Gene Technology in Winemaking: New Approaches to an Ancient Art

Isak S. PRETORIUS

SUMMARY

For the last century, the availability of pure culture yeast has improved reproducibility in wine fermentations and product quality. However, there is not a single wine yeast strain that possesses an ideal combination of oenological characteristics that are optimised for the task set by today's leading winemakers. With new developments in modern winemaking there has arisen an urgent need to modify wine yeast strains in order to take full advantage of technology and to satisfy the demands of the sophisticated wine consumers. The combined use of mutagenesis, hybridisation and recombinant DNA methods have significantly increased the genetic diversity that can be introduced into *Saccharomyces cerevisiae* strains. The overall aim of the strain development programmes extends far beyond the primary role of wine yeast to catalyse the rapid and complete conversion of grape sugars into alcohol and carbon dioxide without distorting the flavour of the final product. Starter cultures of *S. cerevisiae* must now possess a range of other properties that differ with the type and style of wine to be made and the technical requirements of the winery. Our strain development programme focuses on a number of targets that are amenable to a genetic approach, including strain security and quality control, the increase of fermentation and processing efficiencies, and the enhancement of the sensorial quality and health properties of wine and other grape-based beverages. However, successful commercialisation of transgenic wine yeasts will depend on a multitude of scientific, technical, economic, marketing, safety, regulatory, legal and ethical issues. Therefore, it would be foolish to entertain unrealistic expectations over rapid commercialisation and short-term benefits. However, it will be equally unwise to deny the potential advantages of genetically improved wine yeasts to both the winemaker and consumer in the third millennium.

KEY WORDS

Saccharomyces cerevisiae, wine yeast, genetic improvement

Institute for Wine Biotechnology, University of Stellenbosch
Stellenbosch 7600, South Africa

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INTRODUCTION

Yeasts have been used in the production of wine and other fermented beverages and foods for several thousand years. Originally, yeast species and strains present on the grapes and cellar equipment were responsible for the "spontaneous" and unpredictable fermentation which took place. But, over the last century, *Saccharomyces cerevisiae* strains have been selected on the basis of their fermentation behaviour and this has led to significant improvements in both fermentation control and wine quality.

No controlled breeding of wine yeasts has been attempted until relatively recently. This is largely because the yeasts used in most research laboratories differ significantly from those used in winemaking and, as a consequence, the techniques developed with laboratory-bred strains cannot be applied directly to wine yeasts. For the same reason studies of the genetics of wine yeasts have lagged behind investigations of laboratory strains of *S. cerevisiae.* In addition to these technical difficulties that restrict the progress with strain development, it is not surprising that with traditional fermentation methods and products there was initially little need to change the yeast strain; in fact, with local wines with their own unique character there was every reason for preserving the strain or strains then in use. However, today's fierce competition for market share in a world where there is an over supply of wine at several price points, fundamental innovations in viticultural and winemaking practices will undoubtedly continue to revolutionise the wine industry during the 21st century. These forces of *market-pull* and *technology-push* are therefore expected to challenge the tension between the tradition and innovation. Clearly, "traditional" wine yeasts are far from optimised for the demanding task which they are set by modern day's innovative winemakers and sophisticated consumers.

There is an ever-growing demand for new and improved wine yeast strains. In addition to the primary role of wine yeast to catalyse the efficient and complete conversion of grape sugars to alcohol without the development of off-flavours, starter culture strains of *S. cerevisiae* must now possess a range of other properties. The importance of these additional yeast characteristics differs with the type and style of wine to be made and the technical requirements of the winery. The need is for *S. cerevisiae* strains that are better adapted to the different wine-producing regions of the world with their respective grape varietals, viticultural practices and winemaking techniques.

GENETIC TECHNIQUES FOR THE ANALYSIS AND DEVELOPMENT OF WINE YEASTS

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

The classical genetic techniques all have value in strain development programmes, but these methods lack the specificity required to modify wine yeasts in a well-controlled fashion. It may not be possible to define precisely the change required using these genetic techniques, and a new strain may bring an improvement in some aspects, while compromising other desired characteristics. Gene cloning and transformation offer the possibility to alter the characteristics of wine yeasts with surgical precision: the modification of an existing property, the introduction of a new characteristic without adversely affecting other desirable properties, or the elimination of an unwanted trait. By using such procedures it is possible to construct new wine yeast strains that differ from the original only in single specific characteristics.

TARGETS FOR STRAIN DEVELOPMENT

It is well established that wine yeasts vary markedly in their winemaking abilities. Some of the properties required by many leading winemakers are complex and difficult to define genetically without a better understanding of the biochemistry and physiology involved. To date, no wine yeast in commercial use possesses an ideal combination of oenological characteristics. While some degree of variation can be achieved by altering the fermentation conditions, a major source of variation is the genetic constitution of the wine yeasts. Fortunately, recombinant DNA prosedures are now available which allow this problem to be addressed. Many possible targets for genetic modification have been identified and reviewed extensively by Pretorius (2000). The following sections are an excerpt from this recent review.

Improved quality control and strain handling

Strain maintenance

One of the main objectives for using pure cultures in winemaking is to ensure reproducible fermentation performance and product quality. It is therefore important to maintain the genetic identity of wine yeasts and to slow down the rate of strain evolution caused by sporulation and mating, mutations, gene conversions and genetic transpositions. Total prevention of heterogeneity in pure cultures is impossible, since homothallism, inability to sporulate and mate, and polyploidy (multiple gene structure) only protect against genetic drift caused by sexual reproduction and mutation, but not against that caused by gene

conversion and transposition. Even stringently-controlled conditions for maintenance of culture collections (*i.e.*, freeze-dried cultures, cultures preserved in liquid nitrogen or in silica gel) will not render full protection against genetic drift in pure yeast cultures. In fact, freeze-drying (lyophilisation) for long-term maintenance of yeast stock cultures causes phase transitions in membrane lipids and cell death during freeze-thawing and may also induce respiratory deficient variants. In an attempt to improve resistance to cryo-damage, anti-freeze peptides from polar fish were successfully expressed in *S. cerevisiae*. Cryoprotectants and osmoprotectants such as cellular trehalose and glycerol also alleviate freeze-thaw and water stress. Trehalose appears to stabilise cell membranes of lyophilised or cryopreserved stock cultures as well as their cellular proteins by replacing water and forming a hydration shell around proteins. Glycerol accumulation seems to control intracellular solute potential relative to that of the culture medium thereby counteracting the deleterious effects of dehydration on lyophilised yeast cells.

A better understanding of how yeast cells precisely acquire cryotolerance and osmotolerance may lead to genetic modification of starter culture strains with greater robustness for industrial fermentations. However, at present, fermentation trials, continuous strain evaluation and early detection of genetic changes using comparative molecular techniques are the only practical ways to limit possible economic loss.

Molecular marking

The potential for genetic markers in wine yeast identification has been recognised and deliberately marked oenological strains were developed as an aid to monitor the kinetics of yeast populations during wine fermentations. Genetic labeling could also be regarded as a quality control tool in general yeast culture management as well as in trouble-shooting, particularly for wineries using more than one yeast strain, as the genomes of commercial wine yeasts can be tagged. This would also discourage illegitimate use of (patented) commercial wine yeast strains by 'pirate' yeast and wine producers.

The marking of wine yeast strains usually entails the integration of specific genetic markers into their genomes. This could take the form of synthetic oligonucleotides or foreign genes of known nucleotide sequences. These DNA sequences can then be used as 'diagnostic probes' to identify specific wine yeast strains. In one instance a wine yeast was double-marked with diuron and erythromycin resistance genes. A more sophisticated manner of marking was the expression of the *Escherichia coli* β-glucuronidase (GUS) gene (*uidA*) under control of the yeast alcohol dehydrogenase I promoter and terminator sequences. The *GUS* construct was integrated into the *ILV2* gene of *S. cerevisiae* and a simple assay procedure was devised to detect GUS activity in yeast cells or colonies. In a GMO ('genetically modified organism') risk assessment experiment, this yeast is currently being used to monitor the dissemination of transgenic yeast strains on vines cultivated in a biologically contained glass house. This will undoubtedly provide an insight into the kinetics of transgenic and native yeast populations on vines.

Improvement of fermentation performance

The primary selection criteria applied to most strain development programmes relate to the overall objective of achieving a better than 98% conversion of grape sugar to alcohol and carbon dioxide, at a controlled rate and without the development of off-flavours. The growth and fermentation properties of wine yeasts have, however, yet to be genetically defined. What makes the genetic definition of these attributes even more complex is the fact that lag phase, rate and efficiency of sugar conversion, resistance to inhibitory substances and total time of fermentation are strongly affected by the physiological condition of the yeast as well as by the physicochemical and nutrient properties of grape must.

Generally, sugar catabolism and fermentation proceed at a rate greater than desired, and are usually controlled by lowering the fermentation temperature. Occasionally, wine fermentation ceases prematurely or proceeds too slowly. Measures to rescue such 'sluggish' or 'stuck' fermentations include the increase of fermentation temperature, addition of vitamin supplements, limited aeration by pumping over and re-inoculation. The commercial implications of 'runaway' wine fermentations arise from the fact that fermentor space is reduced because of foaming and volatile aroma compounds are lost by entrainment with the evolving carbon dioxide. Conversely, financial losses through sluggish or incomplete wine fermentations are usually attributed to inefficient utilisation of fermentor space and wine spoilage resulting from the low rate of protective carbon dioxide evolution and high residual sugar content. Optimal performance of wine yeasts in white wine fermentations, conducted at cooler temperatures (10-15^{0}C) so as to minimise the loss of aromatic volatiles, and red wine fermentations, performed at higher temperatures (18-30^{0}C) to enhance extraction of anthocyanin pigments, is therefore of critical importance to wine quality and cost-effectiveness.

Fermentation predictability and wine quality is directly dependent on wine yeast attributes that assist in the rapid establishment of numerical and metabolic dominance in the early phase of wine fermentation, and that determine the ability to conduct an even and efficient fermentation with a desirable residual sugar level. A wide range of factors affect the fermentation performance of wine yeasts. Apart from a successful inoculation with the appropriate starter culture strain, the physiological condition of such an active dried wine yeast culture, and its ability to adapt to and cope with nutritional deficiency and the pres-

ence of inhibitory substances, is of vital importance to fermentation performance.

Successful yeast cellular adaptation to changes in extracellular parameters during wine fermentation requires the timely perception (sensing) of chemical or physical environmental parameters, followed by accurate transmission of the information to the relevant compartments of the cell. Chemical signals emanating during wine fermentations include the availability/concentration of certain nutrients (*e.g.*, fermentable sugars, assimilable nitrogen, oxygen, vitamins, minerals, ergosterol and unsaturated fatty acids) and the presence of inhibitory substances (*e.g.*, ethanol, acetic acid, fatty acids, sulphite, agrochemical residues and killer toxins). Signals of a physical nature include factors such as temperature, pH, agitation and osmotic pressure. As an example, physiological and morphological modifications in response to a limited supply of essential nutrients such as carbon and nitrogen sources include a shift in transcription patterns, the modification of the cell cycle, a change in budding pattern and strongly polarised growth. It is becoming clear that a complex network of interconnected and cross-talking signal transduction pathways, relying on a limited number of signaling modules, governs the required adaptive responses to changes that occur as the fermentation progresses.

This complexity explains why it is so difficult to define all the key genetic determinants of a yeast's fermentation performance that may be candidates for genetic engineering. However, general targets include increased tolerance to desiccation and viability of active dried yeast; improved grape sugar uptake and assimilation; increased ethanol tolerance; improved nitrogen assimilation; enhanced resistance to microbial metabolites and toxins; resistance to heavy metals and agrochemical residues; tolerance to sulphite; and reduced foam formation.

