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Review

Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking

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Abstract

Yeasts are predominant in the ancient and complex process of winemaking. In spontaneous fermentations, there is a progressive growth pattern of indigenous yeasts, with the final stages invariably being dominated by the alcohol-tolerant strains of *Saccharomyces cerevisiae*. This species is universally known as the 'wine yeast' and is widely preferred for initiating wine fermentations. The primary role of wine yeast is to catalyze the rapid, complete and efficient conversion of grape sugars to ethanol, carbon dioxide and other minor, but important, metabolites without the development of off-flavours. However, due to the demanding nature of modern winemaking practices and sophisticated wine markets, there is an ever-growing quest for specialized wine yeast strains possessing a wide range of optimized, improved or novel oenological properties. This review highlights the wealth of untapped indigenous yeasts with oenological potential, the complexity of wine yeasts' genetic features and the genetic techniques often used in strain development. The current status of genetically improved wine yeasts and potential targets for further strain development are outlined. In light of the limited knowledge of industrial wine yeasts' complex genomes and the daunting challenges to comply with strict statutory regulations and consumer demands regarding the future use of genetically modified strains, this review cautions against unrealistic expectations over the short term. However, the staggering potential advantages of improved wine yeasts to both the winemaker and consumer in the third millennium are pointed out.

Keywords: ***Saccharomyces cerevisiae*; wine yeast; genetic improvement**

Contents

Introduction

The history of winemaking parallels that of civilization: historians believe that wine was being made in the Caucasus and Mesopotamia as early as 6000 BC [131]. References to wine have been found in Egypt and Phoenicia dating as far back as 5000 BC and, by 2000 BC, wine was being produced in Greece and Crete. Colonization by the Romans spread winemaking all around the Mediterranean; by 500 BC, wine was being produced in Sicily, Italy, France, Spain, Portugal and northern Africa. Cultivation of the vine also spread into the Balkan States, and the Romans took it into Germany and other parts of northern Europe, eventually reaching as far as Britain (Figure 1) [131].

European explorers in the sixteenth century introduced the vine into the New World. In 1530 the Spanish conquistadors planted *Vitis vinifera* in Mexico, Argentina, Peru and Chile. In 1655 Dutch settlers in South Africa planted French vine cuttings on the lower slopes of the Cape of Good Hope's

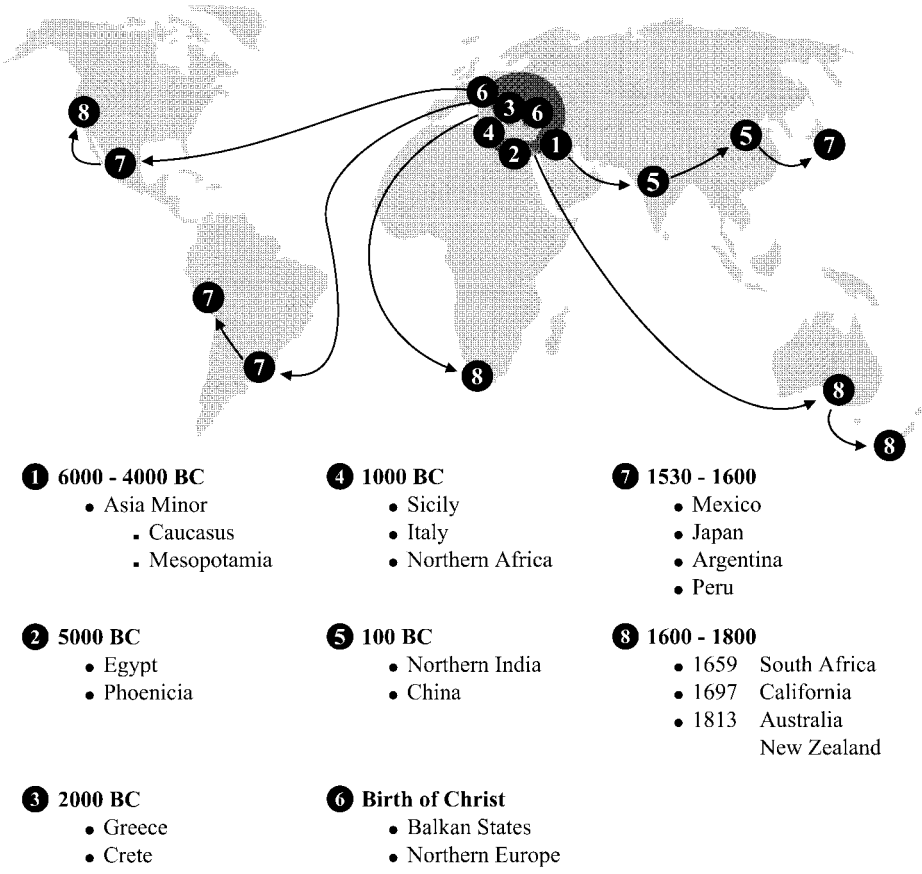

Figure 1. The early spreading and world distribution of the vine and winemaking technology

majestic Table Mountain. Planting in California followed soon thereafter, and in Australia and New Zealand more than a century later, in 1813 [131].

Disaster struck the world of wine in the 1870s, when the root-eating insect *Phyloxera vastatrix* (*Dactylasphaera vitifoliae*) threatened almost every vine in Europe and the New World. They were pulled up and replaced with new *V. vinifera* vines grafted onto phyloxera-resistant rootstocks from the native American vine. Despite attacks by phyloxera and the spread of other diseases, such as downy mildew (*Plasmopara viticola*) and powdery mildew (*Oidium tuckerii*), the Office International de la Vigne et du Vin (OIV) in Paris reports that today there are some 8 million hectares of vineyards across the world, mainly concentrated within the earth's temperate zones. Each of these vineyards reflects the *terroir*, history, culture and traditions of its region.

Until the early years of the seventeenth century, wine was considered to be the only wholesome readily storable (to a point) beverage, accounting for the rapid global increase of wine fermentation technology. Today wine is synonymous with culture and a convivial lifestyle around the world, complementing food, entertainment and the arts. Wine plays a major role in the economies of many nations, which produce more than 26 billion litres of wine annually. Modern winemakers supply a wide variety of wines year round independent of location and time of consumption. Fierce competition for market share has led to increased diversity (Figure 2) and innovation within the wine industry, much to the benefit of the consumer.

A look at the early days of winemaking makes it obvious that, while different techniques produced varied styles of wine, the basic principles changed very little (Figure 3). During the last 150 years or so, however, the scientific basis of winemaking has

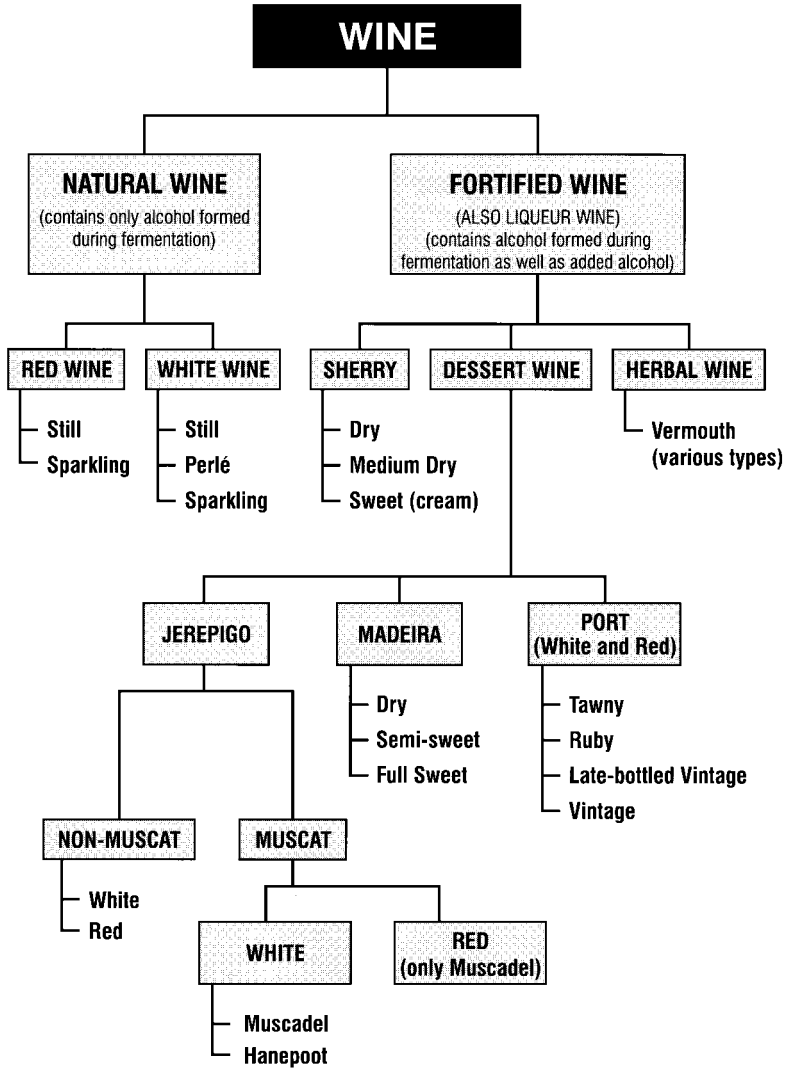

Figure 2. Diversity of natural (table) and fortified wines produced in South Africa

gradually become clearer, and many practices once thought impossible have now become routine.

In 1863, Louis Pasteur revealed for the first time the hidden world of microbial activity during wine fermentation. He proved conclusively that yeast is the primary catalyst in wine fermentation, basing his work upon Antonie van Leeuwenhoek's first microscopic observation (in 1680) of yeast cells and the claims by three other independent pioneers, Cagniard-Latour, Kützing and Schwann (in the late 1830s) that these cells are living organisms [6]. With the knowledge that yeast was responsible for the biotransformation of grape sugars (mainly glucose and fructose) into alcohol and carbon dioxide, winemakers could control the process from vineyard to bottling plant. Later, yeasts with improved characteristics were selected and, by 1890, Müller-Thurgau introduced the concept of inoculating wine fermentations with pure yeast cultures. As a result, the quality and quantity of wine production were vastly improved.

These fundamental innovations in winemaking practices revolutionized the wine industry, and today the forces of *market-pull* and *technology-push* continue to challenge the tension between tradition and innovation [118]. There will continue

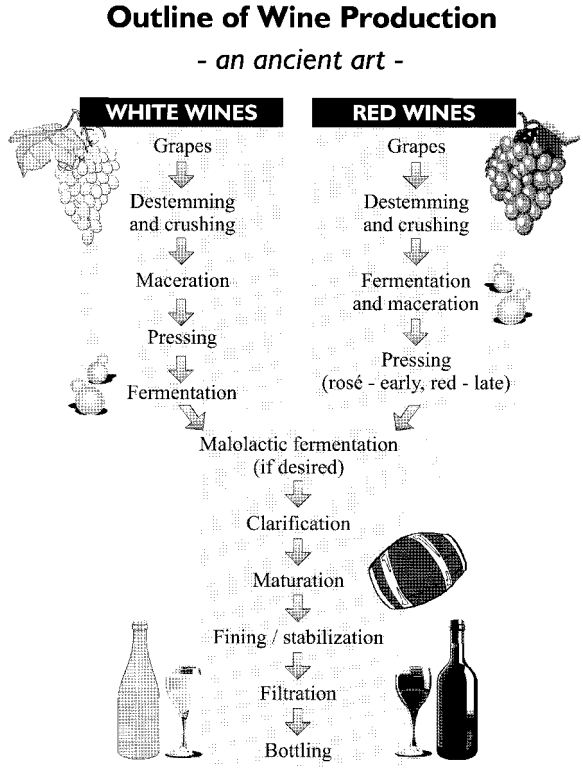

Figure 3. The main steps in wine production (adapted from Walker [176])

to be further improvements in winemaking by refining viticultural and oenological practices. These factors will remain important to the improvement of the overall quality and endless variety of wine.

But today there is a new (and, for the moment, controversial) focal point for innovation in winemaking—the genetic modification of the two main organisms involved, the grape cultivar and wine yeast. The diversity of yeast species associated with winemaking, the tailoring of wine yeast, and the possible use of strains expressing novel designer genes make possible exciting new approaches to winemaking in the twenty-first century.

Yeast diversity associated with grapes and winemaking

Yeast biodiversity and ecology

Yeast diversity and important taxa

Louis Pasteur's simple biochemical process of converting grape juice into wine by allowing the yeasts to ferment the grape sugars spontaneously to ethanol, carbon dioxide and other metabolites, is today vastly more complex and sophisticated. The fermentation of grape must and production of premium quality wines is a complex ecological and biochemical process involving the sequential development of microbial species, as affected by a particular environment. The process includes the interaction of fungi, yeasts, lactic acid bacteria, acetic acid bacteria, as well as the mycoviruses and bacteriophages affecting these grape-associated microorganisms [35,36]. Of all these, yeasts are at the heart of the biochemical interaction with the musts derived from the varieties of *V. vinifera* and other grape species.

Yeasts are defined as unicellular ascomycetous or basidiomycetous fungi whose vegetative growth results predominantly from budding or fission, and which do not form their sexual states within or upon a fruiting body [80]. Of the 100 yeast genera representing over 700 species described in the latest edition of the monographic series, *The Yeasts, A Taxonomic Study* [79], 15 are associated with winemaking: *Brettanomyces* and its sexual ('perfect') equivalent *Dekkera*; *Candida*; *Cryptococcus*; *Debaryomyces; Hanseniaspora* and its asexual counterpart *Kloeckera*; *Kluyveromyces*; *Metschnikowia*; *Pichia*; *Rhodotorula*; *Saccharomyces; Saccharomycodes*; *Schizosaccharomyces;* and *Zygosaccharomyces* [121]. Despite the striking growth in the number of described yeast species over the last 50 years, [77,79,94,95], it is generally accepted that the wealth of yeast biodiversity with hidden oenological potential is still largely untapped.

Yeast ecology

With respect to the vineyard and winery niche habitats, some of these yeasts are considered as 'autochthonous' (essential) and others as 'allochthonous' (transient or fortuitous) members of the communities found in these environments. Their successful coexistence depends on the sum of all physical, chemical and biotic factors that pertain to vineyards and wineries [84]. 'Generalist' yeasts are endowed with a broad niche and occupy many habitats, whereas 'specialist' yeasts occur in unique habitats [176].

The microflora of grapes vary according to the grape variety; temperature, rainfall and other climatic influences; soil, fertilization, irrigation and viticultural practices (e.g. vine canopy manage-

ment); development stage at which grapes are examined; physical damage caused by mould, insects and birds; and fungicides applied to vineyards [121]. It is also important to note that harvesting equipment, including mechanical harvesters, picking baskets and other infrequently cleaned delivery containers can also represent sites for yeast accumulation and microbiological activity before grapes reach the winery [41]. This becomes more important as travel time to the winery increases.

Kloeckera (e.g. *K. apiculata*) and *Hanseniaspora* (e.g. *H. uvarum*) are the predominant species on the surface of grape berries, accounting for roughly 50–75% of the total yeast population [35,36]. Numerically less prevalent than these apiculate yeasts are species of *Candida* (e.g. *C. stellata* and *C. pulcherrima*), *Brettanomyces* (e.g. *B. intermedius, B. lambicus* and *B. custeri*), *Cryptococcus*, *Kluyveromyces*, *Metschnikowia* (e.g. the sexual equivalent of *C. pulcherrima, M. pulcherrima*), *Pichia* (e.g. the so-called film yeast, *P. membranaefaciens*, as well as those species that were previously assigned to the genus *Hansenula,* i.e. *H. anomala*) and the pink yeast *Rhodotorula* (e.g. *R. minuta*) [35,36,161].

