Succinic acid production by wine yeasts

by

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DECLARATION

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SUMMARY

One of the most striking qualities of wine is its tart, sour taste. The sensory perception of sourness is mainly attributed to the presence of hydrogen ions (protons) at high concentrations. Large amounts of weak carboxylic acids (organic acids) are the main sources of these ions within wine. Once wine enters a person's mouth, the dissociable protons of the weak organic acids within wine are partially neutralized or, in other words, titrated by the saliva secreted inside one's mouth. This explains why the duration and intensity of a wine's sourness is related to its titratable acidity content. The sour taste of wine is usually considered refreshing and it helps balance wine flavour. In fact, wines become watery when its titratable acidity content is too low.

After alcoholic fermentation, the titratable acidity of wine will usually be less than that of the grape juice from which was made due to ethanol-induced precipitation of potassium bitartrate crystals and partial consumption of malic acid by fermenting wine yeasts. Occasionally however, increases in titratable acidity are observed during alcoholic fermentation. If wine is produced from grape juice with optimum levels of titratable acidity, unforeseen increases in titratable acidity during alcoholic fermentation can be detrimental to the quality of the final product.

Although the net production of malic acid by wine yeasts contributes to increases in titratable acidity seen during grape juice fermentations, the production of succinic acid is regarded as the primary contributor. In fact, succinic acid accounts for approximately 90% of the non-volatile acids produced during fermentation of grape juice. Between 0.5 and 1.5 g/L succinic acid is normally found in wine, but higher concentrations thereof (up to 3.0 g/L) have been detected within certain red wines.

Acidity adjustments should preferably be carried out before the onset of alcoholic fermentation to allow better integration of the added compound(s) and to ensure that conditions during fermentation favour the quality and microbial stability of the final product. In doing so unfortunately, winemakers run the risk of ending up with wines that may taste too sour if they are unable to accurately predict and take into consideration the amount of succinic acid produced during alcoholic fermentation. Knowledge with regard to the factors involved in succinic acid's production by fermenting wine yeasts is therefore required in order to manage the titratable acidity of wines more accurately.

Ever since Louis Pasteur first noticed succinic acid amongst the by-products of alcoholic fermentation, attempts have been made to determine the metabolic pathways and factors involved in its production by fermenting wine yeasts. Up until now however, it remains unclear why wines sometimes end up with exceptionally high levels of succinic acid.

For these reasons it was decided to investigate the possible causes of very high succinic acid concentrations within wine. Due to complexity of grape juice's chemical composition and the problems associated with sterilizing grape juice, fermentation experiments were conducted within a chemically defined grape juice-like medium.

Succinic acid production by nine different industrial wine yeast strains was studied under various conditions with regard to the nutrient status of the synthetic grape juice, temperature and availability of molecular oxygen during alcoholic fermentation.

The amount of succinic acid produced during alcoholic fermentation was found to depend on the yeast strain, fermentation temperature and chemical composition of the synthetic grape juice. Out of the nine commercial yeast strains selected for this study, strain WE372 produced the largest amount of succinic acid in synthetic grape juice at 28°C. Strain WE372 produced significantly smaller amounts of acetic acid than the other yeast strains of this study and very little acetic acid at 28°C, which indicated that strain WE372 may have less acetaldehyde dehydroganase activity than the other yeast strains of this study under the conditions tested. The effect this has on NAD: NADH balance is the probable cause for its ability to form more glycerol, succinic and malic acid than the other strains.

Results from our study show that succinic acid production is influenced primarily by the metabolizable fraction of YAN, which we termed metabolically available nitrogen (MAN). Succinic acid production by fermenting yeasts will be favoured by moderate to high fermentation temperatures (20°C to 28°C) in grape juice with a nicotinic acid and/ or nicotinamide deficiency, high sugar content (200 g/L to 240 g/L), moderate amounts of metabolically available nitrogen (300 ± 50 mg/L MAN), the presence of flavonoids and large supplies of unsaturated long-chain fatty acids. Even higher concentrations of succinic acid were produced when oxygen was made available to fermenting yeasts by aerating the fermenting grape juice. Fermentation temperatures below 18°C, too much metabolizable nitrogen (> 450 mg/L MAN), very high concentrations of fermentable sugar (> 240 g/L), lipid deficiencies and a lack of pantothenic acid, thiamine, biotin or pyridoxine will decrease the amount of succinic acid produced fermenting yeasts.

PREFACE

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the journal Microbiology to which Chapter 3 is to be submitted for publication.

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Chapter 1

INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 GENERAL INTRODUCTION

Acidity is the spine of wine. It supports the other flavours of wine and gives it a refreshing taste. A lack of acidity will cause wine to become watery, which can be described as dull, flat or insipid (Fischer, 2001). In fact, wine's acidity can be regarded as the single most important factor that influences the quality and stability of wines. Acidity influences chemical and enzymatic oxidation of grape juice and wine constituents, the metabolism of micro-organisms, the solubility of proteins, polysaccharides and potassium bitartrate, wine colour and the effectiveness of sulphur dioxide, fining agents and pectin degrading enzymes (Boulton *et al*., 1998a).

Different measures of acidity are used to describe the acidity of grape juice and wine. These measurements are all related, but the information that they convey to the oenologist or winemaker differs (Boulton, 1980; Boulton *et al*., 1998a). The two most important measures of acidity to the wine industry are pH and titratable acidity. The pH is a measure of the hydrogen ion concentration or more correctly, the hydrogen ion activity. Often referred to as real acidity, pH affects all aspects of winemaking as discussed in the previous paragraph. Titratable acidity on the other hand, refers to the concentration of hydrogen ions that are recovered from acids when an acidic solution is titrated with a strong base to a specified endpoint. A pH of 8.2 is normally chosen as the titration endpoint, which corresponds with the pH at which phenolphthalein's colour change occurs (Boulton *et al*., 1998a). In South Africa, titratable acidity of grape juice or wine is expressed as the equivalent concentration tartaric acid (g/L). The term total acidity is sometimes erroneously used to describe titratable acidity (Ribéreau-Gayon *et al*., 2000a). Total acidity is the hydrogen ion equivalent of all the organic acid anions within a solution. The titratable acidity of grape juice will always be less than its total acidity, because some of the hydrogen ions from organic acids in grapes are exchanged for metal cations during ripening (Boulton, 1980; Boulton *et al*., 1998a). In the case of wine or juice from mould-infected grapes, it is often necessary to differentiate between the two components of total acidity, *i.e*. volatile acidity and fixed acidity. Acetic acid is the principle component of wine's volatile acidity, but wine also contains other important volatile acids, such as sulphurous acid (H_2SO_3) and carbonic acid (H_2CO_3) (Ribéreau-Gayon *et al*., 2000a).

High concentrations of weak carboxylic acids (organic acids) are the primary cause of wine's tart, acidic taste and low pH. The main organic acids in grapes are L-tartaric acid and L-malic acid (Boulton *et al*., 1998a). Together, these two organic acids represent up to 90% of grape juice's total acid content (Kliewer, 1966; Ruffner, 1982; Jackson, 1994). The third most important organic acid in grapes is L-citric acid. Mature grapes contain 1-7 g/L L-tartaric acid, 1-4 g/L L-malic acid and 150-300 mg/L L-citric acid (Margalit, 1997). It is also possible to find small amounts of

oxalic acid (< 100 mg/L) within juice from healthy grapes if harvesting occurs before the majority of grapes are ripe (Amerine & Cruess, 1960a; Bombardelli & Morazzoni, 1995). Various other acidic compounds can contribute to the acidity of healthy grapes. Examples thereof include ascorbic acid, phenolic acids, amino acids and inorganic acids (Boulton *et al*., 1998a; Ribéreau-Gayon *et al.*, 2000a).

Grapes infected by *Botrytis cinerea* can contain considerable amounts of sugar acids. Gluconic-, galacturonic and galactaric acid (mucic acid) are the most important sugar acids in grape juice from mould-infected grapes (Radler, 1975, 1986; Sponholz *et al*., 1987; Ribéreau-Gayon *et al*., 2000a). *Botrytis*-growth lowers the malic- and tartaric acid contents of grapes (Shimazu *et al*., 1984; Sponholz *et al*., 1987), and creates suitable growth conditions for yeasts and other pathogenic microorganisms. As a result, grape juice from mouldy grapes often contains abnormally high levels of formic-, acetic, lactic and succinic acid (Sponholz *et al*., 1987).

Wine's organic acid composition differs noticeably from that of grapes. Various physico-chemical and biochemical reactions are responsible this. One of the most noticeable changes to the original organic acid content of grape juice is the appearance of high succinic acid concentrations in wine. Considerable amounts of succinic acid can be found in unripe *V. rotundifolia* (muscadine) grapes (Lamikanra *et al*., 1995) and grapes infected by moulds, but grape juice from healthy *V. vinifera* grapes contains only traces of succinic acid (Sponholtz & Dittrich, 1977; Sponholtz *et al*., 1987). *B. cinerea* and other moulds produce succinic acid from the sugars in grapes (Rankine, 1986). The maximum amount of succinic acid that was found in botrytisized grape juice was 783 mg/L (Sponholz *et al*., 1987). Considerable amounts of succinic acid can be formed from malic acid by the fumarases and fumarate reductases of grapes when intact grapes are subjected to the anaerobic conditions employed during carbonic maceration (Flanzy *et al*., 1976; Romieu *et al*., 1988). Carbonic maceration is a method of extracting the pigments and tannins from the skins of intact red grapes by keeping them under an atmosphere of carbon dioxide or nitrogen gas (Boulton *et al*, 1998b; Ribéreau-Gayon *et al*., 2000b). Malic acid can also be converted into succinic acid by some heterofermentative *Lactobacillus* and *Oenococcus oeni* (ex *Leuconostoc oenos*) strains (Carr & Whiting, 1956; Herrero *et al*., 1999). These lactic acid bacteria can convert more than half of the original malic acid content into succinic acid at pH values greater than or equal to 4.4. However, only traces of succinic acid are produced by lactic acid bacteria at pH values below 3.6 (Carr & Whiting, 1956). Malo-succinic fermentation by lactic acid bacteria is favoured by temperatures below 15°C or temperatures higher than 27°C (Herrero *et al*., 1999). Succinic acid can also be formed from tartaric acid by a few strains of *Lactobacillus brevis*, *L. bucheri* and *L. pastorianus* (Krumperman & Vaughn, 1966; Radler & Yannissis, 1972), but these strains are very sensitive towards sulphur dioxide and find it hard to grow at the low pH of grape juice and wine (Ribéreau-Gayon *et al*., 2000c). In other words, most succinic acid in wine

is usually derived from the fermentative metabolism of yeasts when the juice is obtained from fully matured and healthy grapes. In fact, yeasts are capable of producing very high concentrations of succinic acid (> 1.0 g/L) during alcoholic fermentation (Heerde & Radler, 1978; Lamikanra, 1997; Margalit, 1997; Boulton *et al*., 1998a).

1.2 RATIONALE

Even though, succinic acid is quantitatively the second most important non-volatile by-product of alcoholic fermentation by yeasts (Oura, 1977), its importance in wine is often overlooked due to its weak acidity. The pK_a values for succinic acid's two dissociable protons are 4.21 and 5.64 when it is dissolved in water (Usseglio-Tomasset & Bosia, 1978; Margalit, 1997; Boulton *et al*., 1998a). Succinic acid is an even weaker acid in wine due to its high ethanol content, which decreases the dielectric character of aqueous solutions (Usseglio-Tomasset & Bosia, 1978; Boulton *et al*., 1998a). In addition, wines have high buffering capacities, which usually vary between 35 and 50 mM/ L/ pH (Boulton *et al*., 1998a). This implies that even large quantities of succinic acid will have little effect on wine's pH.

Like any other organic acid though, succinic acid will increase the buffering capacity of an aqueous solution at pH values within two units from its pK_a values (Christian, 1994; Dartiguenave *et al*., 2000). The buffering capacity of wine is defined as its ability to resist changes in pH, when it is diluted with water or titrated with small amounts of alkali (base) or acid (Christian, 1994; Boulton *et al*., 1998a). In other words, wine's titratable acidity is directly related to its buffering capacity. Wine's titratable acidity will be increased by 1.3 times the concentration of succinic acid in solution when it is expressed as gram equivalents tartaric acid per litre. The sensory perception of sourness is mainly attributed to hydrogen ions and their concentration (Paul, 1917; Amerine *et al*., 1959; Clarke & Bakker, 2004). When wine is tasted, its acids are partially neutralized (titrated) by saliva inside ones mouth (Boulton *et al*., 1998a), which explains why the duration and intensity of wine's sourness is related to its buffering capacity (Usseglio-Tomasset, 1993; Dartiguenave *et al*., 2000) or, in other words, related to its titratable acidity (Amerine *et al*. 1959, 1965). Thus, succinic acid increases the sour taste of wine by increasing its buffering capacity (titratable acidity).

Succinic acid tastes salty and bitter in addition to being sour (Amerine & Cruess, 1960b; Coulter *et al*., 2004). Tasters at the AWRI found succinic acid's taste unpleasant compared to tartaric acid's taste and indicated that succinic acid's unusual taste lingered for some time after expectoration (Coulter *et al*., 2004). The taste threshold of succinic acid dissolved in water ranges from 34-35 mg/L (Berg *et al*., 1955; Amerine *et al*, 1959), but wines with very high levels of succinic acid were not identifiably salty or bitter according to the results of informal tasting held at the AWRI (Coulter *et al*., 2004).

Succinic acid is also involved in the formation of aroma-active esters. Significant amounts of ethyl succinate have been detected in the aroma extracts of different types of sherry wines (Webb *et al*., 1964). Ethyl succinate's aroma is pleasant, but mild (Webb *et al*., 1964). Diethyl succinate are present at high concentrations in aged wines, wines with high alcohol contents, sherry wines and wines produced by *Saccharomyces bayanus* var. *uvarum* yeasts (Webb *et al*., 1962, 1964; Antonelli *et al*., 1999; Francioli *et al*., 2003; Alves *et al.*, 2005). Diethyl succinate's aroma is also mild, but fruity and reminiscent of watermelon (Jordan *et al*., 2002). Large quantities of methyl succinate contribute to the characteristic aroma of muscadine (*Vitis rotundifolia*) wines (Lamikanra, 1987; Lamikanra *et al.*, 1996). Wines will contain higher levels of succinate esters if glycosidic enzyme preparations are used to enhance the varietal character of the grapes in wine, because these products often contain esterase activity (Tambora *et al*., 2004).

Sometimes it is necessary to adjust the acidity of grape juice or wine to levels that will be more beneficial to the overall quality and stability of wine. It is preferable to carry out such adjustments as early on as possible during the winemaking process to ensure better integration of any added compound(s) and to provide early on control over all the factors that influence wine's quality and stability. However, wines produced from grape juice with optimal levels of acidity, sometimes end up with undesirable high levels of titratable acidity. Succinic acid production by fermenting yeasts is seen as the primary cause for increases in titratable acidity during wine production (Thoukis *et al.* 1965; Ough *et al*., 1969; Lamikanra, 1997; Coulter *et al*., 2004).

Ever since Pasteur (1860) first noticed succinic acid amongst the fermentation by-products of yeasts, attempts have been made to determine the metabolic pathways and factors involved in succinic acid's formation by fermenting yeasts (Genevois, 1936; Kleinzeller, 1941; Ribéreau-Gayon *et al.*, 1956; Stoppani *et al*., 1958; Mayer *et al*., 1964; Thoukis *et al.*, 1965; Heerde & Radler, 1978; Wakai *et al*., 1980; Muratsubaki, 1987; Arikawa *et al*., 1999; Camarasa *et al*., 2003). Up until now however, it remains unclear why wines sometimes end up with exceptionally high levels of succinic acid (Coulter *et al*., 2004). It is important to determine the factors involved in succinic acid's production during grape juice fermentations so that one would know which factors to manipulate in order to control the levels of succinic acid in wine. Knowledge in this regard will also help us to predict how much succinic acid will be formed during grape juice fermentations so that the predicted values can be taken into account when adjustments to the acidity of grape juice are made.

1.3 RESEARCH OBJECTIVES

This study forms part of a larger research project, which is aimed at predicting changes in acidity during the various stages of wine production. A computer software program (ACID®) was specifically written for this purpose and was subsequently tested at Stellenbosch University's Institute for Wine Biotechnology (SU-IWBT).

Assessment of the software program's accuracy revealed that it occasionally did not live up to expectations. It was especially difficult to predict the titratable acidity of certain red wines accurately with this program. The possible causes for the observed discrepancies between the actual and predicted values were investigated and it was found that the ACID® program was unable to predict the acidity of wines accurately when high levels of succinic acid were produced during alcoholic fermentation. Therefore, it was decided to investigate the possible causes of high succinic acid concentrations in wines. The specific aims of the current study were the following:

I. to determine the succinic acid producing capabilities of commercial wine yeast strains, which are used for the production of *méthode champenoise* sparkling wine, white or red table wine.
II. to investigate possible relationships between the production of succinic acid and the production of other fermentation by-products of yeasts.
III. to investigate temperature's influence on succinic acid production by fermenting yeasts.
IV. to investigate the relationship between succinic acid production and the yeast assimilable nitrogen (YAN) content of grape juice.
V. to investigate what influence a grape juice's vitamin B_1 (thiamine) content will have on succinic acid production by yeasts.
VI. to investigate what influence a grape juice's vitamin B_3 (niacin) content will have on succinic acid production by yeasts.
VII. to investigate whether sources of unsaturated long-chain fatty acids in grape juice influence succinic acid production by yeasts.
VIII. to determine whether the presence flavonoids in grape juice influences succinic acid production by yeasts.

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Chapter 2

LITERATURE REVIEW

Succinic Acid Production by Wine Yeasts

2. LITERATURE REVIEW

SUCCINIC ACID PRODUCTION BY WINE YEASTS

Succinic acid, which is also known as 1,4-butanedioic acid, accounts for approximately 90% of the non-volatile acids produced by wine yeasts during alcoholic fermentation (Conway & Brady, 1950; Thoukis *et al*., 1965). Next to tartaric and malic acid, it is the third most important organic acid in wines that have not undergone malolactic fermentation. The succinic acid content of wine usually varies between 0.5 and 1.5 g/L (Margalit, 1997; Rankine, 1998a; Boulton *et al*., 1998a). Other fermented beverages such as beer, cider and rice wine also contain considerable amounts of succinic acid (Coote & Kirsop, 1974; Whiting, 1976; Rhee *et al*., 2003). The highest concentration of succinic acid ever reported for wine amounted to 3.0 g/L and was found in an Australian red wine (Coulter *et al*., 2004). In fact, Australian red wines contain about twice as much succinic acid than Australian white wines (Coulter *et al*., 2004).

2.1 PRODUCTION PERIOD

Most of the succinic acid that is excreted by fermenting yeasts is formed during the early stages of alcoholic fermentation (Thoukis *et al.*, 1965; Heerde & Radler, 1978; Arikawa *et al*. 1999a), before the formation of about 4-5% (v/v) alcohol (Thoukis *et al*., 1965). Initially it was thought that succinic acid is only produced during the exponential growth phase of fermenting yeasts (Heerde & Radler, 1978), but more recently it was shown that fermenting yeasts also produce succinic acid during the stationary phase of alcoholic fermentation (Lamikanra, 1997; Arikawa *et al*., 1999a).

2.2 METABOLIC PATHWAYS INVOLVED IN SUCCINATE PRODUCTION

Succinate is an intermediate of at least four metabolic pathways within yeasts. They are the tricarboxylic acid (TCA) cycle (Krebs- or citric acid cycle), the γ-amino butyric acid (GABA) bypass (shunt), glyoxylic acid bypass and the methylcitric acid (MCA) cycle. The TCA cycle's involvement in succinic acid production by yeasts will be dealt with first, since the other pathways can be seen as modifications thereof.

2.2.1 THE TCA CYCLE

The TCA cycle is usually operational within the mitochondria of eukaryotic cells (Horton *et al*., 1996). Cytoplasmic isoforms of all the TCA cycle enzymes, except α-ketoglutarate dehydrogenase, succinyl-CoA synthetase and succinate dehydrogenase, exists within yeasts (Atzpodien *et al*.1968; Wu & Tzagoloff, 1987; Bisson, 1993; Loftus *et al*., 1994; Boulton *et al*., 1998b). However, the cytosolic isoform of $NADP^+$-dependant isocitrate dehydrogenase (Idp2p) is inactive within yeasts during growth on

fermentable sugars (Loftus *et al*., 1994). In other words, only the mitochondrial $NADP^+$- and NAD^+-specific isoforms of isocitrate dehydrogenases are present within wine yeasts during fermentation (Keys & McAlister-Henn, 1990; Haselbeck & McAlister-Henn, 1991).

Although mitochondria are present within respiring wine yeasts (Vitols *et al*., 1961; Linnane *et al*., 1962; Wallace & Linnane, 1964), these organelles are absent within non-respiring ones (Linnane *et al*., 1962; Polakis *et al*., 1964; Wallace & Linnane, 1964). However, anaerobically-grown yeasts contain mitochondria-like structures called promitochondria when they are grown in the presence of $\Delta^{5,7}$-unsaturated sterols (*e.g.* ergosterol) and/ or unsaturated long-chain fatty acids (Linnane *et al*., 1962; Polakis *et al*., 1964; Morpurgo *et al*., 1964; Wallace *et al*., 1968; Damsky *et al*., 1969; Plattner & Schatz, 1969). Yeast mitochondria are also converted into promitochondria under respiro-fermentative conditions, *i.e* fermentation under aerobic conditions (Polakis *et al*., 1964; Jayaraman *et al*., 1966; Wallace *et al*., 1968). Normally, the α-ketoglutarate dehydrogenase and succinate dehydrogenase complexes are imbedded within the inner membranes of eukaryotic cells' mitochondria (Horton *et al*., 1996). The double membrane structure of mitochondria is well preserved within promitochondria (Plattner & Schatz, 1969). Hence, it can be said that the α-ketoglutarate dehydrogenase and succinate dehydrogenase complexes are imbedded within the inner membranes of fermenting yeasts' promitochondria.