Improved viability and vitality of active dried wine yeast starter cultures

Both the genetic and physiological stability of stock cultures of seed yeast and wine yeast starter cultures are essential to optimal fermentation performance. Physiological stability and 'fitness' of active dried wine yeast cultures relate to the maintenance of cell viability and vitality during the process of yeast manufacturing, including desiccation and storage. The differentiation between yeast 'viability' and 'vitality' is based upon the fact that cells which irreversibly lose their ability to reproduce may still be capable of active metabolism. Therefore 'viability' is defined as the relative proportion of living cells within an active dried starter culture, whereas 'vitality' refers to the measure of metabolic activity and relates to fitness or vigour of a starter culture. Yeast viability can be assessed directly by determining loss of cell reproduction/division (*e.g.*, plate and slide counts) and indirectly by assessing cellular damage (*e.g.*, vital staining with bright-field or fluorochrome stains) or loss of metabolic activity (*e.g.*, ATP bioluminescence and NADH fluorescence). Yeast vitality can be indirectly assessed by measuring metabolic/fermentative activity (*e.g.*, CO_2 evolution in mini-scale fermentations), storage molecules (*e.g.*, glycogen), intracellular/extracellular pH (acidification power) and gaseous exchange coefficients (*e.g.*, respiratory quotients or RQ). Automatic in-line monitoring of yeast cell viability in fermentation plants can be achieved with electrosensors such as capacitance probes or with fluorescent probes coupled with flow cytometry which can rapidly determine cell viability and other aspects of yeast physiology (*e.g.*, stress responses). These techniques generally show varying degrees of correlation with fermentative performance and none of them alone can accurately predict the physiological activity of a active dried wine yeast starter culture.

The manufacturers of active dried wine yeast starter cultures can positively influence the degree of viability and vitality as well as the subsequent fermentation performance of their cultures by the way they cultivate their yeasts. Industrial cultivation of wine yeasts can have a profound effect on the microbiological quality, fermentation rate, production of hydrogen sulphide, ethanol yield and tolerance, resistance to sulphur dioxide as well as tolerance to drying and rehydration. For example, if a protein to phosphate ratio (P_2O_5:N) of 1:3 in a yeast cell is exceeded it would result in an excess of water linked to the protein which would, in turn, negatively affect the drying procedure, viability and final activity of the dry yeast. Due to the roles that trehalose and glycogen play in a yeast cell's response to variations in environmental conditions, it is generally recommended that the manufacturers of active dried wine yeast starter cultures cultivate their yeast in such a way that the maximum amount of these storage carbohydrates is accumulated in the yeast cells.

In *S. cerevisiae*, trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is synthesised from glucose-6-phosphate and UDP-glucose by the *TPS1*-encoded trehalose-6-phosphate synthetase and converted to trehalose by the *TPS2*-encoded trehalose-6-phosphate phosphatase. The regulation of trehalose synthesis and degradation (by trehalase) is mediated by cAMP-dependent phosphorylation mechanisms. Trehalose is associated with nutrient-induced control of cell cycle progression; control of glucose sensing, transport and initial stages of glucose metabolism; as well as stress protection against dehydration, freezing, heating and osmo-stress, and toxic chemicals such as ethanol, oxygen radicals and heavy metals. This storage carbohydrate plays an important role during sporulation, nutrient starvation, growth resumption and growth rate. Trehalose content in the yeast cell is probably one of the most important factors affecting the resistance of yeasts to drying and subsequent rehydration. The accumulation of this disaccharide on both sides of the plasma membrane is thought to

confer stress protection by stabilising the yeast's membrane structure.

Glycogen, another carbohydrate reserve whose accumulation by yeast propagated for drying has also been linked to enhanced viability and vitality upon reactivation, provides a readily mobilisable carbon and energy source during the adaptation phase. The biosynthesis of glycogen (α-1,4-glucan with α-1,6 branches) is effected by glycogen synthase, which catalyses the sequential addition of glucose from UDP-glucose to a polysaccharide acceptor in a linear α-1,4 linkage, while branching enzymes are responsible for the formation of α-1,6 branches. There are two forms of glycogen synthase in *S. cerevisiae*, Gsy1p and Gsy2p. The *GSY1* gene is expressed constitutively at a low level along with growth on glucose, while the level of the *GSY2*-encoded glycogen synthase increases at the end of the exponential phase of growth when glycogen accumulates. This indicates that *GSY2* encodes the major glycogen synthase. Glycogen breakdown, catalysed by glycogen phosphorylase quickly following depletion of nutrients at the end of fermentation, is accompanied by sterol formation. Since sterol is essential for yeast vitality, low levels of accumulated glycogen in active dried wine yeast starter cultures may result in insufficient yeast sterols, which, in turn, may impair yeast performance upon inoculation into grape juice. In this regard, it is important to note that the overexpression of the *SUT1* and *SUT2* genes has been shown to promote the uptake of sterol from the medium under fermentative conditions.

Owing to its multiple roles in increasing survival of *S. cerevisiae* cells exposed to several physical and chemical stresses, trehalose and glycogen have important implications for the viability, vitality and physiological activity of active dried wine yeast starter cultures upon reactivation. Therefore, there is a strong incentive to develop wine yeast strains with a superior trehalose and glycogen accumulation ability. However, due to the complexity of yeast viability, vitality and physiological activity, it is unclear at this stage whether the modification of the expression levels of the *TPS1*, *TPS2*, *GSY1, GSY2, SUT1* and/or *SUT2* genes, would contribute to yeast fitness and fermentation performance of starter culture strains.

Efficient sugar utilisation

In *S. cerevisiae*, glucose and fructose, the main sugars present in grape must, is metabolised to pyruvate via the glycolytic pathway. Pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol. The rate of fermentation and the amount of alcohol produced per unit of sugar during the transformation of grape must into wine is of considerable commercial importance. During wine yeast glycolysis, one molecule of glucose or fructose yield two molecules each of ethanol and carbon dioxide. However, the theoretical conversion of 180 g sugar into 92 g ethanol (51.1%) and 88 g carbon dioxide (48.9%) could only be expected in the absence of any yeast growth, production of other metabolites and loss of ethanol as vapor. In a model fermentation, about 95% of the sugar is converted into ethanol and carbon dioxide, 1% into cellular material and 4% into other products such as glycerol.

The first step to ensure efficient utilisation of grape sugar by wine yeasts is to replace any mutant alleles of genes encoding the key glycolytic enzymes, namely hexokinase (HXK), glucokinase (GLK), phosphoglucose isomerase (PGI), phosphofructokinase (PFK), aldolase (FBA), triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (TDH), phosphoglycerate kinase (PGK), phosphoglycerate mutase (PGM), enolase (ENO), pyruvate kinase (PYK), pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH). The genes encoding PGI, TPI, PGM and PYK appear to be present in single copy in a haploid genome, while multiple forms exist for TDH (three isozymes), ENO (two isozymes) and ENO/GLK (three isozymes).

The assumption that an increase in the dosage of genes encoding these glycolytic enzymes would result in an increase in the efficiency of conversion of grape sugar to alcohol has been disproved; it has been demonstrated that overproduction of the enzymes has no effect on the rate of ethanol formation. This indicates that the step of sugar uptake represents the major control site for the rate of glycolytic flux under anaerobic conditions, whereas the remaining enzymatic steps do not appear to be rate limiting. In other words, the rate of alcohol production by wine yeast is primarily limited by the rate of glucose and fructose uptake. Therefore, in winemaking, the loss of hexose transport toward the end of fermentation may result in reduced alcohol yields.

Sugars enter yeast cells in one of three ways: simple net diffusion, facilitated (carrier-mediated) diffusion and active (energy-dependent) transport. In grape must fermentations where sugar concentrations above 1 M are common, free diffusion may account for a very small proportion of sugar uptake into yeast cells. However, since the plasma membranes of yeast cells are not freely permeable to highly polar sugar molecules, various complex mechanisms are required for efficient translocation of glucose, fructose and other minor grape sugars into the cell. The hexose transporter family of *S. cerevisiae* consists of more than 20 proteins comprising high, intermediate and low affinity transporters and at least two glucose sensors. Many factors affect both the abundance and intrinsic affinities for hexoses of these transporters present in the plasma membrane of wine yeast cells, among them glucose concentration, stage of growth, presence or absence of molecular oxygen, growth rate, rate of flux through the glycolytic pathway and nutrient availability (particularly of nitrogen).

Although the precise mechanisms and regulation of grape sugar transport of wine yeast are still unclear, some aspects about glucose and fructose uptake can

be noted. Glucose uptake is rapid down a concentration gradient, reaching an equilibrium and is therefore not accumulative. Several specific, energy-dependent glucose carriers mediate the process of facilitated diffusion of glucose and proton symport is not involved. Phosphorylation by the the *HXK1*- and *HXK2*-encoded hexokinases and the *GLK1*-encoded glucokinase is linked to high-affinity glucose uptake. Glucose transporters, encoded by *HXT1-HXT18* and *SNF3* are stereospecific for certain hexoses and will translocate glucose, fructose and mannose. Some members of this multigene permease family affect glucose, galactose, glucose and mannose, or glucose, fructose and galactose uptake, but thusfar none has been described as specifically affecting fructose uptake. It appears that in *S. cerevisiae* fructose is transported via facilitated diffusion rather than active transport, whereas related species (*S. bayanus* and *S. pastorianus*) within the *Saccharomyces sensu stricto* group do possess fructose-proton symporters.

Based on the spectacular increase in the amount of information on sugar sensing and their entry into yeast cells that has come to the fore over the last few years, several laboratories have identified this main point of control of glycolytic flux as one of the key targets for the improvement of wine yeasts. For example, in some instances, certain members of the *HXT* permease gene family are being overexpressed in an effort to enhance sugar uptake, thereby improving the fermentative performance of wine yeast strains. However, more in-depth details are required about the complex regulation of glucose and fructose uptake as well as glycolysis as it occurs in grape juice (especially in the presence of high sugar levels during the early phase of fermentation and during the final stages of sugar depletion coupled to nutrient limitation) before it will be possible to devise novel strategies to improve wine yeast's fermentation performance and to prevent sluggish or stuck fermentations.

Improved nitrogen assimilation

Of all nutrients assimilated by yeast during wine fermentations, nitrogen is quantitatively second only to carbon. Carbon-nitrogen imbalances and more specifically, deficiencies in the supply of assimilable nitrogenous compounds, remain the most common causes of poor fermentative performance and sluggish or stuck fermentations. Such problematic and incomplete fermentations occur because nitrogen depletion irreversibly arrests hexose transport. Other problems that are related to the nitrogen composition of grape must include the formation of reduced-sulphur compounds, in particular hydrogen sulphide, and the potential formation of ethyl carbamate from metabolically produced urea.

Unlike grape sugars that are usually present in large excess (often exceeding 20% w/v) to that needed for maximal yeast growth, the total nitrogen content of grape juices ranges 40-fold from 60 to 2400 mg/l and can therefore be growth limiting. The assimilable content of grape must is dependent upon grape cultivar and root stock, as well as several aspects of vineyard management, including nitrogen fertilisation, berry maturation, vine water status, soil type and fungal infection. Grape juices with nitrogen levels below 150 mg/l have a high probability of becoming problem ferments due to inadequate yeast growth and poor fermentative activity. There are two basic strategies to circumvent problems linked to nitrogen deficiency: prevention of nitrogen deficiency in grape juice by optimising vineyard fertility, and more commonly, supplementation with ammonium salts such as diammonium phosphate (DAP). However, the injudicious use of DAP supplements often contravenes the wine industry's desire to minimise its use of additives while producing wines of high quality. Moreover, excessive addition of inorganic nitrogen often results in excessive levels of residual nitrogen, leading to microbial instability and ethyl carbamate (and phosphate in the case of DAP) accumulation in wine. The degree of supplementation of inorganic nitrogen in grape juice is therefore often regulated. This implies that knowledge of the nitrogen content of grape juice and the requirement for nitrogen by yeast are important considerations for optimal fermentation performance and the production of wines that comply with the demands of regulatory authorities and consumers.

The major nitrogenous compounds in the average grape must are proline, arginine, alanine, glutamate, glutamine, serine and threonine, while the ammonium ion levels may also be high, depending on grape variety and vineyard management. Proline and arginine accounts for 30 to 65% of the total amino acid content of grape juices. High proline accumulation in grape must is associated with grapevine stress, in particular with low moisture, whereas high levels of γ-aminobutyrate, another nitrogen compound, may be formed in the grape berries most probably postharvest and prior to processing of the grapes.

