Yeast and bacterial modulation of wine aroma and flavour

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Abstract

Wine is a highly complex mixture of compounds which largely define its appearance, aroma, flavour and mouth-feel properties. The compounds responsible for those attributes have been derived in turn from three major sources, viz. grapes, microbes and, when used, wood (most commonly, oak). The grape-derived compounds provide varietal distinction in addition to giving wine its basic structure. Thus, the floral monoterpenes largely define Muscat-related wines and the fruity volatile thiols define Sauvignon-related wines; the grape acids and tannins, together with alcohol, contribute the palate and mouth-feel properties. Yeast fermentation of sugars not only produces ethanol and carbon dioxide but a range of minor but sensorially important volatile metabolites which give wine its vinous character. These volatile metabolites, which comprise esters, higher alcohols, carbonyls, volatile fatty acids and sulfur compounds, are derived from sugar and amino acid metabolism. The malolactic fermentation, when needed, not only provides deacidification, but can enhance the flavour profile.

The aroma and flavour profile of wine is the result of an almost infinite number of variations in production, whether in the vineyard or the winery. In addition to the obvious, such as the grapes selected, the winemaker employs a variety of techniques and tools to produce wines with specific flavour profiles. One of these tools is the choice of microorganism to conduct fermentation. During alcoholic fermentation, the wine yeast Saccharomyces cerevisiae brings forth the major changes between grape must and wine: modifying aroma, flavour, mouth-feel, colour and chemical complexity. The wine bacterium Oenococcus oeni adds its contribution to wines that undergo malolactic fermentation. Thus flavour-active yeasts and bacterial strains can produce desirable sensory results by helping to extract compounds from the solids in grape must, by modifying grape-derived molecules and by producing flavour-active metabolites. This article reviews some of the most important flavour compounds found in wine, and their microbiological origin.

Keywords: acids, aroma, bacteria, esters, higher alcohols, terpenes, thiols, wine, yeast

1. Introduction

1.1 Wine quality and perceived value

It is surprising that the wine industry attracts so much attention, given its relative size. Vines cover less than 0.5% of the world’s crop land and wine accounts for just 0.4% of global household consumption – but for millions of investors and hundreds of millions of consumers, the industry’s products command intense interest, often bordering on obsession (Anderson 2004). Although there is value addition to the grapevine through the production of wine and its subsequent enticing packaging and intensive marketing, it is still surprising how fascinated people are with this product, one cloaked in mysticism and romanticism. Wine writers often collapse into language like ‘the artistry of bottling poetry, the science of bottling sunshine, and the economics of bottling sustainable consumer satisfaction – a unique artistic masterpiece of individual creativeness, innovative technology and smart ‘bottomlining’ when writing about the product and not the process. Why is this the case? There are as many hypotheses to this question as there are labels on the shelves of a good wine merchant.

Whatever the explanation, the wine industry has come to realise that centre stage is a market-driven space (Figure 1), and today’s consumers vote with their wallets for those wine producers who offer a pleasurable and recognisable ‘sensory experience’. They expect a safe product produced in an environmentally sustainable manner and enjoyable in all sensory aspects (Bisson et al. 2002). The wine industry’s challenge is to respond to these consumer sentiments and deliver products at superior quality/price ratios.

The terms quality and value are widely used in reference to wine; the International Standards Organisation defines quality as the ‘degree to which a set of inherent characteristics fulfils requirements’ (Francis et al. 2005). It is instructive to relate this definition to the different elements involved in the wine production chain. To the consumer who compares wines for purchase, fulfilling requirements is associated with the ‘intrinsic’ sensory quality of the wine, i.e. how the wine pleases on appearance, the nose and the palate, as well as the perceived value.

Value is related to both intrinsic quality and image – the latter derived from many aspects such as how the wine is marketed, awards received, winery environmental sustainability record – and cost (Francis et al. 2005). Thus, a wine with delightful and recognisable sensory attributes and a high perceived image at a competitive price would be considered by consumers as high in value.
This quality/value equation underlies the successes of the Australian wine industry and Brand Australia; producers listen to the consumer. However, in a globalised, market-driven sector with more than 34 countries producing hundreds of thousands of different wine labels and a global annual surplus of about 6 billion litres of unsaleable wine (Pretorius and Bauer 2002), no wine industry can afford to entertain complacency. Meeting quality requirements in the future will require a better understanding of the biology of human perception, olfactory and flavour preferences, the relationship between composition and the sensorial quality of wine, and the production of wine to changing market specifications and sensory preferences.

Wine drinkers’ senses are not uniformly sensitive to the subtle assortment of changing sensations. Some of the diversity in sensory perception and preferences for different wine styles among individuals and populations is cultural, some learned, some genetic (Pretorius et al. 2004). But preferences are also influenced by factors such as gender and age (Figure 2). Fierce competition is forcing wine producers to understand better the expectations and preferences of individuals and populations in their target markets and produce distinctive wines accordingly (Figure 3).

1.2 Organoleptic quality of wine
Above all, wine is supposed to be enjoyed. Four senses are involved in defining the organoleptic quality of wine: sight, smell, taste and touch. Wine jargon has arisen to describe what a wine drinker senses (Forrestal 2000): appearance (sight – e.g. cloudy, hazy, deposit in the bottom of the glass, depth of colour, hue, mousse), nose (smell – e.g. aroma and bouquet) and palate (taste and touch – flavour and mouth-feel). The term aroma is normally used to describe the smell of a young, fresh wine; primary aromas originate during fermentation – typically youthful with upfront fresh fruit notes. Bouquet is the term for an older wine, less fresh but more complex; secondary aromas stem from oak maturation and tertiary aromas originate during bottle ageing – developed fruit showing more age, with stewed or dried fruit and other smells also seeking attention. Wine flavour involves sweetness, acidity, bitterness, saltiness and the taste of umami, mouth-feel relates to the body and texture of wine influenced by factors such as alcohol content (sensation of warmth) and tannins (drying sensation). The structure of a wine includes acidity, sweetness, bitterness (occasionally), tannin (in red wine), alcohol, palate weight and length, mouth-feel, mousse (in sparkling wine), as well as the intensity of fruit aroma and flavour, and complexity (diversity and layers of flavour). These structural elements should be in balance and harmony – they are not assessed in isolation but in relationship to each other. The myriad terms (Forrestal 2000) used to describe different wine styles demonstrate how complex it is to assess and define professionally the organoleptic quality of wine; the scope of this article is limited to the contribution of the most important, but not the only, flavour-active compounds with a stronger emphasis on the aromatic compounds. For the sake of simplicity, the terms aroma and flavour are used interchangeably.

The aroma of wine is due to chemical compounds with low boiling points, which are, therefore, volatile, escaping the glass and detectable by the human nose. Small differences in the concentration of these volatile aroma compounds can mean the difference between a world-class wine and an average, ‘run of the mill drop’. To date, more than 680 volatile compounds have been identified, an indication of the potential complexity of wine aroma (Schreier 1979, Maarse and Vissher 1994, Rapp 1998, Guth and Sies 2002).

1.3 Wine aroma and flavour
The aroma and flavour of wine are one of the main characteristics that define the differences among the vast array of wines and wine styles produced throughout the world. They are affected by the innumerable possible variations in wine’s production, both in viticulture and in wine-making. For example, while some wines have a barely detectable odour, others have fragrance that leaps from the glass.

One of the numerous tools that can assist winemakers...
in producing wines with specific flavour profiles and to market specifications is the choice of microbial starter culture strains to conduct fermentation (Figure 4). During the alcoholic fermentation, yeasts do not only convert sugars to ethanol and carbon dioxide; they also produce a range of minor but sensorially important volatile metabolites that gives wine its vinous character (Schreier 1979, Etiévant 1991, Guth 1998, Rapp 1998, Lambrechts and Pretorius 2000, Romano et al. 2003) (Figure 5). Similarly, during the malolactic fermentation, bacteria do not only provide deacidification when needed but they can also enhance the flavour profile (Henick-Kling 1993, Henschke 1993, Laurent et al. 1994, Bartowsky et al. 2002a).

Wines made from specific grape varieties typically display varietal character, e.g. distinctive aromas which evoke that variety (Dubourdieu 2000, Lambrechts and Pretorius 2000, Guth and Sies 2002, Swiegers and Pretorius 2005). However, while some volatile aroma compounds arise directly from chemical components of the grapes, many grape-derived compounds are released and/or modified by the action of flavour-active yeast and bacteria, and a further substantial portion of wine flavour substances result from the metabolic activities of these wine microbes (Schreier 1979, Simpson 1979, Williams et al. 1989, Etiévant 1991, Guth 1998, Boulton et al. 1998, Rapp 1998, Dubourdieu 2000, Ferreira et al. 2000, Lambrechts...
and Pretorius 2000, Ribéreau-Gayon et al. 2000a,b, Ortega et al. 2001, Guth and Sies 2002). That is why wine has more flavour than the grape juice it was produced from. Therefore, the importance of yeast and, to a lesser extent, bacteria are central to the development of wine flavour.

Many biosynthetic pathways in wine yeast and malolactic bacteria are involved in the formation of wine aroma and are affected by various factors such as the composition and pH of the grape must and the nature and prevailing temperature of the fermentation. In addition, viticultural factors influencing the quality of the grapes and the wine include the cultivar, soil quality, water management, vine canopy management and the ripeness of the grapes. Technological aspects and vinification methods like the method of grape crushing, must treatment and skin contact time also significantly influence the final aroma (Houtman et al. 1980a,b, Henick-Kling 1993, Boulton et al. 1998, Lambrechts and Pretorius 2000, Ribéreau-Gayon et al. 2000a,b, Bartovsky et al. 2002a).

Modern advances in scientific research are giving winemakers tools to shape their wines toward predetermined aroma outcomes. Today, wine yeast and bacteria can be selected to optimally biosynthesise flavour-active compounds, and to release grape-derived flavour compounds and/or modify grape-derived flavour compounds without affecting the general fermentation performance.

2. The modulation of wine flavour by yeast

Though grape must is relatively complete in nutrient content, it can support the growth of only a limited number of microbial species (Henschke 1997). 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 sulfur dioxide, added as an anti-oxidant and anti-microbial preservative, impose additional selection, particularly against undesirable oxidative microbes. 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 (Henschke 1997).

Yeasts are predominant during the complex process of winemaking. Of the 100 yeast genera representing over 700 species, 16 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, Saccharomyces, Schizosaccharomyces, Torulaspora and Zygosaccharomyces (Pretorius et al. 1999).

In spontaneous fermentations there is a progressive growth pattern of indigenous yeasts: 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% (Fleet and Heard 1993). The latter stages of natural wine fermentations are invariably dominated by the alcohol-tolerant strains of Saccharomyces cerevisiae. Other yeasts, such as species of Brettanomyces, Kluyveromyces, Schizosaccharomyces, Torulaspora and Zygosaccharomyces, might also be present during the fermentation and subsequently in the wine, some of which are capable of adversely affecting sensory quality.

The selective pressures prevailing during the winemaking process always favour the yeasts with the most efficient fermentative catabolism, particularly strains of
**Saccharomyces cerevisiae.** For this reason, *Saccharomyces cerevisiae* is almost universally preferred for initiating alcoholic fermentation, and has earned itself the title of the *wine yeast.* The primary role of wine yeast is to catalyse the rapid, complete and efficient conversion of grape sugars to ethanol, carbon dioxide and other minor, but sensorially important metabolites without the development of off-flavours (Figure 5) (Pretorius 2000). A secondary role concerns the modification of grape-derived constituents such as glyco- and cysteine-conjugates, which enhance the wines’ varietal character. In this section, specific attention will be given to the contribution of yeast-derived acids, alcohols, carbonyl compounds, phenols, esters, sulfur compounds and monoterpenoids to the aroma and flavour profile of wine.

### 2.1 Acids

#### 2.1.1 Non-volatile acids

The acidity of grape juice and wine has a direct impact on its sensory quality and physical, biochemical and microbial stability (Fowles 1992, Jackson 1994, Boulton et al. 1998). Acids can have both positive and negative impacts on aroma and flavour, depending on concentration and the type and style of wine. This acidity, particularly pH, influences (i) the survival and growth of all microorganisms; (ii) the effectiveness of anti-oxidants, antimicrobial compounds and enzyme additions; (iii) the solubility of proteins and tartrate salts; (iv) the effectiveness of bentonite treatment; (v) the polymerisation of the colour pigments; (vi) the oxidative and browning reactions; and (vii) the freshness of some wine styles. Wine contains a large number of organic and inorganic acids. The predominant non-volatile organic acids are tartaric acid and malic acid, accounting for 90% of the titratable acidity (TA) of grape juice. Citric acid and lactic acid also contribute to the acidity of grape juice; succinic and keto acids are present only in trace amounts in grapes, but concentrations are higher in wines as a result of fermentation (Whiting 1976, Fowles 1992, Radler 1993, Boulton et al. 1998).

![Figure 5](image-url) A schematic representation of derivation and synthesis of flavour-active compounds from sugar, amino acids and sulfur metabolism by wine yeast.

The main features of wine acidity include the types and concentrations of the acids, the extent of their dissociation, the titratable acidity and pH. Acidity imbalances can result, under certain climatic conditions, from the development of acidic compounds in the grapes and the physical and microbial modification of these compounds during the process of winemaking. Without adjustment of acidity, the wines will be regarded as unbalanced or spoilt.

Chemical adjustment in cooler climates generally means reducing titratable acidity by blending, chemical neutralisation by double salting (addition of calcium carbonate) and precipitation. In warmer viticultural regions with adequate sunshine during the growing season and grape ripening period, malic acid is catabolised at a faster rate than tartaric acid. 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 acid and citric acid, depending on the laws of the country.
As tartaric acid is essentially stable to microbial activity, little change in its concentration occurs during fermentation. Most yeasts can utilise significant concentrations of malic acid. Whereas wine strains of *Saccharomyces cerevisiae* typically degrade 3–45% of malic acid during fermentation most strains of *Schizosaccharomyces pombe* and *Schizosaccharomyces malidevorans* can completely degrade it to ethanol and CO$_2$ (Rankine and Fornachon 1964, Radler 1993). Whilst the formation of malic acid is restricted to only some strains of *Saccharomyces cerevisiae*, it appears to be widespread amongst *Saccharomyces uvarum* strains (Radler 1993, Guidici et al. 1995). In one study, it was reported that a commercial wine strain of *Saccharomyces cerevisiae*, Enoferm M2, increased the malic acid concentration of Cabernet Sauvignon wines by up to 1.5 g/L, whereas another commercial wine yeast, ICV D254, consumed 0.5 g/L (Holgate 1997).

