (Cox, 1983), while the others (REP1 and REP2) are required for stable replication (Broach, 1982). Their function in the yeast cell has not been established and since phenotypically normal yeast strains have been identified that lack the 2 µm plasmid (Livingston, 1977), they are not required for cell viability. Other than its own maintenance, the 2 µm plasmid appears to confer no advantage on the host cell.

Although most of this information has been obtained with laboratory strains, a 2µm DNA of similar structure is found in wine yeast strains. The 2µm DNA serves an important tool in the genetic manipulation of wine yeasts, as many plasmid vectors are based on the 2µm origin of replication.

Mitochondrial DNA

In laboratory strains, mitochondrial DNA has a molecular weight of about 50 x 10^6 Da and consists of a 75 kbp circular molecule. It is present at 10-40 molecules per haploid cell and represents between 5 and 20% of the total cell DNA. It is very A-T rich compared with chromosomal DNA resulting in a lower bouyant density (Fangman and Zakian, 1981). Mitochondrial DNA shows typical cytoplasmic inheritance and its replication is independent of nuclear control, taking place throughout the cell cycle (Newlon and Fangman, 1975). The mitochondrial genome carries the genetic information for only a few essential mitochondrial components; more than 90% of mitochondrial proteins are coded by nuclear genes (Dujon, 1981). Mutations in mitochondrial DNA produce petite strains which are unable to utilize non-fermentable substrates. Such respiratory-deficient mutations can range from point mutations (mit^-) through deletion mutations (ρ^-) to complete elimination of mitochondrial DNA (ρ^o).

The generation of petite mutants of wine yeasts occurs spontaneously at quite high rates. It is important to note, however, that yeasts with different mtDNAs can differ in their flocculation characteristics, lipid metabolism, higher alcohol production and the formation of flavour compounds (Lewis *et al.*, 1976; Hammmond and Eckersly, 1984). This indicates the importance of mtDNA encoded functions. For this reason, petite strains are not used for wine making.

Killer factor

Many strains of *Saccharomyces* yeast contain cytoplasmic double-stranded RNA molecules (dsRNA), encapsidated in virus-like particles (Tipper and Bostian, 1984). There are two main varieties of dsRNA molecules with characteristic properties. L dsRNA is present in most yeast strains and encodes the capsid protein of the virus-like particles. M dsRNA is present only in killer strains of *Saccharomyces* (Wickner, 1983) and has been

shown to encode both a killer toxin, which is lethal to sensitive strains, and the immunity factor which prevents self-killing. Both L and M dsRNA molecules are linear but they have little sequence homology. In killer strains there are normally about 12 copies of M dsRNA per cell and about 100 copies of L dsRNA per cell. Killer strains can be cured of M dsRNA by growth at elevated temperature or by treatment with cyclohexamide (Fink and Styles, 1973); such cured strains often produce more L dsRNA. The maintenance, regulation and expression of M dsRNA are all regulated by nuclear genes, many of which have been mapped (Wickner, 1983).

Killer activity has been detected in yeasts isolated from established vineyards and wineries in various regions of the world including Europe and Russia (Barre, 1984; Gaia, 1984; Naumov and Naumova, 1973), South Africa (Tredoux *et al.*, 1986) and Australia (Heard and Fleet, 1987a,b). Killer factors are of interest to the wine industry for two reasons. First, van Vuuren and Wingfield (1986) showed that stuck or sluggish wine fermentations can be caused by contaminating killer yeasts. Secondly, in theory, selected killer yeasts could be used as the inoculated strain to suppress growth of undesirable wild strains of *Saccharomyces cerevisiae* during grape juice fermentation.

Other genetic elements

Several minor genetic elements have been described over the years. The ψ factor (Cox, 1965) is a potentiator of nonsense suppression. It is inherited cytoplasmically and has been shown not to be associated with dsRNA, 2μm DNA or the mitochondrial genome. [URE3] allows cells growing on ammonia or glutamate to use ureidosuccinate and so bypass ura2 mutants. It is inherited cytoplasmically but is not distributed to all progeny at meiosis (Aigle and Lacroute, 1975). 20S RNA is a sporulation-specific RNA molecule whose synthesis is controlled by a cytoplasmic genetic element. It is not present in vegetative cells and is only produced by cells under sporulation conditions (Kadowaki and Halvorson, 1971).

Due to the lack of appropriate investigations, none of these minor genetic elements have been described in wine yeast strains.

2.3 GENETIC TECHNIQUES FOR YEAST STRAIN IMPROVEMENT

Several genetic techniques can be used in yeast improvement programs. Some of these techniques are used to recombine or rearrange the entire genome, whereas others alter specific regions of the genome. Techniques having the greatest potential in the genetic improvement of wine yeast strains include clonal selection; clonal selection after mutagenesis; hybridization; spheroplast fusion; rare-mating; and recombinant DNA technology. All of these methods have been used with industrial strains (either brewing, distilling or wine yeasts) and will be described below.

2.3.1 Clonal selection

This method takes advantage of the natural genetic variation present in wine yeast strains. Some degree of heterozygosity is almost certain to be present in all yeast strains and new substrains may arise, possibly through mutation, but more likely through mitotic recombination during vegetative growth. The selection procedure requires the testing of large numbers of clones derived from single cells of the parental strain. An example of the successful use of clonal selection in which variants of an Epernay yeast with improved fermentation characteristics were isolated has already been cited (Zimmerman, 1978). It has also been used to select non-foaming wine yeast mutants (Ouchi and Akiyama, 1971; Eschenbruch and Rassell, 1975); variants with improved ethanol tolerance (Brown and Oliver, 1982); and strains with reduced H_2S production (Rupela and Taura, 1984).

2.3.2 Clonal selection after mutagenesis

Selection after mutagen treatment has been used in the improvement of wine, brewing, baking and distiller's yeasts. The common mutagens used with wine yeasts have been UV or X-rays, ethyl methane sulfonate (EMS), N-Methyl-N-nitro-N-nitrosoguanidine (NTG), N-nitrosourea, or diethylstilbestrol (Tubb and Hammond, 1987). Since many of the desirable mutations are recessive, their expression in diploid or polyploid wine strains has resulted from homozygosity brought about by gene conversion or mitotic crossing over.

Molzahn (1977) employed mutagenesis to successfully isolate brewing yeast mutants with increased flocculation, and with modified abilities to produce diacetyl and hydrogen sulfide. Ingraham and Guymon (1960) used ultraviolet light to generate isoleucine and valine requiring mutants that produced only trace amounts of isoamyl alcohol and isobutyl alcohol respectively. From EMS treated wine yeasts, Rous *et al.* (1983) isolated leucine auxotrophic recessive mutants that also produced reduced concentrations of higher alcohols.

Mutagenesis has the potential to disrupt or eliminate undesirable characteristics and to enhance favourable properties in wine yeasts. However, the heavy mutagen treatment frequently used could produce mutations in addition to the one of interest, which is a possible disadvantage of this method.

2.3.3 Hybridization

Hybridization involves the mating of haploids of opposite mating-types to yield a heterozygous diploid. Recombinant progeny are recovered by sporulating the diploid, recovering individual haploid ascospores and repeating the mating/sporulation cycle. The fact that wine yeasts are generally homothallic complicates the use of this procedure.

However, this problem can be overcome by direct spore-cell mating, as mating type switching does not occur until the third or fourth generation of growth after spore germination (Takano and Oshima, 1970).

Thornton (1982) used selective hybridization of pure culture wine yeasts to significantly improve fermentation efficiency (from 84 to 93%). Also, useful killer wine yeast strains have been bred by hybridization of a killer sake yeast with mesophilic (Hara *et al.*, 1980) and cryophilic (Hara *et al.*, 1981) wine yeasts. Hybridization programs are also hampered by the poor sporulation and low spore viability of wine yeast strains. Consequently, this breeding method has not been widely employed in the improvement of wine yeasts.

2.3.4 Rare mating

Industrial yeast strains which fail to show a mating-type can be force-mated with haploid a or α strains. In this procedure, known as rare mating, a large number of cells of the parental strains are mixed together and a strong positive selective pressure is applied to identify the rare hybrids. Hybrids are usually selected as respiratory-sufficient prototrophs from crosses between a respiratory-deficient mutant of the industrial strain and an auxotrophic haploid strain (Gunge and Nakatomi, 1971). Hybrids produced by rare mating are often able to sporulate (Spencer and Spencer, 1977) providing another possible route for the genetic analysis of industrially important characteristics. By rare mating, Tubb *et al.* (1981) constructed brewing strains with the ability to ferment dextrins.

An important development has been the use of rare mating to generate progeny ('cytoductants' or 'heteroplasmons') which receive cytoplasmic contributions from both parents but retain the nuclear genome of only one of them. This form of strain construction has been termed cytoduction. The technique has become highly efficient through the use of haploid strains which carry the kar1 mutation and are, therefore, defective in nuclear fusion

(Conde and Fink, 1976). Such strains can be used as donors of cytoplasmic genetic material to industrial strains. For example, transfer of the double stranded (ds) RNA determinants for the K1 zymocin (and associated immunity) has been used to produce brewing strains with anti-contamination properties (Young, 1981,1983; Hammond and Eckersley, 1984).

2.3.5 Spheroplast fusion

Spheroplast fusion provides another direct asexual technique for manipulating industrial yeasts genetically and, like rare mating, can be used to produce either hybrids or cytoductants. The procedure was first described by van Solingen and van der Platt (1977). Spheroplasts are formed by removal of the cell wall with an appropriate lytic enzyme preparation such as Zymolyase (a glucanase from *Arthrobacter luteus*) in a medium containing an osmotic stabilizer (usually 1.0 M sorbitol) to prevent cell lysis. Spheroplasts from different strains are fused together in the presence of polyethylene glycol and calcium ions, and then allowed to regenerate their cell walls in an osmotically stabilized agar medium. Spheroplast fusion can also be obtained by electroporation in a weak inhomogeneous alternating electric field. Fusion of the aligned cells can then be induced by applying a higher-intensity electric field (Tubb and Hammond, 1987).

Spheroplast fusion of non-sporulating yeast strains serves to remove the natural barriers to hybridisation. Cells of different species, or levels of ploidy, can be fused. The use of spheroplast fusion to modify an industrial yeast is illustrated by the construction of brewing strains able to ferment dextrins (Freeman, 1981; Russell *et al.*, 1983). Yokomori *et al.* (1989) produced cytoductants of a sake wine yeast by spheroplast fusion that exhibited good fermentation performances and produced quality wine with low volatile acids. Spheroplast fusion has also been used to create yeast strains with improved tolerance to ethanol (Seki *et al.*, 1983).

2.3.6 Recombinant DNA technology

The various mating procedures described above result in parental strains contributing major portions of their respective genomes. This hybridization is essentially empirical and a lengthy selection procedure is often required to obtain a suitable strain from the large number of recombinant types produced. Recombinant DNA technology, however, provides the opportunity to specifically alter single characteristics in wine yeast strains. Furthermore, genetic engineering permits introduction of genes from any source into wine yeasts.

The addition of exogenous DNA to yeast and its subsequent incorporation into the genetic framework of the cell, resulting in the acquisition of a novel characteristic, is termed transformation (described in Section 4.1). Transformation has not yet been used in the improvement of wine yeast strains but has been successfully applied to brewing yeasts. For example, enhanced degradation of starch and sugar utilization by brewing yeasts has been achieved by cloning starch-degrading enzymes into brewing yeasts (Stewart, 1981; Meaden *et al.*, 1985). A β-glucanase gene from *Bacillus* has also been introduced and expressed in brewing yeasts (Cantwell *et al.*, 1986). The transformed yeasts effectively secrete substantial levels of β-glucanase enzyme which degrades β-glucan in wort.

In addition to the introduction of specific genes into wine yeasts, recombinant DNA approaches offer wider applicability. For example, the potential exists to eliminate specific undesirable strain characteristics by gene inactivation. Also, it may be possible to develop new gene products with modified characteristics by site directed mutagenesis.

2.4 TARGETS FOR WINE YEAST STRAIN DEVELOPMENT

Although the techniques involved in the genetic manipulation of industrial yeasts are quite well established, the wine industry has been slow to engage in yeast genetics and

strain development programmes (Thornton, 1983). This is due in part to the technical difficulties involved in such programmes, and partly to the fact that the requirements of the wine industry have not been defined in genetic terms. Despite these obstacles, the genetic basis for several characteristics of oenological importance has been determined and there are a number of examples of wine yeast strain improvement. These examples are included below in a list of specific targets for yeast genetics in wine-making.

2.4.1 Sedimentation and flocculation

The aggregation of dispersed yeast cells into flocs towards the end of fermentation is called flocculation. Non-flocculent yeasts settle slowly following fermentation and form a fine sediment which is easily disturbed on racking. This property may necessitate longer settling times, centrifugation or the use of fining agents to clarify the wine with a consequent increase in production costs. Flocculent yeasts, however, form a heavy sediment and a clear wine which can be easily racked close to the lees.

The nature of the interactions among flocculent yeast cells is poorly understood, and basically two models for the mechanism exist. Those based on physicochemical principles propose cooperative bonding between cell surface polysaccharides (Mill, 1964). The observation that protease treatment leads to an irreversible loss of flocculation supports this theory (Miki *et al.*, 1980) . Alternatively, flocculation interactions may be mediated by a specific cell surface recognition mechanism, involving lectin-like binding of surface proteins to polysaccharides on adjacent cells (Taylor and Orton, 1978).

Genetic studies of yeast flocculation were first reported by Gilliland (1951) and Thorne (1951). Since then, a number of genes have been reported for the flocculence phenotype in *Saccharomyces* species: FLO1, flo3, FLO5, flo6, flo7, FLO8, fsu1, fsu2 and tup1 (Johnston and Reader, 1983; Yamashita and Fukui, 1983; Lipke and Hull-Pilsbury, 1984). Of these genes, the dominant flocculation gene, FLO1, which is 37cM distal to

ade1 on chromosome 1, has been most extensively analysed both genetically and biochemically (Johnston and Reader, 1983; Miki *et al.*, 1982 a,b).

The property of flocculation was introduced into a powdery wine yeast strain MD26 by mating spores of MD26 with a haploid laboratory yeast strain which carried the dominant FLO1 gene (Thornton, 1985). Following the hybridization step, a series of backcrosses to the original wine yeast parent were performed - the outcome of this genetic modification program was the conversion of the powdery yeast MD26 to a flocculent yeast while retaining its positive winemaking properties. More recently, molecular cloning of the FLO1 gene (Watari *et al.*, 1989) has provided the opportunity for specific introduction of the flocculation characteristic into industrial yeast strains by transformation.

2.4.2 Non-foaming mutants

The production of froth-head during fermentation is an undesirable trait of wine yeasts as up to 5% of the capacity of the fermentation vessel may have to be reserved to prevent the froth from spilling out. Selection of non-foaming mutants of the widely used Kyokai No. 7 strain of sake yeast was achieved by Ouchi and Akiyama (1971) by either a cell agglutination or a froth flotation method.

The cell agglutination method is based on the observation that a normal sake yeast was agglutinated when mixed in acid solution with certain species of lactobacilli, whereas a non-foaming strain was not (Momose *et al.*, 1968). Ouchi and Akiyama (1971) were able to enrich for non-foaming mutants by a series of repeated selection cycles. Washed cells from a culture of normal Kyokai No. 7 were mixed with cells of *Lactobacillus plantarum* in dilute citric acid. After mixing and allowing to settle, an aliquot of the upper part of the suspension was used as the inoculum for another round of yeast growth. After the ninth selection step most of the clones sampled were non-foaming. When cells of the parental

strain were mutaganized by exposure to UV light prior to selection cycles, 100% of the sampled clones at the seventh cycle were non-foamers.

Selection by the froth flotation method was described by Akiyama *et al.* (1971), who found that cells from the normal foaming strain adhered to the CO_2 gas bubbles formed during fermentation, while cells of a non-foaming strain did not. Non-foaming mutants were progressively enriched in repeated selection cycles by bubbling air through the culture. Aliqouts of the culture, now enriched for non-foaming mutants, were used as the inoculum for the next round. After nine selection cycles, approximately 50% of the tested clones were non-foamers; when the series was started with UV-irradiated cells, about 80% of the sampled clones were non-foamers after seven cycles. Using the froth flotation technique, Eschenbruch and Rassell (1975) were able to select non-foaming mutants from two strains of New Zealand wine yeasts.

In studies of the nature of cell walls, Ouchi and Nunokawa (1973) found that non-foaming mutants have fewer hydrophobic groups on their surface, probably because of masking by phosphomannan. The genetic basis for foaming has been investigated by Kasahara *et al.* (1974), who found that the non-foaming sake mutations were recessive. Tetrad analysis revealed that the foaming character was under the control of at least two genes. Using wine yeasts, Thornton (1978 a,b) also showed that two dominant genes (designated *FRO1* and *FRO2*) control foaming, that they are allelic to the sake yeast genes and that they are linked on chromosome VII, 21 cM from one another and near *ade3*.

2.4.3 Sulfite and Sulfide Production

The formation of SO_2 and H_2S by wine yeasts greatly affects the quality of wine. Sulfur dioxide is regularly added to disinfect fermentation equipment, to control organisms that would compete with the yeast fermentation and to prevent excessive oxidation of the wine. Health concerns have led to efforts to restrict its use as an additive. Hence the

production of SO_2 itself has become a matter of some importance. Although SO_2, when properly used, has beneficial effects, the same cannot be said of H_2S. From an oenological perspective, H_2S is one of the most undesirable yeast metabolites affecting the smell and taste of wines.

The formation of these two substances during wine making has been well reviewed by Eschenbruch (1974). Both are complex processes that are not yet fully understood. From a genetic viewpoint, however, it is important to note that wide variation has been found between yeast strains. Most *S. cerevisiae* strains produce between 10 and 30 mg/liter of SO_2 when tested under comparable conditions but some form as little as 10 mg while others form in excess of 100 mg/liter (Eschenbruch, 1974; Eschenbruch and Bonish, 1976b). Strain variation in H_2S production has also been revealed (Zambonelli, 1964 a,b; Rankine, 1968; and Eschenbruch *et al.*, 1978).

Studies into the sulfur metabolism of high and low sulfite-producing strains have revealed considerable differences in the levels of activity of sulfate permease (Dott *et al.*, 1977), ATP-sulphurylase (Heinzel and Truper, 1978) and sulfite reductase (Dott and Truper, 1976). These differences suggest that it may be possible to introduce specific properties from low sulfite producing yeasts into selected wine yeast strains.

Sulfide can be formed from sulfate or sulfite, elemental sulfur applied to grapes as a fungicide or from cysteine (Eschenbruch, 1974). Its formation can be indirectly influenced by the amount of yeast growth, pantothenate or pyridoxine deficiencies or excess levels of certain amino acids, metal ions and yeast cell autolysis (Snow, 1983). The reduction of elemental sulfur may occur both enzymatically or by reaction with thiol groups.

The variation and genetic control of H_2S production has been investigated by Zambonelli (1964 a,b; 1965 a,b,c). In screening 100 strains for H_2S production from sulfate or sulfite, he found four strains that produced none under all conditions tested; the

rest formed varying amounts, from traces to over 200 μg/50ml culture medium. When H_2S positive strains were crossed with negative strains, the hybrid produced H_2S and spores segregated for H_2S production at a ratio of 2:2. However, since the amount of H_2S produced by the positive clones varied considerably, segregation of modifying genes was indicated.

In two later papers, Zambonelli *et al.* (1975) and Romano *et al.* (1976) reported genetic results with prototrophic mutants of sulfite reductase. This enzyme is essential for the biosynthesis of the sulfur amino acids and the prototrophy of the reductase-negative strains was ascribed to leakiness of the reductase mutation. From a survey of several strains, it was concluded that a number of factors influence the production of sulfide from sulfate: inhibition of sulfite reductase by endogenous factors, reduced function of the reductase caused by mutation, enzymatic blockage after the reductase step causing methionine auxotrophy and the state of genetic heterozygosity of the cell.

Given these various sources of sulfide and the number of influences on its formation, one could not expect to find a single gene that would eliminate it. However, the fact that strains do vary in their capacity to produce it suggests that there is considerable natural genetic heterogeneity that could be exploited. The specific introduction of mutations in certain enzymes of the sulfur, sulfur amino acids and pantothenate and pyridoxine pathways may result in reduced production of sulfide (Snow, 1983).

2.4.4 Ethanol tolerance

The inhibitory action of ethanol produced in the course of fermentation or added externally, is complex. A number of parameters have been used as indicators of the relative sensitivity or tolerance of yeast strains to the alcohol. These include fermentation rate; biomass yield; growth rate and cell viability (Oliver, 1987).

One of the major target sites of ethanol in the yeast cell is the plasma membrane, as well as the membrane of the various cellular organelles (Thomas and Rose, 1979). The damage caused by ethanol to the cell membrane results in altered membrane organization and permeability. It has been shown that ethanol causes the leakage of essential cofactors and coenzymes from *Zymomonas mobilis* (Osman and Ingram, 1985). The leakage of these components, which are essential for the activity of enzymes involved in glycolysis and alcohol production, was sufficient to explain the inhibitory effect of ethanol on fermentation in *Z. mobilis* as well as in yeasts (Ingram and Buttke, 1984).

There have been many other mechanisms proposed for the inhibitory effects of ethanol. These include the inhibition and denaturation of various intracellular proteins and glycolytic enzymes (Nagodawithana *et al.*, 1977), inhibition solute transport systems (van Uden, 1985), inhibition of glucose-induced proton fluxes (Juroszek *et al.*, 1987), accelerated passive re-entry of protons in a manner resembling the action of an uncoupler (Leao and van Uden, 1984, Cartwright *et al.* 1986), derepression of the optimum and maximum temperature for growth (Sa-Correia and van Uden, 1983) and the enhancement of thermal death (Leao and van Uden, 1982) and petite mutations in yeast (Cabeca-Silva *et al.*, 1982). Furthermore, the inhibitory effects of alcohols were observed to increase with increasing carbon number, suggesting that the potency of alcohols is related to lipid solubility (van Uden, 1984). In short, ethanol has a complex inhibitory action. This has been reviewed by van Uden (1985).

Given the pleiotropic nature of the effect of ethanol on yeast, it is most unlikely that any single gene will be responsible for the sensitivity or tolerance of the organism to the alcohol. A number of mutant nuclear genes have been found to confer an ethanol-sensitive phenotype on yeast (Jones, 1977; Sugden and Oliver, 1983). However, attempts to isolate ethanol-tolerant mutants by conventional agar plate screening methods, have failed (Ismail and Ali, 1971a,b). This failure is not surprising given the nature of ethanol toxicity - it is likely that the mutation of a number of genes will be required to improve the ethanol

tolerance tolerance of yeast and, furthermore, such improvements are likely to be small, since *S. cerevisiae* is already a highly tolerant organism. In this situation, when only small quantitative increases are likely to be obtained as a result of multiple mutations, the use of continuous selection is preferred. Brown and Oliver (1982) adopted a system in which the intensity of selection was determined by the yeast culture itself via a feedback control circuit. Using this system, they successfully isolated yeast mutants with increased ethanol tolerance.

2.4.5 Higher alcohols

Higher alcohols are alcohols with carbon numbers greater then that of ethanol, such as isobutyl and isoamyl alcohol. They are formed from either sugar metabolism or intermediates in the branched chain amino acids pathway leading to leucine, isoleucine and valine by transamination, decarboxylation and reduction (Webb and Ingraham, 1963). Although they have undesirable flavor and odor characteristics, they are usually present in wines below the flavor threshold and may, in some cases, contribute to wine quality (Kunkee and Amerine, 1970). However, their reduction in wines that are to be distilled (for example, for brandy production) could be of considerable importance, since they are concentrated by the distilling process (Snow, 1983).

Ingraham and Guymon (1960) and Ingraham *et al.* (1961) were able to produce unusually low levels of isobutanol and iso-amyl alcohols in fermentations carried out with valine, isoleucine, and leucine mutants. However, these mutants were of no commercial use as their growth rate and fermentation rate were compromised. A leu- mutant derived from the widely used Montrachet wine yeast (UCD, Enology 522) was reported to produce more than 50% less isoamyl alcohol during fermentation than the prototrophic parent (Snow, 1983). Taste panel trials indicated no difference between wines produced with the mutant and the Montrachet parent strain.

2.4.6 Killer factor

Killer strains of yeast were first recognised by Bevan and Makower (1963). Killer yeasts secrete polypeptide toxins which kill sensitive strains of the same genus and less frequently, strains of different genera (Philliskirk and Young, 1975; Tipper and Bostian, 1984). The oenological significance of killer yeasts is still largely speculative. It is considered that such strains could be used to resrict the growth of undesirable wild strains of *S. cerevisiae* and other closely related *Saccharomyces* species during and after alcoholic fermentation.

Ouchi and Akiyama (1976) introduced the killer plasmid into sake and wine yeast by crossing them with a wild killer strain isolated as a contaminant from a sake mash. Repeated backcrossing with a selection at each generation for particular characteristics of the sake strain gave killer hybrids that produced sake or grape wine of comparable quality to the parent. This backcrossing program of Ouich and Akiyama was hampered by poor sporulation and spore viability. To overcome these problems, Ouchi *et al.* (1979) employed a donor of killer character that was deficient in nuclear fusion, mated this with a haploid (derived from a sake yeast), and selected for sake strains containing cytoplasmic elements of both strains. This strain gave results in trial fermentations that were better than the parental strain with regard to rapidity of fermentation, volatile flavour components and acidity.

2.4.7 Malolactic fermentation

Malolactic fermentation involves the decarboxylation of L-malate to L-lactate and CO_2 and is carried out by several species of lactic acid bacteria. These species all belong to one of three genera: Lactobacillus, Leuconostoc or Pediococcus (Snow, 1983). This process performs three significant roles for the winemaker: i) reduction in acidity; ii) microbiological stability following growth of the bacteria; and iii) changes in wine flavour

caused by products of the bacterial fermentation (Kunkee and Goswell, 1977). The fermentation can be brought about by holding the wine under conditions that are favourable for the growth of the bacteria already present or by inoculation with the appropriate bacterial species.

An advantage to the winemaker would be to have the malolactic fermentation occur during or shortly after the alcoholic fermentation so the wine can be adjusted for cellar storage without risk of becoming spoiled. This could be achieved if the wine yeast were able to carry out the malolactic fermentation. To this end, attempts have been made to transfer the genetic information necessary for the malolactic fermentation from lactic acid bacteria to a wine yeast. Fusions between *S. bailli*, *S. rouxii* and *Schiz. pombe* with *S. cerevisiae* have been made, but the resultant hybrids had less ability to ferment malate than the parent strains (Subden and Osothsilp, 1987). The cloning and expression of the malolactic gene (L-malate:NAD carboxylase) from *Lactobacillus delbrueckii* (Williams *et al.*, 1984) and *Leuconnostoc oenos* (Lautensach and Subden, 1984) in *Escherichia coli* and in *S. cerevisiae* has been reported. However, in both cases, the level of conversion of malate to lactate by the engineered yeast strain was insufficient to be of practical benefit. This lack of success may be due to problems with expression of the cloned genes, or to the limited ability of the yeast host to take up the malate. In an attempt to overcome these problems, current research is directed towards the cloning and introduction of genes encoding malate permease and the malic enzyme from *Schiz. pombe* into *S. cerevisiae* (Subden and Osothsilp, 1987).

2.4.8 Genetic marking

As an aid to yeast management, particularly for wineries using more than one yeast strain, the genomes of commercial wine yeasts can be tagged. A marking system assists in monitoring yeast strains used in fermentations and discourages the illegal use of commercial

wine yeast strains. A deliberately marked oenological strain was developed by Vezinhet and colleagues (Vezinhet and Lacroix, 1984; Vezinhet, 1985) by selecting for natural mutants in a population of the Lalvin V yeast. The strain, which is now commercialized as K1, is double marked with two antibiotic markers, diuron and erythromycin. An extensive survey of yeasts for resistance to these antibiotics demonstrated that few strains are naturally resistant to both drugs simultaneously.

2.5 CONCLUSIONS

Limited success has been achieved to date in the genetic improvement of wine yeast strains. For example, Thornton (1983) was able to introduce the flocculation character from a laboratory strain into a wine yeast by hybridization and a series of subsequent backcrosses to the wine yeast parent. Also, a leucine auxotroph derived from a widely used Montrachet wine yeast was reported to produce at least 50% less isoamyl alcohol during fermentation than the prototrophic parent (Snow, 1983). Seki *et al.* (1985) were able to construct a killer wine yeast by spheroplast fusion and showed that the growth of sensitive cells in grape juice was inhibited by the killer fusant. Success was achieved in the selection of a genetically marked wine yeast strain (Vezinhet, 1985), which has provided an insight into the kinetics of yeast populations during fermentation (Delteil and Aizac, 1988).

Most of the attempts to develop improved wine yeast strains have relied on traditional genetic techniques such as mutation and selection, hybridization, rare mating and spheroplast fusion. These techniques are problematic because they involve unspecific alterations or exchanges of genetic material in yeast strains. These problems can be avoided by the use of recominant DNA technology in yeast improvement programs. Recombinant DNA technology has not yet been employed in the production of an improved wine yeast strain. However, classical genetic studies have provided background information which could be used in genetic engineering programs. For example, the dominant flocculation gene, FLO1, which has been extensively analysed both genetically and biochemically

(Johnston and Reader, 1983; Miki *et al.*, 1982 a,b), could be introduced into wine yeasts to produce flocculent strains. An alternative to the use of hybridization and cytoduction to introduce the killer character into wine yeasts would be to clone the toxin and immunity genes into wine yeast strains. Both the toxin and immunity genes reside on the same M-dsRNA molecule, and reverse transcription has already been used to produce a cDNA molecule of these two genes (Bostian *et al.*, 1984).

Genetic engineering could also be used to eliminate or reduce undesirable characteristics by gene disruption. Snow (1983) suggested that the deliberate introduction of mutations in certain enzymes of the sulfur, sulfur amino acids, pantothenate and pyridoxine pathways might enable stepwise elimination of these characteristics and hence a reduction in sulfide production in wine yeasts. Integrative disruption of specific *ILE*, *LEU* amd *VAL* genes of wine yeasts may result in lower concentrations of higher alcohols. Recombinant DNA techniques could also be used to eliminate the foaming charateristics of wine yeast strains by specific disruption of the *FRO1* and *FRO2* genes on chromosome VII.

The extent to which recombinant DNA technology can influence the breeding of improved wine yeast strains will largely depend upon the requirements of the wine industry and advances in the field of wine biochemistry. The true potential of this technology will become apparent once the research is under way.

Chapter 3 Materials and Methods

3.1 STRAINS AND MEDIA

The *Escherichia coli* strain used for bacterial transformations was DH5α [F^-, *end*A1, *hsd*R17, *sup*E44, *thi*-1, λ^-, *rec*A1, *gyr*A96, *reL*A1, Δ (*arg*F-*lac*ZYA) U169, ø80*lacz*ΔM15]. Haploid yeast strain O11 (MAT-α, *his* 3-11, 3-15, *leu* 2-3, 2-112, *ura* 3-251, 3-373) was obtained from Dr. H.B. Lukins, Department of Biochemistry, Monash University. Wine yeast strains used in this study were obtained from The Australian Wine Research Institute Collection and are listed in Table 3.1.

Bacterial growth media was LB [1% bacto-tryptone (Difco), 0.5% yeast extract (Difco), 1% NaCl]. Ampicillin (100 μg/ml) was added to molten media at 50°C. Yeast growth media was YPD [1% yeast extract (Difco), 2% bacto-peptone (Difco), 2% glucose], SD [0.67% Bacto yeast nitrogen base without amino acids (Difco), 2% glucose], or YEPG [1% yeast extract (Difco), 2% bacto-peptone (Difco), 2% glycerol]. Chloramphenicol (dissolved in ethanol) and cycloheximide were added to molten media (YEPG and YPD respectively) just prior to pouring plates. Sulfometuron methyl (SM) (obtained from DuPont deNemours and Co.) was dissolved in acetone and added to molten SD medium just prior to pouring plates. As SM is light sensitive, plates containing SM were incubated in the dark.

3.2 YEAST TRANSFORMATION METHODS

3.2.1 Alkali cation transformation

Alkali cation transformation of yeast was performed according to the method of Ito *et al.* (1983) with slight modification. Yeast cells were grown to late logarithmic phase (OD

Table 3.1. Wine yeasts strains obtained from The Australian Wine Research Institute Culture Collection.

Yeast Strain	Source	Description
AWRI 1A (350)	Thomas Hardy & Son, South Australia	Good flocculation properties and produces no H_2S. The yeast produces a significant level of higher alcohols and esters which give rise to a floral character.
AWRI 2A (729)	Lindeman's winery, South Australia	Tendancy to accumulate acetic acid when fermenting some musts, particularly at low temperatures and in the presence of high sugar concentration. Very active yeast with a fast rate of sugar attenuation at low cell numbers. Low formation of higher alcohols and esters. Used for red and white wine production.
AWRI 3A (796)	R. Eschenbruch (1975)	Originally isolated in South Africa, used for preparing red and white wine. It is a low SO_2 and H_2S producer, and has the K_2 killer property.
AWRI 5A (138)	Roseworthy Agricultural College (1945)	A flocculent yeast producing esters which gives a fruit aroma to wine which decreases on storage. Generally used for champagne production, it has a low tolerance to SO_2.
AWRI 6A (348)	Pasteur Institute, Tunis (1950)	Reported to produce low levels of higher alcohols. Limited use for the production of fortified wines and tolerates high fermentation temperatures.
AWRI 7A (833)	Penfold's Winery, Barossa Valley (1979)	Isolated from a starter culture in 1979 where it exhibited rapid fermentation at low temperature. Laboratory fermentations showed low volatile acidity and SO_2 formation. It has been examined commercially on a small scale with acceptable results.
AWRI 9A (81)	N.M. Bretez, Victoria	No information available.

.....cont'd

AWRI 8A (834)	Unknown	Selected as suitable for secondary fermentation in sparkling wine production following comparison with several other yeasts in small-scale commercial trials. A killer (K_2) yeast.
AWRI 10A (835)	Dept. of Agriculture, Western Australia (1971)	Similar properties to strain AWRI 2A (729), except that it accumulates less acetic acid. It has killer (K_2) properties.
AWRI 11A (R2)	Petaluma winery, South Australia	Formerly *Saccharomyces bayanus*. It is a vigorous fermenter when propagated correctly. Produces a highly aromatic bouquet and is most suited to white wine production. It has killer (K_2) properties.
AWRI 12A (143)	Te Kawata	Formerly *Saccharomyces roseii*. Demonstrated tolerance for high baume musts and produces a low level of acetic acid under those conditions. It ferments relatively slowly and produces neutral aroma components. This yeast can ferment to 15% alcohol.

$_{610}$ 4-8) at 28°C in liquid YPD. Cells were harvested, washed once in 10 ml TE buffer (10mM Tris, pH7.5, 1mM EDTA), suspended in 20 ml LiOAc/TE buffer (10mM Tris-HCl, pH7.5, 0.1M LiOac, 1mM EDTA) and mixed gently by shaking at 30°C for 1 hour. Approximately 8 x 10^{7} cells were harvested and suspended in 100 μl of LiOAc/TE buffer. Plasmid DNA (10μg) [and sometimes carrier DNA (salmon sperm, Sigma) was added to 5 μg] was added in a total volume <10 μl and the suspension was incubated at 28°C for 30 mins. Seven times the volume of filter -sterilized PEG reagent (40% polyethylene glycol 4000, 0.1M LiOAc, 10mM Tris-HCl, pH7.5, 1mM EDTA) was added and the mixture was vortexed before incubation at 28°C for 1 hour. Cells were then heat shocked by incubation at 42°C for 5 mins, harvested, and resuspended in 1 ml sterile water.

For the selection of auxotrophic markers in the transformation of strain O11, 100 μl of cell suspension was spread on SD media containing 50 μg of amino acid per ml (L-histidine and L-leucine for plasmid pCP2-4-10; L-histidine and uracil for plasmids pAW219 and pRIM-C3). Selection of the *CAT* gene on plasmid pAW119 was performed by spreading 100 μl of the cell suspension on YEPG media containing 5 mg/ml chloramphenicol. The *RIM-C* gene of plasmid pRIM-C3 was selected by plating 100 μl of the cell suspension on YPD containing 2 μg/ml of cycloheximide. In order to select for the *SMR1* gene on plasmids pCP2-4-10 and pWX509, 100 μl aliquots of the cell suspension was plated on solid SD media containing 10 μg/ml sulfometuron methyl (and 50 μg/ml of L-histidine, L-leucine and uracil when selecting O11 transformants).

3.2.2 Spheroplast fusion method

The sheroplast fusion method was performed essentially as described in Burgers and Percival (1987). Cells were grown overnight with vigorous aeration in 50 ml YPD to approximately 3 x 10^{7} cells/ml. After harvesting, cells were washed successively with 20 ml of sterile water and 20 ml of 1.2M sorbitol, followed by 5 min spins. They were resuspended in 20 ml SCEM (1.2M sorbitol, 0.1M sodium citrate, pH 5.8, 10mM EDTA,

30mM β-mercaptoethanol), 1 mg/ml zymolyase 20000 (Seikagaku Koguo Co. LTD) was added, and the cells were incubated at 30°C with occasional inversions. Spheroplasting was monitored by measuring the decrease in turbidity at 800 nm of a 10-fold dilution of spheroplasts in water. When spheroplasting had proceeded to 90% (about 20-30 mins) the spheroplasts were harvested by centrifugation at 300*g* for 5 mins. They were gently resuspended in 20 ml of 1.2M sorbitol and pelleted for 5 mins at 300*g*. The spheroplasts were gently resuspended in 20 ml of STC (1.2M sorbitol, 10mM Tris-HCl, pH 7.5, 10mM $CaCl_2$) and pelleted again for 5 mins. This pellet was resuspended in 2 ml of STC. Aliquots (100 μl) were mixed with plasmid DNA (10 μg) [and sometimes carrier DNA (salmon sperm, Sigma) was added to 5 μg] in a total volume <10 μl in a 10ml plastic tube. After 10 minutes at room temperature, 1 ml of PEG (10mM Tris-HCl, pH 7.5, 10mM $CaCl_2$, 20% polyethylene glycol 8000; filter sterilized) was added and mixed gently. After a further 10 min room temperature incubation spheroplasts were harvested by a 4 min centrifugation. The pellet was gently resuspended in 150 μl of SOS (1.2M sorbitol, 6.5mM $CaCl_2$, 0.25% yeast extract, 0.5% bactopeptone; filter sterilized) and left at 30°C for 20-40 mins. Eight milliliters of TOP (1.2M sorbitol, 2.5% agar in SD medium) kept at 45°C was added. The tube was inverted quickly several times to mix and plated immediately on SORB plates [SD plates containing 0.9M sorbitol, 3% glucose and sulfometuron methyl (10μg/ml)].