S. cerevisiae is incapable of adequately hydrolysing grape proteins to supplement nitrogen-deficient musts, and relies therefore on the ammonium and amino acids present in the juice. Wine yeasts can distinguish between readily and poorly used nitrogen sources. Ammonium is the preferred nitrogen source and as it is consumed, the amino acids are taken up in a pattern determined by their concentration relative to yeast's requirements for biosynthesis and to total nitrogen availability. When a readily used nitrogen such as ammonium, glutamine and asparagine is present, genes involved in the uptake and catabolism of poorly utilised nitrogen sources (including proline) are repressed. This nitrogen catabolite repression exerted upon nonpreferred nitrogenous compounds rigorously impairs the assimilation of proline as well as arginine since both amino acids depend on the proline utilisation pathway. Since the proline content of wine is generally not less than

grape juice, it appears that proline is not taken up by wine yeast under anaerobic fermentative conditions. Proline is transported into *S. cerevisiae* by the general amino acid permease and the *PUT4*-encoded proline-specific permease. Once inside the yeast cell, proline is converted to glutamate in mitochondria by the *PUT1*-encoded proline oxidase and *PUT2*-encoded pyrroline-5-carboxylate dehydrogenase. The expression of both *PUT1* and *PUT2* is regulated by the *PUT3*-encoded activator and the *URE2*-encoded repressor. Ure2p represses transcription of *PUT1* and *PUT2* under nitrogen-limiting conditions, while the *GLN3*-encoded regulator has no effect on these genes.

Since wine yeast strains vary widely in their nitrogen requirement, an obvious target for strain improvement is to select or develop starter strains that are more nitrogen efficient for use in low nitrogen musts. To achieve this a thorough understanding of the regulation of nitrogen assimilation by yeast under fermentative conditions is required. In an effort to develop wine yeast strains that are relieved from nitrogen catabolite repression and that are capable of utilising proline more efficiently under winemaking conditions, a mutant containing a *ure2* recessive allele was constructed. It was demonstrated that this mutation strongly deregulates the proline utilisation pathway, thereby improving the overall fermentation performance of the *ure2*-carrying yeast. This may be the first step towards the development of wine yeasts that are able to efficiently assimilate the abundant supply of proline in grape juice under fermentative conditions.

Improved ethanol tolerance

The winemaker is confronted by the dilemma that, while ethyl alcohol is the major desired metabolic product of grape juice fermentation, it is also a potent chemical stress factor that is often the underlying cause of sluggish or stuck fermentations. Apart from the inhibitory effect of excessive sugar content on yeast growth and vinification fermentation, the production of excessive amounts of ethanol, coming from harvest of over-ripe grapes, is known to inhibit the uptake of solutes (*e.g.*, sugars and amino acids) and to inhibit yeast growth rate, viability and fermentation capacity.

The physiological basis of ethanol toxicity is complex and not well understood, but it appears that ethanol mainly impacts upon membrane structural integrity and membrane permeability. The chief sites of ethanol action include the yeast cell's plasma membrane, hydrophobic proteins of mitochondrial membranes, nuclear membrane, vacuolar membrane, endoplasmic reticulum and cytosolic hydrophilic proteins. Increased membrane fluidity and permeability due to ethanol challenge seem to result in futile cycling of protons and dissipation of ATP energy. However, the dissipation of the proton gradient across the membrane and ATP is not only affected by increased permeability to protons, but ethanol may also directly affect the expression of the ATPase-encoding genes (*PMA1* and *PMA2*) and membrane ATPase activity. This explains the interference of ethanol with energy-coupled solute transport in yeast cells.

Several intrinsic and environmental factors are known to synergistically enhance the inhibitory effects of ethanol. These factors include high fermentation temperatures, nutrient limitation (especially oxygen, nitrogen, lipids and magnesium ions) and metabolic by-products such as other alcohols, aldehydes, esters, organic acids (especially octanoic and decanoic acids), certain fatty acids and carbonyl and phenolic compounds. By manipulating the physicochemical environment during the cultivation and manufacturing of active dried wine yeast starter cultures and during the actual vinification process, the yeast cells' self-protective adaptations can be promoted. Prior exposure of yeast cells to ethanol (physiological pre-conditioning) elicits adaptive stress responses that confer a degree of resistance to subsequent exposure to high levels of ethanol. Furthermore, osmotic pressure, media composition, modes of substrate feeding and by-product formation play important roles in dictating how yeast cells tolerate ethanol during vinification. Most of the so-called survival factors (*e.g.*, certain unsaturated long chain fatty acids and sterols) are formed only in the presence of molecular oxygen which in part explains the great success in the use of commercial starter cultures that are cultivated under highly aerobic conditions and in low glucose concentrations. These starter yeast cells contain high levels of the survival factors that can be passed onto the progeny cells during the six or seven generations of growth in a typical wine fermentation.

Wine yeast strains usually contain higher levels of survival factors than nonwine *Saccharomyces* strains. The physiological response of wine yeast to ethanol challenge is also greater than is the case with nonwine strains. These defensive adaptations of wine yeasts, conferring enhanced ethanol tolerance, range from alterations in membrane fluidity to synthesis of detoxification enzymes. Responses include a decrease in membrane saturated fatty acids (*e.g.*, palmitic acid); an increase in membrane unsaturated long chain fatty acids (*e.g.*, oleic acid); phosphatidylinositol biosynthesis (thereby increasing the phospholipid: protein ratio in the membrane); elevated levels of cellular trehalose that neutralise the membrane-damaging effects of ethanol; stimulation of stress protein biosynthesis; enhanced mitochondrial superoxide dismutase activity that countereffects ethanol-induced free radical synthesis; increased synthesis of cytochrome P450, alcohol dehydrogenase activity and ethanol metabolism.

From this it is clear that the genetics of ethanol tolerance are polyvalent and very complex. It is speculated that more than 250 genes are involved in the control of ethanol tolerance in yeast. Nevertheless, some reports claim that continuous culture of yeasts in a feedback system in which the ethanol was controlled by

the rate of carbon dioxide evolution, enabled the selection of viable mutants with improved ethanol tolerance and fermentation capabilities. Dramatic increases in ethanol tolerance, however, seem to elude researchers. It therefore appears that for the time being, ethanol tolerance in wine yeasts will be addressed by 'cell engineering' rather than 'genetic engineering'.

Increased tolerance to antimicrobial compounds

Besides the various yeast metabolites such as alcohols, acetic acid and medium chain length fatty acids (*e.g.*, decanoic acid) that can interfere with efficient grape must fermentations, there are several other antimicrobial compounds that can impede the fermentation performance of wine yeasts. These compounds include killer toxins, chemical preservatives (especially sulphite) and agrochemicals containing heavy metals (*e.g.*, copper). Since *S. cerevisiae* strains vary widely in their ability to resist or tolerate these compounds, the differences may lend themselves as targets for strain development.

Killer toxins are proteins produced by some yeasts that are lethal to sensitive wine yeast strains. The killers themselves, however, are immune to these mycovirus-associated toxins. Whether the growth and zymocidal activity of some wild killer yeasts have the potential to delay the onset of fermentation, cause sluggish or stuck fermentations and produce wines with increased levels of acetaldehyde, lactic acid, acetic acid and other undesirable sensory qualities is still a matter of controversy. However, it appears that under certain conditions (*e.g.*, inefficient inoculation with highly sensitive starter cultures in low-nitrogen musts) that favour the development of killer yeast contamination of grape juice, potent zymocidal yeasts may indeed contribute to incomplete fermentations. While zymocidal toxins produced by killer strains (K_1, K_2, K_3, K_{28}) of *S. cerevisiae* are lethal only to sensitive strains of the same species, those produced by non-*Saccharomyces* killer species (K_4 to K_{11}) may be toxic to a wider range of wine yeast strains and other wild yeasts. The killing of sensitive wine yeasts by the two *S. cerevisiae* killer toxins that function at wine pH, K_2 and K_{28}, occur via two different mechanisms: the K_2 toxin acts as an ionophore affecting membrane permeability and leakage of protons, potassium cations, ATP and amino acids, whereas the K_{28} toxin inhibits DNA synthesis.

An unfortunate consequence of ignorance regarding the role of killer yeasts in wine fermentations is that some winemakers use co-cultures to inoculate fermentations; one strain being a killer and the other a sensitive strain. The advantage of using killer or neutral wine yeasts should therefore not be underestimated. For this reason the aim of many strain development programmmes is to incorporate the mycoviruses from killer yeasts into commercial wine strains. Mycoviruses are readily transmitted by cytoplasmic fusion and have been used to transfer the killer character into commercial yeasts. In most cases, however, the mixing of the genomes of commercial strains and donor strains containing the killer character would prove undesirable even though repeated back-crossing could be used to minimise the unwanted effects.

One way to circumvent this problem is cytoduction between a donor killer strain deficient in nuclear fusion and a haploid derived from a commercial wine strain. Another means is to cross a haploid derived from a killer wine yeast with haploid cells or ascospores from a sensitive wine yeast. An alternative to the use of cytoduction and hybridisation to develop broad spectrum zymocidal resistance into wine yeasts would be to clone and introduce the toxin-immunity genes from non-*Saccharomyces* killer yeasts into wine yeasts.

Sulphur dioxide is widely used in wineries to suppress the growth of unwanted microbes, and tolerance to sulphite forms the basis of selective implantation of active dried wine yeast starter cultures into grape must. Membrane transport of sulphite in wine yeasts is by simple diffusion of liberated sulphur dioxide rather than being carrier-mediated. SO_2 dissociates within the cell to SO_3^{2-} and HSO_3^- and the resulting decline in intracellular pH forms the basis of the inhibitory action. Although *S. cerevisiae* tolerates much higher levels of sulphite than most unwanted yeasts and bacteria, excessive SO_2 dosages may cause sluggish or stuck fermentations.

Wine yeasts vary widely in their tolerance of sulphite, and the underlying mechanism of tolerance as well as the genetic basis for resistance are still unclear. Once these have been better defined, it may be advantageous to engineer wine yeast starter strains with elevated SO_2 tolerance. This, however, should not replace efforts to lower the the levels of chemical preservatives in wine.

Improper application of copper-containing fungal pesticides (copper oxychloride) to control downy mildew (*Plasmopara viticola*) and, to a lesser extent, dead arm (*Phomopsis viticola*) and anthracnose (*Gloeosporium ampelophagum*) could lead to copper residues in musts that may cause lagging fermentation and affect wine quality detrimentally. Copper toxicity towards wine yeast cells involves the disruption of plasma membrane integrity and perhaps also intracellular interaction between copper and nucleic acids and enzymes. Several copper uptake, efflux and chelation strategies have been developed by yeasts to control copper ion homeostasis. One such protective mechanism relates sequestration of copper by the *CUP1*-encoded copper-binding protein, copper-chelatin. Such metallothein proteins are generally synthesised when *S. cerevisiae* cells are exposed to potentially lethal levels of toxic metals. The copper resistance level of a given yeast strain correlates directly with the *CUP1* copy number. One way to engineer wine yeasts resistant to copper would be to clone and integrate the *CUP1* gene at multiple sites

into their genomes. This would enable the wine yeast to tolerate higher concentrations of copper residues in musts. Copper-resistant wine yeasts should, however, not be used to encourage disrespect for recommended fungicide withholding periods.

Reduced foam formation

Excessive foaming, caused by certain wine yeast strains during the early stages of wine fermentation, can result in the loss of grape juice. Moreover, formation of a froth-head can reduce fermenter capacity, as part of the fermentation vessel may have to be reserved to prevent the froth from spilling over. In some cases foaming may also reduce the suspended yeast cell density in the fermenting must.

Froth generation varies widely among *S. cerevisiae* strains. Genetic analysis of the foaming characteristic suggests that this trait is under the control of at least two dominant genes, *FRO1* and *FRO2*. Apparently these genes, located on chromosome VII, code for proteins that interact with the grape juice thereby causing foaming. Several researchers have successfully used intragenomic hybridisation to cross out the genes that are responsible for foaming. However, the *FRO1* and *FRO2* genes have yet to be cloned and their encoded proteins characterised. Once this is done, the regulation of *FRO1* and *FRO2* can be unravelled. Gene disruption through targeted homologous recombination would then also become possible which would eliminate the foaming characteristic of wine yeast strains without changing the remainder of their genetic backgrounds.