Reactions of the TCA cycle (**Figure 2.1**) produce reduced coenzymes, which can be used by the mitochondrial electron-transport chain to generate a proton gradient across a mitochondrion's inner membrane. The ATP synthases (F_0F_1-ATPases), imbedded within the inner membranes of mitochondria, utilize the energy that is stored within this proton gradient (proton motive force) to drive the phosphorylation of adenosine diphosphate (ADP). In other words, chemo-osmotic potential energy is converted into chemical-potential energy in the form of adenosine triphosphate (ATP). This process is known as oxidative phosphorylation (Horton *et al*., 1996). Even under aerobic conditions, fermenting yeasts will be very limited in their ability to produce ATP by means of oxidative phosphorylation (respire), because promitochondria lack an integrated electron transfer chain and functional oxidative phosphorylation system (Criddle & Schatz, 1969; Cartledge & Lloyd, 1972; Lowdon *et al*., 1972; Salmon *et al*., 1998). Despite this, reactions of the TCA cycle are still needed within fermenting yeasts for biosynthetic purposes.

The cyclic functioning of the TCA metabolic pathway is interrupted within fermenting wine yeasts (Machado *et al*., 1975; Arikawa *et al*., 1999b; Gombert *et al*., 2001; Maaheimo *et al*., 2001; Camarasa *et al*., 2003). Citrate synthases, aconitases, isocitrate dehydrogenases, fumarases and malate dehydrogenases are always present within fermenting wine yeasts (Polakis *et al*., 1964; Beck & von Meyenburg, 1968; Chapman & Bartley, 1968; Machado *et al*., 1975; Wales *et al*., 1980). However, the specific activities of these TCA cycle enzymes are decreased by 60-89% within wine yeasts under respiro-fermentative conditions (Wales *et al*., 1980) and even more so under anaerobic conditions (Chapman & Bartley, 1968; Wales *et al*., 1980). The α-ketoglutarate dehydrogenase and succinyl-CoA synthetase complexes are present at

extremely low levels or not detected at all within fermenting yeasts when they are grown under anaerobic conditions and/ or in media without glutamate, glutamine or threonine as the principle sources of yeast assimilable nitrogen (Machado *et al*., 1975; Schwartz *et al*., 1983; Wales *et al*., 1980; Camarasa *et al*., 2003). Although fermenting yeasts contain slightly higher levels of α-ketoglutarate dehydrogenase activity when they are grown under aerobic conditions (Wales *et al*., 1980; Arikawa *et al*., 1999a, 1999b), the TCA cycle is interrupted to some degree at the point in the pathway where α-ketoglutarate is supposed to be converted into succinyl-CoA. This explains why wines can contain up to 346 mg/L α-ketoglutaric acid (Whiting, 1976). In fact, wine yeasts excrete only 3% more α-ketoglutarate during fermentation of synthetic grape juice after the complete removal of α-ketoglutarate dehydrogenase activity (Camarasa *et al*., 2003) by means of *KGD1* deletion (Arikawa *et al*., 1999a, 1999b). Further evidence in support of the above is given by the fact that the succinate derived from [3-^{13}C]-aspartate was not labelled at its first and fourth carbon positions after fermentation, which indicated that very little or no succinate was formed from oxaloacetate via the formation of succinyl-CoA from α-ketoglutarate (Camarasa *et al*., 2003). The TCA cycle within fermenting yeasts is also interrupted at the point in the pathway where succinate is supposed to be oxidized to fumarate, as is evident from carbon-13 nuclear magnetic resonance (^{13}C-NMR) studies with labelled glutamate, in which shown that no [3-^{13}C]-glutamate was incorporated into malate during anaerobic fermentation (Camarasa *et al*., 2003). All succinate dehydrogenase activity in yeasts can be eliminated by disruption of *SDH1* (Arikawa *et al*., 1999a, 1999b). *SDH1*-deletion mutants and the corresponding wild-type strains produced similar amounts of succinate during anaerobic fermentation (Arikawa *et al*., 1999a, 1999b; Camarasa *et al*., 2003), thus providing further evidence that the TCA cycle is interrupted within fermenting yeasts at the level of the succinate dehydrogenase complex.

Basal levels or no succinate dehydrogenase specific activity are detected within anaerobically-grown yeasts by the time the stationary phase of growth is reached (Lukins *et al*. 1966, Duntze *et al*., 1969; Lowdon *et al*., 1972; Wales *et al*., 1980; Muratsubaki *et al.*, 1987; Rosenfeld *et al*., 2004). Notable higher levels of succinate dehydrogenase are found within respiro-fermenting yeasts (Lukins *et al*., 1966; Lowdon *et al*., 1972; Wales *et al*., 1980), because fermenting yeasts contain more promitochondria when oxygen is available to them (Lukins *et al*., 1966; Rosenfeld *et al*., 2004). However, succinic acid is still excreted into the medium under respiro-fermentative conditions. In fact, succinic acid production is significantly increased if fermentation is conducted in the presence of oxygen (Ribéreau-Gayon *et al*., 1956; Coote & Kirsop 1973; Nagai *et al*., 1992; Magarifuchi *et al*., 1995; Arikawa *et al*., 1999b). In other words, succinate dehydrogenase's *in vivo* activity is inhibited during fermentation.

The succinate dehydrogenase complex (succinate:ubiquinone oxidoreductase) are also part of the mitochondrial electron transport chain, which is why it also known as complex II of the respiratory electron transport chain. Flavin adenine dinucleotide (FAD) serves as coenzyme for the succinate dehydrogenase complex and is reduced when succinate is oxidised to fumarate. The reduced form of FAD ($FADH_2$) must be re-oxidised

before succinate dehydrogenase is able to catalyze the oxidation of another succinate molecule. However, FAD is covalently bonded to the flavoprotein subunit of the succinate dehydrogenase complex in yeasts (Robinson *et al*., 1994; Lemire & Oyedotun, 2002), which is why $FADH_2$'s electrons have to be passed onto a mobile electron carrier in order to convert $FADH_2$ back to FAD. Ubiquinone (coenzyme Q) serves as the mobile electron carrier that accepts electrons from succinate dehydrogenase's reduced prosthetic group ($FADH_2$). Its availability within yeast is therefore crucial for the continued operation of succinate dehydrogenases. Ubiquinone is barely detected within anaerobically-grown yeasts (Lester & Crane, 1959; Lowdon *et al*., 1972) and it is also repressed by the presence of fermentable sugars in the growth medium (Gordon & Steward, 1969; Lowdon *et al*., 1972). Ubiquinone also accepts electrons from the NADH dehydrogenases (NADH:ubiquinone oxidoreductase) of (pro)mitochondria (von Jagow & Klingenberg, 1970; deVries & Marres, 1987; Bakker *et al*., 2001). Even anaerobically-grown glucose-repressed yeasts contain noteworthy levels of NADH dehydrogenase (Criddle & Schatz, 1969; Rosenfeld *et al*., 2004). Ubiquinone is reduced to ubiquinol (QH_2) when it accepts electrons from the reduced prosthetic group of succinate- or NADH dehydrogenases ($FADH_2$). In other words, the already limited supplies of ubiquinone within fermenting yeasts will be removed at an even more rapid rate due to the activity of promitochondrial NADH dehydrogenases, unless ubiquinol can be re-oxidised back to ubiquinone.

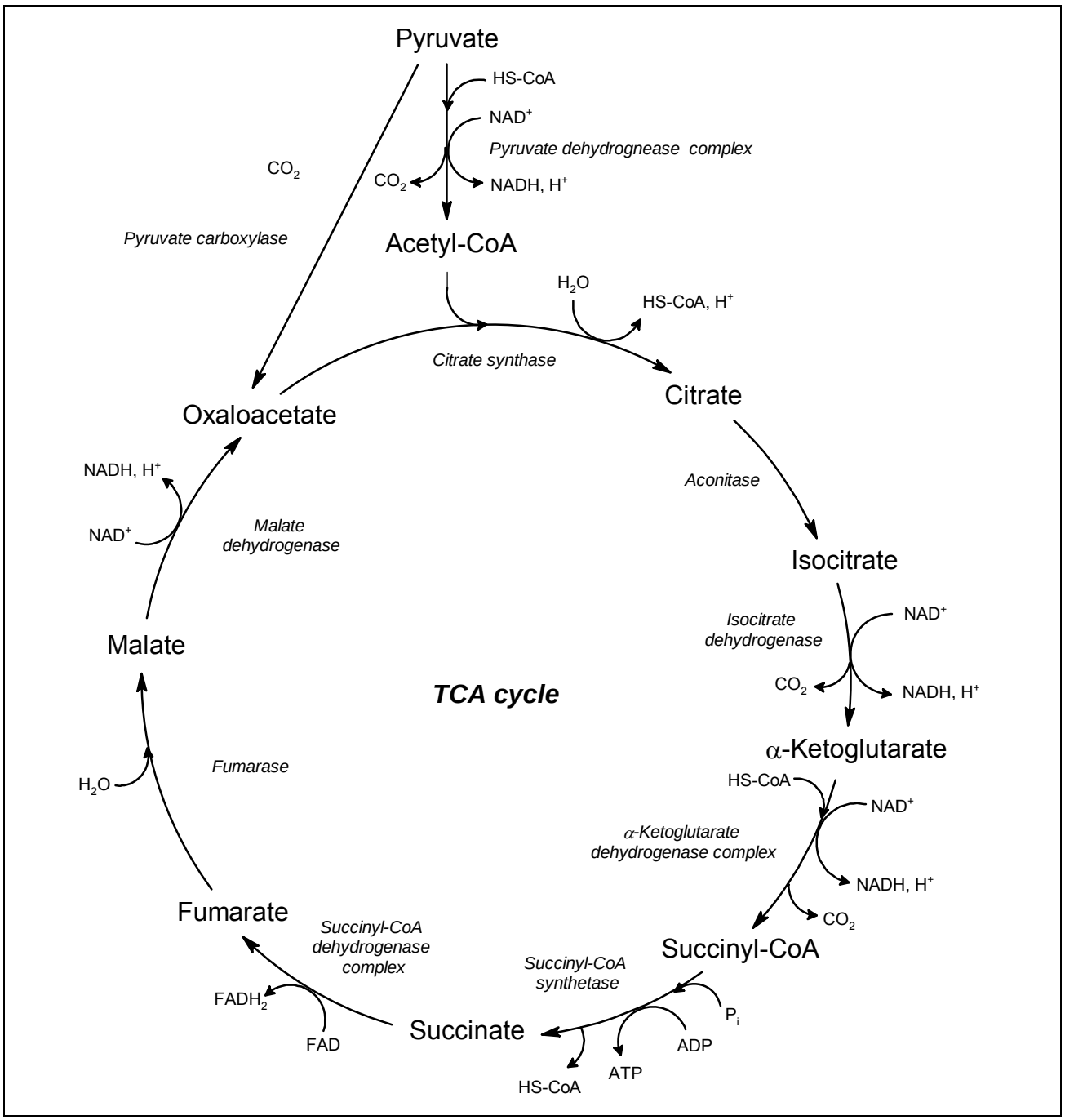

Figure 2.1 The TCA cycle during respiration

The TCA cycle can also function in the reductive direction within fermenting and/ or anaerobically-grown yeasts (Mayer *et al*., 1964; Lupianez *et al*., 1974; Machado *et al*., 1975; Wakai *et al*., 1980; Muratsubaki, 1987; Magarifuchi *et al*., 1995; Arikawa *et al*., 1999a, 1999b; Camarasa *et al*., 2003). Yeasts contain noteworthy levels of malate dehydrogenases during fermentation and/ or anaerobiosis (Chapman & Bartley, 1964; Heerde & Radler, 1978; Wales *et al*., 1980), which is why oxaloacetate can easily be converted into malate under such conditions. However, fumarases have a 17 times higher affinity for fumarate than for malate (Keruchenko *et al*. 1992; Pines *et al*., 1996), which is why considerable amounts of malate are excreted by fermenting yeasts (Radler & Lang,

1982; Schwarz & Radler, 1988; Arikawa *et al*., 1999a, 1999b; Camarasa *et al*., 2003). Fermenting and/ or anaerobically-grown yeasts contain fumarate reductases, which catalyze the irreversible reduction of fumarate to succinate (Rossi *et al*., 1964; Hauber & Singer, 1967; Muratsubaki & Katsume, 1982, 1985). Although fumarases' affinity for fumarate is 1.3 - 4 times higher than fumarate reductases' affinity for fumarate (Hauber & Singer, 1967; Pines *et al*., 1996), fermenting and/ or anaerobically-grown yeasts contain considerably more malate dehydrogenases and fumarate reductases than fumarases (Rossi *et al*., 1964; Hauber & Singer, 1967; Wales *et al*., 1980). In other words, fumarase's very high affinity for fumarate does not prevent malate from being converted into fumarate during fermentation due to high intracellular concentrations of malate and the rapid consumption of fumarate by fumarate reductases. The reduction of fumarate to succinate can also be catalyzed by the succinate dehydrogenase complex when its prosthetic group is in the reduced state (Singer *et al*, 1957; Robinson *et al*., 1994), but its specific activity within fermenting and/ or anaerobically-grown yeasts is very low (Heerde & Radler, 1978; Wales *et al*., 1980; Muratsubaki *et al.*, 1987) and its affinity for fumarate is considerably less than fumarase's and fumarate reductase's affinity for fumarate (Rossi *et al*., 1964; Hauber & Singer, 1967; Keruchenko *et al*. 1992; Pines *et al*., 1996). Theoretically, the reductive branch of the TCA cycle should be functional up to the point in the pathway where succinate is converted into succinyl-CoA, since none of the existing enzymes within yeasts can catalyze the reductive carboxylation of succinyl-CoA to α-ketoglutarate (Horton *et al*., 1996). However, succinyl-CoA synthetase's affinity for succinate is very low and it is absent from fermenting yeasts or present at extremely low levels when the yeasts are grown in media without glutamate, glutamine or threonine as the principle sources of yeast assimilable nitrogen (Schwarz *et al*., 1983). The branched version of the TCA cycle is shown in **Figure 2.2**.

The fermentation temperature, dissolved oxygen content, composition of the growth medium's yeast assimilable nitrogen (YAN) content and genetic factors (yeast strain) determine each metabolic pathway's contribution to succinic acid production (Heerde & Radler, 1978; Albers *et al*., 1996, 1998a; Arikawa *et al*., 1999a, 1999b; Camarasa *et al*., 2003). The complete removal of α-ketoglutarate dehydrogenase activity from yeasts (disruption of *KGD1*) reduced succinic acid production by 25% during anaerobic fermentation in synthetic grape juice (Camarasa *et al.*, 2003). In other words, only 25% of all the succinic acid produced during fermentation is formed via the oxidative branch of the TCA cycle when fermentation is carried out in a grape juice-like medium.
A similar result (37% less succinic acid) was obtained with another *KGD1*-deletion mutant during anaerobic fermentation of *saké* mash (Arikawa *et al*., 1999a). However, yeasts without α-ketoglutarate dehydrogenase activity produced 72% less succinic acid during anaerobic fermentation when synthetic grape juice contained 300 mg/L glutamate as the only source of YAN (Camarasa *et al.*, 2003).

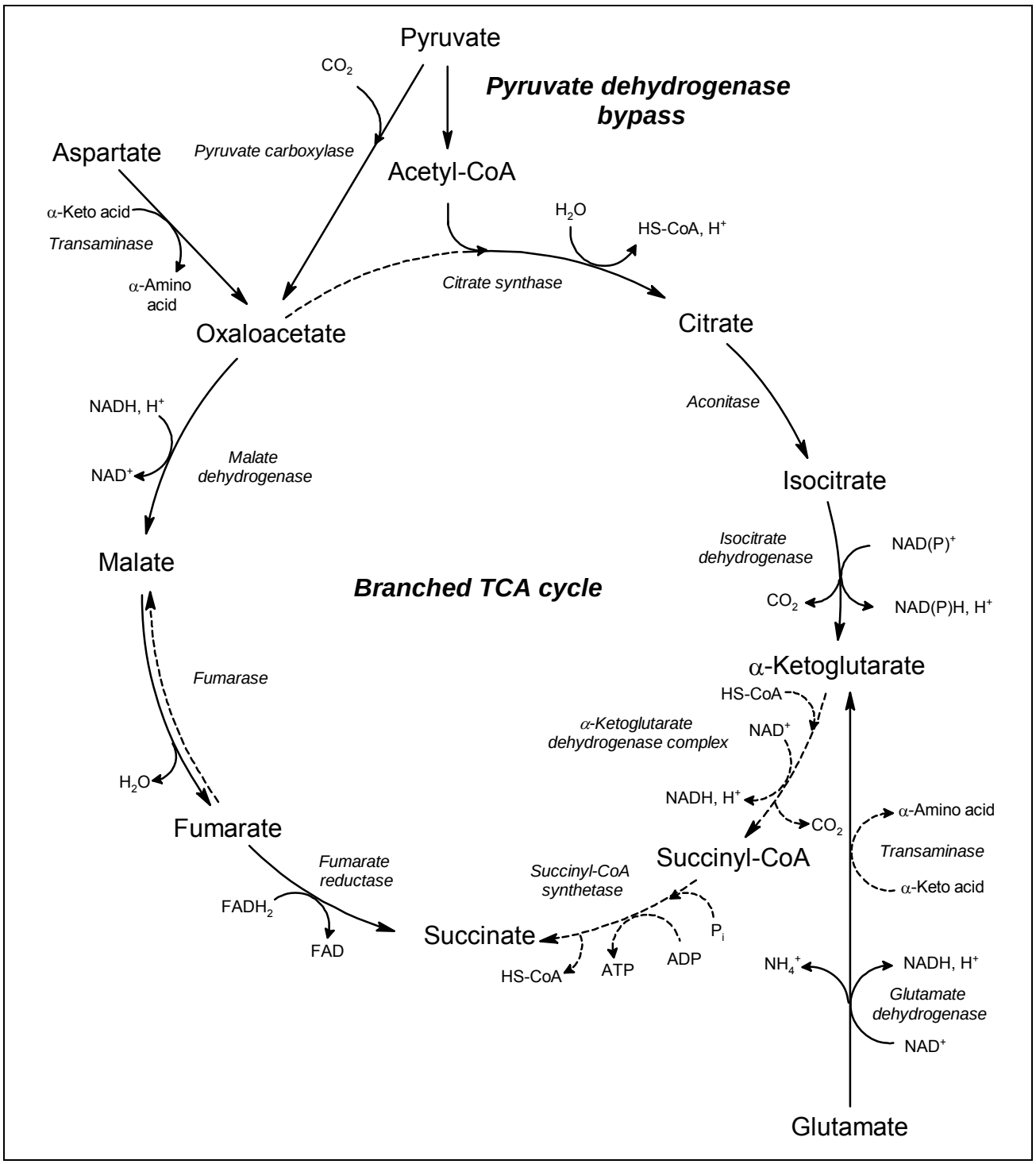

Figure 2.2 Branching of the TCA cycle during fermentation

The specific activities of α-ketoglutarate dehydrogenase and succinyl-CoA synthetase are significantly increased by large quantities of glutamate, glutamine and/ or threonine in the fermentation medium (Heerde & Radler, 1978; Schwartz *et a*l., 1983; Camarasa *et al.*, 2003). This implies that the majority of succinic acid produced by fermenting yeasts will be formed via the oxidative branch of the TCA cycle when the above mentioned amino acids make up the largest portion of the growth medium's YAN content. The disruption of *FUM1* in yeasts eliminates all fumarase activity within yeasts (Arikawa *et al*., 1999a, 1999b) and

was responsible for a 37% decrease in succinic acid production during anaerobic fermentation of *saké* mash (Arikawa *et al*., 1999a). *Saké* mash is similar to grape juice in that most of its YAN is supplied by nitrogen sources other than the amino acids that stimulate the oxidative branch of the TCA cycle within fermenting yeasts (Arikawa *et al*., 1999a). Therefore, it is reasonable to assume that about 40% of all succinic acid produced during anaerobic fermentation of grape juice will be formed via the reductive branch of the TCA cycle. In other words, a small percentage of wine's or rice wine's succinic acid content is unaccounted for by the two branches of the TCA cycle. This implies that another metabolic pathway must be involved in succinic acid's production during grape juice or sake mash fermentations.

2.2.2 THE GLYOXLIC ACID BYPASS

The glyoxylic acid bypass (**Figure 2.3**) consists of two reactions, which modifies the TCA cycle in such a way so that it provides an anabolic alternative for the metabolism of acetyl-CoA. Isocitrate is cleaved into succinate and glyoxylate by isocitrate lyase during the first reaction of the glyoxylic acid bypass, where after acetyl-CoA condenses with glyoxylate to form malate in a reaction catalyzed by malate synthase. Malate can then be converted into glucose via oxaloacetate and the reactions of the gluconeogenic pathway. In other words, the glyoxylate bypass enables yeasts to grow in the absence of sugars when other precursors of acetyl-CoA, such as ethanol or acetic acid, are available (Horton *et al.*, 1996).

The functioning of the glyoxylic acid bypass is in other words unnecessary within fermenting yeasts. Isocitrate lyases and malate synthases of yeasts are therefore strongly repressed by the presence of fermentable sugars in the growth medium (Chapman & Bartley, 1968; Duntze *et al*., 1969; Haarasilta & Oura, 1975; Wales *et al*., 1980;). In fact, the disruption of the isocitrate lyase gene, *ICL1*, in yeasts had no significant effect on succinic or malic acid production during fermentation (Arikawa *et al*., 1999a, 1999b). Both the before mentioned enzymes are also strongly repressed by hypoxia (Chapman & Bartley, 1968; Wales *et al*., 1980), which explains why the fixation of ^{14}C-labelled carbon dioxide resulted in the strong labelling of glutamic acid, but why no labelled glyoxylic acid was detected in the extracts of anaerobically-grown yeasts (Stoppani *et al.*, 1957). Thus, it can be said that the glyoxylic acid bypass is not involved in the formation of succinic acid by fermenting and or anaerobically-grown yeasts.