The production of succinic acid is common amongst yeasts and is the main carboxylic acid produced during fermentation, where it typically accumulates to 2 g/L (Thoukis et al. 1965, Radler 1993, Coulter et al. 2004). Its production is highly variable amongst strains of *Saccharomyces cerevisiae* but *Saccharomyces uvarum* or *Saccharomyces bayanus* strains tend to produce higher concentrations (Heerde and Radler 1978, Giudici et al. 1995, Eglington et al. 2000). Succinic acid has been reported to have an ‘unusual salty, bitter taste’ in wine (Whiting 1976). The most likely pathway for its formation appears to involve the reductive branch (via oxaloacetate and malate) of the tri-carboxylic acid (TCA) cycle during anaerobic fermentation (Roustan and Sablayrolles 2002, Camarasa et al. 2003). As for malic acid, abnormal production of succinic acid is sometimes observed, which can be a problem for the winemaker since it affects the expected wine TA value, and therefore requires further correction after fermentation (Holgate 1997). Abnormal succinic acid accumulation during fermentation has been associated with yeast strain, fermentation temperature, aeration, must clarity and composition, including sugar concentration, nutrient content, pH, titratable acidity and sulfur dioxide concentration (Coulter et al. 2004). γ-Amino butyric acid, whose concentration in must can be affected by post-harvest factors, has been suggested to account for abnormal concentrations of succinic acid in wine (Bach et al. 2004).

The keto acids, principally pyruvic and α-ketoglutaric acid, have implications for wine stability and quality due to their abilities to bind sulfur dioxide and to react with phenols (Rankine 1967, Rankine 1968a,b, Rankine and Pocock 1969). The keto acids are produced either during the early stages of fermentation via sugar metabolism, or from the corresponding amino acids, alanine and glutamate, by the Ehrlich pathway. Strain is the most important factor in determining keto acid production, but nitrogen type and content of the medium also affects the concentration of α-ketoglutaric acid produced (Rankine 1968b). When nitrogen is adequate, α-ketoglutaric acid typically accumulates in wine to less than 50–100 mg/L but, when nitrogen is limited, several hundred mg/L can be produced by yeast (Rankine 1968b, Radler 1993).

Due to its pleasant acidic flavour and its properties as a preservative, lactic acid, the main product of the metabolism of lactic acid bacteria, is widely used as a food acidulant. Lactic acid is stable and, in wine, it might be present in amounts of up to 6 g/L after malolactic fermentation. Due to the inefficiency of the mitochondrial lactic dehydrogenases under fermentation conditions, natural *Saccharomyces cerevisiae* strains produce only traces of lactic acid during alcoholic fermentation (Dequin and Barre 1994).

Researchers have successfully employed genetic engineering to construct *Saccharomyces cerevisiae* strains capable of modulating the concentrations of lactic acid and malic acid. In an attempt to redirect glucose carbon to lactic acid in *Saccharomyces cerevisiae*, the lactic dehydrogenase-encoding genes from *Lactobacillus casei* and bovine sources were expressed in laboratory yeast strains (Dequin and Barre 1994, Porro et al. 1995, Skory 2003). Encouraged by the fact that the *Lactobacillus casei* lactic dehydrogenase gene, expressed under control of the yeast alcohol dehydrogenase gene, converted 20% of the glucose into lactic acid, this construct was also introduced into eight wine yeast strains (Dequin et al. 1999). Although the fermentation rate was slower, wines obtained with these engineered lactic acid-alcoholic fermentation yeasts were effectively acidified.

Unlike *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae* lacks an active malate transport system; malate enters wine yeast by simple diffusion. Once inside the cell, *Saccharomyces cerevisiae*’s own constitutive NAD-dependent malic enzyme converts malate to pyruvate, which, under anaerobic conditions, is converted to ethanol and carbon dioxide. Aerobically, malic acid is decarboxylated into water and carbon dioxide. Although the biochemical mechanism for malate degradation in *Saccharomyces cerevisiae* is the same as in *Schizosaccharomyces pombe*, the substrate specificity of the *Saccharomyces cerevisiae* malic enzyme is about 15-fold lower than that of the *Schizosaccharomyces pombe* malic enzyme (Radler 1993, Ansanay et al. 1996). This low substrate specificity together with the absence of an active malate transport system is responsible for *Saccharomyces cerevisiae*’s inefficient metabolism of malate.

An attempt to produce a yeast for the rapid utilisation of malic acid from highly acidic musts involved the isolation of a mutant strain of *Schizosaccharomyces malidevorans* (Rodriguez and Thornton 1988). The mutant, which was induced by exposure to ultra-violet irradiation, consumed malic acid at a higher rate than the wild-type and had reduced utilisation of glucose in the presence of malic acid (Rodriguez and Thornton 1989). The conversion of malic acid to ethanol and CO$_2$ under anaerobic conditions means that a greater level of deacidification can be achieved than that by malolactic fermentation. Commercial-scale trials under winemaking conditions have shown that the mutant could degrade 3.5–10 g/L malic acid in juices over a 21–73 hour period (Thornton and Rodriguez 1996).

Several groups of researchers have explored genetic engineering of wine yeast to conduct alcoholic fermentation and malate degradation simultaneously (Dequin and...
Barre 1994, Ansanay et al. 1996, Volschenk et al. 1997). To engineer a malo-ethanolic wine yeast, the *mae1* malate permease gene and the *mae2* malic enzyme gene from *Schizosaccharomyces pombe* were co-expressed in *Saccharomyces cerevisiae*. Similarly, to engineer a malolactic pathway in *Saccharomyces cerevisiae*, the malolactic genes (*mleS*) from *Lactococcus lactis* were co-expressed with the *Schizosaccharomyces pombe* *mae1* permease gene (Bony et al. 1997). Malo-ethanolic wine yeast would be favoured for low pH wines from the cooler wine-producing regions, while the malolactic wine yeast would provide the best solution for high pH wines from warmer regions.

During vinification trials, it was shown that these malo-ethanolic and malolactic wine yeasts could degrade all the malic acid in must within three days with no off-flavour (Dequin et al. 1999). The ‘malolactic yeast’ is the first wine yeast to be commercialised by a yeast manufacturing company; it was tested in 2002/2003 in Moldavia. To the best of our knowledge, this represents the first large-scale (20,000 litre) winemaking trial with a genetically modified (GM) wine yeast. Wider use of this GM wine yeast in commercial winemaking is likely to be delayed by several years due to current anti-GMO sentiments.

2.1.2 Volatile acids

Volatile acidity (VA) describes a group of volatile organic acids of short carbon chain-length. The volatile acid content of wine is usually between 500 and 1000 mg/L (10–15% of the total acid content) and of this, acetic acid usually constitutes about 90% of the volatile acids (Fowles 1992, Henschke and Jiranek 1993, Radler 1993). The rest of the volatile acids, principally propionic and hexanoic acids, are produced as the result of fatty acid metabolism by yeast and bacteria.

Acetic acid is of particular importance. At elevated concentrations it imparts a vinegar-like character to wine. Acetic acid becomes objectionable at concentrations of 0.7–1.1 g/L, depending on the style of wine; the optimal concentration is 0.2–0.7 g/L (Corison et al. 1979, Dubois 1994).

Acetic acid production by the strains of *Saccharomyces cerevisiae* used in winemaking has been reported to vary widely and, during fermentation, as little as 100 mg/L and up to 2 g/L are produced (Radler 1993). Strains in current use tend to produce acetic acid concentrations at the lower end of the range for dry wines but tend to higher values for sweet wines (Monk and Cowley 1984, Henschke and Dixon 1990, Millan et al. 1991, Bely et al. 2003, Erasmus et al. 2004). Strains of the related cryotolerant species, *Saccharomyces bayanus* and *Saccharomyces uvarum*, typically produce less acetic acid than *Saccharomyces cerevisiae* (Giudici et al. 1995, Eglington et al. 2000).

Acetate is produced by yeast as an intermediate of the pyruvate dehydrogenase (PDH) bypass, a pathway responsible for the conversion of pyruvate into acetyl-CoA through a series of reactions catalysed by pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase and acetyl-CoA synthase. The PDH bypass supplies the cell with cytosolic acetyl-CoA, which is needed for anabolic processes such as lipid biosynthesis (Flikweert et al. 1996, Pronk et al. 1996). The reaction catalysed by acetaldehyde dehydrogenase also generates reducing equivalents, which are needed in many synthetic pathways and for redox reactions (involving NAD(P)H). Acetaldehyde dehydrogenase forms acetate by oxidising the acetaldehyde produced from pyruvate during the fermentation. The cytosolic acetaldehyde dehydrogenases are encoded by *ALD6*, *ALD2* and *ALD3*, whereas the mitochondrial isoforms are encoded by *ALD4* and *ALD5* (Navarro-Avino et al. 1999). Recently, it has been shown that Ald6p, Ald5p and Ald4p are the main enzymes responsible for acetate formation during the more wine-like anaerobic growth on glucose (Saint-Prix et al. 2004).

Although *Saccharomyces* can produce acetic acid, excessive concentrations in wine are largely the result of the metabolism of ethanol by aerobic acetic acid bacteria, a topic discussed below.

2.2 Alcohols

2.2.1 Ethanol

Modern ‘bottled sunshine’, to continue the metaphor, is usually characterised by full fruit and intense varietal flavours, for which it is becoming common practice to harvest fully ripened grapes (de Barros Lopes et al. 2003). However, sunshine can also burn, and it is the application of smart technology and viticulture that harnesses its good points while negating detrimental influences. To ‘bottle the sunshine’, grape must is typically prepared from fully matured grapes, making for high flavour intensity, but also a considerable concentration of sugar. This much sugar invariably leads to the production of wines with high levels of ethanol, sometimes reaching concentrations above 15% (v/v) (de Barros Lopes et al. 2003).

The presence of ethanol is essential to enhance the sensory attributes of other wine components. Excessive ethanol, however, can produce a perceived ‘hotness’ and mask the overall aroma and flavour of the wine (Guth and Sies 2002). This, along with heightened health consciousness, stricter drinking and driving laws, and increased tax rates associated with high ethanol wines, have increased the demand for wines with reduced alcohol concentrations, putting pressure on wine producers, particularly those in warm climates where grape sugar levels can become high (Day et al. 2002).

The removal or reduction of alcohol in wine can be achieved by various physical processes, which are sometimes used in combination, and include reverse osmosis, adsorption, distillation, centrifugation, evaporation, extraction, freeze concentration, membrane, and partial fermentation. These methods, which are generally effective and allow easy control of the amount of alcohol being removed can, however, involve expensive equipment and processing. There are restrictions on the use of some of these techniques in some countries according to their food laws and regulations. Loss or modification of aroma and flavour compounds during processing is an important consideration for several of these techniques.

Several biological solutions are being developed to overcome some of the limitations imposed by the physi-
cal techniques. As the initial sugar concentration of grape must is an important target for achieving wines with lower alcohol content, glucose oxidase (GOX) provides one approach for reducing the glucose content of must (Pickering 1999 a, b, c). Glucose is converted by GOX to D-glucono-δ-lactone and gluconic acid, rendering it unavailable for alcohol formation during fermentation. Pure enzyme preparations are effective on an industrial scale, but further work is needed to establish the impact on wine sensory properties. The high cost of enzyme preparations would be a deterrent, at least, at this stage.

Genetic modification of wine yeast offers another approach to decreasing ethanol concentration in wine. A decrease of almost 2% in ethanol concentration has been achieved by the expression of the GOX1 glucose oxidase gene of a food-grade fungus (Aspergillus niger) in yeast (Malherbe et al. 2003). Similarly, a significant decrease in ethanol concentration (up to 2%) and a concomitant increase in extracellularly accumulated glycerol have been achieved by the overexpression of either of the authentic GPD1- or GPD2-encoded glycerol-3-phosphate dehydrogenase isozymes of Saccharomyces cerevisiae (Michnick et al. 1997, Remize et al. 1999, de Barros Lopes et al. 2000). Theoretically, a combination of these two strategies should lower the alcohol content by more than 4% (v/v). This research is in progress.

2.2.2 Glycerol
Glycerol is a major product of alcoholic fermentation (Gancedo et al. 1968, Pront et al. 1996, Scanes et al. 1998). Chemically, glycerol is a polyol with a colourless, odourless and highly viscous character. It tastes slightly sweet, as well as having an oily and heavy mouth-feel. Glycerol is present in dry and semi-sweet wines in concentrations ranging from 5 to 14 g/L. Red wines typically have higher concentrations of glycerol than white wines (6.82 g/L versus 10.49 g/L; Nieuwoudt et al. 2002), and botrytised wines frequently have concentrations up to 25 g/L (Rankine and Bridson 1971, Ough et al. 1972, Nieuwoudt et al. 2002). Although this non-volatile triol has no direct impact on the aromatic characteristics of wine, glycerol can, depending on its concentration and the style of wine, have a noticeable effect on apparent sweetness (Noble and Bursick 1984). Sensory tests have shown that glycerol imparts sweetness at a threshold of about 5.2 g/L in dry white wine.

Only at high concentration does glycerol affect the apparent viscosity of wine; a concentration of more than 25.8 g/L would be needed to produce a difference in viscosity (Noble and Bursick 1984). Furthermore, no relationship exists between glycerol concentration and the tears that sometimes form on the inside of a wine glass. In the usual concentrations that it is found in dry wine, glycerol has no effect on the perceived aroma intensity of wine (Nieuwoudt 2004).

Glycerol metabolism by yeasts plays several important roles during the anaerobic fermentation of sugars: (i) it provides precursors for the synthesis of phospholipids, which are components of cell membranes, during the period of yeast growth; (ii) glycerol formation helps to maintain the cell’s redox balance, necessary for ATP energy generation and cell growth; and (iii) glycerol protects yeast from high osmotic stress caused by high sugar concentrations (Pront et al. 1996). This latter role explains the higher concentrations of glycerol found in sweet wines.