3.2.3 Electroporation of intact yeast cells

Transformation of yeast cells by electroporation was performed according to the method of Hashimoto *et al.*, 1985. Yeast cells were grown overnight in 100 ml YPD at 28°C. Logarithmic phase cells were harvested, washed in sterile distilled water and resuspended in 2 ml of the water. A 50 μl aliquot of this cell suspension was transferred to a 1.5 ml Eppendorf tube. Plasmid DNA (10 μg) and 60 μl of 70% PEG4000 were added and mixed thoroughly by vortexing. After standing for 1 hour at room temperature, 40 μl of the cell suspension was placed in a parallel-electrode chamber. This electrofusion

chamber was equipped with two platinum plate electrodes 2 mm apart. Electric field pulses were applied by an electric capacitor discharge method using a BioRad Gene Pulser™ unit. Three successive pulses of an initial intensity of 5 KV/cm were applied with a capacitance of 1 μF. After application of the pulses, the cell-DNA mixture was left to stand for 1 hour. The cell suspension was then transferred to an Eppendorf tube. One milliliter of sterile water was added to the tube and cells were sedimented by centrifugation. The sedimented cells were resuspended in 200 μl sterile water, and 100 μl aliquots were plated onto SD media containing 10 μg/ml sulfometuron methyl.

3.3 RECOMBINANT DNA TECHNIQUES

3.3.1 Restriction digests, fragment isolation and ligation

Restriction enzymes were obtained from Boehringer Mannheim and digests were performed in buffers supplied by the manufacturer. All digests were performed for 1-2 hours at 37°C. DNA fragments were isolated with a GENE CLEAN (BIO 101) kit following supplier's instructions. Dephosphorylation of fragment ends was achieved by incubation of DNA with 1 unit of calf-intestinal alkaline phosphatase (Boehringer-Mannheim) for 30 minutes at 37°C in buffer as recommended by supplier. DNA ligations were carried out using T4 DNA ligase (Boehringer-Mannheim) in the recommended buffer.

3.3.2 Electrophoresis of DNA

Agarose gel elctrophoresis (described in Maniatis *et al.*, 1982) was used for the separation of DNA molecules up to 20 kbp in size. Samples were electrophoresed in 0.8 - 1.0% agarose gels in TAE buffer (40mM Tris-acetate, pH 8.0, 1mM EDTA) at a constant current of approximately 50mA. One part of gel-loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll [type 400]) was added to five parts of DNA sample prior to gel loading. After electrophoresis, gels were stained with ethidium bromide and

visualized on a 254 nm UV light box. DNA fragments required for cloning were observed on a 360 nm UV light box.

3.3.3 Bacterial transformation

Transformation of the *E. coli* strain DH5α was performed according to the method of Hanahan (1983).

3.3.4 Plasmid isolation

Rapid small-scale plasmid isolation was as described by Ish-Horowicz and Burke (1981).

3.3.5 Yeast DNA isolation

Total DNA was isolated by a procedure based on that described by Davis *et al.* (1980). The yeast culture was grown in 10ml YPD to late log phase. Cells were pelleted by centrifugation at 3000*g* for 5 mins, washed once in TE buffer and resuspended in 0.8 ml of 1.2M sorbitol, 0.1M EDTA, pH7.4, and 14mM β-mercaptoethanol. Zymolyase 20000 (Seikagaku Kogyo Co. LTD) was added to a total concentration of 1 mg/ml and the suspension was incubated for 20 mins at 30°C. Spheroplasts were pelleted by centrifugation at 3000*g* for 5 mins, then resuspended in 0.8 ml TE buffer. To this suspension 80 µl of 1 M Tris-HCl, pH 7.4, 80 µl of 0.5 M EDTA, and 40 µl of a 10% SDS solution were added. After mixing, the suspension was incubated at 65°C for 30 mins. A 4M potassium acetate solution (0.25 ml) was then added and the suspension incubated on ice for 1 hour. After centrifugation at 15000*g* for 25 mins, the supernatant was carefully decanted into a fresh tube. An equal volume of 99.5% ethanol was added to this supernant, and after mixing the solution was centrifuged at 10000*g* for 10 mins. The pellet was then dried and resuspended in 500 µl TE buffer, and RNase A (Boehringer

Mannheim) was added to a final concentration of 100μg/ml. The suspension was incubated at 37°C for 30 mins, then extracted twice with and equal volume of phenol/chloroform. The salt concentration of the aqueous phase was adjusted to 0.3M by the addition of 3M sodium acetate, and 2 volumes of cold 99.5% ethanol were added. After mixing the solution was placed at -20°C for 2 hours or longer. DNA was pelleted by centrifugation at 10000*g* for 10 mins. The pellet was washed twice in 75% ethanol, dried and resuspended in 50 -100 μl TE buffer.

3.3.6 Southern hybridization

Southern blotting and hybridizations were carried out by the procedure of Southern (1975) with minor modifications. The membrane used was Hybond N+ (Amersham), and DNA fixation was achieved by exposing the membrane to UV light for 5 mins. Hybridizations were performed at 42°C in solutions consisting of 4% polyethylene glycol 4000, 2 x SSPE, 1% SDS, 50% formamide, 0.5% blotto and carrier DNA (0.5 mg/ml final concentration). DNA probes were prepared with an oligolabeling kit from Amersham. Autoradiography was performed by exposure of the membrane to X-ray film (Fuji RX) between two intensifying screens overnight at -80°C.

3.4 PROTEIN SYNTHESIS ANALYSIS

3.4.1 Incorporation of radioactivity into yeast proteins

A sample of yeast cells removed from the ferment (500 μl) was incubated with ^{3}H-Leucine (5 μCi) for 2 hours at 30°C. Cells were then pelleted by centrifugation in a microfuge and resuspended in a 2% SDS solution (500 μl). Glass beads were added to 1/2 volume and the suspension was vortexed for 20 minutes. At five minute intervals the suspension was placed on ice to avoid over heating. The solution was then centrifuged for 5 mins in a microfuge, the supernatant was removed and placed in a fresh tube. Proteins

were precipitated by the addition of 5 volumes of cold acetone and incubation at -20°C for 2 hours or longer.

3.4.2 Electrophoresis of proteins

Protein profiles were obtained by SDS-poylacrylamide gel electrophoresis on 10% polyacrylamide separating gels according to the procedure of Laemmli (1970). Fluorography of the gel was achieved by soaking the gel in glacial acetic acid for 5 mins, and then in a solution of 20% PPO (2,5 diphenyloxazole) in glacial acetic acid for 1 hour. The gel was then rinsed in water for 1 hour and dried under vaccuum. Autoradiography was performed by exposing the dried gel to X-ray film (Fuji RX) between two intensifying screens at -80°C for 4-6 weeks.

3.5 PULSED FIELD GEL ELECTROPHORESIS

Transverse alternating field electrophoresis (TAFE) was carried out using a Geneline (Beckman) unit. Yeast chromosomes were prepared in agarose plugs essentially according to supplier's recommendations. Yeast cells were grown to early stationary phase in YPD media and approximately 1 x 10^9 cells were harvested by centrifugation at 3000*g* for 5 mins. To minimize problems with degradation of DNA, cells were processed as quickly as possible after harvesting. Cells were washed twice in ET buffer (50 mM EDTA, 1 mM Tris-HCl, pH 8.0) and resuspended in 2 ml ET buffer. Two milliliters of low melting point agarose mixture (1% low melting point agarose (AdeLab Scientific) in 0.1M EDTA, 10 mM Tris, pH 8.0, 20 mM NaCl) at 50°C was added, along with 0.2 ml of a zymolyase solution. The suspension was mixed by vortexing and immediately loaded into a beckman plug mold by pasteur pipette. The plugs were solidified by refrigeration for 30 mins, then carefully pushed from the mold into 10 ml of 10X ET buffer. An additional 0.15 ml zymolyase solution (10 mg/ml) was added, and the plugs were incubated for 48 hours at 37°C with one change of buffer. Plugs were then transferred to 10 ml of 10X ET

buffer containing 1% sodium lauroyl sarcosinate and 1 mg/ml Proteinase K. The plugs were then incubated at 50°C for 48 hours, with one change of buffer and enzyme replacement. The resulting plugs were then washed 4 times in ET buffer, and stored in ET buffer at 4°C.

Chromosomal DNA molecules were separated by elecrophoresis in a 1% Low endoosmosis (LE) (AdeLab Scientific) agarose gel in TAFE buffer (10mM Tris, 4.35mM acetic acid, 0.5mM EDTA). Electrophoresis was carried out at 12°C with a constant current of 150 mA; pulse times were 60 seconds for 18 hours, then 35 seconds for 6 hours.

3.6 β-GLUCURONIDASE (GUS) ASSAYS

3.6.1 Enzyme activity assays

Yeast cells were grown to log phase, then 1 ml of the culture was pelleted by centrifugation, and the cells resuspended in 1 ml GUS extraction buffer (50mM Na_2HPO_4, pH7.0, 10mM beta-mercaptoethanol, 10mM Na_2EDTA, 0.1% sarcosyl, 0.1% triton X-100). Glass beads (Sigma, 1000-1050 microns) were then added to approximately half the volume, and the suspension was vortexed for 10 mins at 1000 r.p.m. After centrifugation, the supernatant was removed and used as the cell extract. 1-50 μl extract was added to 0.5 ml assay buffer (1mM MUG in extraction buffer) and incubated at 37°C. At various time intervals, 100 μl samples were removed from the assay mix and added to 900μl stop buffer (0.2M Na_2CO_3). Solutions were then assayed in a spectrophotofluorometer with xenon lamp (Aminco SPF-125 ™), excitation 365 nm, emission 455 nm. Standard solutions of 4-methylumbelliferone (MU) (Sigma) in the range of 100 nM to 1 μM were prepared for reference values. 1μM MU corresponded to 100 relative units.

Protein content of samples was determined using a bicinchoninic acid protein assay kit (Sigma Co.).

3.6.2 Agar plate assays

Yeast colonies were grown on solid YPD media containing 50-100 μg/ml X-GLUC. After approximately 36 hours growth, a solution containing 0.1M Na_2HPO_4, pH7.0, 1% sarcosyl, 50 μg/ml X-GLUC and 0.7% agarose was poured as a thin overlay on the plate and allowed to set. After 4-5 hours incubation at 37°C a blue precipitate could be detected in the transformed colonies.

3.6.3 Microscopic visualization

Yeast cells were grown overnight in YPD media, harvested and resuspended in a solution of 0.1M Na_2HPO_4, pH 7.0, 1% sarkosyl and 50-100 μg/ml X-GLUC. This suspension was incubated at 37°C for 2-6 hours or until a deep blue colour had formed. Aliquots of the suspension were then visualized under a microscope.

3.7 KILLER YEAST MANIPULATIONS

3.7.1 Curing of killer strain 3AM

A culture of strain 3AM was grown overnight in YPD at 28°C. Serial dilutions were made in 0.9% NaCl and 0.1 ml aliquots (containing approximately 100 cells) were spread on YPD plates and incubated at 37°C. After 48 hours incubation, single colonies were selected at random and assayed for killer activity as described below.

3.7.2 Assay for cured strain

YPD (containing 1% agar) was autoclaved at 120°C for 20 mins. After cooling to 49°C, the medium was buffered to pH 4.2 with a 10% tartrate solution. Methylene blue (to

0.003% w/v) and killer sensitive strain 5A (to 10^5 cells per ml) were added to the medium prior to pouring the plates. Colonies isolated after heat treatment were then patched onto these assay plates and incubated at 18°C for approximately 72 hours. Curing was recognised by the absence of growth inhibition (clear zones) and lack of blue stained cells around the patched colony.

3.7.3 dsRNA isolation

The dsRNA extraction procedure was essentially that described by Fried and Fink (8). Samples of RNA were analysed by electrophoresis on 1.5% agarose slab gels at a constant current of 100 mA. Gels were stained with ethidium bromide and photographed on a short wave UV light box.

3.8 MICROVINIFICATION TRIALS

3.8.1 Fermentation procedure

Starter cultures were prepared by inoculating 10 ml of YPD medium contained in a conical flask with a loopful of yeast and incubated with vigorous aeration at 28°C. After reaching stationary phase, cells were subcultured into YPD and incubated with vigorous aeration at 28°C. After 24 hours, the cell density was determined by microscopic counts. Samples were used to inoculate Riesling must (200 ml) to a density of 5×10^6 cells per ml. The must contained 220 g per litre total sugar and had a pH of 3.1. Fermentations were carried out in 250 ml conical flasks fitted with airlocks. The juice was sterilized by membrane filtration (0.45 μM) prior to inoculation and fermentations were carried out at 18°C with agitation (approximately 100 o.p.m). Samples were removed anaerobically and aseptically during fermentation by needle and syringe through ports covered with rubber septa. They were analysed for the progress of fermentation by refractometer readings and yeast growth was measured spectrophotometrically at 650nm.

3.8.2 Wine analysis

Residual sugars and acetic acid concentrations were determined with appropriate kits from Boehringer-Mannheim. Measurement of sulfur dioxide in wine was as according to Rankine and Pocock (1972). The alcohol content was determined by near infra-red reflectance spectroscopy according to 'Instructions for the Use of the Technicon 260 Infra-analyser', Bran & Luebbe, Australia. The instrument was calibrated according to Sneyd *et al.*, 1990.

3.9 LARGE SCALE LABORATORY FERMENTATIONS

Grape juice was obtained from St. Hallet's winery, Barossa Valley, during the 1991 harvest. *Pedro Ximines* grapes were machine harvested and crushed on the same morning. Immediately after crushing, grape must was pumped into the winery press. Samples of twenty litres were collected in plastic containers (25 litre capacity) which had been fitted with airlocks. The grape must samples were then transported to The Australian Wine Research Institute for fermentation trials. Diammonium orthophosphate (DAP) was added to all samples to 0.3g/L. Sodium metabisulfite (150 mg/L) additions were then made to some samples to achieve a total sulfur dioxide concentration of 100 mg/L. Grape must was then allowed to stand for 4 hours prior to inoculation.

Starter cultures were prepared by inoculating 100 ml of YPD medium contained in a conical flask with a loopful of yeast and incubated with vigorous aeration overnight at 28°C. This culture was used to inoculate 2L of Rhine Riesling grape juice which was incubated for 24 hours at 28°C with vigorous aeration. The cell density of this starter culture was determined by microscopic counts, and grape must samples were inoculated to a density of 4 x 10^6 cells/ml.

Samples (10 ml) were taken from the centre of the vessel at regular times throughout the fermentation. Progress of fermentation was monitored by refractometer readings and yeast growth was measured spectrophotometrically at 650nm. Duplicate aliquots of each sample (0.1 ml) were plated onto solid YPD media and incubated for 48 hours at 28°C. Colonies were then assayed for GUS activity by the method described in Section 3.6.2.

Chapter 4 Establishing a system for the genetic manipulation of wine yeasts

4.1 INTRODUCTION

The components of a transformation system which need to be considered include the selection procedure for isolating transformants, the type of vector used to introduce the foreign DNA, and conditions for cellular uptake of DNA.

4.1.1 Selection systems for identifying transformants

The introduction of recombinant DNA into yeast strains requires some way of detecting the very small proportion of cells (the transformants) which actually receive and express the foreign DNA.

Yeast strains were first transformed with vectors which contained auxotrophic markers such as *LEU2* for selection (Hinnen *et al.*, 1978; Beggs, 1978). Auxotrophic markers are limited in that they require a yeast host strain that is mutated in the corresponding marker gene, and therefore usually has to be a laboratory bred haploid strain. For example, a *leu2* mutant (deficient in the synthesis of leucine) is required as the recipient strain when the wild type *LEU2* gene is included as the selectable marker. Transformants can then be selected for their ability to grow without leucine.

Recessive auxotrophic mutations can not be readily selected in amphiploid or polyploid industrial strains, nor are they likely to allow normal production yields. Therefore 'dominant' markers are required that enable transformants of wild-type wine yeast strains to be selected without prior genetic manipulation. A number of dominant

Table 4.1. Dominant selectable markers used in yeast transformations

Gene	Source	Product	Selection	References
apt	bacterial plasmid (Tn903, Tn5)	kanamycin phosphotransferase	G418 (geneticin) resistance	Jiminez and Davies,1980; Webster and Dickson, 1983; Hadfield et al, 1990.
aph	bacterial plasmid (pCK203)	hygromycin B phosphotransferase	hygromycin B resistance	Gritz and Davies, 1983; Kaster et al, 1984
cat	bacterial plasmid (Tn 9)	chloramphenicol acetyltransferase	chloramphenicol resistance	Cohen et al., 1980 Hadfield et al, 1986
dhfr (cDNA)	mouse	dihydrofolate reductase	methotrexate resistance	Miyajima et al., 1984 Zhu et al, 1985
CUP1	yeast	copper chelatin	copper resistance	Fogel and Welch, 1982 Butt et al., 1984 Henderson et al, 1985; Hinchliffe and Daubney, 1986
MG^R	yeast	not defined	methylglyoxal resistance	Murata et al, 1985
Kil-K1 (cDNA)	yeast	killer toxin	resistance to killer toxin	Bussey and Meaden, 1985
DEX1	yeast	amyloglucosidase	growth on dextrin	Meaden et al, 1985
ble	bacterial plasmid (Tn 5)	not defined	resistance to phleomycin	Gastignol et. al, 1987

..... cont'd

CRY 1	yeast	ribosomal protein 59	resistance to cryptopleurine	Larkin and Woolford, 1983
Tun^R	yeast	UDP-N-acetylglucos-amine 1-P-transferase	resistance to tunicamycin	Rine et al., 1983
$Comp^R$	yeast	HMG-CoA redustase	resistance to compactin	Rine et al., 1983
tcm1	yeast	ribosomal protein L3	resistance to trichodermin	Schultz and Friesen, 1983
RIM-c	yeast	not defined	resistance to cycloheximide	Takagi et al, 1986
SMR1	yeast	acetolactate synthase	resistance to sulfometuron methyl	Casey et al, 1988 Falco, 1986 Yadav et al., 1986

selectable markers have been reported for use with industrial yeast strains and are listed in Table 4.1.

Genes for resistance to chloramphenicol (chloramphenicol acetyltransferase) G418/geneticin (aminoglycosidase phospho-transferase), hygromycin B (hygromycin B phosphotransferase) and phleomycin (DNA scission) all originate from genes carried by *E. coli* transposons. The bacterial control sequences associated with genes involved in chloramphenicol and hygromycin B resistances operate inefficiently in yeast. All transposon genes [chloramphenicol acetlyltransferase (Hadfield et al., 1986); aminoglycosidase phosphotransferase (Yocum, 1986; Hadfield *et al.*, 1990); hygromycin B phophotransferase (Gritz and Davies, 1983); the *ble* gene coded resistance to the DNA scission agent phleomycin (Gastignol *et al.*, 1987) were made to function efficiently as selectable markers by replacement of bacterial DNA with yeast 5' promoter and 3' termination signals. Two of the three bacterial genes, chloramphenicol acetyltransferase (Hadfield et al., 1986) and aminoglycosidase phophotransferase (Sakai and Yamamoto, 1986; Yocum, 1986), were shown to function as selectable markers in industrial strains of *Saccharomyces*.

Resistance to methotrexate was accomplished by modification of the mouse dihydrofolate reductase gene (*dhfr*) for expression in yeast (Miyajima *et al.*, 1984; Zhu *et al.*, 1986); a disadvantage of this resistance marker is that multiple copies are required for selection. Experiments performed by Fogel and Welch (1982) defined the *CUP1* site on chromosome VIII as the coding sequence for methallothionein; however, resistance to copper required the presence of up to 15 tandem nuclear copies of *CUP1*. Meaden and Tubb (1985) used *CUP1* as a selectable marker on a multicopy plasmid in the construction of a dextrin fermenting strain of brewing yeast.

Multicopy plasmids have also been utilized in procedures aimed to identify enzymes inactivated by inhibitors. By using minimal growth limiting concentrations of tunicamycin,

compactin and ethione in a haploid laboratory strain, Rine *et al.* (1983) were able to isolate from a YEp library wild type genes coding for three different enzymes inhibited by these compounds. Multicopy plasmids have also been used to isolate wild type sensitive and recessive resistance alleles of three ribosomal proteins. The recessive alleles *tcm1* (Fried and Warner, 1981), *cyh2* (Fried and Warner, 1982) and *cry1* (Larkin and Woolford, 1983) result in resistance to tricodermin, cycloheximide and cryptopleurine respectively. Although none of the above six genes were used in industrial strains, the methodology used was shown to be applicable to the selection of methylglyoxal resistance that was subsequently expressed in bakers, brewery and sake yeasts (Murata *et al.*, 1985).

Glucoamylase (amyloglucosidase) genes have been cloned from *Saccharomyces diastaticus*: *STA1* and *STA3* (Yamasita and Fukui, 1983); and DEX1 (Meaden *et al.*, 1985). Meaden *et al.* (1985) reported that *DEX1* was used as a selectable marker in haploid laboratory strains by allowing plasmid treated protoplasts to regenerate on glucose-sorbitol medium prior to selection on dextrin medium. However, this protocol was not successful with brewer's yeast (Tubb, 1987), and there have been no reports of successful transformant selection in industrial yeasts using the *STA1* or *STA3* genes.

Bussey and Meaden (1985) developed a transformation protocol for expression of killer K1-cDNA based on medium containing killer toxin. Use of the alkali cation transformation procedure (Ito *et al.*, 1983), 7-9 hours of preincubation prior to selection, and a final screening for the killer phenotype resulted in a transformation efficiency 5-10% of that observed for selection of a prototrophic marker. Two industrial yeasts transformed by this procedure (Bussey and Meaden, 1985) were shown to have stable inheritance of the killer and immunity phenotype.

Two of the most promising dominant markers described to date are the *RIM-C* gene encoding resistance to cycloheximide (Takagi *et al.*, 1986) and *SMR1* which confers resistance to the herbicide sulfometuron methyl (Falco and Dumas, 1985; Casey *et al.*,

1988). Advantages of these two markers are: i) the genes involved are derived from yeasts; ii) both markers result in transformation efficiencies similar to those obtained with prototrophic markers; and iii) both genes are functional selective markers when present as single copies.

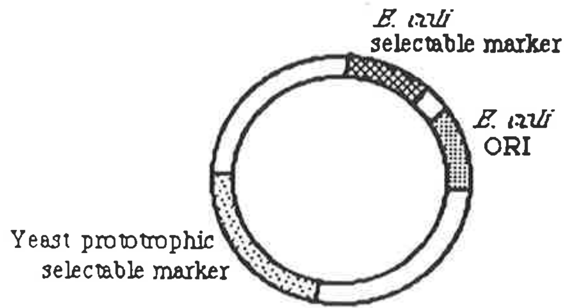
The *RIM-C* gene was cloned from *Candida maltosa* (Takagi *et al.*, 1986) and functions in such a way as to modify ribosomes so that protein synthesis in the cells is no longer inhibited by cycloheximide. The exact mechanism for this modification is not understood. The *SMR1* gene of *S. cerevisiae* provides resistance to sulfometuron methyl (SM) (N-[(4,6 dimethylpyrimidin-2-yl) aminocarbonyl]-2-methoxycarbonyl-benzene-sulfonamide). The target site of SM in *S. cerevisiae* is the enzyme acetolactate synthase, which is involved in the biosynthetic pathway of isoleucine and valine, and is encoded by the *ILV2* gene (Falco and Dumas, 1985; Falco *et al.*, 1985). Sequence analysis has revealed that *ILV2* and *SMR1* are identical alleles, except for a C to T transition mutation at nucleotide 574 of the open reading frame (Falco *et al.*, 1985; Yadav *et al.*, 1986). This mutation results in a proline to serine change in amino acid sequence of acetolactate synthase and confers resistance to inhibition by SM.

4.1.2 Gene transfer vectors

Foreign genes to be expressed in yeast host cells need to become incorporated into a transport vehicle, or vector. The four different types of yeast vectors available are distinguished by their interaction with recipient cells: they are either integrating vectors, designated YIp, replicating vectors (YRp), episomal vectors (YEp) or artificial chromosomes (YAC). Figure 4.1 illustrates the different classes of vectors used in yeast transformations.

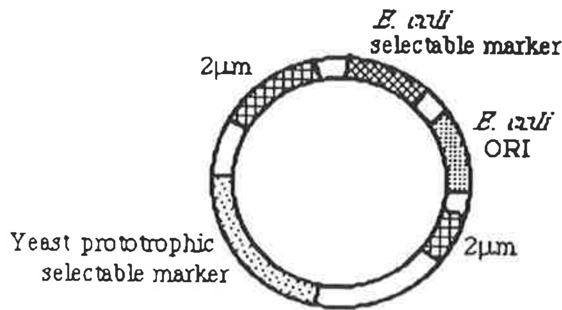
Designation	Structure	Mode of transformation

Yeast integrating vector



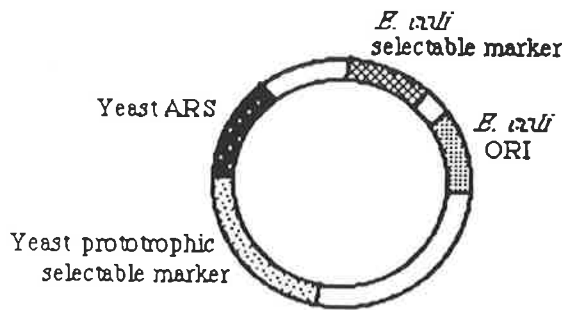
- low frequency
- integrative
- very stable
- one copy per cell

Yeast episomal vector



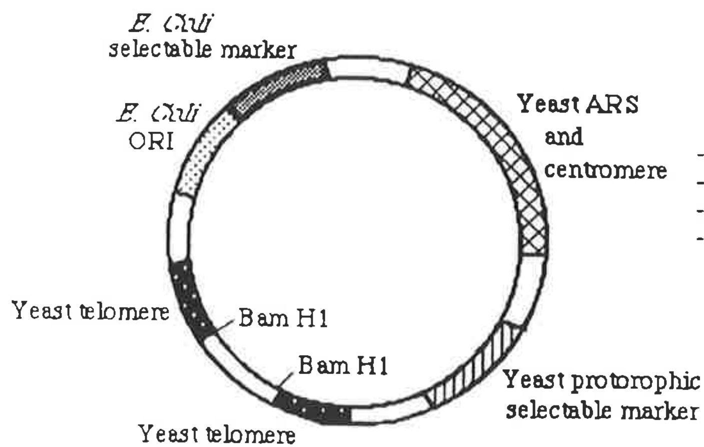
- high frequency
- autonomous
- very unstable
- 5-20 copies per cell

Yeast replicating vector



- high frequency
- autonomous
- moderate stability
- 20-100 copies per cell

Yeast artificial chromosome



- low frequency
- replicates as chromosome
- stable
- 1 copy per cell

Figure 4.1. Classes of cloning vectors available for use in *Saccharomyces cerevisiae.*

Yeast replicating vectors

The YRp (Yeast Replicating Plasmid) vectors which are based on pBR322 and contain the *TRP1* gene and the adjacent chromosomal *ars* (autonomously replicating sequence). These vectors transform yeast at high frequency (1000 to 10000 transformants per µg DNA) and are present at 3-30 copies per cell (Struhl, 1983). The transformants, however, tend to be unstable; about 90% of the cells lose the plasmid in the absence of selection after 10 generations (Struhl *et al.*, 1979). The introduction of chromosomal centromere (CEN) sequences to these vectors stabilizes them considerably, however their copy number is reduced to about 1-2 copies (Clarke and Carbon, 1980).

Yeast episomal vectors

The YEp (Yeast Episomal Plasmid) vectors contain the origin of replication from the naturally occurring 2µm yeast plasmid (Beggs, 1978). 2µm-based plasmids have the advantage that they are usually maintained at high copy number (20-100 copies per cell). Therefore the high dosage of a gene located on such a plasmid can lead to elevated levels of the gene product (Mellor *et al.*, 1985).

Although relatively stable, 2µm-based plasmids are usually lost from yeast cells in the absence of continuous selection for plasmid- encoded characteristics. The frequency of plasmid loss (the failure of plasmid molecules to segregate to a daughter cell) is extremely variable, ranging from less than 1% of cells per generation with the more stable constructs (Beggs, 1981) to as high as 30% in other cases (Struhl, 1983). Most available data, however, relates to haploid strains and it is interesting to note that in continuous culture 2 µm DNA is more stable in diploid cells than haploids (Mead *et al.*, 1986).

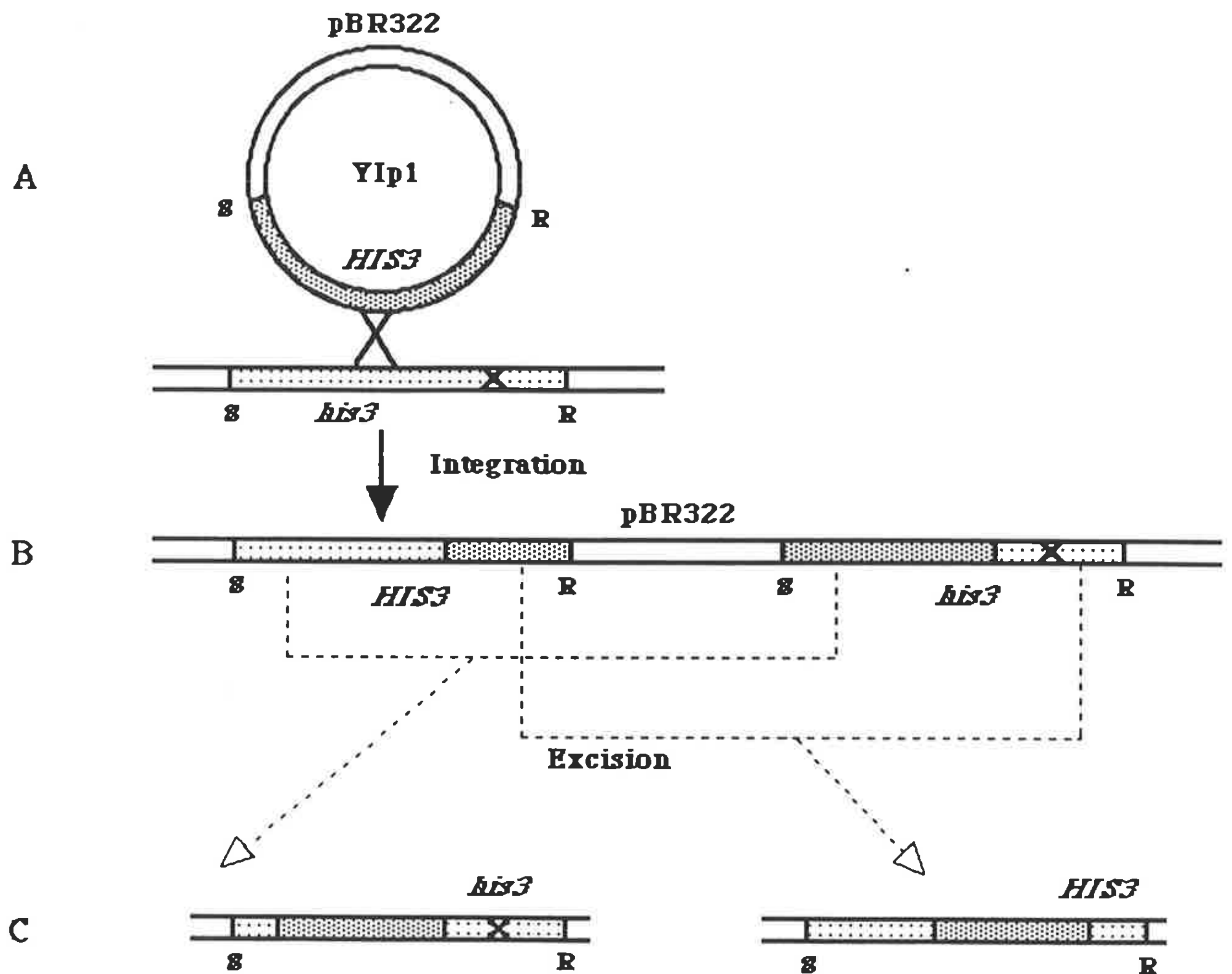


Figure 4.2. Illustration of site specific integration of vector DNA into chromosomal DNA.

(A) Homologous recombination at the *HIS3* region between YIp1 and yeast chromosomal DNA.

(B) Chromosomal structure of the integrated transformant.

(C) Possible structures after excision of the transformed DNA.

Integrative vectors

The YIp (Yeast Integrative Plasmid) vectors can only achieve clonal expression through integration into the genome by homologous recombination. They must carry at least one region homologous to a yeast chromosomal sequence; a selectable marker; and they must lack any *ars* sequence whose presence would confer autonomous replication.

Site specific integration of vector DNA into chromosomal DNA may be mediated by homologous recombination between chromosomal and vector DNA. As shown in Figure 4.2 for the example of the *HIS3* gene region on chromosome XV, homologous recombination results in a duplication of the cloned yeast gene (Struhl *et al.*, 1979). Transformed cells often contain only one copy (sometimes a few copies) of the vector, which replicates with and under the control of chromosomal DNA. The nontandem duplicated structure is not completely stable. After 15 generations of growth in nonselective medium, approximately 1% of the colonies are His^-. This segregation is accompanied by the complete loss of transforming DNA and almost certainly results from a reversal of the original transformation event - ie., excision by homologous recombination.

With yields of approximately 1-10 transformants per µg DNA, transformation frequencies obtained with circular integrating vectors are several orders of magnitude lower than those achieved with self-replicating plasmids. Orr-Weaver *et al.*. (1981) demonstrated that the efficiency of transformation with intregrating plasmids is increased 10- to 1000-fold when the plasmid DNA is first made linear by restriction enzyme digestion within the region of homology to the yeast genome. They also showed that plasmids consistently integrated at the chromosomal location corresponding to the region of the plasmid that had been cleaved by the restriction endonuclease. It was concluded that double-strand breaks in DNA are highly recombinogenic and interact directly with homologous chromosomal sequences by strand invasion and repair synthesis during recombination. This localization

effect has been termed targetting and is the basis for many of the replacement and disruption techniques used to manipulate genes on yeast chromosomes.

Yeast artificial chromosomes

The isolation and cloning of yeast telomeric DNA opened the way for combining these individual functional elements into an artificial chromosome. Murray and Szostak (1983) constructed a series of linear plasmids, all of which contained a selectable marker (eg. *TRP1*, *HIS3*, *URA3*), *ars* replicator, *CEN3* DNA, and telomeres from *Tetrahymena* ribosomal DNA. The plasmids varied in length from 9.8 to 55kb. The very large (>50kb) linear plasmids containing *ars*, CEN and telomeric DNA are present at one or two copies per cell and are more mitotically stable than their circular *ars*, *CEN* counterparts. This stability contrasts with the behaviour of short (<16kb) linear plasmids in which the addition of telomeric DNA reduces the mitotic stability and increases copy number.

The generation of Yeast artificial chromosome (YAC) vectors has provided technology for cloning foreign DNA fragments of several hundred kilobase pairs. This system offers a tenfold increase in the size of DNA molecules than can be cloned in a microbial host, and therefore provides a system for the analysis of complex DNA genomes.

4.1.3 Tranformation procedures

DNA can be introduced into yeast by removing the cell wall enzymatically to produce spheroplasts. Hinnen *et al.* (1978) first succeeded in the transformation of spheroplasts by using a polyethylene glycol-$CaCl_2$ medium which was originally developed for protoplast fusion of plant cells (van Solingen and van der Plaat, 1977). Harashima *et al.*. (1984) have shown that transformants obtained under these conditions were diploids or polyploids in cell size, shape and segregation patterns of genetic markers after crossing with a standard haploid strain, although the strains used as recipients in the transformation were

haploids. It is therefore considered that transformation of spheroplasted yeast cells is directly associated with cell fusion.

An alternative transformation procedure, which avoids cell wall dissolution, involves the treatment of whole cells with alkali cation salts or sulfhydryl reagents and polyethylene glycol (Ito *et al.*, 1983). This is generally less efficient (by 1-2 orders of magnitude) but the overall simplicity of the procedure makes it the prefered method for many applications. Furthermore transformants can be detected after only 36 hours incubation, whereas regeneration of yeast cell walls takes 3-7 days after spheroplast fusion.

Recently, the method of electroporation has become available for yeast transformation. This procedure involves exposure of a suspension of cells and cloned DNA to a high voltage electric discharge (Zimmerman and Vienken, 1982; Potter *et al.*, 1984). In essence, electroporation makes use of the fact that the cell membrane acts as an electical capacitor which is unable (except through ion channels) to pass current. Subjecting membranes to a high voltage electric field results in their temporary breakdown and the formation of pores that are large enough to allow macromolecules to enter or leave the cell. The recovery of membrane integrity is a natural decay process which can be delayed at $0^{o}C$.

In 1985 Karube *et al.* succeeded in using electric field pulses to introduce plasmid DNA into yeast spheroplasts with an efficiency of 10^3 transformants per μg DNA and Hashimoto *et al.* (1985) used electroporation to transform intact cells, although only a low efficiency, of approximately 90 transformants per μg DNA, was achieved. Since then, various modifications of the electroporation method have been described (Simon and McEntee, 1989; Rech *et al.* 1990) and an efficiency of as high as 10^7 transformants per μg DNA has been reported (Meilhoc *et al.* 1990).

Various other yeast transformation procedures have been described including a spheroplast treatment which does not involve cell fusion (Bergers and Percival, 1987);

agitation of whole cells with glass beads (Costanzo and Fox, 1988); and the direct treatment of colonies from agar plates (Keszenman-Pereyra and Hieda, 1988). However, the alkali cation method of *Ito et al.* (1983), remains the favoured protocol for most yeast manipulations.

4.1.4 Expression and secretion of foreign proteins

For a transformed yeast strain to express a foreign gene, the vector must contain various DNA sequences and signals which regualte the processing of a foreign DNA sequence into a protein. These collectively are termed the "expression complex". The expression complex comprises a promoter and terminator surrounding the multiple cloning sites for foreign gene insertion, and it may also contain signals for protein secretion. Promoters originating from yeast genes are used since promoters are fairly host-specific and those from higher eukaryotes tend to have lower efficiency of initiation of transcriptions when used in yeast (Beggs *et al.*, 1980; Rothstein *et al.*, 1984). Table 4.2 gives some common examples of promoter elements derived from yeast.

Promoters for glycolytic enzymes are popular as these enzymes are among the best represented proteins in a yeast cell. Each can represent 1-5% of total cell protein - and it is assumed that this high-level expression is is due to the association of strong promoters with these genes (Goodey *et al.*, 1987). Some of these promoters are inducible or repressible (Brent, 1985) such that by the addition of an inducer or repressor, protein production is either switched on or off. This trait is used to achieve regulated expression of heterologous proteins in yeast. As an example, the transcription of the acid phosphatase gene is tightly repressed when inorganic phosphate is present in the growth medium and induced by depletion of inorganic phosphate. Other examples have been discussed by Kingsman *et al.* (1985) and Goodey *et al.* (1987).

