Yeast and its Importance to Wine Aroma - A Review

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Submitted for publication: June 2000
Accepted for publication: August 2000
Key words: Wine aroma; wine flavour; fermentation bouquet; wine yeast

The most mysterious aspect of wine is the endless variety of flavours that stem from a complex, completely non-linear system of interactions among many hundreds of compounds. In its widest sense, wine flavour refers to the overall impression of both aroma and taste components. Aroma is usually associated with odorous, volatile compounds; the bouquet of wine refers to the more complex flavour compounds which evolve as a result of fermentation, *élevage* and ageing. With the exception of terpenes in the aromatic grape varieties and alkoxypyrazines in the herbaceous cultivars, perceived flavour is the result of absolute amounts and specific ratios of many of these interactive compounds, rather than being attributable to a single "impact" compound. Without underestimating the complexity of these interactive effects or negating the definitive role played by the accumulated secondary grape metabolites in the varietal character of wine, this review will focus mainly on the contribution of yeast fermentation to the sensorial quality of the final product. Yeast and fermentation conditions are claimed to be the most important factors influencing the flavours in wine. Both spontaneous and inoculated wine fermentations are affected by the diversity of yeasts associated with the vineyard and winery. During the primary alcoholic fermentation of sugar, the wine yeast, *Saccharomyces cerevisiae*, together with other indigenous non-*Saccharomyces* species, produce ethanol, carbon dioxide and a number of by-products. Of these yeast-derived metabolites, the alcohols, acetates and C4-C8 πfatty acid ethyl esters are found in the highest concentration in wine. While the volatile metabolites contribute to the fermentation bouquet ubiquitous to all young wines, the production levels of these by-products are variable and yeast strain specific. Therefore, this article also highlights the importance of untapping the hidden wealth of indigenous yeast species present on grapes, and the selection and genetic development of yeast starter culture strains with improved flavour profiles. In the future, some winemakers may prefer to use mixtures of indigenous yeast species and tailored *S. cerevisiae* strains as starter cultures to reflect the biodiversity and stylistic distinctiveness of a given region. This will help winemakers to fullfil the consumer's demand for individual wines with intact local character and to ensure the survival of wine's most enthralling aspect - its endless variety.

INTRODUCTION

Flavour is a wine's most important distinguishing characteristic. A wine's flavour could, in its widest sense, be said to be the overall sensory impression of both aroma (as sensed by nose and from the mouth) and taste compounds, and may therefore incorporate the more measurable aspects of acidity, sweetness, alcoholic strength, fizziness, astringency and bitterness (Robinson, 1994). Whereas flavour refers to the effects of both odour and taste, aroma is purely associated with odorous, volatile compounds, while the bouquet of wine refers to the more complex flavour compounds which evolve as a result of fermentation, *élevage* and ageing. However, most of what is commonly described as wine's flavour is in fact its aroma or alternatively, in the case of older wines, its bouquet (Robinson, 1994). In this review the word *flavour* is used interchangeably with *aroma* and *bouquet*.

Wine flavour is classified according to the sources of the different compounds contributing to it. This includes varietal flavour (flavour compounds originating from the grapes), prefermentative flavour (compounds formed during operations of extraction and conditioning of must), fermentative flavour (produced by yeast and bacteria during alcoholic and malolactic fermentation) and post-fermentative flavour (compounds that appear during the ageing process through enzymatic or physicochemical actions in wood or in the bottle) (Schreier, 1979; Boulton *et al.*, 1995; Rapp, 1998).

It is well known that the secondary metabolites of grapes are responsible for the principal flavour compounds in grape must, and provide the basis of "varietal character" (Schreier, 1979; Rapp & Versini, 1991). Various biosynthetic pathways are interactive during the formation of the aroma of alcoholic beverages. Fermentation increases the chemical and flavour complexity of wine by assisting in the extraction of compounds from solids present in grape must, modifying some grape derived compounds, and producing a substantial amount of yeast metabolites.

Volatiles identified in wines are usually dominated by fermentation products, since these volatiles are present in the highest concentration. In a model fermentation with *Saccharomyces* starting at about 22 to 24% sugar, 95% of the sugar is converted to

Acknowledgements: We are also grateful to Anchor Yeast, Lallemand, the South African wine industry (Winetech) and the National Research Foundation (NRF) for financial support. The authors thank T. Plantinga for critical reading of this manuscript.

ethanol and carbon dioxide, 1% is converted to cellular material, and the remaining 4% is converted to other end products (Fig. 1) (Boulton *et al.*, 1995). The major volatile products of yeast metabolism, ethanol, glycerol and carbon dioxide, make a relatively small, but, nonetheless, fundamental contribution to wine flavour. The main groups of compounds that form the "fermentation bouquet" are the organic acids, higher alcohols and esters and, to a lesser extent, aldehydes (Rapp & Versini, 1991). When present in excess concentrations, some fermentation bouquet compounds may also be regarded as undesirable, such as acetaldehyde, acetic acid, ethyl acetate, higher alcohols and diacetyl. The most "negative" aroma compounds are the reduced sulfur compounds, hydrogen sulfide, organic sulfides and thiols. Fermentative flavour is not only brought about by the conversion of directly fermentable substances, but also by the long-chain fatty acids, organic nitrogen-containing compounds, sulfur-containing compounds and many others. These compounds are able to penetrate from the grape juice medium through the yeast cell wall membrane, where they participate in biochemical reactions producing numerous volatile substances as by-products (Boulton *et al.*, 1995).

There are several yeast genera and species apart from *Saccharomyces cerevisiae* found on the grapes, in the must and in the wine that can contribute to the flavour of wine (Table 1). To ascertain the contribution of these yeasts to the aroma of wine it is necessary to fully understand their roles in the fermentation process. Therefore, it is essential to know (i) the taxonomic identity of each species contributing to the fermentation; (ii) the kinetics of growth of each species; (iii) the biochemical properties of these yeasts and the chemical changes they produce; and (iv) the influences of vinification factors upon the kinetics of growth and chemical change (Fleet & Heard, 1993). Although wine yeasts have been extensively investigated over many years, these studies have lacked a quantitative focus.

The microflora of grapes is highly variable, with a predominance of the low alcohol tolerant strains of *Hanseniaspora*, *Kloeckera* and various species of *Candida*, whereas *S. cerevisiae* is present only at low numbers (Peynaud & Domercq, 1959; Fleet *et al.*, 1984; Heard & Fleet, 1985; Lema *et al.*, 1996). The influence that all of these yeasts will have on the flavour of wine depends on several factors. The method of grape harvest (hand-picked or mechanical), transport (time, temperature and sulfite addition) and the condition of the grapes (temperature, sulfite addition, hygiene, aeration, enzyme treatment, clarification method) all affect the microbial content of grape must. Yeast cell concentrations in freshly prepared must is typically 10^3 to 10^5 cfu/ml, but after processing may vary from near sterility to $>10^6$ yeast cfu/ml. This highly variable content of yeast populations may contribute to chemical and flavour changes that accompany fermentation.

Grape juice fermentation can either be natural, conducted by the flora present on the grapes and in the winery, or inoculated with a commercial strain of *Saccharomyces*. Inoculation minimizes the influence of wild yeasts on wine quality, although it has been shown that such cultures may not necessarily prevent the growth and metabolic activity of indigenous, winery-associated strains of *S. cerevisiae* or other wild yeasts such as *Kloeckera apiculata*, *Hanseniaspora uvarum*, *Candida stellata* and

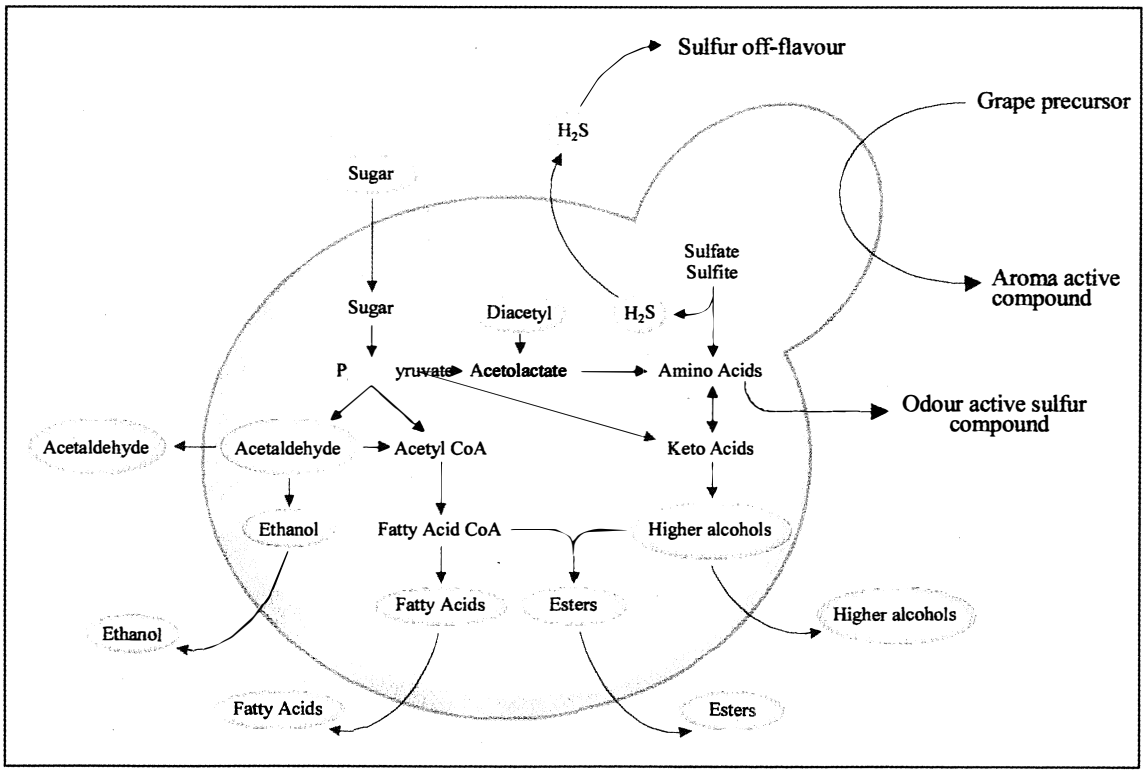

FIGURE 1

A schematic representation of the formation of aroma compounds by yeast (adapted from Henschke & Jiranek, 1993b).

TABLE 1
Wine-related yeasts.

Genus	Species	According to Kurtzman & Fell, 1998
Brettanomyces	*anomalus*	
	bruxellensis	
	intermedius	*Brettanomyces bruxellensis*
Candida	*boidinii*	
	colliculosa	
	guilliermondii	
	hellenica	
	krusei	
	lambica	
	oloephila	
	pelliculosa	
	pulcherrima	
	sorbosa	
	stellata	
	valida	
	vanrijiae	
Cryptococcus	*albidus*	
Debaromyces	*hansenii*	
Dekkera	*anomala*	
	bruxellensis	
Hanseniaspora	*uvarum*	
Hansenula	*anomala*	*Pichia anomala*
	kluyveri	*Pichia kluyveri var. kluyveri*
Kloeckera	*apiculata*	
Kluyveromyces	*marxianus*	
	thermotolerans	
Metschnikowia	*pulcherrima*	
Pichia	*kluyveri*	
	membranifaciens	
Rhodotorula	*glutinis*	
Saccharomyces	*bayanus*	
	beticus	*cerevisiae*
	capensis	*cerevisiae*
	cerevisiae	
	chevalieri	*cerevisiae*
	ellipsoideus	*cerevisiae*
	fermentati	*Torulaspora delbrueckii*
	oviformis	*cerevisiae*
	rosei	*Torulaspora delbrueckii*
	uvarum	*bayanus*
Saccharomycodes	*ludwigii*	
Schizosaccharomyces	*pombe*	
	japonicus	
Torulaspora	*delbrueckii*	
Zygosaccharomyces	*bailii*	
	bisporus	
	florentinus	
	rouxii	

Torulaspora delbrueckii (Heard & Fleet, 1986; Lema *et al.*, 1996; Egli *et al.*, 1998; Henick-Kling *et al.*, 1998). These wild yeasts are capable of anaerobic as well as aerobic growth and may persist during the fermentation, competing with *Saccharomyces* for nutrients, and may produce fatty acid esters and other compounds affecting the fermentation bouquet of the wine. Very few of these yeasts are as ethanol-tolerant as *Saccharomyces,* and therefore are generally undetectable by the end of fermentation. The persistence of these non-*Saccharomyces*, or wild yeasts during fermentation depends upon many factors, such as the temperature of fermentation, nutrient availability, inoculum strength of *Saccharomyces*, use and levels of sulfur dioxide, and the quantity and identity of organisms initially present on the grapes. According to Fleet *et al.* (1984), in a natural selection process, the most suitable indigenous strains of *S. cerevisiae* become established in wineries and vineyards; thus it is assumed that wine character may be influenced by the particular strain conducting the fermentation.

Several researchers have shown that in contrast to *Saccharomyces* species, the non-*Saccharomyces* yeasts produce and secrete several enzymes (esterases, glycosidases, lipases, ß-glucosidases, proteases, cellulases etc.) to the periplasmic space and the medium, where they may interact with grape precursor compounds to produce aroma active compounds, and thus play an important role in varietal aroma (Charoenchai *et al.*, 1997). The activity of these enzymes in must and wine has not been studied extensively and therefore the effect on wine flavour is at present still unclear.

Due to a lack of published hard evidence, the contribution of yeast to the flavour of wine has always been controversial, though there is considerable anecdotal evidence from winemakers and researchers. Studies investigating starter cultures and indigenous yeasts have shown that there are significant differences in the chemical composition of the resultant wines (Mora et *al.*, 1990; Longo *et al.*, 1992; Gafner *et al.*, 1993; Lema *et al.*, 1996). These studies, however, did little to measure sensory differences using tasting panels. More recently, Egli *et al.* (1998) and Henick-Kling *et al.* (1998) clearly showed the effect of indigenous and inoculated yeast populations on the sensory character of Riesling and Chardonnay wines. Similarly, Steger & Lambrechts (2000) showed that different *Saccharomyces cerevisiae* wine yeasts, used to ferment base wines for brandies, produced wines with varying chemical profiles. These profiles conferred to differences in the sensory evaluation of the wines. Furthermore, it was recently discovered that Sauvignon blanc varietal precursors are transformed during alcoholic fermentation by *S. cerevisiae* into aroma active varietal compounds responsible for the overtones of box tree, broom and passion fruit considered typical of this grape variety (Darriet *et al.*, 1995; Tominagà *et al.*, 1998a). These findings suggest that there might be other varietal compounds, liberated or produced by yeasts, still to be discovered.

This review, summarizing the most important aspects of yeast's contribution to wine flavour, illustrates the importance of (i) thorough biological surveys within the ecological framework of the winemaking process; (ii) thorough surveys of the metabolism and enzymatic activities of these yeasts; and (iii) the organoleptic impact of individual or combinations of species on wine flavour.

BIOCHEMICAL COMPOUNDS ARISING FROM YEAST METABOLISM

Yeasts may be categorized by their fermentative properties and growth responses to oxygen as obligately fermentative, facultatively fermentative and non-fermentative. Yeasts exhibit diversity in their modes of energy generation; Table 2 categorizes groups of yeasts with respect to their utilization of respiration and fermentation in ATP production (Walker, 1998).

About one third of the yeast species (over 400) studied by Barnett *et al.* (1979) were categorized as non-fermentative, although such distinctions may be dubious due to the choice of fermentation tests (Scheffers, 1987). During a comparative study on the enzymology of facultatively fermentative and non-fermentative yeast, a variety of yeasts described as non-fermentative contained pyruvate decarboxylase, the key enzyme of alcoholic fermentation. These yeasts were evaluated as to their fermentative capacities. It was found that though these yeasts did not produce gas in the classical Durham-tube test, nearly all strains produced significant amounts of ethanol under the test conditions. It was concluded that the taxonomic test for fermentation capacity, which relies on detection of gas formation in Durham tubes, is unreliable for a physiological classification of yeasts as fermentative or non-fermentative. This might indicate that some of the yeasts shown as non-fermentative in Table 2 might indeed be able to ferment and grow, and therefore contribute to the flavour of wine.

Yeast metabolism refers to the biochemical assimilation (energy consuming) and dissimilation (energy generating) of nutrients by yeast cells (Walker, 1998). These reductive and oxidative processes of anabolism and catabolism are mediated by dehydrogenase enzymes which predominantly use NADP and NAD, respectively as redox co-factors. The formation of NADH and oxidation to NAD are always kept in equilibrum by the cell (the redox balance), thereby preventing the stalling of glycolysis. The formation of aroma compounds by yeast, i.e. esters, fatty acids, fatty acid esters and higher alcohols, is intrinsically linked to the metabolism of yeast cells. Some of these aroma compounds have specific functions in the yeast cell, whereas the function of others is still speculative.

Volatile fatty acids

Alcoholic beverages contain mainly saturated, straight-chain fatty acids, with palmitoleic acid considered to be the only important unsaturated fatty acid. The volatile acid content of wine usually lies between 500-1000 mg/L (10-15% of the total acid content), whereas cognac contains about 200 mg/L. Normally more than 90% of the volatile acid of wine consists of acetic acid (> 0.2 - < 2 g volatile acidity/L) (Henschke & Jiranek, 1993a; Radler, 1993). Acetic acid becomes objectionable near its flavour threshold of 0.7-1.1 g/L, depending on the style of wine, and values between 0.2 and 0.7 g/L are considered optimal (Corison *et al.*, 1979; Dubois, 1994a, b). In contrast Riesen (1992) reported perception values of only 0.1-0.25 g/L and Davis *et al.* (1985) and Henick-Kling (1993) a value of 0.4 g/L in delicately flavoured wines. By law the volatile acidity (expressed as acetic acid) of wines may not be higher than 1.0-1.5 g/L, depending on the country (Eglinton & Henschke, 1999). In the case of base wines for brandy, cognac and armagnac the legal limit is 0.7 g/L. A variety

TABLE 2
Principal modes of sugar catabolism in yeasts (Walker, 1998).