Contrary to popular belief, fermentative species of *Saccharomyces* (e.g. *S. cerevisiae*) occur at extremely low populations on healthy, undamaged grapes and are rarely isolated from intact berries and vineyard soils [100]. In fact, the origin of *S. cerevisiae* is quite controversial; one school of thought claims that the primary source of this commercially important yeast is the vineyard, and the presence or absence of *S. cerevisiae* differs with each plant and grape cluster [157]. Others believe the evidence points to a direct association with artificial, man-made environments such as wineries and fermentation plants, and that a natural origin for *S. cerevisiae* should be excluded [100,168]. In contrast to its low occurrence in natural habitats such as vineyards, *S. cerevisiae* is abundant on the grape juice and must-coated surfaces of winery equipment, forming an important component of a so-called 'residential' or 'winery' yeast flora [37]. In fact, *S. cerevisiae* is by far the most dominant yeast species colonizing surfaces in wineries, demonstrating the selective effects of grape juice and wine as growth substrates. The extent of the development of a winery yeast flora, usually comprising species of *Saccharomyces*, *Candida* and *Brettanomyces*, depends upon the nature of the surface and the degree to which it has been cleaned and sanitized. Irregular, unpolished surfaces such as cracks and welds may support dense populations of winery yeasts [41].

The microbiota of grape must are affected indirectly by all the factors influencing the indigenous grape microflora and the winery flora. Added to these factors are the following: method of grape harvest (handpicked or mechanical), grape temperature, transport from vineyard to cellar (distance/time, initial grape temperature, air temperature, sulphite addition), condition of grapes (time, temperature, sulphite addition) and must pretreatment (cellar hygiene, aeration, enzyme treatment, sulphite addition, clarification method, temperature, inoculation with yeast starter cultures) [61,121].

Though grape must is relatively complete in nutrient content, it can support the growth of only a limited number of microbial species [61]. The low pH and high sugar content of grape must exert strong selective pressure on the microorganisms, such that only a few yeast and bacterial species can proliferate. Concentrations of sulphur dioxide, added as an antioxidant and antimicrobial preservative, impose additional selection, particularly against undesirable oxidative microbes [61]. The selectivity of fermenting must is further strengthened once anaerobic conditions are established; certain nutrients become depleted and the increasing levels of ethanol start to eliminate alcohol-sensitive microbial species [61]. Spontaneous fermentation of grape juice into wine can be regarded as a heterogeneous microbiological process involving the sequential development of various yeasts and other microbiological species, affected by the prevailing fermentation conditions in a particular vat or tank.

When must is used as a culture medium, selective pressures always favour the yeasts with the most efficient fermentative catabolism, particularly strains of *S. cerevisiae* and perhaps strains of closely related species such as *Saccharomyces bayanus*. For this reason, *S. cerevisiae* is almost universally preferred for initiating alcoholic fermentation, and has earned itself the title of 'the wine yeast'. This, however, does not preclude the possibility that, in the future, some winemakers might prefer to use a mixture of indigenous yeast species and strains as starter cultures tailored to reflect the yeast biodiversity of a given region [58].

Wine yeast starter cultures

Spontaneous vs. inoculated wine fermentations

Originally, all wine was made by taking advantage of natural microflora for spontaneous fermentation; no deliberate inoculation was made to start the process. Various yeasts found on the surface of grape skins and the indigenous microbiota associated with winery surfaces participate in these natural wine fermentations. Yeasts of the genera *Kloeckera*, *Hanseniaspora* and *Candida* predominate in the early stages, followed by several species of *Metschnikowia* and *Pichia* in the middle stages, when the ethanol rises to 3–4% [37,105]. The latter stages of natural wine fermentations are invariably dominated by the alcohol-tolerant strains of *S. cerevisiae.* Other yeasts, such as species of *Brettanomyces, Kluyveromyces, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces*, may also be present during the fermentation and subsequently in the wine, some of which are capable of adversely affecting sensory quality.

A breakthrough was made when Hansen of the Carlsberg Brewery in Denmark isolated a pure culture derived from a single yeast cell and, in 1890, Müller-Thurgau from Geisenheim introduced the concept of inoculating wine fermentations with pure yeast starter cultures [119]. In 1965, the first two commercial active dried wine yeast (ADWY) strains were produced for a large Californian winery [27]. These two strains, Montrachet and Pasteur Champagne, were offered worldwide as all-purpose yeasts, with limited success. Today, several yeast-manufacturing companies market a wide variety of dehydrated cultures of various *S. cerevisiae* strains. In guided fermentations, the actively growing starter culture dominates the native yeast species present in grape must. To achieve this, a cell density upon inoculation of 1–3 million colony forming units (cfu)/ml is usually recommended [41].

The practice of spontaneous fermentations remained prevalent in 'Old World' wine-producing areas until the 1980s because of the popular belief that superior yeast strains associated with specific vineyards gave a distinctive style and quality to wine. Even today, winemakers at many 'boutique' wineries accept the potentially staggering risks involved in spontaneous fermentations to achieve stylistic distinction and vintage variability. Conversion of grape sugars to alcohol and other end products by mixed populations of yeast may undoubtedly yield wines with distinct sensorial quality, often described as wine with a fuller, rounder palate structure. This may well be the consequence of higher concentrations of glycerol and other polyols produced by indigenous yeasts. Furthermore, the extended lag phase before the onset of vigorous fermentation allows for the reaction of oxygen with anthocyanins and other phenols in the absence of ethanol, which is thought to enhance colour stability in red wine as well as accelerating phenol polymerization [189].

However, the cast of stylistic characters and the individual and collective contribution of indigenous yeasts to the wine vary. The outcome of spontaneous fermentation depends not only on the numbers and diversity of yeasts present in must, but also upon grape chemistry and processing protocol. The combined effect makes the outcome difficult to predict. This lack of predictability/reproducibility is most troublesome when comparing spontaneous fermentation with that resulting from active dried yeast starters.

Notwithstanding the fact that spontaneous fermentations usually take longer than most winemakers are willing to accept, and that the outcome is not always what was anticipated, there is no consensus among the world winemakers about using yeast starters [41]. At one extreme are those who continue to use solely indigenous yeasts, believing that the unique contributions of diverse yeast species confer a complexity upon wine not seen in inoculated and guided fermentations. Others prefer to begin with native yeasts and later inoculate with a commercial yeast starter. Still others initiate their wine fermentation with starters but at lower than recommended inoculum levels.

In large-scale wine production, however, where rapid and reliable fermentations are essential for consistent wine flavour and predictable quality, the use of selected pure yeast inocula of known ability is preferred. These large wineries will be the main beneficiaries of programmes aimed at producing new yeast strains with even more reliable performance, reducing processing inputs, and facilitating the production of affordable high-quality wines.

Industrial-taxonomic relationship for wine yeast starter cultures

The first 'all-purpose' commercial wine yeast strains were only partially successful. As the modern

winemaking community was slowly adopting the use of starter cultures in preference to the 'gambling' tradition of spontaneous fermentations, the need became evident for separate *Saccharomyces* strains with specific characteristics for different types of wine. The strains of *Saccharomyces* became classified into several different species or varieties, including *S. bayanus*, *S. beticus, S. capensis, S. chevaleri*, *S. ellipsoideus*, *S. fermentati*, *S. oviformis, S. rosei* and *S. vini* [169].

The characteristics of some of the yeasts used for the production of specific wine types were so marked that a strong taxonomic linkage was believed to exist [61]. For example, while *S. ellipsoideus* was widely used for the production of dry wine, ethanol-tolerant and flocculent strains with autolytic properties (e.g. *S. bayanus* and *S. oviformis*) were preferred for the production of bottle-fermented sparkling wine, film-forming strains with strong oxidative capabilities (e.g. *S. beticus* and *S. capensis*) for the production of flor sherry, and osmotolerant strains forming little or no volatile acids (e.g. *S. rosei*) for sweet wines [61].

Over the years, successive editions of *The Yeasts, A Taxonomic Study* re-defined and re-grouped species of *Saccharomyces* so that the 16 and 21 species that were described in the first and second editions, respectively, were assigned to a single species in the third edition and designated as *S. cerevisiae* [77,94,95]. In the fourth edition, the single species has been separated into four species [79].

Despite the considerable phenotypic differences among the various wine yeast strains, most of them are now considered to be physiological strains of *S. cerevisiae*. Of all these wine yeasts, only *S. fermentati* and *S. rosei* were not re-classified as *Saccharomyces* but rather as *Torulaspora delbrueckii* [79]. The assignment of most of the traditional wine yeast strains to a single species does not, however, imply that all strains of *S. cerevisiae* are equally suitable for the various wine fermentations; they differ significantly in their fermentation performance and their contribution to the final bouquet and quality of wine and distillates [61,121]. It is therefore not surprising that several molecular methods have been developed for wine yeast strain differentiation [25,26,160] (Table 1).

A quest for new wine yeast strains

While the old saying 'the best wines are made in the vineyard' is still valid, oenologists have also come to recognize the importance of specific *S. cerevisiae* starter culture strains to the type and style of product. With the importance of *S. cerevisiae*'s role in winemaking now firmly established, there is an ever-growing demand for new and improved wine yeast strains. In addition to the primary role of wine yeast to catalyze 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, such as those listed in Table 2. 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. Leading winemakers are now translating the adage "horses for courses" into "special yeasts for special treats".

Genetic constitution of wine yeasts

Wine yeast cytology and reproduction

S. cerevisiae cells are generally ellipsoidal in shape. The subcellular compartmentalization in this eukaryote is schematically presented in Figure 4. Under optimal nutritional and cultural conditions, *S. cerevisiae* doubles its mass every 90 min. The cell division cycle is presented in Figure 5 and the basic life cycles of heterothallic and homothallic strains are shown in Figure 6.

Chromosomal DNA and ploidy

S. cerevisiae has a relatively small genome, a large number of chromosomes, little repetitive DNA and few introns [116]. Haploid strains contain approximately 12–13 megabases (mb) of nuclear DNA, distributed along 16 linear chromosomes. Each chromosome is a single deoxyribonucleic acid (DNA) molecule approximately 200–2200 kilobases (kb) long. The genome of a laboratory strain of *S. cerevisiae* has been completely sequenced and found to contain roughly 6000 protein-encoding genes. The *S. cerevisiae* genome, which is relatively rich in guanine and cytosine content (%G + C of 39–41) is much more compact when compared with the genomes of other eukaryotic cells.

Table 1. Molecular methods for wine yeast strain differentiation (adapted from Walker [186])

Method	Description
Chromatography	Pyrolysis-gas chromatography or gas chromatography of long-chain fatty acid methyl esters
Polyacrylamide gel electrophoresis (*PAGE*)	Total soluble yeast proteins are electrophoresed and banding patterns analyzed by computer
Restriction enzyme analysis (DNA fingerprinting)	Total, ribosomal or mitochondrial DNA is digested with restriction endo-nucleases and specific fragments hybridized after electrophoretic separation with multi-locus DNA probes such as the *Ty1* retrotransposon; restriction fragment length polymorphisms (*RFLP*s) are detected
Electrophoretic karyotyping (chromosome fingerprinting)	Whole yeast chromosomes are separated electrophoretically using pulse-field techniques; chromosome length polymorphisms (*CLP*s)
Polymerase chain reaction (*PCR*)	Specific DNA sequences are exponentially propagated *in vitro* and the amplified products analyzed after electrophoretic separation. Randomly amplified polymorphic DNA (*RAPD*) and amplified fragment length polymorphisms (*AFLP*s) can also be analyzed by PCR
Genetic tagging	Specific genetic sequences, including selectable markers, are introduced into yeasts to facilitate their recognition (e.g. replacement of chloramphenicol resistance sequences with a 'tag' which confers sensitivity to the antibiotic)

Most laboratory-bred strains of *S. cerevisiae* are either haploid or diploid. However, industrial wine yeast strains are predominantly diploid or aneuploid, and occasionally polyploid [142]. It is not yet clear whether polyploidy in industrial yeast strains is advantageous. When a series of homozygous and heterozygous strains with ploidy from one to eight were constructed, it was found that the heterozygous triploids and tetraploids were more efficient in fermentation than the homozygous strains of higher or lower ploidy. Based on these results it was concluded that heterosis rather than ploidy is responsible for improvement of fermentation performance [56]. However, other researchers claim that the polyploid state might enable industrial yeasts to harbour a high dosage of genes important for efficient fermentation. When a heat-induced endomitotic polyploidization procedure was used to construct an isogenic ploidy series (2N to 4N) from an industrial wine yeast strain, it was found that the physical and metabolic differences observed among these strains were due to differences in gene dosage alone, and not to heterosis [132]. These reports only emphasize the fact that the relationship between the fermentation ability and the ploidy of a yeast strain is rather complicated.

Table 2. Desirable characteristics of wine yeast

Fermentation properties	Technological properties
Rapid initiation of fermentation	High genetic stability
High fermentation efficiency	High sulphite tolerance
High ethanol tolerance	Low sulphite binding activity
High osmotolerance	Low foam formation
Low temperature optimum	Flocculation properties
Moderate biomass production	Compacts sediment
Flavour characteristics	Resistance to desiccation
Low sulphide/DMS/thiol formation	Zymocidal (killer) properties
Low volatile acidity production	Genetic marking
Low higher alcohol production	Proteolytic acitivity
Liberation of glycosylated flavour precursors	Low nitrogen demand
High glycerol production	Metabolic properties with health implications
Hydrolytic activity	Low sulphite formation
Enhanced autolysis	Low biogenic amine formation
Modified esterase activity	Low ethyl carbamate (urea) potential