Promoter element	Abbreviation	Reference
acid phosphatase	PHO5	Kramer et al., 1984
		Meyhack et al., 1982
alcohol dehydrogenase	ADH1	Hitzeman et al., 1981
		Bennetzen and Hall, 1982
galactokinase	GAL1	Stepien et al., 1983
		Goff et al., 1984
glyceraldehyde-3-phosphate dehydgrogenase	GAP3	Bitter and Egan, 1884
		Holland and Holland, 1980
Mating factor-α	MFα	Bitter et al., 1984
		Kurjan and Herskowitz, 1982
Phosphoglycerate kinase	PGK	Dobson et al., 1982
		Tuite et al., 1982
triose phophate isomerase	TPI	Alber and Kawasaki, 1982

Table 4.2. Some of the common promoter elements used for foreign gene expression vectors in yeast.

The biological activity of a protein also depends on its correct processing which includes proteolytic cleavage of signal sequences, glycosylating, and tertiary structure folding. Several reviews have covered heterologous protein processing from yeast (Kingsman *et al.*1988; Smith *et al.*, 1985; Goodey *et al.*, 1987).

Generally, it is useful to have the protein product of foreign genes secreted from the yeast cell. The mechanism of secretion appears to be similar to that of higher eukaryotes (Schekman and Novmic, 1982). However, heterologous gene products derived form higher eukaryotes and produced in yeast are not secreted in high amounts indicating that the secretion signals for these genes are not correctly recognised in the yeast cell (Kingsman *et al.*, 1985). Therefore signal sequences from proteins naturally secreted from yeast are

usually used, such as that for acid phosphatase (Smith *et al.*, 1985), the a-mating type factor (Julius *et al.*, 1984), and invertase (Perlman and Halvorsen, 1983).

4.1.5 Chapter aims

The question arises as to which of these systems is most useful to the wine industry, and what are the limitations or advantages of particular vectors. A desirable vector should have the following attributes: i) good transformation efficiency, ii) stable inheritance of transferred genes; and iii) usefulness in a wide range of industrial strains. It is also imperative that the vector or the transformation procedure does not adversely affect the characteristics of the yeast strain under fermentation conditions.

A current limiting factor in the commercial application of recombinant DNA technology to industrial food and beverage yeasts is the presence of non-food yeast nucleotide sequences in the transformants. In light of regulatory and market requirements then, it is important to consider the possibility of producing transformants containing only yeast sequences when assessing the advantages of vector systems.

The aim of this chapter is to investigate a number of selectable markers, vector systems and transformation protocols to establish a system (which meets the criteria discussed above) for the application of recombinant DNA technology to wine yeasts.

4.2 RESULTS

4.2.1 Choice of a selectable marker

In order to choose an efficient dominant selectable marker for use in wine yeast transformations, an experiment was performed to test three available markers.

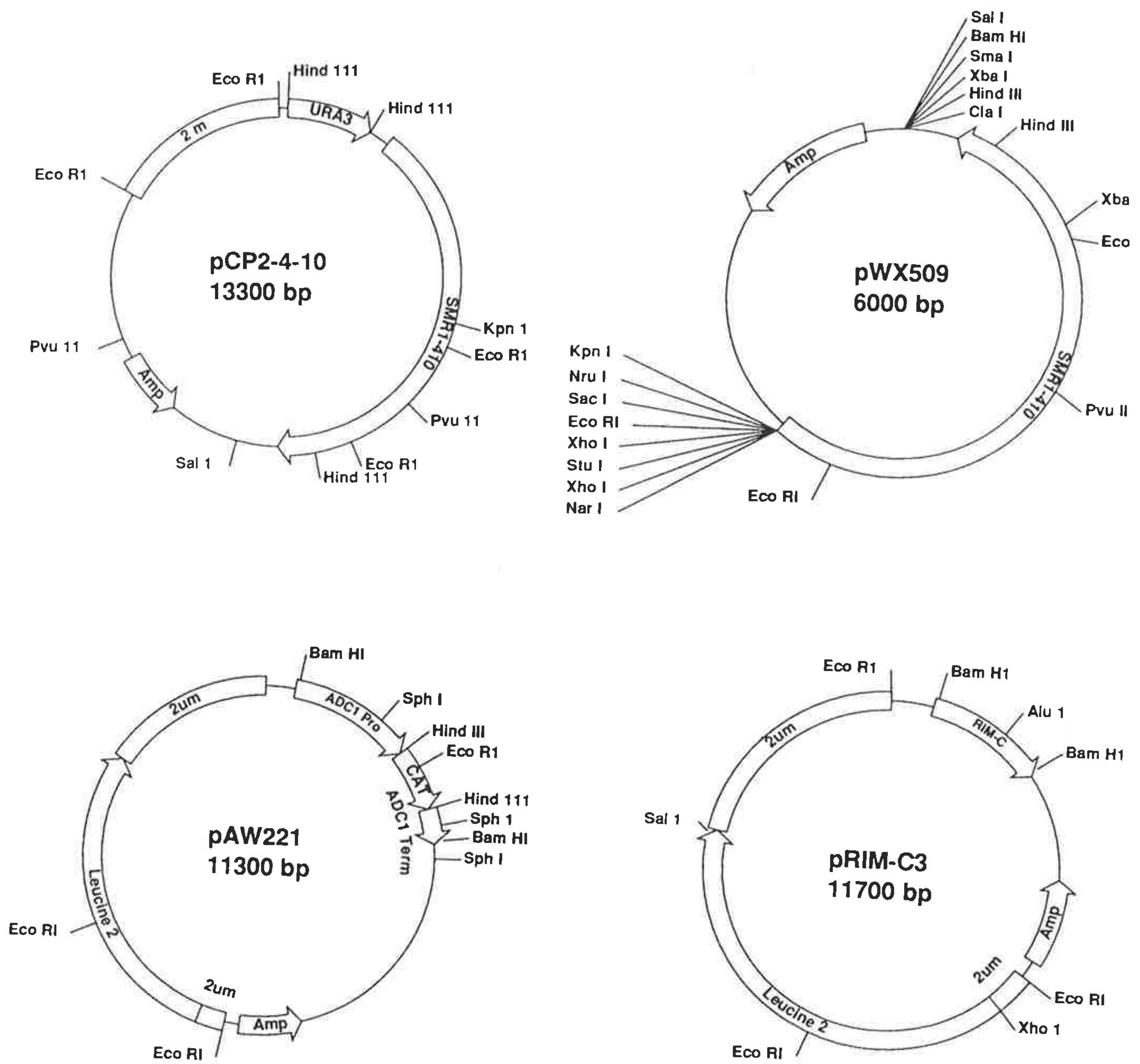


Figure 4.3. Cloning vectors used in transformation experiments.

i) Chloramphenicol Resistance

Chloramphenicol (Cm) inhibits protein synthesis carried out by 70S ribosomes, as found in procaryotes and mitochondria, but not by 80S ribosomes, found in the cytoplasm of eukaryotic cells. Eukaryotic cells may be inhibited by Cm if their growth depends upon utilising a carbon source that can only be assimilated via mitochondrial-dependent aerobic metabolism. Resistance to Cm by certain bacteria is mediated by plasmid encoded chloramphenicol acetyl transferase (*CAT*) (Shaw, 1983). This enzyme causes inactivation of the antibiotic by acetylation derived from acetyl-CoA.

The *CAT* coding sequence from Tn9 has been shown to confer resistance to chloramphenicol in yeast when present as a single copy per cell (Hadfield *et al.*, 1986). The sequence, however requires yeast promoter and terminator signals for expression. Therefore, the *CAT* coding region was cloned into the HindIII site of the vector AAH5 (Ammerer, 1983) which contains the yeast alcohol dehydrogenase 1 (*ADC1*) promoter and terminator sequences. A clone with the coding sequence in correct orientation with respect to the promoter (pAW 221) (Figure 4.3) was selected for transformation experiments.

ii) Cycloheximide

The cycloheximide resistance gene from *Candida maltosa* (*RIM-C*) has already been described. The plasmid pRIM-C3 (Figure 4.3) which contains the resistance gene was obtained from Dr. M. Takagi for transformation trials.

iii) Sulfometuron methyl

Plasmid pCP2-4-10 which contains the *SMR1-410* gene was obtained from Dr. G. Casey for transformation studies.

In order to test the suitability of each of these selectable markers for use in the transformation of wine yeasts, it was first necessary to determine the minimum inhibitory levels of the relevant chemicals on a range of yeast strains. Yeast growth (colony size) was assessed on solid media containing the inhibitors. Table 4.3 depicts the growth responses of yeast strains to a range of concentrations of chloramphenicol, sulfometuron methyl and cycloheximide. It should be noted that the minimal inhibitory concentration of cycloheximide was not accurately determined, as complete growth inhibition of all strains was achieved with the lowest concentration tested (2 µg/ml). However, as successful selection of pRIMC-3 transformants had been reported using 10 µg/ml cycloheximide (Takagi *et al.*, 1986), it was decided that 2 µg/ml would be a suitable concentration for transformation experiments. Complete growth inhibition of all strains tested was achieved at a concentration of 10 µg/ml sulfometuron methyl and 5 mg/ml chloramphenicol. These concentrations were used in transformation studies.

The efficiency of each marker was tested in transformation experiments using each respective plasmid on the haploid laboratory yeast strain Ol1. This strain is auxotrophic for both uracil and leucine. Approximately 5 µg of plasmid DNA was used to transform OL1 using the lithium acetate method (Ito *et al.*, 1983). Aliquots of the transformation mix were then plated out on different selection media for direct comparison of the auxotrophic and dominant markers on each plasmid. Table 4.4 shows the numbers of transformants obtained in each case.

These results indicate that under conditions described here transformants of plasmids pAW221 and pRIM-C3 can be selected directly for their auxotrophic marker (leucine), but not for their dominant markers (chloramphenicol and cycloheximide, respectively). Plamsid pCP2-4-10, however, resulted in an equal number of transformants when selected by either the auxotrophic (uracil) or the dominant (sulfometuron methyl) markers.

chemical concentation	YEAST STRAIN GROWTH				
	OL1	1A	2A	3A	5A
sulfometuron methyl (μg/ml)					
0	++++	++++	++++	++++	++++
2	++	++	+++	++	++
5	+	-	-	+	-
10	-	-	-	-	-
20	-	-	-	-	-
cycloheximide (μg/ml)					
0	++++	++++	++++	++++	++++
2	-	-	-	-	-
5	-	-	-	-	-
10	-	-	-	-	-
20	-	-	-	-	-
chloramphenicol (mg/ml)					
0	++++	++++	++++	++++	++++
1	+++	++	++	++++	+++
2	++	+	+	++	++
3	-	-	+	+	+
4	-	-	-	+	-
5	-	-	-	-	-

Table 4.3. Responses of a number of yeast strains to a range of concentrations of chemicals to be used in the selection process for yeast transformants. + designates one arbitrary unit of growth after 4 days incubation.

Plasmid	Transformants per 10μg DNA	
	Auxotrophic marker	Dominant marker
pAW221	847	0
pRIM-C3	915	0
pCP2-4-10	763	785

Table 4.4. Efficiencies of auxotrophic and dominant selectable markers used in transformation of strain Ol1.

Transformation method	pCP2-4-10 transformants	pWX509 transformants	Incubation time (hours)
Alkali cation (Ito *et al.*, 1983)	825	31	36
Alkali cation + carrier DNA	2500	160	36
Spheroplast fusion (Burgers and Percival, 1987)	1160	93	48-72
Spheroplast fusion + carrier DNA	4250	340	48-72
Electroporation (Hashimoto *et al.*, 1985)	300	15	36

Table 4.5. Comparison of yeast transformation methods.

In order to confirm appropriate levels of expression of the dominant markers on plasmids pAW221 and pRIM-C3, transformants which had been selected by their auxotrophic markers were streaked onto the relevant dominant selection media. Both pAW221 and pRIM-C3 transformants showed positive growth on the dominant selection media, thus they displayed an indirect resistance phenotype. One can conclude that appropriate levels of the *RIM-C* and *CAT* genes are achieved in *S. cerevisiae*, but that direct exposure of transformants to either chloramphenicol or cycloheximide is not feasible. It is possible that post-transformation incubation prior to selection would result in isolation of transformants based on cycloheximide or chloramphenicol resistance. In fact, the transformation method described by Takagi *et al.* (1986) includes an overnight incubation of transformants prior to exposure to cycloheximide. Transformation of O11 using pRIM-C3 and pAW221 was therefore repeated with an altered selection procedure. Following transformation, cells were plated directly onto non-selective media, incubated overnight at 28°C, and then overlayed with selective media. This procedure, however, again failed to yield transformants with either plasmid.

Due to ease of selection and a transformation comparable efficiency with the *URA3* marker, the *SMR1* gene was used in further transformation experiments.

4.2.2 Choice of a transformation procedure

A number of transformation methods were tested to find one most suitable for manipulation of wine yeast strains:

i) the alkali cation method of Ito *et al.* (1983);

ii) the alkali cation method of Ito *et al.* (1983) with the addition of carrier DNA to the plasmid DNA;

iii) the spheroplast fusion method of Burgers and Percival (1987);

iv) the spheroplast fusion method of Burgers and Percival (1987) with the addition of carrier DNA to the plasmid DNA ; and

v) the electroporation of intact cells as described by Hashimoto *et al.* (1985).

Wine yeast strain 5A was transformed with plasmids pCP2-4-10 and linearised pWX509 (digested with PvuII). Transformants were selected by resistance to sulfometuron methyl. Each transformation experiment was performed in triplicate - the results of these transformation experiments are presented in Table 4.5. Success of the method is determined by the average number of transformants, and the expediency with which transformants are obtained.

The use of carrier DNA in alkali cation transformations and in the spheroplast fusion method increased efficiencies approximately three fold. This observation may be explained by the fact that foreign DNA is susceptible to nuclease attack in the host cell, and that the carrier DNA provides an alternative substate for these nucleases thus 'protecting', to some extent, the plasmid DNA.

Direct comparison of all methods tested shows that the highest yields of transformants were obtained with the spheroplast fusion/carrier DNA procedure, and that electroporation proved to be the least efficient. Electroporation, however is the quickest and easiest method to perform (although expensive equipment is required) and transformants are visible after approximately 36 hours incubation. The alkali cation method is relatively inexpensive and expedient, and again, transformants can be detected after appproximately 36 hours incubation. The spheroplast fusion method is the most time-consuming to perform, and 2-4 days are required for detection of transformants.

AWRI strain	Transformants per 10μg DNA	
	pCP2-4-10	linear pWX509
7A	5000	560
8A	3000	320
10A	3500	150
11A	3000	300
3A	2500	160
6A	300	50
5A	450	30
2A	400	80

Table 4.6. Transformants obtained using the alkali cation/carrier DNA method on a range of commercially popular wine yeast strains.

Considering all factors, the alkali cation/ carrier DNA method was selected. This method was used to transform a number of wine yeasts from the AWRI collection with plasmids pCP2-4-10 and pWX509, selecting directly for resistance to sulfometuron methyl. Results from this experiment (Table 4.6) verify that the selectable marker and transformation method are suitable for a wide range of wine yeasts strains. Figure 4.4 shows the results of the transformation of one of these wine yeast strains (7A) with pCP2-4-10, circular pWX509 and linear pWX509. Plate A shows the transformants obtained with pCP2-4-10; an efficiency of 5000 transformants per 10 µg DNA was obtained. Plate B is the control transformation in which no DNA was present in the transformation mixture. Five colonies are present on this plate and represent the background spontaneous mutants which are resistant to sulfometuron methyl. Plate C shows the transformants obtained with linearised pWX509 - an efficiency of 560 transformants per 10µg DNA was achieved. Plate D shows the results of transformation with circular pWX509, and in this case the efficiency was similar to that obtained in the control transformation (Plate B). One can conclude, therefore, that transformation with circular pWX509 was not successful.

4.3.3 Analysis of transformants

Transformants of strain 5A were analysed for the presence of plasmids pCP2-4-10 or pWX509. Total DNA was isolated from 3 transformants of pCP2-4-10 (5A-pCP2-4-10), 3 transformants of pWX509 (5A-pWX509) and a control, untransformed colony of 5A. The DNA was digested with PvuII, electrophoresed on a 0.8% agarose gel and transferred by Southern blotting to a nylon membrane. Both plasmids contain sequences of pBR322. Therefore, the presence of each plasmid could be detected by hybridization of the membrane with radiolabeled pBR322.

Figure 4.5 depicts the result of this hybridization experiment. As expected, no hybridization signal is apparent in the lanes containing DNA from the untransformed colony

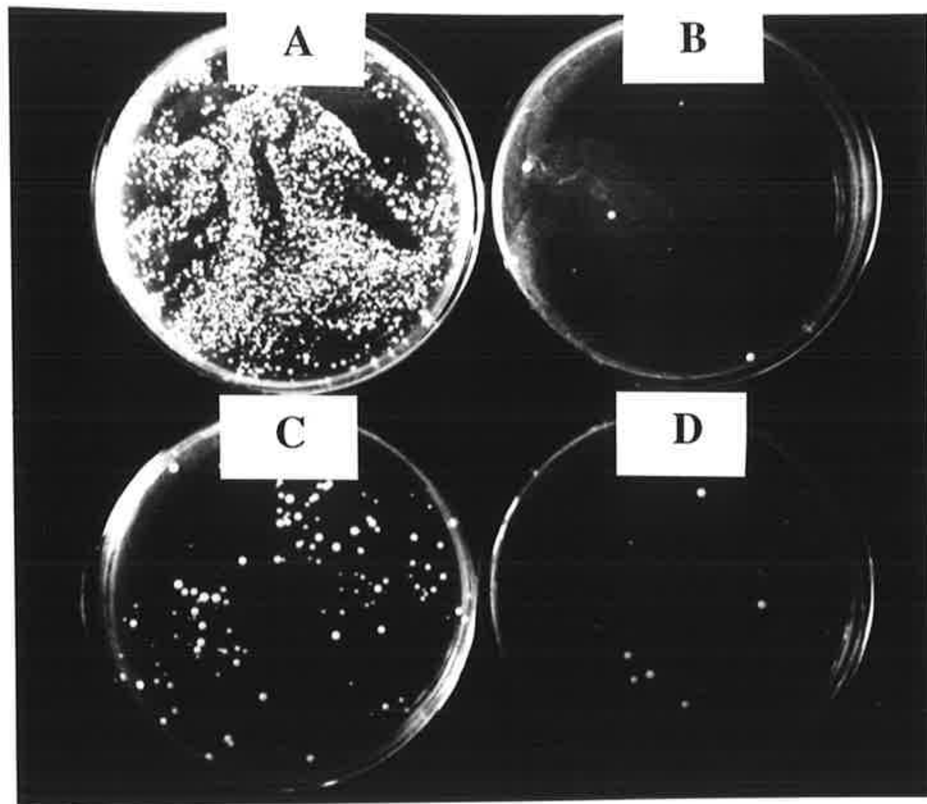


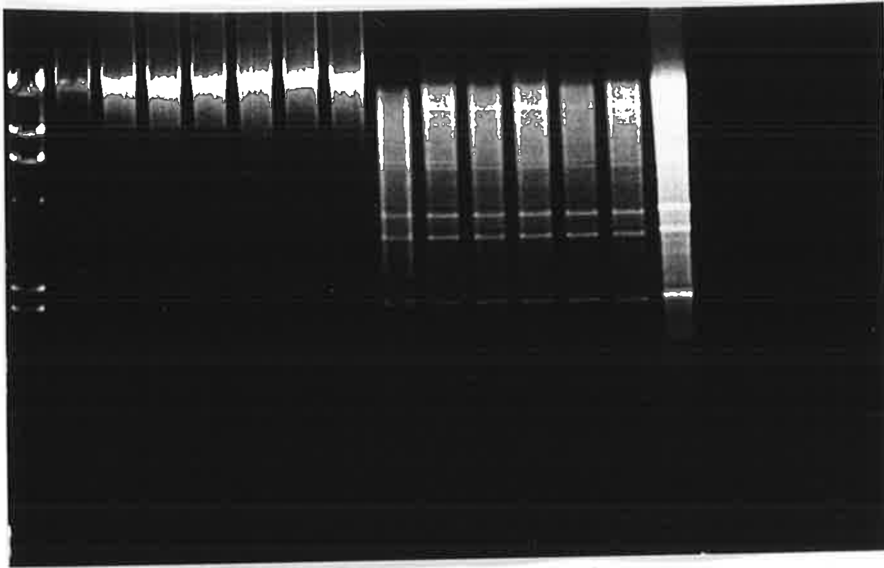
Figure 4.4. Transformation of wine yeast strain AWRI 7A with the following plasmids: Plate A - pCP2-4-10; Plate B - no DNA control; Plate C - pWX509 linearised at the Pvu II site; Plate D - circular pWX509.

Figure 4.5. Southern hybridization detecting plasmids pCP-2-4-10 and pWX509 in transformants of strain 5A. Panel A: Total yeast DNA samples electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualised on a UV light box. Panel B: Autoradiogram of gel depicted in Panel A, after hybridization with a radiolabeled sample of pBR322.

Lanes:		
	1	λ hind III markers
	2	5A
	3-5	5A -pCP2-4-10
	6-8	5A-pWX509
	9	5A digested with Pvu II
	10-12	5A-pCP2-4-10 digested with Pvu II
	13-15	5A-pWX509 digested with Pvu II
	16	pCP2-4-10
	17	pCP2-4-10 digested with Pvu II
	18	pWX509
	19	pWX509 digested with Pvu II

A

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



B

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

(lanes 2 and 9). The undigested DNA from transformants obtained with plasmid pCP2-4-10 (lanes 3-5) show a strong signal at the position of the chromosomal DNA, and a weaker signal at approximately 9.8kb, which correlates to the size of the undigested plasmid pCP2-4-10 (lane 16). The signal at the position of the chromosomal DNA is unexpected as the plasmid is a self-replicating vector and is not expected to be associated with genomic DNA. The PvuII digested DNA samples (lanes 10-12) show strong hybridization signals to fragments of approximately 10kb and 3kb. These correspond to the fragments generated by a PvuII digest of pCP2-4-10 (lane 17). Three other weaker signals are apparent at approximately 4.0, 3.8 and 2.6kb respectively.

The undigested DNA samples from transformants of the pWX509 vector (lanes 6-8) show weak signals at the chromosomal DNA position. As pWX509 is an integrating vector, the hybridization signal is expected to be associated with genomic DNA. The strength of the signal is indicative of the low copy number (possibly only one copy per genome) of integrating plasmids. The PvuII digested samples (lanes 13-15) give rise to signals of 6.0kb, which corresponds to the size of the plasmid after excision from the genome. Another band of approximately equal intensity is also present at a position which represents a fragment size of 2.6kb. This signal is common to both pCP2-4-10 and pWX509 transformants.

As plasmid pWX509 was linearised at the PvuII site prior to transformation, the highly recombinogenic ends of the plasmid molecule will target the vector to integrate directly into the PvuII site of the *ILV2* gene on chromosome XIII (Figure 4.6). The position of plasmid pWX509 in the genome was analysed by pulsed field gel electrophoresis and Southern hybridization. Chromosomes were isolated from a control untransformed 5A colony and a pWX509 transformant. They were then separated by electrophoresis using the Transverse Alternating Field Electrophoresis (TAFE) system (Gardiner *et al.* 1986) and transferred by Southern blotting to a nylon membrane. A radiolabeled EcoR1 fragment of the *SMR1* gene was hybridized to the membrane to locate

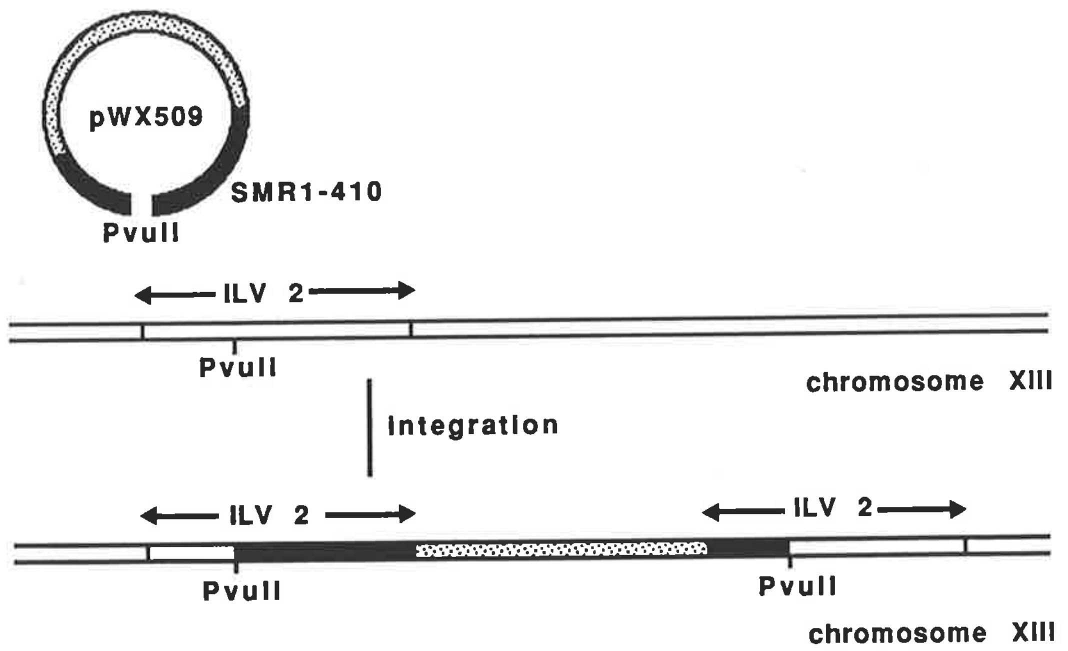


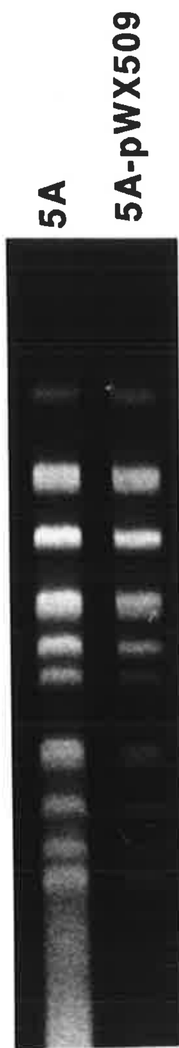
Figure 4.6. Integration pattern of pWX509 into yeast chromosome XIII.

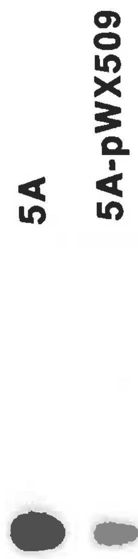
the *ILV2* gene. The signal resulting from this hybridization (Figure 4.7) is present in both the untransformed and transformed strains and, as the *ILV2* locus has previously been mapped to chromosome XIII (Mortimer *et al.*, 1989), this band represents chromosome XIII. The membrane was then stripped of the *SMR1* probe and rehybridized with a labeled pBR322 plasmid to identify the position of the integrated plasmid. Figure 4.7 shows that the pBR322 probe does not hybridize to the untransformed strain but, in the tranformant, has hybridized to the same band as the *SMR1* probe, indicating that plasmid pWX509 has been successfully targetted to chromosome XIII.

An experiment was performed to determine whether plasmid pWX509 had integrated into every copy of the *ILV2* gene in the transformant. Given that wine yeast strains are diploid or polyploid, there will be more than one copy of the *ILV2* gene present in the nucleus of strain 5A. One application of the transformation system described in this chapter could be to disrupt (and therefore inactivate) undesirable genes in wine yeasts. In order to achieve gene inactivation, it is necessary to disrupt every copy of that particular gene in the cell. It is therefore important to know whether this transformation procedure targets plasmid pWX509 to every copy of the *ILV2* sequence.

Total DNA was isolated from control strain 5A and from strain 5A transformed with pWX509 (5A-pWX509). Both DNA samples were digested with EcoRV. There are no EcoRV sites in plasmid pWX509. Therefore EcoRV generated fragments of chromosome XIII in which pWX509 has integrated in the *ILV2* gene will be 6kb longer than fragments which contain the intact *ILV2* gene. The DNA samples were electophoresed on a 1% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a radioactively labeled sequence of the *ILV2* gene. Results of this hybridization are presented in Figure 4.8. A fragment of approximately 5.9kb is detected in the untransformed strain 5A DNA (Panel B). This band represents EcoRV fragments containing intact *ILV2* genes. In the transformant DNA (5A-pWX509) two fragments were highlighted - one at 5.9kb and the other at approximately 12kb. The 12kb fragment represents EcoRV fragments which

Figure 4.7. Southern hybridization depicting the chromosomal location of the pWX509 vector in transformed strain 5A-pWX509. Panel A: Yeast chromosomes separated on a 1% agarose gel by transverse alternating field electrophoresis. Panel B: Autoradiogram of gel depicted in Panel A probed with a radiolabeled sequence of the *ILV2* gene. Panel C: Autoradiogram of gel depicted in Panel A probed with a radiolabeled sequence of pBR322.


5A
5A-pWX509
A


5A
5A-pWX509
B

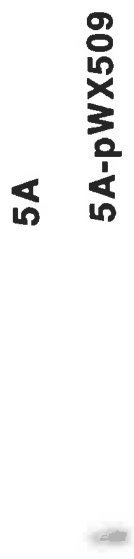
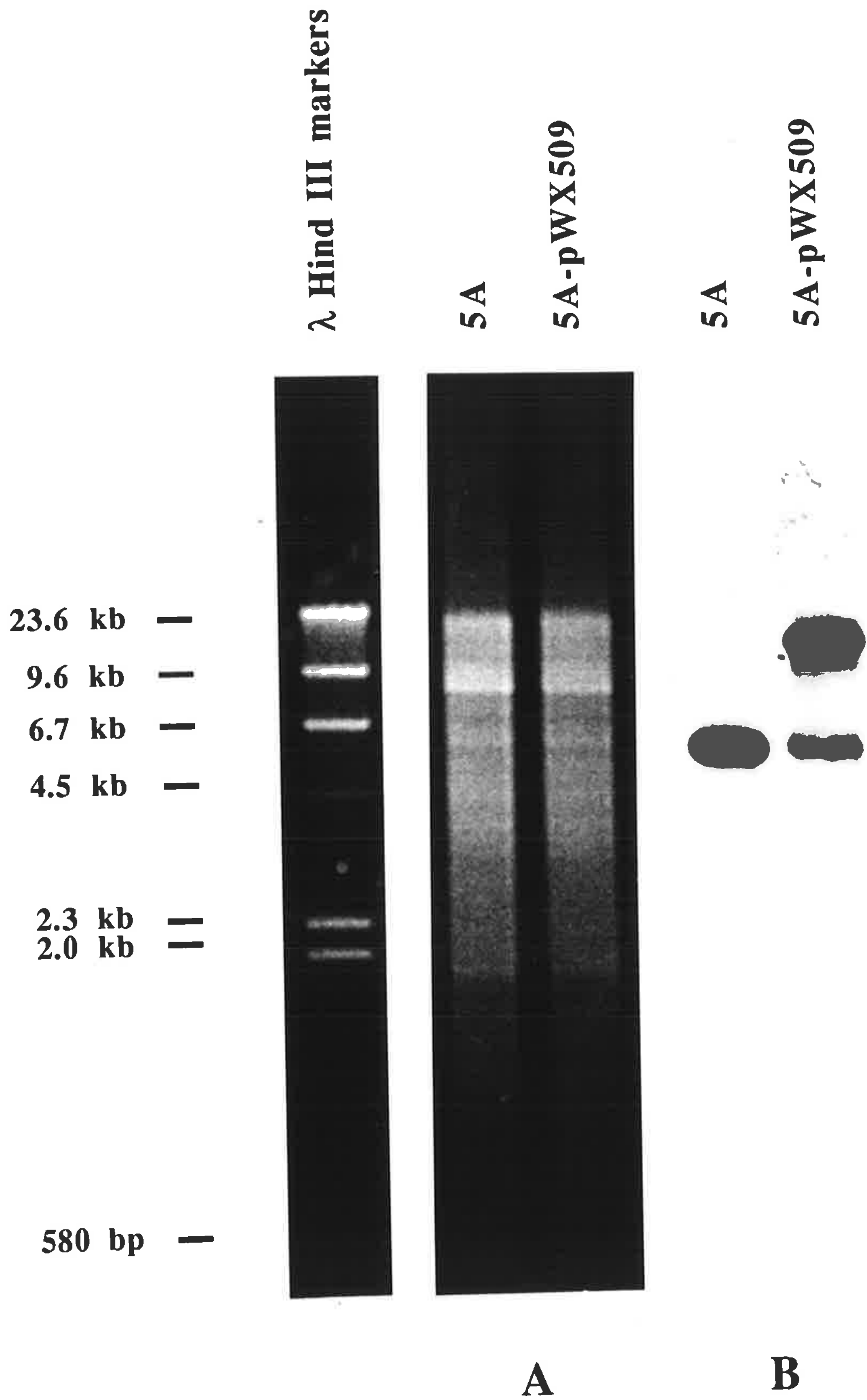

5A
5A-pWX509
C
chromosome XIII

Figure 4.8. Southern hybridization to detect copy number of integrated plasmid pWX509 in transformant 5A-pWX509. Panel A: Total cellular DNA digested with Eco RV, electrophored on a 1% agarose gel, stained with ethidium bromide and visualised on a UV light box. Panel B: Autoradiogram of gel depicted in Panel A probed with a radiolabeled sequence of the *ILV2* gene.



λ Hind III markers
5A
5A-pWX509
5A
5A-pWX509
23.6 kb
9.6 kb
6.7 kb
4.5 kb
2.3 kb
2.0 kb
580 bp
A
B

contain pWX509 integrated into the *ILV2* gene. The 5.9kb fragment detected in the transformant DNA sample indicate that there are some intact copies of the *ILV2* gene present in this strain. Therefore plasmid pWX509 has not integrated into every available copy of the *ILV2* gene.

It is interesting to note that the hybridization signal of fragment 12kb is approximately twice as intense as the signal of the 5.9kb fragment in the 5A-pWX509 DNA . There are two possible explanations for this observation. First, there may be twice as many copies of the *ILV2* gene which have integrated copies of pWX509 as there are of intact *ILV2* genes. If this is the case, it may reflect the ploidy level of chromosome XIII of strain 5A; that is, the strain may be triploid for chromosome XIII. Secondly, there may be more than one copy of the plasmid pWX509 integrated into one particular *ILV2* site. If this event has occurred, it is not possible to draw any conclusions about the ploidy level of chromosome XIII.

4.3.4 Fermentation trials

Transformants of strain 5A were subjected to fermentation trials in order to determine whether the introduction of foreign DNA had a deleterious effect on yeast performance. Trials were conducted on:

i) a control untransformed 5A;

ii) 5A transformed with the self-replicating plasmid (5A-pCP2-4-10); and

iii) 5A transformed with the integrating plasmid (5A-pWX509).

Three colonies of each type of strain were inoculated in duplicate into Riesling grape juice and fermented at 25°C. Samples were taken at regular intervals and assayed for sugar content (by refractometer) and yeast growth (by optical density at 650nm). Average readings were plotted on graphs (Figure 4.9). No significant differences were found between the three strains.

The pH and percent alcohol (vol/vol) were also measured for each of the ferments. Average readings are presented in Table 4.7. Again, there are no significant differences in data between the three strains.

On completion of each ferment, aliquots of the yeast population were appropriately diluted and equal volumes were plated onto selective (SD containing 10 μg/ml sulfometuron methyl) and non-selective (YPD) media in order to analyse stability of the introduced plasmids. The number of colonies per plate after 48 hours incubation at 28°C are recorded in Table 4.8 The number of colonies on the YPD media represents the total colony forming units present at the end of the ferment. The colonies growing on selective media represent colony forming units which have retained the plasmid.

These results indicate that approximately 85% of the cells of the strain transformed with the self-replicating plasmid (pCP2-4-10) have retained the plasmid throughout fermentation. No plasmid loss is evident from the strain transformed with the integrative vector (pWX509).

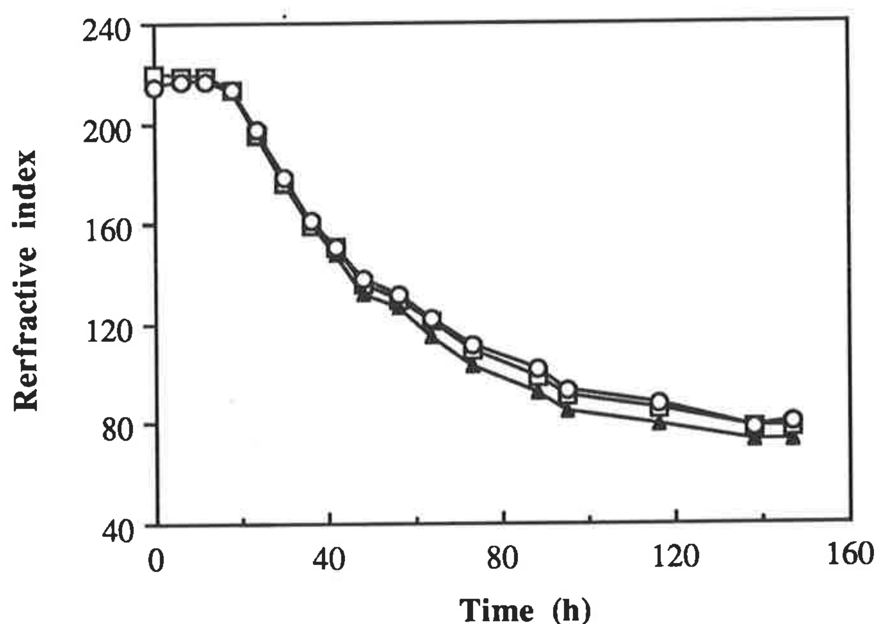


Figure 4.9. Fermentation curves of the control untransformed (AWRI 5A) (□) strain, the strain transformed with the replicating vector (5A-pCP2-4-10) (▲) and the strain transformed with the integrating vector (5A-pWX509) (O).

Strain	pH	Alcohol (% vol/vol)
AWRI 5A	2.96 ± 0.01	13.12 ± 0.17
5A (pCP2-4-10)	2.96 ± 0.01	13.33 ± 0.08
5A (pWX509)	2.96 ± 0.01	13.03 ± 0.08

Table 4.7. pH values and alcohol content of wines produced by transformed strains.

STRAIN	VIABLE COLONIES per ml	HERBICIDE RESISTANT COLONIES per ml	% HERBICIDE RESISTANT CELLS
AWRI 5A	$9.5 \pm 0.85 \times 10^6$	0	0
5A (pCP2-4-10)	$1.5 \pm 0.13 \times 10^7$	$1.2 \pm 0.14 \times 10^7$	85
5A (pWX509)	$1.2 \pm 0.12 \times 10^7$	$1.2 \pm 0.15 \times 10^7$	100

Table 4.8. Stability of introduced plasmids in transformed yeast strains during fermentation.