Improvement of processing efficiency

Improved protein and polysaccharide clarification

The main objectives of fining and clarification during wine processing include the removal of excess levels of certain components to achieve clarity and ensure the physicochemical stability of the end product. The need for fining and clarification depends on the composition of the must and the winemaking practices that have been employed. Fining of wine entails the deliberate addition of an adsorptive compound, followed by the settling or precipitation of partially soluble components from the wine. Further clarification is usually achieved by sedimentation and racking, centrifugation and filtration. Wine filtration involves a wide range of objectives, from the partial removal of large suspended solids by various grades of diatomaceous earth or filter sheets to the complete retention of microbes by perpendicular flow polymeric membranes. While clarification of wine is generally thought to produce insignificant compositional changes, fining is intended to bring about changes that will prevent further precipitation. Fining can therefore be used to modify the sensory attributes of wine even though existing clarity may not be a problem.

Fining reactions include the the removal of colloids such as partially soluble, haze-forming proteins, filter-clogging polysaccharides as well as complexes between proteins and phenols, and between proteins and polysaccharides. The removal of tannic or brown polymeric phenols is usually achieved by proteinaceous fining agents (*e.g.*, casein, isinglas, albumin and gelatin), whereas the depletion of monomeric and small polymeric phenols is reached by treatment with polyamide materials (*e.g.*, polyvinylpolypyrrolidone or PVPP). Haze-forming proteins are removed by exchanging clays such as bentonites, while the removal of fine colloidal particles and incipient precipitates is achieved by the sieving effect of other gelatinous materials.

The slow development of protein hazes in white wine is considered to be the next most common physical instability after the precipitation of potassium bitartrate. Protein instability, occurring after bottling and shelf storage, is induced by high ethanol and low pH. Protein haze is not dependent upon total protein content but rather upon specific grape-derived proteins whose size or isoelectric properties make them particularly susceptible to solubility limitations. Protein instability is presumably associated with pathogenesis-related (PR) proteins produced in grape berries when challenged by fungal attack. Although the removal of these haze-forming proteins by bentonite treatment is effective, the non-specific nature of this diatomaceous clay can result in the loss of important aroma and flavour compounds, thereby altering the sensory characteristics of the wine. Furthermore, bentonite fining is an expensive and laborious practice that generates large volumes of lees for disposal and causes a 5 to 20% loss of wine.

To omit the bentonite treatment, an application of an appropriate acid protease to hydrolyse the grape PR-proteins has been suggested. However, the search for fungal enzymes that could degrade these haze-forming proteins has so far remained unsuccessful.

We have investigated the feasibility of engineering a proteolytic wine yeast that could facilitate protein haze reduction. Proteolytic activities of *S. cerevisiae* include the acid endoprotease, protease ysc A; the endo serine-sulphydryl protease, protease ysc B; the serine exopeptidase, carboxypeptidase ysc Y; and the four metallo exopeptidases, namely carboxypeptidase ysc S, aminopeptidase ysc I, aminopeptidase ysc II and aminopeptidase ysc Co. However, the vacuolar protease A, encoded by the *PEP4* gene, is the only one that is active at the low pH of wine. Furthermore, it has been reported that the prolonged storage of wine on the lees after the completion of the alcoholic fermentation renders a wine more protein stable. This phenomenon was attributed to the action of proteinase A during autolysis.

This acid endoprotease is synthesised as a preprotein in *S. cerevisiae*. The prepeptide is cleaved early in the secretory pathway and the propeptide is cleaved upon entrance of proteinase A into the vacuole. The

propeptide contains the vacuolar targeting information and serves as an inhibitor to keep protease A inactive during transport through the secretory pathway. The *PEP4* gene was cloned and expressed in a wine yeast by using different combinations of several promotor, leader and termination sequences. Northern blot analysis indicated the presence of these *PEP4* transcripts in the various transformants. Upon replacing the *PEP4*-encoded prepro-region (vacuolar localisation signal) with the yeast mating pheromone a-factor (*MFa1*-encoded) prepro-region (secretion signal), no extracellular protease activity was detected. However, western blot analysis revealed the presence of extracellular protease A when the *PEP4* gene was overexpressed under control of the constitutive yeast alcohol dehydrogenase I promoter ($ADH1_p$) and terminator ($ADH1_T$) signals. Casein agar test plates confirmed that these transformants secreted biologically active protease A. Overexpression of *PEP4* in *S. cerevisiae* seems to have saturated the vacuolar targeting machinery and resulted in secretion of biologically active protease.

Later, it became known, however, that bentonite fining is unlikely to be replaced by the addition of proteolytic enzymes to wine or by engineering a proteolytic wine yeast. This is not because these proteases are inactive in must and wine, but because the haze-forming proteins in wine are inherently resistant to proteolysis. Their resistance is not due to protection by other wine components in wine nor is it due to covalently bound sugars (glycosylation) or associated phenolic compounds. It appears that protein conformation bestows stability to these PR-proteins and that appropriate viticultural practices, rather than postharvest processing, may hold the key to controlling the concentrations of protein in wine.

Like grape proteins, polysaccharides also influence the clarification and stabilisation of must and wine. Polysaccharides, found in wines at levels between 300 and 1000 mg/l, originate in the grape itself, the fungi on the grape and the microorganisms present during winemaking. The main polysaccharides responsible for turbidity, viscosity and filter stoppages are pectins, glucans (a component of cellulose) and, to a lesser extent, hemicellulose (mainly xylans). Grape pectic substances are heteroplosaccharides consisting of partially methylated α-1,4-D-galacturonan chains linked to L-rhamnopyranose units carrying neutral side chains. Glucans such as β-1,3-1,6-glucan produced by the grey mould *Botrytis cinerea* in botrytised grape juice, comprise β-D-glucopyranose units with a high degree of polymerisation. Xylans are complex polymers consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl and glucuronosyl side chains. Enzymatic breakdown of pectic polymers occurs by the de-esterifying action of pectinesterase, releasing the metyl ester groups of the pectin molecule, and by the hydrolase or lyase action of the depolymerases (pectin lyase, pectate lyase and polygalacturonase), splitting the α-1,4-glycosidic linkages in the polygalacturonate chain. Glucans are hydrolysed by endoglucanases (β-1,4-D-glucan glucanohydrolase), exoglucanases (β-1,4-D-glucan cellobiohydrolase), cellodextrinases and cellobiases (β-1,4-D-glucoside glucohydrolase, a member of the β-glucosidase family). Enzymatic degradation of xylans is catalysed by the synergistic actions of endo-β-1,4-D-xylanases, β-D-xylosidases and α-L-arabinofuranosidases.

The endogenous pectinase, glucanase, xylanase and arabinofuranosidase activities of grapes and yeasts are often neither efficient nor sufficient under winemaking conditions to prevent polysaccharide hazes and filterstoppages. Industrial enzyme preparations are widely used to supplement these polysaccharide-degrading activities. Most commercial pectinase and glucanase preparations are derived from *Aspergillus* and *Trichoderma*, respectively.

Since the addition of these commercial enzyme preparations can be quite expensive, some researchers are looking at the native pectinases and glucanases of *S. cerevisiae*. Certain strains of *S. cerevisiae* were reported to produce pectinesterase, polygalacturonase and pectin lyase, while all strains of *S. cerevisiae* show some form of glucanase activity. All of these glucanase genes have been cloned and characterised. The *EXG1* (*BGL1*) gene encodes a protein whose differential glycosylation accounts for the two main extracellular exo-β-1,3-glucanases (EXGI and EXGII), while *EXG2* encodes a minor exo-β-1,3-glucanase (EXGIII). *BGL2* encodes a cell wall associated endo-β-1,3-glucanase, while *SSG1* (*SPR1*) codes for a sporulation-specific exo-β-1,3-glucanase.

Since these endogenous pectinolytic and glucanolytic activities of *S. cerevisiae* are not sufficient to avoid clarification and filtration problems, we have introduced a wide variety of heterologous pectinase, glucanase, xylanase and arabinofuranosidase genes into *S. cerevisiae*. A pectinolytic wine yeast was developed by co-expressing the *Erwinia chrysanthemi* pectate lyase gene (*pelE*) and the *Erwinia carotovora* polygalacturonase gene (*peh1*) in *S. cerevisiae*. Both these bacterial genes were inserted in the $ADH1_P$-$MF\alpha1_S$-$ADH1_T$ yeast expression-secretion cassette. The pectinase gene cassette, consisting of $ADH1_P$-$MF\alpha1_S$-*pelE*-$ADH1_T$ (designated *PEL5*) and $ADH1_P$-$MF\alpha1_S$-*peh1*-$ADH1_T$(designated *PEH1*) enabled wine yeast strains of *S. cerevisiae* to degrade polypectate efficiently. Likewise, our laboratory has also constructed a glucanase gene cassette comprising the *Butyrivibrio fibrisolvens* endo-β-1,4-glucanase gene (*END1*), the *Bacillus subtilis* endo-β-1,3-1,4-glucanase (*BEG1*), the *Ruminococcus flavefaciens* cellodextrinase gene (*CEL1*), the *Phanerochaeta chrysosporium* cellobiohydrolase gene (*CBH1*) and the *Saccharomycopsis fibuliger* cellobiase gene (*BGL1*). Upon introduction of this glucanase gene cassette, *S. cerevisiae* transformants were able to degrade glucans efficiently. We have also successfully

expressed in *S. cerevisiae* the endo-β-xylanase genes from *Aspergillus kawachii* (*XYN1*), *Aspergillus niger* (*XYN4* and *XYN5*) and *Trichoderma reesei* (*XYN2*) as well as the *Bacillus pumilus* xylosidase (*XLO1*) and the *A. niger* α-L-arabinofuranosidase gene (*ABF2*). The xlnA and xlnB genes from *Aspergillus nidulans* were also reported to be expressed in *S. cerevisiae*.

It is hoped that these efforts will lay the foundation for developing pectolytic, glucanolytic and xylanolytic wine yeasts that would contribute to the clarification of wine and replace or reduce the levels of commercial enzyme preparations needed. Furthermore, polysaccharide-degrading enzymes secreted by wine yeasts may also improve liquefaction of the grapes, thereby increasing the juice yield. Since much of the flavour compounds are trapped in the grape skins, pectolysis, glucanolysis and xylanolysis may release more of these aromatic compounds during skin contact in red wine fermentations and make a positive contribution to the wine bouquet.

Controlled cell sedimentation and flocculation

S. cerevisiae adapts its growth pattern in response to a wide range of physical and chemical signals sensed by the cells. These changes include yeast filamentation, agglomeration, flocculation and flotation, influenced by a variety of genetic, physiological and biochemical factors which are not always clearly understood.

Filamentous growth and the formation of pseudohyphae and hyphal-like structures often result in dimorphism, known to be affected by nutrient limitation and the availability of oxygen. The phenomenon of agglomeration involves an extensive, non-reversible cell aggregation process; flocculation refers to an asexual cellular aggregation when yeast cells adhere, reversibly, to one another to form microscopic flocs which sediment out of suspension. Yeast cell flotation, the converse of flocculation, defines the ability of non-aggregated yeast cells to trap CO_2 bubbles in a fermenting liquid and form a film or vellum at the top of fermentation vessels. All these phenomena are highly relevant to the production of several yeast-fermented products. Grittiness, caused by agglomerated baker's yeast strains and the concomitant appearance of granular material, is detrimental since it results in inadequate mixing into bread dough leading to limited leavening ability. Yeast flocculation, on the other hand, is often exploited in the production of lager beer and wine (especially bottle-fermented sparkling wine). The flocs that settle to the bottom of the fermentor by the end of the primary fermentation can easily be removed from the fermentation product, thereby allowing for rapid and efficient clarification and reduced handling of wine. Yeast flotation is important for the production of traditional ale beer by top-fermenting strains, and flor sherry by vellum-forming strains.