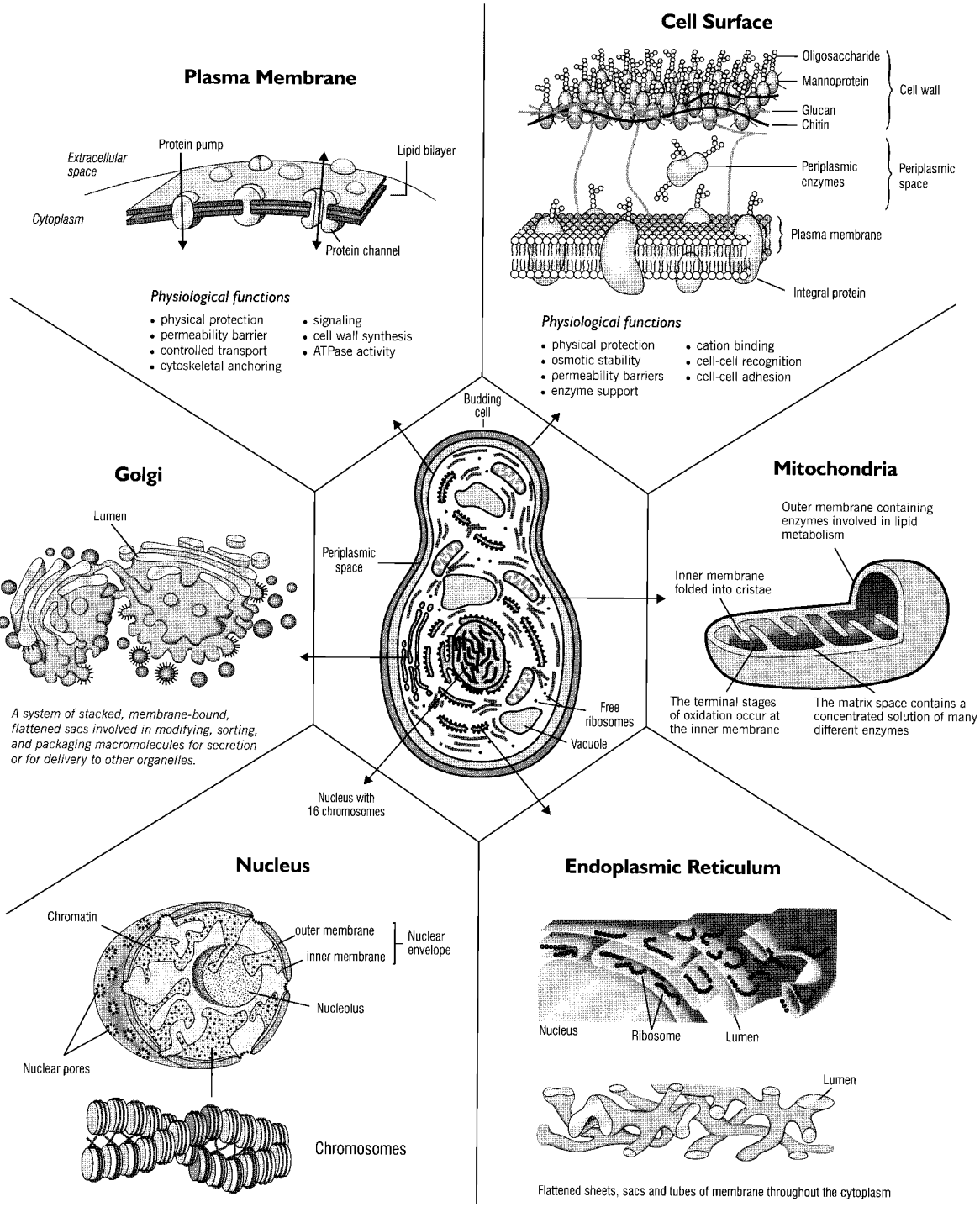

Figure 4. A schematic representation of the subcellular compartmentalization of a wine yeast cell (adapted from Pretorius and Van der Westhuizen [119]). The cell envelope, comprising a cell wall, periplasm and plasma membrane, surrounds and encases the yeast cytoplasm. The structural organization of the intracellular milieu, containing organelles such as the nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria and vacuoles, is maintained by a cytoskeleton. Several of these organelles derive from an extended intramembranous system and are not completely independent of each other

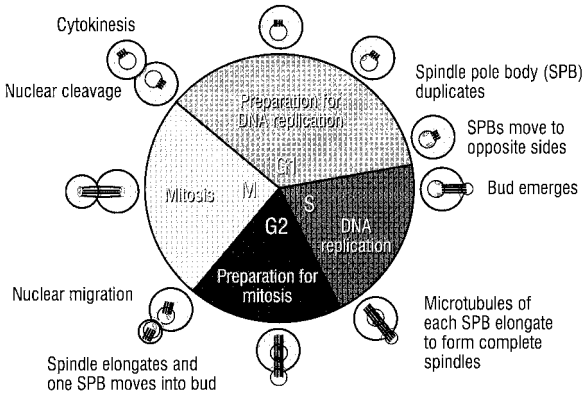

Figure 5. A schematic representation of the cell cycle of a budding wine yeast cell. Buds may arise at any point on the mother cell surface, but never again at the same site. Branched chaining (pseudohyphae) may occasionally follow multilateral budding when buds fail to separate. Under optimal nutritional and cultural conditions *S. cerevisiae* doubles its mass every 90 min. The cell division cycle consists of four phases, G_1, S, G_2 and M

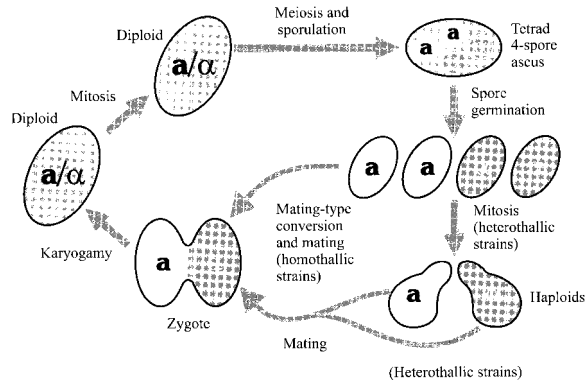

Figure 6. A schematic representation of the life cycle of heterothallic and homothallic wine yeast strains (adapted from Hammond [56]). Haploid cells of the *MAT*α mating type produce a 13 amino acid-long α factor; while the **a** mating type cells produce a peptide of 12 amino acids, the a factor. A *MAT***a**/*MAT*α diploid cell formed by mating by two haploid cells of opposite mating types can neither produce nor respond to mating pheromones and will under satisfactory nutritional and cultural conditions grow and divide, maintaining the diploid state. Upon nutritional starvation, the *MAT***a**/*MAT*α diploid cell undergoes meiosis, generating four haploid ascospores (two *MAT***a** and two *MAT*α ascospores) encapsulated within an ascus. When released from the ascus, the ascospores germinate to commence new rounds of haploid existence. Strains that can be maintained stably for many generations as haploids are termed heterothallic. Strains in which sex reversals, cell fusion and diploid formation occur are termed homothallic

Extrachromosomal elements

Several non-Mendelian genetic elements are known to exist in the nucleus (e.g. transposons and 2 μm plasmid DNA), mitochondria and cytoplasm (e.g. viral-like particles and prion-like elements such as ψ, η and URE3). The genome of *S. cerevisiae* contains approximately 35–55 copies of retrotransposons (Ty elements). These transposable elements move from one genomic location to another via an RNA intermediate using reverse transcriptase. The 2 μm plasmid DNA is the only naturally occurring, stably maintained, circular nuclear plasmid in *S. cerevisiae*. This 6.3 kb extrachromosomal element is also inherited in a non-Mendelian fashion and, although most strains of *S. cerevisiae* contain 50-100 copies of 2 μm DNA per cell, its biological function has not yet been discovered.

Mitochondrial DNA

Mitochondria possess their own genetic system and their own protein synthetic machinery. *S. cerevisiae* has among the largest mitochondrial DNAs (mtDNAs) of any organism, consisting of 75 kb circularly permuted molecules [55]. However, the mitochondrial genome of *S. cerevisiae* is adenine-thymine (A–T) rich, carrying the genetic information for only a few, essential, mitochondrial components, and does not even code for the majority of the enzymes involved in the generation of ATP during aerobic growth. Unlike the replication of nuclear DNA, replication of mtDNA is not limited to the S phase; it takes place throughout the cell cycle. The mtDNA polymerase also lacks proofreading (exonuclease) activity, resulting in a much higher mutation rate within the mtDNA than within nuclear genes, so mtDNA can evolve extremely rapidly [55]. This lack of an error repair mechanism during mtDNA replication is partly compensated for by the abundance of mitochondrial DNA molecules in a single cell [179].

With a genome that is much larger than required, the yeast mtDNA encodes proteins that perform only a few activities. One explanation for the persistence of this large mitochondrial genome is that, in yeast, it could play the additional role of a reservoir of genetic diversity, capable of serving the nuclear genome by contributing evolved sequences [55]. This could be one contributing factor to the

observed genetic heterogeneity of pure cultures of wine yeasts.

Unlike other eukaryotic cells, yeasts can survive without its mtDNA. Mitochondrial mutants usually lack vital oxidative enzymes, rendering them unable to generate ATP oxidatively. As a result, mitochondrial mutants grow slowly and form smaller (*petite*) colonies on solid agar surfaces than the wild-type (*grande*) cells. Petite mutants are respiratory-deficient and unable to utilize non-fermentable substrates. The term 'cytoplasmic petite mutant' describes respiratory-defective strains with cytoplasmically inherited mutations, ranging from point mutations (Mit^-) through deletion mutations (Rho^-) to complete elimination of the mtDNA (Rho°) [55]. To distinguish cytoplasmic petite mutants from respiratory-deficient strains with genetic lesions in nuclear genes, the latter are referred to as nuclear petite or *pet* mutants [55].

The mitochondrial genome is also involved in cell functions other than respiratory metabolism. Since the generation of petite mutants of wine yeasts occurs spontaneously at quite high rates, it is important to note that yeasts with different mtDNAs could differ in their flocculation characteristics, lipid metabolism, higher alcohol production and formation of flavour compounds [176]. Thus, although wine yeasts are not required to respire during fermentation of grape must, some mtDNA-encoded functions are important and for this reason petite strains are not used for winemaking.

Killer factors

The killer phenomenon in *S. cerevisiae* is associated with the presence of non-infectious, intracellular virus-like particles (VLP). VLPs in killer (zymocidal) yeasts that are cytoplasmically inherited contain two major linear double-stranded ribonucleic acid (dsRNA) types, the L and M genomes [182]. The L genome encodes an RNA-dependent RNA polymerase and the viral coat protein that encapsulates both genomes. The M genome encodes both a proteinaceous toxin (zymocin) and an immunity factor. The zymocin is secreted by the zymocidal strains and is lethal to sensitive strains of the same species. Five types of *S. cerevisiae* killers, K_1, K_2, K_3, K_{28} and K_{3GR1} have been described [40,182,185,186,187]. It now seems that K_3 and K_{3GR1} are only variants from the K_2 type. The size of the L genome is 4.5 kb, whereas the different M dsRNA genomes vary between 1.3 and 2 kb. Some yeast strains are immune to these zymocins but do not produce active toxin. These so-called neutral strains contain L and M dsRNA genomes, but the M genomes code only for the production of the immunity factor and not for the production of an active zymocin. Zymocidal strains isolated from fermenting grape musts are usually of the K_2 or K_{28} type, most probably because of the low pH optimum for their zymocidal activity which is favoured by the acidic conditions of grape must (pH 2.8–3.8) [136,182]. Apart from sporadic reports which imply that the presence of killer contaminants could, under certain conditions, contribute towards stuck (incomplete) or sluggish wine fermentations [167], no real effects on the wholesomeness and sensorial quality of wines are produced by zymocidal *S. cerevisiae* strains.

Genetic techniques for the analysis and development of wine yeast strains

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 [8,56,119,123]. Techniques having the greatest potential in genetic programming of wine yeast strains are: clonal selection of variants, mutation and selection, hybridization, rare-mating, spheroplast fusion and gene cloning and transformation. The combined use of tetrad analysis, replica-plating, mutagenesis, hybridization and recombinant DNA methods have dramatically increased the genetic diversity that can be introduced into yeast cells.

Selection of variants

Genetic drift

The maintenance of the genetic identity of strains in a pure culture is complex. Although the term 'pure culture' denotes that it has been derived from a single cell, it does not imply that the culture is genetically uniform [142]. Even under closely controlled conditions of growth, a yeast strain reveals slow but distinct changes after many generations. This might be due to a number of different processes, including spontaneous mutation, Ty-promoted chromosomal translocations and, more frequently, mitotic crossing-over or gene conversion. Over the years, the phenomena of hetero-

geneity in pure yeast cultures and genetic drifting were harnessed to improve wine yeast strains [142]. It was shown that successive single-cell cultures of commercial wine yeast strains could result in strains with considerably improved characteristics. It is well known that the sporulation and spore viability of pure yeast cultures are generally poor and that there is considerable variation in growth rate between spore clones [142]. Some of this genetic heterozygosity of pure cultures is undoubtedly due to segregation of aneuploid chromosome complements from a polyploid or aneuploid parental strain; the remaining variation probably reflects the segregation of lethal genes or genes compromising efficient growth. Mating between *MAT**a*** and *MAT*α ascospores, generated by sporulation, can also cause genetic instability. Increased homozygosity in polyploid yeasts is expected to confer greater genetic stability. It has also been reported that the rate of genetic drift of yeast strains increases with ploidy. This finding is contrary to a long-held belief that the polyploid state protects against mutation and genetic variability. Since wine yeasts most probably harbour recessive mutations, genetic stability is likely to be a function of the frequency of segregational events leading to expression of mutant genes, rather than the frequency of mutation itself [56]. It has also been established that, although at a much lower frequency than in laboratory strains under strong selective pressure, Ty-driven ectopic recombinations are more commonly responsible for karyotypic variability in wine yeast than was initially expected [124]. It would seem unwise to assume *a priori* that all wine yeast strains are genetically stable. It is not yet clear what the contributions of mitochondrial genomes are to the genetic drift in wine yeasts. However, this genetic heterogeneity serves as a rich, natural gene pool from which desirable variants can be selected and further improved. Selection of variants is, therefore, a direct means of strain development and successful isolation of variants with desirable characteristics depends on the frequency at which mitotic recombination, Ty-driven remodelling of the genome and spontaneous mutation occur, as well as the availability of selection procedures to identify those variants exhibiting improved oenological traits.

Genome renewal

For many years, strains of *S. cerevisiae* were isolated from vineyards and wine fermentations, and selected to be used as commercial starter cultures. It is now believed that strains of *S. cerevisiae* indigenous to vineyards and wineries tend to be homozygous for most of the genes by a process known as 'genome renewal' [105]. This phenomenon is based on the ability of homothallic haploid *S. cerevisiae* cells to switch their mating-type from **a** to α and vice versa through the *HO*-controlled cassette model (Figure 7), and to conjugate with cells of the same single-spore colony. Continued propagation of yeast cells in their natural (e.g. vineyard) or man-made (e.g. winery) habitats leads to strains of *S. cerevisiae* accumulating heterozygous recessive mutations, and the concomitant heterozygotes can change to completely homozygous diploids by sporulation and homothallic switching of individual haploid spores [105].

This process would eliminate the recessive lethal or deleterious genes that adversely affect yeast fitness (e.g. slower growth, lower fermentation rate, reduced spore viability, etc.). Genome renewal could also be responsible for the replacement of the parental heterozygous strains by the new homozygous diploids bearing new recessive alleles that increase fitness (Figure 8). Perhaps this is the reason why most indigenous strains of *S. cerevisiae* isolated from grapes, wineries and must are homothallic, since homothallism, together with the capability to sporulate, would provide the yeast community with a mechanism by which cells carrying deleterious recessive mutations could be eliminated, thereby enabling them to adapt efficiently to changing environmental conditions [121].

The practical implications of genome renewal and yeast population dynamics in the vineyards and wineries (and even within yeast starter cultures) are far-reaching, whether winemakers rely on spontaneous fermentation of grape juice or whether they inoculate grape must with selected wine yeast strains. Although dramatic improvements in most characteristics cannot be expected, intra-strain selection has been used for decades to obtain improved wine yeast strains [119].