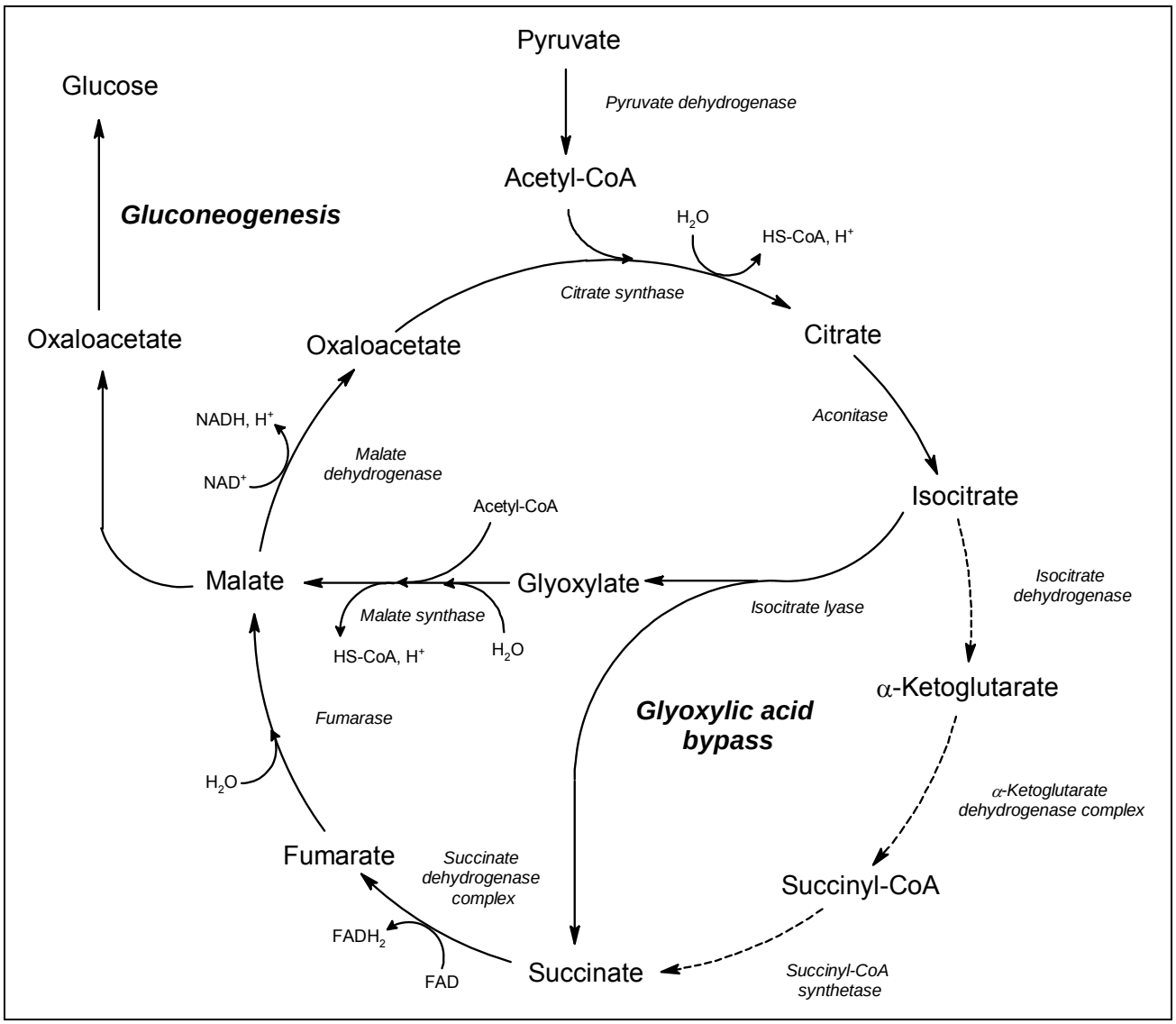

Figure 2.3 TCA cycle and Glyoxylic acid bypass

2.2.3. THE GABA BYPASS

The GABA bypass (**Figure 2.4**) circumvents the two reactions of the TCA cycle that convert α-ketoglutarate into succinate. During the first step of the GABA bypass, α-ketoglutarate is turned into glutamate by reacting with ammonia and/ or by accepting either glutamine's amide group or another α-amino acid's α-amino group. The reaction between α-ketoglutarate and ammonium is catalyzed by $NADPH_2$-dependant glutamate dehydrogenase and the amination of α-ketoglutarate by glutamine is catalyzed by glutamate synthase (Horton *et al*., 1996). In the subsequent reaction of the GABA bypass, glutamate decarboxylase converts glutamate into γ-aminobutyrate (GABA), which in turn is deaminated by GABA transaminase to succinate semialdehyde. Succinate semialdehyde is then oxidized to succinate by NAD^+-dependant succinate semialdehyde dehydrogenase (Satya Narayan & Nair, 1990; Shelp *et al*., 1999; Coleman *et al.*, 2001).

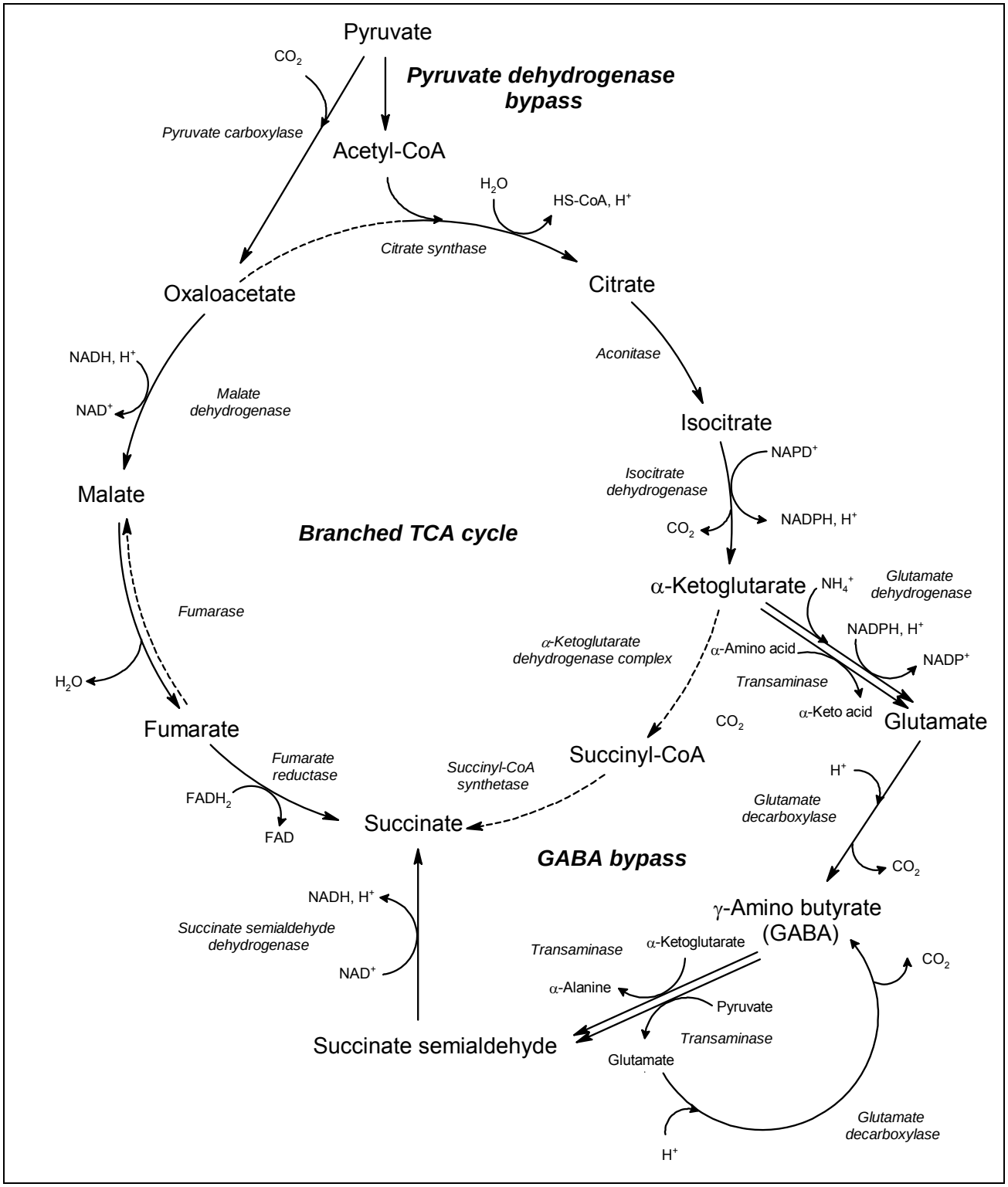

Figure 2.4 Branched TCA cycle with the GABA bypass

Besides the TCA cycle's reductive branch, the GABA bypass is the only metabolic pathway within yeasts that can convert pyruvate-derived acetyl-CoA into succinic acid during alcoholic fermentation. Unfortunately though, this metabolic pathway's possible involvement in succinic acid production by yeasts has not been investigated yet.

2.2.4 THE MCA CYCLE

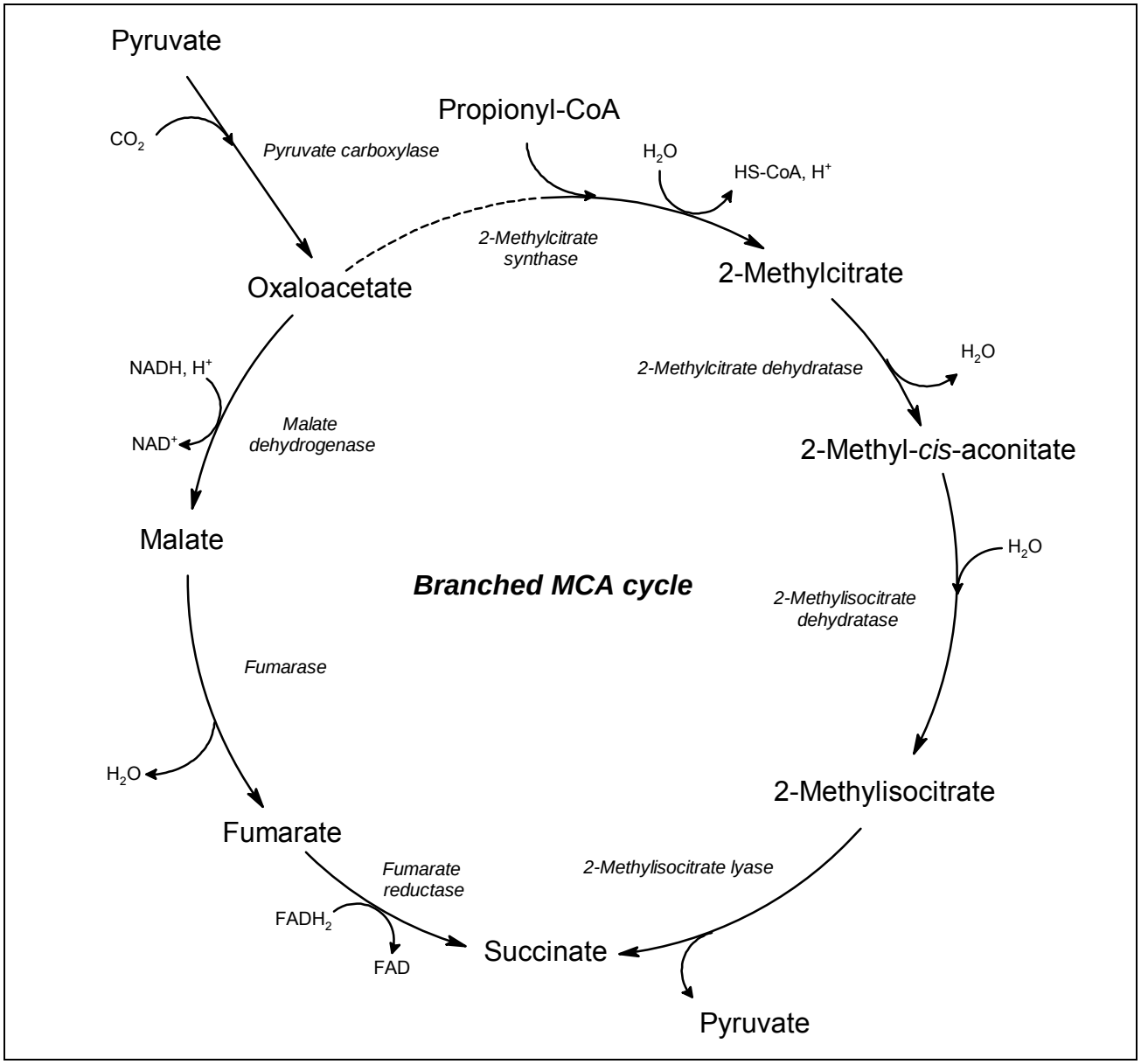

Figure 2.5 Branched MCA cycle

Wine yeasts (*S. cerevisiae*) make use of the methylcitric acid cycle (**Figure 2.5**) to metabolize propionyl-CoA derived from branched-chain amino acids and odd-chain fatty acids (Shindo *et al*., 1993; Pronk *et al*., 1994; Luttik *et al.*, 2000). Reduced coenzymes generated by this metabolic pathway can be used to produce ATP via mitochondrial electron transport chain. In other words, the MCA cycle enables yeasts to generate chemical potential energy (ATP) from substrates that are precursors of propionyl-CoA.

The reaction sequence of the MCA cycle is similar to the glyoxylic acid cycle, but oxaloacetate reacts with propionyl-CoA, in stead of acetyl-CoA during the first reaction of the MCA cycle. Condensation between oxaloacetate and propionyl-CoA results in the formation of 2-methylcitrate, which is catalyzed by 2-methylcitrate synthase.

2-Methylcitrate is then isomerized to 2-methylisocitrate, which is cleaved into succinate and pyruvate by 2-methylisocitrate lyase. The rest of the MCA cycle's reactions are identical to that of the TCA cycle (Pronk *et al*., 1994).

Like the TCA cycle though, the MCA cycle is interrupted at the level of the succinate dehydrogenase complex within fermenting yeasts and therefore contributes to the production of succinic acid when the fermentation medium contains branched-chain amino acids and/ or odd-chain fatty acids (Shindo *et al*., 1993).

2.3 PHYSIOLOGICAL ROLE OF SUCCINIC ACID PRODUCTION

2.3.1 REDOX BALANCE

Intracellular supplies of nicotinamide adenine dinucleotide, NAD(H), and other coenzymes are limited. Enzymatic reactions that cause the reduction of a particular coenzyme are therefore balanced by ones that convert them back to their oxidized state. The intracellular balance, which exists between the reduced and oxidised forms of each coenzyme is referred to as the redox (reduction-oxidation) balance of the cell and must be maintained at all times to ensure continuous operation of metabolic processes.

Respiratory-deficient mutants without mitochondrial DNA (rho^- mutants, petite mutants) possess the fumarate reductase content than normal cells that were grown under fermentative and/ or anaerobic conditions (Hauber & Singer, 1967). Wine yeasts (*S. cerevisiae*) contain two isoforms of fumarate reductase. Fumarate reductases encoded by *FRDS* (fumarate reductase I) are located within the cytosol of yeasts (Enomoto *et al*., 1996), whereas fumarate reductases encoded by *OSM1* (fumarate reductase II) are found within the (pro)-mitochondrial matrix of yeasts (Muratsubaki *et al*., 1998). Yeasts in which both fumarate reductase genes have been disrupted (*FRDS*Δ*OSM1*Δ-mutants) are unable to grow under anaerobic conditions (Arikawa *et al*., 1998, 1999b; Enomoto *et al*., 2002). These findings imply that fumarate reductases are essential for yeast growth when they are unable to respire. The arrested growth of the before mentioned *FRDS*Δ*OSM1*Δ-mutant was temporarily restored in the absence of oxygen by the addition of chemical compounds capable of oxidizing NADH back to NAD^+ (methylene blue or phenazine methosulphate) (Enomoto *et al*., 2002). This finding indicates that yeasts require fumarate reductases for the reoxidation of intracellular NADH during anaerobic growth. In other words, reductive formation of succinic acid is necessary for maintaining the NADH:NAD^+ balance within yeasts during anaerobiosis.

NADH can be reoxidized by mitochondrial NADH dehydrogenases (NADH:ubiquinone oxidoreductase) during respiration (deVries & Marres, 1987; Bakker *et al*, 2001). Yeasts possess two types of NADH dehydrogenase, which are both located within the inner membrane of mitochondria (von Jagow & Klingenberg, 1970; deVries & Marres, 1987; Marres *et al*., 1991; Small & McAlister, 1998; Bakker *et al*., 2001). The one type catalyzes the reoxidation of cytosolic NADH (external NADH dehydrogenases) and has active sites that face the inter-membrane space of

mitochondria (Small & McAlister, 1998), whereas the other one catalyzes the reoxidation of intramitochondrial NADH (internal NADH dehydrogenases) and therefore possesses active sites directed towards the mitochondrial matrix (Marres *et al*, 1991). The external NADH dehydrogenases of yeasts are encoded by *NDE1* and *NDE2* (Luttik *et al*., 1998; Bakker *et al*, 2001), while internal NADH dehydrogenases are encoded by *NDI1* (Marres *et al*., 1991; Bakker *et al*, 2001). *S. cerevisiae*'s NADH dehydrogenases are subject to carbon-catabolite repression (de Vries & Grivell, 1988; de Risi *et al*., 1997; Bakker *et al*, 2001) and are also repressed by hypoxia (ter Linde *et al*., 1999; Bakker *et al*, 2001). However, noteworthy levels of NADH dehydrogenase (NADH:ferricyanide reductase) are found within fermenting yeasts, even when they grow under completely anaerobic conditions (Criddle & Schatz, 1969; Rosenfeld *et al*., 2004). *In vivo* reoxidation of NADH by NADH dehydrogenases causes the reduction of ubiquinone to ubiquinol (de Vries & Grivell, 1988; Bakker *et a*l., 2001). As mentioned before however, ubiquinone is almost entirely absent within anaerobically-grown yeasts (Lester & Crane, 1959; Lowden *et al*., 1972) and once it is reduced, cannot be converted back to its original form due to the absence of cytochromes *b*, c_1 and *c*, cytochrome c oxidase and molecular oxygen (Criddle & Schatz, 1969; Cartledge & Lloyd, 1972; Salmon *et al*., 1998; Rosenfeld *et al*., 2004). Considerable amounts of ubiquinone are found within the promitochondrial extracts of respiro-fermenting yeasts (Gordon & Steward, 1969; Lowden *et al*., 1972). Nonetheless, intracellular levels of ubiquinone will probably be very limited during fermentation, because wine yeasts do not contain NADH oxidases (Criddle & Schatz, 1969; Bakker *et al*., 2001) and although ubiquinol can give up its reducing equivalents to oxygen via the bc_1-complex during fermentation (Salmon *et al*., 1998), this particular component of the electron transport chain is subject to carbon-catabolite repression (Klein *et al*., 1998) and dissolved oxygen is constantly being displaced by carbon dioxide and lost to the atmosphere. The scarcity of ubiquinone within fermenting yeasts is made worse by the fact that NADH dehydrogenases have to compete with succinate dehydrogenases for the same substrate, *i.e*. ubiquinone. To summarize, NADH cannot be reoxidized by NADH dehydrogenases during anaerobiosis and their ability to reoxidise NADH is extremely limited during aerobic fermentation. So in stead of oxygen, organic molecules serve as the final acceptors of reducing equivalents from NADH during alcoholic fermentation and/ or anaerobiosis.

No attempt has yet been made to determine whether *S. cerevisiae* yeasts contain transhydrogenase specific activity. However, none of the wine-related yeasts that were investigated contained transhydrogenase specific activity (Bruinenberg *et al*., 1983a, 1985; Camougrand *et al*., 1988). In other words, it is unlikely for $NADP^+$ to serve as final acceptor of reducing equivalents from NADH within fermenting wine yeasts.

Acetaldehyde is the most important acceptor molecule of reducing equivalents from NADH when yeasts grow on fermentable sugars as carbon and energy sources. This explains why ethanol is the main product of yeasts when they are grown on hexoses. Alcoholic fermentation is a redox-neutral process (Oura, 1977; van Dijken & Scheffers, 1986; Albers *et al*., 1998b; Bakker *et al*., 2001), which means that for every molecule of

NADH formed during glycolysis, one is oxidised during the reduction of acetaldehyde to ethanol (**Figure 2.6**). However, glycolysis is not the only source of NADH. Lipid biosynthesis results in the net production of NADH (Albers *et al*., 1998b). During anaerobiosis though, yeasts must attain most of the lipids they need from the medium in which they grow (Snoek & Steensma, 2007). The production of yeast biomass from assimilated nutrients results in a net consumption of NADPH and a net production of production of NADH, even if lipid biosynthesis is not taken into account (Gommers *et al.*, 1988; Verduyn et al., 1990; Albers *et al*., 1998b; Bakker *et al*., 2001). In other words, not enough acetaldehyde is available within fermenting yeast to get rid of any extra NADH that results from biomass formation (van Dijken & Scheffers, 1986; Albers *et al*., 1998b; Bakker *et al*., 2001).

In addition, the rate of ethanol formation is very slow during the first few hours of carbon-catabolite repressed growth of aerobically-grown yeasts (Peña *et al*., 1972; Boulton *et al*., 1998b). This period of metabolic adaptation is known as the lag phase of alcoholic fermentation and is caused by the low initial concentrations of cytosolic pyruvate decarboxylase and alcohol dehydrogenase (Adh1p) within respiring yeasts (Denis *et al.*, 1983; Schmitt *et al.*, 1983; Sharma & Tauro, 1986; Boulton *et al*., 1998b). Hence, acetaldehyde is not the only intermediate of sugar metabolism in yeasts that accepts reducing equivalents from NADH during carbon-catabolite repression (fermentation).

The glycolytic intermediate dihydroxyacetone phosphate (**Figure 2.6**), is the second most important acceptor of reducing equivalents from NADH within fermenting and/ or anaerobically-grown yeasts (Oura, 1977; Radler & Schütz, 1982; van Dijken & Scheffers, 1986; Albers *et al*., 1998b; Bakker *et al*., 2001). In fact, yeasts are unable to grow under anaerobic conditions when the enzymes responsible for the NADH-dependant reduction of dihydroxyacetone phosphate are removed from yeasts by the disruption of their structural genes (Ansell *et al.*, 1997; Björkqvist *et al.*, 1997). The reduction of dihydroxyacetone phosphate to glycerol-3-phosphate is catalyzed by NADH-dependant glycerol-3-phosphate dehydrogenase. Glycerol-3-phosphate is converted into glycerol after the removal of its phosphate group by glycerol-3-phosphatase (Nevoigt & Stahl, 1997; Prior & Hohmann, 1997; Bakker *et al*., 2001). However, glycerol production is energetically expensive to fermenting yeasts, because the stoichiometric conversion of glucose to pyruvate and glycerol does not lead a net production of ATP. Reducing equivalents from excess NADH within fermenting yeasts must therefore also be passed on to other organic molecules besides acetaldehyde and dihydroxyacetone phosphate.