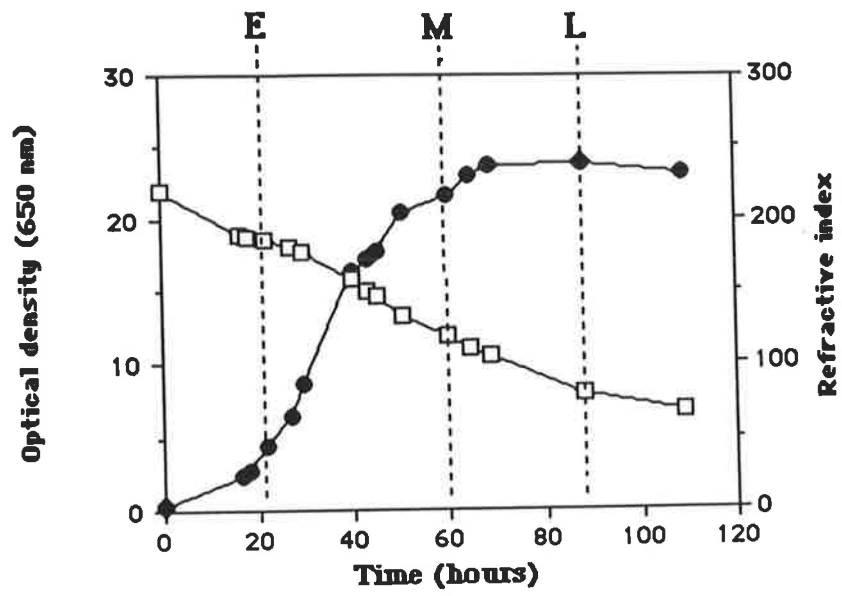
4.3.5 Gene regulation during fermentation

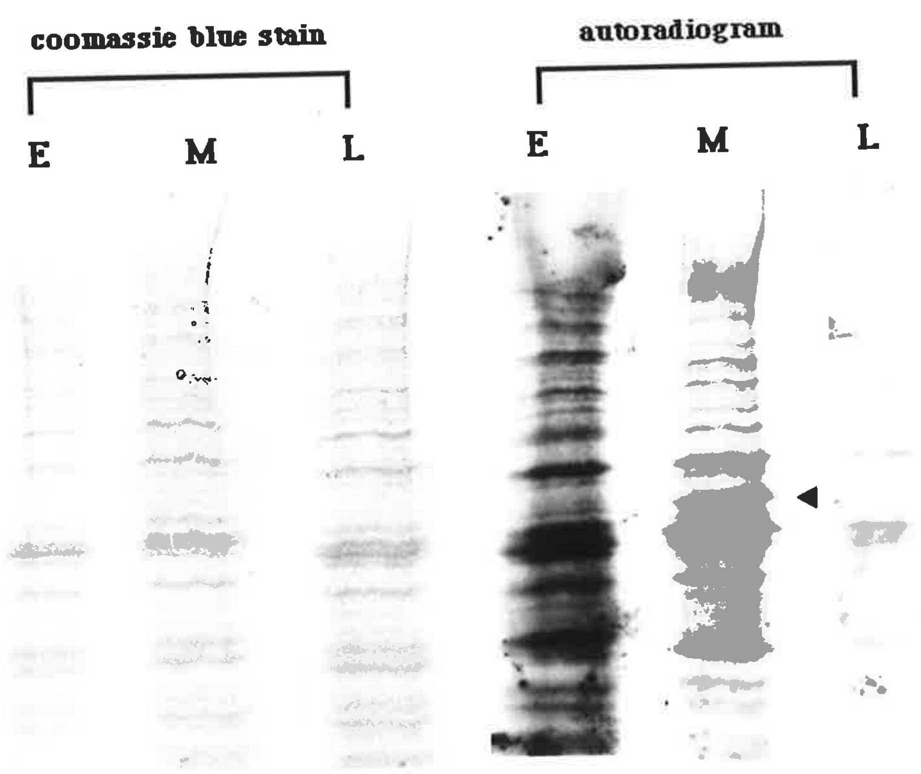
The possibility of isolating promoters which are 'switched on' at specific stages of fermentation was investigated. This investigation involved the analysis of protein synthesis in a wine yeast strain at different times of a fermentation. Variations in the profile of proteins synthesized at different fermentation stages would reflect specific promoter regulation. For example, the presence of a protein late in the ferment that had been absent in early stages would indicate that the promoter associated with this protein had been 'switched on' late in the fermentation.

Riesling grape juice was inoculated with an overnight culture of strain AWRI 5A. Fermentation was conducted at 18°C under anaerobic conditions with gentle agitation. Samples were removed aseptically and analysed for yeast growth (by optical density) and sugar utilization (by refractive index). At three stages of the fermentation - early phase (E), mid phase (M), and late phase (L) - samples were removed and incubated with radioactively labeled leucine for two hours. These three stages of fermentation are depicted in Figure 4.10 (Panel A). Following each incubation, total cellular proteins were isolated from the suspension and stored for analysis. Protein samples were later electrophoresed on a polyacrylamide gel, stained with coomassie blue and treated by fluorography. The fluoragraphic treatment is performed prior to autoradiography, and enhances the detection of radioactivity.

Results of the coomassie blue staining and autoradiography are shown in Figure 4.10 (Panel B). The autoradiogram indicates that the highest level of incorporation of radioactive leucine occurred in the early phase of fermentaion (and yeast growth). The level of incorporation decreased as the fermentation progressed and by late phase is difficult to detect by autoradiography. Although definition of the bands is poor, it appears that some bands my be specific to the mid or late phases of fermentation. An example of such a band

Figure 4.10. Protein synthesis during fermentation. Top panel: Fermentation progress of strain AWRI 5A depicted by yeast growth (●) and sugar utilization (□). Samples were removed from the ferment at early (E), middle (M) and late (L) stages as indicated. Lower panel: Protein profiles of each different sample after coomassie blue staining and fluorography.


E
M
L
30
20
10
0
300
200
100
0
0
20
40
60
80
100
120
Optical density (650 nm)
Refractive index
Time (hours)


coomassie blue stain
autoradiogram
E
M
L
E
M
L

is indicated by an arrow. This band may represent a protein which is selectively expressed in the later stages of fermentation.

4.3 DISCUSSION

A system has been described for the stable introduction of foreign DNA to a range of wine yeast strains. This system utilizes the *SMR1* gene (Casey *et al.*, 1988) as a dominant selectable marker. Advantages of this marker over others that have previously been reported include the fact that the gene is derived from a natural *ILV2* yeast gene, and therefore shares sufficient homology with sequences on the yeast genome to target direct integration. Only one copy of the gene is required to confer resistance to the herbicide sulfometuron methyl upon transformed cells. Moreover, the use of genetically engineered organisms in the wine industry is likely to be more acceptable if the introduced DNA comprises naturally occurring yeast sequences.

The most efficient transformation method tested in this study, with respect to yield of transformants, cost and expediency was a modification of the alkali cation method (Ito *et al.*, 1983). This modification included the incubation of competent yeast cells with carrier DNA and plasmid DNA at the time of cellular uptake. This method was shown to be successful with a range of commercially popular wine yeast strains.

Southern hybridization analysis of transformants confirmed the presence of both the self-replicating (pCP2-4-10) and integrating (pWX509) vectors. Plasmid pWX509 was linearised at the PvuII site of the *SMR1* gene prior to transformation. This was designed to target the plasmid to the *ILV2* gene on chromosome XIII. TAFE separation and Southern hybridization of chromosomes from the pWX509 transformant revealed that the plasmid had been successfully targetted to chromosome XIII.

Fermentation trials indicated that there were no significant changes in the growth or fermenation rates between the original strain 5A and the strains transformed with either the self-replicating plasmid (pCP2-4-10) or the integrating vector (pWX509). Similarly, there were no significant differences in the pH measurements or the alcohol content of the resultant wines.

Stability of the introduced plasmids throughout fermentation was measured by comparing colony forming units (cfu) in aliquots of the finished ferment on both selective and non-selective media. These results indicated that the self-replicating plasmid displayed instability, with approximately 85% of the total yeast cells retaining the plasmid throughout fermentation. No plasmid loss was detected in the transformed strain which contained the integrating vector.

Southern analysis of the pWX509 transformants showed that the vector did not integrate into every copy of the *ILV2* gene. It should be noted that only one transformant was analysed in this experiment, so it is possible that all copies of the gene are disrupted in some cases. Also, different transformation conditions may encourage integration into all possible sites. For example, increasing the amount of plasmid DNA in the transformation mixture may result in disruption of all homologous sites. Nevertheless one can conclude from this result that under the transformation conditions described in this chapter, integration will not necessarily occur in all copies of the targeted gene.

This is an important observation considering that one possible application of this transformation system may be to inactivate undesirable genes. For example, Suizu *et al.* (1990) recently inactivated the *CAR1* gene of a laboratory haploid *S. cerevisiae* strain by homologous integration. The disruption of this gene, which codes for arginase, resulted in a yeast strain which did not produce urea. The chemical reaction of urea and ethyl alcohol in alcoholic beverages produces ethyl carbamate, which is a suspected carcinogen and, therefore, an undesirable compound in wine. The *CAR1* gene disruption of a wine yeast

strain may be desirable in terms of reducing potential levels of ethyl carbamate in wine. However, to achieve gene disruption, integration would be required into every copy of the *CAR1* gene of the polyploid wine yeast cells. Results of this chapter show that this may not occur in a one-step transformation procedure. Multiple transformation events, each one requiring a different selectable marker, may provide a means for gene inactivation in wine yeast strains.

Conclusive results about regulated gene expression during fermentation were not obtained in this study. Although protein synthesis investigations indicated that some genes may be transcribed at specific stages of fermentation, the search for regulated promoters will require a more thorough and detailed analysis. For example, mRNA species should be isolated from different stages of fermentation and used to produce cDNA libraries. Differential screening of these libraries may reveal stage-specific clones. These clones could then be used to screen a genomic library in order to identify the associated promoter elements.

The identification of promoters in this type of study would increase the scope for designing efficient strategies for foreign gene expression during fermentation. For example, the products of some introduced genes may interfere with growth of the yeast strain. Switching on foreign gene expression towards the end of a fermentation after a high yeast biomass has been reached may result in more economical yields of the foreign product.

It can be concluded that the system described in this chapter can be used to target foreign DNA to specific regions of the yeast genome such that it will be maintained stably in the strain throughout fermentation. The transformation procedure is applicable to a wide range of wine yeast strains and does not adversely affect the fermentation performance of the yeast.

Chapter 5 Development of a system for wine yeast strain marking and identification

5.1 INTRODUCTION

Winemakers have recognized the importance of the yeast strain in determining the flavour and quality of wine, and the economics of its production (Rankine, 1968). Pure culture inoculation now dominates the industry, particularly in the newer wine producing regions. This practice provides greater reliablility and control of the fermentation through more rapid onset and completion resulting in wines with fewer flavour defects (Rankine, 1977). Furthermore, this technology enables strain differences to be utilized in the production of a wide variety of wines. The selection and characterization of wine yeasts by The Australian Wine Research Institute supported and enhanced the utility of pure culture inoculation technology (Rankine, 1968). More recently, the availability of active dried yeasts have given the winemaker even greater scope for exploiting the oenological properties of different strains. These factors have led to the use of several yeast cultures in the winery; a practice which addresses the need for a suitable yeast identification scheme.

The process of oenology employs a raw material containing an unknown load of indigenous yeasts, some of which are capable of producing off-flavours and spoiling wine. Natural grape must will undergo a spontaneous fermentation by the indigenous yeasts to produce wine. In the case of wines produced by pure culture inoculation, it is considered that the addition of $S0_2$ will suppress the indigenous yeast population and thereby encourage fermentation by the inoculated strain (Kunkee and Amerine, 1970; Benda, 1982; Kunkee, 1984).

Only recently have quantitative kinetic studies of the ecology of grape juice fermentation been made (Fleet *et al.* 1984; Heard and Fleet, 1985, 1986a). These

investigations, made with both inoculated and uninoculated grape juices under a range of oenological conditions, provide evidence which challenges the concept that *Saccharomyces* necessarily suppresses non-*Saccharomyces* yeasts to establish itself as the dominant organism. Fermentation conducted at low temperature, for example $10^{o}C$, and to a lesser extent at high pH, for example pH 3.5, resulted in greater growth and survival of *Kloeckera apiculata* and several *Candida* species than previously believed, and in some cases approached that of *Saccharomyces* yeasts (Heard and Fleet, 1988). Furthermore, it was shown that SO_2 did not necessarily suppress the growth of non-*Saccharomyces* yeasts (Heard and Fleet, 1988a).

These findings, therefore, call into question the effectiveness of some oenological practices for controlling the growth and survival of indigenous yeasts. Relevant studies have been hindered by the lack of suitable techniques for the differential quantitation of the inoculated strain and wild yeast. While methods for differentiating between *Saccharomyces* and many non-*Saccharomyces* yeasts have recently been developed (Heard and Fleet, 1986b), few methods are yet available to distinguish between the inoculated strain and wild strains of *Saccharomyces*. The reason for this is simply that oenological strains are merely wild strains of *Saccharomyces* selected for desirable properties. Therefore, no classical taxonomic differences exist which would permit strain differentiation.

Research presented in this chapter involves two different approaches to monitor yeast in the production of wine. The first investigates a method for identifying yeasts to the strain level by producing a chromosomal fingerprint. The second approach involves tagging a wine yeast with a genetic marker enabling the efficiency of oenological practices to be determined.

5.1.1 Yeast chromosomal fingerprints

Saccharomyces cerevisiae may be identified by classical methods including cell shape and mode of reproduction; sugars fermented; carbon and nitrogen compounds assimilated; vitamin requirements; sensitivity to inhibitors; co-enzyme Q system and DNA base composition (Kreger-van Rij, 1984; Kreger-van Rij, 1987), or by the pattern of responses to some 80 physiological tests (Barnett *et al.*, 1983). A considerable input of expertise and time precludes the application of these methods to routine identification.

Furthermore, these methods are not useful in distinguishing strains of *S. cerevisiae*. Two fundamentally different approaches to identifying strains of yeast have been pursued, namely phenotypic and genotypic analysis. In the first, a yeast characteristic or family of components is measured or profiled. Giant colony morphology has proved useful but may require at least 2-4 weeks for conclusive results (Heard and Fleet, 1987). Serology (Nishikawa *et al.*, 1979) is rapid but generally lacks specificity and resolution. Fatty acid composition as measured by gas chromatography is relatively rapid and a generally accessible technique (Oosthuizen *et al.*, 1987) but its potential for strain identification still needs to be confirmed. Yeast protein fingerprinting in the form of electrophoretic patterns of selected enzymes (Subden *et al.*, 1982), total soluble cell protein (van Vuuren and van der Meer, 1987) and extracellular yeast proteins (Ciolfi, 1988) indicate high specificity and sensitivity. All three protein methods generated unique fingerprints enabling identification of closely related strains. An important disadvantage of all phenotypic methods is the rigorous requirement for control of cultural conditions in order to minimize the variation of results.

In the genotypic approach, DNA composition and structure is analysed. The principal advantage of DNA analysis is the complete independence of results on yeast cultural conditions. Two early methods which have been widely applied to yeast systematics are DNA base composition and DNA homology (Price *et al.*, 1978; Kurtzman

and Phaff, 1987). DNA base composition is measured by either thermal denaturation procedures or by bouyant density measurements and gives only a tentative indication of strain identity. DNA homology, which measures the degree of affinity or association between fragments of single-stranded DNA from two yeasts, is a measure of strain relatedness but generally is not sufficiently sensitive to reveal strain identity.

Of greater interest, however, is characterization of DNA by the techniques of restriction analysis and pulsed field gel electrophoresis. Restriction endonucleases cut DNA at specific points. This action generates a unique range of DNA fragments whose size is readily determined by agarose gel electrophoresis resulting in a characteristic pattern of bands in the gel. This technique has only recently been applied to yeast and is becoming a critical technique in yeast systematics (Kreger-van Rij, 1984). Both nuclear (Proffitt *et al.*, 1984; Panchall *et al.*, 1987) and mitochondrial DNA (mtDNA) (Lee *et al.*, 1985) have been characterised. Restriction of nuclear DNA yields a large number of poorly resolved fragments. Therefore, specific probes which only hybridize to sequences found in one or a few of the fragments are used to generate simpler patterns. Ciriacy and Grossman (1988) screened eight wine yeasts with three probes constructed from the alcohol dehydrogenase gene (ADH), 2 µM DNA and Ty sequence. The ADH and 2 µM DNA probes revealed little difference between strains, while Ty, a dispersed repetitive sequence, produced unique fingerprints.

Analysis of mtDNA purified from strains of yeast isolated from wine has been reported by Dubourdieu *et al.* (1987). Digestion of mtDNA with the restriction enzyme EcoR1 yielded from 4 to 10 fragments in the 23 strains assayed. All but two strains showed unique fingerprints. The technique, therefore, provides unequivocal identification of wine yeast but the complexity of mtDNA isolation precludes application to routine analysis.

A recently developed technique, pulsed field electrophoresis, has extended the size range of DNA molecules which can be fractionated by the electrophoretic technique (Shwartz and Cantor, 1984). Utilizing this technique, it is now possible to electrophoretically separate intact yeast chromosomes on a gel. Chromosomes from *S.cerevisiae* have been shown to range in size from 150kbp (chromosome I) to approximately 2,500 kbp (chromosome XII) (Carle and Olson, 1985; DeJonge et al., 1986). The method involves the application of an alternating electric field to the slab gel which forces the chromosomes to periodically change their direction or mobility; it is thought that the smaller chromosomes migrate more rapidly through the gel because they can reorientate themselves more rapidly to the changing electric field through the gel and become stuck in the gel matrix less frequently. A variety of electrode arrangements have been used and many are available as commercial units.

Field inversion gel elctrophoresis (FIGE) is the least complex arrangement (Carle *et al.*, 1986). A standard horizontal submarine gel is used but the electric field is alternatively inverted and the frequency of inversion is controlled. Unequal periods of field inverson produces a net migration in one direction, while the duration and frequency of the electric pulses affects migration rates of different sized macromolecules. This method allows the resolution of DNA molecules of several hundred thousand base pairs. However, this is not adequate for yeast chromosomal DNA which extends into the megabase (mbp) range.

Orthogonal field alternation gel electrophoresis (OFAGE) differs from FIGE essentially by the angle betweeen the two alternating electric fields. In FIGE, the field is inverted by 180° whereas the inversion in OFAGE is 120° in the plane of the gel. The orthogonal field causes the DNA molecules to zigzag across the plane of the gel. The resolution range, which also depends on the pulse frequency, is extended to the 9,000 kbp range (Carle and Olson, 1984). A disadvantage of OFAGE is that the lanes of DNA migration are not straight, making comparisons between samples difficult.

In 1986, two new concepts were published: Transverse Alternating Field Electrophoresis (TAFE) and Contour-clamped Homogenous Electric Field (CHEF) electrophoresis. In TAFE, the gel is oriented vertically with a simple four electrode array placed on either side of the gel (Gardiner and Patterson, 1986). Sample molecules are forced to zigzag through the thickness of the gel. As all lanes experience the same electrical field effects the bands remain straight. As the molecules move down the gel, they are subjected to continual variations in field strength and reorientation angle - but to all lanes equally.

Also in 1986, Chu *et al.* demonstrated that the critical factor in resolving large DNA is keeping the angle between the two directions of alternating fields greater than 90 degrees. Field gradients were not necessary for high resolution. To avoid field distortions arising from the finite size of an electrophoresis chamber, the researchers designed a hexagonal array that placed pairs of electrodes at a 120° angle. In their CHEF system, rather than use long electrodes, the researchers broke the electrodes up into short segments. Then, using resistors (and later transistors), they clamped and forced the elctrodes to their ideal voltages. The samples in the CHEF system experience a large, highly uniform electric field with no edge effects.

The chromosome banding patterns of *S. cerevisiae* (Carle and Olson, 1985; De Jonge *et al.*, 1986), *Candida albicans* (Snell and Wilkens, 1986) and *Schizosaccharomyces pombe* (Smith et al., 1987; Vollrath and Davis, 1987) 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 the one species (De Jonge *et al.*, 1986). The technique of pulsed field gel electrophoresis, therefore, offers an improved system for the identification of wine yeast strains.

5.1.2 Marked strains

Determining the efficiency of winemaking practices (such as monitoring yeast propagation and fermentation for contamination) is hampered by the absence of suitable methods to differentially quantify the inoculated wine yeast strain and indigenous yeasts. Some studies have exploited the naturally occurring killer or zymocidal marker present in some strains as a tool for such analysis (Heard and Fleet, 1988). This marker can be used in either of two ways: (i) samples can be plated onto nutrient media for a total count and replica plated onto media containing toxin to determine the proportion of marked strain; or (ii) the proportion of the marked strain can be determined by replica plating onto a lawn of sensitive yeast.

However, a deliberately marked oenological strain as developed by Vezinhet and colleagues (Vezinhet and Lacroix, 1984; Vezinhet, 1985) provides a more powerful technique for investigations of this type. The strain, which is now commercialized as K1, is double marked with two antibiotic markers, diuron and erythromycin. An extensive survey of yeasts for resistance to these antibiotics demonstrated that few strains are naturally resistant to both drugs simultaneously. K1 was developed by selecting for natural mutants in a population of the Lalvin V yeast.

The results of extensive winery trials using this marked strain have already been published (Delteil and Aizac, 1988). The main emphasis of these trials was to investigate the efficiency of various inoculation techniques, including method of yeast propagation, direct inoculation, inoculum level and timing of inoculation. The marked strain enabled data to be accumulated on the effect of winery procedures by the extent of K1 domination during fermentation. Recommendations could then be given for improving the efficiency of fermentation control.

The marked strain, K1, provides an insight into the kinetics of yeast populations during fermentation and therefore represents a major advance in the control of wine production. However, a major limitation exists in the fact that wine yeast strains of choice cannot be easily marked.

A procedure is described here which employs recombinant DNA technology to introduce the *Escherichia coli* β–glucuronidase (GUS) gene as a marker into any desired yeast strain. The GUS gene was developed as a reporter gene system for use in nematodes and more recently in the study of plant gene expression (Jeffersen et al., 1986, 1987). The advantages of the GUS system as a marker in yeast strains include its low background levels in *S.cerevisiae* and other yeasts associated with grape must fermentation and its ease of assay by fluorimetry, spectrophotometry and agar plate tests.

5.2 RESULTS

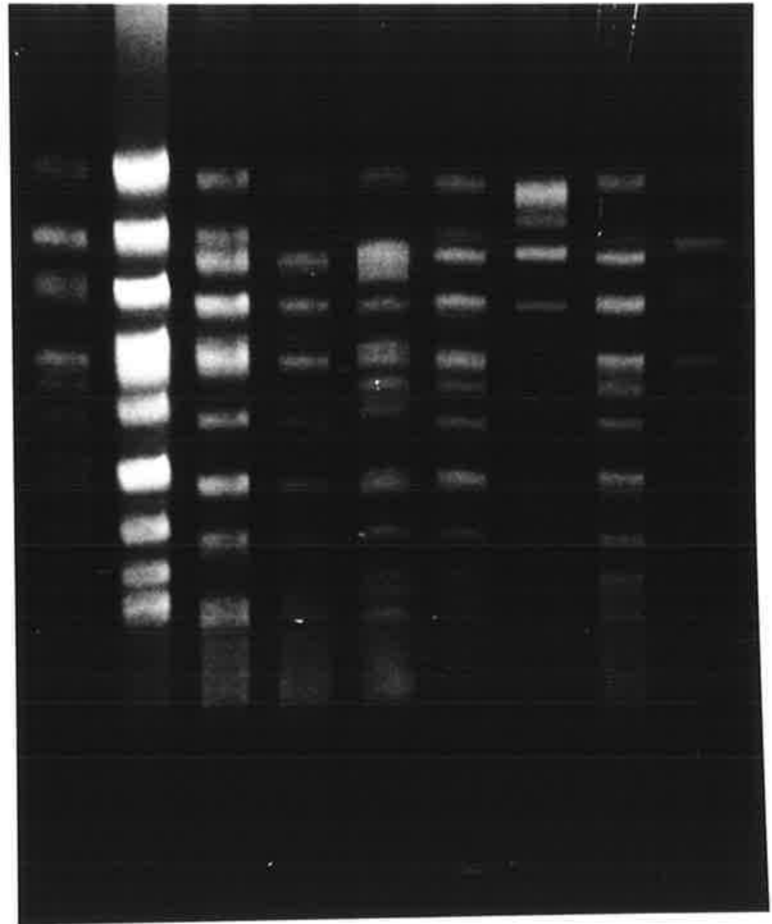
5.2.1 Wine yeast chromosome fingerprinting

The TAFE gel banding patterns of twelve winemaking yeasts were compared with that of a standard strain of *S. cerevisiae* (Beckman 334). Seven strains are shown in Figure 5.1. The standard strain produced 13 bands (numbered from bottom of the gel), although bands 10 and 11 were usually fused. Bands 9 and 12, which stained more intensely, are doublets. On the basis of data from genetic maps of *S. cerevisiae* (Mortimer and Schild, 1985) and chromosome physical size (Anand, 1986), Beckman have assigned the following chromosome numbers to the bands starting from the bottom of the gel: chromosome number I, VI,III,IX,VIII,V,XI,X, doublet of II and XIV, XIII, XVI, doublet of VII and XV, and IV. Chromosomes XII and XVII were not detected. The largest, chromosome IV (band 13), was estimated to be in the range of 1500 to 2500 kbp and the smallest detected, chromosome I, at 245 kbp. The two pairs of co-migrating chromosomes, II and XIV (band 9), and VII and XV (band 12) are apparent as more intensively stained bands.

Figure 5.1. Chromosome banding pattern of AWRI winemaking yeasts produced by transverse alternating field electrophoresis. The chromosomes for *Saccharomyces cerevisiae* strain 334 are labeled according to data provided by Beckman Instruments, California.

Lanes:

1	*Saccharomyces cerevisiae* 334
2	1A (350)
3	2A (729)
4	8A (834)
5	9A (J7)
6	11A (R2)
7	*Torulaspora delbrueckii* 12A (143)
8	Lalvin EC1118
9	*Saccharomyces cerevisiae* 334


1 2 3 4 5 6 7 8 9
IV
VII, XV
XVI
XIII
II, XIV
X
XI
V
VIII
IX
III
VI
I

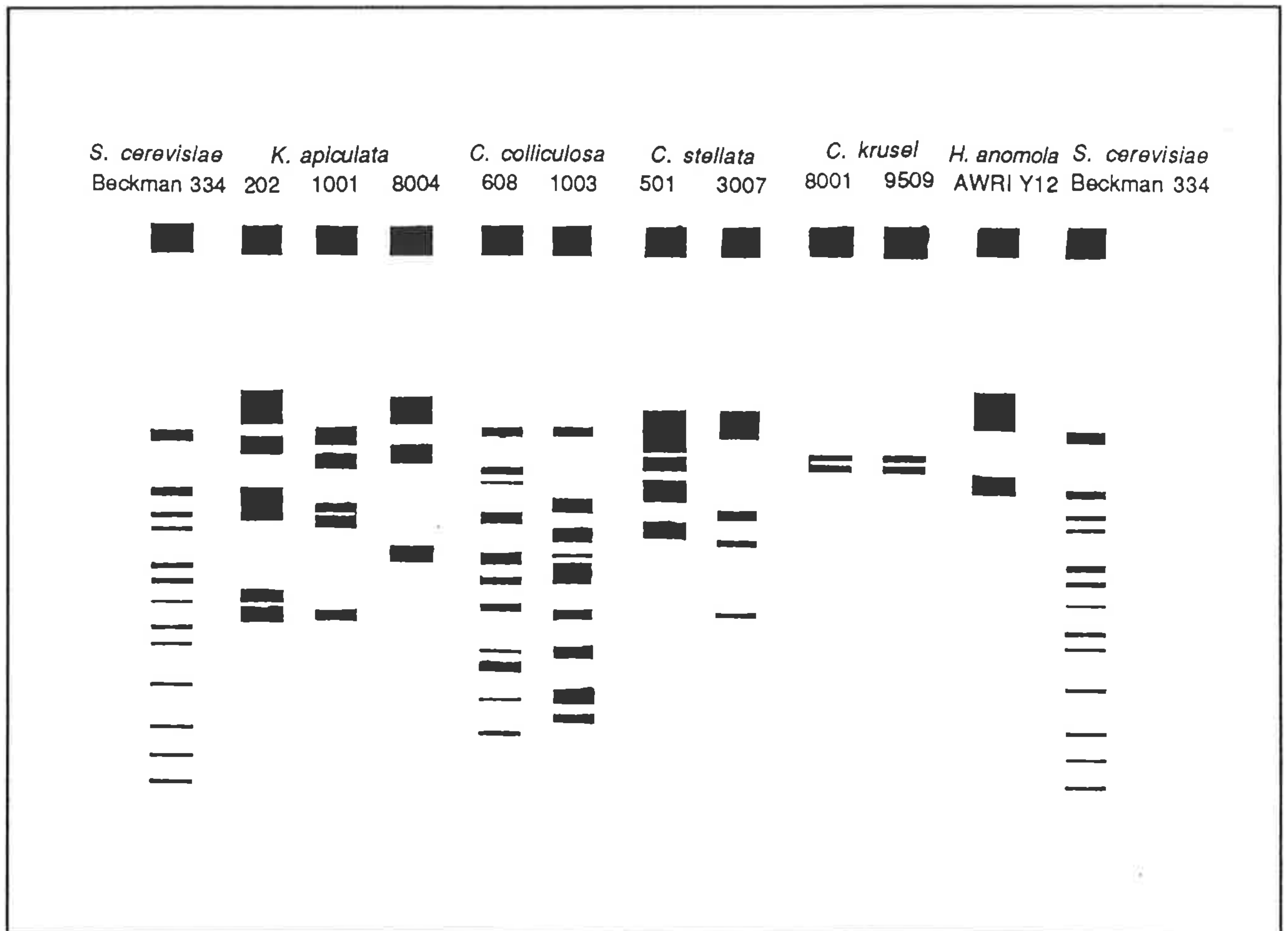


Figure 5.2. Diagramatic representation of chromosomal patterns obtained from yeast strains after transverse alternating field electrophoresis.

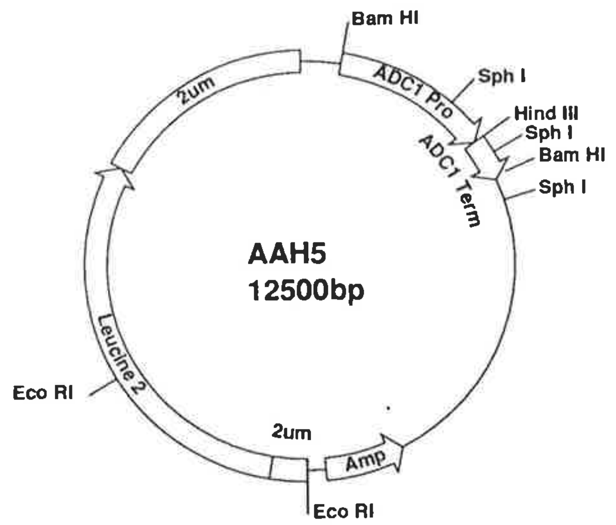
The twelve wine strains investigated revealed banding patterns different from that of the standard strain. Only one strain, 12A, showed a pattern that was dramatically different from the standard. This strain will be discussed separately below. Three variations are apparent: position, number and intensity of bands. Band position on the gel provides an estimate of chromosome physical size. All eleven strains showed various degrees of chromosome polymorphism except for chromosomes IV (band 13), XI (band 7) and IX (band 4). Considerable chromosome polymorphism has been reported in *Saccharomyces* strains from other sources (De Jonge *et al.*, 1986; Casey *et al.*, 1988b). The number of bands does not necessarily correlate with the number of chromosomes as already indicated by the standard strain. Most of the wine yeasts exhibit less than 13 bands indicating that several groups of chromosomes were not resolved under these conditions. Such groups of chromosomes may be identified by broad or more intensely stained bands.

The banding pattern for strain 12A *Torulospora delbrueckii* was fundamentally different when compared to those of *Saccharomyces* yeasts, suggesting a greatly reduced number of chromosomes. Several non-*Saccharomyces* yeasts isolated from fermenting grape must and wine were analysed and are depicted diagramatically in Figure 5.2. The chromosome fingerprints of these yeasts are generally quite different to those of *Saccharomyces* strains, in particular, strains of *Kloeckera apiculata, Candida stellata, Candida krusei* and *Hanseniaspora anomala* did not show small chromosomes. These results suggest that this method may readily distinguish between *Saccharomyces* and non-*Saccharomyces* strains.

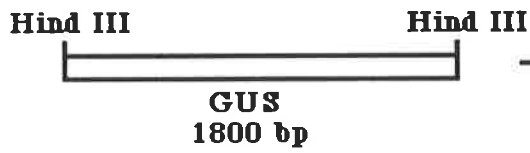
5.2.2 GUS-Vector construct

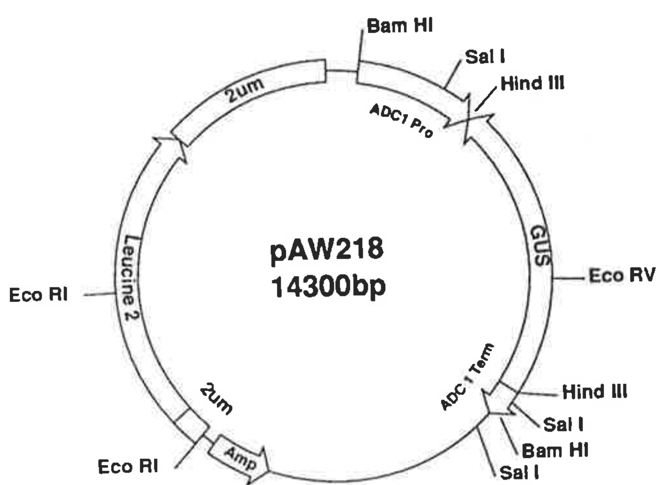
The efficiency of the *E. coli* ß-glucuronidase gene as a marker gene in yeasts is dependent upon maintenance of the gene in a growing population and efficient gene expression.

Figure 5.3. Construction of plasmids pAW218 and pAW219. The 1.8kb Hind III GUS fragment was ligated with a Hind III digested AAH5 plasmid. This ligation gave rise to the AAH5 plasmid containing the GUS gene in either the wrong orientation with respect to the promoter (pAW218), or the correct orientation with respect to the promoter (pAW219).



Digest with
Hind III





+

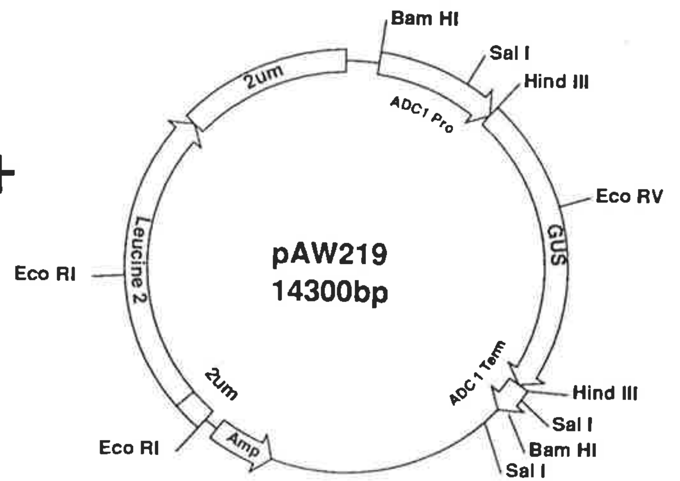
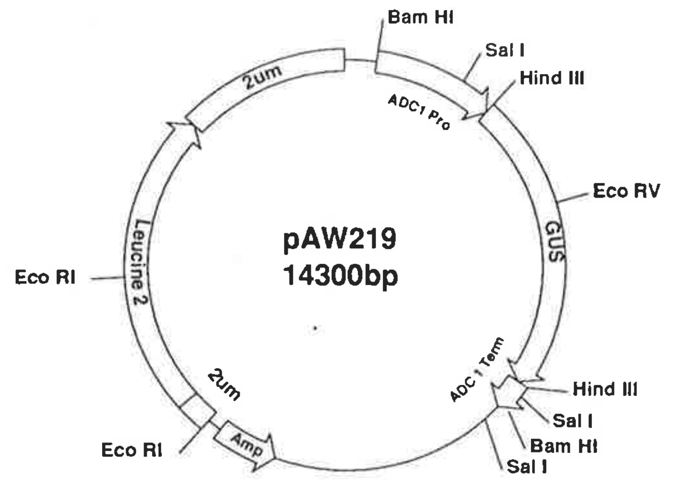
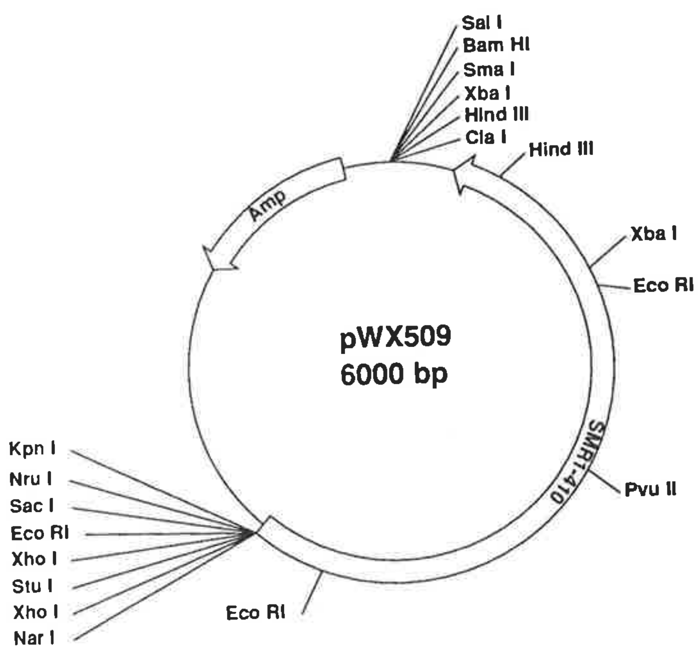
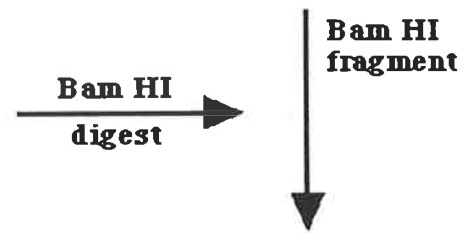
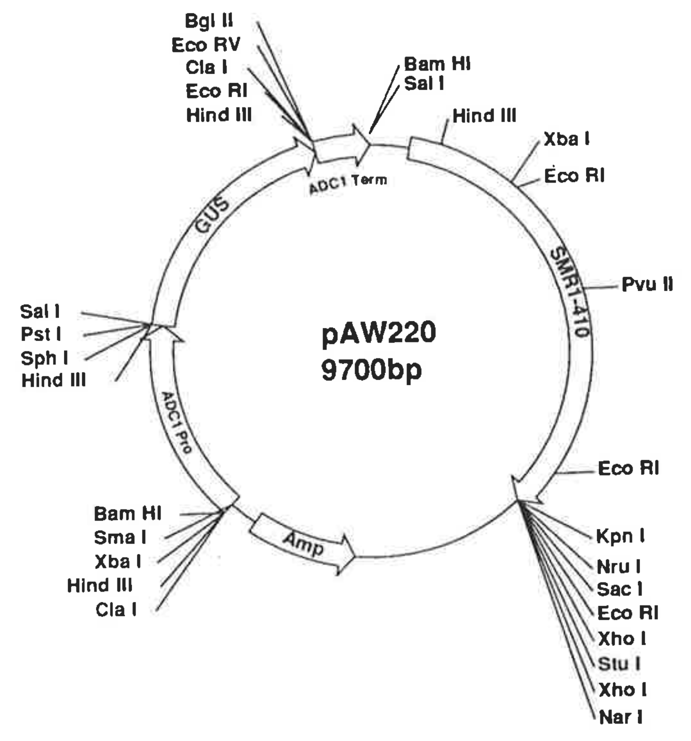


Figure 5.4. Construction of plasmid pAW220. The 3.7kb Bam H1 fragment of pAW219 was ligated with the Bam H1 digested pWX509 to give rise to pAW220.