Mutagenesis and selection

The average spontaneous mutation frequency in *S. cerevisiae* at any particular locus is approximately 10^{-6} per generation [119]. The use of mutagens greatly increases the frequency of mutations in a

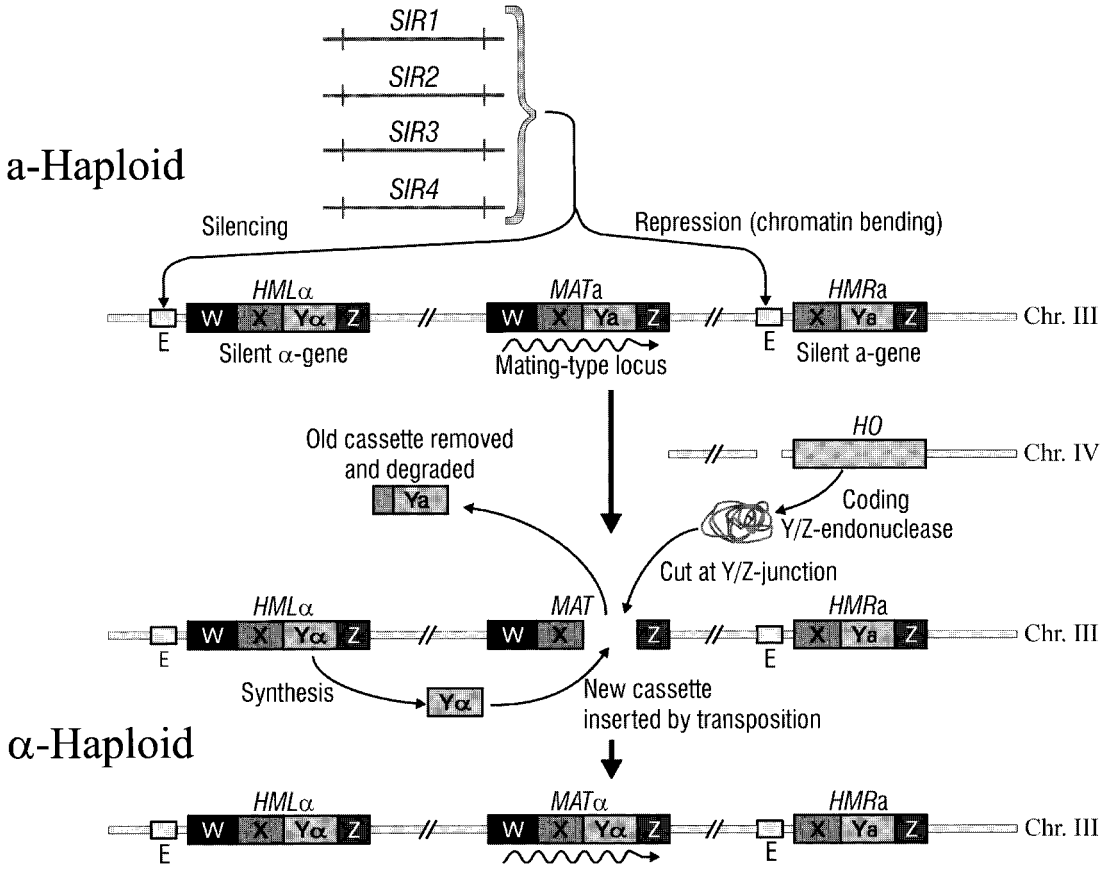

Figure 7. The cassette model of mating-type switching in homothallic wine yeast strains (adapted from Pretorius and Van der Westhuizen [119])

wine yeast population. Mutation and selection (often through replica-plating on selective agar media) appear to be a rational approach to strain development when a large number of performance parameters are to be kept constant while only one is to be changed.

However, mutation of wine yeasts can lead to improvement of certain traits with the simultaneous debilitation of other characteristics [56]. Although mutations are probably induced with the same frequency in haploids, diploids or polyploids, they are not as easily detected in diploid and polyploid cells because of the presence of non-mutated alleles. Only if the mutation is dominant is a phenotypic effect detected without the need for additional alterations. Therefore, haploid strains of wine yeasts are preferred, though not essential, when inducing mutations. Successful mutation and breeding is usually associated with mutations in meiotic segregants, where the two mating parents of a genetically stable hybrid provide a good basis for the introduction of recessive mutations. Mutagenesis has the potential to disrupt or eliminate undesirable characteristics and to enhance favourable properties of wine yeasts. Though the use of mutagens for directed strain development is limited, the method could be applied to isolate new variants of wine yeast strains prior to further genetic manipulation.

Hybridization

Mating

Intra-species hybridization involves the mating of haploids of opposite mating-types to yield a heterozygous diploid (Figure 9). Recombinant progeny are recovered by sporulating the diploid, recovering individual haploid ascospores and repeating the mating/sporulation cycle as required [56]. Haploid strains from different parental diploids, possessing

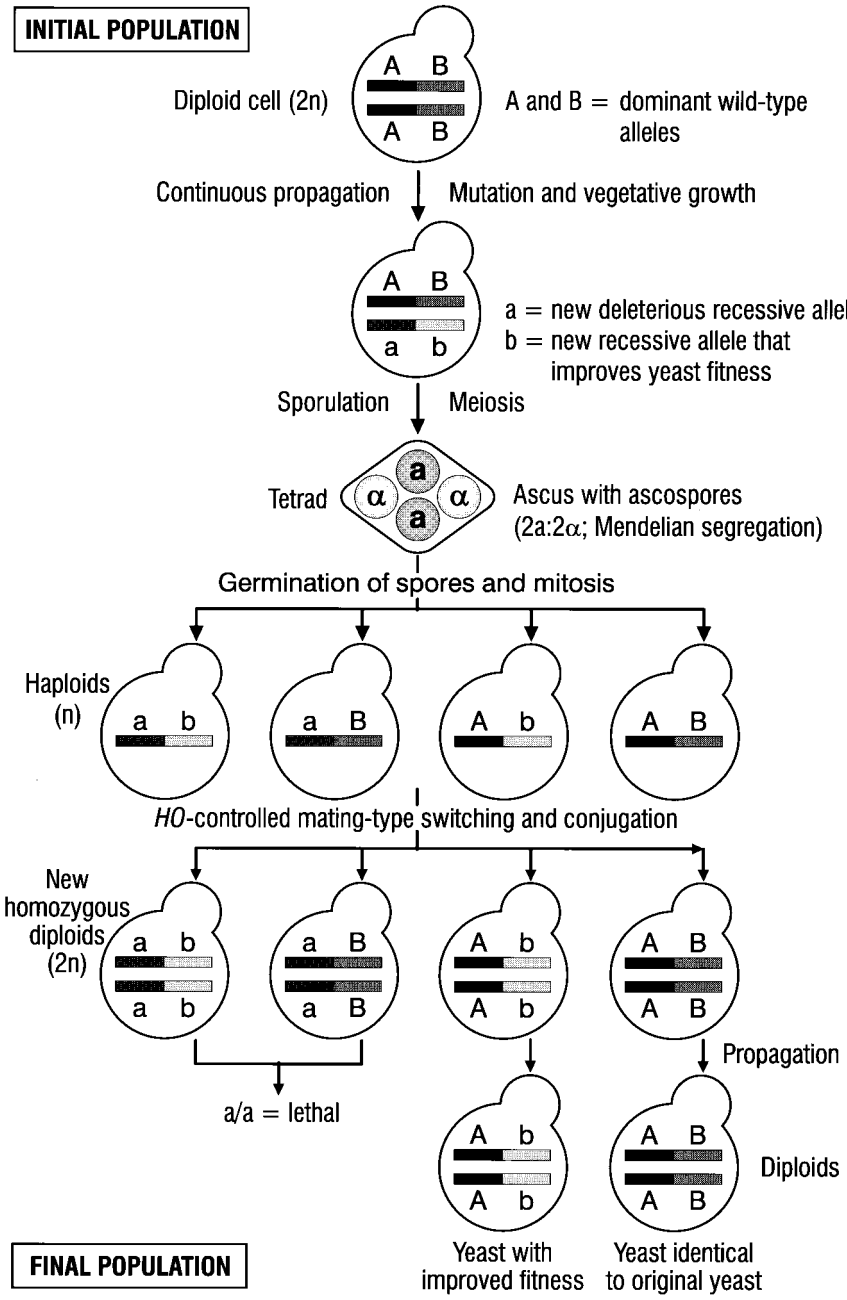

Figure 8. A hypothetical scheme to describe a possible succession of events leading to the replacement of one *Saccharomyces* population by another through the process of 'genome renewal' (adapted from Mortimer *et al.* [105])

different genotypes, can be mated to form a diploid strain with properties different from that of either parental strain. Thus, in theory, crossbreeding can permit the selection of desirable characteristics and the elimination of undesirable ones [8].

Unfortunately, many wine yeasts are homothallic and the use of hybridization techniques for development of wine yeast strains has proved difficult. However, this problem can be circumvented by direct spore-cell mating using a micromanipulator, in which four homothallic ascospores from the same ascus are placed into direct contact with

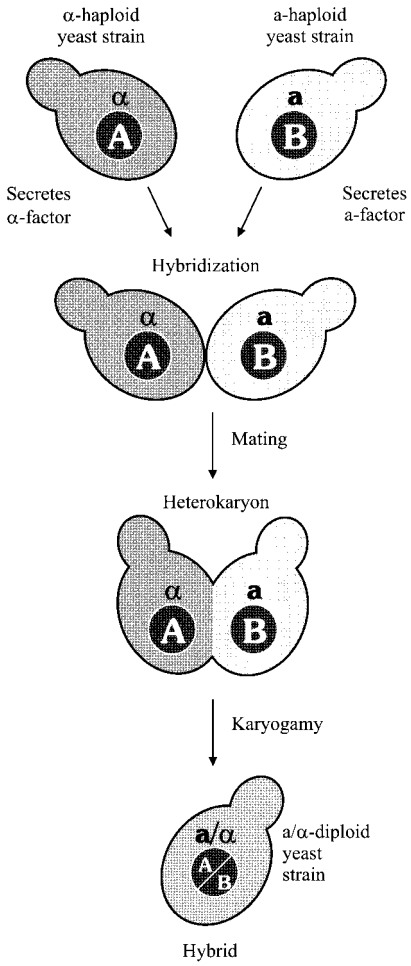

Figure 9. Hybridization (mating) between haploids of two opposite mating-types of wine yeast (adapted from Hammond [56])

heterothallic haploid cells (Figure 10). Mating takes place between compatible ascospores and cells. Elimination or inclusion of a specific property can thus be achieved relatively quickly by hybridization, provided that it has a simple genetic basis, for example one or two genes [119]. Unfortunately, many desirable wine yeast characteristics are specified by several genes or are the result of several gene systems interacting with one another.

Rare mating

Wine yeast strains that fail to express a mating-type can be force-mated (rare-mating) with haploid *MATa* and MATα strains (Figure 11). Typically, a large number of cells of the parental strains are mixed and a strong positive selection procedure is

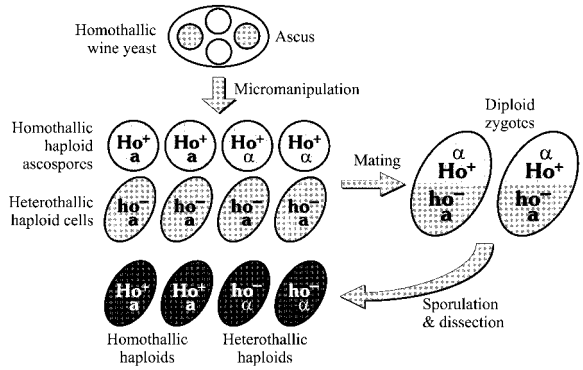

Figure 10. The isolation of haploid strains from a homothallic wine yeast by spore-cell mating. Four ascospores from the same ascus are micromanipulated into direct contact with heterothallic haploids yeast cells. Mating takes place between compatible spores and cells. The resulting diploid is sporulated. Since two spores in each ascus are homothallic and two spores are heterothallic, stable haploids can be isolated from the sporulated diploids (adapted from Pretorius and Van der Westhuizen [119])

applied to obtain the rare hybrids formed. For instance, industrial strains that have a defective form of, or lack, mtDNA (respiratory-deficient mutants) can be force-mated with auxotrophic haploid strains having normal respiratory characteristics [56]. Mixing of these non-mating strains at high cell density will generate only a few respiratory-sufficient prototrophs. These true hybrids with fused nuclei can then be induced to sporulate for further genetic analysis and crossbreeding.

Cytoduction

Rare-mating is also used to introduce cytoplasmic genetic elements into wine yeasts without the transfer of nuclear genes from the non-wine yeast parent (Figure 11). This method of strain development is termed cytoduction. Cytoductants (or heteroplasmons) receive cytoplasmic contributions from both parents but retain the nuclear integrity of only one [56]. Cytoduction requires that a haploid mating strain carry the *kar1* mutation; i.e. a mutation that impedes karyogamy (nuclear fusion) after mating. This more specific form of strain construction can, for example, be used to introduce the dsRNA determinants for the K_2 zymocin and associated immunity into a particular wine yeast.

Cytoduction can also be used to substitute the mitochondrial genome of a wine yeast or to introduce a plasmid encoding desirable genetic

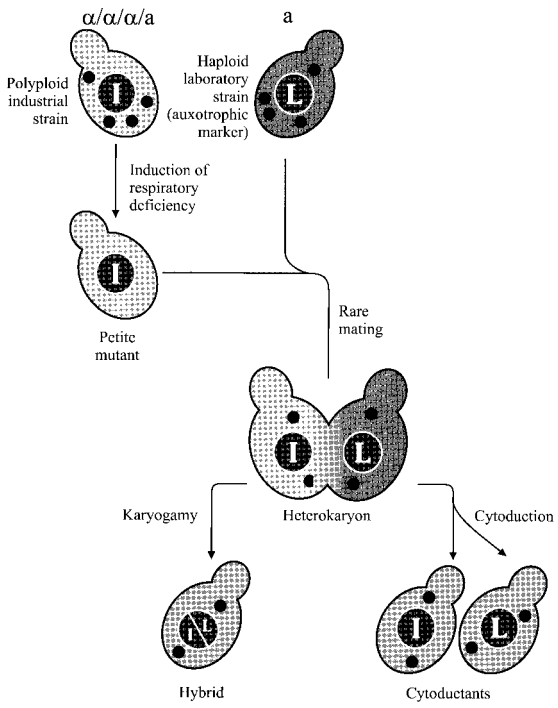

Figure 11. Rare-mating between industrial and laboratory strains of wine yeast. Industrial strains that fail to show a mating type are force-mated with haploid strains, exhibiting a or α mating type. A large number of cells of the parental strains are mixed and the rare hybrids or cytoductants are selected as respiratory-sufficient prototrophs from crosses between a respiratory-deficient mutant of the industrial strains and an auxotrophic haploid laboratory strain (adapted from Hammond [56])

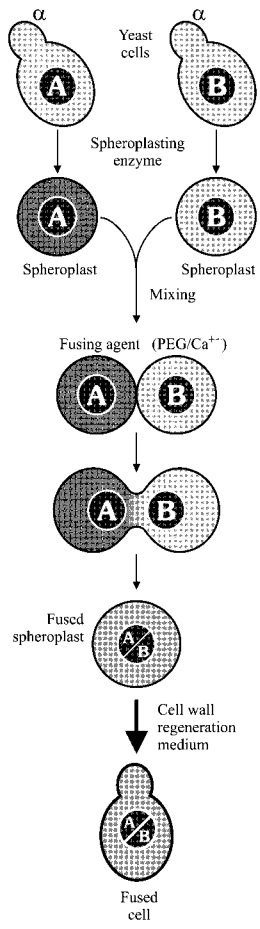

Figure 12. Spheroplast fusion between two different yeast cells is a direct asexual technique to produce either hybrids or cytoductants. Spheroplasts are formed by removing the cell wall with an appropriate lytic enzyme preparation in an osmotically stabilized medium to prevent lysis. Spheroplasts from two different strains are mixed together in the presence of polyethylene glycol and calcium ions to fuse. The fused cells are allowed to regenerate their cell walls in an osmotically stabilized agar medium (adapted from Hammond [56])

characteristics into specific wine yeast strains. Mating between strains, one of which carries the *kar1* allele, occasionally generates progeny that contain the nuclear genotype of one parent together with an additional chromosome from the other parent [119]. The donation of a single chromosome from an industrial strain to a haploid *kar1* recipient is termed 'single-chromosome transfer', and is used to examine individual chromosomes of industrial yeast strains in detail [56].