Group name	Examples	Respiration	Fermentation	Anaerobic growth
Obligate respirers	*Rhodotorula* spp. and *Cryptococcus* spp.	Yes	No	No
Aerobic respires	*Candida* spp., *Kluyveromyces* spp., *Pichia stipitis* and *Pachysolen tannophilus*	Yes	Anaerobic in pregrown cells	No
Aerobic fermenters	*Schizosaccharomyces pombe*	Limited	Aerobic and anaerobic	No
Facultative aerobic	*Saccharomyces cerevisiae*	Limited	Aerobic and anaerobic	Facultative, but no growth in absence of sterols and fatty acids
Obligate fermenters	*Torulopsis* (*Candida*) *pintolopesii*	No	Anaerobic	Yes

of other fatty acids are present in small amounts in wine. The short-chain fatty acids, acetic, propanoic and butanoic acids, are by-products of fermentation, although the presence of propanoic and butanoic acid are associated with the intervention of bacteria (Ribéreau-Gayon *et al.*, 1998). Acetic and lactic acid bacteria can also be associated with high levels of acetic acid.

The long-chain fatty acids (C16 and C18) are essential precursors in the synthesis of many lipid components found in yeast, including mono-, di- and tri-acylglycerols, phospholipids, sphingolipids and a variety of glycolipids (Ratledge & Evans, 1989). These fatty acids do not occur in a free form in living cells but are normally found as esters, usually of glycerol or sterols (Paltauf *et al.*, 1992). Unsaturated long chain fatty acids and sterols are an integral part of the yeast plasma membrane, where they are reported to regulate the movement of various compounds into and out of the yeast cell, enhance the ability of yeast to resist high ethanol concentrations and assist the activities of membrane-bound enzymes in their function (Thurston *et al.*, 1981; Alexandre & Charpentier, 1998). The unsaturated fatty acids, which play important roles in yeast membrane integrity and ethanol tolerance, include palmitoleic (16:1) and oleic (18:1) acids. Together, these two compounds constitute the bulk (ca. 70%) of the fatty acids in *S. cerevisiae* cell membranes. The others consist mainly of the saturated fatty acids, primarily palmitic acid (16:0), with lesser amounts of stearic (18:0) and myristic (14:0) acids (Ratledge & Evans, 1989). These fatty acids are not normally found in wine as products from the yeast, but will be found in distilled products in which the distillation is carried out with yeast lees. Residues of linoleic and occasionally linolenic acid can usually be attributed to fatty acids inadvertently present in the original medium.

Medium-chain (C8, C10 and C12), volatile fatty acids are produced by yeasts as intermediates in the biosynthesis of long-chain fatty acids, rather than as a result of acid catabolism. Medium-chain fatty acids and their ethyl esters are natural components of alcoholic beverages. These fatty acids can also act as potential inhibitors of alcoholic fermentation. This inhibiting power on yeast growth is directly proportional to their solubility, which is strictly dependent upon the content of ethanol in the fermentation medium (Walenga & Lands, 1975; Lafon-Lafourcade *et al.*, 1984; Sa-Correia *et al.*, 1989; Viegas *et al.*, 1989). Table 3 lists the odours associated with some of the more prominent volatile fatty acids and their average concentration levels in wine as produced by yeast during fermentation.

In considering the total lipid content of yeasts, Ratledge & Evans (1989) classified yeast as either oleaginous or non-oleaginous, with the somewhat arbitrary division at about a 20 to 25% lipid content. The non-oleaginous group encompasses the vast majority of yeasts and includes *S. cerevisiae*. The oleaginous group is much smaller, and includes species of *Lipomyces* and *Rhodotorula*. A third category of lipid-producing yeasts could be considered for those yeasts that produce extracellular lipids. Although the non-*Saccharomyces* yeasts found on grapes have not been tested for their lipid content, most if not all would probably fall into the non-oleaginous group.

***Biosynthesis of fatty acids by yeasts*:** Pathways leading to fatty acid synthesis have been intensively studied for *S. cerevisiae* (for recent reviews see: Ratledge & Evans, 1989; Paltauf *et al.*, 1992). The formation of acetyl coenzyme A (acetyl-CoA) is the first step in the biosynthesis of fatty acids (Fig. 2) (Lynen, 1967). Acetyl-CoA is formed by the oxidative decarboxylation of pyruvic acid. Further synthesis of long-chain saturated fatty acids occurs via two enzyme systems: acetyl-CoA carboxylase and fatty acid synthetase. Acetyl-CoA carboxylase first converts acetyl-CoA into malonyl-CoA, which is utilized by the fatty acid synthetase complex that carries out repetitive condensation between enzyme-bound acetyl-CoA and malonyl-CoA for the synthesis of saturated fatty acids and for chain elongation (Ratledge & Evans, 1989; Paltauf *et al.*, 1992). Propionyl-CoA replaces acetyl-CoA in the biosynthesis of the odd numbered fatty acids.

TABLE 3

Some volatile fatty acids produced by yeast and their concentrations, threshold values and odours in wine (Salo, 1970a; Ribéreau-Gayon *et al.*, 1982; Baumes *et al.*, 1986, 1988; Miranda-Lopez *et al.*, 1992; Riesen, 1992; Dubois, 1994a, b).

Compound	Conc. in wine (mg/L)	Threshold (mg/L)	Odour
Formic acid	50		
Acetic acid	150-900	700-1000 100-125 400	Vinegar, pungent
Propionic acid	Traces	20.0*	Rancid, slightly pungent
Butyric acid	Traces	2.2/4.0*	Pungent
Isobutyric acid	Traces	8.1*	Pungent, less than butyric acid
Valeric acid	Traces		Unpleasant
Isovaleric acid	< 3	0.7*	rancid, cheese, sweaty, at times putrid, stinky
2-Methylbutyric acid	?		
Hexanoic acid	Traces-37	8 8.8*	sour, vinegar, cheese, sweaty, rancid, fatty, pungent
Heptanoic acid	Traces		
Octanoic acid	Traces-41	13 15*	oily, fatty, rancid, soapy, sweet, faint fruity, butter
Nonanoic acid	?		
Decanoic acid	Traces-54	10 8.2*	fatty, unpleasant, rancid, citrus, phenolic
Tridecanoic acid	15-118		fatty, citrus, unpleasant

* Percentage-above-chance-scores of 50% in grain spirit solutions of 9.4% (w/w)

The synthesis of long-chain, mono-unsaturated fatty acids in yeasts involves an oxidative desaturation by a fatty acid desaturase, to introduce double bonds into the saturated fatty acid. The requirement for oxygen in the desaturase-mediated synthesis of essential unsaturated fatty acids means that *S. cerevisiae* cannot grow strictly anaerobically in the absence of these compounds (Walker, 1998).

Under certain growth conditions (as in the case of a wine fermentation), yeast cells depend on the uptake of exogenous unsaturated fatty acids or ergosterol. Such is the case when fatty acid desaturation or sterol biosynthesis is suppressed by the absence of oxygen or by mutations affecting the synthesis of unsaturated fatty acids and/or sterols (Ratledge & Evans, 1989). *S. cerevisiae* is normally unable to degrade fatty acids by ß-oxidation (Johnston & Paltauf, 1970). A peroxisomal ß-oxidation system can, however, be induced by incubating yeast in the presence of oleic acid (Veenhuis *et al.*, 1987).

The grape contains 0.15 to 0.24% (wet weight basis) of total lipids. Among the fatty acids present in grapes, unsaturated fatty acids represent the major component of total lipids. Linoleic acid is the most abundant, followed by oleic, linolenic and palmitoleic acid (Gallender & Peng, 1980; Castella *et al.*, 1985). Among saturated fatty acids, palmitic acid is the most abundant. The fatty acid content varies according to technological procedures applied to the must. Some researchers have found that clarification drastically reduces the content of fatty acids in the resultant wine and can result in stuck fermentations (Delfini & Costa, 1993). Others have demonstrated that the addition of lipids, especially ergosterol and unsaturated long-chain fatty acids, can have a pronounced effect on the growth and metabolism of yeast (Andreasen & Stier, 1954; Maurico *et al.*, 1990; Rosi & Bertuccioli, 1992).

Delfini *et al.* (1992) also investigated the evolution of fatty acids, acetaldehyde, acetic and pyruvic acid production during fermentation. They noted that, among the two *S. cerevisiae* strains tested, a large amount of fatty acids is released during the first days of alcoholic fermentation, reaching maximum levels on the 4^{th} and 5^{th} day. This was followed by a decrease around the 7^{th} and 8^{th} days, and a levelling off at lower values, although still rather high, until the end of fermentation. For both strains tested in the study, the production of acetic acid was very high during the first phases of fermentation, reaching a maximum the 5^{th} to 7^{th} days.

Houtman & Du Plessis (1981) found that the yeast cell obtains

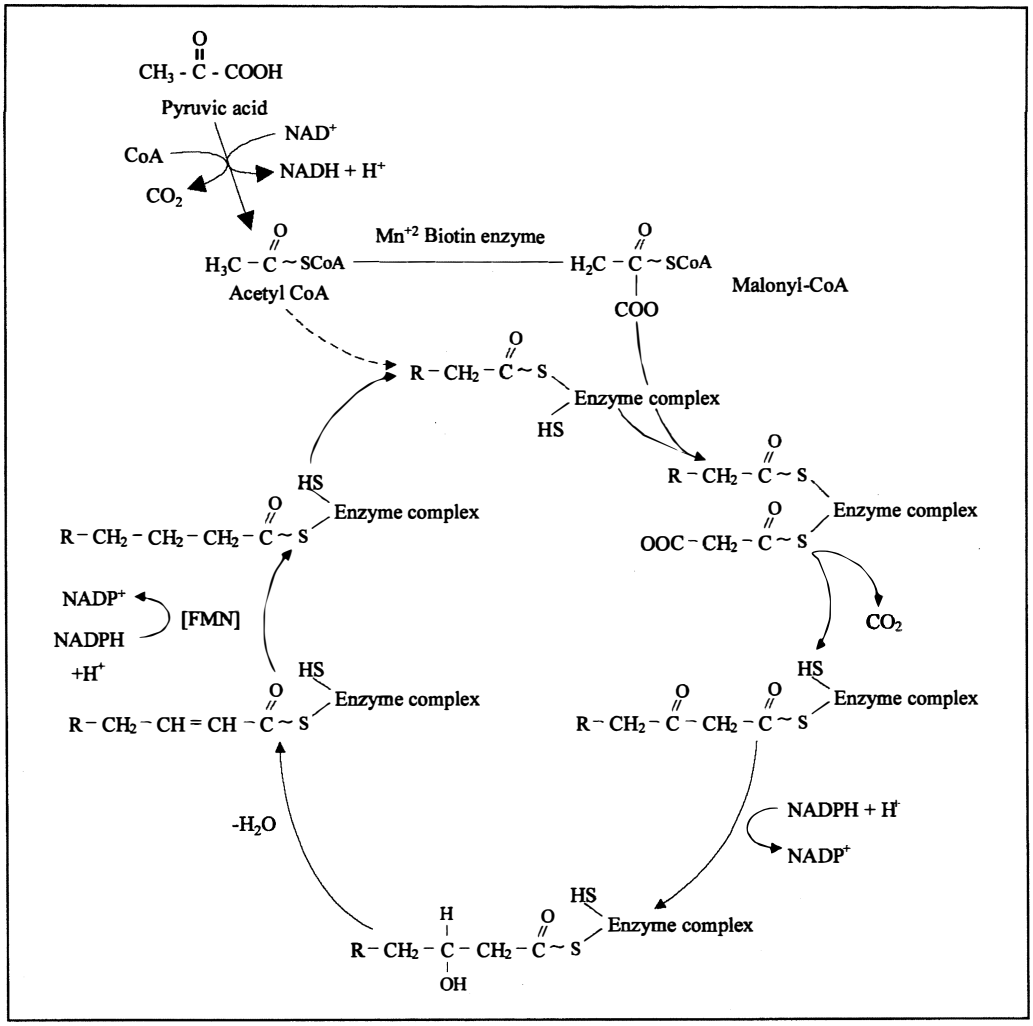

FIGURE 2

A schematic representation of fatty acid biosynthesis (adapted from Lynen, 1967).

substances from the grape juice clarification deposit causing it to limit or even prevent the release of acetic acid in the fermenting medium. These ill-defined substances increase in the must, especially with pressing of skins, and their utilization by yeasts seems to be mediated by enzymes belonging to the yeasts and/or to musts. This was also observed by Delfini & Cervetti (1987). Furthermore, amounts greater than 1 g/L of acetic acid are synthesized in free run musts which were clarified by cooling, adjuvants, decantation, centrifugation or filtration. They also showed that the ammonium concentration has no influence on acetic acid production; however, a small decrease can be observed by addition of methionine, alanine and cysteine.

Rosi & Bertuccioli (1992) tested the effect of fatty acid and triglyceride mixtures on the fatty acid composition of yeast cells under simulated vinification conditions. Their results showed a significant relationship between fatty acid composition of cells and secretion into the medium of esters, higher alcohols and medium-chain fatty acids. During the first six days of fermentation, the content of linoleic and linolenic acid of yeast was correlated positively to the secretion of isobutanol and isoamyl alcohol, and negatively to isoamyl acetate and octanoic and decanoic acid of fermented samples. During the latter part of fermentation the content of octanoic, decanoic, dodecanoic and *cis*-9-hexadecanoic acids of yeast cells was positively correlated to the secretion of ethyl octanoate, ethyl decanoate, ethyl acetate, isoamyl acetate, and medium-chain fatty acids and negatively correlated to isobutanol, active amyl alcohol and isoamyl alcohol. The different pattern of flavour active compounds also correlated to the descriptive sensory analysis of the four aroma attributes (banana, artificial fruit, fusel alcohol, soapy). Therefore, any environmental or genetic manipulation of yeast which brings about a specific change in lipid composition is a potential method for controlling the sensory quality of wine.

Yeasts synthesize much the same fatty acids irrespective of the nature of raw materials used. However, the fatty-acyl composition of yeasts is highly variable; changes in growth substrate and minor alterations in growth conditions (pH value, temperature, dissolved oxygen tension, presence/absence of major or minor nutrients) as well as the growth rate of the organism itself may affect the relative proportions of the individual components

(Paltauf *et al.*, 1992). Furthermore, the amount and portion of medium-chain fatty acids released into the fermentation medium is strictly dependent on yeast strain, the composition of the medium, and fermentation conditions such as pH, temperature, and degree of aeration during fermentation.

***Yeast strain effect on volatile fatty acid production*:** Although acetic acid and lactic acid bacteria can be responsible for high levels of acetic acid in wine, so can the yeasts involved in the winemaking process. Acetic acid formation by strains of *S. cerevisiae* is affected by sugar concentration, pH and nitrogen (Shimazu & Watanabe, 1981, as quoted by Henschke & Jiranek, 1993b; Hannemann, 1985). This was confirmed by Monk & Cowley (1984), who also found that the addition of 5 mg/L nicotinic acid to a synthetic medium enhanced the formation of acetic acid at low and high sugar concentrations. Hanneman (1985) found that among 100 strains of various genera, 13 strains of *S. cerevisiae* produced more than 1 g/L of acetic acid in a synthetic medium. Acetic acid is formed early in fermentation and its production stops as soon as the sugar is fermented. Generally, more acetic acid is produced under conditions that are growth limiting. Increasing amounts of acetic acid is produced below pH 3.2 and at values more neutral than pH 4 (Hanneman, 1985; Delfini & Cervetti, 1987).

Delfini & Cervetti (1991) separated *Saccharomyces* strains into three distinct classes: low (0.0 to 0.3 g/L), medium (0.31 to 0.60 g/L) and high (more than 0.61 g/L) acetic acid producers. In particular, all *Saccharomyces uvarum* strains produced very low amounts of acetic acid. These strains also produced the lowest levels of volatile acidity (ca. 0.1 g/L) of the 23 strains tested. Several studies have shown that strains of, for example, *K. apiculata* (1-2.5 g/L), *C. stellata* (1-1.3 g/L), *Metschnikovia pulcherrima* (0.1-0.15 g/L), *C. krusei* (1 g/L) and *H. anomala* (1-2 g/L) have the potential to produce greater or lesser concentrations of acetic acid than *S. cerevisiae* (0.3-1.2 g/L) (Hanneman, 1985; Fleet & Heard, 1993).

Ravaglia & Delfini (1993) also classified 48 yeast strains into three homogeneous groups with regard to their C8 and C10 fatty acid production (Table 4). Few fatty acids were produced by *Zygosaccharomyces bailii, K. apiculata* and *Schizosaccharomyces japonicus*. *Saccharomyces cerevisiae* var. *cerevisiae* and *S. cerevisiae* var. *bayanus* produced the highest amounts, recording 0.9-7 mg/L of octanoic acid. The only strains that produced detectable amounts of ethyl caprylate were *S. cerevisiae* var. *bayanus* and *S. japonicus*. Thus, the content of fatty acids as well as their ethyl esters present in wine depend on the strain used during alcoholic fermentation.