Bam HI
Sal I
Hind III
ADC1 Pro
Eco RV
GUS
pAW219
14300bp
Eco RI
Leucine 2
2um
ADC1 Term
Hind III
Sal I
Bam HI
Sal I
Eco RI
Amp
2um


Sal I
Bam HI
Sma I
Xba I
Hind III
Cla I
Hind III
Amp
Xba I
Eco RI
pWX509
6000 bp
SMR1-410
Pvu II
Kpn I
Nru I
Sac I
Eco RI
Xho I
Stu I
Xho I
Nar I
Eco RI


Bam HI
digest
Bam HI
fragment


Bgl II
Eco RV
Cla I
Eco RI
Hind III
Bam HI
Sal I
Hind III
Xba I
Eco RI
ADC1 Term
GUS
SMR1-410
Pvu II
pAW220
9700bp
Sal I
Pst I
Sph I
Hind III
ADC1 Pro
Eco RI
Bam HI
Sma I
Xba I
Hind III
Cla I
Amp
Kpn I
Nru I
Sac I
Eco RI
Xho I
Stu I
Xho I
Nar I

Expression of the GUS coding region was achieved by use of the yeast alcohol dehydrogenase (*ADC*1) promoter and terminator sequences. The GUS gene was isolated as a Hind III fragment from the plasmid pKLG4. This was ligated into the Hind III site of the vector AAH5 (Ammerer, 1983). Clones with the GUS coding region, in both orientations with respect to the *ADC*1 promoter, were obtained (Figure 5.3). A clone with the GUS coding region in the correct orientation with respect to the *ADC*1 promoter was identified (plasmid pAW219). Plasmid pAW219 was then digested with Bam H1. This digestion results in excision of the GUS gene flanked by the *ADC*1 expression signals. The GUS coding region with attached *ADC*1 signals cassette was cloned into the Bam H1 site of vector pWX509 (Casey *et al.*, 1988a) - giving rise to plasmid pAW220 (Figure 5.4).

5.2.3 Transformation and Southern analysis

The *SMR1-410* gene on plasmid pAW220 is almost identical in sequence to the *ILV2* gene - a single base point mutation leads to resistance to the herbicide sulfometuron methyl. Therefore, sufficient homology exists between the two sequences to target integration to the *ILV2* gene via homologous recombination with *SMR1-410*. Recombination is enhanced by digesting plasmid pAW220 with Pvu II prior to transformation. This gives rise to a linear molecule with *SMR1-410* sequences at either end. The DNA ends are highly recombinogenic and integration is most likely to occur at the Pvu II site in the *ILV 2* gene. The result of this event will be two *ILV2* genes flanking the GUS cassette, one of which will contain the *SMR1-410* mutation conferring herbicide resistance upon the transformed cell (see Figure 5.5).

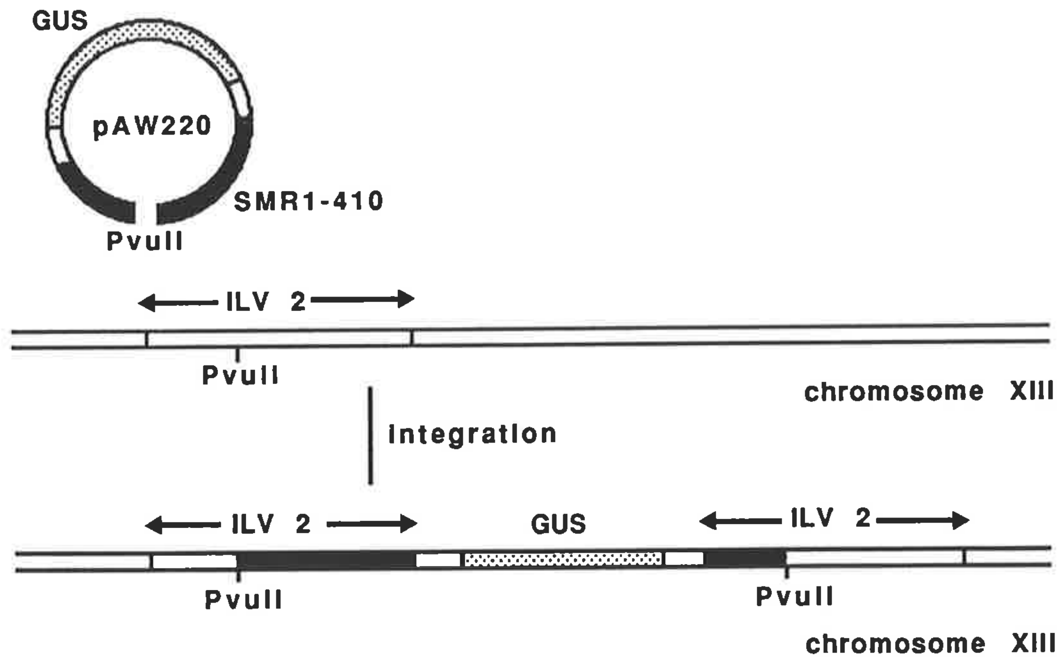


Figure 5.5. Integration pattern of pAW220 into yeast chromosome XIII.

Figure 5.6. Southern hybridization detecting the 9.7kb GUS vector in transformed strain 3AM. Panel A: Hind III molecular weight markers. Panel B: Total yeast DNA digested with Pvu II and electrophoresed on a 1% agarose gel. The gel was stained with ethidium bromide and visualized on a UV light box. Panel C: Southern hybridization performed on samples depicted in Panel B, probed with a radiolabeled sequence of the GUS gene.

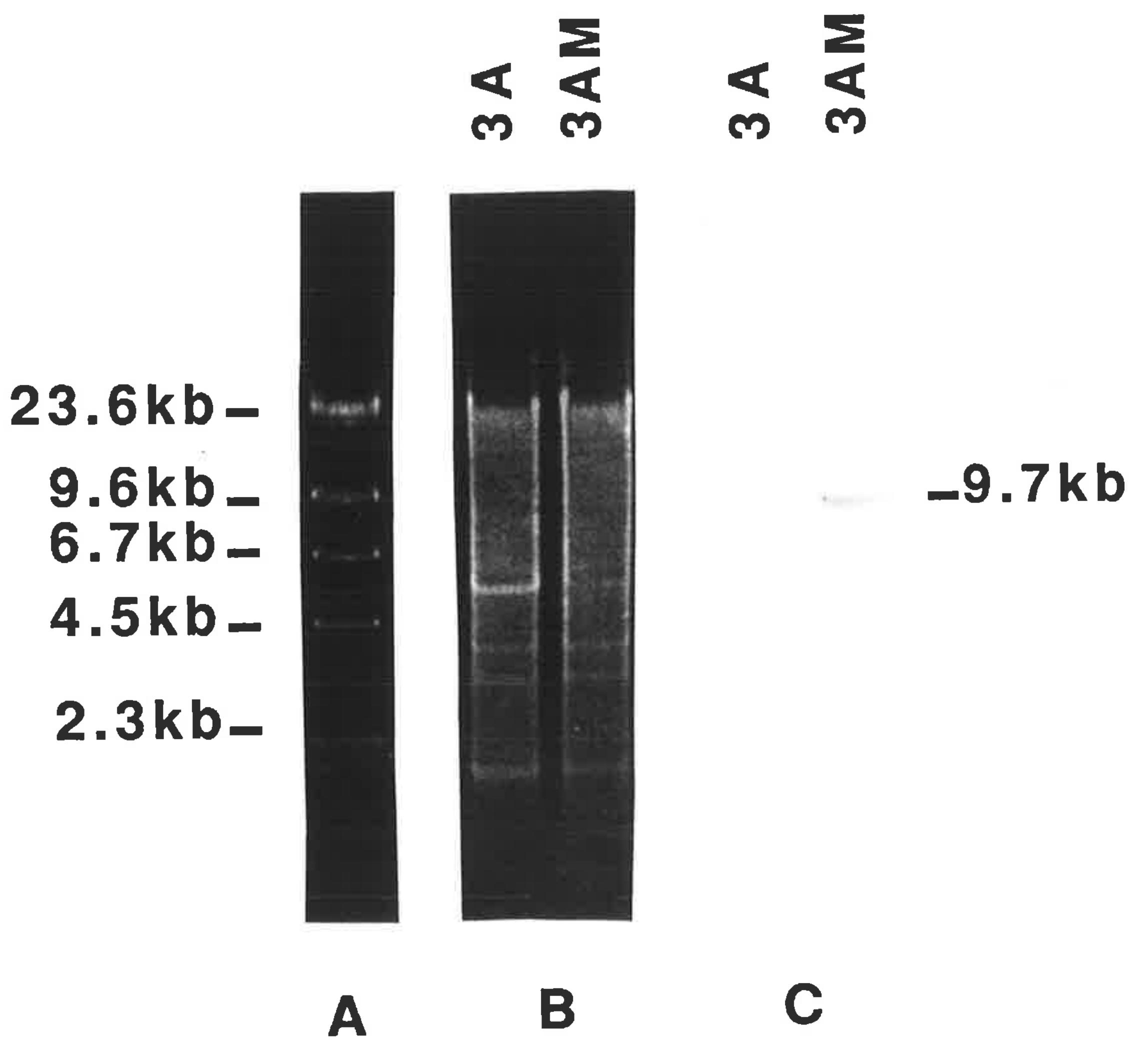
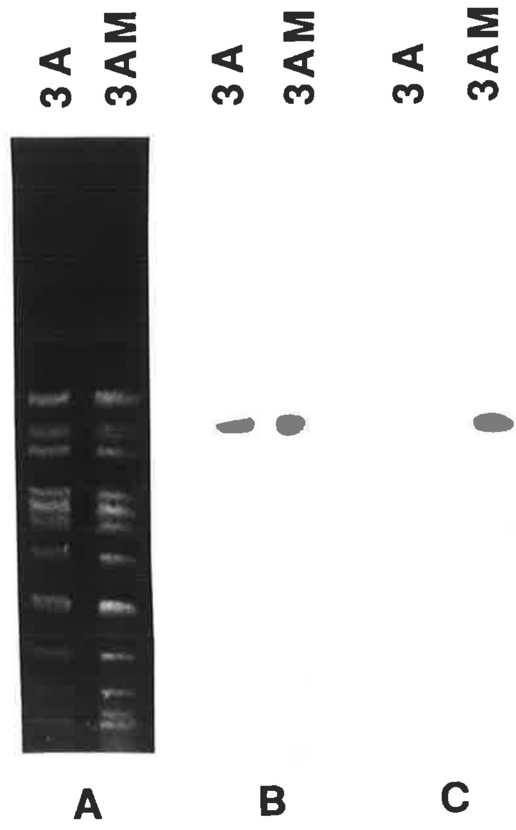

3A
3AM
3A
3AM
23.6kb
9.6kb
6.7kb
4.5kb
2.3kb
9.7kb
A
B
C

Figure 5.7. Southern hybridization depicting the chromosomal location of the GUS vector in strain 3AM. Panel A: Yeast chromosomes separated on a 1% agarose gel by transverse alternating field electrophoresis. Panel B: Southern hybridization of chromosome patterns depicted in Panel A probed with a sequence of the ILV2 gene. Panel C: Chromosome patterns depicted in Panel A probed with a radiolabeled sequence of the GUS gene.

chromosome XIII –



Pvu II digested plasmid, pAW220, was introduced into wine yeast strain 3A by the method of Ito *et al.* (1983). Transformants were selected for resistance to the herbicide sulfometuron methyl (at 10 μg/ml). A transformed colony (designated 3AM) was then screened for the presence of the GUS construct. Total DNA was isolated and digested with Pvu II, thereby releasing the GUS-vector construct intact from the chromosomal DNA. A Southern hybridization was then performed using the Hind III fragment from plasmid pKLG4 to probe for the GUS sequence (Figure 5.6). A band of approximately 9.7 kbp was evident in the transformed strain indicating the presence of the GUS construct.

The chromosomal location of the GUS construct in the transformed strain was determined. Intact chromosomes were prepared from strain 3AM, separated by pulsed field gel elecrophoresis on a TAFE unit and screened by Southern hybridization with the GUS sequence. Results of this analysis (Figure 5.7) show that the GUS construct has integrated into chromosome (XIII). This chromosome also contains the *ILV2* gene; the expected site of integration.

5.2.4 <u>Development of a GUS assay for yeasts</u>

A number of substrates are available commercially for GUS detection assays. Two of these substrates were selected for development of a GUS assay for yeasts:

i) 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-GLUC). This substrate is available for histochemical localization of β-glucuronidase activity in tissues and cells. The X-GLUC molecule is cleaved by the β-glucuronidase enzyme to produce an indoxyl derivative which, upon oxidation, gives rise to an insoluble and highly coloured indigo dye.

ii) 4-methyl umbelliferyl glucuronide (MUG). This fluorogenic substrate has been described in the literature for assay of β-glucuronidase activity (Jefferson, 1987). The compound is not fluorescent until cleaved by β-glucuronidase to release 4-methyl umbelliferone which is fluorescent only when the hydroxyl group is ionized. The PK_a of

the hydroxyl is between 8 and 9 but maximal fluorescence will only be obtained if the product is in solution at a pH greater than the PK_a.

Previous reports of GUS activity in yeast had not been documented, therefore an empirical investigation was necessary to determine appropriate conditions for detection of the GUS construct in *Saccharomyces* strains. GUS activity had been detected in filamentous fungi by growth on agar media containing X-GLUC; transformed strains giving rise to blue colonies (Roberts *et al.*, 1989). An initial experiment was performed to determine whether GUS activity could be detected in yeast cells simply by incubation with the substrate X-GLUC.

Plasmids pAW218 and pAW219 were transformed into the *Saccharomyces* lab strain O11. Both types of transformants were grown to stationary phase in liquid culture and after harvesting, were resuspended in 0.1M Na_2HPO_4, pH 7.0. The substrate X-GLUC (50μg/ml) was added and the suspensions were incubated at 30^oC. At regular intervals, the cells were checked for development of blue colour. After a 24 hour incubation, no colour was evident in either of the transformants.

Possible explanations for this negative response include: i) the substrate is not entering the cell and therefore is not exposed to the β-glucuronidase enzyme; or ii) the *ADC1*-GUS construct is not directing sufficient expression of the GUS coding region and therefore, inadequate levels of the β-glucuronidase enzyme exist in the yeast cell.

To investigate the possibility that the substrate is not coming into contact with the enzyme, the experiment was repeated but this time the yeast cell wall was physically disrupted by agitation with glass beads. The result of this experiment is presented in Figure 5.8 (A). The positive response obtained after physical disruption of the yeast strain containing pAW219 indicates that sufficient expression of the GUS gene is achieved with the ADH-GUS construct. The positive response was only recorded with the transformant

in which the GUS coding region is oriented correctly with respect to the ADH promoter. This confirms that fortuitous expression of the GUS gene was not sufficient to generate a detectable level of GUS activity. Furthermore, it can be concluded that over the time course of this experiment, the X-GLUC was not taken up by the cells nor could it diffuse into intact cells.

A number of other treatments were tested for the ability to induce permeation of the yeast cell wall and to facilitate detection of GUS activity. These treatments included:

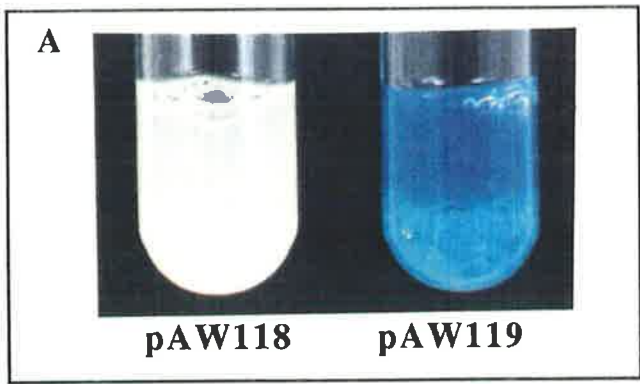
i) addition of the ionic detergents sarkosyl or SDS (sodium dodecyl sulphate) to the cell suspension (total concentrations ranging from 0 - 5%);

ii) addition of the non-ionic detergent triton-X to the cell suspension (total concentration ranging from 0 -5%); and

iii) addition of the sulphydral reagent β-mercaptoethanol to the cell suspension (total concentration ranging from 0 - 100 mM).

Cells of strain Ol1 containing plasmid pAW219 were suspended in 0.1M Na_2HPO_4 containing 50 mM X-GLUC prior to the addition of each compound. Each suspension was then incubated at 37°C for 4 hours. Results of these treatments are presented in Figure 5.8 (B). Addition of the ionic detergents sarkosyl and SDS (at concentrations of 1%) resulted in the fastest colour development, and after 4 hours these suspensions were the deepest blue. A decrease in rate of colour development was evident in samples which contained the detergent in concentrations higher than 1%. This could be due to a partial denaturation of the β-glucuronidase enzyme, which would result in a decrease in enzyme activity. Addition of triton-X was also successful in inducing leakiness in the yeast cells, however, colour development was slower with this treatment. Treatment of the suspensions with β-mercaptoethanol (up to 100mM) was not succesful, in the time course of this experiment, in facilitating detection of GUS activity. It can be concluded, therefore, that treatment of cells

Figure 5.8. Visual assays to determine suitable conditions for the detection of GUS activity in yeast cultures. Panel A: *Saccharomyces cerevisiae* strain OL1 transformed with pAW218 and pAW219. Cells were suspended in 0.1M Na_2HPO_4, pH 7.0 and vortexed for five minutes with glass beads. Panel B: *Saccharomyces cerevisiae* strain Ol1 transformed with pAW219. Cells were suspended in 0.1M Na_2HPO_4 and substances were added as indicated.



B

sarkosyl

0%	0.5%	1%	2%	5%

SDS

0%	0.5%	1%	2%	5%

triton-X

0%	0.5%	1%	2%	5%

β-mercaptoethanol

0 mM	10 mM	50 mM	100 mM

with an ionic detergent (sarkosyl or SDS) at a concentration of 1% induces permeability of the yeast cell and allows detection of GUS activity in marked cells.

The MUG substrate was then investigated as a potential detection system for marked yeast strains. Cell suspensions of 3A and 3AM were prepared in 0.1M Na_2HPO_4, pH 7.0, 1% sarkosyl and 1mM MUG, and incubated with shaking at 37^oC. After 2 hours incubation, the suspensions were viewed on a short wave UV light box (Figure 5.9). Fluorescence is easily detected in the 3AM suspension, while strain 3A gives a negative response.

The potential use of a marked strain in the wine industry relies on the ability to quantify the marked cells in a mixed population of yeasts. Therefore, a procedure in which individual cells can be assayed for GUS activity is required. In light of the observations described above, one premise is that such a procedure will involve artificial induction of permeability of the substrate to the yeast cells. The direct counting of colonies on agar medium is an accurate method for quantifying yeast cells and was chosen as the basis of an assay procedure. A method was sought which disrupted the yeast colony permeability barrier, without disturbing the distinct colony formations (and therefore the counting of these colonies). This was achieved by allowing colonies to grow on nutrient agar media containing X-GLUC (50µg/ml). The colonies were then overlayed with a molten 0.8% agarose solution (at approximately 45^oC) containing 0.1M Na_2HPO_4, pH 7.0 and 1% sarkosyl. With a thin overlay, the agarose solution solidified within 2 minutes, and colonies remained intact and distinct. The sarkosyl present in the overlay induced leakiness of the yeast cell wall/membrane and after 4-6 hours incubation at 37^oC, colonies of the marked strain appeared blue.

Results of this assay are depicted in Figure 5.10. Mixed populations of yeast cells containing various proportions of the marked strain 3AM were prepared as follows. Strains 3AM and 3A were grown separately in liquid culture to stationary phase where similar

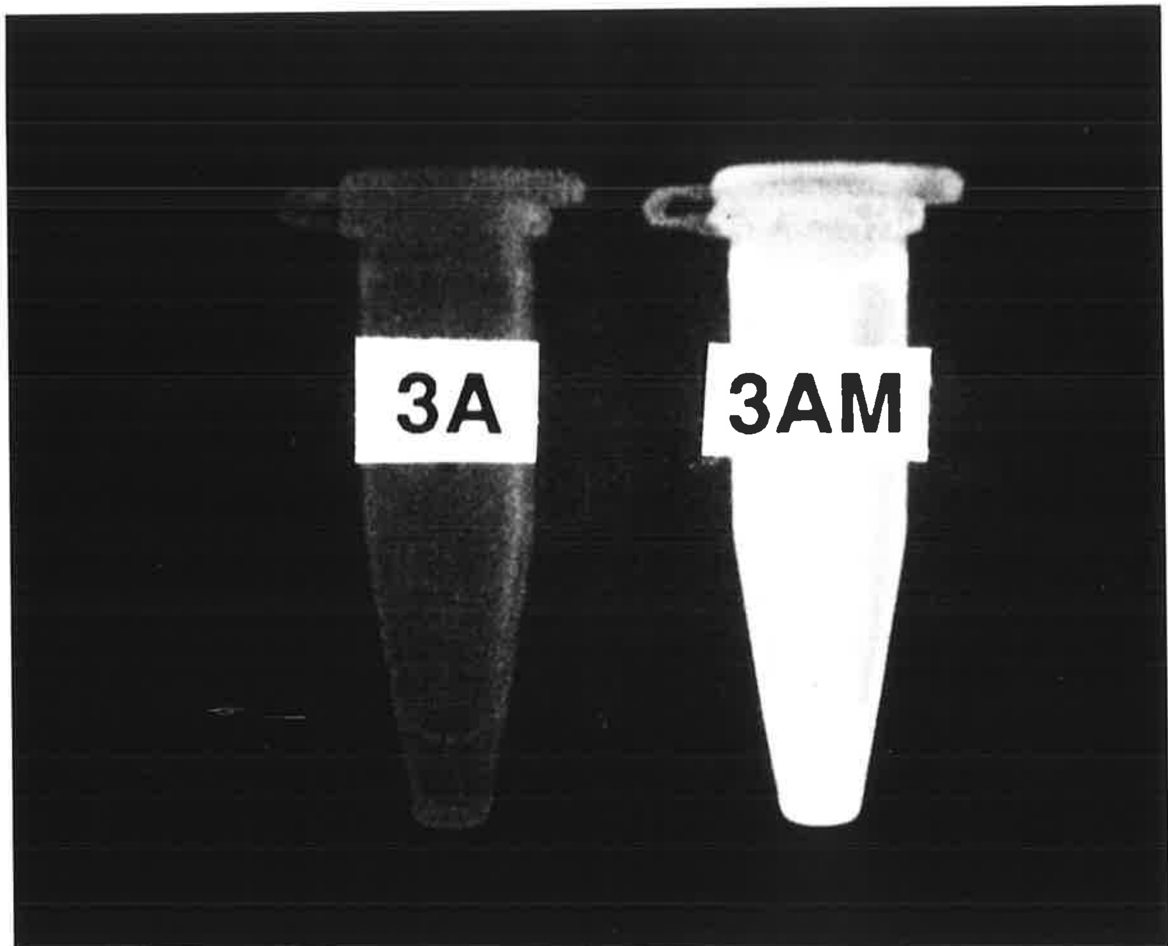


Figure 5.9. Fluorescence assay for detecting GUS activity. Yeast cell extracts containing 1mM MUG were incubated at 37°C for two hours and the visualized on a UV light box.

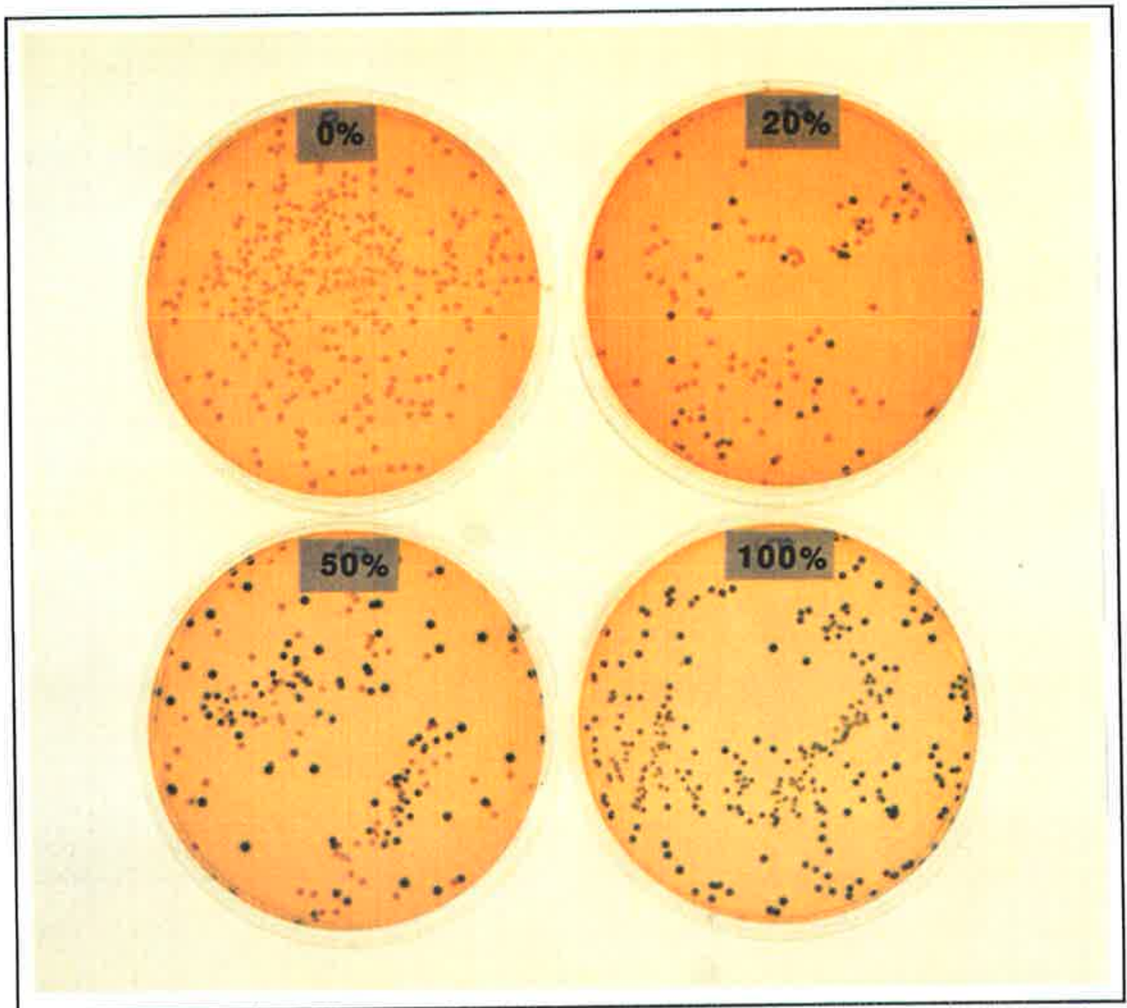


Figure 5.10. Agar plate assays to detect GUS activity in a yeast population containing 0, 20, 50 and 100% strain 3AM.

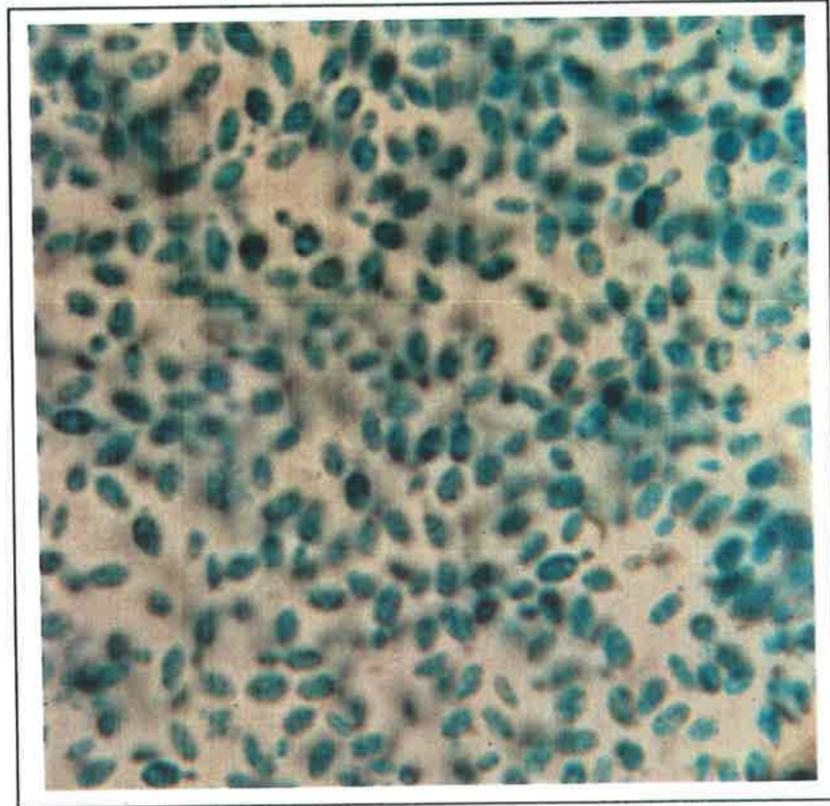


Figure 5.11. Microscopic visualisation of GUS activity in cells of strain 3AM.

optical density and cell number per ml were achieved. These strains were then mixed together in different ratios (vol/vol), resulting in a range of mixed populations in which the percentage of strain 3AM was 0, 20, 50 and 100%. Samples from each population were serially diluted in saline solution (0.85% w/v) and plated onto YPD media containing X-GLUC (50 mg/ml) before being assayed as described above. In each case, the percentage of blue colonies per plate was approximately equal to the percentage of the 3AM culture (vol/vol) in the prepared mixed population.

An attempt was also made to visualize GUS activity at the cellular level. A suspension of 3AM cells containing 0.1M Na_2HPO_4 pH 7.0, 1% sarkosyl and X-GLUC (50 μg/ml), was incubated at 37°C until a deep blue colour had developed (2-4 hours). A sample of this suspension was then viewed under a microscope (Figure 5.11). The blue precipitate was detected inside the marked cells. This result indicates that mixed populations of yeasts can be analysed for presence of the marked strain within two to four hours of sampling.

5.2.5 β-Glucuronidase enzyme activity

The β-glucuronidase activity in the marked strain was measured using the fluorogenic substrate 4-methyl umbelliferyl glucuronide (MUG) (Jefferson, 1987). The detection of fluorescent molecules offers a very high signal-to-noise ratio because the incident excitation light does not impinge on the detection apparatus, and has a spectrum distinct and separable from that of emission. The use of fluorescence measurements to detect enzyme activity allows two to four orders of magnitude greater sensitivity than methods that rely on spectrophotometric determination of product concentration.

Preparation of yeast cell extracts for enzyme assays involved vortexing a suspension of cells in GUS extraction buffer (50 mM Na_2HPO_4, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% triton X-100) with glass beads (to half the

volume). Throughout the vortexing, the cell suspension was periodically placed on ice to avoid overheating. A time course experiment was performed to determine the minimum period of votexing required to release the maximum amount of protein from the cells. At various intervals samples were removed from the vortexing suspension and cell debris was pelleted by centrifugation. The supernatant was then stored for analysis of protein content. Results of this experiment are presented in figure 5.12. The maximal release of protein from the cell suspension was achieved after 20 minutes of vortexing.

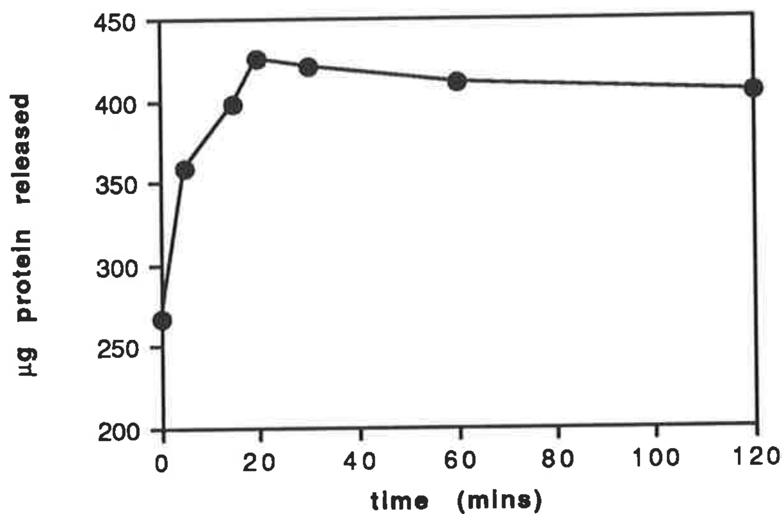


Figure 5.12. Time course analysis of the amount of protein released from a suspension of yeast cells after vortexing with glass beads.

In order to analyse β-glucuronidase activity, strains 3AM and 3A were grown to early stationary phase in liquid YPD media. Cell extracts were prepared as described above by vortexing with glass beads for 20 minutes. Both 5 and 50 µl samples of extract were assayed using the MUG substrate as described in Section 3.6.1. At the same time a standard curve of 4-methylumbelliferone (MU) was prepared, so that the relative fluorescence readings from the extract samples could be interpreted as concentration of MU and then converted to nmoles of MU produced. This experiment was performed twice and

average readings are presented graphically in Figure 5.13. The protein content of each extract was found to be 5 mg/ml for strain 3AM and 4.4 mg/ml for the 3A extract. No enzyme activity was detected in strain 3A, while the average β-glucuronidase activity of strain 3AM was calculated to be 0.65 nmoles MU/min/mg protein.

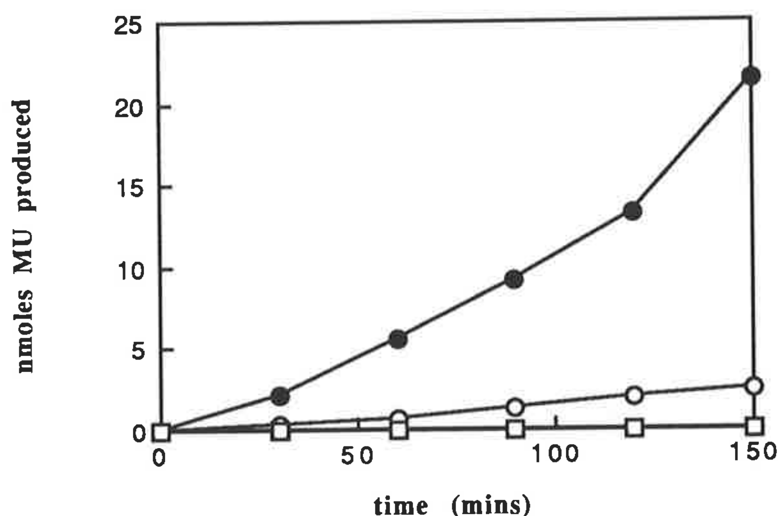


Figure 5.13. β-Glucuronidase enzyme assays: (●) 50 µl strain 3AM extract; (O)5µl strain 3AM extract; (□) 50 µl strain 3A extract.

5.2.6 Fermentation trials

The 3AM strain was used in a fermentation trial to monitor the effects of transformation on the yeast oenological properties. Three different transformants were isolated and used to inoculate separate starter cultures. Three colonies of control, un-transformed 3A yeast were also inoculated into starter cultures. Each of these six cultures was inoculated in duplicate into flasks of Riesling grape juice at a concentration of 4×10^6 cells/ml. Fermentations were carried out under anaerobic conditions at 18°C. Samples were taken at regular intervals and assayed for yeast growth (by measuring optical density

at 650 nm) and progress of fermentation (by refractive index). Refractive indices were averaged and the two resulting fermentation

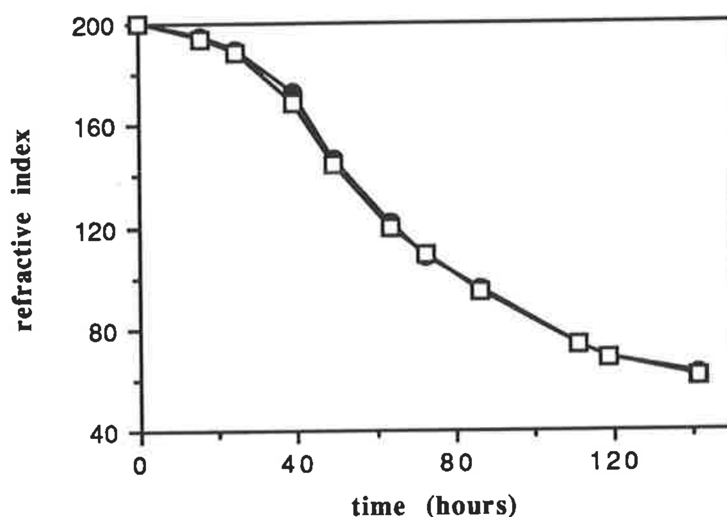


Figure 5.14. Fermentation curves of the marked strain 3AM (□) and parent strain 3A (●).

Wine analysis	Yeast strain		Statistical analysis	
	3A	3AM	V.R. ($F_{1,9}$)#	F. Prob.*
pH	3.32	3.35	4.24	0.07
Residual sugars (g/L)	1.43	1.90	0.66	0.4
SO2 (total) (mg/L)	31.0	23.8	2.09	0.18
SO2 (free) (mg/L)	1.33	0.67	1.13	0.31
Acetic acid (g/L)	0.46	0.48	0.95	0.36

Variance ratio between the two strains.

* F. Prob. indicates the probability of the associated variance ratio.

Table 5.1. Analyses of wines produced from the original yeast strain (3A) and the marked strain (3AM).

curves were plotted (Figure 5.14). A log transformation of the data indicated that the V.R. (F1,99) is 1.01, and the associated F probability is 0.317. Therefore, statistical analysis has revealed no significant difference between the fermentation curves of strains 3A and 3AM.