Flocculation in *S. cerevisiae* is thought to be mediated by specific calcium-activated lectins, the *FLO*-gene encoded flocculins which are surface glycoproteins capable of directly binding mannoproteins of adjacent cells. Proteinaceous 'hairy' protrutions called 'fimbrae' often emanate from the cell surface of flocculent *S. cerevisiae* cells. Cell surface charge and hydrophobicity have also been implicated in a primary or complementary role with lectins to facilitate the onset of flocculation. Environmental factors that may influence the level of flocculent *S. cerevisiae* strains include temperature, pH, calcium and zinc ions, certain inhibitors, oxygen content, sugar and inositol depletion, growth phase and cell density.

Several dominant, semi-dominant and recessive genes are known to be involved in flocculation, and distinct flocculation phenotypes have been identified based on their sensitivities to sugar inhibition and proteolylitic enzymes. These phenotypes, designated Flo and NewFlo, also display different sensitivities to yeast growth conditions, most notably temperature, acidity of the culture medium and glucose availablity. The flocculation genes include *FLO1, FLO2, flo3, FLO4, FLO5, flo6, flo7, FLO9, FLO10* and *FLO11/MUC1*. The *FLO1/MUC1* gene was also shown to be involved in pseudohyphal development and invasive growth, while *FLO8* was reported to encode a transcriptional activator of *FLO1* and *FLO11/MUC1*. Apparently, Flo8p inactivates the *TUP1* and CYC8/SSN6 encoded cascade which represses flocculation and pseudohyphal differentiation in certain strains. However, when the expression of *FLO1* and *FLO11/MUC1* was investigated in 25 commercial wine yeast strains, it was found that they are not co-regulated. Furthermore, it is unclear what the advantage would be to the yeast cell by co-regulating the expression of *FLO11/MUC1* and three glucoamylase-encoding genes (*STA1, STA2* and *STA3*) involved in starch metabolism. In fact, the unusually long (> 3 kb) promoter sequences of *FLO11/MUC1* and *STA1-3* are almost identical, and we have shown that several transcriptional activators (*e.g.*, Flo8p, Msn1p and Mss11p) co-regulate *FLO11/MUC1*-mediated filamentous growth and *STA1-STA3*-facilitated starch assimilation.

The overall structure of the *FLO11/MUC1*-encoded cell wall associated protein is similar to those of the Flo1p, Flo5p and Flo10p. All these flocculins comprise an amino-terminal domain containing a hydrophobic signal sequence and a carboxyl-terminal domain with homology to the glycosyl-phosphatidylinositol-anchor-containing proteins, separated by a central domain of highly repeated sequences rich in serine and threonine residues. Of all the flocculation genes, *FLO1* is considered to be the best studied and perhaps most important, capable of conferring flocculation when transformed into non-flocculent *S. cerevisiae* strains.

Regulated expression of the flocculation genes is important in wine production, because yeast must perform conflicting roles; during fermentation of grape must, a high suspended yeast count ensures a rapid fermentation rate, while at completion of sugar con-

version, efficient settling is needed to minimise problems with wine clarification. Moreover, flocculation has also been linked to enhanced ester production. For these reasons we have linked the *FLO1* gene to the *HSP30* gene promoter. It is known that the *HSP30* promoter induces high gene expression during late stationary phase. We have shown that the expression of *FLO1*, linked to the late-fermentation *HSP30* promoter, can be induced by a heat-shock treatment, confirming that controlled flocculation is indeed possible during fermentation.

Controlled cell flotation and flor formation

Flor sherry is produced using certain strains of *S. cerevisiae* (formerly known as *S. beticus* and *S. capensis*) capable of forming a yeast film on the surface (flor) of a base wine exposed to air. These strains are known for their high ethanol tolerance, superior film-forming ability and desirable oxidative metabolism. Flor sherry is characterised by a high ethanol (> 15%), low sugar and high aldehyde content. The typical nutty character of flor sherry can be ascribed to the partial oxidation of ethanol to acetaldehyde and to the specific contribution made by the flor strains of *S. cerevisiae.*

Although initial reports indicated that the vellum-forming trait segregated according to Mendelian rules in asci of sherry yeasts, it now seems unlikely that the flor trait is controlled by a single dominant gene. Several genes encoding cell wall associated, hydrophobic proteins have been implicated in vellum-formation. Since few yeasts capable of growth on wine are suitable for flor sherry production, the genotype of sherry yeasts is likely to be more complex than originally expected. However, once the most important genes responsible for film formation and the characteristic nutty bouquet have been identified, the relevant genetic and metabolic mechanisms that would allow for controlled vellum formation in flor sherry production may be brought to light.

Improvement of wine flavour and other sensory qualities

The single most important factor in winemaking is the organoleptic quality of the final product. A wine's bouquet is determined by the presence of desirable flavour compounds and metabolites in a well-balanced ratio, and the absence of undesirable ones.

Many variables contribute to the distinctive flavour of wine, brandy and other grape-derived alcoholic beverages. Grape variety, viticultural practices, and *terroir* affect vine development and berry composition, and exert major influences on the distinctiveness of wine and brandy flavour. Oenological practices, including the yeast and fermentation conditions, have a prominent effect on the primary flavours of *V. vinifera* wines. The volatile profile of wines is dominated by those components that are formed during fermentation, since these compounds are present in the highest concentrations. In brandy, the character is further changed as distillation alters the absolute and relative amounts of volatiles.

The flavour of wine and brandy immediately after fermentation or distillation only approximates that of the finished product. After the sudden and dramatic changes in composition during fermentation and distillation, chemical constituents generally react slowly during aging to move to their equilibria, resulting in gradual changes in flavour. The harmonious complexity of wine and brandy can subsequently be further increased by volatile extraction during oak barrel aging.

Despite the extensive information published on flavour chemistry, odour thresholds and aroma descriptions, the flavour of complex products such as wine and brandy cannot be predicted. With a few exceptions (*e.g.*, terpenes in the aromatic varieties and alkoxypyrazines in the vegetative or herbaceous cultivars), perceived flavour is the result of specific ratios of many compounds rather than being attributable to a single 'impact' compound. In wines and brandies, the major products of yeast fermentation, esters and alcohols, contribute to a generic background flavour, whereas subtle combinations of trace components derived from the grapes usually elicit the characteristic aroma notes of these complex beverages.

Enhanced liberation of grape terpenoids

Varietal flavour of grapes is mainly determined by the accumulation and profile of volatile secondary metabolites in *V. vinifera*. However, a high percentage of these metabolites occur as their respective, non-volatile *O*-glycosides. Several studies have shown that increased enzymatic hydrolysis of aroma precursors present in grape juice can liberate the aglycon to intensify the varietal character of wines. For instance, terpenols such as geraniol and nerol can be released from terpenyl-glycosides by the grape-derived β-D-glycosidase activity present in muscat grape juice. However, grape glycosidases are unable to hydrolyse sugar conjugates of tertiary alcohols such as linalool. Moreover, these grape enzyme activities are inhibited by glucose and exhibit poor stability at the low pH and high ethanol levels of wine. Thanks to these limiting characteristics of grape-derived glycosidases and the fact that certain processing steps during the clarification of must and wine profoundly reduce their activity, these endogenous enzymes of grapes have a minimal effect in enhancing varietal aroma during winemaking.

As an alternative to the inefficient grape glycosidases, aroma-liberating β-glucosidases from *Aspergillus* and other fungal species have been developed as components of commercial enzyme preparations to be added to fermented juice (as soon as the glucose has been consumed by the yeast) or to young wine. The addition of exogenous enzyme preparations to wine, however, is an expensive practice, and is viewed by

many purists as an 'artificial' or 'unnatural' intervention by the winemaker. This has led to renewed interest in the more active β-glucosidases produced by certain strains of *S. cerevisiae* and other wine associated yeasts such as *Candida*, *Hanseniaspora* and *Pichia* (formerly *Hansenula*) species.

Unlike the grape β-glycosidases, yeast β-glucosidases are not inhibited by glucose, and the liberation of terpenols during fermentation can be ascribed to their action on the terpenyl-glycoside precursors. Since these b-glucosidases are absent in most *S. cerevisiae* starter culture strains, we have functionally expressed the b-glucosidase gene (*BGL1*) of the yeast *Saccharomycopsis fibuliger* in *S. cerevisiae*. When the β-1,4-glucanase gene from *Trichoderma longibrachiatum* was expressed in wine yeast the aroma intensity of wine increased, presumably due to the hydrolysis of glycosylated flavour precursors. Likewise, we have overexpressed the *S. cerevisiae* exo-β-1,3-glucanase gene (*EXG1*) and introduced the endo-β-1,4-glucanase gene (*END1*) from *Butyrivibrio fibrisolvens*, the endo-β-1,3-1,4-glucanase (*BEG1*) from *Bacillus subtilis* and the α-arabinofuranosidase (*ABF2*) in *S. cerevisiae*. Further trials are underway to determine the effect of these transgenic yeasts on the varietal character of muscat wines.

Another intriguing discovery gives yeast the potential to modify the 'impact' compound profile of low-flavoured wines. Certain mutants of the yeast sterol biosynthetic pathway are able to produce monoterpenes (geraniol, citronelol and linalool) similar to those of the muscat grape cultivars.

Enhanced production of desirable volatile esters

During the primary or alcoholic fermentation of grape sugars, wine yeast produces ethanol, carbon dioxide and a number of by-products including esters, of which alcohol acetates and C_4-C_{10} fatty acid ethyl esters are found in the highest concentration in wine and brandy. Although these compounds are ubiquitous to all wines and brandies, the level of esters formed varies significantly. Apart from factors such as grape cultivar, rootstock and grape maturity, the ester concentration produced during fermentation is dependent on the yeast strain, fermentation temperature, insoluble material in the grape must, vinification methods, skin contact, must pH, the amount of sulphur dioxide, amino acids present in the must and malolactic fermentation. Furthermore, the ester content of distilled beverages is greatly dependent on whether the yeast lees is present during distillation.

The characteristic fruity odours of wine are primarily due to a mixture of hexyl acetate, ethyl caproate and caprylate (apple-like aroma), iso-amyl acetate (banana-like aroma), and 2-phenylethyl acetate (fruity, flowery flavour with a honey note). The synthesis of acetate esters such as iso-amyl acetate and ethyl acetate in *S. cerevisiae* is ascribed to at least three acetyltransferase activities: alcohol acetyltransferase (AAT), ethanol acetyltransferase (EAT) and iso-amyl alcohol acetyltransferase (IAT). These acetyltransferases are sulfhydryl (SH) enzymes which react with acetyl coenzyme A (acetyl-CoA) and, depending on the degree of affinity, with various higher alcohols to produce esters. It has also been shown that these enzymatic activities are strongly repressed under aerobic conditions and by the addition of unsaturated fatty acids to a culture.

The *ATF1*-encoded alcohol acetyltransferase activity (AAT) is the best-studied acetyltransferase in *S. cerevisiae*. It has been reported that the 61-kDa *ATF1* gene product (Atf1p) is located within the yeast's cellular vacuomes, and that it plays a major role in the production of iso-amyl acetate and to a lesser extent ethyl acetate during beer fermentation. To investigate the role of AAT in wine and brandy composition we have cloned the *ATF1* gene from a widely used commercial wine yeast strain (VIN13) and placed it under control of the constitutive yeast phosphoglycerate kinase gene (*PGK1*) promoter and terminator. Integration of this modified copy of *ATF1* into the genomes of three commercial wine yeast strains (VIN7, VIN13 and WE228) resulted in the overexpression of AAT activity and increased levels of ethyl acetate, iso-amyl acetate and 2-phenylethyl acetate in wine and distillates. The concentration of ethyl caprate, ethyl caprylate and hexyl acetate showed only minor changes, whereas the acetic acid concentration decreased by more than half. These changes in the wine and distillate composition had a pronounced effect on the solvent/chemical (associated with ethyl acetate and iso-amyl acetate), herbaceous and heads-associated aroma of the final distillate and the solvent/chemical and fruity/flowery character of Chenin blanc wines. This study established the concept that the over-expression of acetyltransferase genes such as *ATF1* could profoundly affect the flavour profiles of wines and distillates deficient in aroma, thereby paving the way for the production of products maintaining a fruitier character for longer periods after bottling.