Herraiz *et al.* (1990) studied the influence of pure, mixed and sequential cultures of *K. apiculata, T. delbrueckii* and *S. cerevisiae* on the volatile composition of wines obtained without the addition of sulphur dioxide. Their data (Table 5) show clearly that *S. cerevisiae* produces high amounts of caproic, caprylic and capric acids; *T. delbrueckii* and *K. apiculata* producing lower amounts. However, *K. apiculata* gives rise to notable amounts of lauric, myristic, palmitic and palmitoleic acids, and *T. delbrueckii* produces high levels of isobutyric acid (Herraiz *et al.*, 1990).

TABLE 4
Production of fatty acids and esters by wine yeasts in a synthetic nutrient medium (adapted from Ravaglia & Delfini, 1993).

Strains	C_8 (µg/L)	C_{10} (µg/L)	C_{12} (µg/L)	Eth-C_8 (µg/L)	Eth-C_{10} (µg/L)	Eth-C_{12} (µg/L)
High Producers						
S. bayanus	3094	1126	0	250	0	0
S. bayanus	3425	1568	0	258	0	35
S. cerevisiae	3693	2532	81	59	0	0
S. cerevisiae	7066	1327	46	29	34	0
Medium Producers						
S. bayanus	1980	546	0	597	0	0
S. fermentatii	1813	285	0	0	0	0
S. exiguus	1364	160	0	0	0	0
S. chevalieri	1769	250	0	0	0	0
Low Producers						
S. cerevisiae	286	137	0	0	0	0
S. ludwigii	255	142	0	0	0	0
Z. bailii	40	0	0	0	0	0
K. apiculata	23	110	0	0	0	0
S. japonicus	0	21	0	275	13	0

Higher alcohols

The term higher alcohols refers to those possessing more than two carbon atoms with a higher molecular weight and boiling point than ethanol. Higher alcohols are quantitatively the largest group of aroma compounds in alcoholic beverages, and are secondary products of alcoholic fermentation (Amerine *et al.*, 1980). Higher alcohols, also known as fusel alcohols, can be recognized by their strong, pungent smell and taste and can have a significant influence on the taste and character of wine and brandy. Although they exhibit a harsh, unpleasant aroma at the concentrations generally found in wine (unless the must contains a high level of suspended solids), below 300 mg/L they usually contribute to the desirable complexity of wine. When their concentrations exceed 400 mg/L, the higher alcohols are regarded as a negative influence on the quality of the wine (Rapp & Mandery, 1986). Their overall presence in wine covers a wide range: from a concentration slightly less than 100 mg/L to a concentration higher than 500 mg/L (Nykänen, 1986). Generally white wine varieties contain lower fusel alcohol concentrations than red varieties, but some white varieties, such as Semillon, almost always yield high concentrations (Guymon, 1972). Higher alcohols can be of major importance in wine distillates, in which they are much more concentrated (Boulton *et al.*, 1995).

Higher alcohols are composed of aliphatic and aromatic alcohols (Nykänen *et al.*, 1977). Table 6 lists some of the more prominent higher alcohols produced by yeast during fermentation. The aliphatic alcohols include propanol, isobutyl alcohol, active amyl alcohol and isoamyl alcohol; the aromatic alcohols of which phenethyl alcohol is the most important. Besides phenethyl alcohol, the other major phenolic alcohol in wine is tyrosol. This yeast-synthesized phenol has a mild, beeswax, honey-like odour. Whether it plays a role in the honey-like bouquet of certain wines, such as botrytised wines, is unknown (Dubois, 1983). Isoamyl alcohol is the main aliphatic fusel alcohol synthesized by yeast during fermentation. Depending on the nature of the beverage, it comprises 40-70% of the total fusel alcohol fraction. Other important higher alcohols are propanol, isobutyl alcohol and optically active amyl alcohol. The two amyl alcohols constitute the most abundant minor components or congeners of brandy and other distilled spirits (Nykänen *et al.*, 1977). Viticultural conditions and the use of different yeasts contribute to considerable variations in the quantity of higher alcohols (Giudici *et al.*, 1990). Higher alcohols are also important precursors for ester formation (Soles *et al.*, 1982), and the esters of higher alcohols are associated with pleasant aromas.

Rankine (1967) determined the taste threshold levels of some prominent higher alcohols (isoamyl alcohol, isobutyl alcohol and 1-propanol) in wine and a model solution, showing that differences in amounts of isoamyl alcohol capable of being formed by *Saccharomyces* can influence the taste of wines, depending on the taster. Differences in the amounts of isobutyl alcohol and 1-propanol produced by different yeasts do not affect wine aroma; however this does not take into account the other constituents in wine or the synergism between these compounds contributing to a pungent smell.

***Biosynthesis of higher (fusel) alcohols by yeast*:** Higher alcohols are produced by yeasts during alcoholic fermentation through the conversion of the branched chain amino acids present in the medium: valine, leucine, isoleucine, threonine and phenylalanine (Table 6). They are also produced *de novo* from a sugar substrate (Fig. 3a, b) (Äyrapää, 1961, 1962, 1965, 1967, 1968 & 1972). The Ehrlich mechanism or catabolic formation of higher alcohols involves the initial transamination between an amino acid and an α-keto acid with subsequent decarboxylation and reduction. The α-keto acids produced in the first step may be secreted by yeast or they may be decarboxylated, and subsequently reduced to the fusel alcohol (Zoecklein *et al.*, 1995). The final reduction step involves the reoxidation of $NADH + H^+$ to NAD^+, and thus helps to maintain the redox balance within the cell (Van Dijken & Scheffers, 1986; Quain, 1988; Zoecklein *et al.*, 1995). However,

TABLE 5

Fatty acid composition in wines resulting from fermentation with *K. apiculata* (A), *T. delbrueckii* (B) and *S. cerevisiae* (C) in pure, mixed (AB, AC, BC, ABC) and sequential inoculations (A+C, A+B+C) (Herraiz *et al.*, 1990).

Compound (mg/L)	A	B	C	AB	AC	BC	ABC	A+C	A+B+C
Butyric acid	0.05	0.1	0.11	0.25	0.17	0.21	0.19	0.11	0.06
Isobutyric acid	0.48	3.42	0.4	6.13	0.61	1.18	0.95	0.74	0.74
Isovaleric acid	0.26	2.15	2.08	3.05	3.22	2.52	1.83	0.62	0.54
Hexanoic acid	0.45	0.19	3.18	0.16	2.5	3.23	2.54	0.71	0.41
Octanoic acid	0.38	0.75	4.21	0.7	3.92	4.55	3.96	0.98	0.69
Decanoic acid	0.24	0.16	0.99	0.12	1.29	1.21	0.91	0.25	0.19
Dodecanoic acid	0.26	0.04	0.1	0.05	0.14	0.1	0.1	0.04	0.07
Tetradecanoic acid	0.1	0.08	0.04	0.08	0.09	0.09	0.06	0.04	0.07
Hexadecanoic acid	0.4	0.19	0.1	0.17	0.19	0.26	0.24	0.23	0.22
Cis-9-hexadecanoic acid	0.15	0.02	0.03	0.02	0.14	0.03	0.04	0.02	0.14

TABLE 6

Some higher alcohols produced by yeast and their concentrations, threshold values and odours in wine (Rankine, 1968; Salo, 1972; Shinohara & Watanabe, 1976; Ribéreau-Gayon *et al.*, 1982; Baumes *et al.*, 1986; Nykänen, 1986; Renger *et al.*, 1992; Dubois, 1994; Zoeklein *et al.*, 1995; Cabanis & Cabanis, 1998)

Compound	Amino acids	Conc. in wine mg/L	Threshold value (mg/L)	Odour
Propanol	Threonine/2-Amino-butyric acid	9 – 68 125	500† 800‡	Stupefying
Butanol	-	0.5 – 8.5		Fusel odour
Isobutyl alcohol	Valine	9 – 28 (100) 140`	500† 75.0* 200‡	Alcoholic
Active amyl alcohol	Isoleucine	15 – 150	65‡	Marzipan
Isoamyl alcohol	Leucine	45 – 490	300† 7.0* 70‡	Marzipan
Hexanol	-	0.3 – 12	5.2* 4‡	
Tyrosol	Tyrosine			Bees wax, honey-like
Tryptophol	Tryptophan			
Phenethyl alcohol	Phenylalanine	10 – 180	7.5* 125‡	Floral, rose

* Percentage-above-chance-scores of 50% in grain spirit solutions of 9.4% (w/w)
†In a wine solution
‡In beer

according to Boulton *et al.* (1995) the exact function of higher alcohols is unknown and this reaction is not considered to be an important means for the reoxidation of NADH, since, they reason, there appears to be enough acetaldehyde to help maintain the redox balance. Higher alcohol formation may simply serve to detoxify any aldehydes produced during amino acid catabolism or may be involved in the regulation of amino acid anabolism (Boulton *et al.* 1995). Physiologically, oxidative deamination provides the yeast with a mechanism for obtaining more nitrogen when the pool has become depleted (Vollbrecht & Radler, 1973).

The metabolism of the amino acids to higher alcohols is restricted to the exponential growth phase (Vollbrecht & Radler, 1973). Guymon *et al.* (1961) have shown that propanol and the branched chain C4 and C5 alcohols, all of which appear as major components in alcoholic beverages, are formed from valine, leucine and isoleucine (Table 6). ∝-Ketobutyric acid acts as an intermediate in the formation of propanol, and it is also an intermediate in the formation of active amyl alcohol. Reazin *et al.* (1973) investigated the effect of threonine and isoleucine on the formation of higher alcohols. They showed that in *S. cerevisiae,* isoleucine forms active amyl alcohol as almost the only product, whereas threonine produces propanol, active amyl alcohol and isoamyl alcohol. Webb & Ingraham (1963) reported that isobutyl alcohol is formed from pyruvic acid via ∝-keto-isovaleric acid.

Parts of the enzymatic pathways needed for formation of the corresponding amino acids are utilized for the anabolic formation of higher alcohols from sugars. Peynaud & Guimberteau (1962) estimated that no more than one sixth of the leucine and valine in grape musts assimilated during fermentation gave rise to isoamyl and isobutyl alcohols respectively. Peynaud & Guimberteau (1962) felt that since these amino acids are low in grape musts, the formation of higher alcohols by amino acid degradation is negligible. Instead, nearly all of the higher alcohols are derived from carbohydrate degradation. All routes for the formation of higher alcohols lead to ∝-keto acids which can, apart from being decarboxylated to an aldehyde and reduced to the corresponding alcohol (Ehrlich - Neubauer and Fromherz degradation), participate in one or more of several other reactions. Using radioactive tracers, Reazin *et al.* (1970) found that 35% of higher alcohols arose from carbohydrates.

The amino acids in a medium are among the most important factors influencing fusel alcohol formation. They are able to alter the yield of higher alcohols in several different ways (Schulthess & Ettlinger, 1978). Amino acids contribute to the total nitrogen content of the medium, and the amount of fusel alcohols formed by the anabolic pathway (from carbohydrates) depends to a great

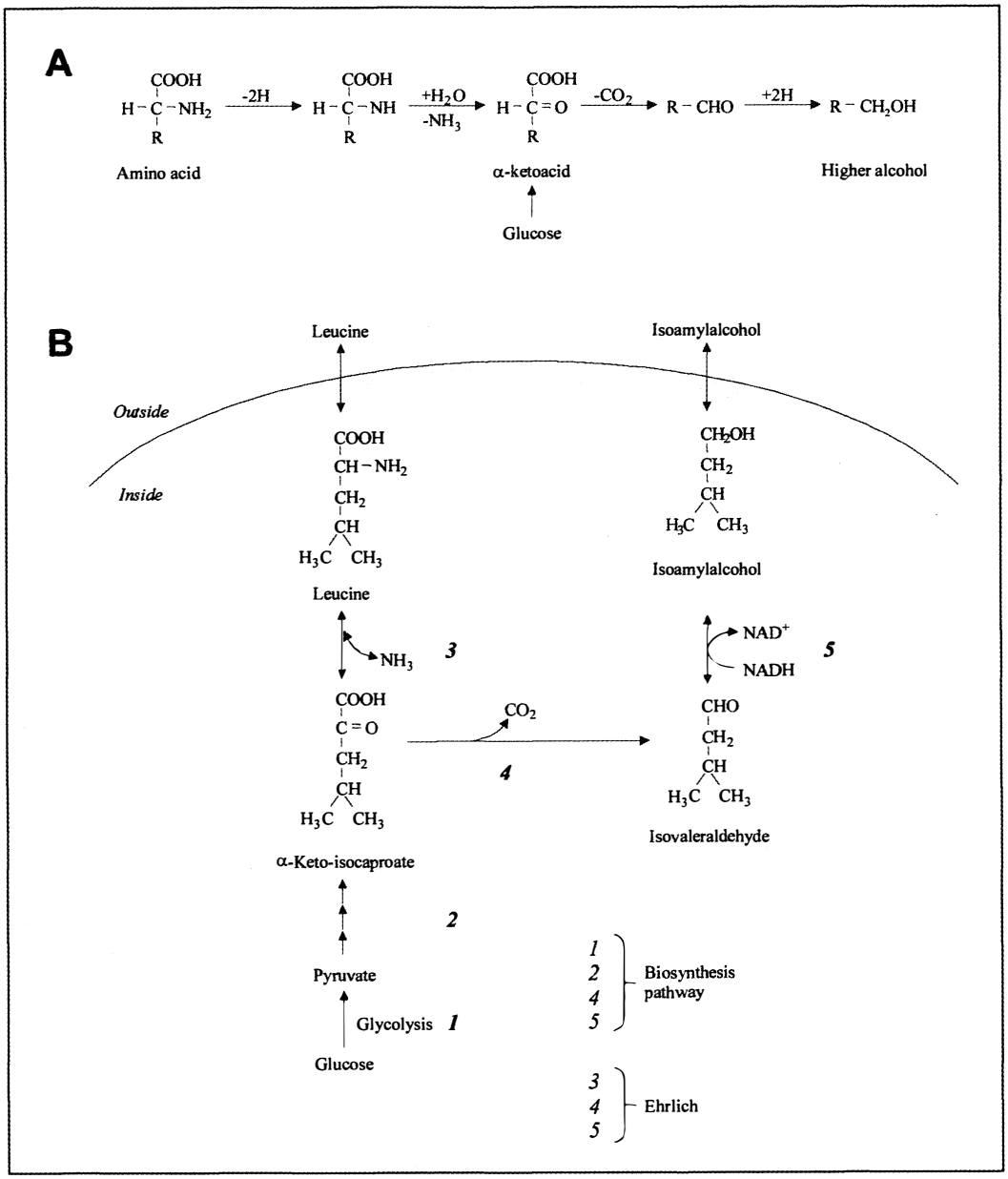

FIGURE 3

A) Generalized pathway for formation of higher alcohols (fusel oils) from amino acid and ∝-keto acid precursors. B) Formation of isoamyl alcohol via the anabolic and catabolic pathways (adapted from Boulton *et al.*, 1995; Walker, 1998).

extent on the nitrogen level. Äyrapää (1968) showed that the closer the nitrogen concentration is to the growth-limiting level, the higher is the yield of anabolic fusel alcohols. Both catabolic and anabolic synthesis can be decreased by the addition of nitrogen. The total yield of higher alcohols can also be diminished, even with the addition of branched chain amino acids, if the total nitrogen content at the beginning of fermentation is low. On the other hand, the addition of valine, leucine or isoleucine to a fermentation medium may increase the total formation of the corresponding higher alcohols, although obviously the influence of nitrogen in such a case is of a second order (Pierce, 1987).

Amino acids can influence the anabolic formation of their corresponding higher alcohols by inhibiting the biosynthetic enzymes. This has been shown for the formation of isobutyl, active amyl and isoamyl alcohol on the biosynthetic pathway of valine, isoleucine and leucine (Boulton *et al.*, 1995). Amino acids can also be converted directly into higher alcohols. This catabolic pathway is known as the Ehrlich mechanism.

The question arises as to what extent the catabolic and anabolic formation of fusel alcohol changes, if the medium, in addition to possessing a sufficient amount of nitrogen, is supplied with branched chain amino acids. Schulthess & Ettlinger (1978) showed that the total and catabolic production of higher alcohols increases with increasing concentrations of the corresponding amino acids, and that the anabolic pathway can be suppressed by high levels of the corresponding amino acid. Giudici *et al.* (1993)

observed that the differences in the amount of higher alcohols in various wines, irrespective of the yeast strain used to ferment the original juice, could be due to the must composition in general or, in particular, to differences in the amino acid content of the juices. They found that, despite the fact that low amounts of amino acids were present with respect to the quantity of corresponding higher alcohols formed (via the Ehrlich reaction), the amino acids may play a role in controlling the pathways of their own formation and thus influence the anabolic formation of the higher alcohols. This was shown to be the case for isobutanol, active amyl and isoamyl alcohol (Schulthess & Ettlinger, 1978).

Yeast strain, yeast growth, ethanol production, fermentation temperature, must pH, aeration, level of solids, grape variety and maturity and skin contact time all affect the production of each alcohol in different ways (Fleet & Heard, 1993).