On completion of fermentation, pH, residual sugars, sulfur dioxide, alcohol and acetic acid concentrations were measured for all twelve samples. Averages were calculated for each parameter (Table 5.1). Again, no significant differences were evident between the two strains.

5.2.7 Stability of GUS construct

An experiment was performed to measure the stability, or maintenance, of the GUS construct over successive generations of strain 3AM. Under fermentation conditions there is an absence of selection for the GUS gene, therfore it is important to determine the spontanous rate of loss of the gene in a dividing population. An overnight culture of strain 3AM was generated from a single colony. This culture was then diluted by a factor of 10^{-3} in a sub-culturing process (by inoculating 100 μl into 100 ml YPD) and grown to stationary phase. This sub-culturing was repeated seven times. Samples were removed at stationary phase of each sub-culture and assayed for GUS activity by the agar plate method. The number of blue and white colonies were scored and recorded in Table 5.2. An instability of the gene in less than 1% of the total population was recorded at each sampling and did not increase significantly over time.

Sub-culture	Dilution effect	Blue colonies	White colonies	Total colonies	% white colonies
-	-	565	2	567	0.35
1	10^{-3}	780	5	678	0.74
2	10^{-6}	764	6	770	0.78
3	10^{-9}	673	5	782	0.64
4	10^{-12}	868	8	876	0.91
5	10^{-15}	725	6	731	0.82
6	10^{-18}	540	5	545	0.92
7	10^{-21}	615	5	620	0.81

Table 5.2. Stability test of GUS construct in strain 3AM after successive sub-culturing. The agar plate GUS assay was performed at each sub-culture step to detect the number of negative (white) colonies.

5.3 DISCUSSION

Close examination of pulsed field gel electrophoresis fingerprints of twelve wine yeasts indicate that none of the strains have identical electrophoretic chromosomal profiles. Similar results have recently been reported by Vezinhet *et al.* (1990). In an analysis of 22 wine yeasts isolated from various wine growing regions, only 3 strains (originating from the same vineyard) could not be differentiated.

These differences in profiles result from chromosome rearrangements which have taken place in yeast strains during the course of evolution. The high degree of polymorphism among wine yeast strains is perhaps not surprising in view of their diverse origins. It is likely that the strains held in The Australian Wine Research Institute Yeast Collection have undergone reproductive isolation for some time by virtue of their localization in different viticultural regions of the world.

Pulsed field gel electrophoresis can be used to gain a better understanding of the processes which generate chromosome-length polymorphisms between strains. For example, the FIGE and OFAGE systems have been used to show that certain chromosome-length polymorphisms segregate in a 2:2 ratio, indicating single structural alterations of the chromosomes (Ono and Ishino-Arao, 1988). Chromosome-length polymorphisms, however, can also result from two or more structural alterations per chromosome and are not restricted to specific chromosomes. The TAFE system has been used for the analysis of chromosomal segregants and inheritance (Bilinski and Casey, 1989), and Viljoen *et al.* (1989) used the OFAGE system to establish possible amamorph/telemorph relations in yeasts. Another application of pulsed-field gel electrophoresis is the localization of specific genes and the distinction of two yeast strains that differ only in the chromosomal localisation of specific genes. Pretorius and Marmur (1988) probed a Southern blot of an OFAGE gel containing the resolved chromosomes of four *S. cerevisiae* strains with the

cloned *STA2* glucoamylase gene and showed that the strains differed only in the presence and/or chromosomal location of the gene.

Results obtained with chromosome fingerprinting of wine yeasts reveal an immediate application of TAFE technology to Research and Development and Quality Control in wine fermentations. At fixed pulse and electrophoresis times the pattern for any strain is highly reproducible and results in a unique signature easily distinguished from other contaminating strains. The relatively rapid technique of electrophoretic karyotyping of yeasts may therefore be applied to improvement of yeasts by clonal selection during successive rounds of fermentation. This method would ensure that contaminant strains of *S. cerevisiae* were not inadvertently selected. Processes which cause chromosome damage could also be identified using this techmique (Contopoulou *et al.*, 1987). The effect of environmental selection pressures (such as storage conditions or culture media) on the genomes of wine yeasts may also be determined. Such information could have significant impact on the preparation of starter cultures and the mode of storage of wine yeasts. In addition, in developing new strains for industrial applications, TAFE analyses can be useful for monitoring chromosome profiles of parent and progeny strains employed in such research. Fingerprints that examine all chromosomes within a yeast strain can also be viewed as genetic 'snapshots' of that yeast at a fixed point in time and therefore can be used to monitor chromosome stability. This system is, therefore, useful for routinely monitoring the stability of a yeast strain used within a winery as well as for distinguishing strains used in different wineries.

However, for quantitative studies of yeast growth kinetics during fermentation where large numbers of cells need to be monitored for statistical analyses, the TAFE identification system is unsuitable. The time involved in the preparation of plugs, and the number of gels required for the analysis of large numbers of colonies precludes the TAFE system from this application. For this reason, the GUS marking system was developed. Results presented in this chapter show that expression of the *E.coli* GUS gene can be

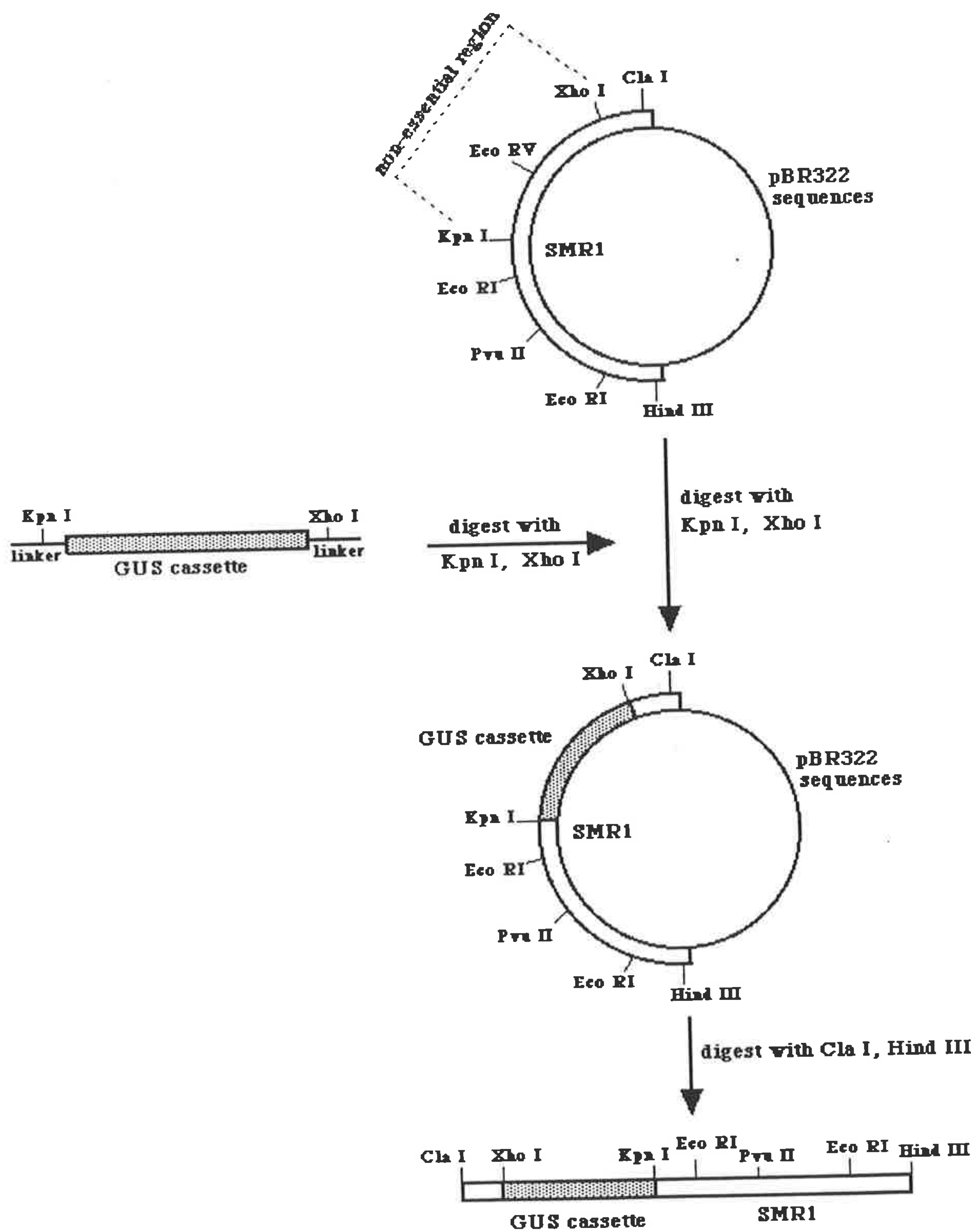
achieved in *Saccharomyces* strains by a construct in which the GUS coding region is linked to the yeast alcohol dehydrogenase (*ADC1*) promoter and teminator sequences. The construct was successfully targeted to the site of the *ILV2* gene on chromosome XIII of wine yeast strain 3A. Fermentation trials indicated that the transformation procedure did not significantly alter vinification properties of wine yeast strain 3A. Commercial ferments would need to be carried out to confirm that the transformation procedure does not produce organoleptic changes in the wine.

The transformation system used to introduce the construct has been used successfully to transform a range of strains in The Australian Wine Research Institute yeast collection (see Section 4.2.2). The implications are, therefore, that the GUS marking system can be used to tag any wine yeast strain of choice. In this respect the GUS constuct offers a unique marking system for wine yeasts. The marked oenological strain K1 (Vezinhet and Lacroix, 1984; Vezinhet, 1985) was developed by selecting natural mutants in a population of the Lalvin V yeast. This procedure is a time consuming and non-specific method of genetic manipulation and, therefore, it is not readily applied to wine yeasts of interest.

Assaying for the GUS marker can be achieved by fluorimetry, spectrophotometry, or by agar plate method. Although natural transport of X-GLUC or MUG substrates did not occur across yeast cell membranes in the time course of experiments described here, this problem was overcome by inducing artificial permeation in assay procedures.

Analysis of a large sample of colonies during the successive sub-culturing of strain 3AM revealed an instability of the GUS construct. This instability was detected by the presence of white colonies in the agar plate assay procedure. The frequency of colonies which responded negatively to the GUS plate assay was always less than 1% of the total plate count and did not increase significantly over the period of sub-culturing. Occassionally a sectored colony was detected, suggesting either excision of the gene by

Figure 5.15. Theoretical steps involved in the development of a GUS-*SMR1* construct for gene replacement. Polylinkers could be added to the 3.7kb GUS cassette (derived from pAW219) to produce flanking Kpn 1 and Xho 1 ends. This cassette could then replace the 2.3kb Xho 1-Kpn 1 fragment in the non-essential region of the *SMR1* gene (Xiao and Rank, 1989). The resulting plasmid would contain the GUS cassette flanked by *SMR1* sequences. A Cla 1 + Hind III digestion would then generate a GUS-*SMR1* construct suitable for gene replacement.


non-essential region
Cla I
Xho I
Eco RV
pBR322
sequences
Kpn I
SMR1
Eco RI
Pvu II
Eco RI
Hind III
digest with
Kpn I, Xho I
Kpn I
Xho I
linker
GUS cassette
linker
digest with
Kpn I, Xho I
Cla I
Xho I
GUS cassette
pBR322
sequences
Kpn I
SMR1
Eco RI
Pvu II
Eco RI
Hind III
digest with Cla I, Hind III
Cla I
Xho I
Kpn I
Eco RI
Pvu II
Eco RI
Hind III
GUS cassette
SMR1

homologous recombination (Struhl *et al.*, 1979) or loss of the gene after mitotic crossing over (Roeder *et al.*, 1988). The implications of this instability of the GUS gene require consideration. In mixed-population kinetic studies involving a GUS-marked strain, the proportion of the marked strain will be underestimated due to the 'false negative' cells which have lost the marker gene. This underestimation can be corrected if the frequency of instability is calculated by appropriate control experiments.

The instability detected in this chapter indicate that statistical analysis of herbicide resistant colonies performed in section 4.3.4 was not sensitive enough to detect a <1% loss of the integrative vector. That experiment could be improved by analysing a larger number of colonies. Furthermore, determination of the proportion of herbicide resistant colonies may be better achieved by replica-plating colonies from non-selective media directly onto selective media.

Loss of the gene through excision by homologous recombination may have been avoided by adopting an alternative method of targeting the GUS constuct to the *ILV2* gene. For example, instead of introducing the plasmid pAW221 by homologous recombination, the GUS construct could be integrated to the genome of yeast by the process of gene replacement (Rothstein, 1983). It would be possible to clone the GUS construct into a non-essential region of the *SMR1* coding region (Xiao and Rank, 1989), thus producing a chimaeric GUS-*SMRI* cassette where the GUS construct is flanked by *SMR1* upstream sequences. The release of this cassette by restriction enzyme digestion would generate a fragment with both recombinogenic ends homologous to yeast chromosome XIII DNA at the *ILV2* locus. Transformation of yeast with this fragment would result in integration by gene replacement at the *ILV2* locus (Figure 5.15). The use of this method to introduce the GUS construct to yeast strains offers the prospect of greater stability.

In determining suitable procedures for the introduction of a stable GUS construct in wine yeasts, it is also important to consider the phenomenon of mitotic recombination and

gene conversion (Zimmerman, 1990). This source of instability in wine yeasts can be overcome by a procedure which involves sporulation and isolation of clones from single ascospores. The haploid homothallic spore diploidizes shortly after germination giving rise to a completely homozygous diploid. A problem with this technique, however lies in poor sporulation ability of wine yeasts, and in the laborious selection procedures involved in obtaining a spore with identical properties to the original parent.

The GUS system described in this chapter will enable a wide range of yeast strains to be marked, providing the means for unequivocal identification and monitoring during fermentation. Recent investigations by Heard and Fleet (1985,1986,1988) made with both inoculated and uninoculated grape juices under a range of fermentation conditions suggest that *Saccharomyces* strains are not necessarily the dominant organism during vinification. These studies, along with a lack of knowledge regarding incidence and importance of wild strains of *Saccharomyces* in fermenting grape juice, call for detailed monitoring of inoculated strains under various oenological conditions. The agar plate method will find application in the monitoring of starter cultures, effect of prefermentation processes on the microbial populations of must, and the efficiency of inoculation. Furthermore, the cell suspension assay has the advantage of obtaining results from the starter culture or ferment populations within two to four hours of sampling.

It is important to note, however, that the GUS system has been developed using recombinant DNA technology and utilizes a gene derived from *E. coli*. Consequently, yeast strains marked with the GUS gene are not immediately available for use in the Australian Wine Industry. It is envisaged, however, that public perception of genetically engineered organisms and legislations limiting their release will be less restricting over time. The benefits of recombinant DNA technology have been widely reported and microorganisms have been successfully exploited for the production of a range of pharmaceutical products such as interferon, insulin and human growth hormone (Bloom, 1980; Woodruff, 1980). Genetically engineered organisms are being constructed for a variety of environmental

applications. These include their use in agriculture as pesticides (Bishop *et al.*, 1988); or for agronomic crop production; pollution control of toxic waste in land filled sites, wastewater treatment facilities or after accidental spillages; and in mining and the petro-chemical industry for enhancing oil and mineral recovery (Keeler, 1988). A genetically engineered agrobacterium which controls crown gall in plants has recently been released in Australia (Kerr, 1989). These advances will lead to a more informed general public on matters involving genetically engineered organisms and, perhaps, will smooth the way for others.

In conclusion, two systems for the identification of yeast strains have been described. Advantages of TAFE system are that it facilitates a better understanding of processes involved in yeast genome rearrangements, and can be used to monitor chromosome stability in wine yeasts. The GUS system enables the monitoring of specific wine yeast strains in commercial fermentations which contain an unknown load of indigenous yeasts.

Chapter 6 Determination of killer toxin activity in fermenting grape juice using a marked *Saccharomyces* strain

6.1. INTRODUCTION

Killer activity in yeasts was first reported in strains of *S. cerevisiae* in 1963 by Bevan and Makower. Killer yeasts secrete polypeptide toxins which kill sensitive strains of the same genus and less frequently, strains of different genera (Philliskirk and Young, 1975; Tipper and Bostian, 1984). Previous studies indicate that the toxin of *Saccharomyces* is a protein which binds to a receptor on the cell wall of the sensitive yeast, disrupting the electrochemical gradient across the cell membrane and hence the intracellular ionic balance (De la Pena *et al.*, 1981; Skipper and Bussey, 1977).

Production of the toxin and immunity to it are determined by a cytoplasmically inherited double stranded (ds) RNA plasmid, otherwise known as the M-genome (Bostian *et al.*, 1980) which is found only in cells containing an additional dsRNA species designated the L-genome. Both types of dsRNA exist in virus-like particles and require a protein encoded by the L-dsRNA for encapsidation (Bostian *et al.*, 1980; Harris, 1978).

Based upon properties of the toxin, killer yeasts have been classified into eleven groups (K_1 through K_{11}) (Naumov and Naumova, 1973; Young and Yagiu, 1978). Those unique to *Saccharomyces* fall into the first three (K_1, K_2 and K_3). The *Saccharomyces* toxin is reversibly inactivated at low pH (2.0) and irreversibly inactivated at pH in excess of 5.0 (Young and Yagiu, 1978). More specifically, the biological activity of K_1 is optimal between pH 4.6 and 4.8, while K_2 shows optimal activity between 4.2 and 4.7 (Shimazu *et al.*, 1985). Compared with K_1, the K_2 toxin is stable over a wider pH range (2.8 to 4.8) (Rogers and Bevan, 1978) and is therefore more relevant in wine fermentation.

Killer activity has been detected in yeasts isolated from established vineyards and wineries in various regions of the world including Europe and Russia (Barre, 1984; Gaia, 1984; Naumov and Naumova, 1973), South Africa (Tredoux *et al.*, 1986) and Australia (Heard and Fleet, 1987a,b). This widespread occurrence has prompted interest in the oenological significance of killer wine yeasts. In theory, selected killer yeasts could be used as the inoculated strain to suppress growth of undesirable wild strains of *S. cerevisiae* during grape juice fermentation. In addition, as killer interactions have been reported to occur between yeasts of different genera (Radler *et al.*, 1985; Rosini, 1985a), the possibility exists to genetically engineer broad spectrum killer strains of *S. cerevisiae* (Boone *et al.*, 1990).

Studies have been conducted to assess the efficiency of killer toxin on sensitive yeast strains. However, reports have been contradictory on the expression of killer activity under fermentation conditions (Cuinier and Gros, 1983; Delteil and Aizac, 1988; Lafon-Lafourcade and Ribereau-Gayon, 1984). Attempts to determine the population kinetics of killer and sensitive strains during wine fermentation have been restricted because of the difficulty involved in identifying the two types when grown in mixed cultures. Approaches used to date include i) choice of killer and sensitive strains that can be distinguished by their growth rates (Barre, 1984) or production of hydrogen sulfide (Rosini, 1985b); ii) use of auxotrophic and respiratory deficient mutants of killer strains and appropriate plating conditions under which they can be identified (Hara *et al.*, 1980, 1981; Seki *et al.*, 1985); iii) use of killer and sensitive strains which can be distinguished by differences in colony morphology (Heard and Fleet, 1987a); and iv) assaying colonies directly for killer activity (Longo *et al.*, 1990). All of these methods are limited by the fact that the assays involved are laborious and time-consuming, or that only killer strains with specific characteristics can be studied.

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This chapter describes the use of a marked *S. cerevisiae* killer strain in a mixed culture inoculum to quantify directly the effect of killer toxin on a sensitive *S. cerevisiae* strain under fermentation conditions. As a wide range of yeast strains can be readily and stably marked, this system of analysis is unlimited in application and provides a simple and unequivocal means of quantifying killer yeast strains in mixed culture ferments.

6.2 RESULTS

6.2.1 Curing of Strain 3AM

In order to specifically analyse the effect of killer toxin in fermentations, an experiment was designed to compare two isogenic strains which differ only in the presence of the M-dsRNA genome and therefore, in their ability to produce killer toxin.

Killer strain 3AM has previously been marked with the *Escherichia coli* β-Glucuronidase (GUS) gene (see Chapter 5). This system allows the marked strain to be readily identified in a mixed population by a simple plate assay which results in the formation of a blue precipitate in marked colonies. Strain 3AM was cured of its M-dsRNA plasmid by heat treatment (Wickner, 1974), the cured or sensitive colonies being identified by killer plate assays. Figure 6.1 shows the response of strain 3AM and an isolated cured derivative (designated 3AMC) to the killer plate assay. The zone of inhibition clearly evident around strain 3AM is absent around 3AMC, indicating that strain 3AMC is not producing killer toxin. As strain 3AMC is derived from 3AM, it inherits the GUS gene and therefore is also a marked strain.

Double stranded RNA species were isolated from strains 3AM and 3AMC and analysed by standard electrophoresis techniques (Figure 6.2). A band representing the M-dsRNA genome is present in strain 3AM, and absent in strain 3AMC.

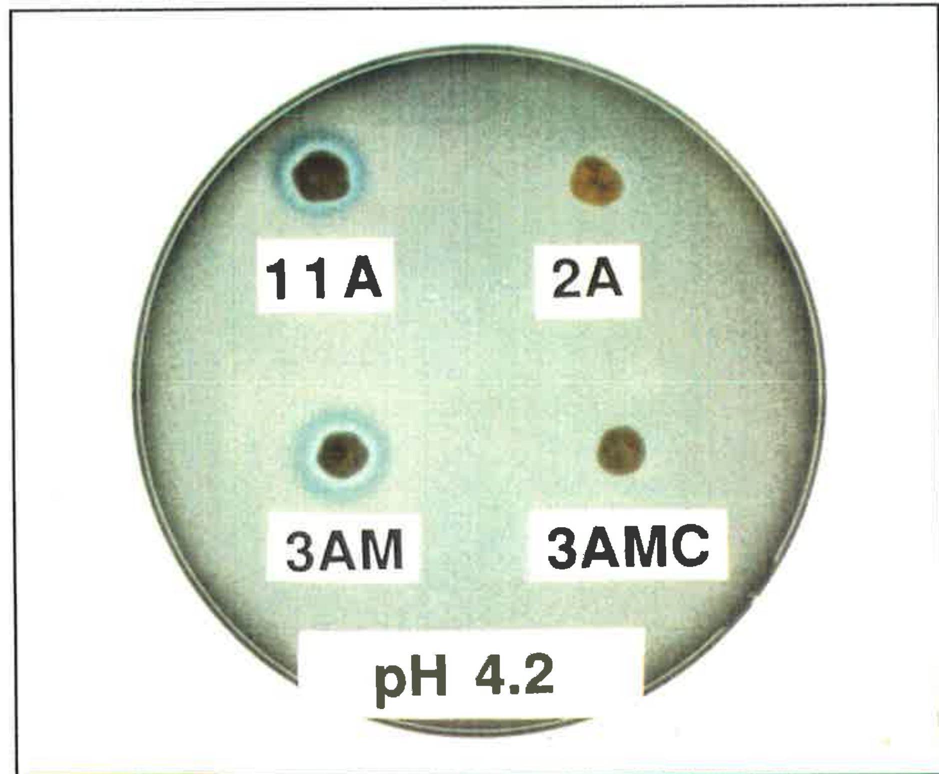


Figure 6.1. Agar plate assay for killer activity. The agar (pH 4.2, 0.003% methylene blue) is seeded with an overnight culture of strain 3AMC, and strains to be tested for killer activity are patched onto the solid media. 11A is a known killer strain, and 2A is a known sensitive strain.

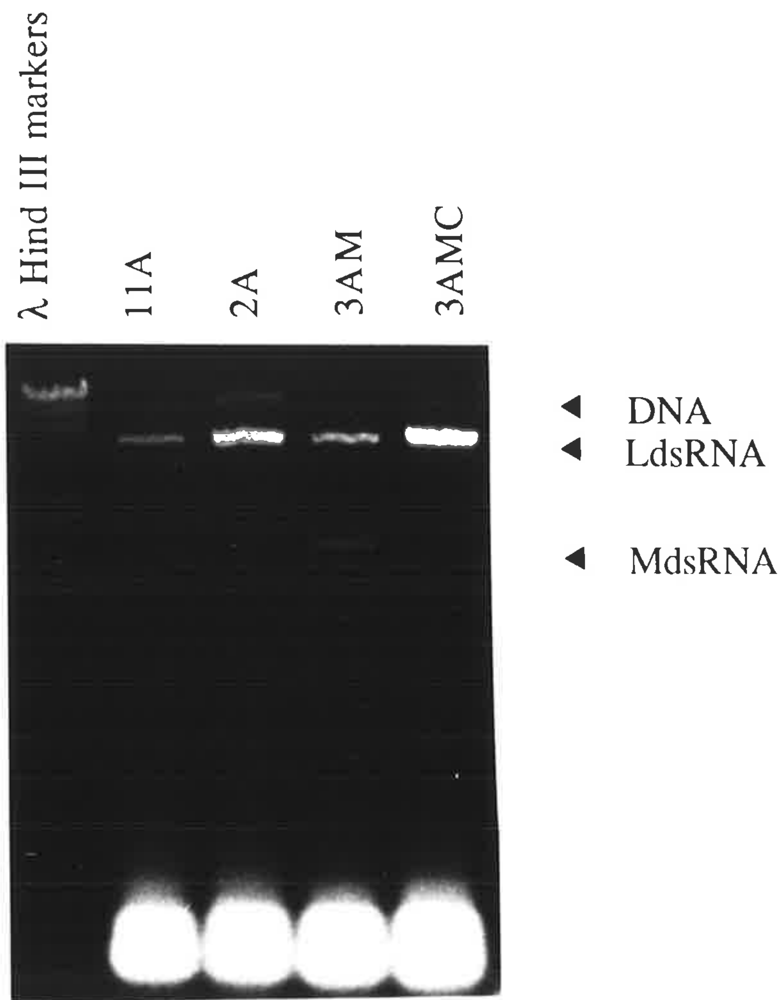


Figure 6.2 Species of dsRNA isolated from strains 11A, 2A, 3AM and 3AMC.

Fermentation trials were then performed on strains 3AM and 3AMC to determine the effect of the curing procedure on yeast growth and fermentation rates. Starter cultures of each strain were inoculated in triplicate into flasks of Riesling grape juice at a concentration of 5 x 10^6 cell per ml. Samples were taken at regular intervals and asssayed for yeast growth and progress of fermentation. The average readings for each strain were plotted over time (Figure 6.3). There are no significant differences in the growth or fermentation rates between strains 3AM and 3AMC.

6.2.2 Analysis of killer activity during fermentation

Strains 3AM and 3AMC were analysed for killer activity in Riesling juice by co-inoculating each strain with the sensitive *Saccharomyces* strain 5A. Control ferments of each strain (3AM, 3AMC and 5A) as pure inoculums were also performed. Each ferment was conducted in duplicate at 18°C with gentle agitation under anaerobic conditions. GUS plate assays were then performed to identify the marked strain (3AM or 3AMC). Colonies of the marked strain turn a deep blue colour as a result of this assay, allowing simple identification.

GUS plate assays were also performed on the control ferments to confirm the validity of the assay. Plate assays on the control 5A ferment were consistently negative, highlighting the absence of background GUS activity in natural yeast cells. However, control 3AM and 3AMC ferments gave values of between 99 - 100% of total colonies per plate for the marked strain count. This level of instability corresponds to that determined in Chapter 5. The background reversion frequency was taken into account throughout the analysis.

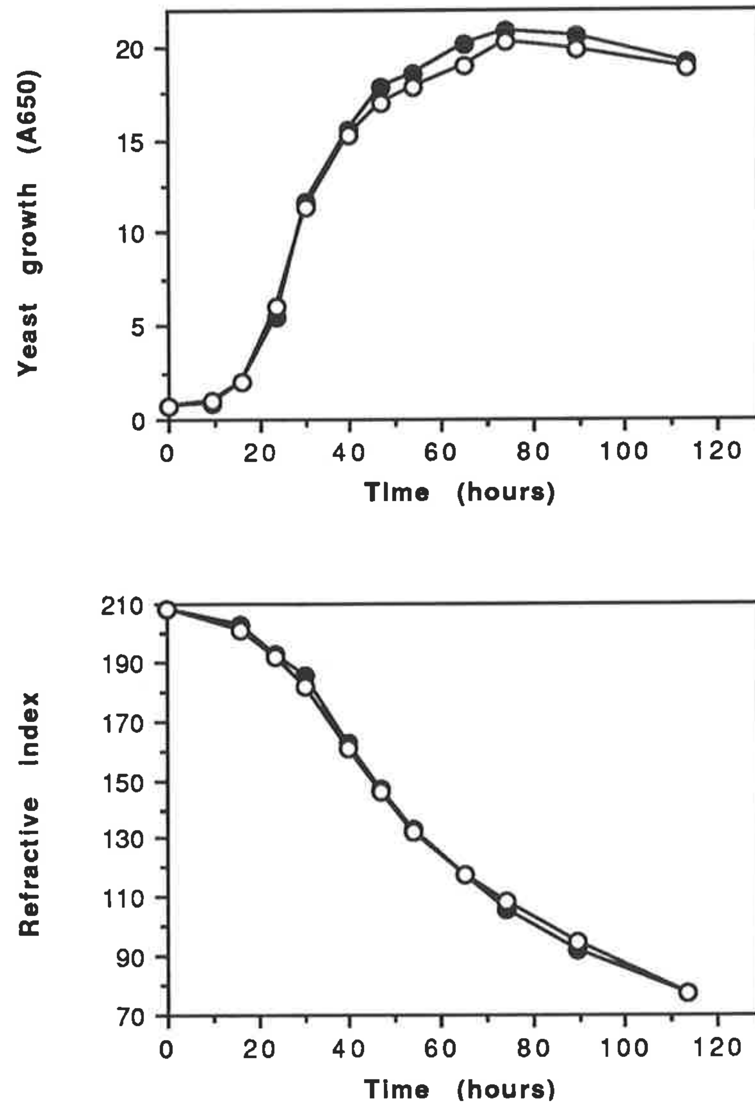


Figure 6.3. Yeast growth (Panel A) and sugar utilisation (Panel B) curves of strains 3AM (●) and 3AMC (O).

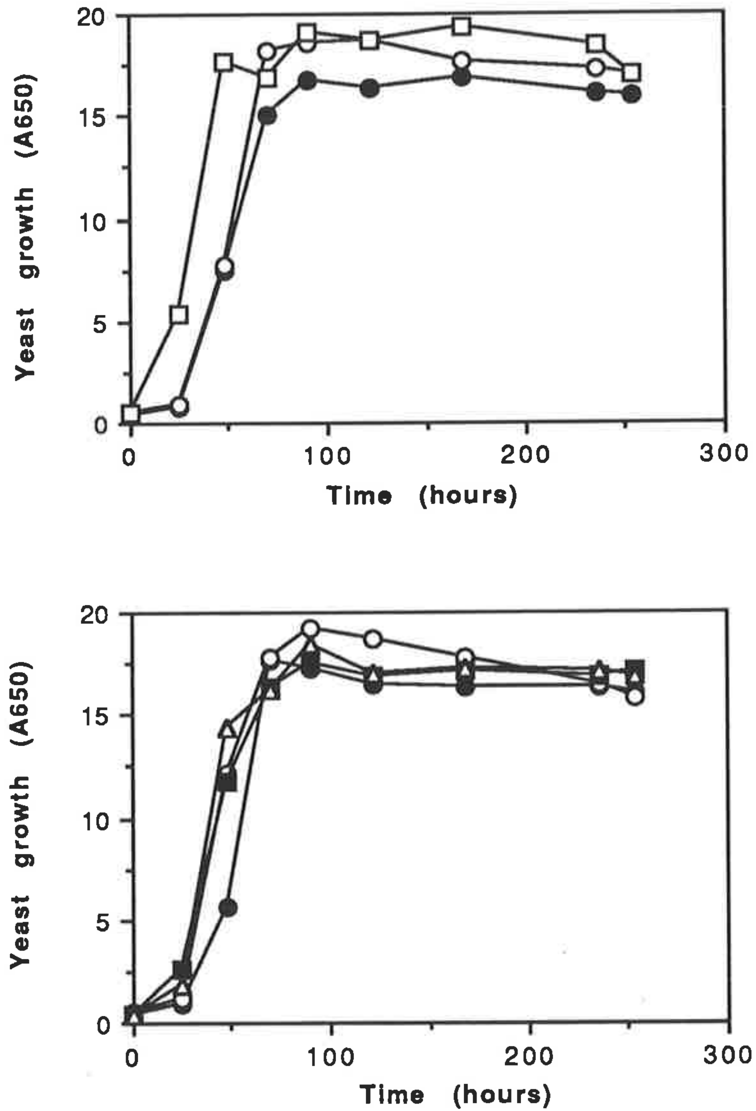


Figure 6.4. Panel A: Growth curves of control single monoculture ferments. Symbols: ● 3AM; O 3AMC; □ 5A. Panel B: Growth curves of mixed culture ferments. Symbols: ● 3AM and 5A at an inoculum ratio of 2:1; O 3AMC and 5A at an inoculation ratio of 2:1; ■ 3AM and 5A at an inoculum ratio of 1:1; Δ 3AMC and 5A at an inoculum ratio of 1:1.

The following mixed culture ferments were carried out:

i) 3AM and 5A at an inoculum ratio of 1:1;

ii) 3AMC and 5A at an inoculum ratio of 1:1;

iii) 3AM and 5A at an inoculum ratio of 2:1; and

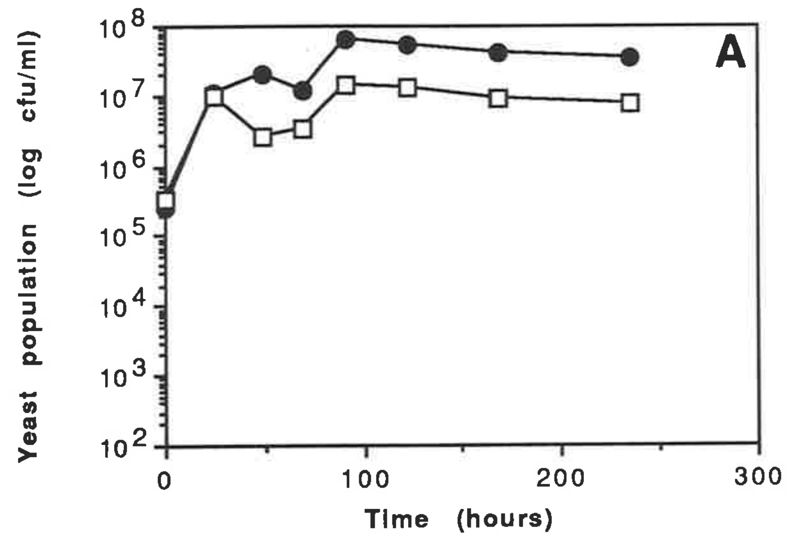
iv) 3AMC and 5A at an inoculum ratio of 2:1.

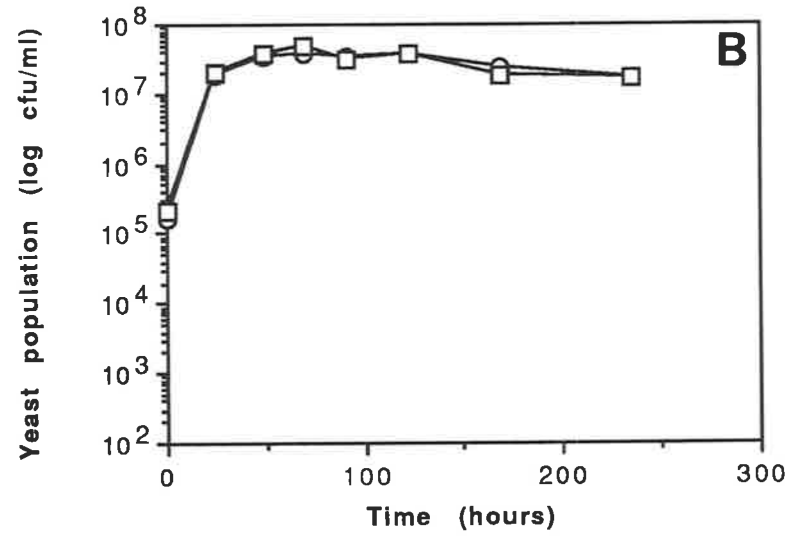
These mixed ferments exhibited normal growth kinetics, as did the three control ferments (Figure 6.4).

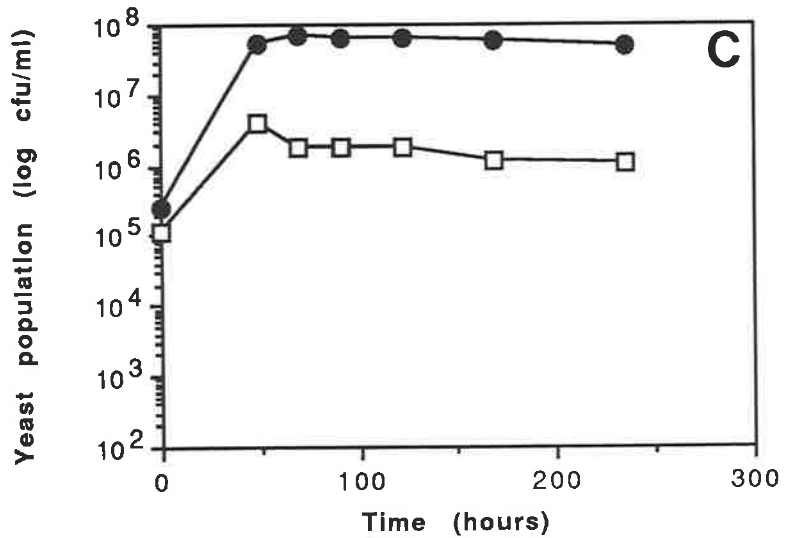
The time course of growth (colony forming units per ml) of each strain in the mixed culture ferments is plotted in Figure 6.5. At inoculum ratios of 1:1, there was a notable increase in the proportion of killer strain 3AM, whereas the cured strain 3AMC fails to exert any dominance over the sensitive strain under otherwise identical conditions. Statistical analysis was used to test the null hypothesis that the ratio of killer : sensitive cells remains 1:1 throughout the ferment. A goodness of fit test (normal test) rejected the null hypothesis, with p-value $<< 0.001$. However, identical analysis of the cured : sensitive strain ferment accepted the null hypothesis that the ratio of the two strains remains at 1:1 throughout the ferment. With an increased proportion of strains 3AM and 3AMC in the inoculum (ratio 2:1), the dominating effect of strain 3AM was more pronounced, whereas strain 3AMC again showed no apparent change in proportion over time. The dominance of strain 3AM in mixed culture ferments is illustrated more clearly when the percentage of each strain is plotted over the time of the ferment (Figure 6.6). For an inoculum ratio of 1:1, 3AM increased to approximately 80% after 3 days but for a higher inoculum ratio of 2:1, 3AM eventually accounted for 97% of the total yeast population. It is important to note that the strain 5A persisted, albeit at low levels, throughout the ferments.