Spheroplast fusion

Spheroplast fusion is a direct, asexual technique employed in crossbreeding as a supplement to mating. Like rare-mating, spheroplast fusion can be used to produce either hybrids or cytoductants (Figure 12). Both these procedures overcome the requirement for opposite mating types to be crossed, thereby extending the number of crosses that can be done [56]. Cell walls of yeasts can be removed by lytic enzymes in the presence of an osmotic stabilizer to prevent osmolysis of the resulting spheroplasts. Spheroplasts from the different parental strains are mixed together in the presence of a fusion agent, polyethylene glycol and calcium ions, and then allowed to regenerate their cell walls in an osmotically-stabilized selective-agar medium.

Spheroplast fusion of non-sporulating industrial

yeast strains removes the natural barriers to hybridization. The desirable (and undesirable) characteristics of both parental strains will recombine in the offspring. Cells of different levels of ploidy can be fused. For instance, a diploid wine yeast strain can be fused to a haploid strain to generate triploid strains. Alternatively, two diploid wine yeasts with complementing desirable characteristics can be fused to generate a tetraploid strain with all the genetic backgrounds of the two parental wine yeasts [119].

Gene cloning and transformation

Clonal selection, mutagenesis, hybridization, rare-mating and spheroplast fusion all have value in strain development programmes, but these methods lack the specificity required to modify wine yeasts in a well-controlled fashion [119]. 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 (Figure 13) offer the possibility of altering 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 (Figure 14). By using such procedures, it is possible to construct new wine yeast strains that differ from the original only in single specific characteristics.

In addition to the introduction of specific genes into wine yeasts, recombinant DNA approaches offer wider applicability, including: (a) amplification of gene expression by maintaining a gene on a multi-copy plasmid, integration of a gene at multiple sites within chromosomal DNA or splicing a structural gene to a highly efficient promoter sequence; (b) releasing enzyme synthesis from a particular metabolic control or subjecting it to a new one; (c) in-frame splicing of a structural gene to a secretion signal to engineer secretion of a particular gene product into the culture medium; (d) developing gene products with modified characteristics by site-directed mutagenesis; (e) eliminating specific undesirable strain characteristics by gene disruption; (f) incorporation of genetic information from diverse organisms such as fungi, bacteria, animals and plants [56,119].

Genetic techniques of mutation, hybridization, cytoduction and transformation will most likely be used in combination for commercial wine yeast improvement. Strain modification has been revolutionized by DNA transformation strategies, but it remains difficult to clone unidentified genes. Thus, mutation and selection will persist as an integral part of many breeding programmes. Furthermore, although recombinant DNA methods are the most precise way of introducing novel traits encoded by single genes into commercial wine yeast strains, hybridization remains the most effective method for improving and combining traits under polygenic control.

Targets for wine yeast strain development

Some of the requirements listed in Table 2 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 has all the characteristics listed, and it is well established that wine yeasts vary in their winemaking abilities. While some degree of variation can be achieved by altering the fermentation conditions, a major source of variation is the genetic constitution of the wine yeasts.

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 provide full protection against genetic drift in pure yeast cultures. In fact, freeze-drying (lyophilization) for long-term maintenance

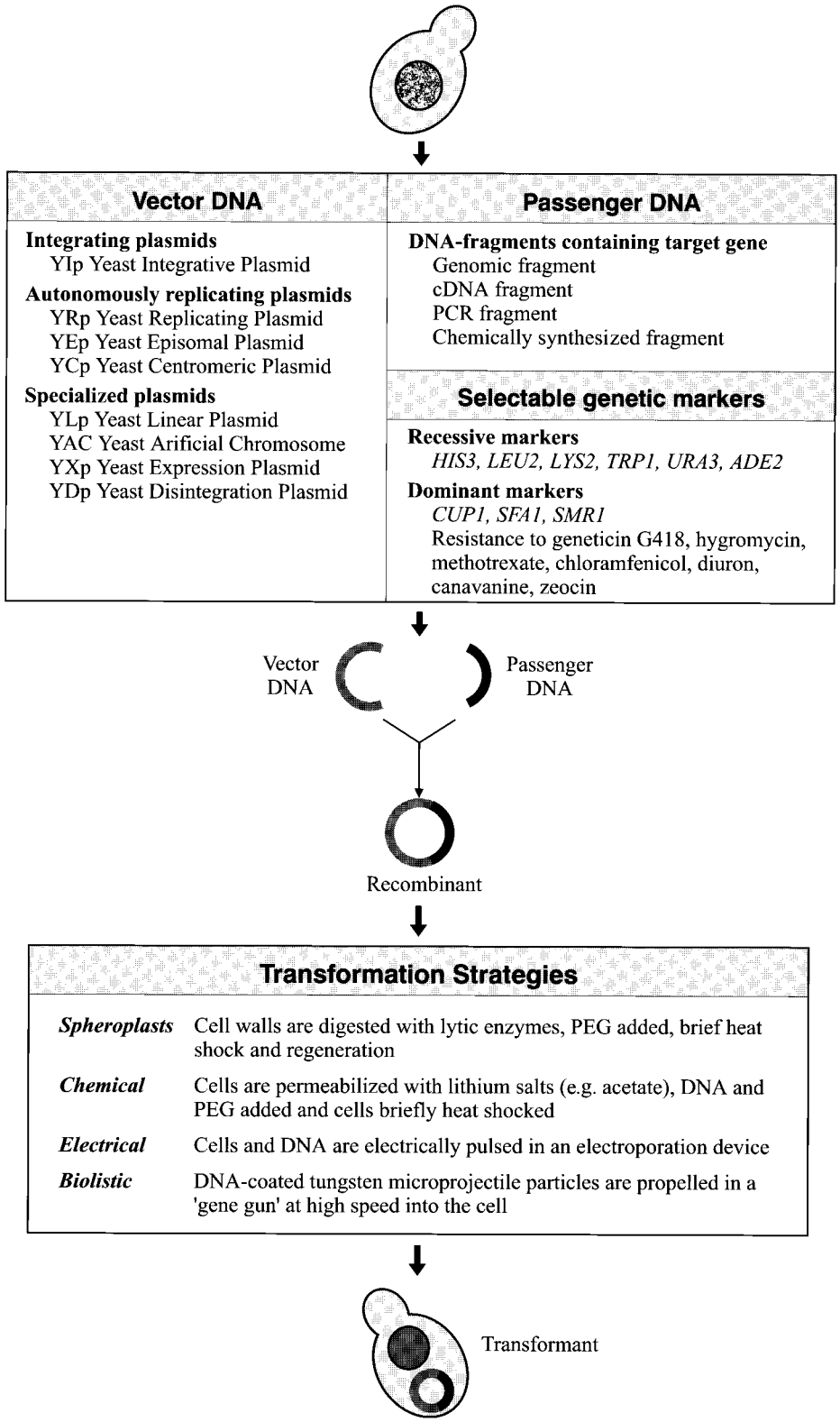

Figure 13. Gene cloning and transformation are used to introduce recombinant DNA molecules (e.g. possessing a useful gene) into wine yeasts

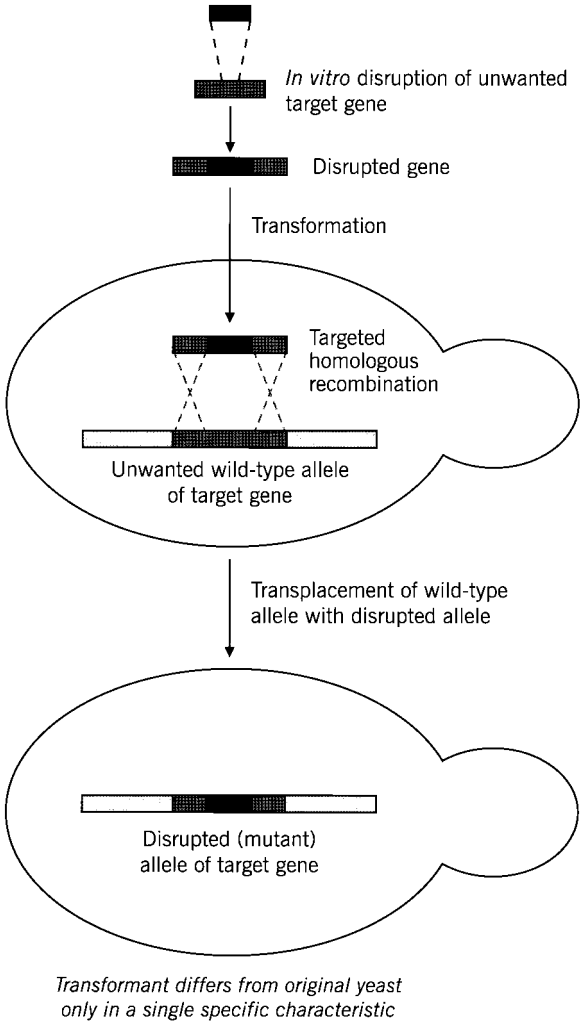

Figure 14. Homologous recombination can be used to transfer mutations into and out of a given locus on a yeast chromosome by the process of gene transplacement

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* [31]. Cryoprotectants and osmoprotectants, such as cellular trehalose and glycerol, also alleviate freeze–thaw and water stress [176]. Trehalose appears to stabilize cell membranes of lyophilized 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 lyophilized yeast cells.

A better understanding of precisely how yeast cells 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 exploiting genetic markers in wine yeast identification has been recognized, and deliberately marked oenological strains were developed as an aid to monitor the kinetics of yeast populations during wine fermentations [116,172]. Genetic labelling 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. The genomes of commercial wine yeasts can be tagged so as to 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 [172]. 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 [116]. 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 glasshouse. 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 [61]. 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 [61].

Generally, sugar catabolism and fermentation proceed at a rate greater than desired, and are usually controlled by lowering the fermentation temperature [62]. 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 [62]. 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 [61]. Conversely, financial losses through sluggish or incomplete wine fermentations are usually attributed to inefficient utilization of fermentor space and wine spoilage resulting from the low rate of protective carbon dioxide evolution and high residual sugar content [62]. Optimal performance of wine yeasts in white wine fermentations, conducted at cooler temperatures (10–15°C) so as to minimize the loss of aromatic volatiles, and red wine fermentations, performed at higher temperatures (18–30°C) to enhance extraction of anthocyanin pigments, is therefore of critical importance to wine quality and cost-effectiveness [61].

Fermentation predictability and wine quality are 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 presence of inhibitory substances, are 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 (Figure 15). 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 polarized growth. It is becoming clear that a complex network of interconnected and cross-talking signal transduction pathways, relying on a limited number of signalling modules, governs the required adaptive responses to changes that occur as the fermentation progresses [9].

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

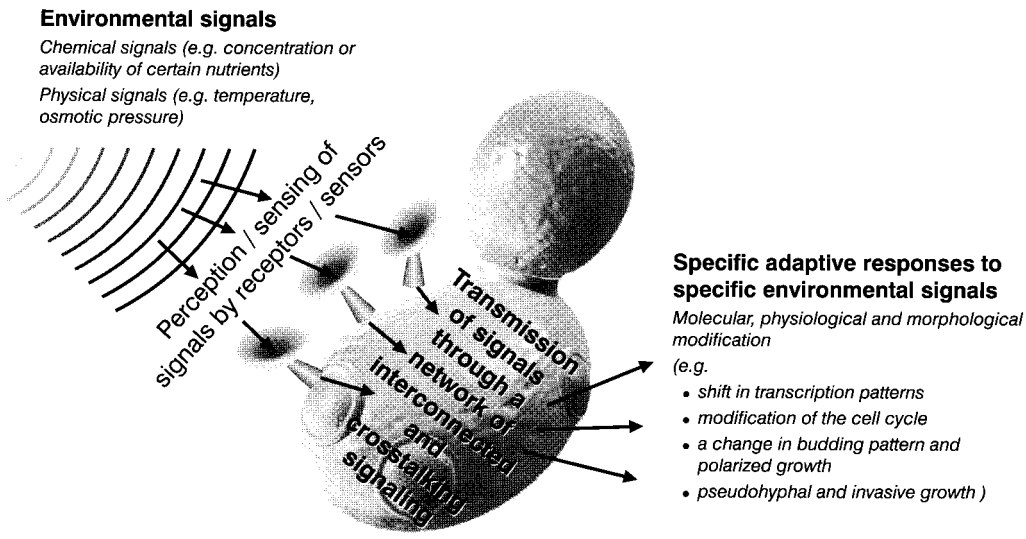

Figure 15. A schematic representation of how environmental signals are transmitted through a network of interconnected signal transduction pathways within a yeast cell. The ability of yeast cells to adapt to changing environmental conditions during yeast manufacturing or alcoholic fermentation is essential for their survival and physiological activity

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 the fitness or vigour of a starter culture [176]. 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) [176]. These techniques generally show varying degrees of correlation with fermentative performance and none of them, alone, can accurately predict the physiological activity of an 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 [27]. 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 [27]. 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 synthesized 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 (Figure 16) [39]. The regulation of trehalose synthesis and degradation (by trehalase) is mediated by cAMP-dependent phosphorylation mechanisms [152,153]. 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; toxic chemicals, such as ethanol, oxygen radicals and heavy metals [152]. 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 [27]. The accumulation of this disaccharide on both sides of the plasma membrane is thought to confer stress protection by stabilizing the yeast's membrane structure.