***Yeast strain effect on higher alcohol formation*:** Early studies by Webb & Kepner (1961) showed that Burgundy, Jerez and Montrachet yeasts differed considerably in the relative proportion of specific higher alcohols produced. Rankine (1967) examined several species of *Saccharomyces* as well as native yeast species with respect to higher alcohol production in wine (Table 7). In the case of pure culture *Saccharomyces* fermentations, significant differences in the concentrations of various higher alcohol fractions (most notably 1-propanol) were observed. In mixed culture fermentations where the population of the native yeast was low, fusel alcohol levels were not substantially different from those of pure culture *Saccharomyces* fermentations. Giudici *et al.* (1990) examined one hundred strains of *S. cerevisiae* for the ability to produce higher alcohols. The production of higher alcohols was found to be an individual strain characteristic (strains were divided into high and low producers), and as such was statistically significant. The characteristics of the strains used were found to be uncorrelated to isobutanol and isoamyl alcohol production, whereas the production of high levels of 1-propanol was found to be related to the inability to produce H_2S. The ability of strains to produce different amounts of higher alcohols can be used as a determining character in yeast selection for industrial purposes. For the production of isoamyl alcohol, isobutanol and propanol the concentrations ranged from 5 to 18 mg/L, 3 to 11 mg/L and 5 to 58 mg/L, respectively. Similar results were obtained by Giudici *et al.* (1993) under enological conditions.

Antonelli *et al.* (1999) studied 13 different yeasts for their secondary product formation during alcoholic fermentation. They found two *S. cerevisiae* yeasts that produced high quantities of 3-ethoxypropanol, correlated to a high propanol content. Di Stefano *et al.* (1981) reported this compound as typical of certain yeast strains, and its concentration was recently shown to increase significantly during flor ageing of Vernaccia di Oristano wine. Herraiz *et al.* (1990) found comparable amounts of this alcohol produced by *T. delbrueckii*, but with no correlation to *n*-propanol content.

Herraiz *et al.* (1990) investigated the influence of pure, mixed and sequential cultures of *K. apiculata*, *T. delbrueckii* (formerly *Saccharomyces rosei*) and *S. cerevisiae* on the volatile composition of wines. The results clearly showed that growth of *K. apiculata* and *T. delbrueckii* prior to the inoculation of *S. cerevisiae*, as well as their growth without such inoculation, produced wines whose volatile compositions were essentially different from those of wines arising from the use of *S. cerevisiae* to start the fermentation. They also noted that the ratio of isoamyl alcohol to active amyl alcohol and isobutanol to propanol, are in general, characteristic for each yeast. Their results showed that apiculate yeasts are important contributors to the chemical composition and quality of wine. Longo *et al.* (1992) also showed that dodecanol and tetradecanol production is highly yeast strain characteristic. Mateo *et al.* (1991) studied the contribution of different yeasts (*C. valida*, *Brettanomyces bruxellensis*, *Rhodotorula aurantica*, *H. uvarum*, *K. apiculata*, *Dekkera intermedia*, *S. cerevisiae* var. *capensis*, *S. cerevisiae* var. *chevalieri*, *S. cerevisiae* var. *bayanus* and *S. cerevisiae* var. *cerevisiae*) isolated from musts of Monastrell grapes to the aroma of wine. Their results also clearly showed the variation in higher alcohol production among the different yeasts. Gil *et al.* (1996) and Herraiz *et al.* (1990) found a 3-fold higher production of higher alcohols in wines fermented

TABLE 7

Higher alcohol formation (mg/L) by *Saccharomyces* yeast in 3 varieties of grape juice (Rankine, 1967).

Yeast	Pedro Ximinez pH 3.3			Tokay pH 3.3			Ugni blanc pH 3.42		
	n-PrOH	*i*-BuOH	AmOH*	*n*-PrOH	*i*-BuOH	AmOH	*n*-PrOH	*i*-BuOH	AmOH
S. fructum	20	11	140	107	9	142	52	8	145
S. cerevisiae a	16	23	95	41	14	126	21	15	125
S. chevalieri	12	39	207	26	30	256	10	34	280
S. cerevisiae b	56	21	151	170	9	151	92	9	264
S. oviformis	18	12	111	38	10	128	16	11	146
S. cerevisiae c	13	41	259	25	38	270	14	31	257
S. cerevisiae d	13	22	171	24	18	216	18	15	173
S. bayanus	9	30	166	19	35	249	11	26	195

* AmOH = iso + active amyl alcohol

TABLE 8

Variation of higher alcohol concentrations (mg/L) in wines produced by different yeasts (adapted from Gil *et al.*, 1996)

	Pure culture wines[a]					Mixed culture wines[b]				
	A48	**L22**	**T73**	**HAN**	**KLO**	**M1**	**M2**	**M3**	**M4**	**M5**
Propanol	10.1	12.1	10.0	3.7	9.7	16.8	10.5	14.5	19.2	14.1
Isobutyl alcohol	62.0	44.7	35.5	9.8	18.0	72.4	68.9	78.7	60.0	87.6
Butanol	2.2	2.1	1.5	0.6	1.2	2.4	3.0	2.6	3.6	3.6
Isoamyl alcohol	166	182	202	29.5	44.7	247	220	253	209	235
Hexanol	2.2	2.3	2.4	2.5	2.4	2.3	2.3	2.4	2.4	2.2
Phenethyl alcohol	19.2	26.0	38.0	12.3	7.00	39.5	33.8	44.0	34.6	28.1
Total	**261.7**	**269.2**	**289.4**	**58.4**	**83.0**	**380.4**	**338.5**	**395.2**	**328.8**	**370.6**

[a] A48 = *S. cerevisiae* A48 strain; L22 = *S. cerevisiae* L2226 strain; T73 = *S. bayanus* T73 strain; HAN = *Hanseniaspora uvarum*; KLO = *Kloeckera apiculata.*

[b] M1 = 47.5% KLO + 47.5% HAN + 1% T73 + 4% A48; M2 = 47.5% KLO + 47.5% HAN + 1% T73 + 4% L22; M3 = 5% KLO + 5% HAN + 89% T73 + 1% A48; M4 = 5% KLO + 5% HAN + 5% T73 + 85% L22; M5 = 5% KLO + 5% HAN + 90% A48.

with *Saccharomyces* spp. than in those fermented with pure cultures of apiculate yeasts (Table 8). They also found that wines inoculated with mixed cultures of *Saccharomyces* spp. and apiculate yeasts produced a higher total concentration of higher alcohols and other aroma compounds than wine inoculated with pure cultures of *Saccharomyces* spp. Cluster analysis revealed two clearly defined groups of wines (Gil *et al.*, 1996). One major group consisted of the pure culture wines, which in turn was separated into two subgroups, one with *Saccharomyces* yeasts and the other with apiculate yeasts. The second major group was formed by the mixed culture wines.

Formation of higher alcohols, particularly isobutyl alcohol, increases in aerated musts (Zoecklein *et al.*, 1995). Under oxidative conditions, yeasts with limited fermentative capabilities (*Pichia* sp., *H. anomala*, and *Candida* sp.) produce substantial quantities of fusel alcohols from fermentable sugars (Zoecklein *et al.*, 1995). The effect of aeration is probably stimulated growth and biosynthetic activity of yeast, hence the increase in demand for nitrogenous nutrients. A study done by Mauricio *et al.* (1997) showed that ethanol, isoamyl alcohol, isobutyl alcohol, phenethyl alcohol and isoamyl, butyl and hexyl acetates were produced in greater concentrations in semi-aerobic conditions, mainly due to cellular growth. 1-Butanol and 1-pentanol were produced in greater levels in anaerobic conditions, when cellular growth was lower.

The higher alcohol, phenethyl alcohol, has the unmistakable odour of roses and is also believed to play a sensory role in the perception of body (Zoecklein *et al.*, 1995). Rankine & Pocock (1969) found a 4-fold difference among yeasts in the amounts of phenethyl alcohol produced under comparable conditions. It was also shown by Massoutier *et al.* (1998) that regardless of fermentation temperature, cryotolerant yeasts (*S. uvarum*) produce 4-fold (216 mg/L compared to 45 mg/L) more phenethyl alcohol than mesophilic *Saccharomyces* yeasts. These cryotolerant yeasts also produced twice as much isobutyl and isoamyl alcohols as the mesophilic yeasts.

Esters

Esters are a group of volatile compounds that impart a mostly pleasant smell (Table 9). Most esters found in alcoholic beverages are produced by yeasts during fermentation as secondary products of sugar metabolism and constitute one of the largest and most important groups of compounds affecting flavour (Engan, 1974; Peddie, 1990; Fujii *et al.*, 1994). However, a particular aroma property can only rarely be associated with a specific ester (Van Rooyen *et al.*, 1982). The concentration of esters usually found in wine is generally well above their sensory threshold levels and they make up numerically the largest group of aroma compounds in alcoholic beverages (Salo, 1970a, b; Nykänen *et al.*, 1977; De Wet, 1978; Simpson & Miller, 1984). Thus it is not surprising that some of the descriptors used in sensory evaluation of wine and alcoholic beverages coincide with the associated aroma of these compounds (Maarse & Visscher, 1989; Etievant, 1991). The fresh, fruity aroma of young wines derives in large part from the presence of the mixture of esters produced during fermentation, which is why it is usually called *fermentation aroma/bouquet*. Fermentation compounds, especially the acetate esters, are responsible for the desirably fruity, ester-like character of young wines from neutral cultivars such as Chenin blanc and Colombar (Marais & Pool, 1980).

The esters of alcoholic beverages can be divided into three fractions of different boiling ranges. The first, light fraction consists of compounds eluting before isoamyl-alcohol in a gas chromatogram. The main components are ethyl, isobutyl and isoamyl esters of short chain fatty acids. The middle fraction comprises compounds appearing between ethyl caproate and phenethyl alcohol, and contains ethyl esters of caprylic and capric acids as main components. The third, heavy fraction contains the compounds eluting after phenethyl alcohol. These components include the ethyl esters of tetradecanoic, hexadecanoic and *cis*-9-hexadecanoic acids (Leaute, 1990).

Baumes *et al.* (1986) divided esters into two groups, based on odour evaluation. The first group he termed apolar. The apolar esters have fairly low detection odour thresholds, and with the

TABLE 9

Some esters produced by yeast and their concentrations, threshold values and odours in wine (Salo, 1972; Riesen, 1992; Boulton *et al.*, 1995).

Compound	Conc. in wine (mg/L)	Threshold value (mg/L)	Odour
Ethyl acetate		17.62* 12.3	Varnish, nail polish, fruity
Isoamyl acetate	0.03 – 8.1	0.26*	Banana, pear
2-Phenethyl acetate	0.01 – 4.5		Rose, honey, fruity, flowery
Ethyl isovalerate	ND – 0.7		Apple, fruity
Isobutyl acetate	0.01 – 0.8		Banana
Ethyl butanoate	0.01 – 1.8 0.01 – 3	0.4 (beer)	Floral, fruity
Ethyl 2-methyl-butanoate	ND – 0.9		Strawberry, pineapple
Hexyl acetate			
Ethyl hexanoate	Trace – 3.4	0.08	Apple, banana, violets
Ethyl octanoate	0.05 – 3.8	0.58 0.258*	Pineapple, pear
Ethyl decanoate	Trace – 2.1	0.5	Floral

* Percentage-above-chance-scores of 50% in grain spirit solutions of 9.4% (w/w)

exception of ethyl acetate, contribute fruit and flower notes to wine aroma. Apolar esters are present in wine in lesser amounts than polar esters, and include ethyl acetate, isoamyl acetate, ethyl octanoate, ethyl hexanoate, ethyl propanoate, ethyl butanoate and 2-phenethyl acetate. Polar esters have proved to be relatively insignificant contributors to wine aroma, contributing more to the body of a wine. They include 2-ethyl-hydroxypropionate, diethyl succinate, ethyl-4-hydroxybutanoate, diethyl malate, isopentyl-2-hydroxypropionate and ethyl-3-hydroxy butanoate. Ethyl hexanoate has an odour reminiscent of apple and violets, ethyl octanoate an odour reminiscent of pineapple and pear, and ethyl decanoate has a floral odour (Boulton *et al.*, 1995). Many esters can be formed, but the most significant ones are acetate esters of higher alcohols: ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl hexanoate and 2-phenylethyl acetate; and ethyl esters of straight-chain, saturated fatty acids: ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and ethyl dodecanoate (Thurston *et al.*, 1981; Marais, 1990; Fujii *et al.*, 1994). Table 9 lists some of the more prominent esters produced by yeast during fermentation. Ethyl acetate is the main ester occurring in wine. The proportion of the fatty acid ethyl esters transferred from yeast to the medium decreases with increasing chain length: 100% in the medium for ethyl hexanoate, 54-68% for ethyl octanoate, 8-17% for ethyl decanoate; from ethyl dodecanoate 100% is retained inside the cell (Nykänen *et al.*, 1977).

The volatile character of "acetic nose" is not exclusively the result of acetic acid. Ethyl acetate contributes significantly to this defect, and levels of 150 to 200 mg/L impart spoilage character to wine (Amerine & Cruess, 1960). High acetic acid content does not always confer a spoilage character to wine; this depends on the acetic acid to ethyl acetate ratio. Rejection levels determined for ethyl acetate in white and red wines were identified at 170 and 160 mg/L, respectively (Corison *et al.*, 1979).

The valuable contribution of acetates of higher alcohols to wine aroma has been known for some time. These compounds are synthesized during must fermentation in concentrations usually higher than those theoretically to be expected from their hydrolysis/synthesis equilibria. This means that they are hydrolized to a significant degree in the early stages of wine maturation, following kinetic behaviour well described by Ramey & Ough (1980). Thus, they are particularly important in young wine bouquet, though they still can play a significant role in an aged wine.

Young red table wines of the cultivar Pinotage have a distinct fermentation ("duco") character (Van Wyk *et al.*, 1979). This bouquet is not present in either the Pinotage grapes or must, and is known to be formed during fermentation. Isoamyl acetate, when present in relatively large concentrations, was shown to be the "impact" compound for this typical bouquet. The presence of this bouquet is evidently considered to be within limits a positive quality-enhancing factor. At present, winemakers actively avoid preparing young wines with excessive duco character. During ageing this bouquet gradually decreases in intensity and finally disappears. This change is accompanied by a concurrent decrease in isoamyl acetate concentration.

To determine the contribution of esters to wine aroma, Van der Merwe & Van Wyk (1981) were able to reproduce the quality and intensity of a dearomatised wine by addition of esters. Short-chain esters produced fruity characters, while longer chain esters were responsible for soap-like characters. Ferreira *et al.* (1995)

investigated the roles played by ethyl esters of fatty acids and by acetates of higher alcohols on the aroma of young wines from neutral grape varieties. They found that the roles played by these compounds depends on the type of wine. In white wines their main role was in creating the perception of tree fruit and tropical fruit notes. It has been demonstrated that the former notes are linked to ethyl esters, while the latter are linked mainly to acetates of higher alcohols. In red wines, these compounds did not determine the intensity of fruit aromas, and they only play a modulating role on aroma quality (Ferreira *et al.*, 1995).

There are several factors that influence ester production by yeast during fermentation, such as grape maturity and sugar content (Houtman *et al.*, 1980a, b), the strain used, fermentation temperature (Daudt & Ough, 1973; Engan, 1974; Piendl & Geiger, 1980), vinification methods (Herraiz & Ough, 1993; Gômez *et al.*, 1994), insoluble materials in the grape must (Houtman *et al.* 1980a, b; Edwards *et al.*, 1985), skin contact time (Falqué & Fernández, 1996), cultivar, must pH (Marais, 1978) and sulfur dioxide (Daudt & Ough, 1973; Herraiz *et al.*, 1989, 1990). There are also several factors that influence the concentrations of esters during the ageing process after fermentation. However, although esters are ubiquitous to all wines and brandies, the levels formed are yeast strain dependent (Mateo *et al.*, 1992). Numerous chemical changes also occur in wines during storage and ageing, and these changes may drastically affect aroma and quality. Depending on the storage temperature, the fruity character of young wines can disappear quite rapidly (Marais & Pool, 1980; Ramey & Ough, 1980). Thus, all of the above mentioned factors together determine the ester concentration present in the alcoholic beverage. The final concentration of an ester in a wine is dependent on its formation and destruction during the fermentation and ageing process and its distribution between the wine and yeast (Houtman *et al.*, 1980a, b; Marais & Pool, 1980).

***Biosynthesis of esters by yeast*:** The direct, enzyme-free formation of esters is an equilibrium reaction between an alcohol and an acid, as for example, the formation of ethyl acetate from acetic acid and ethanol ($CH_3COOH + C_2H_5OH \leftrightarrow CH_3COOC_2H_5 + H_2O$) (Engan, 1974). Although a great number of alcohols and acids are formed during fermentation, some are present in the raw material, *e.g.* grapes. All of the alcohols and acids may react to form esters and therefore the theoretical number of esters in wine is very large. Direct ester formation is, however, too slow to account for the ester concentrations found in alcoholic beverages. According to Nordström (1964a), the formation of ethyl acetate during fermentation proceeds according to the following reaction: $CH_3CO\text{-}SCoA + C_2H_5OH \leftrightarrow CH_3COOC_2H_5 + CoASH$. Alcohols become esterified by reacting with fatty acids which have undergone a previous activation by combining with coenzyme A (CoASH). Although acetyl-CoA can be formed by the oxidative decarboxylation of pyruvate, most of the other acyl-CoA compounds come from acylation of CoASH by the action of acyl-CoA synthetase. It is in these activated forms that acyl-CoA compounds can act as the acyl donor. As a result of the need for activation, ester synthesis is an energy-requiring process (Fig. 4). Furthermore, in ester as well as in fatty acid synthesis, the lengthening of the carbon chain of the acid moiety occurs so that malonyl-CoA binds with acyl-CoA in the enzyme complex, bringing two more carbon atoms into the chain of the acid. In the final step an ester is produced in the presence of alcohol, whereas when water is present the result is a free fatty acid. Acetate esters are synthesized by an enzyme called alcohol acetyltransferase, which uses as substrates an alcohol and acetyl-CoA (Peddie, 1990). Acetyl-CoA is formed partly by activation of acetic acid (requiring ATP) and partly by oxidative decarboxylation of pyruvate (requiring lipoic acid and thiamine pyrophosphate) (Piendl & Geiger, 1980). It is important to stress the central role played by acetyl-CoA in ester synthesis, since it is involved in many other reactions within the yeast cell (Fig. 5); e.g. lipid biosynthesis, amino acid biosynthesis, fatty acid biosynthesis and the TCA cycle (Nordström, 1964c; Piendl & Geiger, 1980; Yoshioka & Hashimoto, 1983). Factors which govern the anabolism and catabolism of this compound are crucial for ester production (Peddie, 1990; Dufour & Malcorps, 1994). In a general sense, acetyl-CoA can react with higher alcohols to yield the acetate esters, and acyl-CoA compounds can react with ethanol to yield the ethanol esters. Since acetyl-CoA/acetic acid and ethanol are the most abundant acids and alcohols present in the fermentation, ethyl acetate is normally the most abundant ester. However, if effective analytical techniques are used, almost every combination of acyl-CoA and alcohol can be detected as esters in the fermentation product.