Acetoin (acetylmethylcarbinol) is probably the third most important redox-sink within fermenting yeasts. The formation of acetoin from pyruvate is a redox-neutral process (Romano & Suzzi, 1996; Riberéau-Gayon *et al*., 2000b).

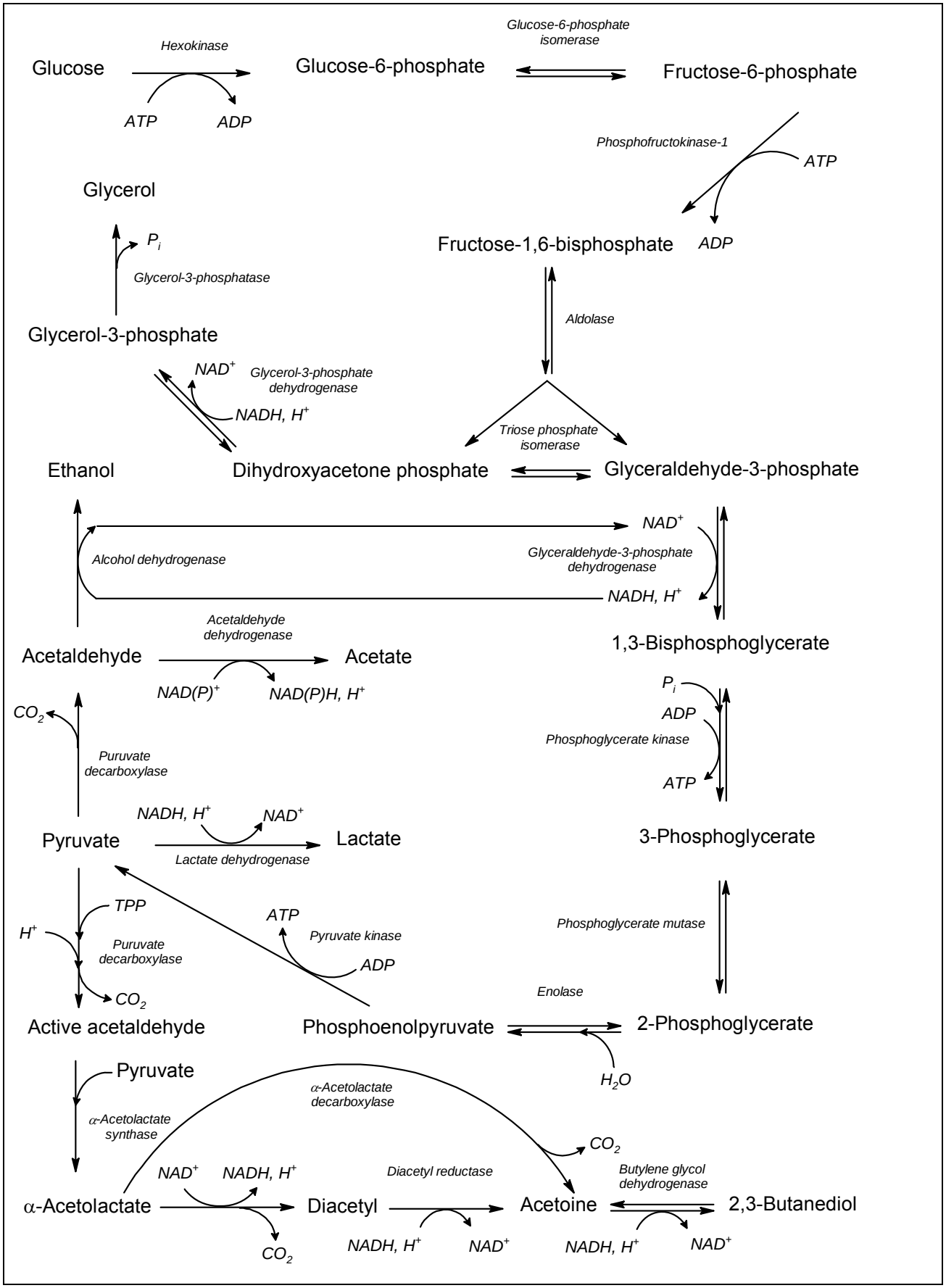

Figure 2.6 Glycolysis and fermentative sugar catabolism

The reduction of acetoin to butane-2,3-diol therefore results in the net regeneration of NAD^+ from NADH (**Figure 2.6**). Between 300 and 1800 mg/L butane-2,3-diol (2,3-butylene glycol) can be formed from acetoin during fermentation of grape juice (Margalit, 1997; Ribereau-Gayon *et al*., 2000b).

Pyruvate can accept reducing equivalents from molecules that are more reduced than it. The reduction of pyruvate results in the formation of lactic acid and is catalyzed by lactate dehydrogenases, which are also capable of catalyzing the reverse reaction. Wine yeasts (*S. cerevisiae*) contain different types of lactate dehydrogenases, depending on the growth conditions (de Vries & Marres, 1987). The lactate dehydrogenases of yeasts are specific for either D- or L-lactate, which explains why both lactic acid isomers are produced during alcoholic fermentation. Most wine yeasts though, produce considerably more of the former than of the latter mentioned lactic acid isomer (Whiting, 1976). The most important lactate dehydrogenases of *S. cerevisiae* yeasts are linked to ferricytochrome *c* of the respiratory electron transport chain (Guiard, 1985; Lodi & Ferrero, 1993; Chelstowska *et al.*, 1999; Bakker *et al*., 2001) and are therefore also known as lactate:ferricytochrome *c* oxidoreductases. Cytochrome *c*-linked lactate dehydrogenases are induced under aerobic conditions by the presence of lactic acid in the growth medium (Ferrero *et al*., 1981; de Vries & Marres, 1987), but are strongly repressed by fermentable sugars (Polakis *et al*., 1965) and hypoxia (Ferrero *et al*., 1981). These enzymes are not, in other words, responsible for the direct reoxidation of NADH, but are needed for the oxidation of lactic acid to pyruvate during respiratory growth on lactic acid. *S. cerevisiae* yeasts also contain NADH-dependant lactate dehydrogenases, which are induced by high concentrations of fermentable sugars in the growth medium or conditions that inhibit respiration (Genga *et al*., 1983; de Vries & Marres, 1987). NADH-dependant lactate dehydrogenases catalyze the reduction of pyruvate to lactic acid and are responsible for the direct reoxidation of NADH back to NAD^+. However, very little lactic acid (~ 200 mg/L) is produced by wine yeasts during fermentation of grape juice (Whiting, 1976). The reduction of pyruvate therefore contributes little to the reoxidation of NADH within fermenting yeasts.

The aldehydes that can be formed from the α-keto acid precursors of certain α-amino acids also serve as final acceptors of reducing equivalents from NADH within yeasts during alcoholic fermentation (Webb & Ingraham, 1963; Nykanen, 1986). However, the production of higher alcohols (fusel-oils) via the so called Ehrlich pathway plays a minor role in the regeneration of NAD^+ from NADH within fermenting yeasts (Boulton *et al*., 1998b), because the majority thereof is formed from hexoses and not from amino acids (Webb & Ingraham, 1963).

Succinic acid production via the reductive branch of the TCA cycle consists of three reactions that are respectively catalyzed by malate dehydrogenases, fumarases and fumarate reductases (**Figure 2.2**). Respiring cells of *S. cerevisiae* contain three isoforms of malate dehydrogenase (Atzpodien *et al*., 1968; Minard & McAlister-Henn, 1991). The mitochondrial malate dehydrogenase isoenzyme (Thompson *et al*., 1988) is absent from anaerobically-grown fermenting yeasts (Atzpodien *et al*., 1968). This is also true for the

MDH2-encoded cytoplasmic malate dehydrogenase (Atzpodien, 1968 Minard & McAlister-Henn, 1992). However, yeasts also contain a constitutively expressed cytoplasmic isoform of malate dehydrogenase (Atzpodien *et al*., 1968), which is encoded by *MDH3* (Steffan & McAlister-Henn, 1992). Malate dehydrogenases require NADH as coenzyme to reduce oxaloacetate to fumarate (Horton *et al*., 1996). In other words, cytoplasmic NADH is reoxidized during the reduction of oxaloacetate to malate. The malate that is formed within the yeast cytoplasm is then converted into fumarate by cytoplasmic fumarases (Wu & Tzagoloff, 1987). Fumarate can then be reduced to succinate within the cytoplasm of yeasts by the *FRDS*-encoded cytoplasmic isoform of fumarate reductase (Enomoto *et al.*, 1996). However, yeast fumarate reductases require reduced FAD as coenzyme to catalyze the reduction of fumarate to succinate (Rossi *et al*., 1964; Hauber & Singer, 1967; Muratsubaki & Katsume, 1982, 1985). FAD is not covalently bonded to yeast fumarate reductases (Rossi *et al*., 1964; Hauber & Singer, 1967; Muratsubaki & Katsume, 1982) and can therefore dissociate from the enzyme in order to go bind to one of the external NADH dehydrogenases of a (pro)-mitochondrion (Small & McAlister, 1998), which are known to be present within anaerobically-grown fermenting yeast (Criddle & Schatz, 1969; Rosenfeld *et al*., 2004). The external NADH dehydrogenases of yeast (pro)mitochondria oxidizes NADH within the cytoplasm back to NAD^+ (de Vries & Marres, 1987; Small & McAlister, 1998; Bakker *et al*., 2001), which will result in the reduction of FAD^+. The reduced FAD groups of yeast mitochondria's external NADH dehydrogenases pass their reducing equivalents on to ubiquinone molecules during respiration (de Vries & Marres, 1987; Small & McAlister, 1998; Bakker *et al*., 2001), but ubiquinone is almost absent from anaerobically-grown fermenting yeasts (Lester & Crane, 1959; Lowden *et al*., 1972). FAD molecules are not covalently bonded to the NADH dehydrogenases of (pro)-mitochondria (de Vries & Marres, 1987; de Vries & Grivell, 1988). Thus, $FADH_2$ molecules are capable of dissociating from the external NADH dehydrogenases of yeast (pro)-mitochondria, after which they will be free to once again bind to the fumarate reductases within the yeast cytoplasm. In other words, two cytoplasmic NADH molecules can be reoxidized for every molecule of oxaloacetate that is converted into succinate within via the reductive branch of the TCA.

The inner membrane of (pro)-mitochondria is virtually impermeable to pyrimidine nucleotides (von Jagow & Klingenberg, 1970; Albers *et al*., 1998b; Bakker *et al*., 2001). Consequently, NADH must be reoxidized in the cellular compartment where it is generated. NADH turnover is needed in both the cytosol and (pro)-mitochondrial matrix (Bruinenberg *et al*., 1983b; Albers *et al.*, 1998b; Bakker *et al*., 2001). Three isoforms of glycerol-3-phosphate dehydrogenase have been identified within *S. cerevisiae* yeasts. The *GUT2*-encoded isoform of glycerol-3-phosphate dehydrogenase is localized within the inner membranes of yeast mitochondria (Rønnow & Kielland-Brandt, 1993). However, the mitochondrial glycerol-3-phosphate dehydrogenase (glycerol-3-phosphate:ubiquinone oxidoreductase) requires FAD as coenzyme and catalyzes the oxidation of cytosolic glycerol-3-phosphate by passing on its electrons to ubiquinone of the electron transport chain. In other words, Gut2p is not involved in glycerol formation, but

forms part of the glycerol-3-phosphate shuttle, which serves as a bypass for the reoxidation of cytosolic NADH by mitochondria's external NADH dehydrogenases during aerobic growth on ethanol (Larsson *et al*., 1998; Bakker *et al*., 2001). In fact, Gut2p is repressed by fermentable sugars in the growth medium (Sprague & Cronan, 1977). Two isoforms of NADH-dependant glycerol-3-phosphate dehydrogenases can be identified within *S. cerevisiae* yeasts. The *GPD1*-encoded isoform of glycerol-3-phosphate dehydrogenase is predominantly involved in the adaptation of *S. cerevisiae* yeasts to osmotic stress (Albertyn *et al*., 1994; Eriksson *et al*., 1995; Ansell *et al.*, 1997 Nevoigt & Stahl, 1997), while the *GPD2*-encoded isoform is responsible for maintaining the NAD^+:NADH balance within yeasts under anaerobic conditions (Norbeck *et al*., 1996; Ansell *et al.*, 1997; Nevoigt & Stahl, 1997). Gpd1p is located within the cytoplasm of yeasts (Larsson *et al*., 1993) and although the actual localization of Gpd2p has not been established yet, it is unlikely to be found within mitochondria (Eriksson *et al*., 1995; Albers *et al*., 1998b). Two types of glycerol-3-phosphatase are found within *S. cerevisiae* yeasts. One of them is encoded by *GPP1* and the other one is encoded by *GPP2* (Norbeck *et al*., 1996). The cellular location of Gpp1p and Gpp2p has not been investigated yet, but both glycerol-3-phosphatases isoforms probably are probably also localized within the cytoplasm of yeasts (Bakker *et al*., 2001). In other words, glycerol can only be formed within the cytoplasm of yeasts during alcoholic fermentation, which implies that promitochondrial NADH cannot be reoxidized by means of glycerol formation. Shuttle mechanisms are therefore needed for the export of reducing equivalents from the NADH within promitochondria to the cytosol.

In addition to the *ADH1* and *ADH2* encoded cytoplasmic isoforms of alcohol dehydrogenase (Lutstorf & Megnet, 1968; Ciriaci, 1997), *S. cerevisiae* yeasts also contain at least one (pro)mitochondrial alcohol dehydrogenase that is encoded by *ADH3* (Young & Pilgram, 1985; Ciriaci, 1997). Expression of Adh1p is increased 5-10 fold by the presence of fermentable sugars in the growth medium (Denis *et al*., 1983), but Adh2p is absent from yeasts during alcoholic fermentation (Lutstorf & Megnet, 1968; Ciriaci, 1997). The mitochondrial alcohol dehydrogenases of wine yeasts are also repressed fermentable sugars (Drewke *et al*., 1990; Albers *et al*., 1998b), but low levels of adh3p specific activity have been found within anaerobically-grown, glucose-limited cultures of *S. cerevisiae* (Nissen *et al.*, 1997). In other words, it is possible that the oxidation of ethanol in the cytosol can be coupled to the reduction of acetaldehyde in yeast promitochondria as a means of regenerating NAD^+ from promitochondrial NADH (von Jagow & Klingenberg, 1970; Albers *et al*., 1998b; Bakker *et al.*, 2001). The above described mechanism for the reoxidation of promitochondrial NADH is referred to as the ethanol-acetaldehyde mitochondrial redox shuttle (**Figure 2.7**) and has been shown to be active within anaerobically-grown glucose-limited chemostat cultures of *S. cerevisiae* (Nissen *et al.*, 1997). However, it might not be active within fermenting yeasts during the stationary phase of alcoholic fermentation in grape juice due to strong repression of Adh3p by high concentrations of fermentable sugar.

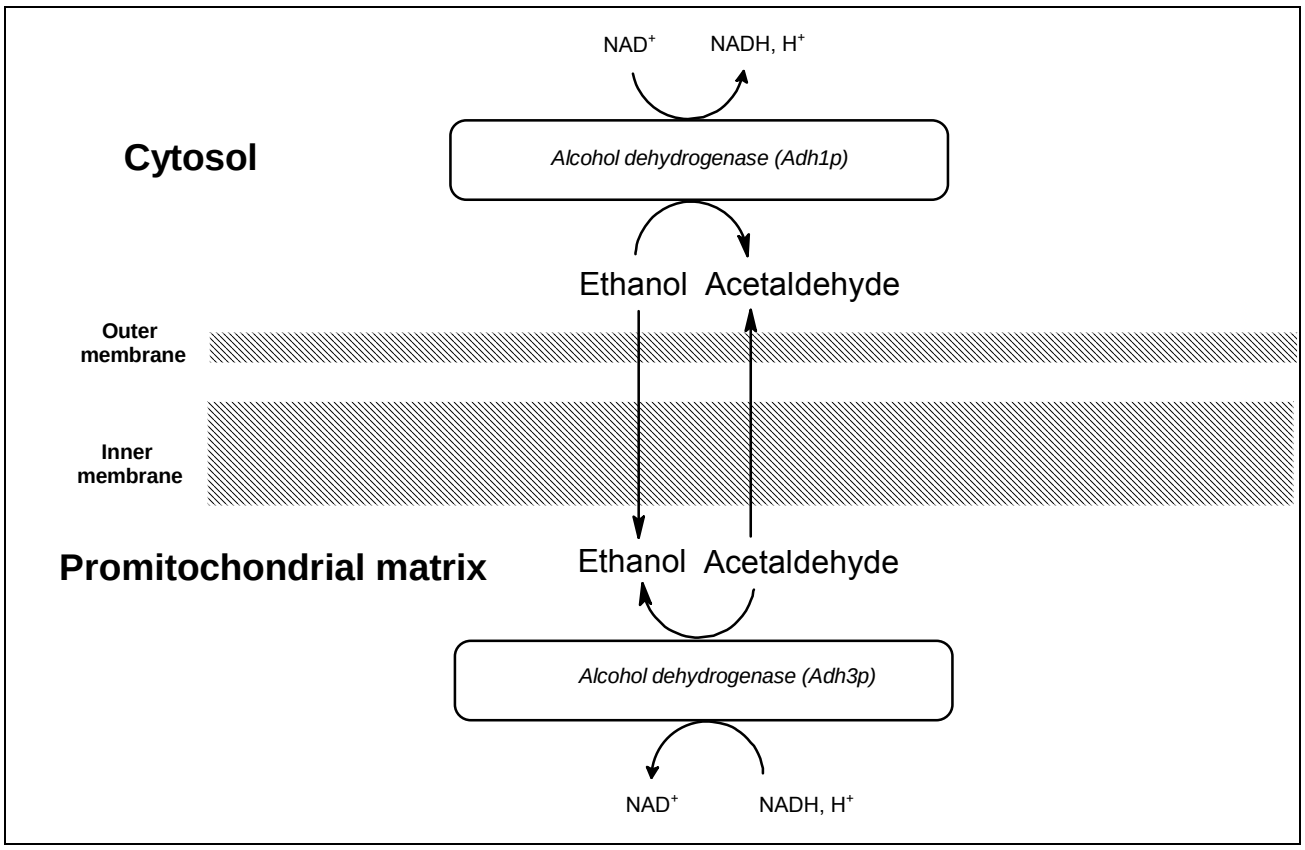

Figure 2.7 Ethanol-acetaldehyde promitochondrial redox shuttle

Although it is not reported elsewhere, it is possible that the reductive formation of succinate is involved the exchange of redox equivalents between promitochondria and the cytosol of fermenting yeasts. An *ACR1*-encoded permease (fumarate-succinate transporter) can transport fumarate formed within the yeast cytoplasm across the double membrane of a (pro)-mitochondrion in exchange for succinate (Palmieri *et al*., 1997). Fumarate can then be reduced to succinate by an *OSM1*-encoded fumarate reductase within the promitochondrial matrix (Muratsubaki & Enomoto, 1998), which will result in the oxidation of the enzyme's $FADH_2$ group (Rossi *et al*., 1964; Hauber & Singer, 1967; Muratsubaki & Katsume, 1982, 1985). The formed succinate will be transported to the cytoplasm in exchange for the next fumarate molecule that enters the promitochondrium. Once it is in the oxidised form, fumarate reductase's FAD group must first be reduced to $FADH_2$ before the enzyme will be able to catalyze the reduction of the next fumarate molecule that enters the promitochondrion. As mentioned before, FAD^+ can dissociate from the fumarate reductases of *S. cerevisiae* yeasts, since they are not covalently bonded to the enzyme (Rossi *et al*., 1964; Hauber & Singer, 1967; Muratsubaki & Katsume, 1982, 1985). Once it is free, FAD^+ can go bind to one of the internal NADH dehydrogenases (Ndi1p) of a (pro)mitochondrion, which are embedded within its inner membrane (de Vries & Marres, 1987; de Vries & Grivell, 1988; Marres *et al*., 1991; Bakker *et al*., 2001). Also already mentioned, is the fact that internal NADH dehydrogenases are present within anaerobically-grown fermenting yeast (Criddle & Schatz, 1969; Rosenfeld *et al*., 2004). This component of the electron transport chain can oxidize NADH of the

promitochondrial matrix back to NAD^+, which will also result in the reduction of FAD^+ to $FADH_2$ (de Vries & Marres, 1987; Marres *et al*., 1991; Bakker *et al*., 2001). During respiration, the reduced FAD groups of internal NADH dehydrogenases pass their reducing equivalents onto ubiquinone of the electron transport chain (de Vries & Marres, 1987; Marres *et al*., 1991; Bakker *et al*., 2001), but ubiquinone is almost absent within the promitochondria of anaerobically-grown fermenting yeasts (Lester & Crane, 1959; Lowden *et al*., 1972). Since the bond between FAD and a (pro)-mitochondrion's internal NADH dehydrogenase is not covalent (de Vries & Marres, 1987; de Vries & Grivell, 1988), $FADH_2$ can dissociate from this particular enzyme in order to go bind to one of the fumarate reductases within the promitochondrial matrix. We will refer to the above-describe redox shuttle as the fumarate-succinate promitochondrial redox-shuttle (**Figure 2.8**).