Experiments were conducted to determine the lowest inoculum ratio of killer to sensitive cells at which significant killer activity can be observed. Mixed ferments of strain 3AM and 5A at inoculum ratios of 1:2 and 1:4 respectively were carried out

Figure 6.5. Growth curves of each strain in mixed-culture ferments expressed as cfu per ml. (A) Mixed ferment of 3AM (●) and 5A (□) at an inoculum ratio of 1:1. (B) Mixed ferment of 3AMC (O) and 5A (□) at an inoculum ratio of 1:1. (C) Mixed ferment of 3AM (●) and 5A (□) at an inoculum ratio of 2:1. (D) Mixed ferment of 3AMC (O) and 5A (□) at an inoculum ratio of 2:1.


A
Yeast population (log cfu/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
100
200
300
Time (hours)


B
Yeast population (log cfu/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
100
200
300
Time (hours)


C
Yeast population (log cfu/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
100
200
300
Time (hours)

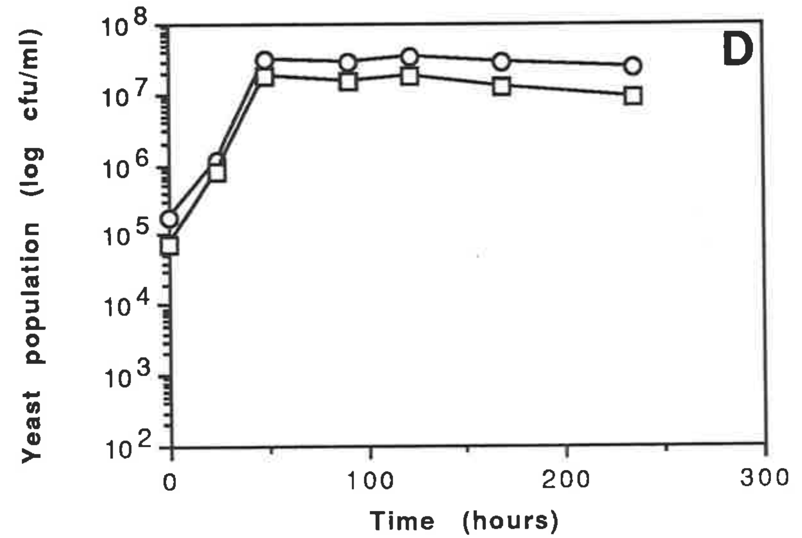
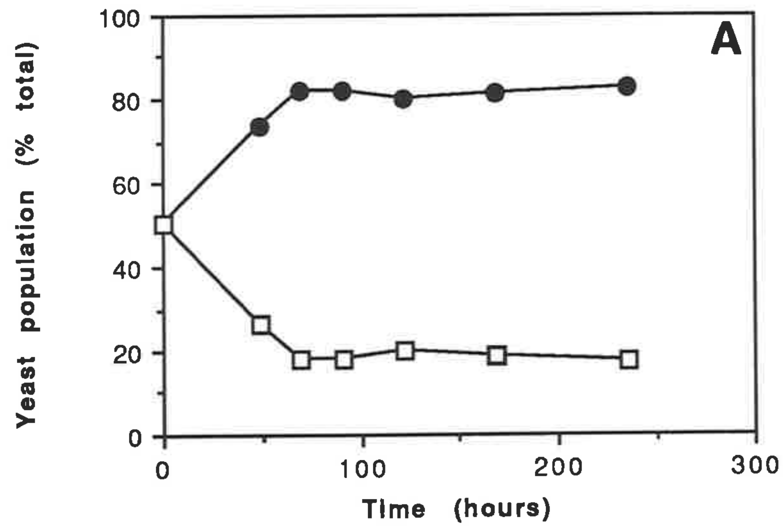
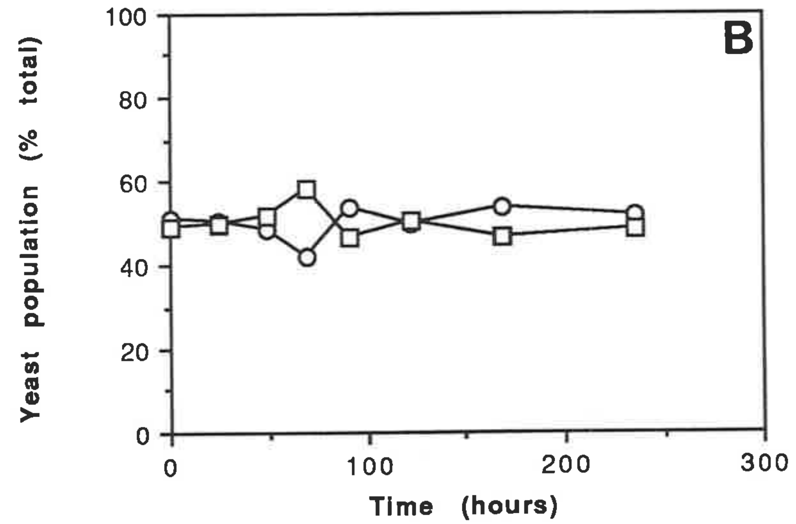
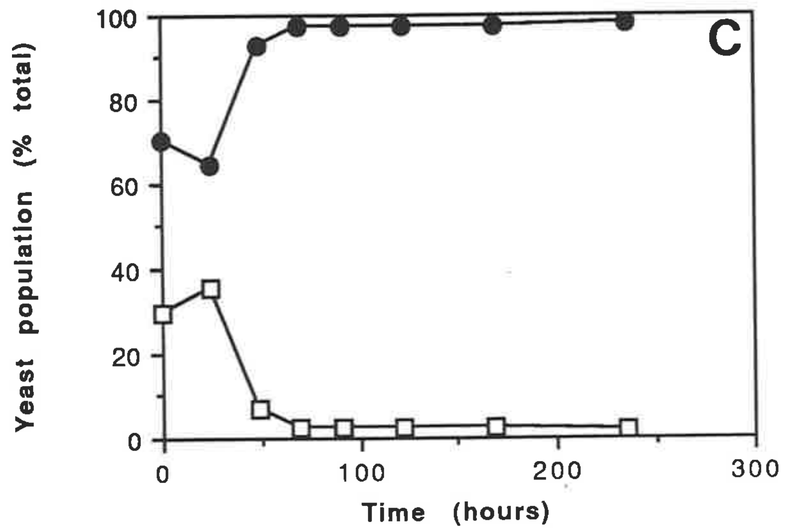
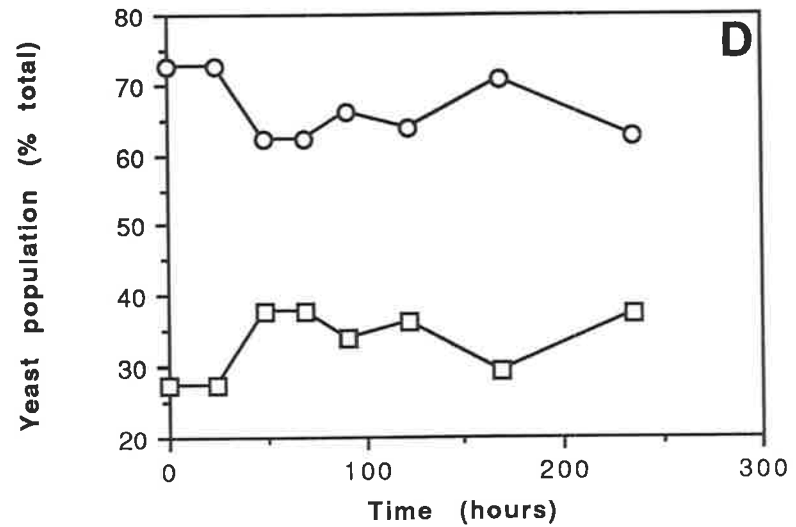

D
Yeast population (log cfu/ml)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
100
200
300
Time (hours)

Figure 6.6. Proportions of each strain in mixed-culture ferments expressed as percentage of the total yeast population. (A) Mixed ferment of 3AM (●) and 5A (□) at an inoculum ratio of 1:1. (B) Mixed ferment of 3AMC (O) and 5A (□) at an inoculum ratio of 1:1. (C) Mixed ferment of 3AM (●) and 5A (□) at an inoculum ratio of 2:1. (D) Mixed ferment of 3AMC (O) and 5A (□) at an inoculum ratio of 2:1.


A
Yeast population (% total)
Time (hours)
0
20
40
60
80
100
0
100
200
300


B
Yeast population (% total)
Time (hours)
0
20
40
60
80
100
0
100
200
300


C
Yeast population (% total)
Time (hours)
0
20
40
60
80
100
0
100
200
300


D
Yeast population (% total)
Time (hours)
20
30
40
50
60
70
80
0
100
200
300

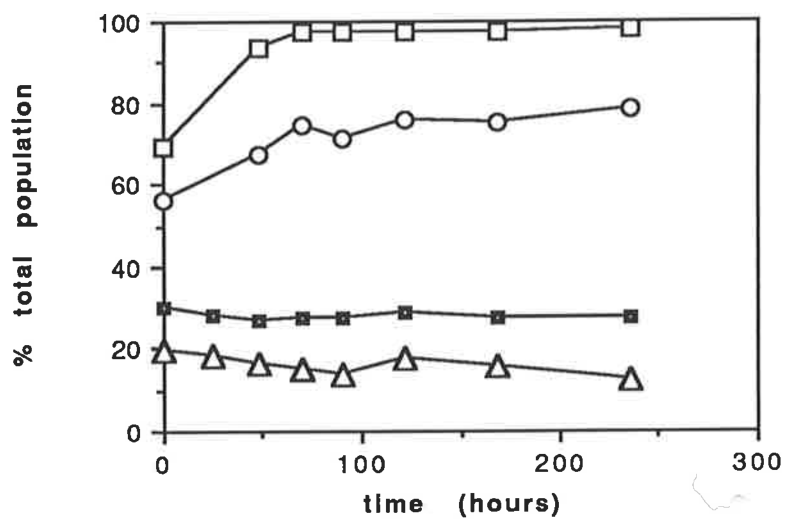


Figure 6.7. The time course in the proportion of killer strain 3AM in the total population of a mixed culture ferment with strain 5A for different inoculum ratios. Symbols: ratio 3AM to 5A □ 2:1; O 1:1; ■ 1:2; Δ 1:4.

under conditions described above. No change from the initial proportion of strain 3AM was detected in either of these ferments. The results of all mixed culture ferments involving strain 3AM are summarised in Figure 6.7.

6.3 DISCUSSION

The GUS marking system has enabled a direct comparison to be made between the inoculation efficiency of a killer strain (3AM) and a cured isogenic derivative (3AMC) in fermenting grape juice. At a ratio of killer to sensitive cells of 1:1 the cured strain 3AMC remained at 50% of the total population while the killer strain increased to 80%. The ability of strain 3AM to dominate 5A during fermentation is likely to be due to the production of killer toxin by strain 3AM and not to a difference in respective growth rates favouring the killer strain. We can conclude, therefore, that the killer toxin has displayed significant activity under these fermentation conditions. This result is of particular interest to the oenologist since the K_2 toxin produced by strain 3A is reported to show maximum activity at pH 4.2 (Rogers and Bevan, 1978), which is 0.5 to 1 pH unit higher than generally found in grape musts.

In cases where killer activity has been reported in fermenting grape juice, a discrepancy exists as to whether effective killing action occurs when the proportion of killer cells is less than 50% of a mixed culture ferment. Heard and Fleet (1987a) did not observe killer action when the ratio of killer to sensitive cells was approximately 1:7 whereas others have reported killer activity with killer to sensitive cell ratios of 1:10 and lower (Barre, 1984; Hara *et al.*, 1980, 1981). Our results showed that an increase in ratio of killer to sensitive cells to approximately 2:1 resulted in a pronounced dominance of the fermentation by strain 3AM to 97% of the total mixed population by the end of the fermentation. However, with killer to sensitive cell ratios of 1:2 or 1:4, no effective killer action was evident. It is possible that differences in either composition of medium, fermentation

conditions or strain sensitivity may account for discrepancies in reports of killer toxin efficiency.

The relevance of killer strains in wine making has been the focus of attention in countries where selected yeast cultures are inoculated into musts to induce fermentation. This focus has intensified since the observations that yeasts which are naturally present in the must also play significant roles in supposedly "pure" culture fermentations (Heard and Fleet, 1985; Lafon-Lafourcade and Ribereau-Gayon, 1984). These natural yeasts include species from the genera *Kloeckera, Candida, Hansenula* and *Saccharomyces*. Killer *Saccharomyces* wine yeast strains may be effective in suppressing natural *Saccharomyces* yeasts during fermentation and the possibility exists to engineer broad range killer yeasts to control strains from other genera. For these reasons, further study is needed to determine appropriate fermentation conditions for effective killer activity.

The GUS marking system provides a method which allows a broad range of killer strains to be rapidly and unequivocally identified in a mixed culture. This system can be employed to gain a better understanding of killer activity during fermentation.

Chapter 7 Comparison of fermentation conditions by use of a marked strain

7.1 INTRODUCTION

One of the most significant technological advances in winemaking has been the commercial availability of selected yeasts, usually strains of *S. cerevisiae*, for inoculation into the juice (Reed and Nagodawaithana, 1988). It is assumed that the inoculated *S. cerevisiae* will suppress and outgrow the indigenous yeasts and dominate the fermentation. Although this assumption is widely accepted, there is little documented evidence of its validity.

In studies conducted at several Australian wineries, Heard and Fleet (1985) showed that growth of *Kloeckera apiculata* and *Candida* species was not suppressed in fermentations inoculated with commercial strains of *S. cerevisiae*. Similar observations have been reported by Martinez *et al.* (1989). Therefore, it can be concluded that inoculation of grape juice with a high population of *S. cerevisiae* will not necessarily prevent growth of indigenous non-*Saccharomyces* yeasts.

The assumed dominance of inoculated strains of *S. cerevisiae* over indigenous strains of this species, has also been called to question, highlighted by the fact that conventional cultural techniques for identifying wine yeasts do not distinguish between strain types. Using electrophoretic methods to distinguish between strains of *S. cerevisiae*, Bouix *et al.* (1981) showed that the inoculated strain did not always dominate the fermentation. Martinez *et al.* (1989) used hydrogen sulfide production as a marker to differentiate between strains of *S. cerevisiae* and demonstrated that the inoculated strain tended to dominate but that indigenous strains remained in significant numbers throughout the fermentation. Loiseau *et al.* (1987) and Delteil and Aizac (1988) conducted

investigations using genetically marked strains of *S. cerevisiae* that could be followed independently of indigenous strains. They concluded that dominance of the inoculated strain was not always assured and depended on the specific conditions of fermentation (for example, method of inoculation).

In light of these observations, one must conclude that inoculation of any particular strain of *S. cerevisiae* does not necessarily guarantee its dominance or exclusive contribution to the fermentation. Clearly, further studies on the subject of strain dominance are required. An experiment was devised, therefore, to monitor the growth of an inoculated strain under different conditions of fermentation. The use of a genetically marked strain provided the means for accurate identification and quantitation of the inoculated strain throughout the fermentation. Conditions of fermentation to be investigated included production of killer toxin by the inoculated strain, pre-treatment of the must with SO_2, and temperature of fermentation. These conditions of fermentation are discussed briefly below.

7.1.1 Killer yeast inoculation

The subject of killer yeasts has already been addressed in Chapter 6 and therefore will not be discussed here in detail. Several studies have now demonstrated that killer-producing and killer sensitive strains of *S. cerevisiae* may occur as part of the natural flora of wine fermentations (Heard and Fleet, 1987a,b). Laboratory experiments have demonstrated that killer strains can inhibit sensitive strains and become the dominant strain in mixed-culture wine fermentations (Heard and Fleet, 1987a,b; Longo *et al.*, 1990). Two important reasons exist for killer yeasts to be of interest to winemakers. First, they may be responsible for a number of stuck or undesirable fermentations. Inoculated *S. cerevisiae* strains could be destroyed by indigenous killer strains of *S. cerevisiae* or non-*Saccharomyces* species, leading to premature termination of the fermentation, slow fermentation or completion of the fermentation by a less desirable strain. Secondly, there may be some advantage in conducting the fermentation with desired killer strains of *S.*

cerevisiae; the expectation being that the growth of less desired indigenous strains would be supressed. Killer strains of *S. cerevisiae* are now commercially available to winemakers but little evidence exists to assure their activity against indigenous yeasts in any particular winery.

7.1.2 Addition of sulfur dioxide to grape must

Addition of SO_2 to grape juice for the purposes of controlling oxidation and restricting growth of indigenous microflora is a well established practice in winemaking (Beech and Thomas, 1985). In the case of wines produced by natural fermentation, it is considered that the addition of SO_2 will suppress the growth of indigenous non-*Saccharomyces* yeasts and encourage dominance of fermentation by the more SO_2 tolerant strains of *S. cerevisiae* (Ribereau-Gayon *et al.*, 1975). For wines produced by inoculation, it is assumed that added SO_2 will control indigenous non-*Saccharomyces*, as well as indigenous *S. cerevisiae* and encourage fermentation by the inoculated strain. However, experimental evidence supporting these effects of SO_2 on yeast ecology in ferments is scarce. Growth of indigenous yeasts in commercial wine ferments has been found where the usual concentrations of SO_2 (50-100 mg/L) have been added to the juice (Fleet *et al.*, 1984; Heard and Fleet, 1985, 1986a). More specifically, growth of *K. apiculata*, one of the main indigenous yeasts, was not inhibited by total added SO_2 concentrations of 100-150 mg/L (Heard and Fleet, 1988a). These findings contradict the assumption that SO_2 controls indigenous yeasts and challenge one of the reasons for using SO_2 in winemaking.

7.1.3 Temperature of fermentation

Temperature control has become an important practice in modern winemaking. In recent years there has been a trend to ferment white wines in particular, at lower temperatures (10-15°C) to encourage the formation of volatile flavours such as esters and to reduce losses of products, including ethanol, by evaporation (Killian and Ough, 1979;

Kunkee, 1984). The effect of temperature on the ecology and kinetics of fermentation, therefore requires consideration.

Heard and Fleet (1988b) demonstrated that temperature can have a dramatic effect on the ecology of the fermentation. Decreasing the temperature below 20°C substantially increased the contribution of the non-*Saccharomyces* yeasts to the fermentation. *Kloeckera apiculata* and *C. stellata*, for example, remained at high populations (10^7-10^8 cells/ml) throughout the fermentation and in fact, *K. apiculata* replaced *S. cerevisiae* as the dominant yeast. It appeared that some of the indigenous non-*Saccharomyces* species grew faster than *S. cerevisiae* at lower temperatures and, in addition, have enhanced ability to tolerate ethanol (Gao and Fleet, 1988). This change in ecology with fermentation at lower temperature could lead to production of wines with altered chemical and sensory compostion. However, research has not yet been conducted to examine this correlation - further studies of yeast population dynamics at lower temperatures are required.

7.2 RESULTS

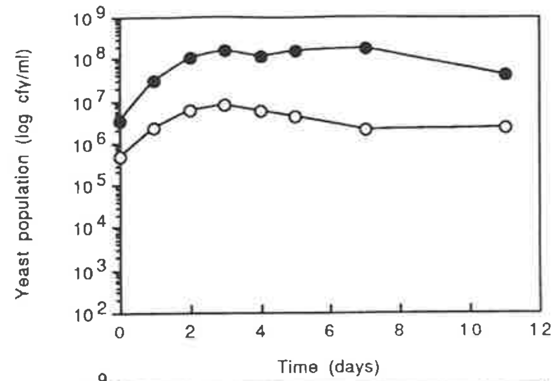
Grape must was collected from St. Hallet's winery in the Barossa Valley. Grapes were crushed and pressed immediately after harvesting, and must was collected directly from the winery press. Details of the must are presented below:

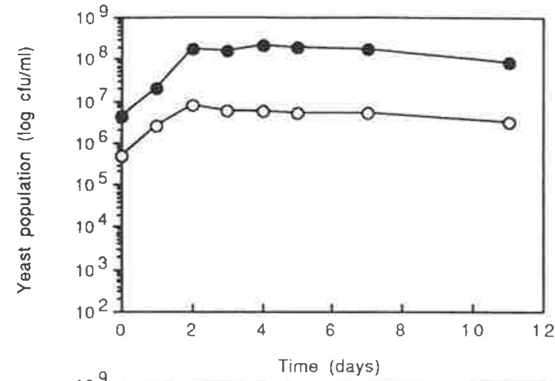
Grape variety:	*Pedro Ximines*
Harvesting Mode:	Mechanical
Date of harvesting:	9th April, 1991
pH of must:	3.4
Glucose + fructose:	229 g/L
Free SO_2:	0 mg/L
Total SO_2:	0 mg/L
Indigenous yeast level:	5 x 10^5 cells/mL

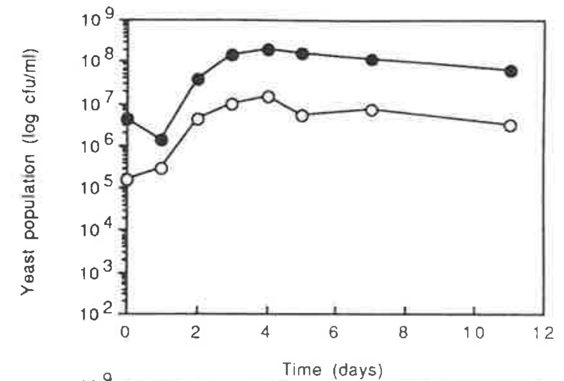
Sample	SO_2 added	Inoculated yeast strain	Temperature of fermentation (^oC)	Time of inoculation (hrs)
1	-	3AMC	20	6
2	-	3AMC	20	30
3	-	3AMC	20	50
4	-	3AM	20	6
5	-	3AM	20	30
6	-	3AM	20	50
7	100 mg/L	3AM	20	6
8	100 mg/L	3AM	20	30
9	100 mg/L	3AM	20	50
10	-	3AM	10	6
11	-	3AM	10	30
12	-	3AM	10	50
13	100 mg/L	3AM	10	6
14	100 mg/L	3AM	10	30
15	100 mg/L	3AM	10	50

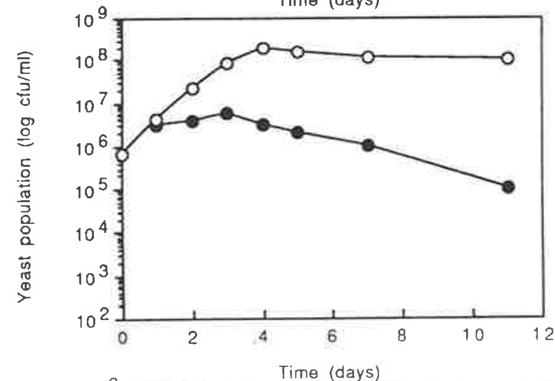
Table 7.1. Conditions under which 15 different fermentations were performed.

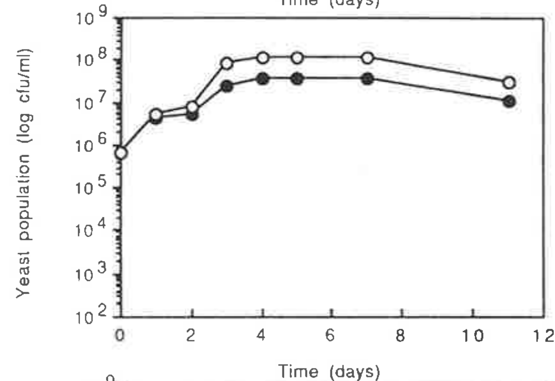
Figure 7.1. Growth curves of the (●) inoculated strain and (O) indigenous population in ferments conducted at 20°C (samples 1 - 9 in Table 7.1).

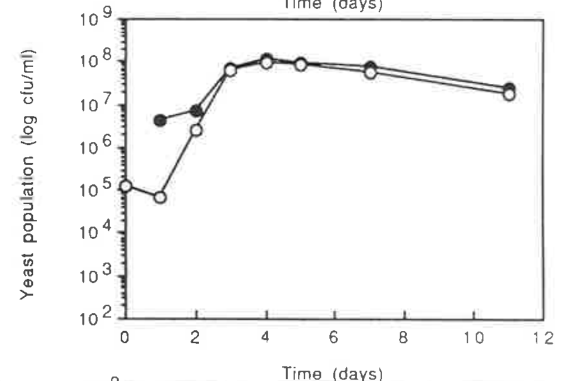

Yeast population (log cfy/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)

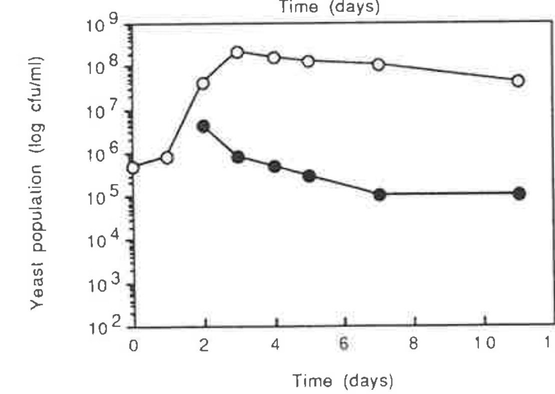

Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)

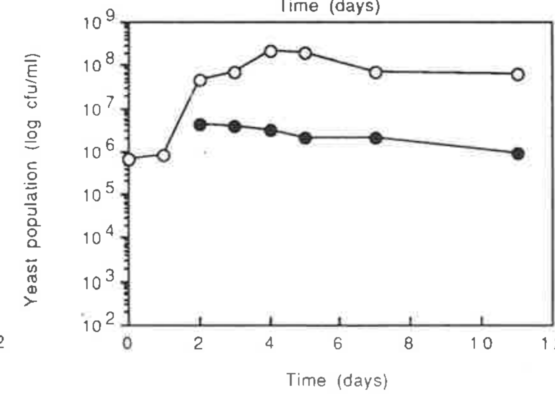

Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)


Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)


Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)


Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)


Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)


Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)

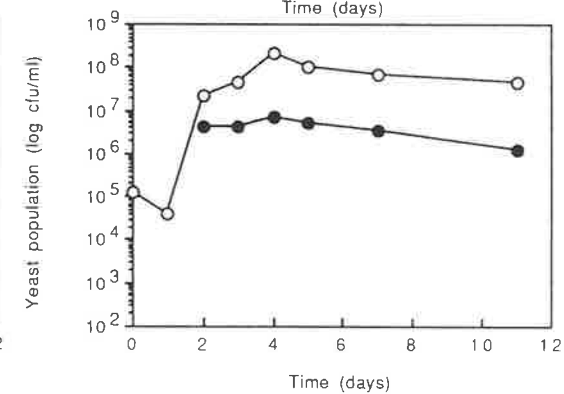
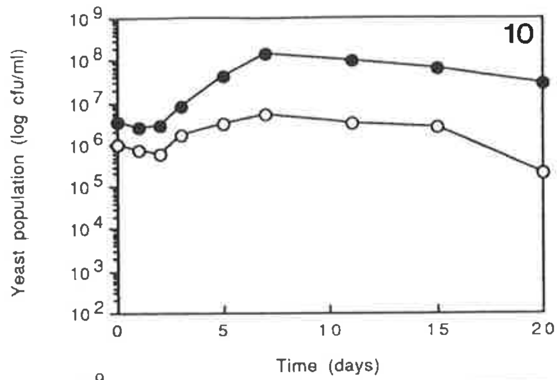
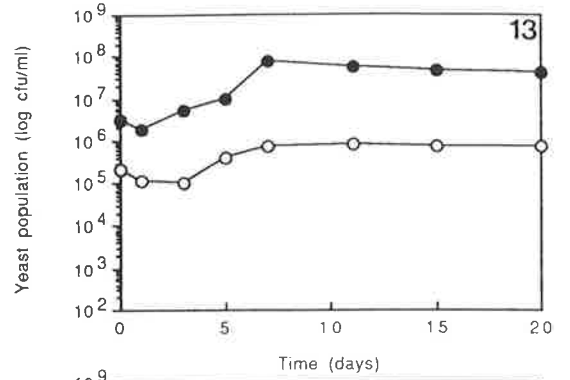
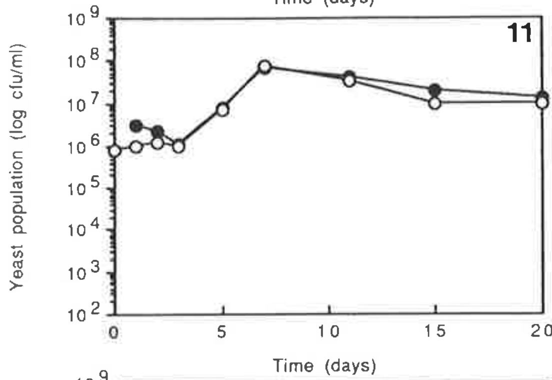
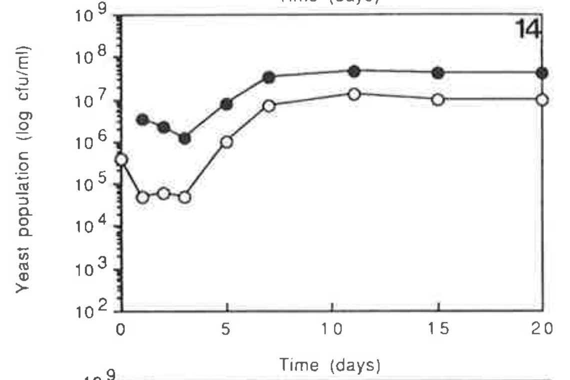

Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
Time (days)

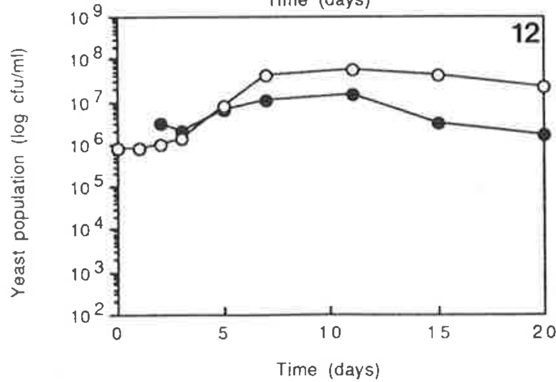
Figure 7.2. Growth curves of the (●) inoculated strain and (O) indigenous population in ferments conducted at 10°C (samples 10-15 in Table 7.1).

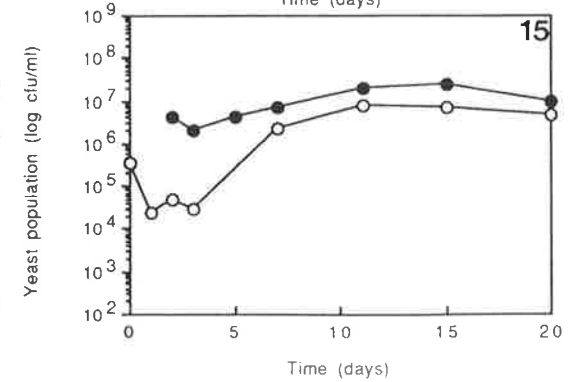

10
Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
5
10
15
20
Time (days)


13
Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
5
10
15
20
Time (days)


11
Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
5
10
15
20
Time (days)


14
Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
5
10
15
20
Time (days)


12
Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
5
10
15
20
Time (days)


15
Yeast population (log cfu/ml)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
0
5
10
15
20
Time (days)

Fifteen must samples (of 20 litre volume each) were fermented under the conditions described in Table 7.1. Aliquots were removed from the centre of the fermentation vessel at regular intervals and plated in duplicate onto YPD media. Resultant colonies were assayed for GUS activity by the agar plate method. The number of blue colonies (representing the inoculated strain) and the number of white colonies (representing the indigenous yeast strains) were counted to determine the proportion of the inoculated strain.

7.2.1 Fermentations conducted at 20ºC

Results of the ferments conducted at 20ºC, which comprise three different sets of conditions, are depicted in Figure 7.1. The effect of killer toxin on the indigenous yeast population in untreated must was analysed. This analysis was achieved by conducting identical ferments, one with killer strain 3AM and one with its cured, sensitive derivative 3AMC. These strains were shown to have similar growth kinetics during fermentation (Chapter 6) and therefore differences observed in these experiments can be attributed to the production of killer toxin. Also, the effect of SO_2 addition to grape must prior to inoculation was investigated. Under each of these fermentation conditions, different times of must inoculation were studied. These times were 6, 30, and 50 hours after grape pressing. Results of each different set of fermentation conditions will be discussed separately.

i) Inoculation with killer sensitive strain 3AMC (samples 1, 2 and 3)

At the inoculation time of 6 hours after pressing (sample 1) indigenous yeasts were present in the must at a level of 5×10^5 cells/ml, and the inoculum was added to 4×10^6 cells/ml. Therefore, strain 3AMC had an eight-fold increase in population size over the indigenous yeasts at the time of inoculation. Strain 3AMC clearly dominated this ferment,

reaching 1.5 x 10^8 cells/ml and accounting for 95% of the population by day three of the ferment.

When inoculation was delayed until 30 hours after pressing of the grapes (sample 2), the indigenous yeast population had reached a cell density of 5 x 10^6 cells/ml just prior to the addition of strain 3AMC. At this inoculation time, then, 3AMC represented only 45% of the total population. Under these conditions, the indigenous yeasts reached a level of 1.9 x 10^8 cells/ml (95% of the population) on day 4 and clearly dominated throughout the ferment. Strain 3AMC reached a peak cell density on day 3 of only 6 x 10^6 cells/ml, and after this point displayed a steady decrease in population size. By day 11 of the ferment it had decreased to 1 x 10^5 cells/ml, and represented less than 1% of the total population.

In the ferment in which inoculation was performed 50 hours after pressing (sample 3), the indigenous yeast population had entered log phase of growth and reached 3.9 x 10^7 cells/ml by the time of inoculation. The indigenous yeasts therefore had a ten-fold higher population density than the inoculated strain and maintained dominance over the ferment. Strain 3AMC displayed a rapid reduction in population size (or death rate) and by day 4 had dropped to 5 x 10^5 cells/ml and accounted for less than 1% of the total population.

ii) Inoculation with the killer strain 3AM (samples 4, 5, and 6)

The kinetics of killer yeast 3AM in untreated must at an inoculation time of 6 hours after pressing (sample 4) was similar to that of strain 3AMC under identical conditions (sample 1). Strain 3AM reached 1 x 10^8 cells by day 2 of the ferment, accounting for 96% of the total population and clearly dominated throughout the ferment.

At inoculation times of 30 and 50 hours after grape presssing, 3AM was dominated by the indigenous yeast population. However it displayed different growth kinetics to 3AMC. In the 30 hour inoculum (sample 5), 3AM reached a density of 4 x 10^7 cells/ml and

represented 25% of the total population. A fairly constant population size was maintained by 3AM throughout this ferment, and by day 11 it represented 35% of the total population, compared to less than 1% represented by strain 3AMC at the same time.

In the ferment inoculated 50 hours after pressing (sample 6), strain 3AM accounted for only 15% of the total population at the time of inoculation, and was strongly dominated by the indigenous yeasts throughout the ferment. However, it differed from strain 3AMC in that a constant cell density (between 1 and 4 x 10^6 cells/ml) was maintained after inoculation. No dramatic reduction in population size of strain 3AM was evident in this ferment, although growth of the strain was clearly suppressed.

iii) Inoculation with 3AM of must pre-treated with sulfur dioxide (samples 7, 8, and 9)

Addition of SO_2 to the grape must had the expected effect of reducing the indigenous yeast population. Four hours after the addition of SO_2 (and 6 hours after grape pressing), the indigenous yeast level had dropped from 5 x 10^5 cells/ml to 1 x 10^5 cells/ml. At this time the first inoculation of 3AM was performed (sample 7) and a concomitant reduction in the inoculum population occurred. The population density of 3AM dropped from 4 x 10^6 cells/ml at the time of inoculation to 1 x 10^6 cells/ml on day one of the ferment, at which point the inoculum represented 82% of the total population. After day one, both the inoculated strain and the indigenous population had recovered from the SO_2 treatment, and entered log phase of growth. By day four 3AM had reached 2 x 10^8 cells/ml (accounting for 93% of the total population) and clearly dominated the ferment. At this time of inoculation, the addition of SO_2 reduced the efficiency of the inoculum in attaining dominance of the ferment when compared to the untreated must sample (sample 4).

In ferments conducted with an inoculation time of 30 hours after pressing, the addition of SO_2 to the must increased the inoculation efficiency. Under these conditions

(sample 8), the level of inoculated strain was approximately 10-fold higher than the indigenous yeasts at the time of inoculation. However, the indigenous population entered log phase of growth before the inoculated strain and by day three had reached a cell density of 7 x 10^7 cells/ml - approximately equal to that of strain 3AM. From this point of the ferment onwards, strain 3AM was present as approximately 55% of the total population and therefore only narrowly dominated the fermentation. This contrasts to the ferment in untreated must with the same inoculation time (sample 5), in which the indigenous yeast population was dominant.

There was little difference in the kinetics of strain 3AM in both untreated (sample 6) and SO_2 (sample 9) treated must with an inoculation time of 50 hours after pressing. In both cases the indigenous population had reached 2 x 10^8 cells/ml and accounted for 97% of the ferment by day 4 of the ferment. Strain 3AM remained at a steady cell density throughout both of these ferments, with a population of 2 x 10^6 cells/ml by day seven.

7.2.2 Fermentations conducted at 10ºC

Two conditions were compared at a fermentation temperature of 10ºC: no treatment of must prior to inoculation; and treatment with SO_2 (100 mg/L) prior to inoculation. Both conditions were investigated with the marked strain 3AM and are presented graphically in Figure 7.2.

i) Inoculation of untreated must

In the untreated must inoculated 6 hours after pressing (sample 10), the indigenous population and strain 3AM exhibited a two day lag phase, during which 3AM was present as 78% of the total population. By day 7, 3AM peaked at 1 x 10^8 cells/ml and accounted for 96% of the total number of yeast cells. At this time of inoculation, strain 3AM exerted a similar dominance over the ferment as it did in the 20ºC ferment (sample 4).

With an inoculation time of 30 hours after pressing (sample 11) both indigenous yeasts and 3AM showed a lag phase up to day 3, at which point strain 3AM comprised approximately 50% of the total population. On day 7 of the ferment, 3AM reached a peak of 7×10^9 cells/ml and at this point was 48% of the total population. Strain 3AM accounted for approximately 50% of the total population throughout the ferment and therefore inoculation efficiency under these conditions was greater at 10°C than it was in the ferment conducted at 20°C (sample 5).