Thurston *et al.* (1981, 1982) reported a large increase in the specific rate of ester production in the latter half of fermentation, which is concurrent with the cessation of lipid synthesis. This, they concluded, is due to the transient increase of available acetyl-CoA, since in the beginning of fermentation ester synthesis is very slow due to the high metabolic demand for acetyl-CoA for yeast growth (Yoshioka & Hashimoto, 1983). At this point oxygen and acetyl-CoA are rapidly consumed for the production of unsaturated fatty acids and sterols. After monitoring the formation of medium-chain fatty acids and related ethyl esters, Bardi *et al.* (1998) also proposed a model in which ester synthesis is a consequence of the arrest of lipid biosynthesis resulting from a lack of oxygen. Under these conditions, an excess of acyl-CoA is produced, and acyl esters are formed as secondary products of reactions aimed at recovering free CoA. In contrast, Dufour & Malcorps (1994) suggested that ester synthesis is not inhibited by unsaturated fatty acids or oxygen, but is rather modulated by a repression-induction of enzyme synthesis or processing. Data presented by Calderbank & Hammond (1994) support the view that precursor alcohol concentration is very important for ester synthesis and that many of the influences of fermentation conditions on ester synthesis are related to alcohol substrate availability. Because ethanol dominates among the alcohols and acetic acid among the volatile acids, most of the esters will be either ethyl esters or acetates. Only minor amounts of other esters are to be expected, as the corresponding alcohols are present in rather low quantities. The similar behaviour of all examined ester synthesizing enzymes points to the involvement of the same enzymatic system and to the existence of a common regulatory mechanism.

The physiological driving force for ester production remains obscure. Esters could simply be overspill products from sugar metabolism during fermentation and may be of no advantage to the yeast cell. Rainbow (1970) suggested that fatty acids with a chain length between C8 – C14 are toxic to the yeast, exhibit

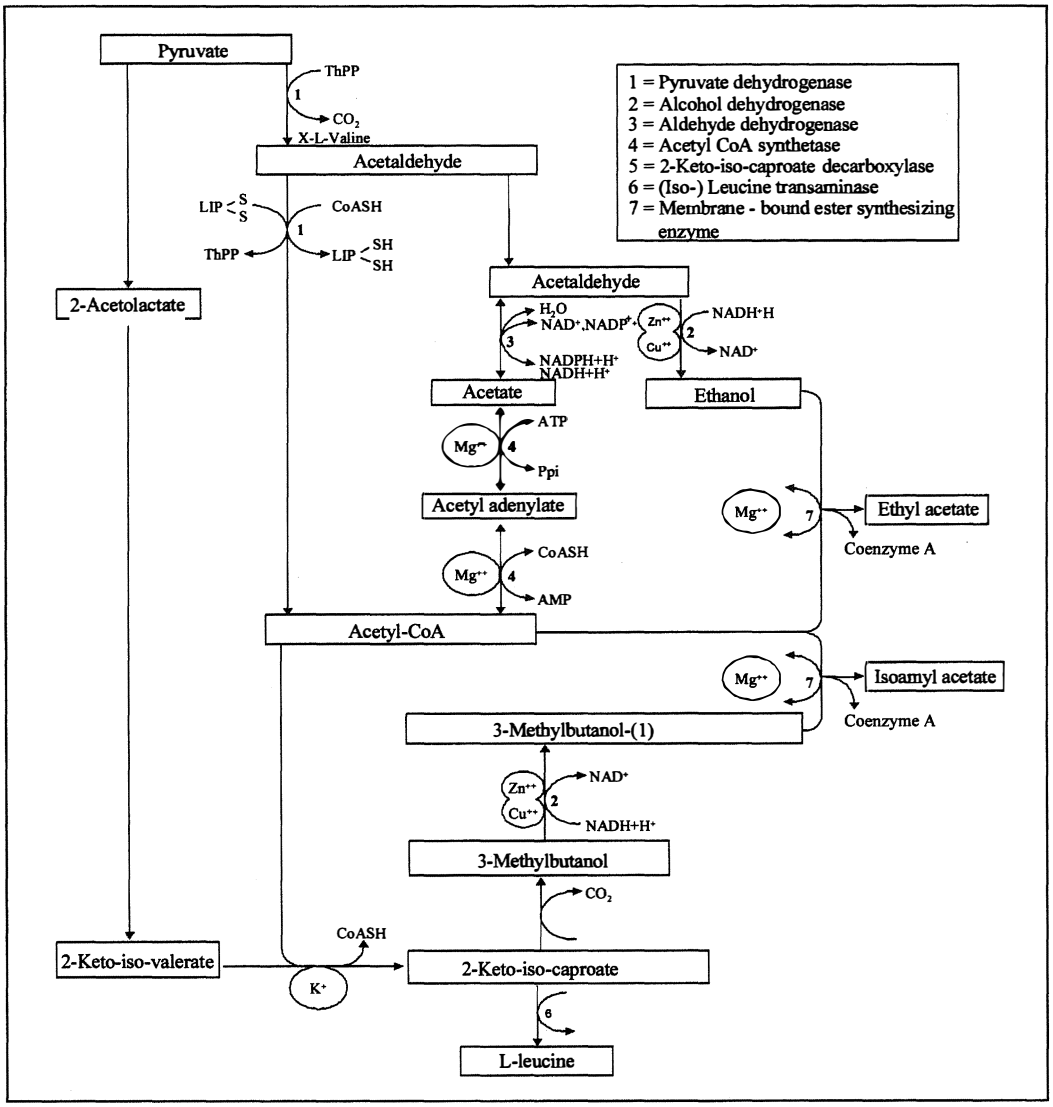

FIGURE 4

Schematic representation of the metabolic regulation of the formation of ethyl acetate and isoamyl acetate (Piendl & Geiger, 1980).

strong anti-microbial activity and, if unsaturated, intensify the effect. Esters may be formed to remove these toxic fatty acids from the yeast cell (Nordström, 1962, 1964b). Esters of shorter chain fatty acids (C2 – C6) would be produced by the same detoxification process. As there are several enzymes involved in the synthesis of esters, this suggests the possibility of different metabolic roles for long-chain and medium-chain alcohol acetylation, and for esterification of long-chain and medium-chain fatty acids with ethanol and ethyl acetate synthesis (Dufour & Malcorps, 1994). A possible role of ethyl acetate synthesis could be to regenerate free coenzyme A from acetyl-CoA without releasing acetic acid. The physiological role of medium-chain aliphatic ester synthesis (isoamyl acetate, ethyl caproate) in yeast remains undetermined. Another reason for ester formation could be to reduce the acetyl charge, as it is essential for the yeast cell to maintain a balance between acetyl-CoA and CoASH. Therefore, yeasts synthesize esters to correct any imbalance of the CoA: acetyl-CoA ratio caused by the cessation of the lipid synthesis pathway through fermentation (Thurston *et al.*, 1981, 1982; Bardi *et al.*, 1998). In contrast, preliminary evidence by Calderbank & Hammond (1994) on *in vitro* alcohol acetyl transferase (AAT) activity (the enzyme involved in ester synthesis) suggests that this enzyme may be primarily responsible for triglyceride or phospholipid synthesis and that ester synthesis is a minor function for it.

***Yeast strain effect on ester formation*:** Particular attention has also been given to ester formation by different yeasts. According to Nykänen (1986), Sponholz *et al.* (1974) showed that *H. anomala* and *C. krusei* yeasts have been found to produce more ethyl acetate than do *S. cerevisiae*, *S. pombe* and *Pichia membranaefaciens*. In another study *Pichia fermentans* produced greater amounts of ethyl acetate than two other *S. cerevisiae* yeast strains, Rankine and Montrachet, which formed the normal amount of ethyl acetate (Ough *et al.*, 1968). On the other hand, of these five

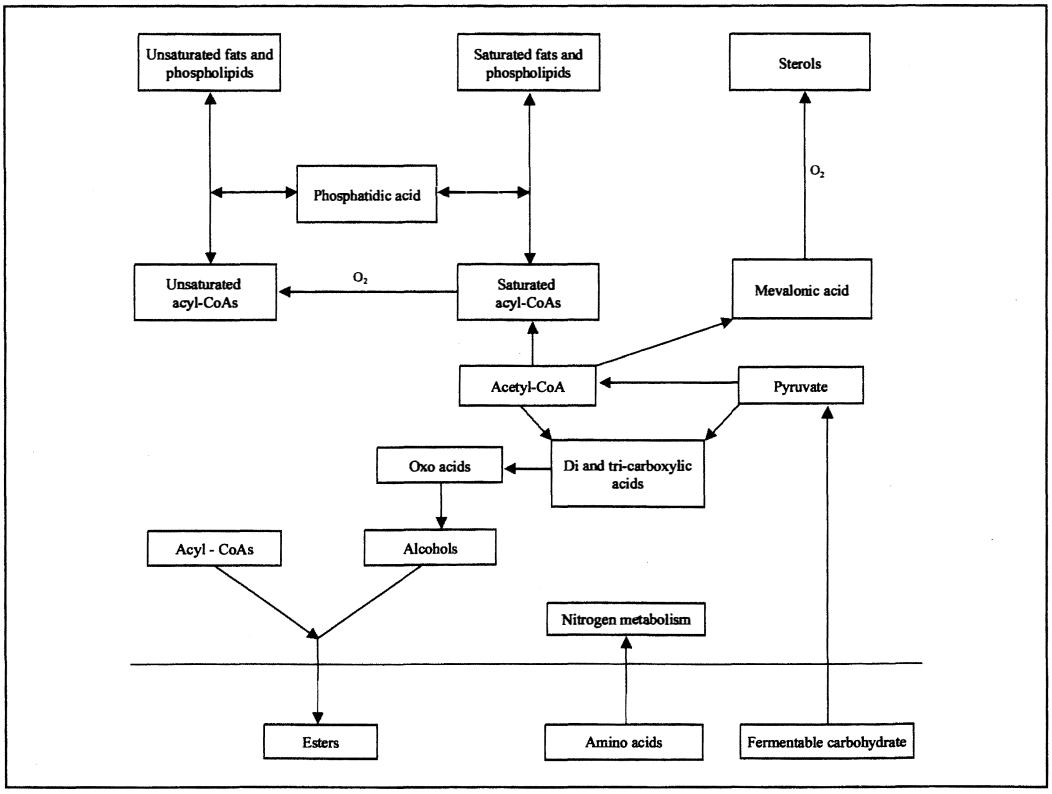

FIGURE 5
Metabolic pathways involved in ester production (García *et al.*, 1994).

yeasts, *H. anomala* and *C. krusei* formed the lowest amounts of octanoic-, decanoic-, and lauric acid ethyl esters. According to Suomalainen & Lehtonen (1979) *Rhodotorula* yeast increases the formation of isoamyl acetate. They have also shown that *H. anomala* and *C. krusei* yeasts produce less esters than, for example, *S. pombe*. Nykänen & Nykänen (1977) also investigated the ability of different yeasts to produce the acetates of isopenthyl and phenethyl alcohol and ethyl esters of the C6-C12 fatty acids in semi-aerobic sugar fermentations. They performed the fermentations with 57 strains of *S. cerevisiae* and three strains of *S. uvarum*; the strains of *S. cerevisiae* were found to produce more isopenthyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and phenethyl acetate than the *S. uvarum* yeasts. A higher proportion of the esters formed appeared to remain in the cells of *S. uvarum* strains than in the cells of *S. cerevisiae*. Suomalainen & Lehtonen (1979) also showed that *S. cerevisiae* produces significantly more isoamyl acetate, ethyl caproate, ethyl caprylate and ethyl caprate than does *S. uvarum*.

Lema *et al.* (1996) studied the dynamics of *Saccharomyces* and non-*Saccharomyces* populations during alcoholic fermentation of Albariño musts from two enological subzones located in Galicia (northwest Spain). They conducted sixteen microvinifications (eight in each must) with five indigenous *S. cerevisiae* strains, two commercial active dry yeast strains and the corresponding spontaneous fermentation. The concentration levels of the compounds analyzed in the wines depended not only on the *S. cerevisiae* strain used in the winemaking, but also on its predominance during alcoholic fermentation, which in turn was directly related to the initial concentration of yeast inoculated into the must and to the origin of the yeast (Lema *et al.*, 1996). A predominance of the *S. cerevisiae* strain inoculated during the fermentative process, along with a notable growth rate of the non-*Saccharomyces* populations in the first days of fermentation, significantly contributed to the production of volatile compounds related to the good aromatic quality of Albariño wines. The indigenous *S. cerevisiae* strain from the "O Rosal" subzone winery inoculated into the must originating from that winery produced higher quantities of ethyl esters, higher alcohol acetates and fatty acids in the resulting wine. This was probably due to its better adaptation to the chemical and microbiological characteristics of the must from this winery (Lema *et al.*, 1996).

Fourteen pure-culture wine yeasts were found to produce white wines with significantly different ester concentrations (Soles *et al.*, 1982). The esters quantified, by glass capillary gas chromatography, were isoamyl acetate (including active amyl acetate), ethyl hexanoate, hexyl acetate, ethyl octanoate, 2-phenylethyl acetate and ethyl decanoate. Wine fermented by the *S. cerevisiae* strain Rankine no. 350 was found to contain statistically more of these six esters in total than any of the other yeast fermentations. The *S. cerevisiae* Rankine no. 350 strain produced significantly more isoamyl acetate than the other yeasts studied. The lowest production level of isoamyl acetate was by the *Saccharomyces fermentati* Flor strain (Soles *et al.*, 1982). Wine fermented by the *S. fermentati* Flor strain contained significantly

more ethyl hexanoate than wine fermented by all the other yeasts except Rankine no. 350 and *S. bayanus*, a Champagne strain from the Institute Pasteur. *S. bayanus* strain California-Champagne, *S. cerevisiae* Rankine no. 350, *Saccharomyces bailii* and an isoleucine-deficient mutant of *S. cerevisiae* produced significantly greater amounts of hexyl acetate than the other yeasts tested except the *S. cerevisiae* Geisenheim strain (Soles *et al.*, 1982). The *S. bayanus* California-Champagne strain produced a significantly higher amount of 2-phenylethyl acetate than eight other yeasts, while the *S. bayanus* Institute Pasteur strain produced wine with more of this ester than seven other yeasts. Ethyl decanoate was produced in significantly different amounts by the yeasts. The *S. bayanus* California-Champagne strain produced significantly higher amounts of ethyl decanoate than nine other yeasts.

In another study Delteil & Jarry (1992) studied the characteristic effects of two commercial yeast strains on Chardonnay wine volatiles. Chardonnay wines fermented with K1M (ICV K1 Marquée, ADWY, batch no: 4254, Lallemand, Canada) contained higher concentrations of isoamyl acetate, ethyl laurate and total esters, and these concentrations were mainly must independent. Chardonnay wines fermented with D47 (ICV D47, ADWY, batch no: 8054, Lallemand, Canada) contained higher concentrations of ethyl caproate and ethyl caprylate, but these concentrations were highly must-dependent. The two major styles of young commercial Chardonnay wines fermented with these two strains reflected differences; high aroma intensities with fresh banana and citrus characters for K1M wines (due to the higher acetate ester levels and higher and better varietal expression), and pineapple and spicy characters for D47 wines due to the lower acetate ester levels (Table 10) (Delteil & Jarry, 1992).

Aroma compounds of wines fermented from sterile grape musts of the *Monastrell* variety inoculated with pure and mixed cultures of apiculate and *Saccharomyces* yeasts were isolated and analyzed (Gil *et al.*, 1996). In wines fermented only with apiculates (*H. uvarum* and *K. apiculata*) which had an elevated amount of residual sugars, ester amounts were detected similar to those found in the wines inoculated with *Saccharomyces* species. Clearly, apiculate yeasts can affect the chemical composition of wines and thus impact on quality (Gil *et al.*, 1996).

The evolution of the cell and must contents of three medium-chain fatty acids (C6, C8, and C10) and their ethyl esters during fermentation with *S. cerevisiae* strains *cerevisiae*, *bayanus* and *capensis* were studied (Zea *et al.*, 1994). The first is a fermentative yeast; the last two are flor film yeasts. Maximum ester concentrations in the cells were attained after 48-72 hours of fermentation. Secretion during this period was quite active, particularly with the flor film yeasts, and musts fermented by these yeasts featured higher maximal ester concentrations than those fermented by *S. cerevisiae* strain *cerevisiae*. In the musts, ethyl octanoate and ethyl decanoate also reached a peak at this point but ethyl hexanoate peaked only after 10 days. After 134 days, *S. cerevisiae* strain *capensis* formed a thick flor film, the strain *bayanus* developed a thin film, and the strain *cerevisiae* formed no film, with non-viable cells accumulating in the bottom of the fermenter. At this point the ethyl ester contents tended to decrease, with the exception of ethyl decanoate, in the fermentations carried out by *S. cerevisiae* strains *cerevisiae* and *bayanus* (Zea *et al.*, 1994). The decrease of these compounds in all wines at the end of this study cannot be attributed only to the flor film yeasts' growth, although this growth induced a greater decrease relative to the fermentative strain in the early ageing period (134 days). The esters already formed and secreted into the wine must disappear due to their chemical hydrolysis at the physical pH of wine, as pointed out by Ramey & Ough (1980), or by hydrolytic processes carried out by enzymes of the grapes and yeasts themselves.