As mentioned, the overexpression of either of the authentic GPD1 or GPD2 genes of Saccharomyces cerevisiae redirects the carbon flow to glycerol formation with the concomitant overproduction of glycerol at the expense of ethanol (Michnick et al. 1997, Scanes et al. 1998, Remize et al. 1999, de Barros Lopes et al. 2000). However, it was found that these high-glycerol-producing prototype strains also increased acetic acid concentrations to unacceptable levels. This negative side effect was circumvented by deleting the ALD6-encoded acetaldehyde dehydrogenase activity, the main contributor to the oxidation of acetaldehyde during fermentation. For example, a laboratory strain of Saccharomyces cerevisiae over-expressing GPD2 and lacking ALD6 had the desired effect of producing more glycerol and less ethanol, without an increase in acetic acid (Remize et al. 2000, Eglington et al. 2002).

2.2.3 Higher alcohols
Higher alcohols (also known as fusel alcohols) are secondary yeast metabolites, and can have both positive and negative impacts on the aroma and flavour of wine (Figure 6). Excessive concentrations of higher alcohols can result in a strong, pungent smell and taste, whereas optimal levels impart fruity characters (Table 1) (Nykänen et al. 1977, Lambrechts and Pretorius 2000, Swiegers and Pretorius 2005).

Higher alcohols are divided into two categories, aliphatic and aromatic alcohols, and are also extremely important in wine and distillates (Nykänen et al. 1977). The aliphatic alcohols include propanol, isoamyl alcohol, isobutanol and active amyl alcohol. The aromatic alcohols consist of 2-phenylethyl alcohol and tyrosol. It has been reported that concentrations below 300 mg/L add a desirable level of complexity to wine, whereas concentrations that exceed 400 mg/L can have a detrimental effect (Rapp and Versini 1991).

The use of different yeast strains during fermentation contributes considerably to variations in higher alcohol profiles and concentrations in wine (Rankine 1968b, Giudici et al. 1990). The concentration of amino acids (the precursors for higher alcohols) in the must also influences higher alcohol production, where the total production of higher alcohols increases as concentrations of the corresponding amino acids increase (Schulthess and Eitlinger 1978). Furthermore, ethanol concentration, fermentation temperature, the pH and composition of grape must, aeration, level of solids, grape variety, maturity and skin contact time also affect the concentration of higher alcohols in the final product (Fleet and Heard 1993). Non-Saccharomyces yeast can also contribute to the levels of higher alcohols. For example, mixed fermentation with Pichia fermentans and Saccharomyces cerevisiae produced a substantial increase in higher alcohols such as 1-propanol, n-butanol and 1-hexanol compared to fermentation with
Figure 6. A schematic representation of the biosynthesis of higher alcohols in wine yeast (based upon Boulton et al. 1998).


<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in wine (mg/L)</th>
<th>Aroma threshold (mg/L)</th>
<th>Aroma descriptor</th>
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<tbody>
<tr>
<td>Ethyl acetate</td>
<td>22.5–63.5</td>
<td>7.5*</td>
<td>VA, nail polish, fruity</td>
</tr>
<tr>
<td>Isoamyl acetate</td>
<td>0.1–3.4</td>
<td>0.03*</td>
<td>Banana, pear</td>
</tr>
<tr>
<td>2-Phenethyl acetate</td>
<td>0–18.5</td>
<td>0.25*</td>
<td>Flowery, rose, fruity</td>
</tr>
<tr>
<td>Isoamyl acetate</td>
<td>0.01–1.6</td>
<td>1.6****</td>
<td>Banana, fruity</td>
</tr>
<tr>
<td>Hexyl acetate</td>
<td>0–4.8</td>
<td>0.7**</td>
<td>Sweet, perfume</td>
</tr>
<tr>
<td>Ethyl butanoate</td>
<td>0.01–1.8</td>
<td>0.02*</td>
<td>Floral, fruity</td>
</tr>
<tr>
<td>Ethyl hexanoate</td>
<td>0.03–3.4</td>
<td>0.05*</td>
<td>Green apple</td>
</tr>
<tr>
<td>Ethyl octanoate</td>
<td>0.05–3.8</td>
<td>0.02*</td>
<td>Sweet soap</td>
</tr>
<tr>
<td>Ethyl decanoate</td>
<td>0–2.1</td>
<td>0.2******</td>
<td>Floral, soap</td>
</tr>
<tr>
<td>Propanol</td>
<td>9.0–68</td>
<td>500**</td>
<td>Pungent, harsh</td>
</tr>
<tr>
<td>Butanol</td>
<td>0.5–8.5</td>
<td>150*</td>
<td>Fusel, spiritous</td>
</tr>
<tr>
<td>Isobutanol</td>
<td>9.0–174</td>
<td>40*</td>
<td>Fusel, spiritous</td>
</tr>
<tr>
<td>Isoamyl alcohol</td>
<td>6.0–490</td>
<td>30*</td>
<td>Harsh, nail polish</td>
</tr>
<tr>
<td>Hexanol</td>
<td>0.3–12.0</td>
<td>4**</td>
<td>Green, grass</td>
</tr>
<tr>
<td>2-Phenylethyl alcohol</td>
<td>4.0–197</td>
<td>10*</td>
<td>Floral, rose</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>100–1150</td>
<td>280*</td>
<td>VA, vinegar</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>10–75</td>
<td>100***</td>
<td>Sherry, nutty, bruised apple</td>
</tr>
<tr>
<td>Diacetyl</td>
<td>&lt;5</td>
<td>0.2** / 2.8***</td>
<td>Buttery</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5–14 g/L</td>
<td>5.2 g/L**</td>
<td>Odourless (slightly sweet taste)</td>
</tr>
<tr>
<td>Linalool</td>
<td>0.0017–0.010</td>
<td>0.0015***/0.025******</td>
<td>Rose</td>
</tr>
<tr>
<td>Geraniol</td>
<td>0.001–0.044</td>
<td>5******/30*</td>
<td>Rose-like</td>
</tr>
<tr>
<td>Citronellol</td>
<td>0.015–0.042</td>
<td>8******/100*</td>
<td>Citronella</td>
</tr>
<tr>
<td>2-acetyl-1-pyrroline (ACPY)</td>
<td>Trace</td>
<td>0.0001******</td>
<td>Mousy</td>
</tr>
<tr>
<td>2-acetylthetahydropyridine (ACPTY)</td>
<td>0.0048–0.1</td>
<td>0.0016******</td>
<td>Mousy</td>
</tr>
<tr>
<td>4-ethylphenol</td>
<td>0.012–6.5</td>
<td>0.14*/0.6***</td>
<td>Medicinal, barnyard</td>
</tr>
<tr>
<td>4-ethyl guaiacol</td>
<td>0.001–0.44</td>
<td>0.033*/0.11***</td>
<td>Phenolic, sweet</td>
</tr>
<tr>
<td>4-vinyl phenol</td>
<td>0.04–0.45</td>
<td>0.02*******</td>
<td>Pharmacetical, sweet</td>
</tr>
<tr>
<td>4-vinyl guaiacol</td>
<td>0.0014–0.71</td>
<td>10******</td>
<td>Clove-like, phenolic</td>
</tr>
</tbody>
</table>

* 10% ethanol, ** wine, *** red wine, **** beer, ***** synthetic wine, ****** water
**Saccharomyces cerevisiae** alone (Clemente-Jimenez et al. 2005).

Branched-chain higher alcohols, isoamyl alcohol, active amyl alcohol and isobutanol, are synthesised in the yeast cell through the Ehrlich pathway, which involves the degradation of the branched-chain amino acids, leucine, isoleucine and valine (Ehrlich 1904). The uptake of branched-chain amino acid by *Saccharomyces cerevisiae* is mediated by at least three transport proteins: the general amino acid permease Gap1p, the branched-chain amino acid permease Bap2p, and one or more unknown permeases (Didion et al. 1996).

The first step in the synthesis of higher alcohols involves the synthesis of α-keto acids, which are formed via the catabolic or Ehrlich pathway or an anabolic pathway involving synthesis of branched-chain amino acids through their biosynthetic pathway from glucose. The first step in the catabolism of branched-chain amino acids is transamination to form the respective α-keto acids (α-ketoisocaproic acid from leucine, α-ketoisovaleric acid from valine, and α-keto-β-methylvaleric acid from isoleucine) (Dickinson and Norte 1993). This reaction is catalysed inside the yeast by mitochondrial and cytosolic branched-chain amino acid aminotransferases encoded by *BAT1* and *BAT2* (Eden et al. 1996, 2001, Kispal et al. 1996). Interestingly, the *BAT1* gene is highly expressed during the logarithmic growth phase and down-regulated during the stationary phase, while the *BAT2* gene shows an inverse pattern of expression (Eden et al. 1996). A pyruvate decarboxylase converts the resulting α-keto acid to the corresponding branched-chain aldehyde with one carbon-less atom, and the alcohol dehydrogenase catalyses the NADH-dependent reduction of this aldehyde to the corresponding fusel alcohol. Alternatively, the aldehyde might be oxidised to an acid (Derrick and Large 1993).

Recently, researchers looked at the effect of increased yeast branched-chain amino acid transaminase activity, in particular Bat1p and Bat2p, on the production of higher alcohols in the flavour profiles of wine and distillates. The *BAT1* and *BAT2* genes were overexpressed under the control of the constitutive *PGK1* (phosphoglycerate kinase 1) regulatory sequences in a widely used commercial yeast wine strain (*VIN13*). It was found that wines and distillates prepared by strains overexpressing *BAT1* increased the concentration of isoamyl alcohol and its corresponding ester isoamyl acetate but to a lesser extent. The concentration of the higher alcohol isobutanol and isobutyric acid also increased. The overexpression of the *BAT2* gene resulted in a substantial increase in the concentration of isobutanol, isobutyric acid and propionic acid production, while the deletion of this gene led to a decrease in the production of these compounds. Sensory analyses indicated that the wines and distillates produced with the strains in which the *BAT1* and *BAT2* genes were overexpressed individually had more fruity characteristics (peach and apricot aromas) than the wines produced by the wild-type strains (Lilly 2004).

### 2.3 Carbonyl compounds

Acetaldehyde is the major carbonyl compound found in wine with concentrations ranging from 10 mg/L to 75 mg/L and a sensory threshold value of 100 mg/L (Schreier 1979, Berg et al. 1955) (Table 1). Aldehydes contribute to flavour with aroma descriptors such as ‘bruised apple’ and ‘nutty’ but can also be a sign of wine oxidation. During fermentation, the most rapid accumulation of acetaldehyde occurs when the rate of carbon dissimilation is at its maximum, after which it falls to a low level at the end of fermentation and then slowly increases over time. Fermentation conditions such as medium composition, nature of insoluble material used to clarify the must, and extreme aerobic growth conditions greatly affect acetaldehyde concentrations (Bennetzen and Hall 1982, Denis et al. 1983, Dellini and Costa 1993).

In wine, the amount of acetaldehyde can increase over time due to oxidation of ethanol, activity of film yeast and aeration (Fleet and Heard 1993). It has also been shown that the use of high concentrations of sulfur dioxide in grape must can result in an accumulation of acetaldehyde by the yeast. Furthermore, it was found that sulphite-resistant strains of *Saccharomyces cerevisiae* produce much more acetaldehyde than parental non-resistant strains (Casalone et al. 1992). Acetaldehyde also increases with increasing fermentation temperature: e.g. a fermentation carried out at 30°C resulted in a significantly higher concentration of acetaldehyde (Romano et al. 1994). However, in previous studies it was shown that temperature does not affect aldehyde concentration at all (Amerine and Ough 1980). Acetaldehyde concentration can also vary considerably (from 6 to 190 mg/L) depending on the yeast strain (Then and Radler 1971).

As the last precursor before ethanol is formed, acetaldehyde is one of the major metabolic intermediates in yeast fermentation. Pyruvate, the end-product of glycolysis, is converted to acetaldehyde through pyruvate decarboxylase enzymes encoded by three genes, *PDC1-3* (Prönk et al. 1996). Acetaldehyde is then converted to ethanol through alcohol dehydrogenase enzymes, the main one being encoded by the *ADH1* gene. This step is crucial for maintaining a redox balance in the cell, as it re-oxidises NADH to NAD⁺, which is required for glycolysis (Prönk et al. 1996).

The presence of acetaldehyde in white wines is an indication of wine oxidation. The process of converting ethanol to acetaldehyde in the presence of oxygen is also referred to as ‘madeirisation’ and this produces a slightly almond flavour that resembles the fortified sweet wine, Madeira. It is usually facilitated by prolonged storage in a barrel at high temperatures and the resulting wine lacks freshness and has a musty taste known as *rancio* (Robinson 1999). Acetaldehyde in red wines can contribute to aroma complexity as long as the concentration does not exceed 100 mg/L.

Acetaldehyde, and indeed other yeast products, including pyruvic acid and vinyl phenol, are involved in reactions with anthocyanins and various wine phenolic compounds to produce stable pigments in red wine (Bakker and Timberlake, 1997, Benadeljalil et al. 2000, Hayasaka and Asenstorfer 2002, Eglinton et al. 2004). Acetaldehyde can react with malvidin 3-O-glucoside to form the vinyl...

Another important carbonyl compound in wine is diacetyl, which produces a ‘butter’ or ‘butterscotch’ aroma. At low concentrations it can be described as nutty or toasty, but it becomes objectionable at concentrations between 1 and 4 mg/L (Sponholz, 1993). Although yeasts biosynthesise some diacetyl (0.2–0.3 mg/L) in wine, most of it originates from the metabolic activities of lactic acid bacteria, as discussed later (Laurent et al. 1991, Bartowsky and Henschke 2004).

2.4 Volatile phenols
Volatile phenols (formed from the hydroxycinnamic acid precursors in the grape must) have a relatively low detection threshold and are, therefore, easily detected. Although volatile phenols can contribute positively to the aroma of some wines, they are better known for their contribution to off-flavours such as ‘Band-aid’, ‘barnyard’ or ‘stable’, which results from high concentrations of ethylphenols (Chatonnet and Henschke 2004).