Glycogen, another carbohydrate reserve whose accumulation by yeast propagated for drying has been linked to enhanced viability and vitality upon reactivation, provides a readily mobilizable 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 catalyzes 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 (Figure 16) [176]. 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 [39]. This indicates that *GSY2* encodes the major glycogen synthase. Glycogen breakdown (catalyzed by glycogen phosphorylase) quickly following depletion of nutrients at the end of fermentation, is accompanied by sterol formation (Figure 17) [39]. Since sterol is essential for yeast vitality, low levels of accumulated glyco-

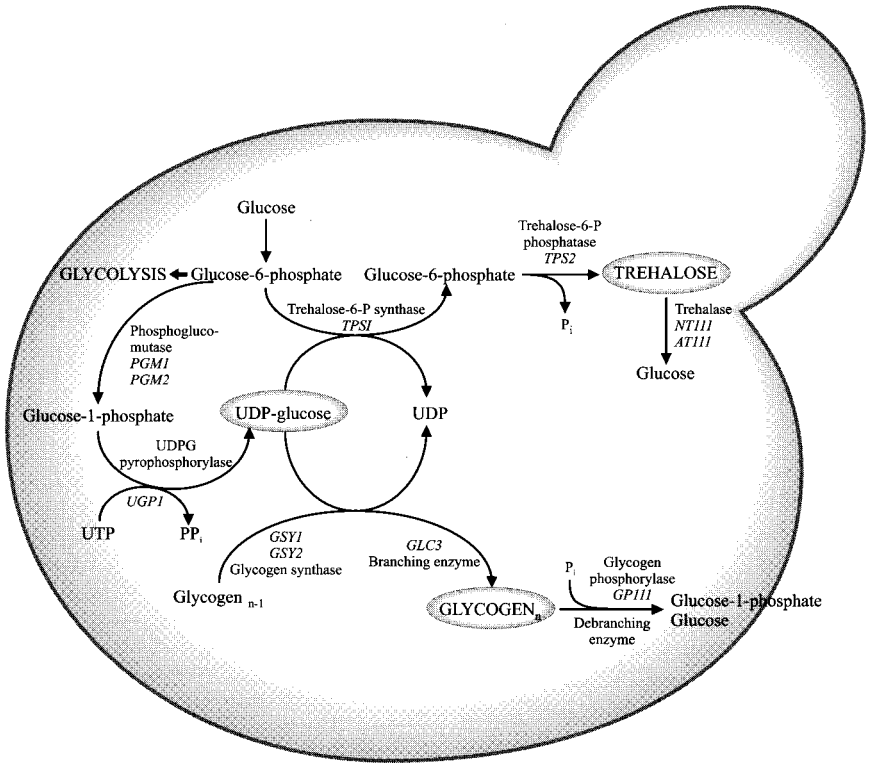

Figure 16. A scheme of the biosynthesis and degradation of glycogen and trehalose (adapted from Francois *et al.* [39])

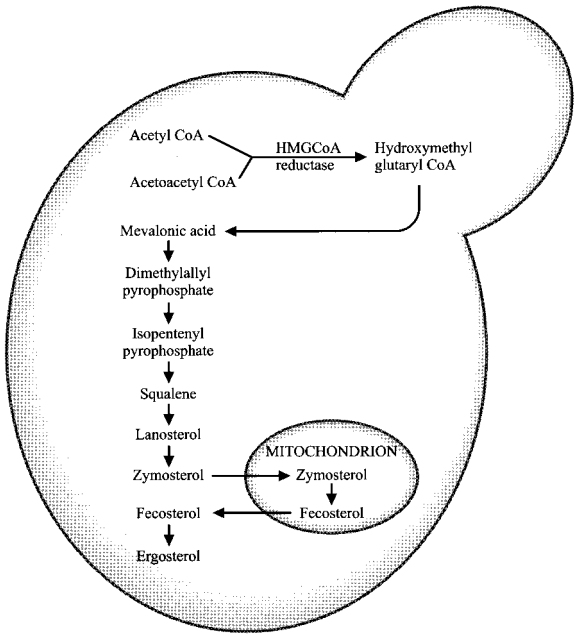

Figure 17. An outline of the main steps leading to ergosterol biosynthesis (adapted from Walker [176])

gen 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 [176]. 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 utilization

In *S. cerevisiae*, glucose and fructose, the main sugars present in grape must, are metabolized 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 yields 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 vapour [13]. 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 [13].

The first step to ensure efficient utilization 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 GLK (three isozymes) (Figure 18) [10].

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 [135]. 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 [13]. 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 towards the end of fermentation may result in reduced alcohol yields [176].

Sugars enter yeast cells in one of three ways: simple net diffusion, facilitated (carrier-mediated)

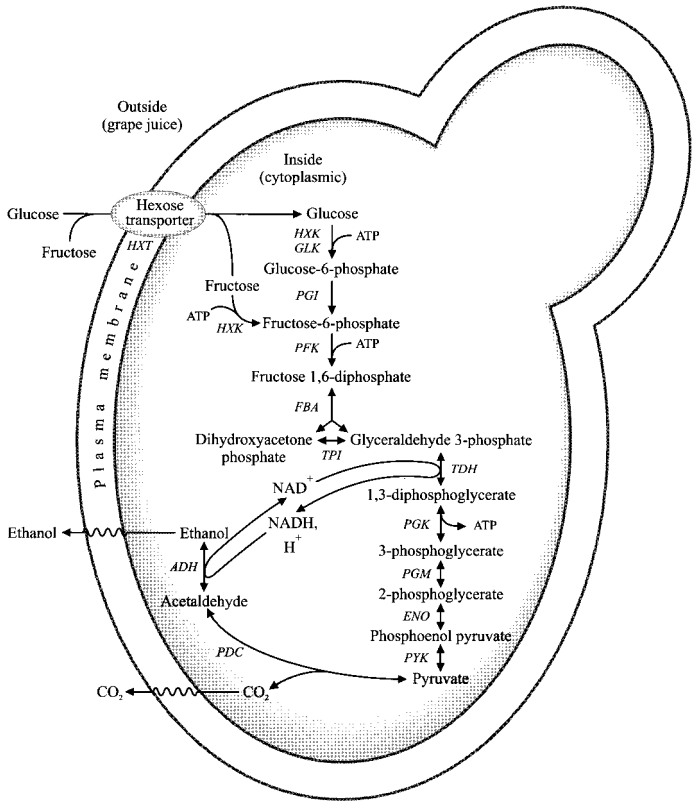

Figure 18. Enzymatic steps of the glycolytic pathway in wine yeast (adapted from Boulton *et al.* [13])

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) [13].

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 [10,19]. Several specific, energy-dependent glucose carriers mediate the process of facilitated diffusion of glucose and proton symport is not involved. Phosphorylation by 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 thus far none has been described as specifically affecting fructose uptake [176]. 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 [62]. 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 [71,72]. Such problematic and incomplete fermentations occur because nitrogen depletion irreversibly arrests hexose transport. Other problems 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 [62]. A schematic overview of nitrogen assimilation by *S. cerevisiae* is presented in Figure 19, while Figure 20 outlines the degradation of nitrogenous compounds in wine yeast.

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 [62]. The assimilable content of grape must is dependent upon grape cultivar and root stock, as well as upon several aspects of vineyard management, including nitrogen fertilization, berry maturation, vine water status, soil type and fungal infection [62]. 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

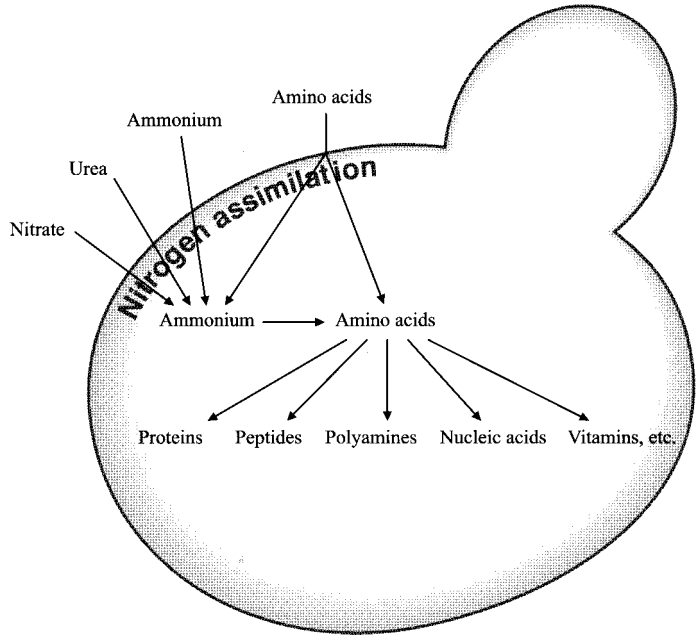

Figure 19. A schematic overview of nitrogen assimilation by wine yeast (adapted from Walker [176])

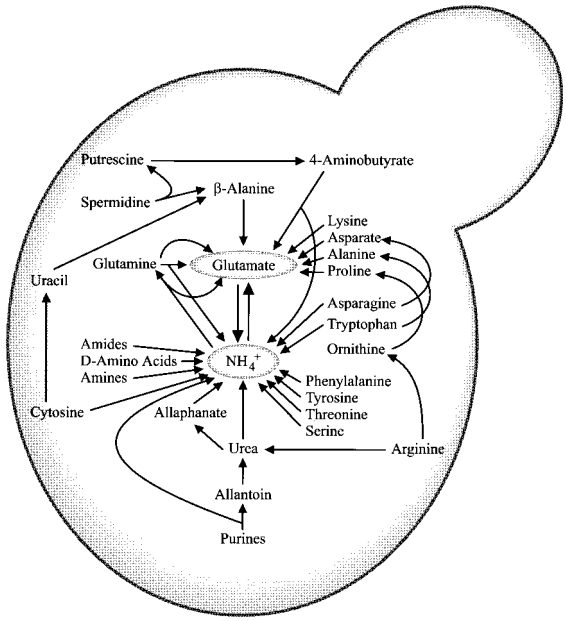

Figure 20. A schematic representation of the degradation of nitrogenous compounds by wine yeast (Henschke & Jiranek [62])

of nitrogen deficiency in grape juice by optimizing 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 minimize 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 [71,72,73]. 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 [13], while the ammonium ion levels may also be high, depending on grape variety and vineyard management. Proline and arginine account for 30–65% 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 post-harvest and prior to processing of the grapes [13].

S. cerevisiae is incapable of adequately hydrolyzing 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 [133]. When a readily used nitrogen source (such as ammonium, glutamine, or asparagines) is present, genes involved in the uptake and catabolism of poorly utilized nitrogen sources (including proline) are repressed. This nitrogen catabolite repression exerted upon non-preferred nitrogenous compounds rigorously impairs the assimilation of proline as well as arginine since both amino acids depend on the proline utilization pathway [133]. 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 [13]. Proline is transported into *S. cerevisiae* by the general amino acid permease and the *PUT4*-encoded proline-specific permease (Figure 21). 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 [133].

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 [61,71,72,73]. 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 utilizing proline

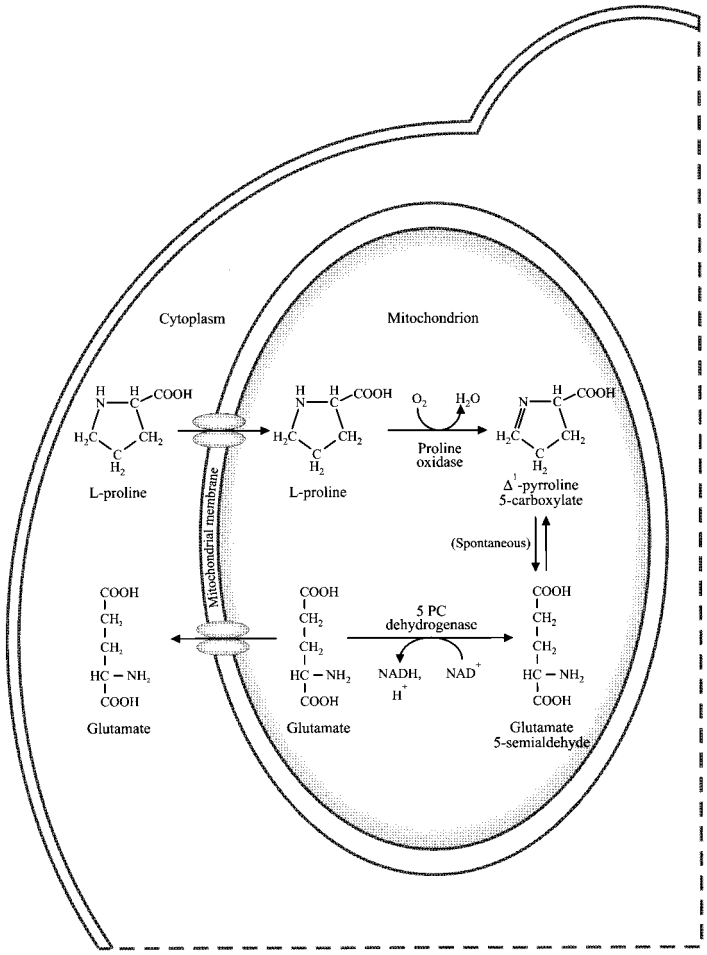

Figure 21. A schematic representation of the pathway for proline degradation in wine yeast (adapted from Boulton *et al.* [13])

more efficiently under winemaking conditions, a mutant containing a *ure2* recessive allele was constructed [133]. It was demonstrated that this mutation strongly deregulates the proline utilization 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 with 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 [13].

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 [13]. 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 [176]. 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 [61]. 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 [32]. 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 [176]. 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 [13].

Wine yeast strains usually contain higher levels of survival factors than non-wine *Saccharomyces* strains [13]. The physiological response of wine yeast to ethanol challenge is also greater than is the case with non-wine 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 neutralize 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 [13,176].

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 [13]. 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 [14]. 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. It remains controversial 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 [138]. 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}, occurs 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 [136].

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 programmes 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 minimize 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 [159]. An alternative to the use of cytoduction and hybridization 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 [61]. Membrane transport of sulphite in wine yeasts is by simple diffusion of liberated sulphur dioxide rather than being carrier-mediated [176]. 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 [13].

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 [158]. 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 [5]. 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 metallothionein proteins are generally synthesized 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 [38]. One way to engineer wine yeasts resistant to copper would be to clone and integrate the *CUP1* gene at multiple sites into their genomes [59]. 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 [142,156]. In some cases, foaming may also reduce the suspended yeast cell density in the fermenting must [61].

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* [74,154,155]. Apparently these genes, located on chromosome VII, code for proteins that interact with the grape juice thereby causing foaming [104]. Several researchers have successfully used intragenomic hybridization to cross out the genes that are responsible for foaming [33,171]. However, the *FRO1* and *FRO2* genes have yet to be cloned and their encoded proteins characterized. 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 [13]. 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 [13]. 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 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, isinglass, albumin and gelatin), whereas the depletion of monomeric and small polymeric phenols is reached by treatment with polyamide materials (e.g. polyvinylpolypyrrolidone or PVPP) [13]. 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 [13].

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 [13]. 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 5–20% loss of wine [15].

To omit the bentonite treatment, an application of an appropriate acid protease to hydrolyze 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 endoprotease, 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 [15]. 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 synthesized 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 promoter, 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 localization signal) with the yeast mating pheromone α-factor (*MFα1*-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, neither 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 post-harvest processing, may hold the key to controlling the concentrations of protein in wine.

Like grape proteins, polysaccharides also influence the clarification and stabilization 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 heteropolysaccharides consisting of partially methylated α-1,4-D-galacturonan chains linked to L-rhamnopyranose units carrying neutral side chains [120]. Glucans such as β-1,3-1,6-glucan produced by the grey mould *Botrytis cinerea* in botrytized grape juice, comprise β-D-glucopyranose units with a high degree of polymerization [120]. Xylans are complex polymers consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl and glucuronosyl side chains [120]. Enzymatic breakdown of pectic polymers occurs by the de-esterifying action of pectinesterase, releasing the methyl 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 hydrolyzed 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 [120].

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 filter stoppages [15]. Industrial enzyme preparations are widely used to supplement these polysaccharide-degrading activities [20]. Most commercial pectinase and glucanase preparations are

derived from *Aspergillus* and *Trichoderma*, respectively [15].

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 [48], while all strains of *S. cerevisiae* show some form of glucanase activity [120]. All of these glucanase genes have been cloned and characterized. 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* [85,86,87]. 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 [87]. 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 gene (*BEG1*), the *Ruminococcus flavefaciens* cellodextrinase gene (*CEL1*), the *Phanerochaete chrysosporium* cellobiohydrolase gene (*CBH1*) and the *Saccharomycopsis fibuligera* cellobiase gene (*BGL1*) [162,163,164,165,166]. 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*) [22,23,81,82,96,103]. The *xlnA* and *xlnB* genes from *Aspergillus nidulans* were also reported to be expressed in *S. cerevisiae* [114].