Sixteen ethyl esters of amino acids have been identified in wines and sherries (Heresztyn, 1984; Herraiz & Ough, 1992). These compounds were found in concentrations of up to 58 mg/L in the wines analyzed; the amounts found indicate that these esters contribute significantly to the pool of basic compounds in wines. Herraiz & Ough (1993) showed that the amino acid ethyl esters are formed during the alcoholic fermentation by yeasts, mainly in the second half of the fermentation, when the concentration of ethanol in the medium was high. It should be expected that high levels of intracellular ethanol in the yeast may increase the formation of ethyl esters formed from amino acids and later liberate them to the surrounding medium. The level of these compounds appeared to increase during yeast autolysis. Some differ-

TABLE 10

Differences in ester production by yeast strains (Delteil & Jarry, 1992).

Compound (mg/L)	ICV K1M	ICV D47
Isoamyl acetate	16.45	9.00
Ethyl dodecanoate	0.09	0.05
Hexyl acetate	0.33	0.53
Ethyl hexanoate	1.13	1.38
Ethyl octanoate	1.54	1.85
Ethyl decanoate	0.6	0.5
Diethyl succinate	0.33	0.41
Total esters	**23.63**	**14.26**

ences were also found between the two yeasts used.

Ethyl esters of alanine, valine, leucine, isoleucine and proline were identified in all the wines studied. Glycine-, γ-aminobutyric-, threonine-, serine-, phenylalanine-, tyrosine- and lysine-ethyl ester were identified in almost all samples, whereas methionine-ethyl ester was identified in eight wines. Two diethyl esters, aspartic acid- and glutamic acid-, were detected only in sherries. Proline ethyl ester was found in the highest amounts; the other amino acid esters found in excess of 1 mg/L were alanine-, glycine- and γ-aminobutyric-ethyl ester. Cysteine ethyl ester was not detected (Herraiz & Ough, 1992).

Carbonyl compounds

Short chain, volatile aldehydes are important to the flavour of a number of foods and beverages, including wine, contributing flavour characteristics ranging from "apple-like" to "citrus-like" to "nutty" depending on the chemical structure (Table 11). Together with the keto-acids, the short-chain aliphatic aldehydes are the key compounds in the biochemical reaction involving the production of higher alcohols from amino acids and sugars by yeast. They are formed in the yeast cell and then transferred to the medium (Nykänen *et al.*, 1977). Because of their low sensory threshold values, aldehydes are important to the aroma and bouquet of wine; among these, acetaldehyde is the major component, constituting more than 90% of the total aldehyde content in wines and spirits (Nykänen *et al.*, 1977).

Acetaldehyde is a normal product of alcoholic fermentation and its amount in wine can vary from 10 mg/L up to 300 mg/L (Schreier, 1979), with a sensory threshold value of 100 mg/L (Berg *et al.*, 1955). In white wines acetaldehyde is regarded as an indicator of the wine oxidation state, whereas in red wines it should be present in amounts up to 100 mg/L. Sherry and Tokay wines contain relatively high amounts of acetaldehyde, due to their production under oxidative conditions.

Normally wines contain 0.2-0.3 mg/L of diacetyl produced by yeast metabolism. This compound with a buttery aroma becomes objectionable at 1-4 mg/L. Such high values will be due to the growth of lactic acid bacteria (Sponholz, 1993); therefore, diacetyl production by bacteria is far more important than the small amounts which might be produced by yeast during vinification.

Mousiness in wines is caused by the nitrogenous carbonyl compounds 2-acetyl-3,4,5,6-tetrahydropyridine, 2-acetyl-1,2,5,6-tetrahydropyridine and 2-ethyl-3,4,5,6-tetrahydropyridine (Heresztyn, 1986; Rapp, 1998). *Lactobacillus* bacteria and yeast from the genus *Brettanomyces* are usually responsible for this odour. These compounds are produced by *Brettanomyces* yeasts from the metabolism of lysine.

***Biosynthesis of carbonyl compounds by yeast*:** The precursors of aldehydes, the 2-keto acids, are formed as intermediates in both the anabolic and catabolic formation of amino acids or higher alcohols. Conditions which favour higher-alcohol production also favour the formation of small quantities of aldehydes (Berry, 1995). These may be secreted but can be reabsorbed and reduced by yeast to the corresponding alcohol during the later stages of the fermentation. Acetaldehyde is an intermediary product formed from pyruvate and is a precursor for acetate, acetoin and ethanol. Oxidation of alcohols, Strecker degradation of amino acids and autoxidation of fatty acids may also produce aldehydes in alcoholic beverages. Acetaldehyde concentrations reach a maximum at the point in fermentation when the most rapid carbohydrate dissimilation takes place. It falls to a low level at the end of fermentation, and thereafter slowly increases again. The

TABLE 11

Some carbonyl compounds produced by yeast and their concentrations, threshold values and odours in wine (Schreier, 1979; Darriet *et al.*, 1995; Ribéreau-Gayon *et al.*, 1998; Ebeler & Spalding, 1999).

Compound	Conc. in wine (mg/L)	Threshold (mg/L)	Odour
Acetaldehyde	10-300	100	Sour, green apple
Benzaldehyde	$0.3 \times 10^2 - 4.1$		Bitter almond
Butanal	Traces		Pungent
Diacetyl	0.05-5	0.15†	Buttery
		2-5*	
Propanal	Traces		Similar to acetaldehyde
Isobutanal	Traces		Slightly apple like
Pentanal	Traces		Cocoa, coffee-like, slightly fruity, choking at high levels
Isovaleraldehyde	Traces		Warm, herbaceous, slightly fruity, nut-like, acrid at high levels
2-acetyltetrahydropyridine	Traces	1.6×10^{-3}	Mousy taint

† Beer

* Values above which an off-flavour will result

autoxidation of vicinal di- and tri-hydroxy phenols also present in the wine leads to the formation of hydrogen peroxide, a strong oxidant, which further initiates a number of oxidation reactions involving oxidation of ethanol to acetaldehyde (Rosi *et al.*, 1989; Romano *et al.*, 1994).

The formation of acetaldehyde also depends on various fermentation conditions. These include extreme aerobic growth conditions (Bennetzen & Hall, 1982), the medium composition (Denis *et al.*, 1983), and the nature of insoluble materials used to clarify musts (Delfini *et al.*, 1993). The amount of acetaldehyde present in wines increases during ageing from oxidation of ethyl alcohol, the activity of film yeasts, or aeration. The use of high concentrations of sulfur dioxide in grape must fermentation causes a considerable increase in acetaldehyde levels formed by the yeast cell. Cassalone *et al.* (1992) found that sulfite-resistant mutants of *S. cerevisiae* accumulate much more acetaldehyde in the medium than does the parental strain.

Fermentation temperature may affect acetaldehyde content, but the reports are mixed. Amerine & Ough (1980) reported that fermentation temperature does not have a significant affect on the final total aldehyde content, whereas other researchers found that aldehyde content increased with increasing fermentation temperature. In a study performed by Romano *et al.* (1994), a fermentation temperature of 30°C considerably increased the amount of acetaldehyde produced

The decarboxylation of pyruvic acid, and complexing with the coenzyme thiamine pyrophosphate, produces a product called active acetaldehyde. The latter further combines with another molecule of pyruvic acid to give α-acetolactic acid. Diacetyl is then formed via the oxidative decarboxylation of α-keto-acetolactate (Boulton *et al.*, 1995). The final concentration in the beverage is determined by the balance between the rate of formation and the rate of degradation. In the later stages of the fermentation, diacetyl can be metabolized by the yeast to acetoin and butane-2,3-dione (Berry, 1995).

***Yeast strain effect on aldehyde content*:** The total aldehyde content varies with the type of yeast strain used. In assays performed by Then & Radler (1971) with 300 different yeasts, the aldehyde content was found to vary from 6 to 190 mg/L. Other data indicate that *S. cerevisiae* strains produce relatively high levels (from 50 to 120 mg/L), whereas other wine yeasts, such as *K. apiculata, C. krusei, C. stellata, H. anomala* and *M. pulcherima*, produce low levels (from non detectable amounts to 40 mg/L) of acetaldehyde (Fleet & Heard, 1993). Romano *et al.* (1994) divided 86 *S. cerevisiae* wine strains into groups producing low, medium and high amounts of acetaldehyde. The low and high phenotypes also differed considerably in the production of acetic acid, acetoin and higher alcohols. Wines obtained with the low acetaldehyde producers had traces of acetoin, lower amounts of acetic acid (<500 mg/L), and a higher total content of higher alcohols (>300 mg/L). Wines obtained with the high producers showed a different pattern, containing detectable amounts of acetoin, elevated amounts of acetic acid (528 to 1185 mg/L), and a lower content of higher alcohols (256 to 270 mg/L). Longo *et al.* (1992) also found variations in the production of acetaldehyde from 13.1 to 24.3 mg/L among 14 strains of *S. cerevisiae*.

Yeasts are able to utilize benzaldehyde in the presence of glucose. Some strains of *S. cerevisiae* and *S. japonicus* can transform benzaldehyde into benzyl alcohol and benzoic acid (Nykänen, 1986). Delfini *et al.* (1991) found that *Schizosaccharomyces* and *Zygosaccharomyces* were strongest in producing benzaldehyde (maximal amount found was 1200 mg/L) and benzyl alcohol [maximally 523 mg/L in a synthetic nutritive medium (MNS) with 10 ppm of benzyl alcohol, benzoic acid, 4-hydroxybenzoic acid and phenylacetic acid added].

Zygosaccharomyces was also noted to be the most effective in the production of benzoic acid (maximally 536 mg/L), followed by *Saccharomyces, Cryptococcus, Kloeckera* and *Torulaspora*. None of the strains tested were able to accumulate benzaldehyde in this particular medium. Delfini *et al.* (1991) also verified that yeasts can be an exogenous source of the benzyl alcohol oxidizing enzyme in grape musts and wines. Wine yeast strains of *Saccharomyces* spp., *Zygosaccharomyces* spp. and *Schizosaccharomyces* spp. fermenting in MNS medium containing 150 g/L glucose, with benzyl alcohol added, transformed the latter into benzoic acid, but not into benzaldehyde, only when glucose was disappearing. No difference was observed between anaerobic and aerobic fermentation conditions. A catabolic repression by glucose was thought to be highly likely, as the uptake of benzyl alcohol was rapid in fermentation assays in the presence of only 10 g/L glucose, and in assimilation assays performed in yeast nitrogen base broth with assimilable carbon compounds added. From an oenological point of view, it is thus important to note that *S. cerevisiae* can utilize benzyl alcohol in grape musts or in wines when it has consumed large amounts of glucose, or while it is utilizing substances with metabolic pathways different from those of glucose. Benzyl alcohol is converted into benzoic acid and no benzaldehyde is formed. Thus catabolic repression of this transformation by glucose also appears likely.

Volatile phenols

Phenolic substances can be very important to the taste, colour and odour of wines (Dubois, 1983). Glycosidic combinations of 4-vinylguiacol, 4-vinylphenol and eugenol may exist in certain grape varieties (Singleton & Esau, 1969). Acetovanillone, ethyl vanillate and methyl vanillate are described as having a vanilla and spicy character. Instead of directly contributing to the varietal aroma of grapes, fruit phenolics appear to be more important as a source of hydroxycinnamic acid esters. In this regard, coumaric and ferulic acid esters are particularly important, as they can be transformed into volatile phenols during fermentation. Vinylphenols (4-vinylguiacol, 4-vinylphenol) in white wines and ethylphenols (4-ethylguiacol, 4-ethylphenol) in red wines are quantitively the most significant volatile phenols identified as classic components of wine aroma (Singleton & Esau, 1969; Etievant, 1981; Chatonnet *et al.*, 1997). They appear at concentrations from 0 to 6047 µg/L in wines and can give phenolic off aromas/odours often described as animal, stable, horse sweat, medical, "elastoplast" when present above their threshold levels (Table 12). The phenolic off odour of red wines most often develops during ageing and especially in wines stored in old barrels and seldom racked (Chatonnet *et al.*, 1992; Chatonnet *et al.*, 1993). *Brettanomyces/Dekkera intermedius* is the yeast species most frequently identified (95%) in Bordeaux red wines with phenolic off-odours (Chatonnet *et al.*, 1992); *Brettanomyces lam-*

TABLE 12

Some vinyl and ethyl phenols produced by yeast and their concentrations, threshold values and odours in wine (Chatonnet *et al.*, 1992, 1993; Rapp, 1998).

Compound	Conc. in wine (µg/L)	Threshold value white wine (µg/L)	Threshold value red wine (µg/L)	Odour
4-Vinylphenol	0-1150	770		Pharmaceutical, elastoplast, gouache
4-Vinylguiacol	0-496	440		Smoky, vanilla, clovelike
4-Vinyl guaiacol + 4-Vinylphenol [1:1]		752		
4-Ethylphenol	0-6047		605	Wet horse
4-Ethylguiacol	0-1561		110	Smoky, vanilla, clovelike
4-Ethylphenol + 4-ethylguiacol [10:1]			369	

bicus is much less common.

The derivatives 4-ethylguaiacol and 4-vinylguaiacol could add smoky, vanilla and clovelike notes to wine (Singleton & Esau, 1969). Beyond a certain concentration, vinylphenols may be responsible for certain sensory faults in wines, commonly referred to as "phenolic" or "pharmaceutical" taints. 4-Vinylphenol is principally responsible for this fault in white wine, to which it confers "heavy", "phenolic" and "medicinal" odours resembling the datura flower, "elastoplast" and gouache. Its presence is always undesirable, even at concentrations lower than its perception threshold, since it masks the fruity nuance of white wines (Dubois, 1983; Chatonnet *et al.*, 1992). It has also been shown that although 500 µg/L 4-ethylphenol, spiked to a faultless neutral wine resulted in medicinal, horse sweat, leathery aromas, 50 µg/L spiked to the same wine already changed the sensory evaluation of the wine. However, this is not the case with 4-vinylguiacol. Despite having a higher limit preference threshold, this molecule can play an important role in the varietal expression of certain grape varieties, such as Gewürztraminer. Nevertheless, the two compounds are always present simultaneously (Dubois, 1983). Concentrations of more than 770 µg/L of a mixture of 4-vinylguaiacol/4-vinylphenol (1:1) in white wines can be responsible for heavy "pharmaceutical" odours reminiscent of "elastoplast" (Chatonnet *et al.*, 1993). Consequently, the quality of white wine is depreciated, in most cases, by concentrations of total vinylphenols above 725 µg/L (Σ vinylphenols/ Limit threshold > 1) (Chatonnet *et al.*, 1992). Over 425 µg/L of a 1:10 blend of 4-ethylguaiacol/4-ethylphenol in red wines results in animal, barnyard or stable off odours (Chatonnet *et al.*, 1990).

***Biosynthesis of volatile phenols by yeast*:** Vinyl- and ethylphenols result from the microbiological transformation of *trans* ferulic and *trans-p*-coumaric acids, the nonvolatile, odourless precursors present in all wines. It was always believed that these compounds were formed due to the development of lactic acid bacteria in wines. Certain lactic acid bacteria can decarboxylate the cinnamic acids and in some cases reduce the vinylphenols to the corresponding ethylphenols (Chatonnet *et al.*, 1992; Cavin *et al.*, 1993). Chatonnet *et al.* (1995) showed that the quantities likely to be formed by these lactic acid bacteria are relatively low by comparison to the quantities easily detected in wines judged to be "phenolic". Chatonnet *et al.* (1997) have recently shown that concentrations above 1 g/L of procyanidins significantly inhibited the ability of lactic acid bacteria in general and *Lactobacillus plantarum* in particular to form ethylphenols. Their results led to the definitive conclusion that contaminating yeasts from the genus *Brettanomyces/Dekkera* sp. were responsible for the development of the "phenolic" character in red wines. It was also shown that polyphenolic compounds found in red wines did not affect the ability of *B. bruxellensis* to synthesize volatile phenols. Once malolactic and alcoholic fermentation are completed, these yeasts grow easily on traces of residual sugars. Only careful hygiene and proper sulfuring of wines and containers can prevent the development of these undesirable yeasts. Furthermore, the presence of tannins in red wine has no influence on synthesis of volatile phenols by *B. bruxellensis*, as opposed to lactic acid bacteria or *S. cerevisiae*.

Certain yeast strains responsible for the alcoholic fermentation of beer and wine, as well as bacteria from various media, are capable of forming vinylphenols such as 4-vinylphenol or 4-vinylguiacol (Goodey & Tubb, 1982). In the case of yeasts, the synthesis of vinylphenols requires a non-oxidative decarboxylation of the phenolic acids (Gramatica *et al.*, 1982). Volatile phenol synthesis by yeast depends on the nature of the strain and on the presence of certain polyphenolic inhibitors (Chatonnet *et al.*, 1993).