Trace amounts of volatile phenols are present in grape must, but they are predominantly produced by yeast during fermentation (Baumes et al. 1988). The nonflavonoid hydroxycinnamic acids, such as p-coumaric acid and ferulic acid, are decarboxylated in a non-oxidative process by Saccharomyces cerevisiae to form the volatile phenols 4-vinylguaiacol and 4-vinylphenol, respectively (Chatonnet et al. 1993). The Brettanomyces/Dekkera spp. yeasts are well-known for their ability to form volatile phenols in wine (Chatonnet et al. 1995, du Toit and Pretorius 2000). These yeasts are associated with the more unpleasant odourous ethylphenols, and are therefore regarded as spoilage organisms resulting in aromas described as ‘Band-aid’, ‘medicinal’, ‘pharmaceutical’, ‘barnyard-like’, ‘horse’, ‘sweaty’, ‘leathery’, ‘mouse urine’, ‘wet dog’, ‘smoky’, ‘spicy’, ‘cheesy’, ‘rancid’ and ‘metallic’ (Chatonnet et al. 1995).

Phenolic acids can also be decarboxylated into volatile phenols, usually first into 4-vinyl derivatives and then reduced to 4-ethyl derivatives through enzymes called phenolic acid decarboxylases (Cavin et al. 1993). Several bacteria and fungi have been found to contain the genes encoding phenolic acid decarboxylases and these genes include PAD1 (also known as POF1) from Saccharomyces cerevisiae, fdc from Bacillus pumilus, pdc from Lactobacillus plantarum, pdc from Bacillus subtilis and padA from Pediococcus pentosaceus (Clausen et al. 1994, Zago et al. 1995, Cavin et al. 1997, Cavin et al. 1998, Barthelmefs et al. 2000b). These enzymes are not inhibited by other grape phenolics and they result in a high transformation of the vinylphenol derivatives to the ethylphenol derivatives.

In addition to the metabolic activity of yeast and bacteria, other factors such as oak maturation can also increase the amount of volatile phenols in wine (Pollnitz et al. 2000). In particular, 4-ethylguaiacol and the 4-ethylphenol concentrations showed a marked increase during oak maturation.


However, Saccharomyces cerevisiae, unlike other organisms, does not use its phenolic acid decarboxylase as its sole defence against phenolic acid toxicity, probably explaining why phenolic acid decarboxylase activity is so low in most Saccharomyces cerevisiae strains (Barthelmefs et al. 2000a).

Tannins do, however, inhibit the cinnamate decarboxylase enzyme of Saccharomyces cerevisiae, which might be a factor in red wines where tannins are abundant (Chatonnet et al. 1993). Pad1p does not commonly cause odour formation in wine, whereas odour formation is very common in beer, which might be due to a lack of tannin inhibitors. Wine yeasts with optimised decarboxylation activity on phenolic acids were developed by overexpressing the Bacillus subtilis phenolic acid decarboxylase gene (padC), the Lactobacillus plantarum p-coumaric acid decarboxylase gene (pdc) and the Saccharomyces cerevisiae phenylacrylic acid decarboxylase gene (PADI/POF1) in a laboratory strain of Saccharomyces cerevisiae (S1278b) (Smit et al. 2003). The overexpression of padC and pdc in Saccharomyces cerevisiae showed high enzyme activity, however, this was not the case for the PADI/POF1-encoded enzyme activity. Subsequently, the padC and pdc genes were also overexpressed in the VIN13 commercial yeast and these strains were compared with both the original VIN13 host strain and a VIN13 mutant in which both alleles of PADI/POF1 were disrupted. Strains overexpressing padC and pdc respectively, gave an approximate twofold increase in volatile phenol formation in a laboratory strain of Saccharomyces cerevisiae (S1278b). Surprisingly, it was also found that the overexpression of the padC gene in wine yeasts resulted in wine with elevated levels of favourable monoterpenes. In wine made with commercial wine yeast VIN13 in which the PADI/POF1 gene was disrupted no volatile phenols could be detected (Smit et al. 2003). Further work might lead to ways through which the concentration of volatile phenols in wine can be controlled.

2.5 Esters
The production of esters by the yeast during fermentation can have a significant effect on the fruity flavours in wine
The most significant esters are ethyl acetate (fruity, solvent-like), isomyl acetate (isopentyl acetate, pear-drops aromas), isobutyl acetate (banana aroma), ethyl caproate (ethyl hexanoate, apple aroma) and 2-phenylethyl acetate (honey, fruity, flowery aromas) (Table 1) (Thurston et al. 1981). Commercial wine strains produce variable amounts of esters, such as isomyl acetate, hexyl acetate, ethyl hexanoate and ethyl octanoate, which have a potential impact on the aroma profile (Rankine 1977, Soles et al. 1982, Lambrechts and Pretorius 2000, Marais 2001). However, there are several non-Saccharomyces wine yeasts that can contribute to the ester aromas of wine. For example, mixed culture fermentations by wild yeasts, such as Hanseniaspora guilliermondii and Pichia anomala, together with Saccharomyces cerevisiae showed increased acetate ester concentrations compared to fermentations with Saccharomyces cerevisiae alone, without significantly affecting acetaldehyde, acetic acid, glycerol and total higher alcohols (Rojas et al. 2003).

Although esters in wine are mainly produced by yeast metabolism (through lipid and acetyl-CoA metabolism), their production can be influenced by the grape variety. In Pinot Noir wines the characteristic fruity flavours of plum, cherry, strawberry, raspberry, blackcurrant and blackberry characters were shown to be influenced by four distinct esters: ethyl anthranilate, ethyl cinnamate, 2,3-dihydrocinnamate, and methyl anthranilate (Moio and Etiévant 1995). These esters are synthesised by the yeast from grape precursors and have distinct aromas: sweet-fruity and grape-like odour (ethyl anthranilate) and cinnamon-like, sweet-balsamic, sweet-fruity, plum and cherry-like flavour (ethyl cinnamate). The aroma of ethyl 2,3-dihydrocinnamate is very similar to ethyl cinnamate, but its contribution to the overall aroma is smaller (Moio and Etiévant 1995).

It has been shown that Chardonnay wines characteristically contain estyl esters such as ethyl-2-methyl propanoate, ethyl-2-butanoate, 3-methyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and the acetate esters hexyl acetate, 2-methylbutyl acetate and 3-methylbutyl acetate. Although, Riesling wines contained similar esters, 3-methyl butanoate and ethyl hexanoate were found to be unimportant to the final aroma of the wines (Smyth et al. 2005). Ester concentrations differed among wine types, and there appears to be a synergy between the grape and the yeast metabolism in establishing the characteristic ester blueprint of different grape varieties.

The synthesis of acetate esters by Saccharomyces cerevisiae is catalysed by a group of enzymes called alcohol acetyltransferases (AAT) by utilising alcohols and acetyl-CoA as substrates (Peddie 1990). Different Saccharomyces AATase encoding genes have been cloned, namely ATF1, ATF2, and LgATF (Fujii et al. 1994, Nagasawa et al. 1998, Yoshimoto et al. 1998, Lilly et al. 2000, Mason and Dufour 2000).

The ATF1 gene was first cloned from Saccharomyces cerevisiae and a brewery lager yeast, Saccharomyces uvarum (Fujii et al. 1994). In this research, yeast strains with multiple copies of the Saccharomyces uvarum ATF1 gene and subsequently elevated enzyme activity showed a 27-fold increase in isoamyl acetate concentration and a nine-fold increase in acetaldehyde, acetic acid, glycerol and total higher alcohols (Rojas et al. 2003).
increase in ethyl acetate concentration while the production of ethanol and other higher alcohols did not change. Furthermore, when the ATF1 gene was disrupted in *Saccharomyces cerevisiae*, AAATase assays using isoamyl alcohol as substrate indicated that, although the AAATase activity of the null mutant was dramatically reduced, 20% of the activity was retained. However, when ethanol was used as the substrate in the AAATase assays, more than 80% of the activity was retained. In support, it has been shown that when overexpressing an ATF1 gene in the VIN13 commercial wine yeast, the concentrations of ethyl acetate, isoamyl acetate and 2-phenylethyl acetate in wine made with this yeast increased up to 10-fold, 12-fold, and 10-fold, respectively (Lilly et al. 2000). These changes in the wine composition had a pronounced effect on the solvent or chemical and fruity or flowery characters of the wines.

Researchers recently investigated the effect of increased ester-synthesising (the ATF1- and ATF2-encoded alcohol acetyltransferases and EHT1-encoded ethanol hexanoyl transferase) and ester-degrading (the IAH1- and TIP1-encoded esterases) enzyme activities on the flavour profile of wine and distillates (Verstrepen et al. 2003, Lilly 2004). Esterases catalyse the reaction $\text{RCOOR} + \text{H}_2\text{O} \rightarrow \text{R}^{\prime}\text{OH} + \text{RCOOH}$ (Peddie 1990). The balance between ester-synthesising enzymes and esterases is important for the net rate of ester accumulation. The ATF1, ATF2, EHT1, IAH1 and TIP1 genes were overexpressed under the control of the constitutive PGK1 regulatory sequences in the VIN13 commercial wine yeast. When the ester concentrations and aroma profiles of wines and distillates prepared with these transformants were compared, it was found that the overexpression of ATF1 and ATF2 increased the concentrations of ethyl acetate, isoamyl acetate, 2-phenylethyl acetate and ethyl caproate, while the overexpression of IAH1 resulted in a significant decrease in the concentrations of ethyl acetate, isoamyl acetate, hexyl acetate and 2-phenylethyl acetate. The overexpression of EHT1 resulted in a marked increase in the concentrations of ethyl caproate, ethyl caprylate and ethyl caprate. The overexpression of TIP1 did not decrease the concentrations of any of the esters. The modification of the ester metabolism by the modified yeast had a pronounced effect on the solvent/chemical and fruity/flowery characters of the wines and distillates. The estery/synthetic fruit flavour was overpowering in the wines fermented with the yeast.

**Figure 8.** A schematic representation of the sulfur metabolism of wine yeast (based upon Wang et al. 2003).
in which \textit{ATF1} was overexpressed, but much more subtle in the strain overexpressing \textit{ATF2}. An intense apple aroma was detected in the wines produced by the yeast in which \textit{EHT1} was overexpressed (Lilly 2004). These investigations into flavour-active esters represent progress towards laying the foundation for the possible development of wine yeast starter strains with optimised ester-producing capabilities.

### 2.6 Sulfur compounds

Sulfur-containing flavour compounds typically occur in wine at very low concentrations, have very low detection thresholds and generally confer a negative sensory contribution to wine (Table 2) (Peppard 1988, Mestres et al. 2000, Vermeulen et al. 2005). On the basis of their chemical structure, sulfur compounds in wine fall into five different categories: sulfides, polysulfides, heterocyclic compounds, thioesters and thiols. These compounds vary widely in their sensory properties. Many sulfur compounds are associated with negative descriptors, which include cabbage, rotten egg, garlic, onion and rubber (Rauhut 1993, Mestres et al. 2000, Vermeulen et al. 2005), whereas some can contribute positive aromas to wine, such as strawberry, passionfruit and grapefruit (Tominaga et al. 1996, 1998a,b).

A variety of biochemical as well as chemical mechanisms are involved in the formation of sulfur compounds in wine and foods, however many of these mechanisms are still poorly defined (Rauhut 1993, Mestres et al. 2000, Vermeulen et al. 2005). The development of these sulfur compounds by yeasts (Figure 8) include (i) the degradation of sulfur-containing amino acids; (ii) the degradation of sulfur-containing pesticides; and (iii) the release and/or the metabolism of grape-derived sulfur-containing precursors (Mestres et al. 2000).

#### 2.6.1 Sulfides

Probably the best known sulfur compound in wine is hydrogen sulfide, a highly volatile thiol which imparts a ‘rotten egg’ aroma and has a very low odour threshold. Due to the frequent occurrence of this compound and the low aroma threshold (50–80 µg/L), it is probably one of the most common problems associated with the winery (Rankine 1963, Acree et al. 1972, Eschenbruch 1974, Vos and Gray 1979, Monk 1986, Henschke and Jiranek 1991, Rauhut 1993). However, the problem is relatively easily dealt with through the use of copper (which results in the formation of copper sulfide) or aeration (resulting in oxidation of the sulfide) (Monk 1986). Nevertheless, elimination of the use of copper salts by wineries is a desirable food processing goal and the presence of oxidised sulfur compounds in young wine could be related to the reductive character in bottled wine.

Hydrogen sulfide can be formed metabolically by yeast from either inorganic sulfur compounds, sulfate and sulfite, or organic sulfur compounds, cysteine and glutathione (Rankine 1963, Eschenbruch 1974, Eschenbruch et al. 1978, Monk 1986, Henschke and Jiranek 1993, Rauhut 1993, Hallinan et al. 1999, Spiropoulis et al. 2000). Cell growth creates a metabolic requirement for the organic sulfur compounds, including cysteine, methionine, \(\delta\)-adenosyl methionine and glutathione. When these organic compounds are absent, the cell must synthesise them from inorganic sulfur compounds accumulated from must. Under certain conditions, sulfide is liberated during the reduction of inorganic sulfur to become detectable by the winemaker. The concentration of hydrogen sulfide produced varies with the availability of sulfur compounds, yeast strain and fermentation conditions, and the nutritional status of the environment (Henschke and Jiranek 1991, Rauhut 1993, Spiropoulis et al. 2000).