Optimised fusel oil production

Alcohols with carbon numbers greater than that of ethanol, such as isobutyl, isoamyl and active amyl alcohol, are termed fusel oil. These higher alcohols are produced by wine yeasts during alcoholic fermentation from intermediates in the branched chain amino acids pathway leading to production of isoleucine, leucine and valine by decarboxylation, transamination and reduction. At high concentrations these higher alcohols have undesirable flavour and odour characteristics. Higher alcohols in wines, however, are usually present at concentration levels below their threshold values and do not affect the taste of wine unfavourably. In some cases, they may even contribute to wine quality. However, since higher alcohols are concentrated by the distilling process, their reduction in wines that are to be distilled for brandy production is of great importance.

Initial attempts to use Ile$^-$, Leu$^-$ and Val$^-$ auxotrophic mutants succeeded in lowering the levels of isobutanol, active amyl alcohol and isoamyl alcohol production in fermentations, but these mutants were of no commercial use as their growth and fermentation rates were compromised. A Leu$^-$ mutant derived from the widely used Montrachet wine yeast was reported to produce more than 50% less isoamyl alcohol during fermentation than the prototrophic parent. It will be of great interest to see whether integrative disruption of specific *ILE*, *LEU* and *VAL* genes of wine yeasts will result in lower levels of fusel oil in wine for distillation.

Enhanced glycerol production

Due to its non-volatile nature, glycerol has no direct impact on the aromatic characteristics of wine. However, this triol imparts certain other sensory qualities; it has a slightly sweet taste, and owing to its viscous nature, also contributes to the smoothness, consistency and overall body of wine.

The amount of glycerol in wines depends on many factors: grape variety, nitrogen composition, degrees of ripeness (sugar levels) and mould infection (during which glycerol is produced), sulfite levels and pH of grape must, fermentation temperature, aeration, and choice of starter culture strain and inoculation level. Typically, under controlled conditions glycerol concentrations are higher in red wines than in white wines, varying from 1 to 15 g/l. The threshold taste level of glycerol is observed at 5.2 g/l in wine, whereas a change in the viscosity is only perceived at a level of 25 g/l. Wine yeast strains overproducing glycerol would therefore be of considerable value in improving the organoleptic quality of wine.

In addition, the overproduction of glycerol at the expense of ethanol could fulfil a growing need for table wine with lower levels of ethanol. About 4 to 10% of the carbon source is usually converted to glycerol, resulting in glycerol levels of 7 to 10% of that of ethanol. Redirecting more of the grape sugars to glycerol would provide a desirable alternative to the current physical ethanol-removing processes that non-specifically alter the sensorial properties of the final product. Conversely, wine yeasts in which the glycerol pathway has been minimised would yield more alcohol, which would be of great value for the production of brandy and other distilled products.

The physiological functions of glycerol synthesis are related to redox balancing, resistance to hyperosmotic and oxidative stress, recycling of cytosolic inorganic phosphate and nitrogen metabolism. Futhermore, glycerol-3-phosphate, the precursor of glycerol, is an essential intermediate in the biosynthesis of membrane lipid. It is also noteworthy that glycerol is not only produced by yeasts, but can also serve as carbon source in aerobically grown cultures.

During wine fermentations, the main role of glycerol synthesis is to supply the yeast cell with an osmotic stress responsive solute and to equilibrate the intracellular redox balance by converting the excess NADH generated during biomass formation to NAD$^+$. Glycerol formation entails the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, a reaction catalysed by glycerol-3-phosphate dehydrogenase and followed by the dephosphorylation of glycerol-3-phosphate to glycerol by glycerol-3-phosphatase. Two cytosolic glycerol-3-phosphate dehydrogenases, considered the key limiting enzymes for glycerol formation in wine, are encoded by *GPD1* and *GPD2*. The expression of *GPD1* is usually increased by hyperosmotic stress, whereas *GPD2* expression is increased by anaerobic conditions. The level of glycerol in *S. cerevisiae* and the expression of both these genes are partially controlled by the HOG (High Osmotic Glycerol) signal transduction pathway when cells are exposed to hyperosmotic stress.

Conversely, the utilisation of glycerol is coupled to respiration via a glycerol kinase. This *GUT1*-encoded glycerol kinase converts glycerol to glycerol-3-phosphate, which is then oxidised to dihydroxyacetone phosphate by the *GUT2*-encoded, flavin-dependent and membrane-bound mitochondrial glycerol-3-phosphate dehydrogenase. *GUT2* is strongly repressed in the presence of glucose. *FPS1* that encodes a channel protein belonging to the MIP family, was shown to act as a glycerol transport facilitator controlling both glycerol influx and efflux.

Slight increases in glycerol production in wine can be achieved by using yeast strains selected or bred for high glycerol production, and by optimising fermentation conditions. More recently it was reported that the overexpression of *GPD1*, together with constitutive expression of *FPS1*, successfully redirected the carbon flux towards glycerol and the extracellular accumulation of glycerol. Depending on the genetic background in these engineered strains, 1.5 to 4-fold increases in glycerol levels were obtained. As a result of redox imbalances resulting from glycerol overproduction, ethanol formation was decreased and the metabolite pattern of these recombinant wine yeasts was considerably changed. A lower biomass concentration was attained in the *GPD1*-overexpressing strains, probably due to high acetaldehyde production during the growth phase. Interestingly, the fermentation rate during the stationary phase of wine fermentation was stimulated in these strains, suggesting that the availability of NAD may be a factor controlling the rate of glycolytic flux. Other side-effects of these glycerol-overproducing yeasts included the accumulation of by-products such as pyruvate, acetate, acetoin, 2,3-butanediol and succinate.

A method was recently devised to overcome the most negative side-effect of glycerol overproduction, namely a marked increase in acetate formation. Since acetaldehyde dehydrogenases were shown to play a prominent role in acetate formation, the *ALD6* and

ALD7 genes encoding a cytosolic Mg^{2+}-activated, NADP-dependent and a mitochondrial K^{+}-activated, NAD(P)-dependent acetaldehyde dehydrogenase, respectively, were disrupted. A wine yeast strain in which *GPD1* was overexpressed in conjunction with the deletion of *ALD6* produced 2- to 3-fold more glycerol and a similar amount of acetate compared to the untransformed strain. The redox balance was maintained in these recombinant wine yeasts by increasing the formation of succinate and 2,3-butanediol to concentrations remaining in the range of that found in wine. These yeasts offer new prospects to improve the quality of wine lacking in smoothness and body, and to production of low-alcohol wines.

Bio-adjustment of wine acidity

The acidity of grape juice and wine plays an important role in many aspects of winemaking and wine quality, including the sensory quality of the wine and its physical, biochemical and microbial stability. The juice and wine acidity, in particular the pH, has a profound influence on the survival and growth of all microorganisms; the effectiveness of anti-oxidants, antimicrobial compounds and enzyme additions, the solubility of proteins and tartrate salts, the effectiveness of bentonite treatment, the polymerisation of the colour pigments, and the oxidative and browning reactions. Wine contains a large number of organic and inorganic acids. The predominant organic acids are tartaric and malic acid, accounting for 90% of the titratable acidity of grapes. The main features of wine acidity include the acids themselves, the extent of their dissociation, the titratable acidity and pH. Factors affecting the pH and titratable acidity of grapes include soil potassium and soil moisture; the nature of the rootstock and characteristics of the root system; viticultural practices such as canopy management and irrigation; climatic conditions and prevailing temperature during ripening; the cultivar and final berry volume at harvest. Of all these factors the climatic conditions and ambient temperature have a critical effect on grape maturation and resulting acidity of the fruit. Under certain climatic conditions, the development of acidic compounds in the grape during maturation and the subsequent physical and microbial modification of these compounds during the process of winemaking can cause imbalances in the acidity of wines. Unless the acidity of such wines with suboptimal pH values is adjusted, the wines will be considered as unbalanced or spoilt. In cooler climates (northern Europe, Canada, north-eastern USA) chemical adjustment generally means a reduction in titratable acidity by physicochemical practices such as blending, chemical neutralisation by double salting (addition of calcium carbonate) and precipitation. These procedures often reduce wine quality and require extensive labour and capital input.

In the warmer viticultural regions of southern Europe, California, South Africa and Australia, blessed with adequate sunshine during the growing season and grape ripening period, malic acid is catabolised at a faster rate. Here, adjustment of wine acidity generally entails increasing the titratable acidity, or more critically, lowering the pH by the addition of tartaric acid, and sometimes malic and citric acids, depending on the laws of the country. Since the addition of calcium carbonate and acids are highly contentious practices that sometimes affect free trade in wine, several laboratories explored biological alternatives in order to minimise such chemical intervention.

At present, biodeacidification of wine is mediated by lactic acid bacteria, in particular *Oenococcus oeni* (formerly *Leuconostoc oenos*). During malolactic fermentation, L-malic acid is decarboxylated to L(+)-lactic acid and carbon dioxide. Malolactic fermentation not only reduces the total acidity of wine, it also enhances microbiological stability and presumably improves the organoleptic quality of wine. However, owing to nutrient limitation, low temperature, acidic pH, and high alcohol and sulphur dioxide levels, the malolactic bacteria often grow poorly in wine, thereby complicating the management of this process. Stuck or sluggish malolactic fermentation often leads to spoilage of wine. Several alternatives were explored, including the possible use of malate-degrading yeasts. During malo-ethanolic fermentations conducted by the fission yeast *Schizosaccharomyces pombe,* malate is effectively converted to ethanol but off-flavours were produced. Attempts to fuse wine yeasts with malate-assimilating yeast also failed. The application of high density cell suspensions of several yeasts including *S. cerevisiae,* in an effort to increase the rate at which L-malate was degraded during fermentation, was unsuccessful.

Their lack of success forced investigators back to the wine yeast itself. The ability of *S. cerevisiae* strains to assimilate L-malate acid varies widely. Unlike *S. pombe*, *S. cerevisiae* lacks an active malate transport system and L-malate enters wine yeast by simple diffusion. Once inside the cell, *S. cerevisiae*'s own constitutive NAD-dependent malic enzyme converts L-malate to pyruvate, which, under anaerobic conditions, will be converted to ethanol and carbon dioxide. Aerobically, malic acid is decarboxylated into water and carbon dioxide. Although the biochemical mechanism for malate degradation in *S. cerevisiae* is the same as in *S. pombe*, the substrate specificity of the *S. cerevisiae* malic enzyme is about 15-fold lower than that of the *S. pombe* malic enzyme. This low substrate specificity together with the absence of an active malate transport system is responsible for *S. cerevisiae*'s inefficient metabolism of malate.

Genetic engineering of wine yeast to conduct alcoholic fermentation and malate degradation simultaneously has been explored by several groups. In order to engineer a malolactic pathway in *S. cerevisiae* the malolactic genes (*mle*S) from *Lactococcus lactis*, *Lactobacillus delbrueckii* and the *mle*A gene from *O. oeni* were cloned and expressed in *S. cerevisiae*. The *mle*S gene encodes a NAD-dependent malolactic

enzyme that converts L-malate to L-lactate and carbon dioxide. However, due to the absence of an active malate transport system in *S. cerevisiae*, these engineered strains could still not metabolise malate efficiently. Efficient malolactic fermentation was achieved only when the *L. lactis mle*S gene was co-expressed with the *S. pombe mae1* gene encoding malate permease.

Similarly, an efficient malo-ethanolic *S. cerevisiae* was constructed by co-expressing *mae1* permease gene and the *mae2* malic enzyme gene from *S. pombe* in *S. cerevisiae*. A functional malolactic wine yeast could replace the unreliable bacterial malolactic fermentation, whereas a malo-ethanolic strain of *S. cerevisiae* would be more useful for the production of fruity floral wines in the cooler wine-producing regions of the world.

Conversely, acidification of high-pH wines produced in the warmer regions with a wine yeast would be an inexpensive and convenient biological alternative. The formation of high levels of L(+) lactic acid by *S. cerevisiae* during alcoholic fermentation would be useful for reducing the pH. In addition to its acidification properties, L(+) lactic acid, the main product of the metabolism of lactic acid bacteria, is stable. Due to its pleasant acidic flavour and its properties as a preservative, lactic acid is widely used as a food acidulant. Moreover, it is naturally present in most fermented products, including wine, where it may be present in amounts of up to 6 g/l after malolactic fermentation.