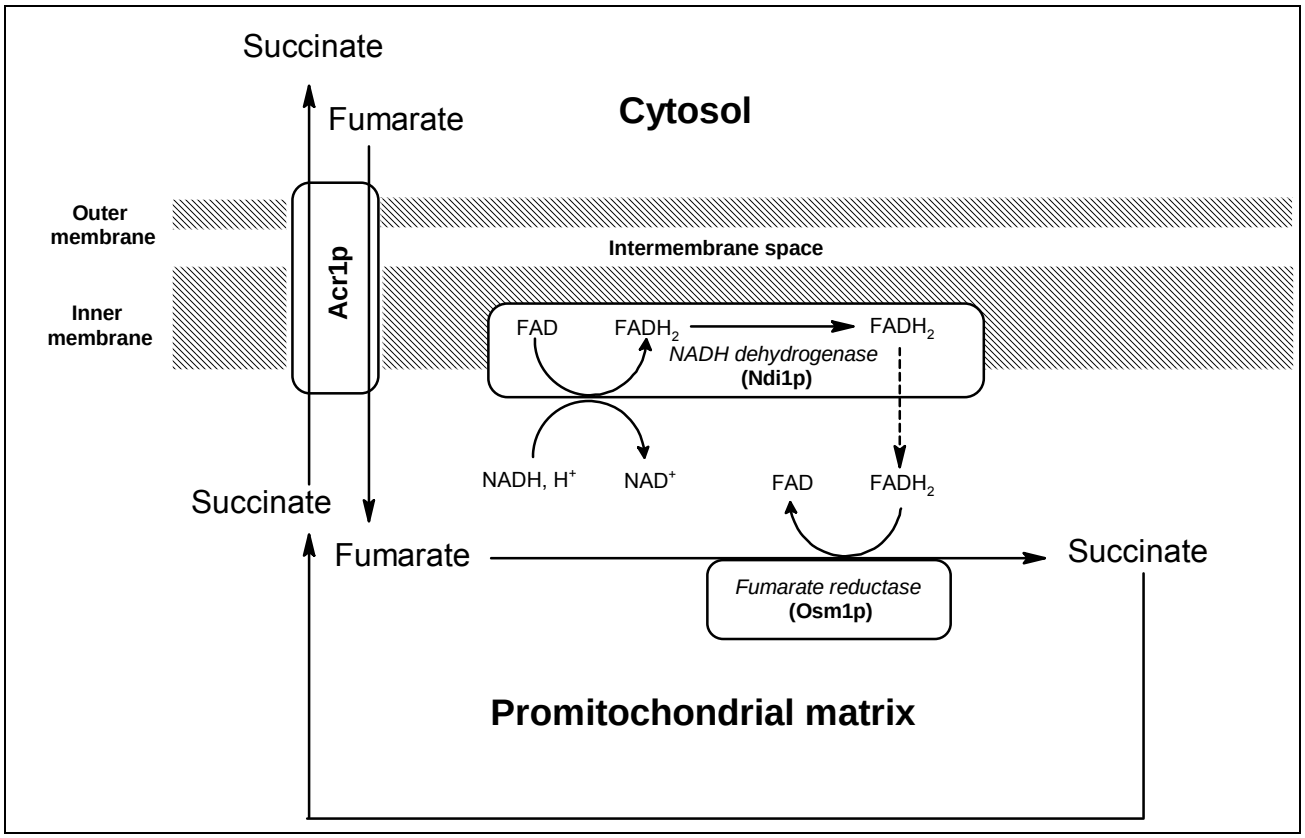

Figure 2.8 Fumarate-succinate promitochondrial redox shuttle

Genevois (1936) noticed a relationship between the amounts of succinate and glycerol produced by yeasts during alcoholic fermentation. He therefore formulated an empirical equation to represent the relationship between glycerol and other quantitatively important by-products of alcoholic fermentation by yeasts (Genevois, 1951; Ribéreau-Gayon *et al*., 1956; Oura, 1976, 1977). The Genevois equation takes on the following form:

$$Glycerol = Acetaldehyde + 2Acetate + 2Acetoin + Butyleneglycol + 5Succinate$$

The coefficient of each variable in this equation happens to be related to the number of NADH molecules that are produced or consumed during the formation of each

fermentation by-product (Oura, 1976; 1977). With 90% accuracy, Genevois' equation (Ribéreau-Gayon *et al*., 1956; Florenzano, 1975; Oura, 1976, 1977) indicates that 5 units of NADH are produced for every molecule of succinate that is produced during alcoholic fermentation. Therefore, it can be concluded that most of the succinic acid produced by fermenting yeasts, are formed from glycolytic pyruvate via the oxidative branch of the TCA cycle (Oura, 1976; 1977). This finding suggests that the reoxidization of NADH is not the principle role of succinic acid production by fermenting yeast. In fact, if the main purpose of succinic acid production was to free yeast cells of excess NADH during anaerobic fermentation, more succinic acid should be produced in media with ammonium as the only nitrogen source than in media with a mixture of amino acids (Albers *et al.*, 1996). Yet, this is not what happens, which is in direct contrast with what happens to glycerol production (Albers *et al.*, 1996).

2.3.2 BIOMASS FORMATION

Muratzubaki (1987) found that succinic acid production is related to the total amount of yeast biomass produced during alcoholic fermentation, which according to him, is caused by an overall increase in the activity of enzymes that are involved in the formation of succinate. This finding was confirmed by others (Verduyn *et al*., 1990; Albers *et al*., 1996), who's data also indicated that increased biomass production resulted in higher concentrations of succinic acid within the fermented media. Heerde & Radler's (1978) result however, showed that the strongest yeast growth (greatest biomass) did not always result in the highest concentrations of succinic acid being produced during alcoholic fermentation, and *vice versa*. Kleinzeller (1941) found that slightly more succinic acid was produced during alcoholic fermentation when the initial yeast population of the must was less dense. If a smaller inoculum of yeast is introduced to the must, yeasts will have to grow more or, in other words, produce more biomass before the maximum cell density is reached. However, Kleinzeller's (1941) findings are in direct contrast with the results obtained by Shindo *et al*. (1993). The latter mentioned authors demonstrated that immobilized yeasts (confined to alginate beads and therefore unable to grow) produced more succinic acid than free actively growing yeasts when the size of the immobilized yeast population were notably larger than the inoculum of free yeast cells.

Other explanation exists for the apparent link between yeast biomass- and succinic acid production during alcoholic fermentation, in addition to the one provided by Muratsubaki (1987). As much as 40 - 60% of yeast cells' dry mass consist of proteins (Verduyn *et al*., 1990; Albers *et al*., 1996; Albers *et al.*, 1998b). Various amino acids are needed for protein biosynthesis during yeast cell proliferation (biomass formation), but even a complex growth medium, such as grape juice, seldom contains adequate amounts of all the required amino acids. The biosynthesis of many of these amino acids involves transamination reactions, in which glutamate usually serve as the amino group donor (Jones & Fink, 1982; Hinnebusch, 1988; Walker, 1998b). Transamination reactions can be divided into two half-reactions. During the first half of a transamination reaction, an amino

group is removed from glutamate (donor) by a transaminase, which results in the formation of α-ketoglutarate. Glutamate's amino group is then transferred from the enzyme onto a different α-keto acid in order to produce a new amino acid. Transaminases catalyze near-equilibrium reactions (Horton *et al*., 1996). The direction in which transamination reactions will proceed inside yeasts are therefore determined by the rate of glutamate formation (substrate concentration) and the rate of α-ketoglutarate removal (reaction product concentration). α-Ketoglutarate formed during transamination reactions can be removed via two metabolic routes in yeasts. Both pathways in yeasts end up in the formation of succinate during alcoholic fermentation and/ or anaerobic growth on sugars due to the intteruption of the TCA cycle at the level of the succinate dehydrogenase complex (Camarasa *et al*., 2003). The one metabolic pathway is known as the GABA bypass (**Section 2.2.3**) and the other one is made up out of two reactions that are respectively catalyzed by α-ketoglutarate dehydrogenase and succinyl-CoA synthetase. In other words, succinate production is linked to biomass formation, because the reactions that ensure the removal of α-ketoglutarate during transamination reactions lead to the formation of succinate. Already mentioned is the fact that yeast biomass formation (protein and nucleic acid biosynthesis) results in a net production of NADH (Gommers *et al*., 1988; Verduyn *et al*., 1990). Most of the NADH produced during the biosynthesis of amino acids and nucleotides occur within yeast (pro)-mitochondria. Rough calculations show that between 30 and 50% of the NADH produced during amino acid biosynthesis is generated within a yeast cell's (pro)-mitochondrial matrix (Nissen *et al*., 1997). The reductive formation of succinate is involved in the reoxidation of yeast promitochondrial NADH during fermentation (**Section 2.3.1**), which helps explain why succinic acid production during alcoholic fermentation appears to be related to yeast biomass formation.

2.3.3 OSMOTIC STRESS

The disruption of *OSM1* in yeasts increases their sensitivity towards osmotic stress (Singh & Sherman, 1978). As mentioned before, *OSM1* in yeasts encodes promitochondrial fumarate reductases (Muratsubaki *et al*., 1998), which catalyze the reductive formation of succinate from fumarate within yeasts. This implies that the reductive formation of succinate is needed within yeasts during growth in media with high osmolarity (low water activity).

Yeasts produce glycerol and retain it inside of them during growth within hypertonic (high osmolarity) media (Blomberg & Adler, 1989; André *et al*., 1991; Nevoigt & Stahl, 1997; Prior & Hohmann, 1997) in order to prevent dehydration. The *FPS1*-encoded glycerol permease of yeast plasma membranes therefore becomes inactive during growth in hyperosmolar (hypertonic) media (Luyten *et al*., 1995; Tamas *et al*., 1999), while the specific activities of the *GPD1*-encoded glycerol-3-phosphate dehydrogenase and *GPP2*-encoded glycerol-3-phosphatase within *S. cerevisiae* yeasts are induced and elevated under such conditions (Varela *et al*., 1992; Albertyn *et al*., 1994; Norbeck *et al*., 1996; Norbeck & Blomberg, 1996; Ansell *et al*., 1997; Erasmus *et al*., 2003). An increase in

glycerol production necessitates an equimolar increase in the formation of NADH within yeast due to a shift in redox balance (van Dijken & Scheffers, 1986; Nevoigt & Stahl, 1997). Under conditions of osmotic stress and growth on fermentable sugars, this requirement seems to be partially met by a decrease in the reduction of acetaldehyde to ethanol on the one hand, and an increase in the oxidation of acetaldehyde to acetate on the other hand (Blomberg & Adler, 1989; Michnick *et al*., 1997; Remize *et al*., 1999; Erasmus *et al*., 2004). As explained in **Section 2.3.1**, the reductive formation of succinate from fumarate by promitochondrial fumarate reductases (Osm1p) forms part of a promitochondrial redox shuttle that is needed for the reoxidation of NADH within promitochondria. However, FAD^+ of promitochondrial fumarate reductases (Osm1p) can bind to either the internal or the external NADH dehydrogenases of (pro)mitochondria, since both types are located within the inner (pro)mitochondrial membrane (de Vries & Marres, 1987; Bakker *et al*., 2001). This implies that the reductive formation of succinate by Osm1p within (pro)-mitochondria can be coupled to the oxidation of cytosolic NADH. An initial increase in glycerol production and decrease in ethanol production during growth in media with high concentrations of fermentable sugar may lead to an overproduction of acetate from acetaldehyde, which can cause a deficiency in a cytosolic NAD^+. Since succinate formation within (pro)-mitochondria can be coupled to the reoxidation of cytoplasmic NADH, redox balance within yeasts can be restored under the above described conditions by the activity of (pro)-mitochondrial fumarate reductase (Osm1p) and external (pro)mitochondrial NADH dehydrogenases. This might explain why *OSM1*-encoded fumarate reductases are needed during growth in hypertonic media, such as grape juice.

2.4 DIFFERENCES BETWEEN VARIOUS YEASTS

Yeasts differ with regard to the amount of succinic acid they produce during alcoholic fermentation (Florenzano, 1975; Heerde & Radler, 1978; Shimazu & Watanabe, 1981; Rainieri *et al*., 1999a). However, most literature that reports the succinic acid producing capabilities of different yeasts contains outdated yeast nomenclature. To make matters even worse, the taxonomic identity of the yeasts mentioned within many of the publications were determined by means morphological and physiological tests, whereas the most recent methods of yeast classification are based on the genetic make-up of the yeasts. In other words, some of the yeasts mentioned within these reports may have belonged to the same species at the time of publication, but are not necessary genetically related. It is often impossible to determine from past literature whether differences between the succinic acid producing capabilities of yeasts are species-dependant or strain-related. Nevertheless, the most important changes to the classification of wine and brewing yeasts are dicussed in the following section in order to make better sense out of it all.

2.4.1 CLASSIFICATION OF WINE AND BREWING YEASTS

Yeasts of various genera are involved in the production of wine from non-sterile grape juice. For the most part however, grape juice fermentations are dominated and carried out to completion by yeasts that belong to the genus *Saccharomyces* (Heard & Fleet, 1985; Boulton *et al*., 1998b; Pretorius *et al*., 1999; Ribéreau-Gayon *et al*., 2000a). Species of *Saccharomyces* have been reclassified several times over the past couple of years. **Table 2.1** summarizes the most important classification and reclassification schemes for *Saccharomyces* between 1952 and 1998.

Oenologists and commercial wine yeast producing companies still make use of Yarrow's classification scheme (1984) to identify and characterize wine yeasts (Boulton *et al*. 1998b; Ribéreau-Gayon *et al*., 2000a). This classification scheme was mainly based on the molar percentages guanine and cytosine in the nuclear deoxyribonucleic acid (DNA) of yeasts (Yarrow, 1975; Yarrow, 1984), which led to the incorporation of seventeen previously distinct species of *Saccharomyces* into one species, namely *S. cerevisiae* (Boulton *et al*., 1998b; Pretorius *et al*., 1999; Ribéreau-Gayon *et al*., 2000a). Oenologists consequently started to differentiate between seventeen different physiological races (syn. varieties, subspecies) of *S. cerevisiae*, based on the ability of yeasts to ferment different sugars (Masneuf *et al*., 1996; Boulton *et al*., 1998b; Ribéreau-Gayon *et al*., 2000a). The nomenclature and sugar fermentation profiles of *S. cerevisiae*'s (Yarrow, 1984) physiological races (p.r.) correspond with that of the previous yeast classification system's (van der Walt, 1970) 17 *Saccharomyces* species that were incorporated into *S. cerevisiae* in 1984 (Boulton *et al*., 1998b; Pretorius *et al*., 1999; Ribéreau-Gayon *et al*., 2000a). A list of *S. cerevisiae*'s different physiological races, along with each one's sugar fermentation profile, is provided in **Table 2.2**. The most important physiological races of *S. cerevisiae* to the winemaking and brewing industries are *S. cerevisiae*'s p.r. *cerevisiae*, *uvarum*, *bayanus*, *steineri* (*italicus*), *capensis* and *chevalieri* (Boulton *et al*., 1998b).

It is important to point out a few wine and beer producing yeasts that were once classified as distinct species of *Saccharomyces* before they were incorporated into *S. cerevisiae*, *S. bayanus* or *S. uvarum* by van der Walt (1970). *S. ellipsoideus* and *S. vini* were incorporated into *S. cerevisiae* and are often used for the production of still table wines (Pretorius *et al*., 1999). Lager brewing strains of *S. carlsbergensis* (Pedersen, 1986; Hansen & Kielland-Brandt, 1994) became part of the specie, *S. uvarum* (Pretorius *et al*., 1999). The *Champagne* (*Prise de Mousse*) yeast *S. oviformis* (Pretorius *et al*., 1999), as well as the lager brewing yeasts *S. monacensis* and *S. pastorianus* (Pedersen, 1986; Hansen & Kielland-Brandt, 1994) were reclassified as *S. bayanus* (Masneuf *et al*., 1998; Boulton & Quain, 2001). Most of the film- or pellicle forming yeasts (flor yeasts) that are used for the production of sherry wines were known as strains of *S. beticus* and *S. cheresiensis* up until 1970 (Pretorius *et al.*, 1999; Boulton *et al*., 1998b), but were then also incorporated into *S. bayanus* (van der Walt, 1970; Pretorius *et al.*, 1999).

The 14 *Saccharomyces* species that appear in the most recent publication of *The Yeast - a taxonomic study* (Vaughan-Martini & Martini, 1998) have been divided up into three groups, called genus complexes. *Saccharomyces*' three genus complexes are *S. sensu stricto*, *S. sensu lato* and *S. kluyveri* (Barnett, 1992; Vaughan-Martini & Martini, 1993b; Ribéreau-Gayon *et al*., 2000a). The reclassification of *Saccharomyces* yeasts by Vaughan-Martini & Martini (1998) was mainly based on the spectrophotometric nuclear-DNA hybridization (reassociation) studies that were used to determine the degree of homology between different yeasts' nuclear DNA (Vaughan-Martini & Kurtzman, 1985, 1988; Vaughan-Martini & Martini, 1987, Vaughan-Martini, 1989). The seventeen physiological races of *S. cerevisiae* were all incorporated into *S. sensu stricto* during reclassification in 1998 (Vaughan-Martini & Martini, 1998; Ribéreau-Gayon *et al*., 2000a).

Most wine and brewing yeasts were therefore incorporated into species of *S. sensu stricto*. Hence, the focus will only be on species that belong to the latter mentioned genus complex. At first, *Saccharomyces. sensu stricto* included only three species, *i.e. S. cerevisiae*, *S. bayanus* and *S. pastorianus* (Vaughan-Martini & Kurtzman, 1985; Vaughan-Martini & Martini, 1987), but *S. paradoxus* was incorporated into *S. sensu stricto* (Vaughan-Martini, 1989) before the latest version of *The Yeast - a taxonomic study* (Vaughan-Martini & Martini, 1998) was released in 1998 (Ribéreau-Gayon *et al*., 2000a). Then in 2000, Naumov *et al.* (2000) identified and characterized three new species of *S. sensu stricto*, *i.e. S. cariocanus*, *S. kudriavzevii* and *S. mikatae*. This brought the total number of *S. sensu stricto* species to seven (Rainieri *et al*., 2003).

S. pastorianus sensu (Vaughan-Martini & Martini, 1987, 1998) consist of *ex S. monacensis* and *S. pastorianus* strains, as well as *ex S. carlsbergensis* strains. *Ex S. oviformis*, *S. beticus* and *S. cheresiensis* strains were not incorporated into *S. bayanus sensu* (Vaughan-Martini & Martini, 1987, 1998) along with all the other strains of *S. bayanus* as defined by van der Walt (1970), but instead became part of *S. cerevisiae sensu* (Vaughan-Martini & Martini, 1987, 1998). S. *globosus*, S. *inusitatus*, S. *heterogenicus* and the non-*S. carlsbergensis* members of *S. uvarum* as defined by van der Walt (1970) were incorporated into *S. bayanus sensu* (Vaughan-Martini & Martini, 1987, 1998) along with the archaic species names *S. tubiformis*, *S. abuliensis* and *S. intermedius* var. *valdensis*. The remaining *Saccharomyces* species as defined by van der Walt (1970) became part of *S. cerevisiae sensu* (Vaughan-Martini & Kurtzman, 1985; Vaughan-Martini & Martini, 1987, 1998).

Table 2.1
Classification of *Saccharomyces* species from 1952 up until 1998.

1952 classification (Lodder & Kreger-van Rij, 1952)	1970 classification (Lodder, 1970)	1984 classification (Kreger-van Rij, 1984)	1998 classification (Kurtzman & Fell, 1998a)
S. bayanus	***S. bayanus*** (syn. ***S. beticus***, *S. cheriensis* ***S. oviformis***, *S. pastorianus*)		
S. oviformis			
S. pastorianus			
S. uvarum	*S. uvarum*		
S. carlsbergensis			
S. logos			
S. cerevisiae (syn. ***S. vini***)	***S. cerevisiae***		
S.c.var. ***ellipsoideus***			
S. willianus			
S. chevalieri	***S. chevalieri***		
S. fructuum			
S. italicus	*S. italicus*	***S. cerevisiae***	***S. bayanus***
S. steineri			*S. pastorianus*
			S. cerevisiae
			S. paradoxus
S. heterogenicus	*S. heterogenicus*		
	S. aceti		
	S. capensis		
	S. coreanus		
	S. diastaticus		
	S. globosus		
	S. hienipiensis		
	S. inusitatus		
	S. norbensis		
	S. oleaceus		
	S. oleaginosus		
	S. prostoserdovii		
S. exiguus	*S. exiguus*	*S. exiguus*	*S. barnettii*
			S. exiguus
			S. spencerorum
S. bailii	*S. bailii*	*Zygosaccharomyces bailii*	*Z. bailii*
S. acidifaciens			
S. elegans			
S. bisporus	*S. bisporus* var. *bisporus*	*Zygosaccharomyces bisporus*	*Z. bisporus*
S. mellis	*S. bisporus* var. *mellis*	*Zygosaccharomyces rouxii*	*Z. rouxii*
S. rouxii	*S. rouxii*		
S. rouxii var. *polymorphus*	*S. bailii* var. *osmophilus*		
	S. inconspicuus		
S. delbrueckii	*S. delbrueckii*		
S. fermentati (syn. ***S. beticus***)	***S. fermentati***		
S. rosei	***S. rosei***	***Torulaspora delbrueckii***	***T. delbrueckii***
	S. saitoanus		
	S. vafer		
	S. microellipsodes var. *osmophilus*		
S. marxianus	*Kluyveromyces marxianus*	*K. marxianus*	*K. marxianus*
S. fragilis	*Kluyveromyces fragilis*		*K. lactis*
S. lactis	*Kluyveromyces lactis*		
S. veronae	*Kluyveromyces veronae*	*K. thermotolerans*	*K. thermotolerans*
	S. amurcae	*Zygosaccharomyces cidri*	*Z. cidri*
	S. cidri		
S. microellipsodes	*S. microellipsodes*	*Zygosaccharomyces microellipsoides*	*Z. microellipsoides*
		S. servazzii	*S. servazzii*
S. pastori	*Pichia pastoris*	*Pichia pastoris*	*Pichia pastoris*
	S. dairenensis	*S. dairenensis*	*S. castellii*
			S. dairenensis
			S. rosinii
S. florentinus	*S. florentinus*	*Zygosaccharomyces florentinus*	*Z. florentinus*
	S. eupagycus		
	S. unisporus	*S. unisporus*	*S. unisporis*
	S. kluyveri	*S. kluyveri*	*S. kluyveri*
	S. telluris	*S. telluris*	*Arxiozyma telluris*
	S. kloeckerianus	*Torulaspora globosa*	*T. globosa*
	S. montanus	*Zygosaccharomyces fermentati*	*Z. fermentati*
	S. mrakii	*Zygosaccharomyces mrakii*	*Z. mrakii*
	S. transvaalensis	*Pachytichospora transvaalensis*	*S. transvaalensis*
	S. pretoriensis	*Torulaspora pretoriensis*	*T. pretoriensis*

[a] Pretorius *et al*., 1999

Table 2.2
Sugar fermentation pattern of *S. cerevisiae*'s (Yarrow, 1984) physiological races

S. cerevisiae p.r.	Galactose	Sucrose	Maltose	Raffinose	Melibiose	Starch
aceti	–	–	–	–	–	–
bayanus	–	+	+	+	–	–
capensis	–	+	–	+	–	–
cerevisiae	+	+	+	+	–	–
chevalieri	+	+	–	+	–	–
coreanus	+	+	–	+	+	–
diastaticus	+	+	+	+	–	+
globosus	+	–	–	–	–	–
heterogenicus	–	+	+	–	–	–
hienipiensis	–	–	+	–	+	–
inusitatus	–	+	+	+	+	–
norbensis	–	–	–	–	+	–
oleaceus	+	–	–	+	+	–
oleaginosus	+	–	+	+	+	–
prostoserdovii	–	–	+	–	–	–
steineri (italicus)	+	+	+	–	–	–
uvarum	+	+	+	+	+	–

[a] Ribéreau-Gayon *et al*., 2000a

DNA (genetic) fingerprinting (Nguyen & Gaillardin, 1997; Nguyen *et al*., 2000; Pulvirenti *et al*., 2000; Nguyen & Gaillardin, 2005; Naumova *et al*., 2005), hybrid fertility studies (Rainieri *et al*., 1999a; Naumov, 2000; Naumova *et al*., 2005), electrophoretic (molecular) karyotyping (Nguyen & Gaillardin, 1997; Rainieri *et al*., 1999a; Nguyen *et al*., 2000) and gene sequencing (Nguyen *et al*., 2000; Nguyen & Gaillardin, 2005; Naumova *et al*., 2005) of *S. bayanus sensu* (Vaughan-Martini & Martini, 1998) strains have shown that it can be divided up into two subgroups. The *ex S. carlsbergensis* strain CBS 1604, and the type strains of *S. uvarum* (CBS 395) and *S. abuliensis* (CBS 7001) were incorporated into *S. bayanus*' subgroup *uvarum*, whereas the type strains of *S. bayanus* (CBS 380), *S. globosus* (CBS 424), *S. heterogenicus* (CBS 425), *S. tubiformis* (CBS 431), *S. inusitatus* (CBS 1546) and *S. intermedius* var. *valdensis* (CBS 1505) became part of *S. bayanus*' subgroup *bayanus*. *S. bayanus*' two subgroups should be considered as varieties of *S. bayanus* according to Naumov (2000) and Naumova *et al*. (2005), but others in the field of yeast taxonomy suggested the reinstatement of *S. uvarum* as a distinct species of *S. sensu stricto* (Rainieri *et al*., 1999a, Nguyen *et al*., 2000; Pulvirenti *et al*., 2000; Nguyen & Gaillardin, 2005). In general however, neither one of the above mentioned two proposals have been accepted yet (Sipiczki, 2008).