Chatonnet *et al.* (1993) showed that *S. cerevisiae* decarboxylates only the cinnamic acids (phenyl-propenoic) into volatile phenols (Fig. 6a). Among the cinnamic acids present in the grape, only ferulic and *p*-coumaric acids can be transformed into 4-vinylguiacol and 4-vinylphenol, respectively. The enzyme responsible for this transformation appears to have a greater affinity for *p*-coumarate than for ferulate. The *S. cerevisiae*

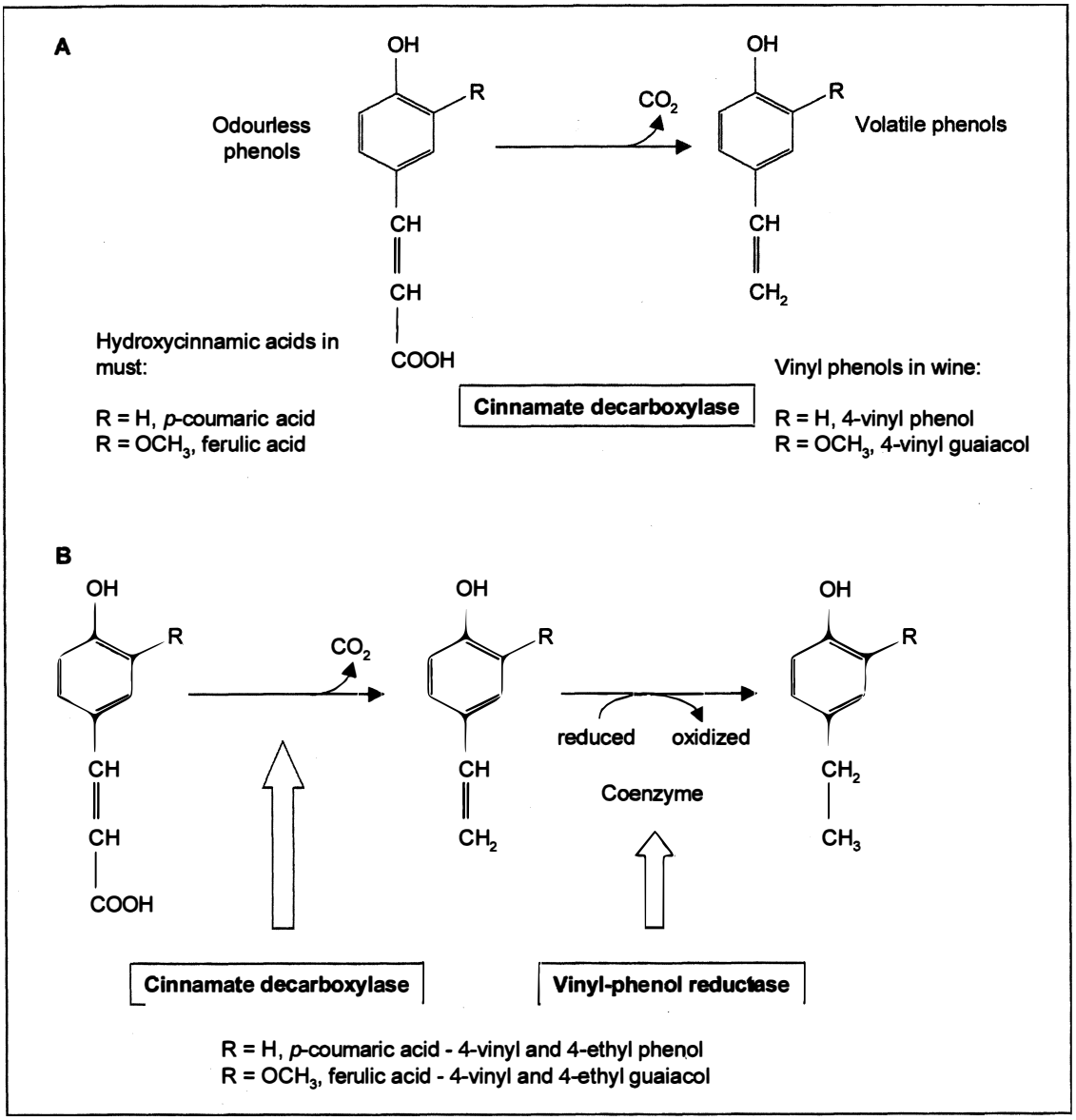

FIGURE 6

A) Decarboxylation of phenolic acids by *S. cerevisiae* during the alcoholic fermentation. B) Enzymatic mechanism for the production of ethyl phenols by *Brettanomyces* sp. (Ribéreau-Gayon, *et al.* 1998).

strains they studied were unable to decarboxylate cinnamic acid to form vinylbenzene (styrene). They concluded that the decarboxylase activity of *S. cerevisiae* is therefore a cinnamate decarboxylase. This decarboxylation of the cinnamic acids is stereospecific and occurs only with the *trans* (E) isomer, generally the form most commonly found in nature. The cinnamate decarboxylase of *S. cerevisiae* is strictly intracellular and has an optimum pH of around 6.5. This activity is constitutive and strictly limited to alcoholic fermentation. No vinylphenol can therefore be synthesized in a dry wine stored in the presence of yeast lees (Goodey & Tubb, 1982; Gramatica *et al.*, 1982).

The biosynthesis of ethylphenols by *Brettanomyces/Dekkera* has shown that a cinnamate decarboxylase transforms certain cinnamic acids into the corresponding vinylphenols, which are then reduced to ethylphenols by a vinylphenol reductase (Fig. 6b) (Chatonnet *et al.*, 1992).

***Yeast strain effect on volatile phenol production*:** Chatonnet *et al.* (1992) tested yeasts isolated from different fermented media (wines, beers pineaux, ciders, vinegars) in a model medium containing *trans-p*-coumaric acid substrate. The microorganims varied in their ability to decarboxylate *p*-coumaric acid into 4-vinylphenol (Table 13). Only the yeasts belonging to the genus *Brettanomyces* and *Dekkera* could transform substantial amounts of *p*-coumaric acid into 4-ethylphenol.

Van Wyk & Rogers (2000) showed that the phenolic and elastoplast off-odours present in some Kerner tabel wines is due to the production of *p*-vinylguaiacol by wine yeast strains during the alcoholic fermentation. They showed that the ability of 18 com-

TABLE 13
Synthesis of volatile phenols from *p*-coumaric acid by different yeasts (Chatonnet *et al.*, 1992)

Microorganisms	Transformation of *p*-coumaric acid[a]	
	4-Vinylphenol	4-Ethylphenol
Candida vini MUCL 27720	-	-
Candida freychussi MUCL 27714	++++	-
Hanseniaspora uvarum MUCL 27770	+	-
Metschnikovia pulcherina MUCL 277876	-	-
Pichia membranefaciens MUCL 27734	-	-
Hansenula anomala MUCL 27753	++++	-
Saccharomyces cerevisiae (*italicus*) ICEB	+++	-
Saccharomyces cerevisiae (*chevalieri*) ICEB	+	-
Saccharomyces cerevisiae (*uvarum*) ICEB	++	-
Saccharomyces cerevisiae (*carlbergensis*) ICEB	-	-
Saccharomyces cerevisiae (*capensis*) ICEB	+	-
Saccharomyces cerevisiae (*cerevisiae*) ICEB	+	-
Saccharomyces cerevisiae (*cerevisiae*) ICEB	++++	-
Saccharomyces cerevisiae (*cerevisiae*) ICEB	+++	-
Saccharomyces cerevisiae (*cerevisiae*) ICEB	+	-
Saccharomyces cerevisiae (*bayanus*) ICEB	++	-
Kluyveromyces thermotolerans MUCL 28822	+	-
Torulaspora delbruchi MUCL 27816	-	-
Pichia carsenis ICEB	+	-
Pichia canadensis MUCL 27722	+	-
Saccharomycopsis fibuligera MUCL 11443	+	-
Zigosaccharomyces bailii ICEB	+	-
Brettanomyces intermedius CBS	+	+++
Dekkera intermedia MUCL 11989	+	+++
Brettanomyces lambicus ICEB SA	+	+++

[a] –, Negative; +/-, very weak ≤ 1% of the substrate; +, 1-20%; ++, 20-40%; +++, 40-60%; ++++, > 60%

mercial wine yeast strains to produce *p*-vinylguaiacol varied significantly (0 – 890 µg/L).

Sulfur compounds

These substances can make a significant contribution to the flavour of wine because of their reactivity and extremely low threshold values. In some circumstances, they are responsible for undesirable sulfurous off-flavours (Schutte, 1975; Peppard, 1988). Many sulfur compounds are extremely flavour active and have thresholds far below 0.002 ppb (Soltoft, 1988); these values vary depending on the wine variety in which they are measured and the taster. Table 14 lists some of the main sulfur compounds found in wine.

The production of hydrogen sulfide (H_2S) has been studied in detail, since its aroma is frequently detected during fermentation because of low nitrogen musts. Only a few reports exist on the synthesis of other flavour-active S-compounds. H_2S has an unpleasant aroma with a low sensory threshold (10 – 100 µg/L); values above its threshold cause an off-flavour reminiscent of rotten eggs. In young wines the production of 20 – 30 µg/L of H_2S may have a positive contribution to "yeasty" flavour (Dittrich & Staudenmayer, 1970). Recent studies show that high amounts of H_2S can also lead to the formation of other undesirable volatile sulfur compounds (Rauhut & Kürbel, 1994a, b). In the past, one of the main sources of H_2S was the reduction of elemental sulfur by wine yeasts from residues originating with applications of dusting sulfur in the vineyard as fungicide (Schütz & Kunkee, 1977). Chemical breakdown of S-containing pesticides (e.g. acephate) can also be the cause of odour-active S-substances like methyl mercaptane (Rauhut *et al.*, 1986).

Analysis of 100 wines showed that wines with a sulfurous off-flavour had increased concentrations of methyl mercaptane

TABLE 14

Some volatile sulfur substances produced by yeast and their concentrations, threshold values and odours in wine (Shreier, 1979; Goniak & Noble, 1987; Rauhut, 1993).

Compound	Conc. in wine (µg/L)	Threshold value (µg/L)	Odour
Hydrogen sulfide	Trace- >80	50-80	Rotten eggs
Dimethyl sulfide	Trace-910	25-60	Asparagus, corn, molasses
Diethyl sulfide	Traces	0.92	Cooked vegetables, onion, garlic
Dimethyl disulfide	Traces-1.6	29	Cooked cabbage, onion-like
Diethyl disulfide	Traces	4.3	Garlic, burnt rubber
Methyl mercaptan	Qualitive	2-12*	Rotten eggs, cabbage
Ethyl mercaptan	Qualitive	1.1	Onion, rubber
S-methyl thioacetate	2-16	300*	Rotten vegetables
S-ethyl thioacetate	Traces-4	40*	Cheesy, burnt
4-Mercapto-4-methylpentan-2-ol	Traces	3×10^{-6}	Box tree, cat urine, guava, conifer

*Beer

(MeSH), ethyl mercaptane (EtSH), dimethyl disulphide (DMDS), methyl ethyl disulfide (MeSSEt), diethyl disulfide (DEDS), thioacetic acid-S-methyl ester (MeSAc) and thioacetic acid-S-ethyl ester (EtSAc), as well as other known and unknown S-compounds (Rauhut & Kürbel, 1996).

Three flavour-active volatile thiols (4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol and 3-mercaptohexan-1-ol) involved in *Vitis vinifera* L. var. Sauvignon blanc wine aroma (box tree, broom, passion fruit, guava and "conifer") were recently identified (Darriet *et al.*, 1995; Tominaga *et al.*, 1998a). These compounds have very low threshold values and are produced by yeast during the alcoholic fermentation from grape precursors.

Tominaga *et al.* (1996) identified 3-mercaptohexyl acetate in Sauvignon wine. This mercapto ester, recently found in passionfruit, exhibits aroma reminiscent of box tree with grapefruit and passion fruit notes. Its perception threshold in water and model solution is around 2-4 ng/L. 3-Mercaptohexyl acetate may contribute to the typical varietal aroma of Sauvignon blanc wines (Tominaga *et al.*, 1996).

***Biosynthesis of sulfur compounds by yeast*:** Several biological and chemical factors affect production of H_2S. As a product of the Sulfate Reduction Sequence (SRS), H_2S is an intermediate in the biosynthesis of all sulfur-containing compounds, including the amino acids, methionine and cysteine, and the methyl-group donor S-adenosylmethionine. Consequently its formation in wine is related to both sulfur and nitrogen metabolism by yeasts (Fig. 7). For detailed reviews see Rauhut (1993), and Henschke & Jiranek (1993b). In a series of regulated steps in the SRS, sulfate is accumulated from the medium and then reduced to sulfide via two ATP activation steps. At this point, sulfide is combined enzymatically with the nitrogen-containing carbon precursors, *O*-acetyl serine and *O*-acetyl homoserine, to ultimately form cysteine and methionine. The SRS is activated to produce sulfide whenever there is a metabolic demand for cysteine and methionine, since only limited amounts are present in grape juice. When a general deficiency of nitrogen coincides with a demand for protein synthesis, an intracellular depletion of nitrogen will ultimately result. A concomitant deficiency of the nitrogenous precursors of sulfur-amino acid biosynthesis, and in turn methionine, cysteine and their regulatory derivatives, will derepress the SRS and H_2S will be produced (Vos & Gray, 1979). Because sulfite freely diffuses into the cell, it essentially bypasses all regulatory steps normally controlling its production; therefore, a high and sustained rate of H_2S production is observed in response to a nitrogen deficiency when cells are grown in the presence of sulfite (Stratford & Rose, 1986).

Increased H_2S production can occur during fermentation at higher temperatures, at higher pH values, in musts that have a higher content of solids, etc. (Rauhut, 1993). A deficiency in a readily available nitrogen supply in the must is an important cause of H_2S formation by yeasts. Rauhut & Kürbel (1994a, b) showed that a high H_2S production caused by elemental sulfur residues was responsible for an increased concentration of other volatile S-compounds during and after fermentation by yeast metabolism. This leads to the formation of high amounts of thioacetic acid-S-methyl ester (MeSAc), thioacetic acid-S-ethyl ester (EtSAc) and other unknown S-substances. These thioacetic acid esters can slowly hydrolyze in wine to methyl and ethyl mercaptane, causing sulfur aroma defects, because in contrast to the thioacetic acid esters (>40 mg/L), the mercaptans have very low threshold values (>5 µg/L). Therefore, sulfur aroma defects can increase or reoccur during storage, since copper sulfate treatment removes only H_2S and mercaptans, while thioacetic acid esters still remain. The mercaptans can in the presence of oxygen be oxidised to disulfides, which are also odour-active compounds. Disulfides are likewise not removable by copper sulfate treatment (Rauhut *et al.*, 1996).

Another source of H_2S is the reduction of elemental sulfur, originating from applications of dusting elemental sulfur in the vineyard as a fungicide, by wine yeasts (Schütz & Kunkee, 1977). The chemical breakdown of S-containing insecticides can

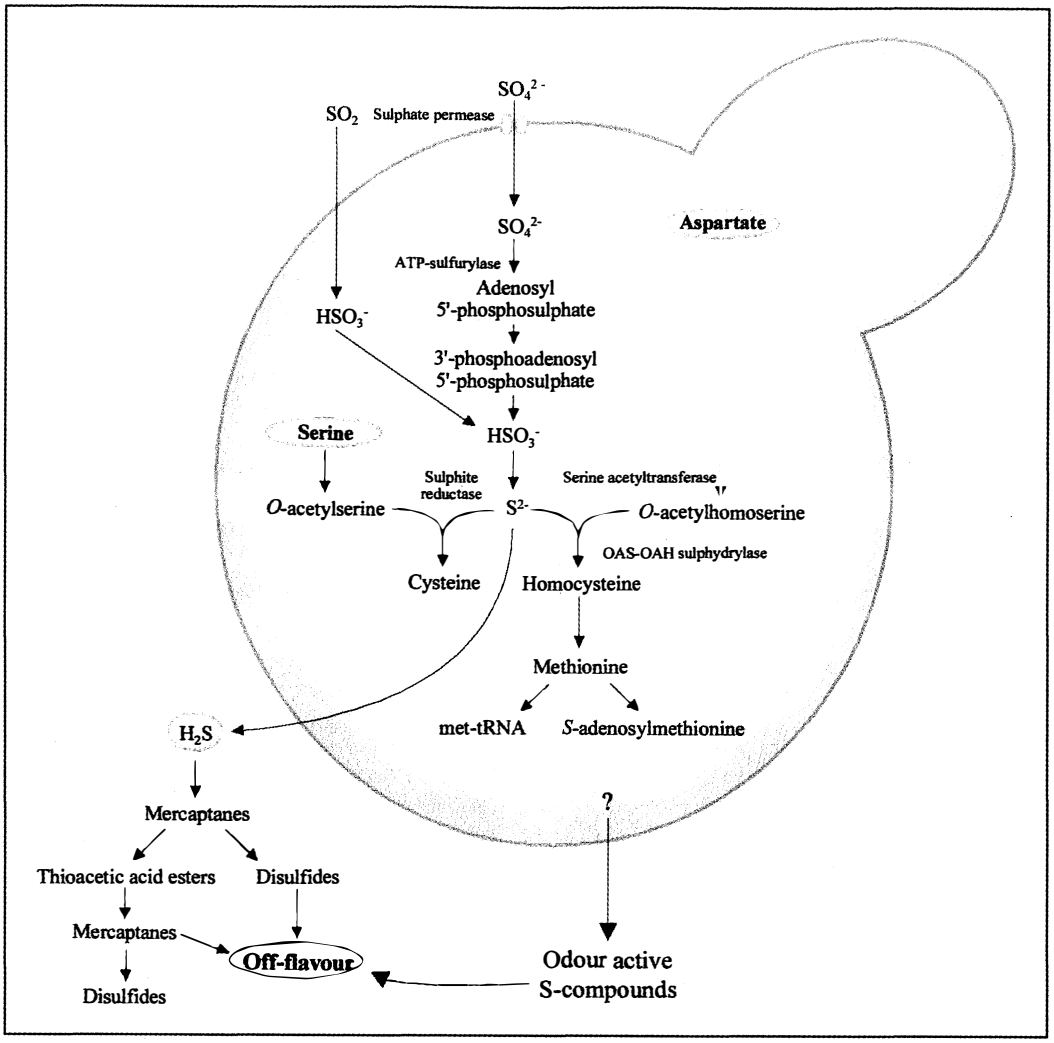

FIGURE 7

A schematic representation of the formation of odour-active S-compounds (Rauhut, 1993; Rauhut *et al.*, 1996).

also be the cause of odour-active S-substances like methylmercaptane (Rauhut *et al.*, 1986).