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 many 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 [119].

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 [52,177]. 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 [151]. Proteinaceous 'hairy' protrusions called 'fimbriae' often emanate from the cell surface of flocculant *S. cerevisiae* cells [139]. Cell surface charge and hydrophobicity have also been implicated in a primary or complementary role with lectins to facilitate the onset of flocculation [150,183]. Environmental factors that may influence the level of flocculant *S. cerevisiae* strains include temperature, pH, calcium and zinc ions, certain inhibitors, oxygen content, sugar and inositol depletion, growth phase and cell density [57].

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 [149]. 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 [143,144,148]. The flocculation genes include *FLO1, FLO2, flo3, FLO4, FLO5, flo6, flo7, FLO9, FLO10* and *FLO11/MUC1* [88,93,151]. The *FLO11/MUC1* gene was also shown to be involved in pseudohyphal development and invasive growth [88], while *FLO8* was reported to encode a transcriptional activator of *FLO1* and *FLO11/MUC1* [46,47,92]. Apparently, Flo8p inactivates the *TUP1* and *CYC8/SSN6*-encoded cascade which represses flocculation and pseudohyphal differentiation in certain strains [76,92]. 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 [18]. Furthermore, it is unclear what the advantage would be to the yeast cell of co-regulating the expression of *FLO11/MUC1* and three glucoamylase-encoding genes (*STA1, STA2* and *STA3*) involved in starch metabolism [174]. In fact, the unusually long (>3 kb) promoter sequences of *FLO11/MUC1* and *STA1-STA3* 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 [46,47,88,181].

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-phosphatidyl-inositol-anchor-containing proteins, separated by a central domain of highly repeated sequences rich in serine and threonine residues [88,93]. 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-flocculant *S. cerevisiae* strains [56,69,178].

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 conversion, efficient settling is needed to minimize problems with wine clarification [61]. Moreover, flocculation has also been linked to enhanced ester production. For these reasons we have linked the *FLO1* gene to the *HSP30* gene promoter [170]. It is known that the *HSP30* promoter induces high gene expression during late stationary phase [130]. 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 [170].

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 [61]. Flor sherry is characterized 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 flavours 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 flavours [21,107]. 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 [54]. In brandy, the character is further changed as distillation alters the absolute and relative amounts of volatiles.

The flavours of wine and brandy immediately after fermentation or distillation only approximate those of the finished product [16]. 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 [16].

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 [21,107]. In wines and brandies, the major products of yeast fermentation, esters and alcohols (Figure 22), 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

The varietal flavour of grapes is mainly determined by the accumulation and profile of volatile secondary metabolites in *V. vinifera* [61]. 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 aglycone to intensify the varietal character of wines [15]. 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 hydrolyze sugar conjugates of tertiary alcohols such as linalool [15]. Moreover, these grape enzyme activities are inhibited by glucose and exhibit poor stability at the low pH and high ethanol levels of wine [61]. 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 [15].

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 [15]. 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

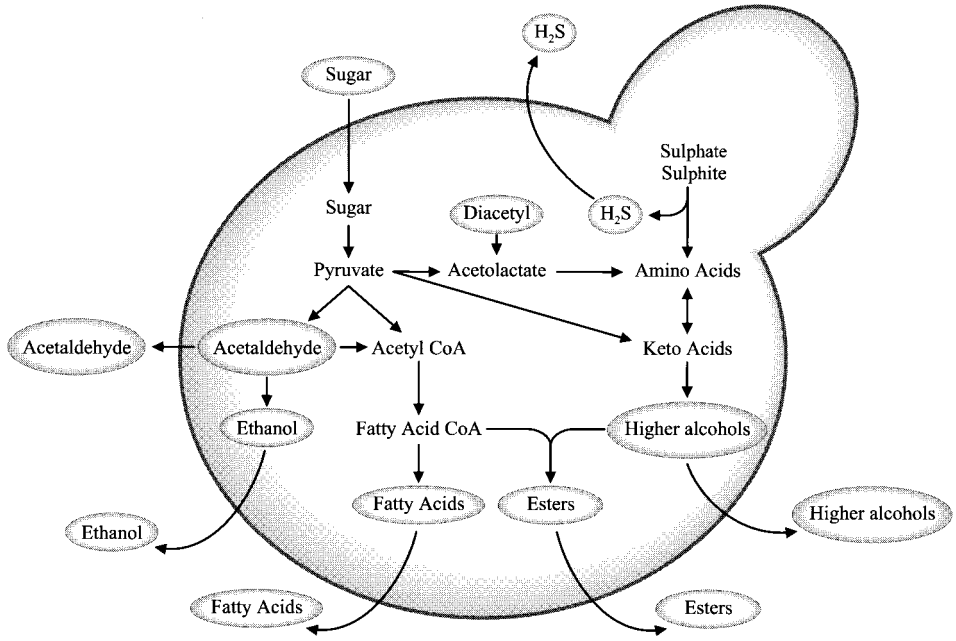

Figure 22. A schematic representation of derivation of flavour compounds from sugar, amino acids and sulphur metabolism by wine yeast (adapted from Henschke and Jiranek [62])

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 [15]. Since these β-glucosidases are absent in most *S. cerevisiae* starter culture strains, we have functionally expressed the β-glucosidase gene (*BGL1*) of the yeast *Saccharomycopsis fibuligera* in *S. cerevisiae* [166]. 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 [115]. 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* [164,165,166]. Further trials are under way 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 [70]. 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 (Figure 22) [63,64,101,108,145,147]. 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 [21,65]. Furthermore, the ester content of distilled beverages is greatly dependent on whether the yeast lees is present during distillation [16,97,108].

The characteristic fruity odours of wine are

primarily due to a mixture of hexyl acetate, ethyl caproate and caprylate (apple-like aroma), isoamyl 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) [98,99]. These acetyltransferases are sulphhydryl (SH) enzymes which react with acetyl co-enzyme A (acetyl-CoA) and, depending on the degree of affinity, with various higher alcohols to produce esters [43,44,45]. 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 vacuoles, 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 [91]. Integration of this modified copy of *ATF1* into the genomes of three commercial wine yeast strains (VIN7, VIN13 and WE228) resulted in the over-expression 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 [91]. 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.

Optimized fusel oil production

Alcohols with carbon numbers greater than that of ethanol, such as isobutyl, isoamyl and active amyl alcohol, are termed fusel oils. 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 [180]. At high concentrations, these higher alcohols have undesirable flavour and odour characteristics [53]. 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 [78]. 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 [142].

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 [67,68]. 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 [142]. 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 [122,134].

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), sulphite levels and pH of grape must, fermentation temperature, aeration and choice of starter culture strain and inoculation level [129,134]. Typically, under controlled conditions, glycerol concentrations are higher in red wines than in white wines, varying

from 1 to 15 g/l [134]. 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 [134]. Wine yeast strains overproducing glycerol would therefore be of considerable value in improving the organoleptic quality of wine [102,129].

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 4–10% of the carbon source is usually converted to glycerol, resulting in glycerol levels of 7–10% of that of ethanol [134]. 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 [129]. Conversely, wine yeasts in which the glycerol pathway has been minimized would yield more alcohol, which would be of great value for the production of brandy and other distilled products [102].

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 [122]. Futhermore, glycerol-3-phosphate, the precursor of glycerol, is an essential intermediate in the biosynthesis of membrane lipids. 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^+ [134]. Glycerol formation entails the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, a reaction catalyzed by glycerol-3-phosphate dehydrogenase and followed by the dephosphorylation of glycerol-3-phosphate to glycerol by glycerol-3-phosphatase [129]. Two cytosolic glycerol-3-phosphate dehydrogenases, considered the key limiting enzymes for glycerol formation in wine, are encoded by *GPD1* and *GPD2* [134]. The expression of *GPD1* is usually increased by hyperosmotic stress, whereas *GPD2* expression is increased by anaerobic conditions [134]. 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 [134].

Conversely, the utilization of glycerol is coupled to respiration via a glycerol kinase. This *GUT1*-encoded glycerol kinase converts glycerol to glycerol-3-phosphate, which is then oxidized to dihydroxyacetone phosphate by the *GUT2*-encoded, flavin-dependent and membrane-bound mitochondrial glycerol-3-phosphate dehydrogenase [134]. *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 [134].

Slight increases in glycerol production in wine can be achieved by using yeast strains selected or bred for high glycerol production, and by optimizing 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 four-fold increases in glycerol levels were obtained [102,129]. 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 (Figure 23). A lower biomass concentration was attained in the *GPD1*-overexpressing strains, probably due to high acetaldehyde production during the growth phase [129]. 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 [129]. Other side-effects of these glycerol-overproducing yeasts included the accumulation of by products such as pyruvate, acetate, acetoin, 2,3-butanediol and succinate [102,129].

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

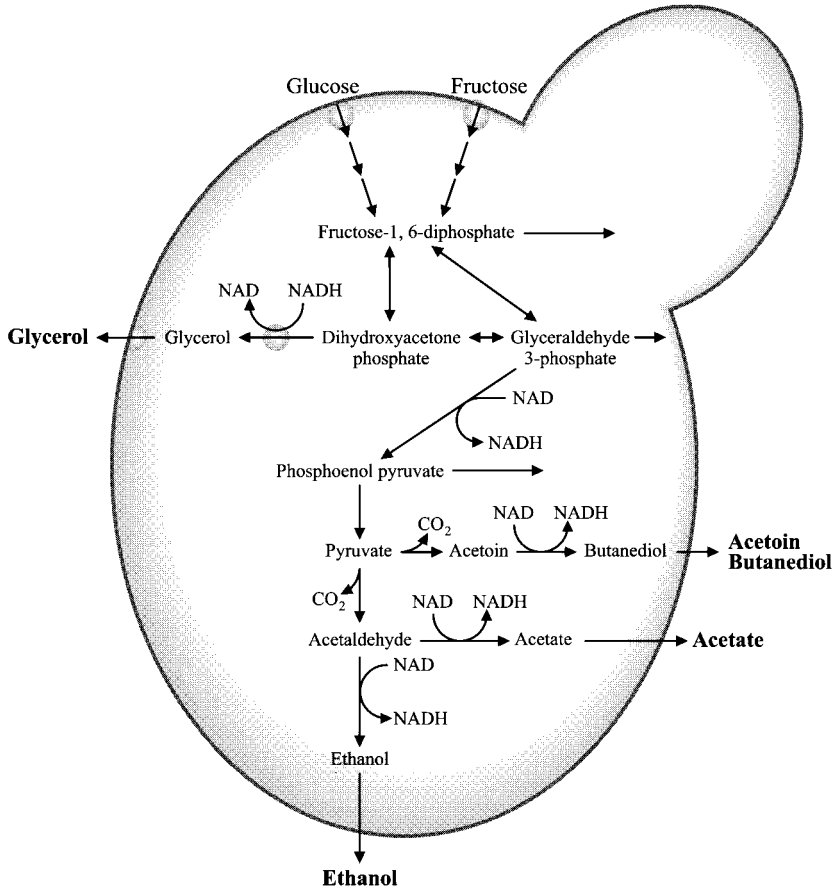

Figure 23. A schematic representation of the overproduction of glycerol in wine yeast resulting in the production of acetate, acetoin and butanediol

ALD6 produced two- to three-fold more glycerol and a similar amount of acetate compared to the untransformed strain [129]. 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 [129].

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 polymerization of the colour pigments, and the oxidative and browning reactions [17]. 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; and the cultivar and final berry volume at harvest [17,90]. Of all these factors the climatic conditions and

ambient temperature have a critical effect on grape maturation and resulting acidity of the fruit [90]. 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 neutralization 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 catabolized 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 minimize 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 [24]. 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 [49]. Attempts to fuse wine yeasts with malate-assimilating yeast also failed [127]. 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 [50].

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 [175]. 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*, [2,3,11,28] *Lactobacillus delbrueckii* [184] and the *mle*A gene from *O. oeni* [83] 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 [28]. However, due to the absence of an active malate transport system in *S. cerevisiae*, these engineered strains could still not metabolize malate efficiently [175]. 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 [175].

Similarly, an efficient malo-ethanolic *S. cerevisiae* was constructed by co-expressing the *mae1* permease gene and the *mae2* malic enzyme gene from *S. pombe* in *S. cerevisiae* [175]. 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 [30].

Due to the inefficiency of the mitochondrial lactate dehydrogenases under fermentation conditions, natural *S. cerevisiae* strains produce only traces of lactic acid during alcoholic fermentation [51]. In an attempt to redirect glucose carbon to lactic acid in *S. cerevisiae,* the lactate dehydrogenase-encoding genes from *Lactobacillus casei* [29] and bovine muscle [117] were expressed in laboratory yeast strains. Encouraged by the fact that the *L. casei* lactate dehydrogenase 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 [30]. Wines obtained with these engineered lactic acid–alcoholic fermentation yeasts were shown to be effectively acidified, but the fermentation rate was slower [30].

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 [61]. 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 non-oxidative fashion to vinylphenols. Perhaps the disruption of *POF1* could provide a way to reduce the content of volatile phenols in, at least, white wines [61].

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 [128]. These substances are formed in grapes during ripening; dusting of vines with fungicides containing elemental sulphur provides 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 [142]. Sulphite is only formed from sulphate, while sulphide is formed from sulphate, sulphite, from elemental sulphur applied as a fungicide, and from cysteine (Figure 24) [128,142]. 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 [128]. 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 [61]. 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 [128]. 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

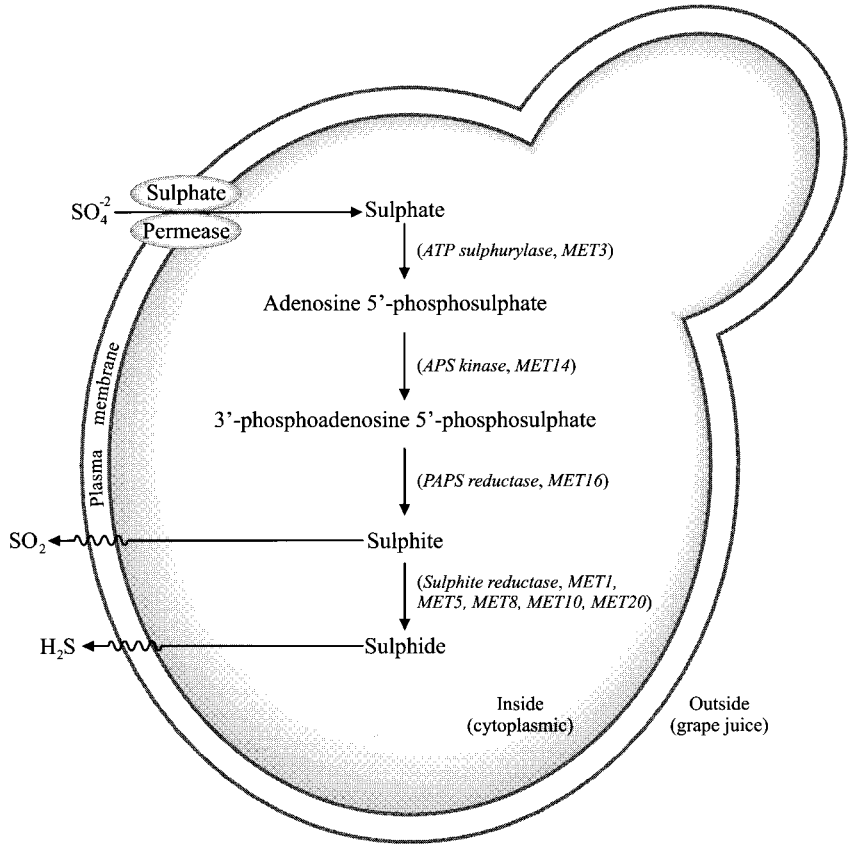

Figure 24. A schematic representation of the pathway for sulphate reduction (adapted from Boulton *et al.* [13])

in the presence of sulphate, sulphite and djencolic-acid [128]. Methionine and cysteine are known to prevent an increase in the levels of sulphite reductase, *O*-acetylserine sulphydrylase and *O*-acetylhomoserine sulphydrylase [128]. 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 [128].