Kurtzman (2003) recently suggested that the *S. sensu stricto* complex should be elevated to genus status, based on the results Kurtzman & Robnett (2003) got with multi-gene sequence analysis. According to his proposal (Kurtzman, 2003), *S. sensu stricto* should again be referred to as *Saccharomyces* and consists of *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. paradoxus*, *S. cariocanus*, *S. kudriavzevii* and *S. mikatae*.

2.4.2 SUCCINIC ACID PRODUCTION BY DIFFERENT WINE YEASTS

Out of all the *Saccharomyces* species investigated by Heerde & Radler (1978) and Shimazu & Watanabe (1981), *S. cerevisiae* strains produced the highest concentration of succinic acid during alcoholic fermentation. As a species however, *S. cerevisiae* did not produce succinic acid concentrations significantly different from that produced by other *Saccharomyces* species (Heerde & Radler, 1978). Florenzano (1975) on the other hand, reported that *S. chevalieri* strains produced more succinic acid on average than *S. cerevisiae* (*S. ellipsoideus*), *S. bayanus* and *S. italicus* strains during fermentation of grape juice.

Cryotolerant (cryophilic) wine yeasts, as first defined by Castellari *et al.* (1992, 1994), tend to produce significantly higher concentrations of succinic acid than mesophilic and thermotolerant wine yeasts during grape juice fermentations at moderate fermentation temperatures (Castellari *et al*., 1994, 1995; Giudici *et al.*, 1995; Bertolini *et al*., 1996; Zambonelli *et al*., 1997; Rainieri *et al*., 1998a, 1998b, 1999b). Cryotolerant wine yeasts grow well at temperatures between 6 and 12°C, but cannot grow at temperatures above 34°C (Walsh & Martin, 1977; Castellari *et al*., 1992; Kishimoto, 1994). In the case of mesophilic wine yeasts though, temperatures become growth limiting below 12°C, as well as between 37 and 40°C (Walsh & Martin, 1977; Castellari *et al*., 1992). The term thermotolerant is used to describe wine yeasts that can still grow at temperatures above 40 and 42°C (Hacking *et al*., 1984; Anderson *et al*., 1988; Rainieri *et al*., 1996).

Most cryotolerant wine yeasts belong to *S. cerevisiae*'s *ex* physiological race *uvarum*, but some of them belong to *S. cerevisiae*'s former physiological race *bayanus* (Castellari *et al*., 1992, 1994). However, Castellari *et al*. (1992) pointed out that the above two mentioned physiological races of *S. cerevisiae sensu* (Yarrow, 1984) included both cryotolerant and non-cryotolerant strains, which confirms Walsh & Martin's (1977) earlier findings. Vaughan-Martini & Martini (1993, 1998) incorporated all of the cold-tolerant *ex S. carlsbergensis* strains of *S. uvarum* (*S. cerevisiae* p.r. *uvarum*) into *S. pastorianus*, while the remaining cold-tolerant *S. uvarum* (*S. cerevisiae* p.r. *uvarum*) strains were incorporated into the *S. sensu stricto* species, *S. bayanus* (Vaughan-Martini & Martini, 1993, 1998). All non-cryotolerant *ex S. uvarum* and *ex S. carlsbergensis* strains (Walsh & Martin, 1977), are now part of *S. cerevisiae sensu* (Vaughan-Martini & Martini, 1993, 1998). In fact, results from Rainieri *et al.*'s (1999a) study have shown that the wine yeasts referred to as "cryotolerant wine yeasts" (Castellari *et al*.., 1992, 1994; 1995; Giudici *et al.*, 1995; Bertolini *et al*., 1996; Zambonelli *et al*., 1997; Rainieri *et al*., 1998a, 1998b, 1999b) only include cold-tolerant strains of *S. uvarum* (*S. cerevisiae* p.r. *uvarum*) that now belong to *S. bayanus*' subgroup *uvarum* (*S. bayanus* var. *uvarum*). In other words, although the growth limiting temperatures for strains of *S. pastorianus* and S*. bayanus* var. *bayanus* are the same than that for the "cryotolerant wine yeasts", their fermentation by-product profiles differ notably from the latter mentioned group of wine yeasts (Rainieri *et al*., 1999a). Strains of the formerly known *S. oviformis* are also cold-tolerant wine yeasts (Vaughan-Martini & Martini, 1993) and belonged to *S. bayanus*

(*S. cerevisiae* p.r. *bayanus*) up until 1998, when they were incorporated into *S. cerevisiae* (Vaughan-Martini & Martini, 1998). Ex-*S. oviformis* (*Champagne*, *Prise de Mousse*) strains are vigorous fermenters (van Wyk & Pretorius, 1990; Henschke, 1997a), whereas cryotolerant wine yeasts *sensu* (Castellari *et al*., 1992; 1994) ferment much slower than mesophilic wine yeasts at moderate to high fermentation temperatures (Kishimoto, 1994). Hence, ex-*S. oviformis* strains should not be referred as "cryotolerant wine yeasts", despite their ability to grow well at cold temperatures (< 12°C).

Wine-related non-*Saccharomyces* yeasts, such as species of *Candida*, can contribute significantly to the fermentation by-product profile of wine (Heard & Fleet, 1985, 1988). Wines with high concentrations of succinic acid are produced when grape juices are fermented by *C. stellata* yeasts (Ciani & Ferraro, 1996; Ferraro *et al*., 2000). This is also true of the wines produced *Saccharomyces bailii* (now *Z. bailii*) yeasts (Shimazu & Watanabe, 1981). Strains of *Saccharomyces rosei* (now *T. delbrueckii*) were often selected for the production of natural sweet wines, since they produce very little acetic acid, even when they ferment grape juices with very high sugar contents (Henschke, 1997a; Pretorius *et al*., 1999). When acetic acid is excreted during fermentation, its formation from pyruvate directs hexose-derived carbon away from the metabolic pathways that lead to the formation of succinic acid during alcoholic fermentation. Hence, it is possible that high concentrations of succinic acid will be produced when grape juices are fermented by strains of *T. delbrueckii*.

2.5 MACRONUTRIENTS' INFLUENCE ON SUCCINIC ACID PRODUCTION

Yeasts require sources of carbon, hydrogen, oxygen, nitrogen, phosphorous, sulphur, potassium and magnesium at macronutrient levels, which approximates at least one millimolar of each element (Walker, 1998a). Although elemental oxygen is a macronutrient of wine yeasts, molecular oxygen may be regarded as a micronutrient of wine yeasts during fermentative growth, since only a few micromolars of it is required for the biosynthesis of essential unsaturated long-chain fatty acids and ergosterol (O'Connor-Cox *et al*., 1996).

2.5.1 YEAST ASSIMILABLE CARBON SOURCES

The most important yeast assimilable carbon and energy (dissimilable carbon) sources from grapes are D-glucose (dextrose) and D-fructose (levulose). Together, these two fermentable sugars make up more than 95% of the total carbohydrate content of grapes (Kliewer, 1965, 1966; Margalit, 1997; Boulton *et al*., 1998c). Glucose and fructose both belong to a class of monosaccharides that are called hexoses, which refers to the six carbon atoms in their respective chemical structures (Horton *et al*., 1996). The sum total of glucose and fructose within juice from mature grapes normally vary between 160 and 260 g/L, which is approximately 18 - 28°Brix (Kliewer, 1967; Jackson, 1994; Margalit, 1997). However, juices from *Botrytis*-infected grapes contain up to 500 g/L sugar due to

increased water losses from injured berries (Erasmus *et al*., 2003). Glucose accumulates earlier within grapes than fructose, but by the time the berry is ripe, the concentrations of glucose and fructose are nearly equal. However, the fructose concentration exceeds that of glucose within overripe berries (Kliewer, 1967; Rankine, 1998). The glucose to fructose ratio of grape juice ranges from 0.7 - 1.1 (Kliewer, 1967; Margalit, 1997). *Vitis vinfera* grapes also contain small quantities of other hexoses, such as rhamnose (0.15 – 0.4 g/L), mannose, galactose and fucose (Kliewer, 1965, 1966; Margalit, 1997; Boulton *et al*., 1998c). However, glucose and fructose are the only hexoses within grape juice that can be fermented by all wine yeasts (Barnett, 1997).

Grapes also contain pentoses (Margalit, 1997; Boulton *et al*., 1998c), which are monosaccharides that are made up out with five carbon atoms (Horton *et al*., 1996). L-Arabinose and D-xylose are the most important pentoses within grapes. The arabinose content of grapes ranges from 0.5 and 1.5 g/L, while the xylose content thereof varies from non-detected to about 0.5 g/L. Very small amounts of D-ribose may also be present within grape juice. On average though, pentoses represent only 1.1% of the carbohydrates found within grapes (Margalit, 1997). Although pentoses can be assimilated by wine yeasts, they are unable to ferment pentoses due to the absence of xylose reductase and xylitol dehydrogenase. Xylose isomerase, an enzyme which converts xylose directly to xylulose, is also not found within wine yeasts (Walker, 1998b).

Sucrose (saccharose) is the most abundant disaccharide within grapes (Kliewer, 1965, 1966; Margalit, 1997). Grapes usually contain between 2 and 10 g/L sucrose, which constitute approximately 3.1% of grapes' carbohydrate content (Margalit, 1997). However, sucrose is readily hydrolyzed to fructose and glucose by yeast- and grape invertases (Barnett, 1997; Walker, 1998b). Negligible amounts of other disaccharides, such as maltose and melibiose, have also been detected within grapes (Kliewer, 1965, 1966; Boulton *et al*., 1998c). The latter two mentioned disaccharides can be utilized by wine yeasts, because they possess the necessary enzymes (invertase and α-galactosidase) to convert them into their compositional monosaccharides (Walker, 1998b). These same enzymes also enable wine yeasts to utilize raffinose (trisaccharide) and stachyose (tetrasaccharide), which are present within grapes in minute quantities (Kliewer, 1965, 1966; Boulton *et al*., 1998c).

Structural polysaccharides, such as cellulose, hemicelluloses and pectin are contained within the walls of cells that make up the various tissues of grapes (Ribéreau-Gayon *et al*., 2000c). The first mentioned two polysaccharides are insoluble in aqueous media and will therefore precipitate out of solution within grape juice. The majority of pectin within grapes is bound to cell walls, but grapes also contain free water-soluble pectin (gums) that increases with ripening (Boulton *et al*., 1998c; Ribéreau-Gayon *et al*., 2000c). Wine yeasts cannot utilize cellulose and hemicelluloses (Walker, 1998b), but some of them are capable of utilizing the D-galacturonic acid that is liberated from pectin by the activities of polygalacturonases (pectinases) from grapes, moulds (*Botrytis cinerea, Aspergillus niger*) and those contained within commercial preparations added by winemakers for clarification purposes (Boulton *et al*., 1998c; Ribéreau-Gayon *et al*., 2000c). The pectin content of

grapes ranges from 0.2 - 4.0 g/L, which represents approximately 0.6% of the carbohydrates within grapes (Margalit, 1997).

The most important organic acids of grapes are tartaric, malic and citric acid (Margalit, 1997; Ribéreau-Gayon *et al*., 2000d). Yeasts cannot metabolize tartaric acid (Radler, 1986; Jackson, 1994) and hardly any citric acid is taken up from the growth medium by fermenting yeasts (Coote & Kirsop, 1974; Shimazu & Watanabe, 1981 Radler, 1986; Jackson, 1994). However, considerable amounts of malic acid can be consumed by fermenting wine yeasts, depending on the fermentation medium's temperature, original malic acid content and pH (Fatichenti *et al*., 1984). Between three and 45% of grape juice's malic acid content can be consumed by wine yeasts during alcoholic fermentation (Rankine, 1966; Fuck & Radler, 1972; Radler, 1993; Fowles, 1992). On the other hand, wine-related yeasts from species such as *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii* can metabolize all the malic acid within grape juice during alcoholic fermentation (Rankine, 1966; Kuzynski & Radler, 1982; Radler, 1986).

2.5.1.1 Hexoses

The concentration of succinic acid produced during alcoholic fermentation is proportional to the amount of sugar fermented (Pasteur, 1860; Kleinzeller, 1941; Coote & Kirsop, 1974; Heerde & Radler, 1978; Shimazu & Watanabe, 1981; Coulter *et al*., 2004). In fact, the amount succinic acid produced by fermenting yeasts is directly related to the growth medium's initial hexose content up to a concentration of about 8% (w/v) hexose (Heerde & Radler, 1978). According to Shimazu & Watanabe's (1981) results though, succinic acid production by wine yeasts increases linearly with increasing hexose concentrations of up to 22% (w/v). At hexose concentrations greater than 22% (w/v), acetic acid production by wine yeasts will increase at the expense of succinic acid production, which explains why there is a sharp decline in succinic acid production at hexose concentrations above 30% (w/v). The type of hexose fermented by wine yeasts has no influence on the amount of succinic acid produced during alcoholic fermentation (Pasteur, 1860; Kleinzeller, 1941).

Increasing levels of fermentable sugars in the growth medium increases the tendency for water to diffuse out of yeast cells. Osmotic stress up regulates the expression of the glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase encoding genes within *S. cerevisiae* (Varela *et al*., 1992; Albertyn *et al*., 1994; Norbeck *et al*., 1996; Norbeck & Blomberg, 1996; Ansell *et al*., 1997; Erasmus *et al.*, 2003). In other words, increasing extracellular concentrations of hexoses up-regulates the production of glycerol by fermenting yeasts (Caridi, 2003; Erasmus *et al*., 2004), which increases the intracellular ratio of NAD^+ to NADH (van Dijken & Scheffers, 1986; Nevoigt & Stahl, 1997). The balance between the reduced and oxidized forms of NAD is restored within fermenting yeasts by a decrease in ethanol production and an increase in acetic acid production (Shimazu & Watanabe, 1981; Blomberg & Adler, 1989; Michnick *et al*., 1997; Remize *et al*., 1999; Caridi, 2003; Erasmus *et al*., 2003, 2004). An increase in acetic acid

production directs large amounts of hexose-derived carbon away from the metabolic pathways that lead to the formation of succinate under fermentative conditions. In other words, sugar induced osmotic stress will decrease the production of succinic acid during fermentation due to an increase in acetic acid production (Caridi, 2003). However, data obtained by Erasmus *et al*. (2003) indicates that succinic acid production by fermenting yeasts should increase when they grow in the presence very high sugar concentrations (≥ 30% w/v). Sugar-induced osmotic stress stimulate the expression of genes that encode hexose-permeases (*HXT1* and *HXT5*), sugar transport-like proteins (*STL1* and *YBR241C*), glycolytic enzymes (*GLK1*, *FBP1*, *TDH1*, *GPM2*, *ENO1*, and *PYK2*), pyruvate carboxylase (*PYC1*) and enzymes of the GABA bypass (*GDH3*, *GAD1*, *UGA1* and *UGA2*). The increased biosynthesis of hexose transporters and glycolytic enzymes will increase the rate of hexose uptake by yeasts and its conversion into pyruvate and ATP, while elevated intracellular levels of pyruvate carboxylases and ATP stimulate pyruvate's conversion into oxaloacetate (Miller & Atkinsin, 1972). Since sugar-induced osmotic stress up-regulates the expression of $NADP^+$-dependant glutamate dehydrogenases, more α-ketoglutarate will then be turned into glutamate, which in turn, will be converted into succinate to a greater extent due to the increased levels of GABA bypass enzymes. These findings are supported by the fact that over-expression of glycerol-3-phosphate dehydrogenases in fermenting yeasts caused an increase in succinic acid production, despite a simultaneous increase in acetic acid production (Michnick *et al*., 1997; Remize *et al*., 1999). However, less succinic acid is produced by yeast in media with very high sugar concentrations, unless the growth medium also contains compounds that inhibit acetic acid production (Caridi, 2003).

By using uniformly ^{14}C-labelled glucose, Thoukis *et al*. (1965) demonstrated that a portion of the hexoses taken up by yeasts is converted into succinic acid during alcoholic fermentation. Calculations show that 1 - 4% of the hexoses consumed by fermenting yeasts are converted into succinic acid (Kleinzeller, 1941; Muratsubaki, 1987). On the other hand, Albers *et al*. (1998a) were unable to detect any labelled succinic acid among the fermentation products of wine yeasts grown in the presence of ^{14}C-labelled glucose. However, as pointed out by Camarasa *et al*. (2003), the initial concentration of ^{14}C-labelled glucose in the fermentation medium used by Albers *et al*. (1998a) was very low. Since most of the carbon from glucose is converted into ethanol, carbon dioxide and glycerol during alcoholic fermentation, undetectable low levels of radioactive carbon were probably incorporated into succinic acid from the small amounts of ^{14}C-labelled glucose.

The cell walls of yeasts are permeable to hexose-sugars, but permeases are required for the transport thereof across their plasma membranes. Glucose and fructose are taken up by means of facilitated diffusion in the case of *S. cerevisiae* yeasts (Kotyk, 1967; Cirillo, 1968; Bisson, 1993; Barnett, 1997; Boulton *et al*., 1998b; Walker, 1998a). Strains of *S. bayanus* and *S. pastorianus* on the other hand, possess an active transport system for the uptake of fructose (Boulton & Quain, 2001b).

Once inside the cell, each hexose molecule is converted into two pyruvate molecules via the reactions of the glycolytic pathway (**Figure 2.6**). Glycolytic enzymes are all located

within the yeast cytoplasm (Boulton *et al*., 1998b; Walker, 1998b). Most hexose-derived pyruvate is transformed into ethanol and carbon dioxide during alcoholic fermentation, but some of it can be converted into intermediates of the TCA cycle (Boulton *et al*., 1998b). Pyruvate joins the reactions of the TCA cycle by being converted into oxaloacetate and acetyl-coenzyme A (acetyl-CoA).

Pyruvate can be converted directly into acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex or it can be converted into acetyl-CoA via the reactions of the pyruvate dehydrogenase bypass (Holzer & Goedde, 1957; Pronk *et al*., 1996; Steensma, 1997; Remize *et al.*, 2000). The latter mentioned metabolic route (**Figure 2.10**) consists of three reactions, which are each catalyzed by a different enzyme. Acetyl-CoA's formation via the pyruvate dehydrogenase bypass starts off with the decarboxylation of pyruvate to acetaldehyde. This reaction is catalyzed by cytosolic pyruvate decarboxylase (van Urk *et al*., 1989; Pronk *et al*., 1996; Steensma, 1997). The pyruvate dehydrogenase complex's affinity for pyruvate is higher than that of pyruvate decarboxylase (Holzer, 1961; Pronk et al., 1996), but these two enzymes do not compete directly for pyruvate, because they are located in different sub-cellular compartments. However, yeast mitochondria's affinity for pyruvate is similar to pyruvate decarboxylase's affinity for pyruvate (van Urk *et al*., 1989). For the most part, the reaction catalyzed by pyruvate decarboxylase is therefore by-passed at low intracellular concentrations of pyruvate, which makes the respiratory dissimilation of pyruvate by the pyruvate dehydrogenase complex possible. At high intracellular concentrations of pyruvate though, most of it will be decarboxylated by pyruvate decarboxylase, thereby triggering the start of alcoholic fermentation (Holzer, 1961; Pronk *et al*., 1996). The E3-subunit of the pyruvate dehydrogenase complex (dihydrolipoamide dehydrogenase) is subject to carbon-catabolite repression (Roy & Dawes, 1987; Pronk *et al*., 1996) and the number of promitochondria in anaerobically-grown fermenting yeasts is much less than the number of mitochondria in respiring yeasts (Lukins *et al*., 1966; Rosenfeld *et al*., 2004). Most acetyl-CoA will therefore be formed via the pyruvate dehydrogenase bypass during alcoholic fermentation. The next step in the pyruvate dehyrogenase bypass involves the oxidation of acetaldehyde to acetate by acetaldehyde dehydrogenases (Holzer & Goedde, 1957; Pronk *et al*., 1996; Steensma, 1997; Remize *et al.*, 2000). Two types of acetaldehyde dehydrogenase exist within *S. cerevisiae* yeasts. Cytosolic acetaldehyde dehydrogenases are activated by Mg^{2+} in wine yeasts (Dickinson, 1996) and are encoded by *ALD2*, *ALD3* and *ALD6* (Navarro-Avino *et al*., 1999). The mitochondrial isoforms of this enzyme on the other hand, are activated by K^+ (Jacobson & Bernofsky, 1974) and are encoded by *ADL4* and *ADL5* (Navarro-Avino *et al*., 1999). However, only cytosolic acetaldehyde dehydrogenases take part in the reactions of the pyruvate dehydrogenase bypass. Those encoded by *ALD2* and *ALD3* make use of NAD^+ as cofactor, but are repressed by fermentable sugars (Navarro-Avino *et al*., 1999). This implies that the principle acetaldehyde dehydrogenase within fermenting wine yeasts' cytoplasm is encoded by *ALD6*. Ald6p can use NAD^+ as cofactor, but it prefers $NADP^+$ as coenzyme (Meaden *et al*., 1997). Acetate is then converted into acetyl-coenzyme A by acetyl-coenzyme A synthetase during the final step

of the pyruvate dehydrogenase bypass. Acetyl-coenzyme A synthetases are encoded by *ACS1* and *ACS2* in *S. cerevisiae*. *ACS2* is constitutively expressed in *S. cerevisiae* yeast, but the expression of *ACS1* is subject to carbon-catabolite repression (Kratzer & Shuller, 1995; van den Berg *et al*., 1996; Steensma, 1997).

