Yeast in the Future: the Role of Genetic Engineering

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Introduction
The process of selecting yeast strains for the improvement of the alcoholic fermentation process during wine making has been occurring for many centuries. Ever since Antonie van Leeuwenhoek’s first descriptions of the ‘wee animalcules’ that he saw through his very crude microscope in 1680 and the work done by Louis Pasteur, Robert Koch and Müller-Thurgau which pioneered the way for the use of pure cultures in winemaking, the selection of yeast strains has been an ongoing process. Saccharomyces cerevisiae is the favoured yeast species for wine making and many of these strains have been selected from famous wine growing regions. Emphasis on the selection of strains over the years has been for strains which are able to tolerate higher ethanol concentrations, can completely ferment the hexoses present in grape must to ethanol and carbon dioxide, exhibit strong growth and fermentation, and have minimal production of undesirable flavours.

With increased understanding of the fermentation process, more specific changes of yeast characteristics are required. For this purpose, molecular genetic techniques are being applied to improve yeast strains since classical techniques, such as strain selection cannot be used to specifically alter a chosen characteristic of a yeast strain. Genetic engineering can be viewed as selectively altering one particular character of the yeast strain while preserving the core properties. In this paper we will describe the process of genetic engineering and discuss specific attributes of wine yeast which are currently being researched by a collaboration between The Australian Wine Research Institute, The University of Adelaide, Cooperative Research Centre for Viticulture and the Grape and Wine Research and Development Corporation.

Strain selection and improvement
Methods for yeast strain improvement have included clonal selection or new isolates of yeast, mutagenesis and selection or hybridization (classical breeding, rare matings, cytoduction or spheroplast fusion). However all of these methods have some disadvantages. Selection of new isolates which is the source of most new strains currently available to wine makers is a long process involving many years of extensive evaluation under a wide range of conditions. Furthermore, this technique suffers from the inability to bring together the required combination of many different oenological properties. The mutagenesis and subsequent selection of a particular yeast strain for the loss of an undesirable attribute may result in the acquisition of unknown characteristics, possibly undesirable. Cross breeding and clonal selection of yeast strains have similar disadvantages and these techniques can be highly time consuming. Unlike all the previously mentioned non-specific techniques genetic engineering can specifically target a single gene which has the advantage that a single pathway can be altered. The parent and transformed yeast strains will be identical except for in that one particular attribute. Recent reviews on genetic engineering of wine yeasts include Barre et al. (1993), Pretorius and Westhuizen (1991) and Henschke (1997).

Transformation
The process of transformation or the transfer of genetic material into a yeast strain is illustrated in Figure 1 and summarized below. The yeast strain used as an example in this figure is unable to grow on a synthetic growth medium unless the amino acid tryptophan is added to the medium, hence the strain is referred to as trp–. The necessary gene for the synthesis of tryptophan can be placed on a separate extra chromosomal piece of DNA, referred to as a plasmid. This plasmid is designated TRP in Figure 1. Such a plasmid can be introduced into the yeast trp– strain by permeabilising the cell wall and membrane so that it will take up the plasmid from the surrounding solution. It is this process which is referred to as transformation. Yeast cells which have taken up this plasmid can be selected for, by growing the yeast on a synthetic medium which does not contain tryptophan (–trp medium). Only those yeast cells which harbour the plasmid will grow on this –trp medium. With this selection procedure two forms of yeast cells can grow, either a transformant or a recombinant. The transformant harbours the plasmid within the cell along with the genome of the yeast.

![Figure 1. Principles of transformation.](image-url)
cell. However, the recombinant which can also grow on the
- trp synthetic medium, does not contain the plasmid, as the
genetic material has recombined with a chromosome
(genome of the yeast cell). These principles are covered in
more detail in several review papers (Barre et al. 1993;
Pretorius and van der Westhuizen 1991).

Areas of current research
The focus of this paper is on the current projects being
undertaken by the Yeast Molecular Biology Group using
genome transfer technology to help our understanding of yeast
metabolism and to improve yeast strains for use in wine
These research projects are listed in Table 1 and discussed
below.

Reduced ethanol production/increased glycerol production
The final alcohol content of wine is dependent on the sugar
content of the grape. However, there are various situations
in which the sugar content of grapes is not always at the
optimum level. In hot summers, for example, delays in grape
harvesting which may or may not be deliberately controlled
can lead to wines with higher levels of alcohol than desired.
In cooler climate regions of Australia, the grapes may be
deliberately left on the vines longer to obtain more intense
fruit flavour. With the increase in fruit flavour, there is also
an increase in the sugar content of the grape giving the
potential for wine with a high alcohol content. For example,
some Australian Chardonnays can have an ethanol content
of 14–16%. These wines are highly flavoured but may be
judged to be imbalanced due to the ‘hotness’ imparted by
the high ethanol content.