<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in wine (µg/L)</th>
<th>Aroma threshold (µg/L)</th>
<th>Aroma descriptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen sulfide</td>
<td>Trace–80</td>
<td>10–80</td>
<td>rotten egg</td>
</tr>
<tr>
<td>Methanethiol (methyl mercaptan)</td>
<td>5.1, 2.1</td>
<td>0.3</td>
<td>cooked cabbage, onion, putrefaction, rubber</td>
</tr>
<tr>
<td>Ethanethiol (ethyl mercaptan)</td>
<td>1.9–18.7</td>
<td>1.1</td>
<td>onion, rubber, natural gas</td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td>1.4–61.9</td>
<td>25</td>
<td>asparagus, corn, molasses</td>
</tr>
<tr>
<td>Diethyl sulfide</td>
<td>4.1–31.8</td>
<td>0.93</td>
<td>cooked vegetables, onion, garlic</td>
</tr>
<tr>
<td>Dimethyl disulfide</td>
<td>2</td>
<td>15, 29</td>
<td>cooked cabbage, intense onion</td>
</tr>
<tr>
<td>Diethyl disulfide</td>
<td>Trace–85</td>
<td>4.3</td>
<td>garlic, burnt rubber</td>
</tr>
<tr>
<td>3-(Methylthio)-1-propanol (methionol)</td>
<td>140–5000</td>
<td>500</td>
<td>cauliflower, cabbage, potato</td>
</tr>
<tr>
<td>Benzothiazole</td>
<td>11</td>
<td>50</td>
<td>rubber</td>
</tr>
<tr>
<td>Thiazole</td>
<td>0–34</td>
<td>38</td>
<td>popcorn, peanut</td>
</tr>
<tr>
<td>4-Methylthiazole</td>
<td>0–11</td>
<td>55</td>
<td>green hazelnut</td>
</tr>
<tr>
<td>2-Furanmethanethiol</td>
<td>0–350 ng/L</td>
<td>1 ng/L</td>
<td>roasted coffee, burnt rubber</td>
</tr>
<tr>
<td>Thiophene-2-thiol</td>
<td>0–11</td>
<td>0.8</td>
<td>burned, burnt rubber, roasted coffee</td>
</tr>
<tr>
<td>4-Mercapto-4-methylpentan-2-one (4MMP)</td>
<td>0–30 ng/L</td>
<td>3 ng/L</td>
<td>cat urine, box tree/ blackcurrant, broom</td>
</tr>
<tr>
<td>3-Mercaptohexyl acetate (3MHA)</td>
<td>1–100 ng/L</td>
<td>4 ng/L</td>
<td>Riesling-type note, passionfruit, box tree</td>
</tr>
</tbody>
</table>
some strains appear to form unregulated amounts of hydrogen sulfide and presumably represent metabolic defects, at least in the wine environment (Jiranek et al. 1995a, b, Spiropoulis et al. 2000, Mendes-Ferreira et al. 2002).


Grape must is typically deficient in organic sulfur compounds (less than 10 mg/L cysteine and methionine), which signals yeast to synthesise organic sulfur compounds from inorganic sources, normally plentiful in grape must (Henschke and Jiranek 1993, Park et al. 2000, Moreira et al. 2002). Hydrogen sulfide is, therefore, a metabolic intermediate in the reduction of sulfate or sulfite needed for the synthesis of organic sulfur compounds (Figure 8). When these reactions proceed in the presence of a suitable nitrogen supply, hydrogen sulfide is sequenced by O-acetyl serine and O-acetyl homoserine, which are derived from nitrogen metabolism, to form the organic sulfur compounds. Under some conditions, however, when insufficient or unsuitable nitrogen sources are available, free hydrogen sulfide can accumulate in the cell and diffuse into the fermenting must (Vos and Gray 1979, Stratford and Rose 1985, Henschke and Jiranek 1991, Giudici and Kunkee 1994, Jiranek et al. 1995a, Jiranek et al. 1996).

In Saccharomyces cerevisiae, hydrogen sulfide is the product of the Sulfate Reduction Sequence (SRS) pathway and acts as an intermediate in the biosynthesis of sulfur-containing amino acids (Yamagata 1989, Rauhut 1993, Thomas and Surdin-Kerjan 1997). The ability of a strain to produce hydrogen sulfide is, at least, partly genetic, since hydrogen sulfide production by different wine strains varies under the same conditions (Thornton and Bunker 1989, Henschke and Jiranek 1993, Jiranek et al. 1995b, Spiropoulis et al. 2000). Mendes-Ferreira et al. (2002) recently screened a large selection of commercial wine yeast, in addition to non-S. cerevisiae yeasts, which, when tested under identical physiological conditions, all had the same growth characteristics but varied in sulfite reductase (the enzyme producing hydrogen sulfide) activity. After fermentation in grape musts, yeast strains could be classified as nonproducers of hydrogen sulfide, must-composition-dependent producers and invariable producers (Mendes-Ferreira et al. 2002).

The first step of the SRS metabolic pathway involves the transport of sulfate from the medium into the yeast cell through the sulfate permease (Figure 8). Sulfate is then reduced to sulfide through a series of steps using the enzymes ATP-sulfurylase (using two ATP molecules) and sulfite reductase. The next step leads to the sequestering of the sulfide: O-acetylserine (from the amino acid serine) combines with sulfide to form cysteine, and O-acetylhomoserine (from the amino acid aspartate) to form homocysteine, which can then be converted to methionine.

A study has recently investigated the role of the bifunctional O-acetylserine/O-acetylhomoserine sulphydrylase as means to modulate hydrogen sulfide production by industrial yeast. Overexpression of the MET17 gene, which encodes O-acetylserine/O-acetylhomoserine sulphydrylase, in a strain of Saccharomyces cerevisiae results in greatly reduced hydrogen sulfide formation. However, this was not the case with another strain, indicating that O-acetylserine/O-acetylhomoserine sulphydrylase activity is not directly related to hydrogen sulfide formation (Spiropoulos and Bisson 2000).

Overexpression of the two genes MET14 and SSU1 have been shown to increase the formation of sulfite (Donalies and Stahl 2002). It has, therefore, been postulated that the deletion of the MET14 adenosylphosphosulphate kinase gene or the MRXI methionine sulfoxide
reductase gene might be the most effective way to prevent wine yeast from producing hydrogen sulfide (Pretorius and Bauer 2002, Pretorius 2003, 2004).

A novel genetic approach that involved modifying the activity of the key enzyme, sulfite reductase, by protein engineering one of the enzyme subunits has been investigated (Sutherland et al. 2003). Sulfite reductase is a heterotetramer, consisting of two α- and two β-subunits, which are encoded by MET10 and MET5 genes, respectively (Kobayashi and Yoshimoto 1982, Hansen et al. 1994, Sutherland et al. 2003). The enzyme, a hemoflavoprotein, binds the cofactors flavin adenine dinucleotide, flavin mononucleotide and siroheme. Mutations were introduced into the MET10 gene such that the α-subunit could no longer bind cofactor but could still form a heterotetramer protein complex with the β-subunit. In this way, overexpression of the mutant met10 gene would produce a nonfunctional subunit which could reduce the proportion of functional sulfite reductase in the cell, and hence reduce sulfide formation. Further work is required to demonstrate whether this genetic strategy will be effective in the wine fermentation.

Because the concentrations of the amino acids cysteine and methionine in grape juices are typically not sufficient to meet the metabolic needs of growing cells, the SRS metabolic pathway is induced in order to meet this demand (Henschke and Jiranek 1993). When adequate nitrogen is also present in the medium, sufficient precursors for these amino acids (O-acetylserine and O-acetylhomoserine) will be available to sequester the sulfide. However, if nitrogen is limiting, insufficient precursors will be available. Therefore, the SRS pathway will be activated and sulfide will accumulate due to the lack of precursors. Surplus sulfide is then liberated from the cell as hydrogen sulfide (Thomas and Surdin-Kerjan 1997). For some strains, the problem can be worse when sulfite is present in the ferment because extracellular sulfite readily diffuses into the cell, resulting in a steady production of hydrogen sulfide. Therefore, in conditions of nitrogen depletion, high and continuous production of hydrogen sulfide is observed in the presence of sulfite (Stratford and Rose, 1985, Jiranek et al. 1995a,b). Sulfate availability can also influence sulfide formation (Hallinan et al. 1999).

Other environmental factors that can affect hydrogen sulfide production include: (i) high residual levels of elemental sulfur; (ii) presence of sulfur dioxide; (iii) presence of sulfur-containing organic compounds; (iv) pantothenate deficiency; (v) high threonine content relative to other amino acids; and (vi) relative methionine to amino acid concentrations (Monk 1986, Henschke and Jiranek 1991, Rauhut 1993, Spiropoulos et al. 2000).

Cells which undergo autolysis after fermentation can also release hydrogen sulfide (Suomalainen and Lehtonen 1991, Rauhut 1993, Ribéreau-Gayon et al. 2000b). Disulfides can be reduced to mercaptans by the action of polysulfides, dimethyl disulfide, dimethyl trisulfide and dimethyl tetrasulfide is believed to involve oxidation of the mercaptans, e.g. oxidation of methyl mercaptan to form dimethyl disulfide. Yeast can reduce disulfides to mercaptans. These compounds, which elicit a ‘rubber’ or ‘garlic’ odour cannot be removed by copper fining.

The mercaptans, including methyl mercaptan and ethyl mercaptan, are highly reactive compounds with low aroma thresholds. The aroma of ethyl mercaptan is described as ‘onion’ or ‘rubber’ with a threshold value of 1.1 µg/L in wine (Goniak and Noble 1987). These mercaptans are observed to form during fermentation in association with hydrogen sulfide. Their suppression by DAP suggests that they are produced as by-products of yeast metabolism of methionine (Rauhut 1993). Thioacetic acid esters of these mercaptans are also observed to form during fermentation, and these can slowly hydrolyse to the parent mercaptan at a later stage (Rauhut et al. 1996).

Disulfides can be reduced to mercaptans by the action of sulfite ions, which can then be removed by copper or silver (not permitted in some countries) fining (Bobet al. 1990). However, the disulfides left as by-products of the reaction cannot be removed by copper ions, and not all of the off-flavours can, therefore, be removed in this way.

Hydrogen sulfide is a highly reactive species, which can take part in a range of reactions to generate compounds that impact on the flavour of a wine (Vermeulen et al. 2005). For example, mercaptans such as ethanethiol can be formed by the reaction of hydrogen sulfide with ethanol or acetaldehyde (Amerine et al. 1980, Rauhut 1993).

The formation of dimethyl sulfide (DMS), which elicits odours described as ‘asparagus’, ‘corn’ and ‘mollases’, is not clear. It could be formed in a similar way to other mercaptans. The concentration of DMS found in wine is well above the sensory threshold of 25 µg/L (white wine) and 60 µg/L (red wine). DMS is considered a beneficial compound in low concentrations, contributing to the aroma of bottle age. The formation of dimethyl sulfide happens during wine maturation, through a yeast mechanism by cleavage of S-methyl-L-methionine to homoserine and dimethyl sulfide. In beer production, heat decomposition during malting of S-methylmethionine produces dimethyl sulfoxide that can reduce to dimethyl sulfide, presumably during storage (Rauhut 1993). DMS formation during fermentation has also variously been linked to cysteine, cystine or glutathione metabolism in yeast (Rauhut 1993, Ribéreau-Gayon et al. 2000b).

One mechanism for formation of the polysulfides, dimethyl disulfide, dimethyl trisulfide and dimethyl tetrasulfide is believed to involve oxidation of the mercaptans, e.g. oxidation of methyl mercaptan to form dimethyl disulfide. Yeast can reduce disulfides to mercaptans. These compounds, which elicit a ‘rubber’ or ‘garlic’ odour cannot be removed by copper fining.

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2.6.2 Sulfur-containing fusel alcohols
Methionine can be metabolised by yeast to form sulfur-containing fusel alcohol, methionol or 3-methylthio-1-propanol, which has ‘cauliflower’ and ‘cabbage’ odours (Mestres et al. 2000). This compound can be converted further to 3-methylthiopropyl acetate, which has a ‘mushroom’ or ‘garlic’ odour. It has also been proposed that 4-methylthio-1-butanol with an onion/garlic odour and 2-mercapto-1-ethanol with a ‘poultry’/‘farmyard’ odour can be biosynthesised by yeast in the same way by using the amino acids homocysteine and cysteine, respectively (Mestres et al. 2000).

2.6.3 Thiols
The volatile thiols are one of the most potent groups of aroma compounds found in wine, some imparting negative aromas, others contributing positively. Furfurylthiol is a potent aroma compound identified in Bordeaux red wines, white Petite Manseng, and also in toasted barrel staves (Tominaga et al. 2000b). Furfurylthiol has also been found in roasted coffee, meat, wheat bread and popcorn, with a perception threshold of 0.4 ng/L (Tominaga et al. 2000b). Its presence in wine has been shown to be the result of yeast transformation of furfural released from toasted oak staves during fermentation (Blanchard et al. 2001). These authors showed that fermentations that have an added nitrogen source, such as asparagine, do not produce as much furfurylthiol. Therefore, production of furfurylthiol is linked to the production of the HS⁻ anion, which is not produced when ammonium sulfate is added in sufficient quantities in a fermentation (Blanchard et al. 2001).

The volatile thiols 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) are of particular importance to wine aroma. These sulfur-containing compounds (thiol referring to the SH functional group) have extremely low perception thresholds: 3 ng/L (4MMP), 60 ng/L (3MH) and 4 ng/L (3MHA). In Sauvignon Blanc wine, these compounds are of particular importance to the varietal character as it imparts box tree (4MMP), passionfruit, grapefruit, gooseberry and guava aromas (3MH and 3MHA) (Dubourdieu et al. 2000). However, 4MMP, 3MH and 3MHA have also been identified in wines made from Colombard, Riesling, Semillon, Merlot and Cabernet Sauvignon in varying concentrations and can, therefore, potentially impact the aroma (Tominaga et al. 2000a, Murat et al. 2001b).

The volatile thiols are almost non-existent in the grape juice and only develop during fermentation. Therefore, it has been proposed that the wine yeast, *Saccharomyces cerevisiae*, is responsible for the formation of volatile thiols. However, it has been shown that 4MMP and 3MH do exist in the grapes but in the form of non-volatile, cysteine bound conjugates and that yeast are responsible for the cleaving of the thiol from the precursor (Darriet et al. 1995).