The indigenous yeast population dominated the untreated must ferment when inoculation of 3AM was performed 50 hours after grape presssing. At the time of inoculation, the indigenous yeast population had reached 9.5×10^5 cells/ml and strain 3AM accounted for 77% of the total yeast population. However, the indigenous yeast population entered log phase of growth before the inoculated strain and by day seven had a density of 4×10^7 cells/ml and represented 79% of the total population. The dominance of the indigenous yeast population was not as great under these conditions as it was in the 20°C ferment (sample 6).

ii) Inoculation of sulfur dioxide treated must

Strain 3AM clearly dominated the ferment when the inoculum was added to the SO_2 treated must 6 hours after grape pressing (sample 13). In contrast to the 20°C ferment inoculated at this time (sample 7), pre-treatment of SO_2 increased the dominance of 3AM. The lower temperature slowed recovery of the indigenous population after addition of SO_2, allowing strain 3AM to enter log phase growth without competition. From day 1 onwards, strain 3AM accounted for greater than 93% of the total population and reached a maximum cell density of 9×10^7 cells/ml (98% of the total population) by day seven.

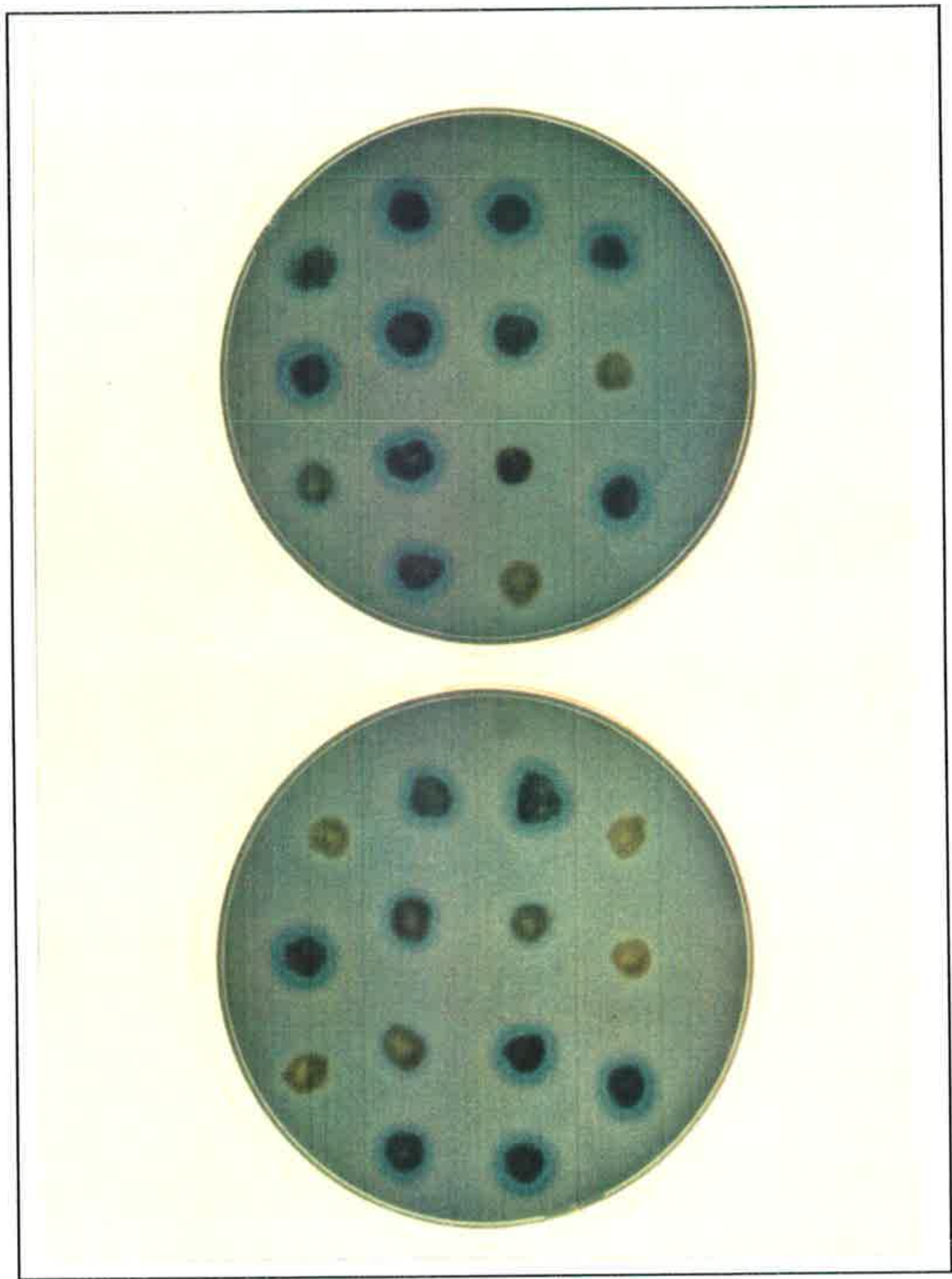


Figure 7.3. Killer assays performed on indigenous yeast colonies isolated on day seven of the fermentation.

When inoculation was performed 30 hours after pressing (sample 14), the level of indigenous yeasts had dropped below 10^5 cells/ml and 3AM dominated the ferment as it comprised approximately 80% of the total population for the duration of the fermentation. At this time of inoculation, strain 3AM achieved greater dominance of the SO_2 treated must at 10°C than it did in the 20°C ferment (sample 8).

When inoculation was delayed for 50 hours after pressing (sample 15), strain 3AM was still able to dominate the ferment in SO_2 treated must at 10°C. At the time of inoculation, strain 3AM accounted for 97% of the total population. The indigenous population, however, recovered from the SO_2 treatment and entered log phase of growth after day 3. From day 5 onwards, strain 3AM represented approximately 70% of the total population. The efficiency of inoculation under these conditions is markedly improved at a temperature of 10°C, compared to similar conditions at 20°C (sample 9) in which the indigenous yeast population was dominant.

7.2.3 Killer activity in the indigenous yeast population

In order to interpret the role of killer activity in the 20°C ferments, killer assays were performed on a number of GUS negative colonies (indigenous yeasts) isolated from SO_2 treated and non-treated ferments. These indigenous yeasts were isolated from samples taken on day seven from ferments No. 2 (non-treated must) and 9 (SO_2 treated must). Assays were performed by testing their ability to kill strain 3AMC and results are presented in Figure 7.3. Twenty eight indigenous yeast colonies were assayed, and of these, twenty (71%) displayed distinct killer activity against strain 3AMC.

7.3 DISCUSSION

General trends have been observed in inoculation efficiency under different conditions. Results presented here clearly demonstrate the importance of the time of

inoculation in ensuring dominance of the ferment by the inoculated strain. Under all conditions examined, the inoculated strain clearly dominated the ferment (accounting for > 90% of the total population by day three) when it was added to the must within six hours of pressing. These results are to be expected, as a delay in inoculation time allows the indigenous population to increase in cell number. Consequently, the indigenous population is more competitive at the time of inoculation.

The killer (3AM) and killer sensitive (3AMC) strains both achieved a similar dominance of the ferment with an inoculation time of six hours after pressing. This result suggests that the greater population size of the selected strain at the time of inoculation is sufficient alone to ensure dominance of the fermentation and that the production of killer toxin does not play an important role under these conditions. It should be noted that the indigenous population did not display a reduction in cell density in the presence of the killer strain. Therefore, it appears that the indigenous yeast cells are being suppressed rather than actively killed by strain 3AM. This observation can be explained by the fact that 75% of the indigenous yeasts isolated from the ferments on day seven displayed killer activity (see Figure 7.3) and therefore are immune to the killer toxin.

Differences were observed, however, between the growth kinetics of the killer and sensitive strains when inoculation was performed at 30 and 50 hours after pressing. Under these conditions, in which the indigenous population was dominant, the sensitive strain showed a marked decrease in population density and appeared to be killed by the indigenous yeasts. The killer strain, however, displayed either an increase in growth (after 30 hour delay in inoculation), or maintained a constant cell density (after a 50 hour delay in inoculation). In interpreting these observations, it is again important to remember that 75% of the indigenous yeasts on day seven displayed killer activity. This killer activity of the indigenous yeasts explains the cell death displayed by the sensitive strain 3AMC in the ferments inoculated 30 and 50 hours after pressing. The fact that killer activity was not evident against strain 3AMC in the ferment inoculated after six hours (sample 1) can be

explained by results obtained in Chapter 6. These results showed that the efficiency of killer activity was dependent on the proportion of killer to sensitive cells in the ferment. When killer cells are present as less than 50% of the population, as they were at inoculation time and throughout this ferment, killer activity was not detected.

The finding that 71% of the indigenous yeast colonies tested displayed killer activity is somewhat surprising considering that Heard and Fleet (1987a) identified only 9 killer yeast from a total of 61 *S. cerevisiae* and 36 non-*Saccharomyces* strains isolated from Australian wineries. However, when one considers the probable species and origin of the colonies tested, this result is perhaps not unexpected. First, it is likely that the colonies tested were strains of *S. cerevisiae*. The colonies were isolated on day seven of 20°C ferments and, invariably, *S. cerevisiae* is the only species isolated from fermenting wine after the first three or four days (Kunkee and Amerine, 1970; Fleet, 1990). Secondly, it is likely that the origin of *S. cerevisiae* strains in wine is the winery and its equipment (Martini and Martini, 1990). Killer strains of *S. cerevisiae* are currently popular choices for use as starter cultures in the Australian wine industry. Of 57 dried wine yeasts currently available to the Australian industry, 21 are killer strains (Henschke, 1990). It is likely therefore, that killer yeasts will be increasingly colonising equipment of Australian wineries and contributing to the indigenous yeast population of freshly extracted grape juice.

The addition of SO_2 had the expected effect of reducing the indigenous yeast population in the grape must of ferments conducted at 20°C. However, when inoculation was performed within six hours of pressing, and four hours after the addition of SO_2, the inoculated yeast strain was also reduced by the added preservative. The pre-treatment of the must with SO_2 did not increase the efficiency of inoculation in this case.

When the inoculum was added 30 hours after pressing, the addition of SO_2 was effective in improving the efficiency of the inoculated strain at 20°C. In this case, the indigenous yeast population was decreased while the inoculated strain did not suffer an

initial reduction in population. However, by 50 hours after pressing, the indigenous population had recovered from the SO_2 treatment, entered log phase of growth and reached a high cell density (2 x 10^7 cells/ml). The inoculated strain was then easily dominated by the indigenous yeasts. Therefore, one can conclude that the addition of SO_2 was not an effective sterilisation process in this case.

The different efficiencies of SO_2 treatment with respect to time of inoculation can be understood by considering the mechanism of SO_2 action on yeast cells. In solution, SO_2 undergoes dissociation to form bisulfite and sulfite ions. The antimicrobial activity is due to penetration of yeast cell membranes by the free, undissociated form of SO_2 and its subsequent inactivation of intracellular constituents (Schmiz, 1980). In the cell membrane, the SO_2 activates ATPase causing a decrease in the ATP concentration. Also, once the molecular SO_2 has entered the cell, it dissociates, because of the pH difference between the grape juice and the cell, and becomes trapped. The combined effects of ATP depletion and the activity of sulfite and bisulfite ions inside the cell lead to cell inactivation and death (Schmiz, 1980; Beech and Thomas, 1985; Stratford and Rose, 1985).

It should also be noted that SO_2 can be bound to grape juice constituents or fermentation products, thus reducing its efficacy as an antimicrobial agent. Binding of SO_2 by grape juice constituents, principally sugars, has been reported by Blouin (1966) and Rankine (1966). The main fermentation products that bind with SO_2 are acetaldehyde, pyruvic acid and 2-ketoglutaric acid and to a lesser extent, galacturonic acid, sugars and anthocyanins. This phenomenon has been reviewed by Beech *et al.* (1979), Beech and Thomas (1985) and Lafon-Lafourcade (1985). The strongest bonding of SO_2 is formed with acetaldehyde. For 50 mg/l of free SO_2, 99% is bound with acetaldehyde in the wine (Lafon-Lafourcade, 1985) and the resulting sulfonate has little inhibitory effect against yeasts (Usseglio-Tomasset *et al.*, 1981; Beech and Thomas, 1985). Because the dissociation constant (1.4 x 10^{-6} to 1.5 x 10^{-6}) of this reaction in wine is high (Rankine,

1966; Burroughs and Sparks, 1973), the binding reaction is not easily reversed and the SO_2 bound by the acetaldehyde is effectively removed from inhibitory action (Rankine, 1966).

Molecular SO_2 in the musts of these ferments then, would either enter the yeast cells and then dissociate, causing death; or become effectively irreversibly bound to constituents in the juice. The result of these processses would be a reduction in the amount of available SO_2 in the ferment over time. This reduction in available molecular SO_2 explains the decrease in efficacy of SO_2 treatment over the three different times of inoculation.

The lower fermentation temperature of 10^oC substantially enhanced the efficiency of SO_2 in its action of suppressing indigenous yeast strains. Even at inoculation times of 30 and 50 hours after pressing, the inoculated strain was able to dominate the ferment although the degree of dominance decreased over time. The increase in antimicrobial activity of SO_2 at 10^oC can be explained by the fact that the lower temperature decreases cell growth and production rate of fermentation products such as acetaldehyde. Therefore the level of available molecular SO_2 (for diffusion into cells of the indigenous yeasts) would be higher over the first few days of the fermentation at 10^oC than at 20^oC.

The direct significance of results presented in this chapter to the wine industry is difficult to assess, as procedures such as cold settling and juice clarification were not conducted prior to inoculation. These procedures are likely to have a major impact on the populations of indigenous yeasts in the juice. The extent of these influences are unclear and require study. Some growth of indigenous yeasts may be expected during settling - the types of species that grow and their rate of growth will depend on the temperature and other conditions.

However, conclusions have beeen drawn from these results regarding the efficiency of inoculation in the presence of different levels of indigenous yeast species. Most importantly, perhaps, this chapter highlights the suitability of the genetically marked strain

in conducting investigations of yeast ecology during fermentation. Very few studies of this type have been conducted due to the difficulties with strain identification. The marked strain provides the means for further studies into, for example, the impact of winery processes such as cold settling and juice clarification on indigenous yeast populations.

Chapter 8 General Conclusions

Results obtained during the course of this project have demonstrated the potential application of molecular genetic yeast manipulations to the wine industry. A procedure which enables the introduction of new genetic material to wine yeasts has been described and, subsequently, employed in the development of a genetic marking system for wine yeast strains. This system utilises the *E.coli* β-glucuronidase (GUS) gene as a marker and, unlike previous marking systems described to date, enables a wide range of wine yeast strains to be readily marked. Furthermore, methods have been developed whereby GUS activity can be detected in single cells or colonies within a two to four hour period. The GUS marking system, therefore, provides a means for rapid and unequivocal identification and monitoring of yeast strains during fermentation.

Marked strains have potential application in monitoring the efficiency of various aspects of wine production on the indigenous yeast load. These aspects include yeast propagation, the preparation of pure starter cultures and prefermentation processes. Marked strains can also play an important role in oenological studies aimed to increase our knowledge and understanding of yeast kinetics, ultimately allowing the optimization of fermentation conditions. This potential role of marked strains has been demonstrated in two separate oenological studies conducted during this project. First, the activity of killer toxin in fermenting grape juice was assessed. Second, an analysis of inoculation efficiency under various fermentation conditions was performed.

In conclusion, this project has three distinct components: the establisment of a wine yeast transformation procedure; use of this transformation procedure in the development of a genetic marking system; and demonstration of the practicality and application of marked strains to the wine industry.

Results described in this thesis also have broader implications. It has been demonstrated that a foreign gene can be stably introduced and expressed at the *ILV2* site in the genome of wine yeast strains without adversely affecting fermentation performance. The *SMR1-410* gene is, therefore, both an appropriate selectable marker and target site for the integration of foreign DNA into wine yeasts. As the *SMR1-410* gene is derived from yeast, it would be possible to construct integrating vectors devoid of bacterial sequences. This would be an important development in facilitating the acceptance of recombinant yeasts in food and beverage production. The challenge now is to define specific targets in the wine-making process to which this technology can be applied.

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Appendix 1 Publications

Petering J., Langridge, P. & Henshke, P. (1988). Fingerprinting wine yeasts: the application of chromosome electrophoresis. *Australian & New Zealand Wine Industry Journal, 3*(3), 48-52.

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Determination of Killer Yeast Activity in Fermenting Grape Juice by Using a Marked *Saccharomyces* Wine Yeast Strain

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The *Escherichia coli* β-glucuronidase gene has been used as a marker gene to monitor a killer *Saccharomyces cerevisiae* strain in mixed-culture ferments. The marked killer strain was cured of its M-dsRNA genome to enable direct assessment of the efficiency of killer toxin under fermentation conditions. Killer activity was clearly evident in fermenting Rhine Riesling grape juice of pH 3.1 at 18°C, but the extent of killing depended on the proportion of killer to sensitive cells at the time of inoculation. Killer activity was detected only when the ratio of killer to sensitive cells exceeded 1:2. At the highest ratio of killer to sensitive cells tested (2:1), complete elimination of sensitive cells was not achieved.

Killer activity in yeasts was first reported for strains of *Saccharomyces cerevisiae* in 1963 by Bevan and Makower (2). Killer yeasts secrete polypeptide toxins which kill sensitive strains of the same genus and, less frequently, strains of different genera (20, 30). Previous studies indicate that the toxin of *Saccharomyces cerevisiae* is a protein which binds to a receptor on the wall of the sensitive yeast cell, disrupting the electrochemical gradient across the cell membrane and hence the intracellular ionic balance (6, 28).

Production of the toxin and immunity to it are determined by a cytoplasmically inherited double-stranded (ds) RNA plasmid, otherwise known as the M genome (4). The M-dsRNA killer plasmids are dependent satellites of L-A-dsRNA, and L-BC-dsRNA exists as a species unrelated to the first or to M. All types of dsRNA exist in virus-like particles and require a protein encoded by the L-A-dsRNA for encapsidation (4, 12, 30).

On the basis of the properties of the toxin, killer yeasts have been classified into 11 groups (K_1 through K_{11}) (18, 33). Those unique to *Saccharomyces* strains fall into the first three groups (K_1, K_2, and K_3). The *Saccharomyces* toxin is reversibly inactivated at low pH (2.0) and irreversibly inactivated at pH in excess of 5.0 (33). More specifically, the biological activity of K_1 is optimal between pH 4.6 and 4.8, while K_2 shows optimal activity between pH 4.2 and 4.7 (27). The K_2 toxin is stable over a wider pH range than the K_1 toxin (2.8 to 4.8) (23) and is therefore more relevant in wine fermentation.

Killer activity has been detected in yeasts isolated from established vineyards and wineries in various regions of the world, including Europe and Russia (1, 9, 18), South Africa (31), and Australia (14, 15). This widespread occurrence has prompted interest in the enological significance of killer wine yeasts. In theory, selected killer yeast strains could be used as the inoculated strain to suppress the growth of undesirable wild strains of *S. cerevisiae* during grape juice fermentation. In addition, as killer interactions have been reported to occur between yeasts of different genera (21, 25), the possibility of genetically engineering broad-spectrum killer strains of *S. cerevisiae* exists (3).

Studies have been conducted to assess the efficiency of killer toxin on sensitive yeast strains. However, reports on the expression of killer activity under fermentation conditions have been contradictory (5, 7, 16). Attempts to determine the population kinetics of killer and sensitive strains during wine fermentation have been restricted because of the difficulty involved in identifying the two types when they are grown in mixed cultures. Approaches used to date include (i) choice of killer and sensitive strains that can be distinguished by their growth rates (1) or their production of hydrogen sulfide (24), (ii) use of auxotrophic and respiratory-deficient mutants of killer strains and appropriate plating conditions under which they can be identified (10, 11, 26), (iii) use of killer and sensitive strains which can be distinguished by differences in colony morphology (14), and (iv) assaying colonies directly for killer activity (17). All of these methods are limited by the fact that the assays involved are laborious and time-consuming or that only killer strains with specific characteristics can be studied.

We describe here the use of a marked *S. cerevisiae* killer strain in a mixed-culture inoculum to quantify directly the effect of killer toxin on a sensitive *S. cerevisiae* strain under fermentation conditions. As a wide range of yeast strains can be readily and stably marked, this system of analysis is unlimited in application and provides a simple and unequivocal means of quantifying killer yeast strains in mixed-culture ferments.

MATERIALS AND METHODS

Strains and media. Sensitive *S. cerevisiae* strains 5A (AWRI 138) and 2A (AWRI 729) and killer (K_2) strain 11A (AWRI 92F) were obtained from the Australian Wine Research Institute. Generation of the marked killer (K_2) strain 3AM (AWRI 796) has been previously described (19). Yeast growth medium was YPD (1% yeast extract [Difco], 2% Bacto Peptone [Difco] and 2% glucose).

Curing of killer strain 3AM. A culture of strain 3AM was grown overnight in YPD at 28°C. Serial dilutions were made in 0.9% NaCl, and 0.1-ml aliquots (containing approximately 100 cells) were spread on YPD plates and incubated at 37°C.

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After 48 h of incubation, single colonies were selected at random and assayed for killer activity as described below.

Assay for cured strain. YPD (containing 1% agar) was autoclaved at 120°C for 20 min. After cooling to 49°C, the medium was buffered to pH 4.2 with a 10% tartrate solution. Methylene blue (to 0.003% [wt/vol]) and killer-sensitive strain 5A (to 10^5 cells per ml) were added to the medium before the medium was poured into the plates. Colonies isolated after heat treatment were then transferred to these assay plates and incubated at 18°C for approximately 72 h. Curing was recognized by the absence of growth inhibition (clear zones) and the lack of blue-stained cells around the colony.

dsRNA isolation. The dsRNA extraction procedure was essentially that described by Fried and Fink (8). Samples of RNA were analyzed by electrophoresis on 1.5% agarose slab gels at a constant current of 100 mA. Gels were stained with ethidium bromide and photographed on a shortwave UV light box.

Fermentation trials. Starter cultures were prepared by inoculating 10 ml of YPD medium contained in a conical flask with a loopful of yeast and incubated with vigorous aeration at 28°C. After 24 h, the cell density was determined by microscopic counts. Samples were used to inoculate Rhine Riesling must (200 ml) to a density of 5×10^6 cells per ml. The must contained 220 g of reducing sugars per liter and had a pH of 3.1. Fermentations were carried out in 250-ml conical flasks fitted with airlocks. The juice was sterilized by membrane filtration (0.45-μM pore size) prior to inoculation, and fermentations were carried out at 18°C with agitation (approximately 100 oscillations per min). Samples were removed anaerobically and aseptically during fermentation by needle and syringe through ports covered with rubber septa.

Samples were analyzed for the progress of fermentation by refractometer readings, and yeast growth was measured spectrophotometrically at 650 nm. For analysis of the proportion of marked strain in the yeast population, serial dilutions of the samples were made in sterile 0.9% NaCl, and 0.1-ml aliquots (containing 200 to 500 cells) were plated on YPD media. The plates were then assayed as described below.

β-Glucuronidase (GUS) plate assays. Yeast colonies were grown on solid YPD medium for approximately 36 h at 28°C. A solution containing 0.1 M Na_2HPO_4 (pH 7.0), 1% sarcosyl, 5-bromo-4-chloro-3-indolyl glucuronide (100 to 150 μg/ml), and 0.7% agarose was then poured as a thin overlay on the plate and allowed to set. After 4 to 6 h of incubation at 37°C, a blue precipitate could be detected in the marked colonies.

RESULTS

Curing of strain 3AM. In order to specifically analyze the effect of killer toxin in fermentations, an experiment was designed to compare two isogenic strains which differ only in the presence of the M-dsRNA genome and therefore in their ability to produce killer toxin.

Killer strain 3AM has previously been marked with the *Escherichia coli* GUS gene (19). This system allows the marked strain to be readily identified in a mixed population by a simple plate assay, which results in the formation of a blue precipitate in marked colonies. Strain 3AM was cured of its M-dsRNA plasmid by heat treatment (32), the cured or sensitive colonies being identified by killer activity plate assays. Figure 1 shows the response of strain 3AM and an isolated cured derivative (designated 3AMC) to the killer activity plate assay. The zone of inhibition evident around strain 3AM is absent around 3AMC, indicating that strain 3AMC is not producing killer toxin. GUS activity was detected in strain 3AMC by the agar plate method (results not shown), indicating that the cured strain is an authentic derivative of strain 3AM.

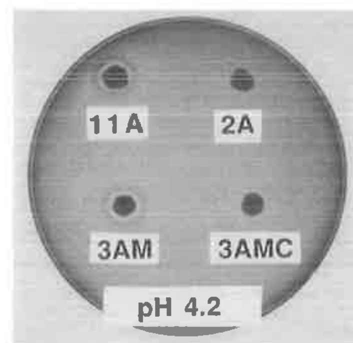

FIG. 1. Agar plate assay for killer activity. The agar (pH 4.2, 0.003% methylene blue) is seeded with an overnight culture of strain 3AMC, and strains to be tested for killer activity are patched onto the solid media. 11A is a known killer strain, and 2A is a known sensitive strain. Strain 3AM displays a response identical to that of killer 11A, with a clear zone and methylene blue-stained border around the patch of growth.

Finally, dsRNA species were isolated from strains 3AM and 3AMC and analyzed by standard electrophoresis techniques (Fig. 2). A band representing the M-dsRNA genome is present in strain 3AM and absent in strain 3AMC.

Fermentation trials were then performed with strains 3AM and 3AMC to determine the effect of the curing procedure on yeast growth and fermentation rates. Starter cultures of each strain were inoculated in triplicate into flasks of Rhine

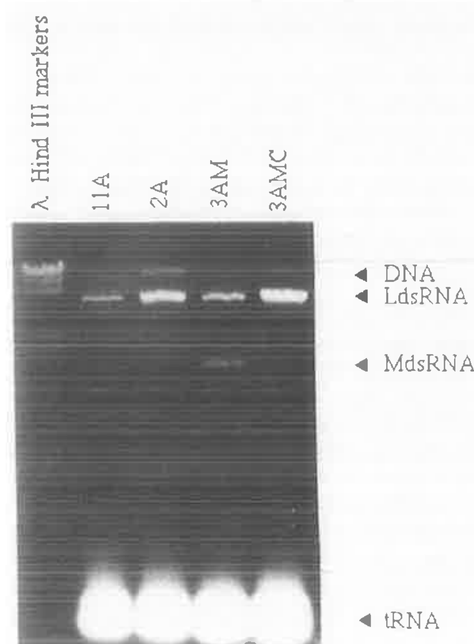

FIG. 2. Electrophoresis of dsRNA species from killer strains 11A and 3AM and sensitive strains 2A and 3AMC. Contaminating DNA and tRNA species are also present.

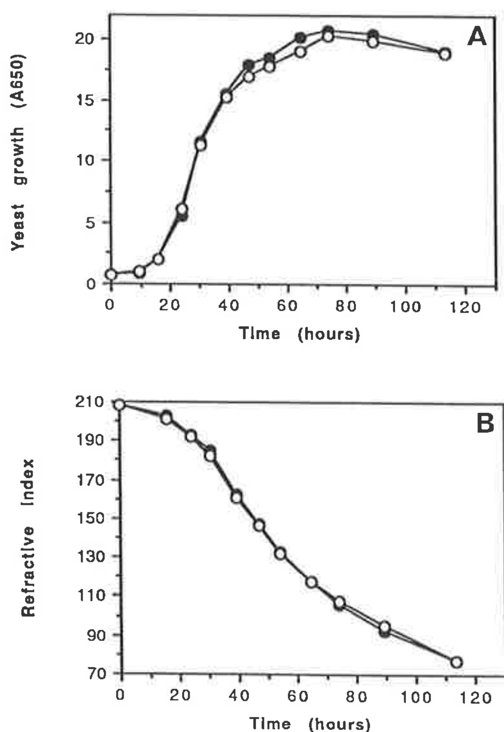

FIG. 3. Yeast growth (A) and sugar utilization (B) curves of strains 3AM (●) and 3AMC (○).

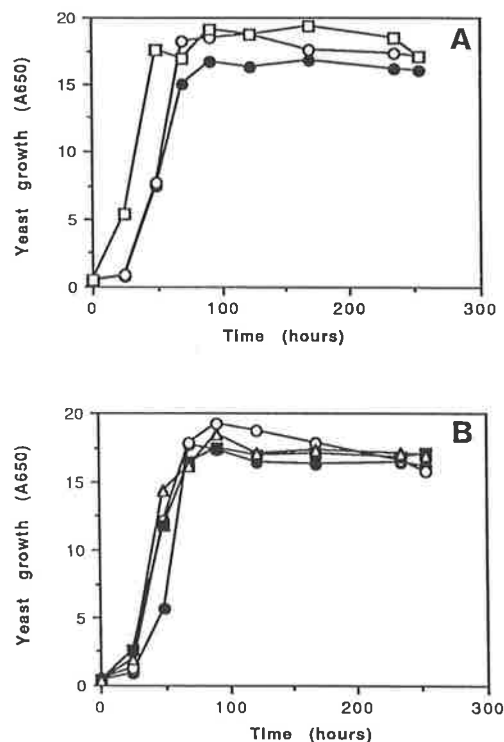

FIG. 4. (A) Growth curves of control single monoculture ferments. Symbols: ●, 3AM; ○, 3AMC; □, 5A. (B) Growth curves of mixed-culture ferments. Symbols: ●, 3AM and 5A at an inoculum ratio of 2:1; ○, 3AMC and 5A at an inoculum ratio of 2:1; ■, 3AM and 5A at an inoculum ratio of 1:1; △, 3AMC and 5A at an inoculum ratio of 1:1.

Riesling grape juice at a concentration of 5×10^6 cells per ml. Samples were taken at regular intervals and assayed for yeast growth and progress of fermentation. The average readings for each strain were plotted over time (Fig. 3). There are no significant differences in the growth or fermentation rates between strains 3AM and 3AMC.

Analysis of killer activity during fermentation. Strains 3AM and 3AMC were analyzed for killer activity in Rhine Riesling juice by coinoculating each strain with the sensitive *S. cerevisiae* strain 5A. Control ferments of each strain (3AM, 3AMC, and 5A) as pure inocula were also performed. Each ferment was conducted in duplicate at 18°C with gentle agitation under anaerobic conditions. GUS plate assays were then performed to identify the marked strain (3AM or 3AMC). Colonies of the marked strain turn a deep blue color as a result of this assay, allowing simple identification.

GUS plate assays were also performed on the control ferments to confirm the validity of the assay. Plate assays on the control 5A ferment were consistently negative, highlighting the absence of background GUS activity in natural yeast cells. However, control 3AM and 3AMC ferments gave values of between 99 and 100% of total colonies per plate for the marked strain count. This observation represents a reversion frequency of less than 1% for the GUS gene.

The following mixed-culture ferments were carried out: (i) 3AM and 5A at an inoculum ratio of 1:1, (ii) 3AMC and 5A at an inoculum ratio of 1:1, (iii) 3AM and 5A at an inoculum ratio of 2:1, and (iv) 3AMC and 5A at an inoculum ratio of 2:1. These mixed ferments exhibited normal growth kinetics, as did the three control ferments (Fig. 4).

The time course of growth (CFU per milliliter) of each strain in the mixed-culture ferments is plotted in Fig. 5. At an inoculum ratio of 1:1, there was a notable increase in the proportion of killer strain 3AM, whereas the cured strain 3AMC failed to exert any dominance over the sensitive strain under otherwise identical conditions. Statistical analysis was used to test the null hypothesis that the ratio of killer to sensitive cells remains 1:1 throughout the ferment. A goodness of fit test (normal test) rejected the null hypothesis, with $P \ll 0.001$. However, identical analysis of the cured to sensitive strain ferment ratio accepted the null hypothesis that the ratio of the two strains remains at 1:1 throughout the ferment. With an increased proportion of strain 3AM in the inoculum (ratio 2:1), the dominating effect of strain 3AM was more pronounced. It is important to note that strain 5A persisted, albeit at low levels, throughout the ferments.

Experiments were conducted to determine the lowest inoculum ratio of killer to sensitive cells at which significant killer activity can be observed. Mixed ferments of strain 3AM and 5A at inoculum ratios of 1:2 and 1:4, respectively, were carried out under the conditions described above. No change from the initial proportion of strain 3AM was detected in either of these ferments. The results of all mixed-culture ferments involving strain 3AM are summarized in Fig. 6.

DISCUSSION

Previous studies have indicated 100% stability of the GUS marker gene in strain 3A throughout fermentation (19). However, analysis of a larger sample of colonies in these experiments has revealed an instability of the construct. This instability was detected in the control fermentations which were inoculated with monocultures of either the marked

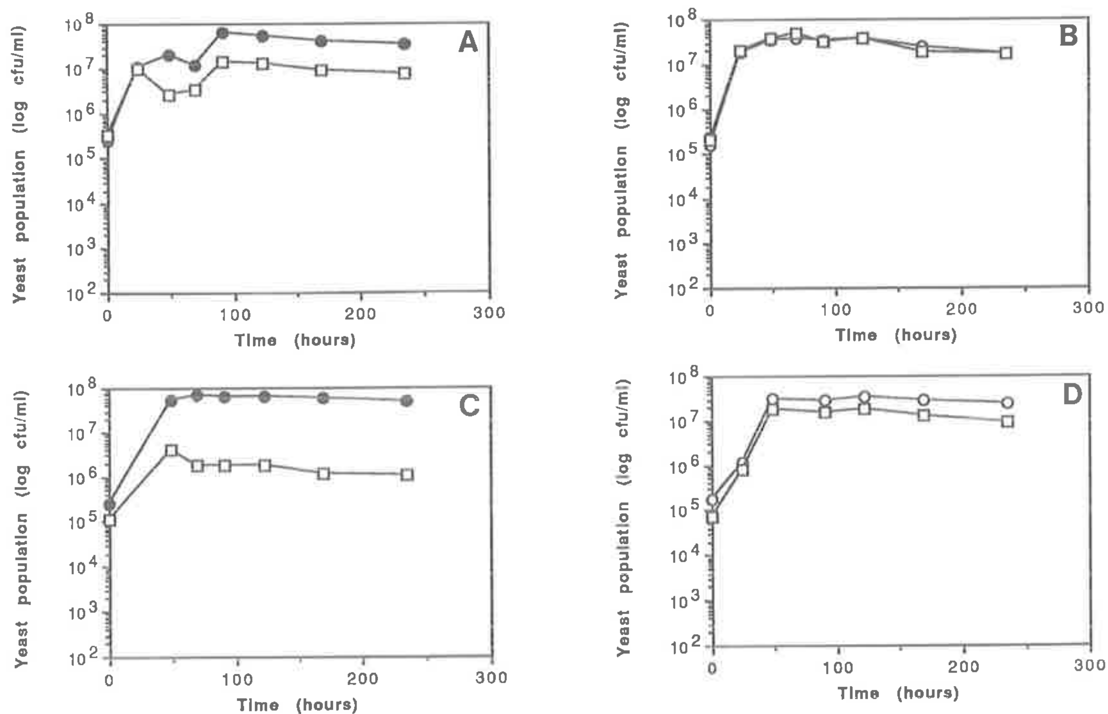

FIG. 5. Growth curves of each strain in mixed-culture ferments expressed as CFU per milliliter. (A) Mixed ferment of 3AM (●) and 5A (□) at an inoculum ratio of 1:1. (B) Mixed ferment of 3AMC (○) and 5A (□) at an inoculum ratio of 1:1. (C) Mixed ferment of 3AM (●) and 5A (□) at an inoculum ratio of 2:1. (D) Mixed ferment of 3AMC (○) and 5A (□) at an inoculum ratio of 2:1.

strain 3AM or 3AMC. Samples from these fermentations gave rise to colonies which responded negatively to the GUS plate assay at a frequency of less than 1% of the total plate count. Occasionally, a colony which was sectored in its response to the assay was detected, suggesting either excision of the gene by homologous recombination (29) or loss of the gene after mitotic crossing-over (22). The frequency of instability did not increase over time during fermentation and could be directly quantified in the control 3AM and 3AMC ferments.

This marking system has enabled a direct comparison to be made between the inoculation efficiency of a killer strain (3AM) and an isogenic cured derivative (strain 3AMC) in fermenting grape juice. At a ratio of killer to sensitive cells of 1:1, the cured strain, 3AMC, remained at 50% of the total population, while the killer strain increased to 80%. The

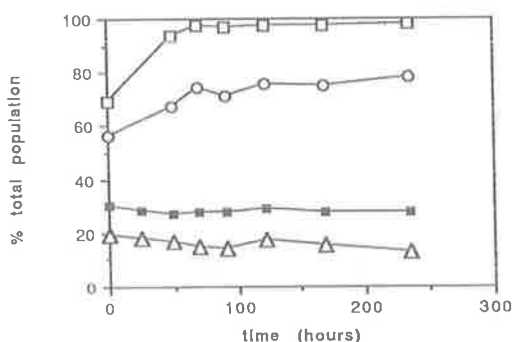

FIG. 6. Time course in the proportion of killer strain 3AM to strain 5A in the total population of a mixed-culture ferment for inoculum ratios of 2:1 (□), 1:1 (○), 1:2 (■), and 1:4 (△).

ability of strain 3AM to dominate strain 5A during fermentation is likely to be due to the production of killer toxin by strain 3AM and not to a difference in respective growth rates favoring the killer strain. We can conclude, therefore, that the killer toxin has displayed significant activity under these fermentation conditions. This result is of particular interest to the enologist, since the K_2 toxin produced by strain 3A is reported to show maximum activity at pH 4.2 (23), which is 0.5 to 1 pH unit higher than generally found in grape musts.

In cases in which killer activity in fermenting grape juice has been reported, a discrepancy as to whether effective killing action occurs when the proportion of killer cells is less than 50% of a mixed-culture ferment exists. Heard and Fleet (14) did not observe killer action when the ratio of killer to sensitive cells was approximately 1:7, whereas others have reported killer activity with killer-to-sensitive-cell ratios of 1:10 and lower (1, 10, 11). Our results showed that an increase in the ratio of killer to sensitive cells to approximately 2:1 resulted in a pronounced dominance of the fermentation by strain 3AM to 97% of the total mixed population by the end of the fermentation. However, with killer-to-sensitive-cell ratios of 1:2 or 1:4, no effective killer action was evident. It is possible that differences in either composition of medium, fermentation conditions, or strain sensitivity may account for discrepancies in reports of killer toxin efficiency.

The relevance of killer strains in wine making has been the focus of attention in countries where selected yeast cultures are inoculated into musts to induce fermentation. This focus has intensified since the observation that yeasts which are naturally present in the must also play significant roles in supposedly "pure" culture fermentations (13, 16). These natural yeasts include species from the genera *Kloeckera*, *Candida*, *Hansenula*, and *Saccharomyces*. Killer *Saccharo-*

myces wine yeast strains may be effective in suppressing natural *Saccharomyces* yeast strains during fermentation, and the possibility of engineering broad-range killer yeast strains to control strains from other genera exists. For these reasons, further study is needed to determine appropriate fermentation conditions for effective killer activity.

The GUS marking system provides a method which allows a broad range of killer strains to be rapidly and unequivocally identified in a mixed culture. This system can be employed to gain a better understanding of killer activity during fermentation.

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