Commercial strains of Sacch. cerevisiae have been selected
for efficient conversion of grape sugars to ethanol and car-
bon dioxide. Unfortunately this characteristic is not desir-
able for the fermentation of grapes of high sugar maturity.
Modifying the sugar conversion efficiency of fermentative
yeast offers an avenue of control. Approaches to reduce the
ethanol potential content of the final wine include redirect-
ing some of the carbon from the grape sugars into other
metabolic compounds, such as cellular reserve compounds
trehalose and glycogen, increasing yeast biomass or increas-
ing the production of organic acids and glycerol. The
approach being studied is to direct some of the carbon from
sugar into glycerol as this compound is regarded to be desir-
able in wine. By altering the carbon balance in the glycolyt-
ic pathway it may be possible to lower ethanol production
(Figure 2). The aim is not to produce wines with very low
ethanol contents but to give the winemaker greater flexibil-
ity in the final alcohol balance of the wine.

The gene for an enzyme in the branch of glycolysis, which
is believed to control glycerol production, has been cloned
into a laboratory yeast. Laboratory fermentation trials have
indicated that wine glycerol content may be increased 2–3
fold. Once this transformed yeast has been characterized and
successfully manipulated it will be tested for other alter-
ations to the glycolytic pathway, such as the production of
ethanol and organic acids. For ease of experimentation ini-
tial tests were made in laboratory yeast strains. Ultimately
the altered gene will be introduced into wine yeast strains by
transformation and homologous recombination to produce
genetically stable strains for large scale winery trials (de
Barros Lopes et al. 1996). Recently, introduction of this
gene into a wine yeast strain was successful and trials are
currently underway to determine glycerol levels in the wine
produced.

Hydrogen sulfide production
Hydrogen sulfide production by yeast during alcoholic fer-
mentation is very undesirable because residual hydrogen sul-
fide can lead to the wine having reduced sensory appeal.
Removal of hydrogen sulfide by treatment with copper salts
is not regarded to be good practice. Hydrogen sulfide can
accumulate in the fermenting must in two stages; during the
active phase of fermentation or at the end of fermentation
when little or no fermentable sugar remains. Hydrogen sul-
fide is often liberated from the yeast cell in response to
nutrient depletion, especially assimilable nitrogen, and pos-
sibly vitamins. Figure 3 illustrates the fate of sulfur in the
yeast cell. Methionine is a sulfur containing amino acid
which is essential for the synthesis of proteins and cell
growth. However, because little methionine is present in
grape juice, yeast must synthesise methionine by first reduc-
ing inorganic sulfur to hydrogen sulfide. When nitrogen in
the form of amino acids and ammonium ion is also limiting,
the hydrogen sulfide is released by the cell into the wine,
hence the addition of DAP to the fermenting grape must

![Figure 2. Sugar metabolism in yeast.](image)

![Figure 3. Sulfur metabolism in yeast.](image)

<table>
<thead>
<tr>
<th>Research topic</th>
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<tbody>
<tr>
<td>Increased glycerol production</td>
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<tr>
<td>Reduced ethanol production</td>
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<tr>
<td>Hydrogen sulfide production</td>
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<tr>
<td>Wine acidification</td>
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<td>Reduced acetate acid production</td>
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<tr>
<td>Proline metabolism</td>
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<td>Zymocidal activity</td>
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Table 1. Targets for yeast improvement by genetic engineering.
may decrease the amount of hydrogen sulfide that is released. This project involves the cloning of the genes for the enzymes involved in the biochemical reduction of sulfate to hydrogen sulfide and the characterization of these genes and their products in order to moderate their activity while allowing optimal fermentative metabolism (Smyl et al. 1996a; Melbourne et al. 1996).

Wine acidification
Many Australian grape musts have low acidity which require adjustment. This is usually done with the addition of tartaric acid which can be a quite a costly exercise. During alcoholic fermentation, wine yeast may produce up to 0.5 g/L L-lactic acid, which is not sufficient to increase the wine acidity to the desired level. The lactate dehydrogenase (LDH) enzyme is responsible for the conversion of pyruvic acid to L-lactic acid in yeast (Figure 2). By manipulating the expression of the LDH gene, the amount of L-lactic acid formed should be altered. The aim of this project is to manipulate the activity of LDH, so as to increase the overall acidity of the wine, and thus reduce the need for acid addition.

Reduced acetic acid production
A cetic acid production during yeast growth can vary considerably, and depends on the strain, sugar concentration and nutrient content of the must. Current wine styles demand a very low acetic acid concentration. Fermentation of grape sugar leads to acetaldehyde formation which is mainly reduced to ethanol (Figure 2). Some acetaldehyde is oxidised to acetic acid, a reaction which is required for the growth of the yeast to maintain the redox balance within the cell. A gene for the enzyme responsible for this oxidation reaction has been cloned in our laboratory. Its role in anaerobic metabolism of acetaldehyde is currently being investigated. The aim of this project is to manipulate the regulation of this gene in order to lower the production of acetic acid by the yeast cell (Eglinton and Henschke 1996; Eglinton et al. 1996b).