A mechanism of thiol release was proposed on the basis of experiments showing that a cell-free enzyme extract of the bacteria *Eubacterium limosum* containing...
carbon-sulfur lyase enzymes can release 4MMP from its precursor S-3-(4-methylpentan-2-one)-L-cysteine (Cys-4MMP) (Tominaga et al. 1995). Therefore, it was suggested that the amplification of Sauvignon Blanc varietal aromas during fermentation occurs through the action of yeast carbon-sulfur lyases (Tominaga et al. 1998a,b). Work at AWRI has investigated how yeasts are affected in their ability to release 4MMP from Cys-4MMP when genes encoding putative yeast carbon sulfur lyases are deleted. Four genes that influence the release of the volatile thiol 4MMP in a laboratory strain were identified, indicating that the mechanism of release probably involves multiple genes. These findings were confirmed in a homoygous derivative of the commercial wine yeast, VL3, showing that deletion of the genes leads to a decrease in the amount of 4MMP released (Howell et al. 2005). In related thiol research, these workers showed that the volatile thiol 3MHA is formed by yeast from 3MH by the action of the ester forming alcohol acetyltransferase, encoded by the ATF1 gene (Swiegers et al. 2005). Hereby, the link in ester and volatile thiol metabolism in yeast was established for the first time.

The laboratory of Dubourdieu has shown in model ferments that when the chemically synthesised precursor, S-3-(hexan-1-ol)-L-cysteine (Cys-3MH) decreases in concentration, 3MH increases. However, only a small fraction (1.6% at day 6 of fermentation) of the cysteine-bound precursor was released as 3MH (Dubourdieu et al. 2000). In Cabernet Sauvignon and Merlot musts, it was shown that the amount of 3MH released was proportional to the Cys-3MH concentration. Therefore, the higher the concentration of the cysteine conjugate thiol precursors in the must are, the higher the volatile thiol concentration in the resulting wine will be (Murat et al. 2001a). However, on average, only 3.2% of the precursor was released during fermentation. It is, therefore, clear that there is a huge, untapped flavour potential remaining in the wine after fermentation but that this source of flavour is not fully utilised due to the metabolic limitations of the yeast cell.

The amount of 4MMP released in wine ferments is dependent on which yeast strain is used to conduct the fermentation (Dubourdieu et al. 2000). Therefore, the genetic and physiological characteristics of the yeast strain have a huge effect on its ability to release thiols. Commericially available wine strains Saccharomyces cerevisiae VL3 and EG8 release more thiols than strains VL1 and 522d. Additionally, Saccharomyces bayanus strains release more 4MMP than Saccharomyces cerevisiae strains VL3 and EG8. Wines made with hybrids produced between Saccharomyces bayanus and Saccharomyces cerevisiae have been shown to contain more of the volatile thiols (Murat et al. 2001b). Work at the AWRI has confirmed these findings by showing that different commercial wine strains have variable abilities to release of 4MMP from the Cys-4MMP precursor in model ferments. Commercial wine yeasts that release even more thiols than VL3 were identified (Howell et al. 2004). Furthermore, the ability of different commercial wine yeast to bioconvert 3MH to 3MHA was also investigated. Large variations in the ability of commercial wine yeast to convert 3MH were observed and in most cases this did not correspond to the ability to release 4MMP (Swiegers et al. 2005). Therefore, yeast strain selection is an important tool that can assist winemakers in creating specific wine styles according to consumer preferences.

2.7 Monoterpenoids
Monoterpenoids are potent aroma compounds that are produced by higher plants, algae, fungi and even some yeast, from a common precursor, geranyl pyrophosphate (GPP) (Figure 9). In particular, two of the plant species that produce monoterpenoids are V. vinifera (grapes) and Humulus lupulus (hops) (King and Dickinson 2000). Some fungal (Penicillium) and yeast species are also able to produce monoterpenoids (Larsen and Frisvad 1994, 1995). Yeast species that produce terpenoids include Kluyveromyces lactis, Torulaspora delbrueckii (formerly Saccharomyces fermentati) and Ambrosiozyma monospora (Drawert and Barton 1978, Fagan et al. 1981, Klingenberg and Sprecher 1985). These compounds could have significant value to the winemaker and brewer.

Although mutant strains of Saccharomyces with a genetic defect in the sterol pathway have been reported, native strains of Saccharomyces cerevisiae are capable of producing only trace amounts relative to concentrations present in wines. A recent survey of native wine strains, isolated in Uruguay, has shown that several are capable of significant production. In order to avoid interference from grape-derived monoterpenes, the experiments were conducted in chemically-defined media free from terpenes or their glycosides. Furthermore, fermentation conditions could be used to enhance monoterpene production. High nitrogen – 400 mg N/L compared with 180 mg N/L – which stimulated fermentation rate but not biomass yield, also stimulated monoterpene production. Interestingly, formation of the sesquiterpenes, nerolidol and farnesol, was not stimulated (Carrau et al. 2005).

To explain these results, Carrau and colleagues (2005) searched the Saccharomyces genome database (www. yeastgenome.org) for the presence of appropriate genes using the web-based BLAST (Basic Local Alignment Search Tool; Altschul et al. 1990) search procedure. From these searches, they hypothesised that monoterpene biosynthesis could proceed by an alternative pathway which does not involve the sterol pathway from which sesquiterpenes appear to be derived. This alternative pathway, which is located in the mitochondrion, involves the conversion of leucine to mevalonic acid. This fact could explain the non-coordinated synthesis of the monoterpene and sesquiterpene groups (Carrau et al. 2005).

This work suggests that some strains of Saccharomyces yeast could contribute to the floral aroma of wine by de novo synthesis of monoterpenes, and this contribution could be augmented by certain fermentation conditions such as musts with higher concentrations of assimilable nitrogen like the ammonium ion, in combination with microaerobic fermentation (Carrau et al. 2005). A schematic representation of the biosynthesis of sterols and terpenes in wine yeast is shown in Figure 9.

The study of the aromatic potential of some fruits,
such as grape, passionfruit, papaya, raspberry, as well as of their fermented products (juice and wine) has revealed that, besides a free fraction of volatile terpenoids, naturally non-odourous and non-volatile precursors exist that represent an important source of fragrant compounds.

The aglycone moiety of the precursor glucoside can be linked to β-D-glucose or to the disaccharides 6-O-α-L-arabinofuranosyl-β-D-glucopyranose, 6-O-α-L-rhamno-pyranosyl-β-D-glucopyranose and 6-O-β-D-apiofuranosyl-β-D-glucopyranose (Günata et al. 1985, Voirin et al. 1990). Terpenols such as linalool, nerol, geraniol, α-terpineol, citronellol, and in some cases linalool oxides and terpene diols and triols, can act as aglycone precursors. Aliphatic or cyclic alcohols, such as hexanol, 2-phenylethanol, benzylalcohol, C_{13}-norisoprenoids and and volatile phenols such as vanillin are also possible precursors (Günata et al. 1985, Park and Noble 1993).

During winemaking, bound terpenoids can be released by the action of glycosidase enzymes which are produced by the grapes, yeast and bacteria. Therefore, increasing glycosidase enzyme activity is a tool for enhancing the terpenoid aromas in wines.

The aromatic grape varieties, such as Muscat, Riesling and Gewürztraminer, contain large amounts of the monoterpene geraniol and nerol. Geraniol has aromas described as rose-like and linalool aromas described as rose, whereas linalool oxides are described as camphorous and nerol oxides as vegetative (Simpson 1979). In general, more bound glycosides are found than the free terpenoids, and the ratios of bound to free terpenoids can also vary amongst different grape cultivars. Muscat of Alexandria grapes, for example, have a ratio of 5:1, whereas some non-Muscat varieties have a ratio of 1:1 (Williams et al. 1984).

Besides enzymatic hydrolysis, chemical acid hydrolysis can be used for the release of the monoterpeneS from their glycosidically-bound, non-volatile precursors. Previously, acid hydrolysis was thought to be an effective method for monoterpene liberation, but studies have shown that high temperature acid hydrolysis results in unwanted rearrangement of the monoterpene aglycones (Usseglio Tomasset and Di Stefano 1980, Williams et al. 1982 a). However, enzymatic hydrolysis is an efficient method to release monoterpeneS and it does not result in modification of the aromatic character (Günata et al. 1985). Enzymatic hydrolysis of monoterpeneS involves two steps. In the first step, an α-L-rhamnosidase and an α-L-arabinofuranosidase or a β-D-apiofuranosidase (depending on the structure of the aglycone moiety) cleave the 1,6-glycosidic linkage, and in the second step the monoterpeneS are liberated from the monoterpene β-D-glycosides by the action of a β-glucosidase (Günata et al. 1988, 1990).

Interestingly, the origin of the enzyme and the structure of the aglycone determine the efficiency of the hydrolysis of monoterpene β-D-glycosides by β-glucosidases. Maturation of grapes results in the cleavage of monoterpene β-D-glycosides by endogenous grape β-glucosidases. However, these enzymes exhibit almost non-existent activity towards grape terpenyl-glycosides in must and wine, probably because they are inhibited by glucose and exhibit poor stability at the low pH and high ethanol concentration (Bayonove et al. 1984, Aryan et al. 1987). Some strains of Saccharomyces cerevisiae possess β-glucosidase activity. However, their activity towards glycoside precursors seems to be very low (Günata et al. 1986, Delcroix et al. 1994, Hernández et al. 2003). Therefore, the addition of functional exogenous β-glucosidase to a fermentation is the most effective way to improve the hydrolysis of the glycoconjugated aroma compounds to enhance wine flavour (Aryan et al. 1987, Shoseyov et al. 1990, Vasserot et al. 1993).

Non-Saccharomyces yeasts such as Brettanomyces/Dekkera, Candida, Debaryomyces, Hanseniaspora and Pichia have been screened for novel β-glucosidases with the desired properties (Vasserot et al. 1989, Rosi et al. 1994, McMahon et al. 1999, Fernández et al. 2000, García et al. 2002). For wine purposes, these glycosidases need to have: (i) high affinity for grape-derived terpeneD aglycones; (ii) optimal activity at wine pH (pH 2.5–3.8); (iii) resistance to glucose inhibition; and (iv) high tolerance to ethanol (Riou et al. 1998). Recently, the β-glucosidase from Debaryomyces pseudopolymeromus was found to be suitable for use under wine conditions (Cordero Otero et al. 2003). It exhibits resistance to wine-associated inhibitory compounds such as glucose, ethanol and sulfur dioxide. Its optimum pH lies within the wine spectrum (pH 2.5–3.8), and it has high substrate affinity and large aglycone-substrate recognition. Addition of the enzyme to Chardonnay fermentations resulted in increased concentrations of citronellol, nerol and geraniol. Due to the financial cost of the addition of exogenous aroma-liberating enzyme preparations to wine efforts have been made to express heterologous glucosidase enzymes in wine yeast. Indeed, the aroma intensity of wine made with a yeast expressing the β-1,4-glucanase gene from Trichoderma longibrachiatum was shown to be more intense than the wild-type (Vilaneuva et al. 2000). For the same reason the β-glucosidase genes (BGL1 and BGL2) of Saccharomyces fibuliger, the β-L-arabinofuranosidase (ABF2) of Aspergillus niger and a glucanase-encoding gene cassette consisting of several glucanase genes (BEG1, EN1 and EXG1) were expressed in wine yeast (Pretorius 2000, van Rensburg and Pretorius 2000, Pretorius 2003, Pretorius and Bauer 2002, Pretorius 2004). Surprisingly, the wines produced by the VIN13 commercial wine yeast transformed with the Saccharomyces fibuli-gera BGL1 and BGL2 β-glucosidase genes also contained increased ester concentrations (van Rensburg et al. 2005). Many of these fragrant compounds, when produced in appropriate concentrations, would contribute to the fermentation bouquet of wine. It is unclear at the moment whether this acquired capacity of the transformed wine yeast is of practical significance in large-scale wine production, but it is fertile soil for further investigation.

C_{13}-norisoprenoids are also considered to be important to the aroma of wine. Although there is no ‘concrete proof’, the role of yeast and bacteria in the release of C_{13}-norisoprenoids is very probable. C_{13}-norisoprenoids are secondary metabolites formed in the grape berry and many accumulate as non-volatile glycosides (Winterhalter et al. 1990, Vassiev et al. 1993).
and Schreier 1994). These C13-norisoprenoids are released from their glycosidic precursors during winemaking. As with monoterpenes, precursor analysis of grapes is often used to identify norisoprenoids in grapes and wine (Ribéreau-Gayon 2000b, Sefton et al. 1993, 1994, 1996, Sefton 1998). Structurally, norisoprenoids can be divided into two main groups, megastigmane and non-megastigmane (Ribéreau-Gayon et al. 2000b).

The megastigmanes have complex aromas. A particularly significant megastigmane, β-damascenone, is thought to have the aroma of flowers, tropical fruit, and stewed apple. β-Damascenone has a particularly low threshold concentration and is thought to be present in all varieties of grapes, often above its threshold concentration. Consequently, this compound is thought to play a role in the aroma of some wines. Analysis of 52 young red wines found that β-damascenone was present in all wines above its threshold concentration (Ferreira et al. 2000). Other megastigmanes such as β-ionone and α-ionone are thought to be important to the aroma of some wines (Ribéreau-Gayon et al. 2000b, Kotseridis et al. 1998). In one study, β-ionone was found above its threshold concentration in a range of young red wines, though α-ionone was found to be below its threshold concentration (Ferreira et al. 2000).

Researchers have identified non-megastigmane norisoprenoids as particularly active aroma compounds in wine; the most important of these is 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), which has a distinctive kerosene aroma (Winterhalter 1991). TDN plays an important role in the aroma of certain old Riesling wines. It is thought that TDN is also present in other wine varieties, although at concentrations below its threshold (Etiévant 1991, Ribéreau-Gayon et al. 2000b, Marais et al. 1992, Simpson and Miller 1983).

3. The modulation of wine flavour by bacteria

Wine is a chemically hostile environment for bacteria. Acetic acid and lactic acid bacteria are the only families of bacteria commonly found in grape juice and wine: lactic acid bacteria play a more important role in winemaking, whereas the acetic acid bacteria are only considered to be spoilage organisms due to the formation of major oxidised products, such as acetaldehyde and acetic acid. Only four genera of the lactic acid bacteria genera – Lactobacillus, Leuconostoc, Oenococcus and Pediococcus – are able to survive the unfavourable conditions (low pH, high ethanol concentration and low nutrients) present in wine to any extent. Oenococcus oeni is the most well adapted wine-associated species and is used almost exclusively for the induction of malolactic fermentation (MLF) in red, white and sparkling base wines (Wibowo et al. 1985, Henick-Kling 1993, Henschke 1993).