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 [61]. 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 (Figure 25) [71]. 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 accumulation [61].

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 synthesize and supply sulphur-containing amino acids to the growing yeast cell [62,71,72,73]. The addition of ammonium salts prevents H_2S accumulation in wine, not by stopping its formation but by enabling the yeast to synthesize amino acid precursor compounds which react with H_2S to form sulphur-containing acids [62]. 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.

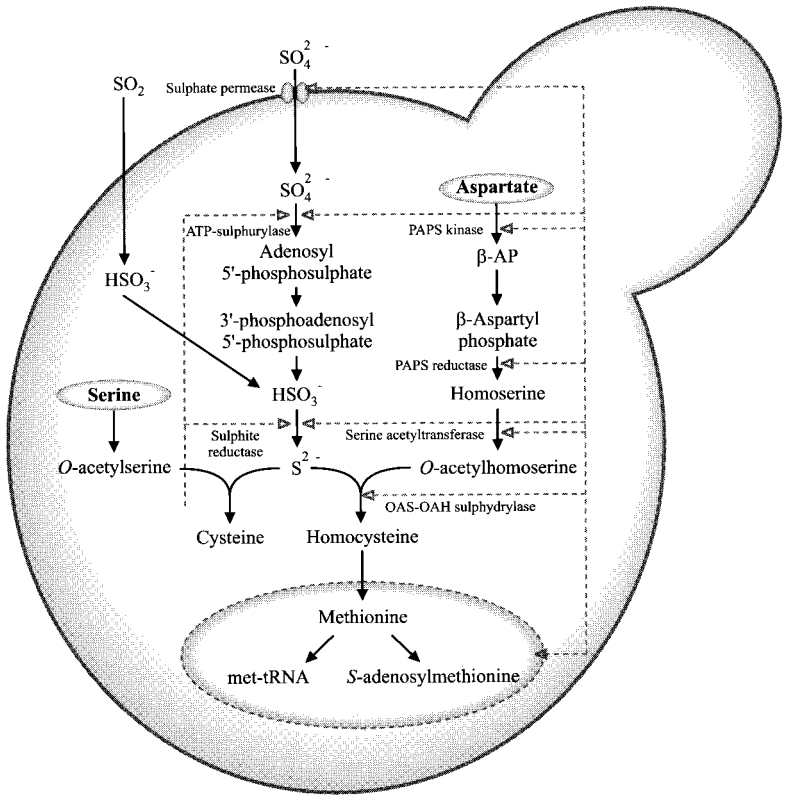

Figure 25. A schematic representation of the repressive regulation of the sulphate reduction sequence and methionine–cysteine biosynthetic pathway (adapted from Henschke and Jiranek [62])

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 [61]. 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 [62].

Yeast strains differ widely in their ability to produce sulphite and sulphide [62]. 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 hybridization. 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 [61]. 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 [71,72,73] and this could also provide a target for down regulation of H_2S formation in wine yeasts.

Improvement of wine wholesomeness

Until the eighteenth 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 twentieth 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 1990s medical science was reporting that moderate consumption, especially of 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 [131]. 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 industrialized 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 induce specific enzymes which metabolize 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 synthesized 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 (*C4H*) and the coenzyme A ligase gene (*4CL216*) in *S. cerevisiae*. The introduction of the grape stilbene synthase gene (*Vst1*) may then catalyze 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 [112]. 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 ageing wines, fortified wines, and brandies by reaction between urea and ethanol [111]. For this reason, excessive application of urea-containing fertilizers 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 [113].

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 (Figure 26). 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 metabolize the external urea. Although all *S. cerevisiae* strains secrete urea, the extent to which they re-absorb the urea differs [1]. *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 [75]. This arginase deletion mutation resulted in a yeast strain that could not metabolize 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 [110]. 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

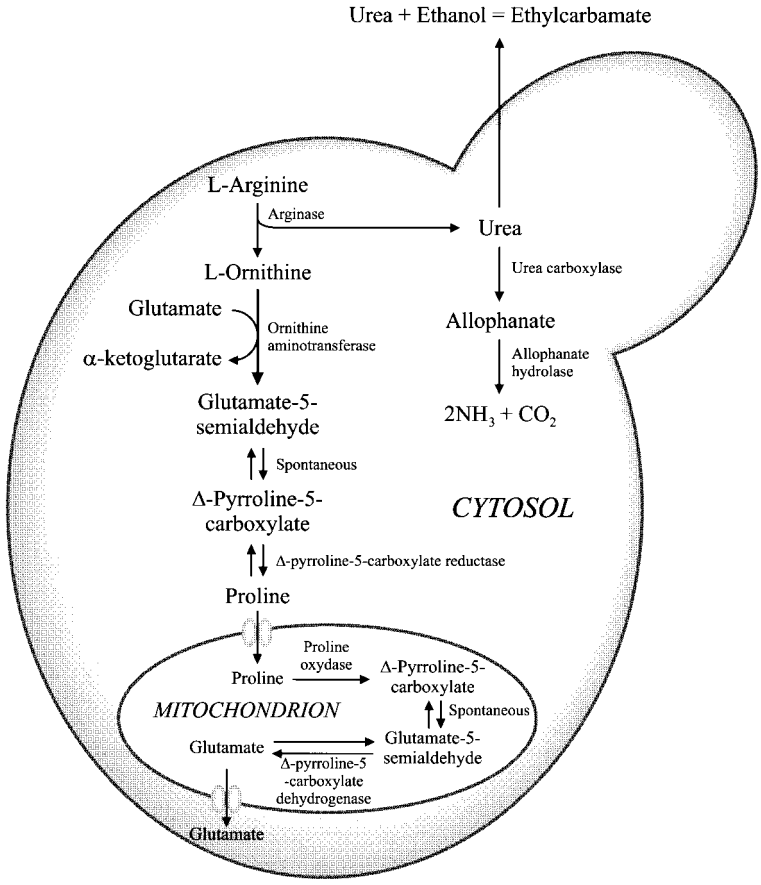

Figure 26. A schematic representation of arginine catabolism and urea formation in wine yeast (adapted from Henschke and Jiranek [62])

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 [173]. 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 [173]. 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 composition 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 [61].

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 [89]. 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 [89].

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 balii* causes turbidity after re-fermentation during storage of wine or after bottling, resulting in sediment formation and reduction in acidity [146]. Wines spoiled by *Pichia membranaefaciens* and *Candida krusei* taste oxidized and less acidic [146].

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* [146]. 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*) [146].

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 [41]. 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 [141]. 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 [4,60,109,125,126]. 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 [109]. 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 pivotal role in the defense mechanisms of host organisms against infection by fungi and bacteria [42]. 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 hydrolyzing 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 [42]. 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 [42].

The OIV has recently approved the use of commercial lysozyme preparations to control malolactic fermentation and to stabilize 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 [106]. 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 [66]. 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 widespread among grape, must, and wine-related yeast genera, including *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Kloeckera, Kluyveromyces, Pichia* and *Rhodotorula* [138]. 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 [138].

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 hybridized 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* [12]. 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 successfully expressed two bacteriocin genes in yeast, the one encoding a pediocin and the other a leucocin [137]. The pediocin gene originates from *Pediococcus*

acidilactici PAC1·0 [188] and the leucocin gene from *Leuconostoc carnosum* B-Ta11a [34].

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) [188]. The leucocin operon of *L. carnosum* comprises two genes: *lcaB* (encoding a 61 amino-acid precursor of the B-Ta11a leucocin) and $lcaB_I$ (encoding a 113 amino-acid immunity factor) [34]. 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 *lcaB* 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 α-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.

Complying with statutory regulation and consumer demands

S. cerevisiae has enjoyed a long and distinguished history in the fermented food and beverage industries; it is without doubt the most important commercial microorganism with GRAS ('generally regarded as safe') status. By brewing beer, leavening bread dough, and sparkling wine, mankind's oldest domesticated organism made possible the world's first biotechnological processes.

With the emergence of modern molecular genetics, *S. cerevisiae* has again been harnessed to shift the frontiers of mankind's newest revolution, genetic engineering. The first approved human vaccine (against hepatitis B) and food product (calf chymosin for cheese making) resulting from recombinant DNA technology were produced with transgenic *S. cerevisiae* strains [7]. *S. cerevisiae* was also the first genetically modified *organism* (GMO), as distinguished from a genetically modified *product*, to be cleared for food use, as a baking and brewing strain [176]. The genetically modified baking strain containing constitutively expressed maltose permease and maltase genes, produces CO_2 faster than conventional baker's yeasts, thereby ensuring that dough rises more rapidly [140]. The novel engineered feature of the pioneer GMO brewer's yeast is a glucoamylase-encoding gene that allows partial hydrolysis of maltodextrins, yielding a lower-carbohydrate beer [140]. Although not yet cleared for commercial use, considerable progress has been made, as detailed in this review, in genetically tailoring wine yeast for specific wine-making processes and products of the vine.

While the scientific case for use of genetic modification in the improvement of food organisms is strong and persuasive, genetically modified baking, brewing, and wine yeasts have not, as yet, been used commercially. The public perception of risk with regard to GM food has, so far, outweighed its view of possible benefits.

Genetic engineering, lauded as a spectacular achievement in science, has unfortunately been repackaged in an emotive, fear-mongering wrapping. Critics are whipping up public alarm, often to fuel political agendas and to protect agricultural markets. The myths of 'Frankenfood' and global havoc caused by GMOs have been spread far enough to masquerade in the cultural folklore as truth. Fears about food and environmental safety spread more readily than good sense or wise science; this has evoked a plethora of strict legislation and regulatory guidelines based far more on emotion than on science.

This mostly irrational debate began with questions about the morality ('unnatural', interfering with evolution, playing God, etc.) and safety (GMOs and GM products are inherently dangerous, toxic to humans and bad for the environment)

of genetic engineering. Over the years, it has become generally agreed that these fears are largely groundless; to date, no scientifically reputable test has shown any of the GM foods currently on the shelf to be in the least toxic.

The initial problems with statutory approval and negative public perception of genetically engineered organisms in food and beverages are now slowly being dissolved by a growing consensus that risk is primarily a function of the characteristics of a product, rather than the use of genetic modification *per se*. Scientists have reached a broad consensus that organisms, whether modified by modern molecular or cellular methods or not, respond to the same physical and biological laws. Therefore, no conceptual distinction exists between modification of yeast and grapevine by classical methods and that by molecular techniques that modify DNA and transfer genes.

Regulations, although differing in detail, are broadly similar in most countries. Guidelines for approval of GM products and the release of GMOs usually require a number of obvious guarantees. These include a complete definition of the DNA sequence introduced, and the elimination of any sequence that is not indispensable for expression of the desired property; the absence of any selective advantage conferred on the transgenic organism that could allow it to become dominant in natural habitats; no danger to human health and/or the environment from the transformed DNA; and a clear advantage to both the producer and the consumer.

The concept of 'substantial equivalence' is widely used in the determination of safety by comparison with analogous conventional food and beverage products [140]. When substantial equivalence can be demonstrated, then usually no further safety considerations are necessary. When substantial equivalence is not convincingly shown, the points of difference must be subjected to further safety scrutiny. However, to date, regulatory authorities appear more willing to approve the use of GMOs than the public is to use them. A significant proportion of the public still suspects that GM food will prove unhealthy in the long term, and that the escape of GMOs with transplanted genes will damage the environment and result in the loss of biodiversity. However, their questions now appear to be growing more specific. Is the product safe to the consumer and the environment? Is there sufficient legal and practical protection against accidents involving GMOs? Will genetic engineering reduce biodiversity and concentrate economic power in the hands of a few large producers? Do patents on living organisms confer an unfair advantage on certain producers? Should products produced by gene technology be specifically labelled?

It is clear that consumer education is essential to remove their fear of the unknown. Scientists must consistently inform the public and remain open about experiments, research and products. The consumer should be reassured of first class, transparent regulatory systems and the meticulous implementation of biosafety legislation with clear technical standards and definitions with respect to GM products. The consumer should be persuaded by proper risk assessment and clear demonstration of safety, and thus empowered to make informed decisions.

There are a number of activities that must be scrupulously avoided: conducting obviously risky experiments; misusing scientific data and exploiting consumer confusion to justify trade bans and technical barriers to free trade; riding the 'backlash' market with labels stating that a particular product is 'GM-free'; suppressing 'inconvenient' scientific data or simply lying about food safety (as has been the case for some governments with distressingly bad biosafety records); and 'force-feeding' GM products and GMOs down consumers' throats for profit when there is no clear advantage for the consumer.

Successful application of recombinant DNA technology in the wine industry will depend on assuring commercial users of genetically modified wine yeasts that existing desirable characteristics have not been damaged, that the requirements of beverage legislation are met, and that the engineered strain will be stable in practice, with suitable procedures for monitoring. The first recombinant wine products should unequivocally demonstrate organoleptic, hygienic and economic advantages for the wine producer and consumer. Furthermore, wine's most enthralling and fascinating aspect, its diversity of style, should never be threatened by the use of tailored wine yeast strains. In fact, gene technology should rather be harnessed to expand the diversity of high quality wines and other grape-derived products.

There is vast potential benefit to the wine

consumer and industry alike, in the application of this exciting new technology. That benefit will be realized, though, only if the application is judicious, systematic, and done with high regard for the unique nature of the product. *In vino veritas!*

Conclusions

Over the last few years considerable progress has been made in developing new wine yeast strains. However, the wine industry, two decades after the first successful yeast transformation, has entered the third millennium without a transgenic wine yeast used on a commercial scale to produce wine.

Successful commercialization 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 about rapid commercialization and short-term benefits. The information and technology that currently exist for laboratory strains of *S. cerevisiae* must be expanded to the much more complex genomes of industrial wine yeast strains before dramatic breakthroughs can be expected. Simultaneously, innovative technical strategies are required to comply with the strict statutory regulations that pertain to the use of GMOs. Credible means must be found to effectively address the concerns of traditionalists within the wine industry and the negative overreaction of some consumer groups.

These are daunting challenges that will have to be overcome during the next few years. But given the availability of the complete genome and proteome of *S. cerevisiae*, the current energetic effort to decipher the function of the roughly 6000 yeast genes, and the eventual understanding of the interaction of gene networks within the yeast cell, there is no question of new and innovative developments that will be of great benefit to both the winemaker and the wine consumer.

With their broad experience in yeast-based fermentations, winemakers are well placed to explore new opportunities offered by the exciting age of molecular yeast genetics and modern biotechnology. I am confident that leading-edge gene technology will be sensibly applied to wine yeast strains and that it will help the wine industry meet the technical challenges of the twenty-first century. Wine yeast toasts the new millennium!

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