Oxaloacetate can be formed from pyruvate or its glycoltic precursor, phosphoenolpyruvate (PEP). The conversion of pyruvate into oxaloacetate is catalyzed by pyruvate carboxylase. Phosphoenolpyruvate on the other hand, is converted into oxaloacetate by PEP carboxykinase (Horton *et al*., 1996). The specific activity of pyruvate carboxylase remains high within yeasts during alcoholic fermentation, but the PEP carboxykinases of wine yeasts are strongly repressed by fermentable sugars (Haarsilta & Oura, 1975). In other words, pyruvate carboxylase is responsible for converting pyruvate into oxaloacetate within wine yeasts during alcoholic fermentation (Losada *et al*., 1964; Gailiusis *et al*., 1964; Pronk *et al*., 1996). Two isoforms of the latter mentioned enzyme have been identified within *S. cerevisiae* yeasts (Stucka *et al*., 1991; Walker *et al*., 1991; Pronk *et al*., 1996), but the *PYC2*-encoded isoform is subject to carbon-catabolite repression (Brewster *et al*., 1994). Both pyruvate carboxylase isoforms are located within the cytosol of wine yeasts (van Urk *et al.*, 1989; Rohde *et al.*, 1991; Walker *et al.*, 1991). The *in vivo* activity of wine yeasts' pyruvate carboxylases is positively related to the intracellular concentrations of the enzyme's substrates, *i.e.* pyruvate, bicarbonate and ATP (Ruiz-Amil *et al*., 1965; Miller & Atkinsin, 1972; Pronk *et al*., 1996). Pyruvate carboxylase's specific activity, as well as its affinity for its substrates, is also stimulated by the presence of fatty acyl-coenzyme A esters, such as acetyl-CoA (Miller & Atkinsin, 1972; Myers *et al*., 1983; Pronk *et al*., 1996). Aspartate is a competitive inhibitor of yeast pyruvate carboxylases (Palacián *et al*., 1966; Myers *et al*., 1983; Pronk *et al*., 1996).

The carboxylation of pyruvate to oxaloacetate, as well as the subsequent condensation thereof with acetyl-CoA is known as anaplerotic (filling-up) reactions of the TCA cycle. Condensation between oxaloacetate and acetyl-CoA results in the formation of citrate (Horton *et al*., 1996). During fermentation and/ or anaerobiosis, both oxaloacetate and citrate can end up in the formation of succinate within yeasts as explained in previous sections of this chapter.

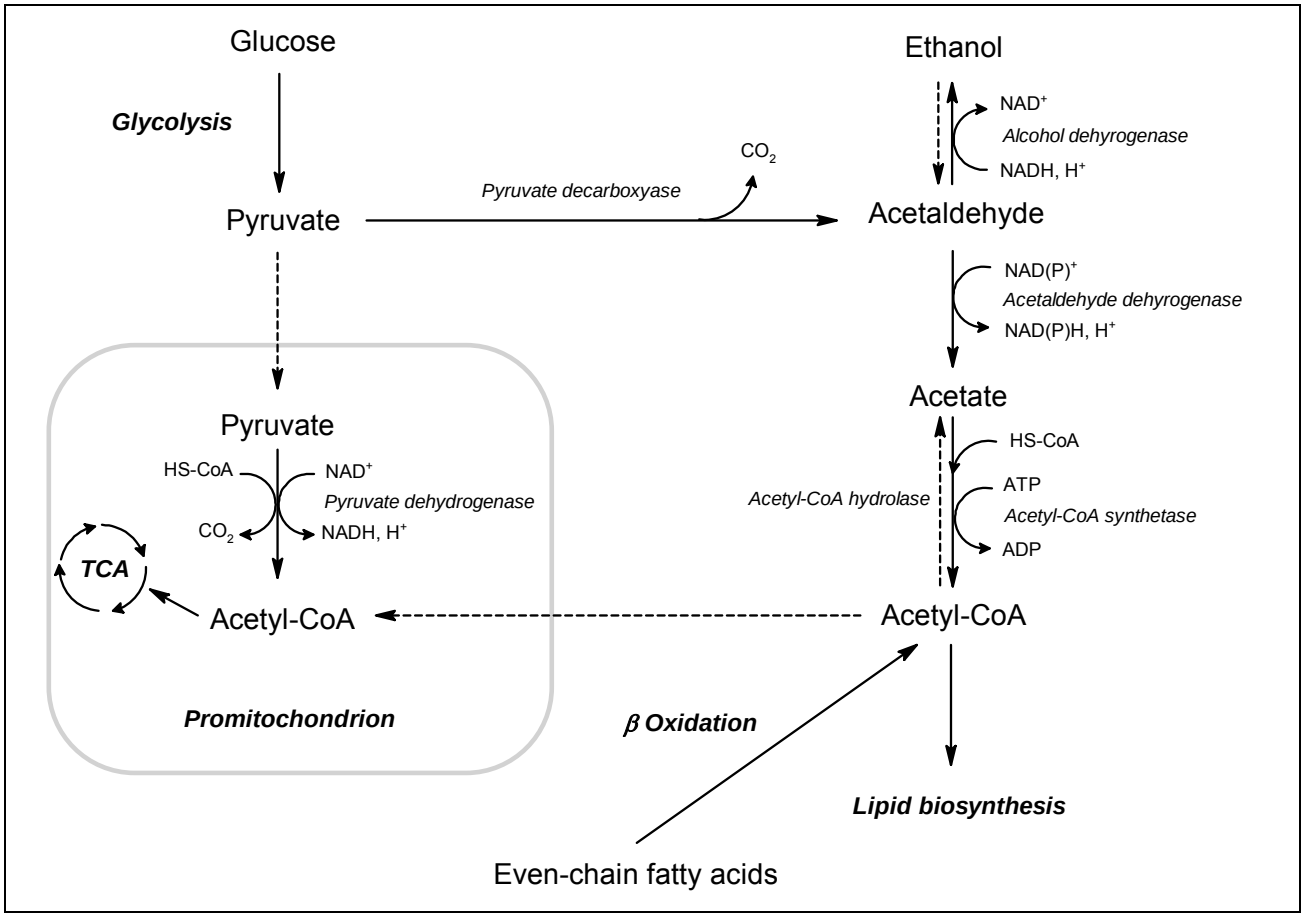

Figure 2.10 The pyruvate dehydrogenase bypass in wine yeasts.

2.5.1.2 Malic Acid

Succinic acid production by fermenting yeasts can be increased by as much as 20% with the inclusion of malic acid in the fermentation medium (Kleinzeller, 1941). In fact, up to 34% of the malic acid taken up by fermenting wine yeasts is converted into succinic acid (Mayer *et al*., 1964). However, the amount of succinic acid formed from malic acid depends on the original malic acid content of the growth medium (Heerde & Radler, 1978), since malic acid can only enter wine yeasts (*S. cerevisiae*) by means of simple diffusion (Delcourt *et al*., 1995). Strains of *Z. bailii* on the other hand, can convert much larger quantities of malic acid into succinic acid during alcoholic fermentation, because the uptake of malic acid is facilitated by specialized permeases imbedded within the plasma membranes of these yeasts (Radler, 1980, 1993). Most of the malic acid that is converted into succinate by fermenting yeasts occurs via the formation of fumarate (Mayer *et al*., 1964; Wakai *et al*., 1980; Salmon *et al.*, 1987). However, succinate can also be formed from malate by means of the TCA cycle's oxidative branch or the GABA bypass within wine yeasts during alcoholic fermentation.

2.5.2 YEAST ASSIMILABLE NITROGEN SOURCES

Yeast assimible nitrogen (YAN) can be defined as the total amount of nitrogen from nitrogen-containing compounds that can be taken up by wine yeasts (*S. cerevisiae sensu*

Yarrow, 1984) and used for the biosynthesis of their own peptides and proteins (Ough *et al*., 1990; Henschke & Jiranek, 1993). Ammonium ions, free amino acids, peptides, proteins and fragments of nucleic acids are quantitatively the most important nitrogen-containing compounds present within grape juice (Henschke & Jiranek, 1993; Boulton *et al*., 1998c). Nitrates also contribute to the nitrogen content of grape juice (Ough & Crowell, 1980), but ammonium ions are the only inorganic nitrogen-containing compounds assimilated by wine yeasts (Ough & Crowell, 1980; Bisson, 1991). In fact, ammonium ions are one of the most preferred sources of assimilable nitrogen to wine yeasts (Bisson, 1991; Henschke & Jiranek, 1993; Jiranek *et al*., 1995). The concentration thereof within grape juice ranges from 5-325 mg/L (Henschke & Jiranek, 1993; Butzke, 1998; Bell & Henschke, 2005) and contributes to between 2 and 53% of grape juice's YAN content (Huang & Ough, 1989; Bell & Henschke, 2005). Peptides of more than five amino acid residues and proteins cannot be utilized by wine yeasts due to the absence of the necessary transport systems and a lack of extracellular proteolytic activity (Bisson, 1991; Henschke & Jiranek, 1993). Although wine yeasts possess the ability to assimilate small peptides, the concentrations thereof within grape musts are still unknown due to the difficulty associated with separating the various peptides and proteins present within grape juice (Henschke & Jiranek, 1993). Free amino acids are the most important organic sources of YAN within grape juice. This group of compounds constitute 60-90% of grape juice's total nitrogen content (Kliewer, 1969; 1970; Henschke & Jiranek, 1993) and account for between 51 and 92% of the YAN within grape must (Bell & Henschke, 2005). Significant amounts of the imino amino acid, proline is normally found within juice from *Vitis vinifera* L. grapes (Ough, 1968; Huang & Ough, 1989, 1991; Henschke & Jiranek, 1993; Bell & Henschke, 2005). However, the utilization of proline by wine yeasts is extremely limited under oenological conditions (Duteurtre *et al*., 1971; Ingledew *et al*., 1987) due to strong nitrogen-catabolite repression of wine yeasts' proline permeases, the extremely low affinity of wine yeasts' general amino acid permease for proline (Lasko & Brandriss, 1981; Horák & Ríhová; 1982; Horák, 1986) and the molecular oxygen requirement for proline oxidases' catalytic activity (Duteurtre *et al*., 1971; Large, 1986). The latter mentioned enzyme is responsible for the first step in proline catabolism within wine yeasts (Duteurtre *et al*., 1971; Large, 1986). This implies that the proline taken up by wine yeasts can only be used for direct incorporation into peptides and proteins under anaerobic conditions. In addition, significant uptake of proline by wine yeasts can only occur once all the nitrogen sources responsible for the repression of wine yeasts' proline permeases are depleted from the growth medium. Generally therefore, proline is ignored as a source of YAN under winemaking conditions (Ough *et al*., 1990; Henschke & Jiranek, 1993). In other words, the most common sources of the YAN within grape juice are free alpha amino acids and ammonium ions (Henschke & Jiranek, 1993; Boulton *et al*., 1998c; Bell & Henschke, 2005). As a result, the YAN content of grape juice is often reported as the sum concentration of free α-amino nitrogen (FAN) and ammonia-calculated (ammoniacal) nitrogen (Henschke & Jiranek, 1993; Sablayrolles *et al*., 1996; Boulton *et al*., 1998c Bell & Henschke, 2005). Other YAN sources within grape juice

include γ-aminobutyric acid, nitrogen-containing vitamins, as well as the purine and pyrimidine bases of nucleic acids with the exception of thymine (Large, 1986; Bisson, 1991; Henschke & Jiranek, 1993).

The free amino acid contents of different grape juices vary considerably. **Table 2.3** lists all the free amino acids that have been found within grapes, along with their reported concentration ranges within grapes and/ or grape juices. The yeast assimilable nitrogen content of grapes is determined by the genetic makeup of the grape vine's scion and rootstock, macroclimatic conditions during ripening of the fruit, the ripeness level of the grapes by the time they are harvested and viticultural practices that influence the availability and accessibility of nitrates and/ or ammonium to the grape vine, microclimatic conditions within the vineyard and the health status of the grapes (Huang & Ough, 1989; Henschke & Jiranek, 1993; Bell & Henschke, 2005). Proline and arginine are usually the predominant free amino acids within *Vitis vinifera* L. grapes (Ough, 1968; Huang & Ough, 1989, 1991; Henschke & Jiranek, 1993; Stines *et al*., 2000; Bell & Henschke, 2005). Differential accumulation of these two amino acids provides a characteristic index, which can help to discriminate between different varieties of *V. vinifera* L. *Pinot noir* for example, is one of the very few *V. vinifera* L. varieties that often produce grapes with a higher arginine than proline content (Kliewer, 1968; Huang & Ough, 1991; Spayed & Andersen-Bagge, 1996; Nicolini *et al.*, 2001). It should be pointed out though; that the ratio of proline to arginine within grapes is not only influenced by the genetic makeup of the grapevine, but also depends on the level of fruit maturity. This is because proline accumulates at the expense of arginine during the late stages of fruit ripening (Kliewer, 1968, 1970). Next to arginine, glutamine, glutamate, alanine, asparagine, serine, threonine and aspartate are the most abundant yeast assimilable amino acids within juice from *V. vinifera* L grapes (Kliewer, 1970; Huang & Ough, 1991; Boulton *et al*., 1998; Hernández-Orte *et al*., 1999; Fukui *et al*., 2002).

The YAN content of grapes is unevenly distributed between the different parts of the grape vine berry (Stines *et al.*, 2000; Bell & Henschke, 2005), as shown by the data presented in **Figure 2.9**. This has important implications for the YAN content of grape must from an oenological point of view when one takes into consideration the different winemaking techniques used to produce different styles of wine. In the case of white wine production, the pulp is the main source of must components except when prolonged contact between the juice and skins is allowed before the onset of alcoholic fermentation. During red wine production though, the skins and seeds are usually in contact with the juice and pulp components during the entire growth period of fermenting yeasts. Although significant amounts of YAN can be found within the combined skins and seeds fraction of grapes (Stines *et al.*, 2000; Bell & Henschke, 2005), only a small percentage thereof can be extracted under normal winemaking conditions according to the data obtained by Fukui *et al* (2002). However, the extraction of YAN from these berry parts can be promoted by higher temperatures and increasing concentrations of ethanol in the must (Fukui *et al*., 2002).

Table 2.3
Concentration ranges of various amino acids within *V. vinerfera* L. grapes.

Amino acid	Concentration range (mg/L)
Alanine	10 – 227
Arginine	20 – 2322
Asparagine	1 – 171
Aspartic acid	10 – 138
Citrulline	0.1 – 83
Cysteine	1 – 8.2
Glutamine	9 – 4499
Glutamic acid	27 – 454
Glycine	1 – 20
Histidine	5 – 197
Isoleucine	1 – 117
Leucine	2 – 160
Lysine	0.7 – 45
Methionine	1 – 33
Ornithine	0.1 – 27.2
Phenylalanine	2.8 – 138
Proline	9 – 2257
Serine	13 – 330
Threonine	9 – 284
Tryptophan	0.2 – 11
Tyrosine	2 – 33
Valine	7 – 116

Data from Bell & Henschke, 2005

Structural damage to grapes can bring sources of YAN in contact with molecular oxygen, light and enzymes. This may cause some of it to be converted into less favoured forms of YAN and/ or compounds that cannot be assimilated by wine yeasts. In addition, it can also provide various micro-organisms access to the YAN sources within grapes before the grapes are crushed and the must inoculated with a pure-wine yeast starter culture. In other words, the total amount and composition of YAN within grape musts can be altered by any damage done to the grapes during harvesting and/ or the transport thereof to the winery. Degradative reactions and unwanted microbial growth during the period that follows the harvesting of grapes and fermentation of the must can be slowed down by harvesting during the night or early morning hours, applying sulphur dioxide to the harvested grapes and transporting or storing the harvested grapes at low temperatures and/ or an anaerobic atmosphere before crushing.

Mechanical stimuli, cold temperatures and anoxic conditions can cause levels of γ-aminobutyric acid to increase within grapes, thereby changing the composition of the available YAN within the resulting must (Flanzy *et al*., 1976; Shelp *et al*., 1999; Kishimoto & Sodeyama, 2003).

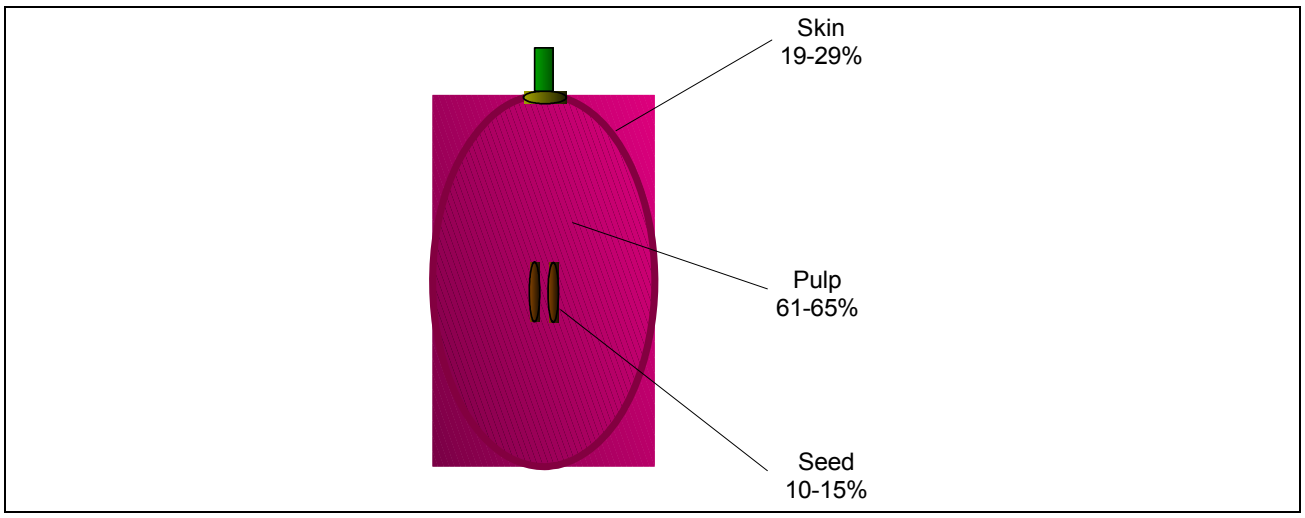

Figure 2.9 Distribution of YAN within *V. vinifera* L. grapes (Bell & Henschke, 2005)

It is possible that the cutting of grape bunches from the vine could trigger the build-up of γ-aminobutyric acid within grapes. If this is the case, it implies that the length of time between harvesting and crushing of grapes influence the γ-aminobutyric acid content of grapes musts. In fact, Bisson (1991) hypothesized that an increase in the time taken to transport harvested grapes to the winery could be held responsible for grape musts with higher than normal levels of γ-aminobutyric acid. A process known as carbonic maceration can be used to extract the colour of red grapes before they are crushed and the must fermented by yeasts (Boulton *et al*, 1998d; Ribéreau-Gayon *et al*., 2000f). This process is carried out by storing whole bunches of grapes under anoxic atmosphere. Carbon dioxide is usually chosen for this purpose, since it is heavier than oxygen, which makes it easier to displace oxygen from the storage container and maintain anoxic conditions during the extraction period. Musts obtained from grapes that underwent carbonic maceration will contain significantly higher concentrations of γ-aminobutyric acid (Kishimoto & Sodeyama, 2003). The same will be true of musts prepared from grapes that were stored at cold temperatures.

Grape juice is clarified before it is inoculated with a yeast starter culture during the production fruity-styled white or rosé wine. The most common way of achieving this, is by allowing particulate matter to settle out of the juice at low temperatures. Pectolytic enzymes (pectinases) from the grapes or added preparations thereof from microbial origins aid this process by degrading some of the suspended colloidal material (pectins) within grape juice. Cold-settled grape juices contain higher concentrations of free amino acids than unclarified grape juice or juices that have been clarified by means of filtration or centrifugation (Ayestarán *et al*., 1995; Ancín *et al*., 1996). Pectinases help release vegetable proteases from berry cells by degrading some of the cell wall components (Ayestarán *et al*., 1995), while cool temperatures increase protease activity due to the increased dissolution of carbon dioxide at decreased temperatures (Kruger *et al.*, 1992). On the other hand, sources of free amino acids like medium-sized

peptides remain inaccessible to wine yeasts in unclarified grape juice or are removed from the juice in case of filtration or centrifugation.

The addition of ammonium salts, such as diammonium hydrogen phosphate (DAP), serve as the most common means of increasing the YAN content of grape musts when a deficiency is suspected or known (Monk, 1982; Bisson, 1991; Henschke & Jiranek, 1993 Monk & Costello, 1994). However, commercial products that contain hydrolysates of yeasts provide a more balanced and complex mixture of YAN sources (Ingledew *et al*., 1986; Henschke, 1997b).