Research in progress is showing that these bacteria can modify some of the components and sensory properties of wine, providing a new opportunity to alter the chemistry and possibly the aroma and flavour perception of wine (Figure 10) (Henick-Kling 1993, Bartowsky et al. 2002b, Matthews et al. 2004). Despite this concentration

![Figure 10](image-url). A schematic representation of the biosynthesis and modulation of flavour-active compounds by malolactic bacteria.
on *Oenococcus oeni*, the contribution by the other wine genera, particularly *Lactobacillus* species, should not be underestimated.

3.1 Acids

3.1.1. Non-volatile acids

Together with tartaric acid, malic acid is one of the major organic acids in wine and is usually present in the concentration range of 2–5 g/L, dependent upon geographical location and climatic conditions. Malic acid metabolism forms the basis of malolactic fermentation and can be carried out by *Oenococcus oeni* and wine-associated species of *Lactobacillus* and *Pediococcus*. The metabolism of organic acids during malolactic fermentation can have a significant impact on the flavour of the wine (Henick-Kling 1993, Bartowsky et al. 2002a).

The metabolism of sugars and organic acids during malolactic fermentation can be divided into three phases (Krieger et al. 2000). During the growth phase (Phase I), sugar catabolism occurs with little production of acetic and lactic acid; minimal citric and malic acid are metabolised in this phase. As the bacterial cell numbers increase above $5 \times 10^6$ cfu/mL during Phase II, the catabolism of sugar ceases and malic acid metabolism proceeds accompanied by production of lactic acid; citric acid remains untouched at this stage, and there is no acetic acid produced during malic acid degradation. Phase III is characterised by the metabolism of citric acid accompanied by an increase in acetic acid. The increase of lactic acid content in the wine results in a softer mouth-feel and the acetic acid contributes to the volatile acidity of the wine (Figure 10).

The decarboxylation of malic acid to lactic acid forms the basis of malolactic fermentation. This reaction is catalysed by the enzyme malate decarboxylase, often referred to as the malolactic enzyme, with the requirement of cofactors NAD$^+$ and Mn$^{2+}$ (Lonvaud-Funel and Strasser de Saad 1982, Caspritz and Radler 1983, Spettoli et al. 1984, Naori et al. 1990, Kunkee 1991) (Figure 10). All wine lactic acid bacteria are able to perform the malolactic reaction; however, *Oenococcus oeni*, the best adapted species to highly acidic wine conditions, is the preferred species.

Tartaric acid is relatively stable to bacterial activity and can only be metabolised aerobically by some *Lactobacillus* species with the production of acetic acid, lactic acid and succinic acid (Kandler 1983, Dittrich 1987). When tartar-
Acidomonas. These bacteria are classified into the genera Acetobacter, Gluconacetobacter and Gluconacetobacter; of these, Gluconobacter oxydans, Acetobacter aceti, Acetobacter pasteurianus, Gluconacetobacter liquefaciens and Gluconacetobacter hansenii are normally associated with grapes and wine (Drysdale and Fleet 1988). The oxidation of ethanol to acetic acid is the best-known characteristic of these wine-associated acetic acid bacteria. In this reaction, a membrane-bound alcohol dehydrogenase oxidises ethanol to acetaldehyde, and is further oxidised to acetic acid by a membrane-bound aldehyde dehydrogenase. The concentration of oxygen required for metabolic activity and survival in wine is now much lower than previously thought; acetic acid bacteria can survive in wine barrels for long periods of low oxygen tension and, somewhat unexpectedly, spoilage of bottled red wine by acetic acid bacteria has been reported (Drysdale and Fleet 1988, Bartowsky et al. 2003).

A small increase in VA is often observed after the completion of malolactic fermentation conducted by malolactic bacteria. Two pathways can be involved. Acetic acid can be produced from residual sugar through heterolactic metabolism (phosphoketolase pathway) (Henick-Kling 1993, Ribéreau-Gayon et al. 2000a) (Figure 10), and the first step in citric acid metabolism produces acetic acid (Cogan 1987, Ramos et al. 1995, Ramos and Santos 1996) (Figure 11). This latter metabolism is discussed further in Section 3.3.2.

3.2 Alcohols (polyols)
Bacteria can modulate the concentrations of alcohols such as glycerol, mannitol and erythritol, and affect wine flavour. Metabolism of glycerol is not widespread amongst the wine lactic acid bacteria (Lactobacillus brevis, Lactobacillus hilgardii and Pediococcus pentosaceus) and results in wine spoilage (Sponholz 1993, Claise and Lonvaud-Funel 2001, Vizoso Pinto et al. 2004). Glycerol can be degraded by two pathways, either via glycerol dehydroylase or glycerol kinase. The glycerol dehydrase converts glycerol to 3-hydroxypropionaldehyde. Spontaneous chemical dehydration of the aldehyde, by heating or by long-term storage in acidic solution, results in the formation of acrolein, which reacts with wine phenolics, particularly in red wines, to form a bitter complex (Figure 10).

Mannitol spoilage of wine (also referred to as mannite disease) can be caused by some heterolactic bacteria. Under certain conditions, these bacteria bring about sliminess and produce a vinegary-estery, slightly sweet taste. A six-carbon sugar alcohol, or polyol, is the end product of fructose reduction (Pilone et al. 1991, Veiga-da-Cunha et al. 1993, von Weymarn et al. 2002, Wisselink et al. 2002). This reduction is catalysed by mannitol-1-phosphatase in homofermentative lactic acid bacteria (Lactobacillus groups I and II and Pediococcus species), whereas in heterofermentative lactic acid bacteria (Lactobacillus Group III, Leuconostoc species and Oenococcus) the mannitol dehydrogenase catalyses the formation of mannitol. In heterofermentative lactic acid bacteria, the reduction of fructose is a mechanism by which the cell can regenerate NAD+, particularly under anaerobic conditions. The homofermentative lactic acid bacteria are also able to utilise mannitol, transporting it into the cell via the mannitol-specific phosphotransferase system and phosphorylating it to mannitol 1-phosphate, which is then oxidised by mannitol 1-phosphate dehydrogenase to the glycolsylic-intermediate fructose 6-phosphate (Figure 10). In general, the homofermentative lactic acid bacteria will only produce small amounts of mannitol, whereas some heterofermentative lactic acid bacteria produce and export substantial amounts of mannitol (Wisselink et al. 2002). Mannitol can serve as a sole carbon source for the homofermentative lactic acid bacteria Lactobacillus plantarum (Davis et al. 1988, Liu et al. 1995, Wisselink et al. 2002).

In Oenococcus oeni fructose can be metabolised by two
3.3 Carbonyls
Acetaldehyde and diacetyl are two of the more important flavour-active carbonyls in wine and both can be metabolised by wine bacteria.

3.3.1 Acetaldehyde
Acetaldehyde, which mainly originates from yeast metabolism (Figure 10), is a highly volatile compound with an apple-like and nutty aroma. It enhances the colour development of red wine by promoting condensation reactions between anthocyanins and catechins to tannins, forming stable polymeric pigments resistant to sulfur dioxide bleaching (Timberlake and Bridle 1976a, Somers and Wescombe 1987). It is, therefore, inevitable that any bacterial activity that affects the concentration of acetaldehyde in wine potentially can affect its colour and flavour.

Some strains of *Oenococcus oeni* and *Lactobacillus* (but not *Pediococcus*) can metabolise acetaldehyde to acetic acid and ethanol (Osborne et al. 2000). The ability to metabolise acetaldehyde bound to sulfur dioxide can inhibit the growth of bacteria by releasing sulfur dioxide, which accumulates to form an inhibitory concentration (Hood 1983) (Figure 10). The chemical and sensory impact of the ethanol and acetic acid formed by the metabolism of acetaldehyde by lactic acid bacteria is believed to be limited, but the reduction in the acetaldehyde pool in wine is believed to influence final wine colour. It has also been suggested that the degradation of acetaldehyde-bound sulfur dioxide by sulfur dioxide-sensitive malolactic bacteria could lead to an incomplete or prolonged malolactic fermentation.

3.3.2 Diacetyl
2,3-Butanediol, commonly referred to as ‘diacetyl’, is a major flavour compound in dairy products. Extensive research has been devoted to this topic, some of which is applicable to the lactic acid bacteria associated with winemaking (Figure 10). When present at a high concentration (exceeding 5–7 mg/L) in wine, diacetyl is regarded by many to be undesirable (Rankine et al. 1969, Davis et al. 1985). At around 1–4 mg/L, however, depending on the style and type of wine, it is considered to contribute a desirable ‘buttery’ or ‘butterscotch’ flavour. The sensory perception of diacetyl in wine is also highly dependent upon the presence of other compounds in the wine, and is influenced by the age, the style and origin of the wine (Rankine et al. 1969, Martineau et al. 1995, Bartowsky et al. 2003).

Yeast and bacteria contribute to the diacetyl content of wine (Figure 10), though the concentration of diacetyl produced by yeast during alcoholic fermentation is usually below its detection threshold (Martineau and Henick-Kling 1995). In contrast, bacteria can produce significant amounts of diacetyl during malolactic fermentation, and diacetyl is one of the most important flavour compounds produced by *Oenococcus oeni*.

The formation and degradation of diacetyl is directly related to the growth of malolactic bacteria and the metabolism of sugar, malic acid and citric acid. It is formed as an intermediate metabolite in the reductive decarboxylation of pyruvic acid to 2,3-butanediol (Figure 11). Pyruvic acid is derived essentially from the metabolism of sugar and citric acid, and the formation of 2,3-butanediol might contribute to the redox balance of cellular metabolism. Theoretically, 1 mol of citrate produces 1 mol of acetic acid, 2 mol of carbon dioxide and 0.5 mol of a mixture consisting of diacetyl, acetoin and 2,3-butanediol (Cogan 1987, Ramos et al. 1995, Ramos and Santos 1996).

A variety of factors, including some that the winemaker can control, affect the concentration of diacetyl in wine, including oxygen exposure, fermentation temperature, sulfur dioxide levels and duration of malolactic fermentation (reviewed by Bartowsky and Henschke 2004).

The conversion of α-acetolactate to diacetyl is a non-enzymatic decarboxylation, enhanced by the presence of oxygen (Figure 10). Although malolactic fermentation is essentially an anaerobic process, it is not greatly affected by limited exposure to air. However, the amount of diacetyl can vary from 2 mg/L under anaerobic conditions to 12 mg/L under semi-aerobic conditions (Nielsen and Richelieu 1999).

Although the optimum temperature in laboratory media for the growth of malolactic bacteria is about 27°C, growth in wine is restricted to 15–25°C with an optimum for most *Oenococcus oeni* cultures of about 20–22°C (Kelly et al. 1989). Malolactic fermentation conducted at lower temperatures, perhaps 18°C rather than 25°C, tends to be slower, but wines accumulate a higher concentration of diacetyl (Lonvaud-Funel et al. 1984, Hart 1997).

The properties of sulfur dioxide, such as antioxidant and antimicrobial, are important to the winemaking process. Sulfur dioxide can interact with carbonyl compounds, including acetaldehyde and diacetyl in a reversible manner. In the presence of sulfur dioxide, the concentration of free diacetyl in wine is lowered, however as the sulfur dioxide content decreases, as for example during ageing, the ratio of free diacetyl will increase again, thus increasing its sensory impact (Nielsen and Richelieu 1999).

The duration of malolactic fermentation is dependent upon numerous factors including bacterial strain, chemical composition of the wine and wine temperature. Wines that undergo a prolonged malolactic fermentation, for
whatever reason, tend to have a higher diacetyl content (McCarthy 2000, Bartowsky et al. 2002a).

Thus, increasing the ‘buttery’ diacetyl impact of a wine can be achieved by using a lower than usual inoculum of a high diacetyl producing strain in the absence of active yeast, such as after racking wine off yeast lees. The diacetyl content should then be stabilised by filtering to remove bacteria (and yeast if present) and prevent re-metabolism, and by adding sufficient sulfur dioxide to prevent further microbial activity. A low diacetyl content can be achieved by using an appropriate strain inoculated during the late stage of alcoholic fermentation, and if necessary maintaining the wine on stirred lees until the diacetyl becomes undetectable (Bartowsky and Henschke 2004).

3.4 Esters

The majority of wine esters are produced by yeast during alcoholic fermentation (Figure 7). Esters can, however, also be derived from the grape, from the chemical esterification of alcohols and from acids during wine ageing (Rapp and Mandery 1986, Etiévant 1991, Younis and Stewart 1998, Lambrechts and Pretorius 2000). Esterase activity of wine-associated bacterial species is not well understood; esterases of dairy-associated species of Lactobacillus and Pediococcus have been observed, and it appears that their growth in grape juice or wine might modify the ester profile of wine (Matthews et al. 2004).

In a survey of wine lactic acid bacteria, over two-thirds of Oenococcus oeni strains, Lactobacillus species and Pediococcus parvulus strains examined demonstrated esterase activity by the hydrolysis of an ester substrate (Davis et al. 1988). Researchers observed increases in ethyl ester concentration in wine following malolactic fermentation, including ethyl acetate, ethyl hexanoate, ethyl lactate, and ethyl octanoate, as well as decreases in some esters (Zeeman et al. 1982, Dittrich 1987, Laurent et al. 1994, de Revel et al. 1999, Delaquis et al. 2000, Gambaro et al. 2001). These variances in ester concentrations during grape vinification suggest that esterases are involved in both the synthesis and hydrolysis of esters. Changes in ester concentration following malolactic fermentation may either enhance or degrade the wine quality, depending on the ester metabolisation.

3.5 Sulfur-containing compounds

Amino acids are the most important source of nitrogen, carbon and sulfur for sulfur-containing amino acids among the wine substrates metabolised by lactic acid bacteria. Except for biogenic amine formation and the catabolism of arginine, the metabolism of amino acids by Oenococcus oeni has not been studied extensively. Though amino acids play an important role in cheese flavour, their role in wine flavour has not been examined to date. Methionine metabolism has been recently shown by Oenococcus oeni strains and wine-associated Lactobacillus species producing methanethiol, dimethyl sulfide, 3-(methylsulfanyl)propan-1-ol and 3-(methylsulfanyl)-propanoic acid (Prippis-Nicolau et al. 2004). In trials with malolactic fermentation induction by four commercial Oenococcus oeni cultures in Merlot wine, elevated concentrations of 3-(methylsulfanyl)propanoic acid were observed, suggesting that Oenococcus oeni can metabolise methionine to form volatile sulfur compounds.