Due to the inefficiency of the mitochondrial lacticodehydrogenases under fermentation conditions, natural *S. cerevisiae* strains produce only traces of lactic acid during alcoholic fermentation. In an attempt to redirect glucose carbon to lactic acid in *S. cerevisiae,* the lacticodehydrogenase-encoding genes from *Lactobacillus casei* and bovine were expressed in laboratory yeast strains. Encouraged by the fact that the *L. casei* lacticodehydrogenase gene, expressed under control of the yeast alcohol dehydrogenase gene, converted 20% of the glucose into L(+) lactic acid, this construct was also introduced into eight wine yeast strains. Wines obtained with these engineered lactic acid-alcoholic fermentation yeasts were shown to be effectively acidified, but the fermentation rate was slower.

Elimination of phenolic off-flavour

Excessive amounts of volatile phenols such as 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol often confer undesirable organoleptic attributes on wine. These phenolic off-flavours can be described as smoky, woody, clove-like, spicy and medicinal. The *POF1* gene in some strains of *S. cerevisiae* encodes a substituted cinnamic acid carboxylase that is able to decarboxylate grape hydroxycinnamic acids in a nonoxidative fashion to vinylphenols. Perhaps the disruption of *POF1* could provide a way to reduce the content of volatile phenols in, at least, white wines.

Reduced sulphite and sulphide production

Owing to their high volatility, reactivity and potency at very low threshold levels, sulphur-containing compounds have a profound effect on the flavour of wine. These substances are formed in grapes during ripening; dusting of vines with fungicides containing elemental sulphur provide another source. During the winemaking process sulphite is deliberately added to most wines as an antioxidant and antimicrobial agent. Health concerns and an unfavourable public perception of sulphite have led to demands for restriction of its use and reassessment of all aspects of sulphite accumulation in wine. Consequently, the production of sulphur-containing compounds by wine yeast itself has become a focal point of research.

Sulphur is essential for yeast growth and *S. cerevisiae* can use sulphate, sulphite and elemental sulphur as sole sources. The formation of sulphite and sulphide by wine yeasts greatly affects the quality of wine. Unlike sulphur dioxide (SO_2), which when properly used, has some beneficial effects, hydrogen sulphide (H_2S) is one of the most undesirable of yeast metabolites, since it causes, above threshold levels of 50-80 g/l, an off-flavour reminiscent of rotten eggs. Sulphite is only formed from sulphate, while sulphide is formed from sulphate, sulphite, from elemental sulphur applied as a fungicide, and from cysteine. The formation of sulphite and sulphide is affected by many factors, including the composition of the fermentation medium.

Apart from strain effect, the nutrient composition of grape juice, the concentration of sulphate, must clarification, the initial pH and temperature all affect sulphite formation by wine yeasts. Defects in sulphate uptake and reduction, which is normally regulated by methionine via its metabolites methionyl-tRNA and *S*-adenosylmethionine, can result in excessive sulphite production. During investigations into the regulation of sulphur metabolism in high and low sulphite-producing wine yeast strains, considerable differences in the levels of activity of sulphate permease, ATP-sulphurylase and sulphite reductase were reported. Sulphate permease, mediating the uptake of sulphate by the yeasts, was shown not to be repressed by methionine in high sulphite-producing strains. ATP-sulphurylase and ADP-sulphurylase are not regulated by sulphur intermediates in high or low sulphite-producing strains. Unlike the high sulphite-producing strains, the low sulphite-producing strains showed an increased biosynthesis of NADPH-dependent sulphite reductase, *O*-acetylserine sulphydrylase and *O*-acetylhomoserine sulphydrylase during the exponentional growth phase in the presence of sulphate, sulphite and djencolic-acid. Methionine and cysteine are known to prevent an increase in the levels of sulphite reductase, *O*-acetylserine sulphydrylase and *O*-acetylhomoserine sulphydrylase.

Since sulphite production is very energy dependent, the cellular metabolism of high SO_2-forming yeast strains is reduced, explaining the decreased production of biomass and slow fermentation rate.

The formation of H_2S by yeast during fermentation is largely in response to nutrient depletion, especially assimilable nitrogen and possibly certain vitamins such as pantothenate or pyridoxine. In the absence of the H_2S sequestering molecules *O*-acetylserine and *O*-acetylhomoserine, as caused by nitrogen starvation, free H_2S accumulates and diffuses from the cell. Depending on soil type and vintage conditions, some grape varieties (*e.g.*, Riesling, Chardonnay and Syrah), tend to have a low nitrogen content. This problem can usually be suppressed by the addition of nitrogen (typically in the form of diammonium phosphate) during active fermentation. However, it has been reported that impaired membrane transport function and intracellular deficiency of certain vitamins can also cause H_2S accumulatation.

The amount of H_2S produced can also be affected by the addition of a high level of SO_2 to the must shortly before inoculating with yeast, and by the strain of yeast involved. Certain yeasts more readily reduce sulphate and SO_2 to H_2S when deprived of nitrogen, in a futile effort to synthesise and supply sulphur-containing amino acids to the growing yeast cell. The addition of ammonium salts prevents H_2S accumulation in wine, not by stopping its formation but by enabling the yeast to synthesise amino acid precursor compounds which react with H_2S to form sulphur-containing acids. Due to higher fermentation temperatures in hot climate red wine production, yeast cells use more nitrogen during rapid fermentations and tend to develop sulphidic smells. Fortunately, H_2S is highly volatile and can usually be removed by the stripping action of CO_2 produced during these rapid high-temperature fermentations. However, H_2S formed towards the end of, or after, fermentation can react with other wine components to form mercaptans, thiols and disulphides, which have pungent garlic, onion and rubber aromas.

Yeast strains differ widely in their ability to produce sulphite and sulphide. One way to take advantage of this fact is to select or develop a wine yeast strain that will either produce less H_2S or that will retain most of the H_2S produced intracellularly. It was amply demonstrated in several laboratories that yeast strains with low H_2S production and improved winemaking properties can be bred by hybridisation. In addition to exploiting the genetic heterogeneity in sulphite and sulphide formation, the deliberate introduction of mutations in certain enzymes of the sulphur, sulphur amino acids, pantothenate and pyridoxine pathways might well enable a stepwise elimination of these characteristics in wine yeasts. The *MET3* gene encoding ATP sulphurylase (the first enzyme in the conversion of intracellular sulphate to sulphite) has been cloned and shown to be regulated at the transcriptional level. This may lead to the elucidation of sulphite and sulphide formation by wine yeasts. H_2S production also appears to be closely related to the activity of sulphite reductase and this could also provide a target for down regulation of H_2S formation in wine yeasts.

Improvement of wine wholesomeness and health properties of wine

Until the 18th century wine played a pivotal role in medical practice, not least because it was a safer drink than most available water. Thanks to its alcohol and acid content, wine inhibits the growth of many spoilage and pathogenic microorganisms. By the second half of the 20th century, though, alcohol consumption, including wine drinking, had become a target of some health campaigners, who, with some success, demanded warning labels on wine bottles. By the 90's medical science was reporting that moderate consumption of especially red wine, can reduce the incidence of heart disease. Today, it is generally accepted that moderate wine drinking can be socially beneficial, and that it can be effective in the management of stress and reducing the risk of coronary heart disease. The prudent wine drinker, however, continues to keep a close eye on what and how he or she drinks to ensure that the benefits exceed the risks. The worldwide decrease of alcohol consumption testifies to this effect.

In developing wine yeast strains, it is therefore of the utmost importance to focus on these health aspects and to develop yeasts that may reduce the risks and enhance the benefits. It is therefore no surprise that, since glycerol and ethanol are inversely related, part of the objective in developing glycerol-overproducing *S. cerevisiae* strains is to reduce alcohol content in the end product. Likewise, research in several laboratories around the world is directed towards the elimination of suspected carcinogenic compounds in wine such as ethyl carbamate, and asthmatic chemical preservatives such as sulphites.

It might even be possible to develop wine yeasts that could increase the levels of phenolic and antioxidative substances (*e.g.*, resveratrol) associated with the so-called 'French paradox', in which, despite the high dietary fat intake of the cheese-loving population of southern France, the death rate from coronary heart disease is significantly lower than in comparable industrialised countries. Several possible explanations have been offered, but the best case for resolving this paradox has been made for red wine phenolics that chemically modify blood lipoproteins in cholesterol-furred arteries.

Resveratrol production

Phytoalexins, including stilbenes such as resveratrol, have been shown to reduce the risk of coronary heart disease. By acting as an antioxidant and as an antimutagen, resveratrol shows cancer chemopreventive activity, as well as the ability to in-

duce specific enzymes which metabolise carcinogenic substances.

Stilbenes are secondary plant products produced through the phenylalanine/ polymalonate pathway. Resveratrol is a stress metabolite produced by *V. vinifera* during fungal infection, wounding or ultra-violet radiation. Resveratrol is synthesised particularly in the skin cells of grape berries and only traces are found in the fruit flesh. Red wine therefore contains a much higher resveratrol concentration than white wine due to skin contact during the first phase of fermentation.

One way to increase the levels of resveratrol in both red and white wine is to develop wine yeasts able to produce resveratrol during fermentation. To achieve this goal, the phenylpropanoid pathway in *S. cerevisiae* will have to be modifed to produce *p*-coumaroyl-coenzyme A, one of the substances for resveratrol synthesis. This can be done by introducing the phenylalanine ammonia-lyase gene (*PAL*), cinnamate 4-hydroxylase gene (*D4H*) and the coenzyme A ligase gene (*4CL216*) in *S. cerevisiae*. The introduction of the grape silbene synthase gene (*Vst1*) may then catalyse the addition of three acetate units from malonyl-coenzyme A, already found in yeast, to *p*-coumaryl-coenzyme A, resulting in the formation of resveratrol. At this stage, however, there is little indication of the chances for success in developing resveratrol-producing wine yeast strains.

Reduced formation of ethyl carbamate

Ethyl carbamate (also known as urethane) is a suspected carcinogen that occurs in most fermented foods and beverages. Given the potential health hazard, there is a growing demand from consumers and liquor control authorities to reduce the allowable limits of ethyl carbamate in wines and related products. Although young wines do not contain measurable levels (< 10 μg/l) of ethyl carbamate, the required precursors are present which can generate a considerable amount of this mutagenic compound when wine is aged or stored at elevated temperatures. High-alcohol beverages such as sherries, dessert wines and distilled products also tend to contain much higher levels of ethyl carbamate than table wine. It is believed that ethyl carbamate forms in aging wines, fortified wines and brandies by reaction between urea and ethanol. For this reason excessive application of urea-containing fertilisers to vines and spraying of urea shortly before harvest to remove leaves are not recommended. Furthermore, the use of urea-containing nutrient supplements for yeast during wine fermentations to avoid stuck or sluggish fermentations is also prohibited. Apart from these factors that could lead to high urea levels and concomitant transgression of ethyl carbamate limits, *S. cerevisiae* strains also vary widely with regard to their urea-forming ability.

In *S. cerevisiae* urea is formed during the breakdown of arginine, one of the main amino acids in grape juice, by the *CAR1*-encoded arginase. During this reaction, arginine is converted to ornithine, ammonia and carbon dioxide, while urea is formed as an intermediate product. Certain yeast strains secrete urea into wine and, depending on fermentation conditions, may be unable to further metabolise the external urea. Although all *S. cerevisiae* strains secrete urea, the extent to which they re-absorb the urea differs. *S. cerevisiae* secretes more urea at higher fermentation temperatures, whereas high ammonia concentrations suppress the re-absorption of urea by the yeast. It is therefore important to inoculate grape must with a low-urea producing wine yeast strain when the juice has a high arginine content.

Strain selection is only one way of reducing the accumulation of urea in wine. As an alternative means of curbing ethyl carbamate formation in the end product, successive disruption of the *CAR1* arginase gene in an industrial saké yeast proved to be successful in eliminating urea accumulation in rice wine. This arginase deletion mutation resulted in a yeast strain that could not metabolise arginine but it also impeded growth, thereby limiting the commercial use of such a strain.