Proline metabolism
Nitrogen is an essential nutrient for yeast growth and its level affects fermentation activity and wine flavour. The main source is however highly variable. A though the total nitrogen content of some grape juices may appear to be sufficient, in Chardonnay and Cabernet Sauvignon grape juices for example, much of this nitrogen is composed of proline. Because Sacch. cerevisiae wine yeast are unable to utilize proline as a nitrogen source during anaerobic growth, the yeast encounters a shortage of usable nitrogen, and most of the proline remains in the wine.

The aim of this research project is to develop a yeast that can metabolize proline and so make use of the nitrogen which is usually trapped in this amino acid. A gene has been cloned that allows proline to be oxidized in the absence of molecular oxygen. It has been demonstrated in a laboratory yeast strain that this cloned gene can be expressed and is functional during anaerobic growth of yeast, so that the strain can utilize proline as a source of nitrogen. Introducing this gene into a wine yeast strain could reduce the necessity for routinely measuring the nitrogen content of grape juice and the addition of DAP to grape must (Smyl et al. 1996b). These procedures add to the cost of wine production. Work is currently underway to introduce this gene into a wine yeast strain and demonstrate that it functions under wine making conditions.

Zymocidal activity
Zymocides are proteins produced by yeast that will kill sensitive yeast present in their immediate environment. Zymocides can also be referred to as microbial toxins, killer factors or zymocins. A ideal yeast would be one that is able to produce a broad spectrum microbial toxin that is lethal to the indigenous yeasts present in grape must and so confer an ecological advantage to the inoculated yeast strain. This project aims to explore the potential of yeast zymocidal activities for controlling the proliferation of indigenous yeast during alcoholic fermentation (Yap et al. 1996). This would reduce the need for sulfur dioxide additions to grape must as an antimicrobial agent.

Consumer acceptance of genetically engineered yeast
The commercial use of genetically engineered wine yeast will depend on their acceptance by both the industry and the consumers. Some genetically engineered foods, such as the FLAVR SAVR tomato are already well received in some countries, whereas others have been some what controversial (Høj and van Heeswijk 1996). Beer has already been brewed on a pilot scale in the United Kingdom but is not yet available for sale. At this point in time we are not aware of the use of genetically engineered yeast in wine production, however several of the major wine producing regions of the world may consider this option in the future.

Table 2. Genetically improved wine yeast research.

<table>
<thead>
<tr>
<th>Function</th>
<th>Country</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Marked yeast</td>
<td>Australia</td>
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</tr>
<tr>
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<td>Canada</td>
<td>Boone et al. (1990)</td>
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<tr>
<td>Malate metabolism</td>
<td>South Africa</td>
<td>Viljoen et al. (1994)</td>
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<tr>
<td>A cidifying yeast</td>
<td>France</td>
<td>Barre et al. (1993)</td>
</tr>
<tr>
<td>Pectinase activity</td>
<td>South Africa</td>
<td>van Vuuren et al. (1996)</td>
</tr>
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<td>Glucosidase hydrolysing yeast</td>
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<td>Barre et al. (1993)</td>
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<tr>
<td>Proline metabolism</td>
<td>Australia</td>
<td>Laing and Pretorius (1992)</td>
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<td>High glycerol</td>
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<td>Pérez-González et al. (1993)</td>
</tr>
<tr>
<td>Reducing acetic acid</td>
<td>Australia</td>
<td>Smyl et al. (1996b)</td>
</tr>
<tr>
<td>Reducing hydrogen sulfide</td>
<td>Australia</td>
<td>de Barros Lopes et al. (1996)</td>
</tr>
<tr>
<td>Zymocidal yeast</td>
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<td>Eglinton et al. (1996)</td>
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<td>Australia</td>
<td>Melbourne et al. (1996)</td>
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<td>Australia</td>
<td>Boone et al. (1990)</td>
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<td>Australia</td>
<td>Yap et al. (1996)</td>
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world are developing strains for future use. Some important recent developments are listed in Table 2. For further information on these yeast consult Barre et al. 1993; Pretorius and van der Westhuizen 1991.

At the present time, genetically engineered microbes, plants and animals are regulated by the Genetic Manipulation Advisory Council (GMAC) and there is no requirement to label foods prepared from such organisms. Recently the Australian and New Zealand Food Authority (ANZFA) has announced that they are drafting new regulations regarding food labelling. It is not yet clear what the implications are for the wine industry.

Conclusions

Research on yeast metabolism is being conducted by the Wine Yeast Molecular Biology Group based at The Australian Wine Research Institute and The University of Adelaide in order to improve or modify the fermentation properties of the wine yeast, Sacch. cerevisiae. The projects being undertaken include: the redirection of some of the sugar carbon into glycerol to increase wine ‘body’ and decrease ethanol content, reduction of hydrogen sulfide formation, increasing the production of L-lactic acid to improve wine acidity, the anaerobic utilization of proline by yeast, and the potential reduction of the use of sulfur dioxide for decrease ethanol content, reduction of hydrogen sulfide formation and improve wine acidity. The anaerobic utilization of proline by yeast has been developed in the brewing and baking industries and have proven successful in trials. Wine yeast research is progressing such that larger scale winery trials can be conducted in the near future.

References


CRCV Annual Report, 1996.