Cysteine can be the precursor of S-containing heterocycles, such as thiazoles. The sulfur-containing amino acid cysteine and the tri-peptide glutathione stimulated the growth of Oenococcus oeni whereas methionine did not (Rauhut et al. 2004). However, none of the three compounds influenced the metabolism of malic acid to lactic acid.

Sulfur-containing amino acids have a high chemical reactivity with carbonyl compounds, in particular with sugars, according to the Maillard reaction. This well-known mechanism occurs at high temperatures and is favoured by dry conditions. However, carbonyl groups have a greater reactivity with electrophiles other than those of sugars, in aqueous media and at ambient temperature.

Cysteine is a particularly interesting amino acid because of its involvement in the varietal flavours of Sauvignon Blanc wines (Tominaga et al. 1998b). A reaction in wine can occur between α-dicarbonyl compounds (including diacetyl) and amino acids, in particular cysteine (Prippis-Nicolau et al. 2004). Various aromas can arise, including sulfury notes, floral, fruity, toasted and roasted notes, depending upon the amino acid involved in the reaction. It has been found that the decomposition rate of cysteine in the presence of diacetyl at pH 3.5 and 25°C is approximately 70% over seven days whereas at pH 8.0, cysteine is completely decomposed within an hour-and-a-half.

These findings highlight the importance of possible reactions arising from carbonyl compounds in the derivation of aromatic products even under unfavourable conditions of low pH and temperatures, such as those encountered during storage and ageing of wine. Of particular interest are reactions involving cysteine and diacetyl that could occur after alcoholic or malolactic fermentations. These reactions have been demonstrated and newly formed compounds identified under wine storage and ageing conditions (low pH and low temperature) (Marchand et al. 2000). Odours developed in solutions with an α-dicarbonyl compound (e.g. diacetyl) were more intense than those developed with a hydroxy ketone (e.g. acetoin). Many of the compounds produced in this way have been identified in wine, and because of their low olfactory thresholds could play an important role in wine aroma and flavour.

3.6 Glycosidic conjugates

The complex aroma and flavour compounds found in wine largely originate from the grape, from yeast metabolism during alcoholic fermentation and from oak when used. Bacterial metabolism during malolactic fermentation might contribute to wine flavour by the formation of additional compounds and the modification of grape-, yeast- and oak-derived compounds.

Grape-derived glycoconjugates constitute a latent pool of volatile aglycones that can be another source of wine aroma and flavour compounds (Williams et al. 1989).
These aglycones are grouped broadly by structure; norisoprenoids (e.g. damascenone), volatile phenols and other benzene derivatives (e.g. raspberry ketone), monoterprenes (e.g. linalool, nerol and geraniol) and aliphatics (e.g. hexanol). In Chardonnay, a non-floral variety, almost 200 different aglycones have been identified in grape juice prior to fermentation (Selton et al. 1993).

The flavourless glycoconjugates are glucosides or disaccharide or trisaccharide glycosides. These all contain a glucosyl moiety, but for the disaccharide glycosides, the glucose is further substituted with α-L-arabinofuranosyl, α-L-rhamnopyranosyl, β-D-xlyopyranosyl or β-D-apiofuranosyl sugars. In the grape, the disaccharide glycosides are the dominant storage form of aroma substances (Williams et al. 1982b). Many of the wine volatile compounds can be released from their flavourless glycoconjugate precursors by either acid or enzymatic hydrolysis.

The glycosidases involved with the enzymatic cleavage of the disaccharide glycosides include α-L-arabinofuranosidase, α-L-rhamnopyranosidase, β-D-xlyopyranosidase, β-apiofuransidase and β-D-glucopyranosidase (also referred to as α-D-glucosidase). The liberation of the volatile aglycone from the disaccharide by yeast involves the sequential release of the sugar moieties, where the first step is the hydrolysis of the inter-sugar link and the second step is a β-glucosidase activity for the cleavage of the remaining β-glucosidic moiety (Günata et al. 1988). This has also been shown to be the case for Oenococcus oeni (D’Incecco et al. 2004).

Wine bacteria, in particular Oenococcus oeni, are able to cleave the glucose moiety from the major red wine anthocyanin, malvidin-3-glucoside, and use it as a carbon source (Vivas et al. 1997). Oenococcus oeni strains possess various glycosidase activities; however, these activities on synthetic glycosides were dependent on wine conditions such as pH, ethanol and residual sugar content (Grimaldi et al. 2000). Another study using arbutin was unable to demonstrate Oenococcus oeni glycosidic activity (McMahon et al. 1999). More recent studies using Tannat wine or an isolated Chardonnay wine glycosidic extract in synthetic wine medium demonstrated that there was some limited release of glycosylated wine volatiles by Oenococcus oeni during malolactic fermentation (Boido et al. 2002, D’Incecco et al. 2004).

The ability of Oenococcus oeni to liberate aroma compounds bound to sugar moieties might depend on the grape variety and conditions of malolactic fermentation. No release could be demonstrated, for example, in a Viognier wine (Mansfield et al. 2002), but release could be shown in a highly aromatic Muscat variety (Ugliano et al. 2003). The degree of release of glycosidically bound aroma compounds appears to be very much strain-dependent (Grimaldi et al. 2000, Boido et al. 2002, Ugliano et al. 2003, D’Incecco et al. 2004). Recently, it was found that species of Lactobacillus and Pediococcus show varying degrees of β- and α-D-glucopyranosidase activity, which in turn is influenced by exposure to ethanol and/or sugars, temperature and pH (Grimaldi et al. 2005).

3.7 Phenols
Phenolic compounds are abundant in wine, originating from the grape (skin, seeds and stalks) and from the wood used for ageing and maturation of the wine. The major groups of phenolic compounds found naturally in white and red grapes are: phenolic acids (hydroxycinnamic and hydroxybenzoic acids and their conjugates), flavanols...
(catechins), proanthocyanidins (grape tannins or condensed tannins) and flavonoids (quercetin). Red grapes also contain the pigmented polyphenolics and anthocyanins. During fermentation of red must many of the grape phenolics compounds are extracted and some are modified, so that red wine contains, in addition to the grape phenolics, pyroanthocyanins (e.g. vitisins), polymeric pigments (conjugates of anthocyanins and proanthocyanidins) and wine tannins (modified proanthocyanidins). Hydrolysable tannins will also be present when wood has been used in the fermentation process.

Of the phenolic compounds present in wine, the phenolic acids are most susceptible to metabolism by many wine lactic acid bacteria and acetic acid bacteria. Phenolic acids can be transported into bacterial cells by active transport, decarboxylated to the vinyl derivatives by hydroxycinnamic acid decarboxylases and enzymatically reduced to the ethyl derivatives (Cavin et al. 1993, 1994) (Figure 10). Identity of the transport systems for the vinyl and ethyl derivatives are not clear. Laboratory studies have demonstrated the ability of various wine lactic acid bacteria strains to produce both vinyl and ethyl phenols from p-coumaric and ferulic acids (Cavin et al. 1993, 1994). However, in white wine, which only contains a low concentration of flavonoids, only the vinylphenol was produced as the major product, and at much diminished concentration compared to that produced by the reference Brettanomyces bruxellensis strain (Chatonnet et al. 1997). Furthermore, the only strain of Oenococcus oeni tested produced much lower concentrations of volatile phenols compared to Pediococcus damnosus and Lactobacillus plantarum. The concentration of (seed) procyanidin tannins was shown to inhibit the formation of volatile phenols by a Lactobacillus plantarum strain, but not by the Brettanomyces bruxellensis strain in wine. Thus, together with Saccharomyces yeast, some lactic acid bacteria appear to have a low capacity to contribute to the accumulation of vinyl phenols but probably not ethyl phenols during wine production.

Studies in a synthetic wine-like medium have shown that hydroxycinnamic acids inhibit the growth of Oenococcus oeni more than do hydroxybenzoic acids, whereas Lactobacillus hilgardii is less affected by these phenolic compounds, with the exception of p-coumaric acid (Campos et al. 2003). Even though numerous Lactobacillus species have genes for the hydroxycinnamic acid (p-coumaric acid) decarboxylase, Lactobacillus hilgardii is unable to decarboxylate p-coumaric acid and/or ferulic acid to 4-vinylphenol and/or 4-vinylguaiacol, respectively (van Beek and Priest 2000). Supporting the inhibitory effect of p-coumaric acid on Lactobacillus hilgardii growth and survival, as well as for Oenococcus oeni, is the inability of these bacteria to decarboxylate, and presumably detoxify, p-coumaric acid.

On the other hand, catechin and gallic acid, which were metabolised by Lactobacillus hilgardii strain 5w, stimulated growth (Alberto et al. 2001, 2004). The production of pyrogallol from gallic acid and catechin metabolism is known to serve as an oxygen scavenger and to reduce the redox potential of media (Vivas and Glories 1995, Alberto et al. 2001). This effect might promote growth in the absence of oxygen. The presence of phenol carboxylic acids (caffeic, ferulic, p-coumaric and gallic acids) and catechin appear to stimulate the growth of Oenococcus oeni, particularly by reducing the initial lag phase and enhancing the metabolism of citric acid (Vivas et al. 1997, Rozes et al. 2003). Though these studies indicate that Oenococcus oeni is stimulated by the presence of ferulic and p-coumaric acids, it remains unclear whether these acids can be metabolised by wine strains of this species to produce sensorially important concentrations of 4-vinylguaiacol and 4-vinylphenol.

3.8 Amino acids and peptides

Oenococcus oeni is a fastidious organism that requires several amino acids and short peptides for growth. In addition to amino acid transport systems Oenococcus oeni has transport systems for peptides, albeit uncharacterised. Proteolytic activity has been observed in selected Oenococcus oeni isolates (Manca de Nadra et al. 1997, Manca de Nadra et al. 1999, Farias and Manca de Nadra 2000). Though the uptake of short peptides by other species of the lactic acid bacteria has been well characterised, both physiologically and genetically, that is not the case with Oenococcus oeni (Kok and de Vos 1994, Christensen et al. 1999, Peltoniemi et al. 2002). Peptides are an important source of amino acids, but they can also contribute to bitterness (Habibi-Najafi and Lee 1996, Desportes et al. 2001).

The metabolism in wine of certain amino acids, notably lysine and ornithine, can lead to the formation of several extremely potent and unpleasant nitrogen-heterocycle ‘mousy’ off-flavour compounds (Heresztyn 1986, Costello et al. 2001) (Figure 12). The compounds are perceived on the back palate as a persistent aftertaste. The heterofermentative lactic acid bacteria, Oenococcus oeni, Leuconostoc mesenteroides and some Lactobacillus species were capable of synthesising the three known sensorially important nitrogen-heterocycle compounds: 2-acetyltetrahydropyridine (ACTPY), 2-acetyl-1-pyrroline (ACPY) and 2-ethylthiethylhydropyridine (ETPY) (Costello et al. 2001). The heterofermentative lactobacilli favoured the formation of ACTPY, Oenococcus oeni the least flavour active ETPY, whereas the homofermentative pediococci favoured the highest flavour active compound, ACYP. Oenococcus oeni strains varied in their ability to form the nitrogen-heterocycles. The homofermentative species, Pediococcus and Lactobacillus plantarum produced little or no detectable off-flavour compounds.

In general, the heterofermentative lactic acid bacteria showed the highest ability to produce nitrogen-heterocycles and mousy-off flavour. Working with a Lactobacillus hilgardii strain it was shown that the presence of ethanol, a fermentable carbohydrate (D-fructose) and Fe²⁺, in addition to L-lysine and L-ornithine, were necessary for the formation of these nitrogen-heterocycle compounds (Costello and Henschke 2002). ACTPY was formed in the absence of the two amino acids but was greatly stimulated by the presence of L-lysine. L-Ornithine was required for ACYP formation. Ethanol and acetaldehyde are
involved in nitrogen-heterocycle compounds formation, possibly by increasing the C-2 acylating pool as side chain precursor (Figure 12).

4. Concluding remarks
One of the grandest research and development aspirations of the Australian wine industry is to develop objective measures for grape and wine quality. Despite good progress, that goal remains elusive (Francis et al. 2005). Not only is the composition of wine challenging to unravel, but consumer and market preferences will dictate the type of measurement that will be important. Nevertheless, it is a common fact that wine flavour is one of the key drivers of consumer choice. Therefore, we need to integrate our research in viticulture and oenology with a ‘grape-to-glass-to-consumer’ focus.

The research outlined in this article demonstrates that the strains of wine yeast and malolactic bacteria with which grape must and wine are inoculated can have an important impact on the aroma and flavour profile of the final product. As our knowledge develops, it will become possible to select specific yeast and bacteria to produce wines of any chosen style and meet the changing demands of consumers. The choice of yeast and bacterial strain to modulate wine flavour according to specifications of a target market will differ with (i) the type and style of wine to be made; (ii) the grape variety and viticultural practices; as well as (iii) winemaking techniques and technical requirements of the winery (reviewed in Pretorius 2000, 2003, 2004, de Barros Lopes et al. 2005, Pretorius et al. 2005, Swiegers and Pretorius 2005).

Future research at The Australian Wine Research Institute and collaborating research and industry partners will focus stronger on the following key issues: (i) development of methodologies to identify sensory characteristics and consumer preferences for aroma and flavour in wine; (ii) development of methodologies to predict wine choice behaviour in key potential export markets; (iii) determination and quantification of grape precursors for key flavour and aroma compounds; (iv) identification of key viticultural factors that influence the levels of grape flavour precursors; and (v) determination of winemaking factors that influence transformation of grape precursors into potent flavour-active compounds and their stability in wine. With this research endeavour we hope to deliver improved consumer preference information, microbial strains, viticultural management and winemaking technologies to the Australian wine industry.

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