Another possibility is adding commercial preparations of acidic urease, enabling the hydrolysis of urea in wine. This practice has recently been approved by the OIV and is used in some wine-producing countries to lower ethyl carbamate levels in their wines and related products. A less expensive route to lower levels of ethyl carbamate would be to develop a wine yeast that produces an extracellular, acidic urease. In one such attempt a novel urease gene was constructed by fusing the α, β and γ subunits of the *Lactobacillus fermentum* urease operon. In addition, jack bean urease linker sequences were inserted between the α and β, as well as the β and γ subunits. Both gene constructs were successfully expressed under the control of the *S. cerevisiae PGK1* promoter and terminator signals in the yeasts *S. cerevisiae* and *S. pombe*. Although the level of transcription in *S. cerevisiae* was much higher than in *S. pombe*, the secretion of urease peptides was extremely low. Unlike the *S. pombe* urease, the *S. cerevisiae*-derived urease was unable to convert urea into ammonia and carbon dioxide. The absence of recombinant urease activity in transformed *S. cerevisiae* cells is probably due to the lack of the essential auxiliary proteins present only in urease-producing species such as *S. pombe*. Without these proteins, *S. cerevisiae* is unable to assemble the various subunits into an active urease. It seems, therefore, that accessory genes of *L. fermentum* will also have to be cloned and expressed in addition to the structural urease genes to enable *S. cerevisiae* to express an active urease.

Improved biological control of wine spoilage microorganisms

Uncontrolled microbial growth before, during or after wine fermentation can alter the chemical compo-

sition of the end product, detracting from its sensory properties of appearance, aroma and flavour. In severe cases of microbial spoilage the wine becomes unpalatable. Owing to the high initial sugar content, low pH, anaerobic fermentation conditions and high alcohol levels at the end of fermentation, only a few spoilage yeasts and bacteria can survive the strong selective pressures present in fermenting grape must and in wine.

Moulds usually spoil wine by infecting the grapes or spoiling cork slabs. These include species of *Penicillium, Anahanocladium, Mucor, Monilia, Trichoderma, Oidiodendron, Botrytis, Rhizopus, Cladosporium* and *Paecilomyces*. *Penicillium glabrum* is considered the major mould on cork slabs, while some strains of *Botrytis cinerea* are associated with grey rot ('pourriture grise') of grapes. They confer mouldiness and cork taints to wine. This earthy, musty, sometimes mushroom-like aroma is associated with the presence of 2,4,6-trichloroanisole in bottled wine.

Spoilage yeasts include species from *Brettanomyces*, the osmotolerant yeast *Zygosaccharomyces* and the film-forming yeast species *Pichia* and *Candida*. *Brettanomyces intermedius* is known to produce haze, turbidity, volatile acidity and a mousy taint; *Zygosaccharomyces baillii* causes turbidity after refermentation during storage of wine or after bottling, resulting in sediment formation and reduction in acidity. Wines spoiled by *Pichia membranaefaciens* and *Candida krusei* taste oxidised and less acid.

Without underestimating the degree of wine spoilage that can be caused by moulds and yeasts, it is widely accepted that bacteria are the primary culprits, especially acetic acid and lactic acid bacteria. A vinegary taint in wine is often associated with the activity of acetic acid bacteria such as *Acetobacter aceti*, *Acetobacter pasteurianus* and *Gluconobacter oxydans*. Although some lactic acid bacteria play a key role in the malolactic fermentation of wine, others may cause serious faults. Excessive volatile acidity, mannitol taint, ropiness, mousiness, acrolein formation and bitterness, tartaric acid degradation, diacetyl overproduction and rancidness, as well as the very unpleasant geranium off-flavour are often the consequence of uncontrolled growth of some species of *Lactabacillus* (*e.g.*, *L. brevis*, *L. hilgardii*, *L. plantarum*), *Leuconostoc* (*e.g.*, *L. mesenteroides*), *Streptococcus* (*S. mucilaginosus*) and *Pediococcus* (*e.g.*, *P. cerevisiae*).

Healthy grapes, cellar hygiene and sound oenological practices (*e.g.*, appropriate pH, fermentation temperature, filtration, application of fining agents, *etc.*) will remain the corner stones of the winemaker's strategy against uncontrolled proliferation of spoilage microbes. But the use of efficient *S. cerevisiae* and *O. oeni* starter cultures at appropriate inoculation levels will usually outcompete undesirable contaminants, thereby limiting the risk of poor quality wine and concomitant financial loss. For additional safety, chemical preservatives such as sulphur dioxide and dimethyl dicarbonate are commonly added to control the growth of unwanted microbial contaminants. However, the excessive use of these chemical preservatives is deleterious to the quality of wine and related fortified and distilled products, and is confronted by mounting consumer resistance.

Consumer concerns have spurred a worldwide search for safe, food-grade preservatives of biological origin. A major focus of these investigations into novel biopreservatives includes the identification and application of effective antimicrobial enzymes (*e.g.*, lysozyme) and peptides (*e.g.*, zymocins and bacteriocins). These efforts have been encouraged by the successful application of lysozyme and nisin to protect beer, wine and fruit brandies from spoilage lactic acid bacteria. But wine is a market-sensitive commodity, and large scale industrial application of purified antibacterial enzymes and bacteriocins is expensive, resulting in an increase in retail costs, as observed in the case of beer production. This may be overcome by developing wine yeast starter culture strains producing appropriate levels of efficient antimicrobial enzymes and peptides.

Wine yeasts producing antimicrobial enzymes

Antimicrobial enzymes are ubiquitous in nature, playing a pivitol role in the defense mechanisms of host organisms against infection by fungi and bacteria. Hydrolytic antimicrobial enzymes such as chitinases, β-glucanases and lysozyme function by degrading key structural components of the cell walls of moulds and bacteria. Chitinases and β-glucanases synergistically attack the main components of fungal cell walls, chitin and β-1,3-glucan. Lysozyme, an *N*-acetylhexosaminidase, lyses the cell walls of certain Gram-positive species of bacteria lacking an outer membrane by hydrolysing the β-1,4-glucosidic linkages of peptidoglycan in the cell wall. Its alkaline nature contributes to the antibacterial activity of lysozyme. Furthermore, Gram-negative bacteria containing an outer membrane are more sensitive to lysozyme in combination with a chelating agent such as EDTA or when lysozyme is modified by perillaldehyde. Conjugation to galactomanan also increases the potency of lysozyme towards Gram-negative bacteria by enabling diffusion of the enzyme across the outer membrane of the target cell.

The OIV has recently approved the use of commercial lysozyme preparations to control malolactic fermentation and to stabilise wine afterwards. However, the general use of lysozyme in winemaking is limited because of its low cost-efficiency. This has encouraged efforts to develop lysozyme-producing *S. cerevisiae* strains. The lysozyme-encoding gene from chicken egg white was successfully expressed in *E. coli* and *S. cerevisiae*. In *E. coli*, the bactericidal action of the recombinant lysozyme against Gram-negative bacteria was enhanced when a pentapeptide was inserted into C-terminus. Research is underway

to express a modified lysozyme gene in wine yeast that would avoid hyperglycosylation and broaden its activity to effectively eliminate spoilage by lactic and acetic acid bacteria.

Wine yeasts producing antimicrobial peptides

The killer phenomenon is wide spread among grape, must and wine related yeast genera, including *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Kloeckera, Kluyveromyces Pichia, and Rhodutorula*. Most zymocidal strains of *S. cerevisiae* associated with wine fermentation produce the K_2 or K_{28} zymocins which are functional at the low pH of grape must and wine. Zymocidal yeast contaminants are implicated as one of the causes of sluggish or stuck fermentations, but they are also promoted for inhibiting the proliferation of unwanted yeast contaminants. However, their efficacy under winemaking conditions has yet to be demonstrated. Furthermore, zymocins produced by *S. cerevisiae* are lethal only to sensitive strains of *S. cerevisiae*, whereas those produced by non-*Saccharomyces* species may be toxic to *S. cerevisiae* as well as non-*Saccharomyces* species.

Several attempts have been made over the years to expand the zymocidal activity of *S. cerevisiae* so that it could also eliminate other yeast contaminants. In some instances different killer types of *S. cerevisiae* were hybridised by mating, cytoduction and spheroplast fusion, while in another case a DNA copy of the K_1 dsRNA was introduced in a K_2 strain of *S. cerevisiae*. However, even a K_1/K_2 double killer *S. cerevisiae* is very limited as to the variety of yeast contaminants that can be eliminated. Rather attention is now focused on the identification of genes encoding more effective zymocins in other yeasts such as *Pichia* and *Hanseniaspora* and their possible introduction into *S. cerevisiae*.

We have investigated the feasibility of controlling spoilage bacteria during wine fermentations by engineering bactericidal strains of *S. cerevisiae*. To test this novel concept, we have succesfully expressed two bacteriocin genes in yeast, the one encoding a pediocin and the other a leucocin. The pediocin gene originates from *Pediococcus acidilactici* PAC1·0 and the leucocin gene from *Leuconostoc carnosum* B-Ta11a.

The pediocin operon of *P. acidilactici* consists of four clustered genes, namely *pedA* (encoding a 62 amino acid precursor of the PA-1 pediocin), *pedB* (encoding an immunity factor), *pedC* (encoding a PA-1 transport protein) and *pedD* (encoding a protein involved in the transport and processing of PA-1). The leucocin operon of *L. carnosum* comprises two genes: *lcaB* (encoding a 61 amino acid precursor of the B-Ta11a leucocin) and $lcaB_1$ (encoding a 113 amino acid immunity factor. Both the *P. acidilactici pedA* and *L. carnosum lcaB* genes were inserted into a yeast expression-secretion cassette and introduced as multicopy episomal plasmids into laboratory strains of *S. cerevisiae*. Northern blot analysis confirmed that the *pedA* and *lca1* structural genes in these constructs ($ADH1_P$-$MF\alpha1_S$-*pedA*-$ADH1_T$, designated *PED1* and $ADH1_P$-$MF\alpha1_S$-*lcaB*-$ADH1_T$, designated *LCA1*), were efficiently expressed under the control of the yeast alcohol dehydrogenase I gene promoter ($ADH1_P$) and terminator ($ADH1_T$). Secretion of the *PED1*-encoded pediocin and *LCA1*-encoded leucocin was directed by the yeast mating pheromone a-factor's secretion signal ($MF\alpha1_S$). The presence of biologically active antimicrobial peptides produced by the *S. cerevisiae* transformants was indicated by agar diffusion assays against sensitive indicator bacteria (*e.g.*, *Listeria monocytogenes* B73). The heterologous peptides were present at relatively low levels in the yeast supernatant but pediocin and leucocin activities were readily detected when intact yeast colonies were used in sensitive strain overlays. These preliminary results indicate that it is indeed possible to develop bactericidal wine yeast strains that could be useful in the production of wine with reduced levels of potentially harmful chemical preservatives.

CONCLUDING REMARKS

Yeast genetics provides both the means to construct improved strains for use in winemaking and a powerful analytical approach to a better understanding of yeast behaviour in industrial fermentations. There is still much to learn of the genetic make-up of industrial yeasts and much to do to demonstrate the advantages of strain development in practice. Nevertheless, over the last few years considerable progress has been made in developing new wine yeast strains. However, for a significant expansion of this effort there is a need for further research into yeast physiology. In particular, it is vital that research continues in the areas of flavour formation and the factors affecting yeast growth under winemaking conditions. Legislation is anothor important factor affecting the development of new wine yeasts. As outlined above, recombinant DNA techniques have been extensively used for the construction of new wine yeast strains. However, the need for approval is a major barrier to commercialise these genetically modified yeast strains. These strict requirements of genetically modified food and beverage regulations are a response to perceived consumer concerns about the risk of genetically modified organisms (GMOs). From several recent surveys in many countries it is apparent that the average consumer is poorly informed about the benefits and risks of biotechnology. It is therefore of key importance that this problem is addressed and that the public is familiarised with the relevant scientific facts and factors involved in the used of GMOs for food and drink production. It is essential to convince public opinion that the risks are small, before wine made from genetically modified grapevine cultivars or fermented with genetically modified yeasts will become generally acceptable. All wine biotechnologists must work hard towards ensuring that any possible risks are seen in the correct perspec-

tive. Otherwise the application of innovative biotechnology in the wine industry will be restricted and many of the benefits will be lost.

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