S-methylmethionine is the main precursor of DMS (Bamforth, 1980). It was also indicated that DMS might be synthesized by yeasts from cystine, cysteine or glutathione (Schreier *et al.*, 1976; De Mora *et al.*, 1986). De Mora *et al.* (1986) have shown that mercaptans are formed from methionine by yeast metabolism.

The volatile thiols 4-mercapto-4-methylpentan-2-one, 4-mercapto-4-methylpentan-2-ol and 3-mercaptohexan-1-ol, which are responsible for box tree, broom, passion fruit, guava and conifer aromas in Sauvignon blanc wines, were shown by Tominaga *et al.* (1998b) to be formed by yeast during the alcoholic fermentation due to the degradation of the corresponding S-cysteine conjugates present in the grapes.

***Yeast strain effect on sulfur aroma compound formation*:** The production of H_2S varies with the strain of *S. cerevisiae*, with some strains producing amounts exceeding 1 mg/L (Acree *et al.*, 1972; Eschenbruch *et al.*, 1978; Vos & Gray, 1979). Rauhut *et al.* (1996) tested the ability of several commercial wine yeasts to produce volatile S-compounds by comparing the sulfur aromagrams from fermentation trials with the same must. Their results clearly showed that *S. cerevisiae* strains differ in their capacity to synthesize S-compounds (Table 15). Some strains were responsible for sulfur off odours in wines correlating to high concentrations of S-compounds. Some strains showed a slight increase in the formation of S-compounds producing wines with no off-flavour, but masking the typical aroma of the variety.

Nearly all studies to date concern the biochemical activities of *S. cerevisiae*; virtually no attention has been given to the production of S-compounds by other species, some of which (e.g. *Kloeckera*, *Hanseniaspora* and *Candida* spp.) can make significant contributions to wine fermentations.

EFFECT OF INDIGENOUS AND INOCULATED YEAST ON WINE AROMA

As mentioned, several studies have shown that various starter cultures and indigenous yeasts produce wines with significant differences in chemical composition, with some of the compounds produced above and others below their sensory threshold

TABLE 15

Influence of the yeasts strain on the concentration of H_2S, methionol and the total amount of volatile S-substances (Rauhut *et al.*, 1996). in ester production by yeast strains (Delteil & Jarry, 1992).

S. cerevisiae strain no.	Intensity of H_2S formation during fermentation[1]	H_2S µg/L at the end of fermentation	Methionol AS/AIS*	Sum of S-substances[2]	Residual sugar g/L	Alcohol g/L
1	1	n.n.	1.3	5.3	12.5	78.9
2	3	17.0	5.4	18.0	2.2	82.1
3	3	14.0	6.7	16.6	2.3	82.9
4	2	5.0	7.1	22.2	2.5	82.4
5	1	4.0	3.6	10.3	27.5	71.9
6	1	14.0	4.4	12.6	2.4	82.9
7	1	4.0	5.0	13.9	6.4	81.0
8	3	12.0	7.0	18.8	1.9	84.1
9	4	8.0	11.0	31.3	4.9	81.9

[1]Intensity of H_2S-formation: weak: 1, middle: 2, strong: 3, very strong: 4
[2]Sum of relative peak area of the S-substances
*AS/AIS: area of the S-substance/area of the internal standard (relative peak area)

values. Although this should result in sensory differences in the resultant wines, little was done in these studies to measure them. Recently two studies were conducted that specifically investigated the effect of indigenous and inoculated yeasts on the sensory character of the resultant wines (Egli *et al.*, 1998; Henick-Kling *et al.*, 1998).

Henick-Kling *et al.* (1998) used Riesling musts, with and without sulfur dioxide added, which were fermented with and without the addition of yeast. There were no significant differences in growth of non-*Saccharomyces* yeasts in uninoculated musts with less than 50 mg/L SO_2 added. The starter culture was completely dominant over indigenous *S. cerevisiae* and strongly inhibitory to non-*Saccharomyces* yeasts. Sensory scores for overall quality indicated that the uninoculated wines were as acceptable as those fermented with a commercial starter culture. Uninoculated fermentations had higher sensory scores ($P > 0.95$) (typical fruity descriptors) for spicy, apple, melon, pear and H_2S, while inoculated wines had higher scores ($P > 0.95$) for paper, oxidized and sweaty, generally considered to be unfavourable. They concluded that the increased fruitiness in the uninoculated wines was due to the contributions by the non-*Saccharomyces* yeasts and by indigenous *Saccharomyces* yeasts. The K-1M starter culture used in the experiment did not promote fruity flavours. Sulfite treatment produced an assortment of significant sensory differences in the finished uninoculated wines, but in inoculated wines the additions of SO_2 to the must had no significant effect on indigenous yeast populations or on flavour.

In another study Egli *et al.* (1998) studied the dynamics of indigenous and inoculated yeast populations (vigorous and slowly fermenting yeast) and their effect on the sensory character of Riesling and Chardonnay wines. Both added starter cultures (strains EC1118 and Assmannshausen) clearly dominated the *Saccharomyces* population from the middle of fermentation until the end. The starter cultures differed in their repression of indigenous non-*Saccharomyces* yeast. Strain EC1118 limited the growth of non-*Saccharomyces* yeasts more strongly than strain Assmanshausen, since it is a more vigorous starter culture. The growth of non-*Saccharomyces* yeasts was further repressed by the addition of sulfite. At the end of the alcoholic fermentation more than one *Saccharomyces* strain was present in each fermentation, with the largest variety in the non-inoculated and the smallest in the EC1118-inoculated fermentation.

Riesling and Chardonnay wines from the three different fermentation types were compared by a trained taste panel. Interestingly, a very similar set of descriptors was chosen in describing the wines produced from the same grape variety (Table 16). Comparison of the scores for the wines revealed significant differences for some of the descriptors used for both the Riesling and Chardonnay wines (Table 16). Furthermore, the flavours that varied depending on the fermentation type did not overlap at all for the Riesling and Chardonnay wines. The wines from non-inoculated fermentations had the majority of high scores for these descriptors, while Riesling wines fermented with strain EC1118 had the lowest scores for these descriptors (Fig. 8). Assmannshausen-inoculated wines were generally between these extremes, but showed high scores for overall fruitiness and acetic, and lowest scores for diacetyl and paper. For both the Riesling and Chardonnay wines, the highest scores for most flavour attributes were given to wines from non-inoculated fermentations. The uninoculated Chardonnay wines were characterized by floral and pear aromas and by strong vegetative, reduced and sweaty aromas. Wines fermented with Assmannshausen tended to have more fruity flavours (floral and pear), and EC1118-inoculated wines were less fruity and had stronger oxidized, astringent and some sweaty and herbaceous flavours. Importantly, these yeast-related differences did not overwhelm the varietal character.

Statistical analysis revealed that the descriptors which were significantly different among Riesling wines were not the same as those which described significant differences in Chardonnay wines (Table 16). This suggests that the different yeasts affected

TABLE 16

Aroma descriptors selected through Free-Choice Profiling, for Riesling and Chardonnay wines, affected by yeast (Egli *et al.*, 1998).

Riesling	**Chardonnay**
Acetic	
Apple	Apple
Astringent/phenolic	**Astringent/phenolic**
Body	
	Caramelized
Citrus	Citrus
Diacetyl/caramelized	
Earthy	Earthy
Flinty	
Floral	**Floral**
Overall fruitiness	
	Herbaceous/vegetative
H_2S	**Reduced sulphur/H_2S**
Melon	Melon
	Mineral/flinty
Oxidized	**Oxidized**
Paper/cardboard	Paper/cardboard
Pear	**Pear**
Pineapple/tropical	
SO_2	SO_2
Spicy	Spicy
Sweaty	**Sweaty**
Yeasty	Yeasty

Descriptors which had significant differences between inoculation treatments (spontaneous, vigorous yeast starter EC1118 and slow fermenting starter Assmannshausen) ($P \geq 0.05$) are in bold.

the flavour compounds variety-specifically. Nevertheless, yeast strain characteristics were also observed (Fig. 8). Assmannshausen-inoculated wines tended to be strongly fruity and spicy in the Riesling, and floral and pear-like in the Chardonnay. Strain EC1118 produced the least fruity wines in this comparison, tending toward aldehyde/phenolic, paper, oxidized and astringent flavours. In non-inoculated wines, most flavour attributes, positive and negative, tended to be highest.

Both Henick-Kling *et al.* (1998) and Egli *et al.* (1998) clearly showed that different vinification parameters such as grape must, sulfite addition, fermentation temperature, starter cultures, etc. that affect the fermentation microflora will have an impact on the sensory characteristics of the resulting wine. The effect of single, co- and sequential *Candida stellata* and *S. cerevisiae* cultures on the aroma of Chardonnay wines has also been illustrated by Soden *et al.* (2000).

GENETIC IMPROVEMENT OF WINE YEASTS

The knowledge of the physiology of genetic reference strains of *S. cerevisiae* becoming available is a great help in directing the modification of industrial yeasts toward practical goals, even though the industrial strains and species are less characterized and their genetic make-up more complex (Hansen & Kielland-Brandt, 1996). Though some work has been done on the genetic make-up of other strains, most detailed studies on industrial strains have been made on lager brewing yeast. Examples include brewers' yeasts producing reduced amounts of diacetyl by over-expression of the *ILV5* gene (Gjermansen *et al.*, 1988; Goossens *et al.*, 1993), or increased amounts of phenethyl alcohol due to a mutated *ARO4* gene on a centromere plasmid (Fukuda *et al.*, 1992).

Wine yeasts have been engineered to degrade malic acid (Ansanay *et al.*, 1996) or to produce lactic acid (Dequin *et al.*, 1999). It has also also been shown that in laboratory *S. cerevisiae* strains, a specific mutation in the *ERG20* gene resulted in geraniol overproduction (Chambon *et al.*, 1990).

In a recent study, Lilly *et al.* (2000) manipulated the expression levels of the *ATF1* gene for its effect on the production of esters, important for the characteristic fruity odours of wine, brandy and

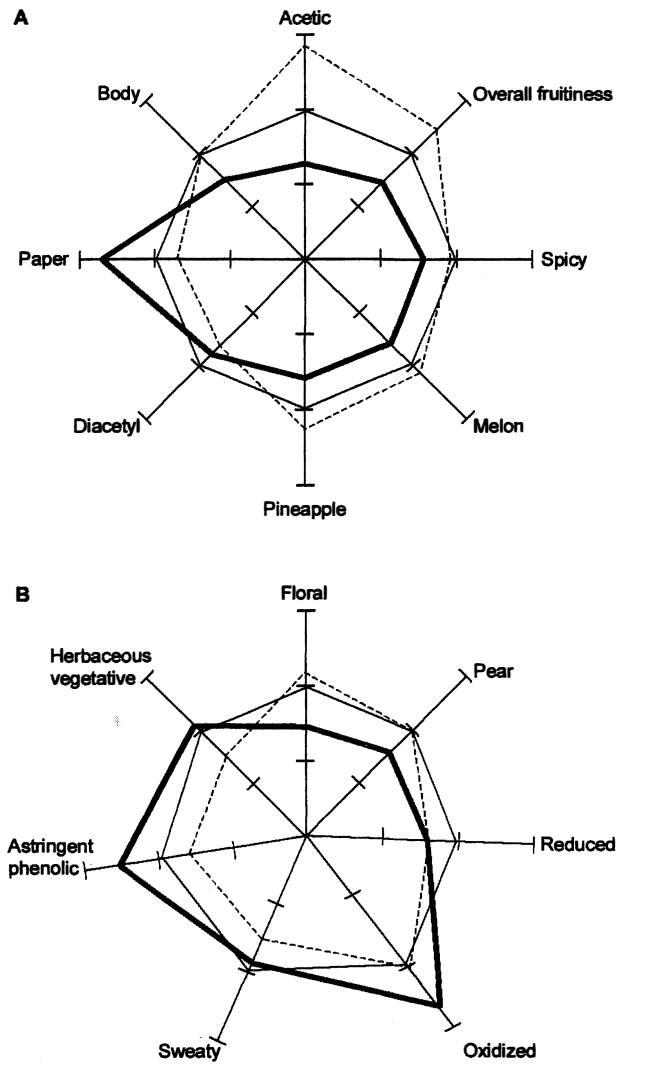

FIGURE 8

Sensory differences in Riesling (a) and Chardonnay (b) wines based on the fermentation type. Comparisons were made by a panel of 11 experienced tasters and data are described using polar graphs following statistical evaluation, (–), strain EC1118; (--), strain Assmannshausen; (-); non-inoculated (Egli *et al.*, 1998).

other alcoholic beverages. The *ATF1*-encoded alcohol acetyl transferase activity is the best studied acetyl transferase activity in *S. cerevisiae*. The *ATF1* gene, located on chromosome XV, was cloned from a widely used commercial wine yeast strain of *S. cerevisiae*, VIN13, placed under the control of the constitutive yeast phosphoglycerate kinase gene (*PGK1*) promoter and terminator, and integrated into the chromosomes of three commercial wine yeast strains. The sensorial evaluation of Chenin blanc wines and distillates from Colombar base wines, fermented with these manipulated wine yeasts, showed that overexpression of a single gene such as *ATF1* could alter ester production significantly, thereby adjusting the aroma profiles of wines and distillates considerably. The significant increases in the levels of ethyl acetate, isoamyl acetate and 2-phenylethyl acetate had pronounced effects on the solvent/chemical and the herbaceous and heads-associated aromas of the final distillate, and the solvent/chemical and fruity or flowery characters of the Chenin blanc wines. This study established the concept that the overexpression of acetyltransferase genes such as *ATF1* could profoundly affect the flavour profiles of wines and distillates deficient in aroma, thereby paving the way for the production of products maintaining a fruitier character for longer periods after bottling.

Considerable progress has been made in developing technology to construct new wine yeast strains over the past few years, although research in wine science has been slow to exploit the vast potential benefit of recombinant DNA technology to the wine consumer and industry alike. A comprehensive review has recently been published by Pretorius (2000).

CONCLUDING REMARKS

Starter cultures have been developed to ferment rapidly and consistently, producing wines with predictable and desirable characteristics. Nevertheless, indigenous non-*Saccharomyces* yeasts may have a significant and favourable effect on flavour development, and they merit consideration as useful tools in managing wine styles. The synergistic interactions among different yeast strains and their effect on wine sensory properties remain to be fully investigated: different *S. cerevisiae* starter cultures might be selected to enhance fruity flavours in wine; yeast combinations of different *S. cerevisiae* strains, and possibly *S. cerevisiae* with selected non-*Saccharomyces* strains, might be used to enhance profiles to produce flavour-unique wines. Since judicious use of sulfite can be used to limit the growth of wild yeasts, mixed or sequential inoculations may ultimately be designed to consistently produce wines with the desired flavours.

Future research might also identify more examples, as in the case of 4-mercapto-4-methylpentan-2-one in Sauvignon blanc aroma, where yeasts contribute to varietal aroma. Recently, King & Dickinson (2000) have shown that *S. cerevisiae* can reduce the monoterpenoid geraniol into *R*-(+)-citronellol, and also isomerizes geraniol into linalool. The yeasts *T. delbreuckii* and *K. lactis* were also shown to convert a variety of monoterpenoids. Although not yet tested in must and wines, such activities should have an effect on wine aroma.

Only when we have a much better understanding of yeast biodiversity, biogeography, ecology and the interaction within yeast communities and their metabolism will we be able to use them optimally in single, mixed or sequential starter cultures and to improve them with gene technology. To achieve this, a comprehensive, long-term biological survey programme has been launched by the Wine and Fermentation Technology Division at the ARC Infruitec-Nietvoorbij, and the Institute for Wine Biotechnology at the University of Stellenbosch (Pretorius *et al.*, 1999; Khan *et al.*, 2000; Van der Westhuizen *et al.*, 2000a, b). This kind of biological survey complements the current international effort to assign a biological function to the products of each of the 6000 genes indentified by computer analysis of the nucleotide sequence of the 16 chromosomes of a laboratory strain of *S. cerevisiae* (Oliver, 1996; Mewes *et al.*, 1997). Some of the new genes will certainly be appropriate targets for modification of metabolic pathways in *S. cerevisiae*, and, due to the close relationship, industrial *Saccharomyces* yeast. Furthermore, a research programme to understand the role of esters and other volatile

compounds and to clone genes from *S. cerevisiae* involved in wine aroma has also been initiated at the Institute for Wine Biotechnology (Lilly *et al.*, 2000). With the important contribution of non-*Saccharomyces* yeasts now fully realised, future research programmes should focus on this group, containing a wide variety of yeasts that have been shown to produce a diverse array of extracellular enzymes compared to *S. cerevisiae* (Charoenchai *et al.*, 1997; Strauss *et al.*, 2000).

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