2.5.2.1 YAN and succinic acid production

The total amount of succinic acid produced by fermenting yeasts depends on both the concentration and source of YAN within the growth medium (Heerde & Radler, 1978; Muratsubaki, 1987). The stimulating effect of YAN on succinic acid production can be seen up to concentrations of about 500 mg/L FAN in the presence of a 100 g/L fermentable sugar (Heerde & Radler, 1978). Compared to ammonium as the only source of YAN, glutamate, glutamine, threonine and aspartate are each, noticeably more favourable to the production of succinic acid by fermenting yeasts (Heerde & Radler, 1978; **Table 2.4**). Fermenting wine yeasts produce the greatest amount of succinic acid in media that contain large amounts of glutamate (Kleinzeller, 1941; Coote & Kirsop, 1974; Heerde & Radler, 1979; Albers *et al*., 1996). In fact, the order in which the before mentioned α-amino acids are listed above, indicates the relative importance of each in relation to the amounts of succinic acid produced by fermenting wine yeasts. Heerde & Radler (1978) found the imino-amino acid, proline, significantly more stimulating to succinic acid production than glutamine. However, it needs to be pointed out that although the before mentioned authors considered the fermentation conditions during their experiments to be anaerobic, the yeasts were in actual fact grown under respiro-fermentative conditions (shaken cultures). As mentioned before, molecular oxygen is required by wine yeasts to liberate the nitrogen contained within proline before it can be used for the biosynthesis of other amino acids and other organic nitrogen-compounds. In other words, proline on its own cannot support wine yeast growth in the total absence of molecular oxygen. Hence, it can be said that large amounts of proline have a large stimulating effect on the production of succinic acid by wine yeasts under repiro-fermentative conditions, which is often the case during red grape must fermentations.

It has been suggested that high levels of γ-aminobutyric acid (GABA) within grape must will encourage the production of succinic acid by fermenting wine yeasts (Bach *et al*., 2004). Kishimoto & Sodeyama (2003) were able to demonstrate this by fermenting a high GABA content grape juice with a wine yeast strain that carried mutations of genes, which code for proteins involved in the uptake and catabolism of GABA. Compared to the wild-type strain, the mutant strain with the inability to assimilate and catabolise GABA produced notably less succinic acid (~200 mg/L) during fermentation of grape juice with a higher than normal GABA content. Kleinzeller (1941) however, reported

no increase in succinic acid production when GABA was added to the fermentation medium, in stead of ammonium.

Large, unforeseen increases in titratable acidity during alcoholic fermentation have been a topic of great concern in the south-eastern wine producing regions of the U.S.A., where the majority of wine is produced from *V. rotundifolia* (muscadine) grapes (Carroll *et al*., 1975; Vine, 1981). In fact, the titratable acidity of juice from *V. rotundifolia* varieties can increase by more than 3 g/L during alcoholic fermentation, which was shown to be caused by the production of succinic acid (Lamikanra, 1997). Notable differences between the YAN contents of *V. rotundifolia* and *V. vinifera* varieties' grapes in general (Marcy *et al*., 1981; Henschke & Jiranke, 1993; Bell & Henschke, 2005) may provide a partial answer as to why exceptionally high levels of succinic acid are produced by yeasts when they ferment musts prepared from *V. rotundifolia* grapes. From a quantitative viewpoint, arginine is usually the most important free α-amino acid within *V. vinifera* grapes (Henscke & Jiranek, 1993; Margalit, 1997; Bell & Henschke, 2005), but it is seldom the most abundant free amino acid within *V. rotundifolia* grapes (Marcy *et al*., 1981). In fact, the grapes of some *V. rotundifolia* varieties like Noble and Dixie contain very little arginine. *V. rotundifolia* grapes always contain high levels of α-alanine and threonine (Marcy *et al*., 1981), which are both noticeably more stimulating to the production of succinic acid during alcoholic fermentation than arginine or ammonium (Heerde & Radler, 1978). It is also important to note that the free amino acid component of YAN is notably higher within *V. rotundifolia* grapes than within *V. vinifera* grapes (Marcy *et al*., 1981; Henscke & Jiranek, 1993; Margalit, 1997; Bell & Henschke, 2005), since much lower levels of proline are found within grapes of *V. rotundifolia* varieties than grapes of the latter mentioned grape vine specie.

2.5.2.2 Mechanisms by which YAN affects succinic acid production

In **Section 2.3.2** it was discussed how the total amount of succinic acid produced during alcoholic fermentation might be related to the size of the living (metabolically active) yeast population (Muratsubaki, 1987; Verduyn *et al*., 1990; Shindo *et al*., 1993; Albers *et al*., 1996). Increasing concentrations of YAN within the growth medium encourages yeast growth (Agenbach, 1977; Radler & Schütz, 1982; Bezenger & Navarro, 1988) logarithmicly (Agenbach, 1977; Henschke & Jiranek, 1993). As a result, Agenbach (1977) found that yeast biomass increased by only 14-40% during alcoholic fermentation when the YAN content of the must was increased from 140-800 mg/L. Despite this, these findings imply that the concentration of YAN within grape juice will have a significant influence on the total number of viable yeasts contributing to production of succinic acid during alcoholic fermentation.

Individual sources of YAN vary with regard to their ability to support yeast growth during alcoholic fermentation, which is well illustrated by the data provided in **Table 2.4** (Heerde & Radler, 1978; Henchke & Jiranek, 1993; Albers *et al*., 1996; Bell & Henschke, 2005). Out of all the YAN sources that are found within grape juice; aspartate, glutamate,

glutamine, asparagine, arginine and ammonium exhibit the most stimulating effect on the proliferation of fermenting wine yeast. Hence, these nitrogen-compounds are regarded as "good" sources of YAN (Watson, 1976; Heerde & Radler, 1978; Henschke & Jiranek, 1993). If succinic acid production by fermenting wine yeasts was determined mainly by the size of the living yeast population, one would expect the levels of produced succinic acid to be greatest if cells were grown in the presence of "good" YAN sources. However, the data presented in **Table 2.4** indicate that, with the exception of glutamic acid, some of the "poorer" YAN sources promote the fermentative production of succinic acid by yeasts more than "good" sources of YAN. In addition, Albers *et al.* (1996) showed that considerably more succinic acid is produced by fermenting wine yeasts when YAN is supplied as glutamic acid in stead of a balanced mixture of amino acids, even though a balanced mixture of amino acids promote the proliferation of wine yeasts noticably more than the same quantity of FAN as glutamic acid. The above mentioned findings therefore suggest that YAN's effect on succinic acid production is not solely related to its influence on yeast growth (biomass production).

Wine yeasts can incorporate amino acids directly into proteins without modification or they can degrade them to compounds that are central to nitrogen- and carbon metabolism (Large, 1986; Henschke & Jiranek, 1993). When an amino acid is metabolized as a source of nitrogen, its amine functional group(s) is either transferred to an α-keto acid or liberated as an ammonium ion. The removal of an amino acid's amine group(s) results in the formation of a carboxylic acid or the coenzyme A ester thereof (**Table 2.6**). Note that the deamination or transamination products of most amino acids are intermediates of metabolic pathways that end up in the formation of succinate when wine yeasts are unable to respire. In other words, the carbon-skeletons of most amino acids are precursors of succinate within fermenting wine yeasts. For example, 17-24% of all the glutamic acid consumed by fermenting wine yeasts is converted into succinic acid via the formation of α-ketoglutarate and succinyl-CoA (Albers *et al.*, 1996; 1998a; Camarasa *et al*., 2003). All α-amino acids can be taken up by wine yeasts and used for the biosynthesis of proteins. Therefore, all α-amino acids are considered as sources of yeast assimilable nitrogen. However, histidine and glycine cannot be degraded by wine yeasts and the catabolism of lysine and cysteine by these micro-organisms are very limited due to the toxicity of certain intermediatary degradation products (Aida *et al*., 1969; Watson, 1976; Pekur *et al*., 1981; Large, 1986; Henschke & Jiranek, 1993). Also note that as in the case of proline, very little tryptophan can be degraded by wine yeasts under winemaking conditions due to the absence or lack of molecular oxygen (Large, 1986).

The different sources of YAN that are found within grape juice are acummulated at different rates from the growth medium by growing wine yeasts (Caster, 1953; Caster & Archer, 1959; Henschke & Jiranek, 1993; Jiranek *et al*., 1995). "Good sources of YAN (aspartate, glutamate, glutamine, asparagine, arginine and ammonium), along with lysine, threonine and serine are preferentially assimilated by proliferating wine yeasts (Rose & Keenan, 1981; Henschke & Jiranek, 1993; Jiranek *et al*., 1995). The selective uptake of YAN sources by wine yeasts is mediated by a mechanism known as nitrogen-

catabolite repression (NCR), whereby expression of certain transport systems (amino acid permeases) are induced, while others are degraded and/ or their expression delayed (Henschke & Jiranek, 1993; ter Schure *et al*., 2000; Magasanik & Kaiser, 2002; Bell & Henschke, 2005). The expression of nitrogen-catabolite repressed amino acid permeases, such as proline uptake transporter IV (Put4p) and the high capacity general amino acid permease I (Gap1p) are only induced when the so-called "preferred" nitrogen sources become depleted from the growth medium. De-repression of these amino acid transporters enables poorer YAN sources, as well as, any remaining "good" YAN sources to be taken up by wine yeasts. In other words, the relative abundance of the "good" YAN sources determines at what stage less-preferred amino acids will be accumulated by growing wine yeasts (Henschke & Jiranek, 1993).

Table 2.4

Different sources of YAN's effect on biomass and succinic acid production by a *S. cerevisiae* strain[a] under respiro-fermentative conditions.

Biomass (dry weight) (g/L)	Succinic acid (mg/L)
Aspartate (6.1)	Glutamate (1560)
Glutamate (5.9)	Proline (610)
Glutamine (5.7)	Glutamine (430)
Asparagine (5.5)	Threonine (330)
Ammonium (5.1)	Aspartate (270)
Arginine (5.0)	Alanine (160)
Tyrosine (3.9)	Serine (140)
Tryptophan (3.3)	Tryptophan (140)
Methionine (3.1)	Asparagine (130)
Alanine (3.0)	Phenylalanine (130)
Threonine (2.8)	Ammonium (110)
Phenylalanine (2.8)	Valine (90)
Serine (2.6)	Arginine (80)
Isoleucine (2.6)	Tyrosine (80)
Leucine (2.4)	Methionine (70)
Valine (1.9)	Leucine (50)
Proline (1.3)	Isoleucine (40)
Histidine (0)	–
Lysine (0)	–
Glycine (0)	–
Cysteine (0)	–

Adapted from Heerde & Radler (1978). [a]*S. cerevisiae* strain GU-MWRI 43 (Gothenburg University Microbiology and Wine Research Institute culture collection, Germany). Fermentation medium contained 100 g/L glucose and an excess YAN (500 mg/L FAN). [b]compounds highlighted in grey cannot be metabolized under anaerobic conditions. [c]compounds highlighted in yellow are "good" sources of YAN. [d]compounds highlighted in blue are "preferred" sources of YAN, along with the ones highlighted in yellow.

The sequential uptake of YAN sources by wine yeasts implies that the "preffered" amino acids will have to be catabolised in order to provide amino-groups for the biosynthesis of less-preferred amino acids (Jones *et al*., 1969; Henschke & Jiranek, 1993). In fact, a significant portion of the carbon skeletons that make up yeast proteins' amino acids are derived from α-keto acids that originated from sugar catabolism (Jones *et al*., 1969; Henschke & Jiranek, 1993). As a result, a considerable proportion of de-aminated derivatives from "preferred" amino acids are transformed into succinate and excreted as succinic acid during alcoholic fermentation.

Out of all the yeast assimilable amino acids, glutamate has by far the most stimulating effect on succinic acid production by fermenting wine yeasts (Heerde & Radler, 1978). High intracellular levels of glutamate increase the specific activity of NAD^+-dependent glutamate dehydrogenase within wine yeasts and down-regulates the expression of the NADPH-dependent isoform (Roon & Even, 1973; Heerde & Radler, 1978). The NAD^+-dependent isoform of this enzyme is responsible for converting glutamate into α-ketoglutarate and ammonium, whereas the one linked to NADPH catalyzes the inverse of this reaction. When considerable amounts of glutamate are de-aminated by NAD^+-dependent glutamate-dehydrogenases a concomitant increase in intracellular α-ketoglutarate levels elevate the specific activities of α-ketoglutarate-dehydrogenase and succinyl-CoA synthase from extremely low to noteworthy levels within fermenting yeasts (Heerde & Radler, 1978; Schwartz *et a*l., 1983; Camarasa *et al.*, 2003). In other words, high levels of glutamate stimulate the production of succinate via the oxidative branch of the TCA cycle. Glutamate can also participate in transamination reactions with α-keto acids for the biosynthesis of other α-amino acids. Although the latter mentioned reaction also results in the formation of α-ketoglutarate, most α-keto acids that act as acceptor molecules during amino-group transfer reactions are precursors of α-ketoglutarate. In other words, α-ketoglutarate's formation during transamination reactions with glutamate as the amino-group donor is balanced by the consumption of a α-ketoglutarate precursor. Hence, it is likely that less succinic acid will be formed when the majority of available glutamate partakes in transamination reactions, in stead of being converted directly into α-ketoglutarate and ammonium by NAD^+-dependant glutamate dehydrogenase. Since glutamate is a "preffered" source of YAN, large quantities thereof will be converted in significant amounts of succinic acid during alcoholic fermentation due to its participation in the biosynthesis of less-preferred amino acids during the early stages of yeast growth. However, notable amounts of ammonium in grape juice will cause a significant delay in the uptake of glutamate by wine yeasts (Henschke & Jiranek, 1993; Jiranek *et al*., 1995), due to repression of the general amino acid permease I and the dicarboxylic amino acid permease II (Grenson & Hou, 1972; Darte & Grenson, 1975; Henschke & Jiranek, 1993). Large amounts of ammonium will also cause less glutamate to be directly converted into succinate via the formation of α-ketoglutarate and succinyl-CoA due to the repression of NAD^+-dependant glutamate dehydrogenase (Roon & Evan, 1973; Coschigano & Magasanik, 1991; ter Schure *et al*., 1995).

Table 2.6
Amino acid catabolism within wine yeasts

AMINO ACID	METABOLISM	END PRODUCTS N-COMPOUND	C-COMPOUND
ALIPHATIC			
ALANINE	TRANSAMINATION	GLUTAMATE	PYRUVATE
VALINE	TRANSAMINATION	GLUTAMATE	ISOBUTYRYL COA
	SUBSEQUENT CARBON METABOLISM		SUCCINYL COA
LEUCINE	TRANSAMINATION	GLUTAMATE	ISOVALERYL COA
	SUBSEQUENT CARBON METABOLISM		ACETYL COA + ASETOACETATE
ISOLEUCINE	TRANSAMINATION	GLUTAMATE	METHYLBUTYRYL COA
	SUBSEQUENT CARBON METABOLISM		ACETYL- + SUCCINYL COA
GLYCINE	NOT METABOLIZABLE AS N-SOURCE	–	–
HYDROXY			
SERINE	DEAMINATION	AMMONIUM	PYRUVATE
THREONINE	DEAMINATION	AMMONIUM	PROPIONYL COA
	SUBSEQUENT CARBON METABOLISM		SUCCINYL COA
ACIDIC			
GLUTAMATE	DEAMINATION	AMMONIUM	α-KETOGLUTARATE
ASPARATE	TRANSAMINATION	GLUTAMATE	OXALOACETATE
AMIDE			
ASPARAGINE	DEAMINATION	AMMONIUM + ASPARTATE	
GLUTAMINE	DEAMINATION	AMMONIUM + GLUTAMATE	
IMINO			
PROLINE	METABOLISM REQUIRES O_2	GLUTAMATE	
BASIC			
ARGININE	ANAEROBIC PRODUCTS	2×AMMONIUM + GLUTAMATE + PROLINE	CO_2
LYSINE	NOT METABOLIZABLE AS N-SOURCE	–	–
HISTIDINE	NOT METABOLIZABLE AS N-SOURCE	–	–
AROMATIC			
TRYPTOPHAN	METABOLISM REQUIRES O_2	ALANINE + AMMONIUM	2×ACETYL COA
PHENYLALANINE	DEAMINATION	AMMONIUM	PHENYLPYRUVATE
TYROSINE	DEAMINATION	AMMONIUM	HYDROXYPHENYL-PYRUVATE
SULPHUR			
METHIONINE	TRANSAMINATION	GLUTAMATE	α-KETOBUTYRATE
	SUBSEQUENT CARBON METABOLISM		SUCCINYL COA
CYSTEINE	NOT METABOLIZABLE AS N-SOURCE	–	–

Adapted from Boulton *et al.* (1998b).

As mentioned before, arginine is usually the most abundant YAN source within juice from *V. vinifera* grapes, as well as one of the preferred YAN sources of wine yeasts. However,

two molecules of ammonium is formed for every arginine molecule catabolised by yeast, which implies that very large concentrations of arginine in grape juice will lead to exceptionally high levels of intracellular ammonium. In view of these findings, one can hypothesize that higher concentrations of succinic acid will be produced by wine yeasts when they ferment grape juices with lower concentrations of arginine and ammonium. In fact, exceptionally high levels of succinic acid are produced when wine yeasts ferment musts with low arginine levels, such the ones prepared from *V. rotundifolia* grapes (Carroll *et al*., 1975; Marcy *et al*., 1981; Vine, 1981; Lamikanra, 1997).

2.5.3 MACRO ELEMENTS

Little or no information is available on what influence different concentrations of the remaining macronutrients of wine yeasts (phosphorous, sulphur, potassium and magnesium) might have on the fermentative production of succinic acid. However, in the subsequent paragraphs of this section an attempt will be made to predict the effect the availability of above mentioned nutrients could have on the production of succinic acid by fermenting wine yeasts.

Orthophosphate ($H_2PO_4^-$) is the most available and accessible form of phosphorous for wine yeasts growing in grape juice. The natural phosphate content of grape juice can range from 70-560 mg/L (Arellano *et al*., 1997; Ribéreau-Gayon *et al*., 2000g). Phosphorous is found in the phosphate groups of phosphorylated adenosine, nucleic acids and membrane phospholipids within yeasts. It will also be present as free orthophosphate in the cytoplasm and vacuoles of yeasts (Okorokov *et al*., 1980; Theobald *et al*., 1996a, 1996b; Walker, 1998a). Phosphate acts as a competitive inhibitor of pyruvate decarboxylase (Boiteux & Hess, 1970; Whiting, 1976), the enzyme responsible for catalyzing the intermediatary reaction during pyruvate's conversion into ethanol (Pronk *et al*., 1996; Hohmann, 1997). In other words, higher concentrations of phosphate in yeasts decrease the rate of pyruvate decarboxylation due to a decrease in pyruvate decarboxylase's affinity for pyruvate. This implies that more pyruvate will become available to pyruvate carboxylase, which means that more thereof can be converted into oxaloacetate during alcoholic fermentation (Pronk *et al*., 1996). Oxaloacetate can be converted into succinate via either branch of the TCA cycle within fermenting wine yeasts. Hence, it can be hypothesized that higher levels of phosphate in the grape juice migth lead an elevated production of succinic acid by fermenting wine yeasts.

Inorganic sulphate and methionine are central to the sulphur metabolism within wine yeasts. Sulphur is primarily required for the biosynthesis of sulphur-containing amino acids, but is also utilized for the biosynthesis of important enzyme cofactors, such as iron-sulphur complexes, lipoic acid, coenzyme A, thiamine pyrophosphate and biotin. Between 100 mg/L and 290 mg/L sulphate is normally found within grape juice (Arellano *et al*., 1997; Ribéreau-Gayon *et al*., 2000g), whereas the methionine content thereof varies between 1 mg/L and 33 mg/L (Bell & Henschke, 2005). The potential effect of grape juice's assimilable sulphur content on succinic acid production by fermenting

wine yeasts have not been investigated as far as it is known. However, it is very unlikely for sulphur deficiencies to occur in grape juice, since bisulphite is usually added to grape musts before fermentation to inhibit oxidation and unwanted microbial growth. Wine yeasts are able to utilize bisulphite as an assimilable source of sulphur (Walker, 1998a).

Wine yeasts have an absolute growth requirement for potassium ions. It is an essential cofactor for a variety of enzymes, involved in the uptake of other nutrients such as phosphate and it acts a non-specific change-balancer and stabilizer of macromolecules and ribosomes within yeasts (Walker, 1998a). The potassium ion content of grape juices can range anything from 200-2200 mg/L (Radvanyi *et al.*, 1980; Juhász *et al*., 1987; Margalit, 1997). In the early 1940's, it was found that fermenting wine yeasts accumulated potassium ions in exchange for protons they extrude in order to maintain their intracellular pH. The inclusion of potassium ions into the fermentation medium diminished the excretion of succinic acid by fermenting wine yeasts. According to the authors that made this finding, succinic acid appear to be a major source of the protons that are exchanged for potassium ions by fermenting wine yeasts. Although protons are still extruded from fermenting yeasts under such conditions, more of the succinate produced within fermenting yeasts is retained as potassium succinate (Conway & Brady, 1950). It is unknown however, whether increasing concentrations of potassium ions at the levels found within grape juice will decrease the production of succinic acid by fermenting wine yeasts.

Magnesium ions are essential for wine yeast growth, because it is a cofactor for various enzymes and plays a structural role by stabilizing macromolecules (Walker, 1998a). The concentration of divalent magnesium ions normally range from 50-105 mg/L within grape juice (Juhász *et al*., 1987; Margalit, 1997). Both pyruvate decarboxylase and pyruvate carboxylase depend on divalent magnesium ions as cofactors (Gailiusis *et al*., 1964; Ruiz-Amil *et al*., 1965; Hübner *et al*., 1992; Pronk *et al*., 1996). While pyruvate decarboxylase directs pyruvate mostly away from the formation of succinate, the opposite is true of pyruvate carboxylase. In view of these facts, it is unlikely that variations in intracellular magnesium ion concentrations will have any effect on the amount of succinic acid produced by wine yeasts during alcoholic fermentation.

2.6 MICRONUTRIENTS' INFLUENCE ON SUCCINIC ACID PRODUCTION

2.6.1 TRACE ELEMENTS

Trace elements refer to mineral elements that are required as nutrients in the micromolar or nanomolar range (Walker, 1998a). Ions of zinc, copper, iron and manganese are regarded as the most important trace elements that are required by wine yeasts (Jones & Greenfield, 1984; Walker, 1998a; Boulton *et al*., 1998b). Other, less important trace elements required by wine yeasts include ions of molydenum, cobalt and calcium (Walker, 1998a). Grape juices usually contain adequate amounts of all the trace elements needed by wine yeasts (Walker, 1998a; Ribéreau-Gayon *et al.*, 2000e). **Table 2.7** lists the

four most essential trace elements required by wine yeasts, as well as the levels at which they occur within grape juice.

Some of the above mentioned trace elements' availability within grape juice might become growth limiting for innoculated cultures of wine yeast if the juice is obtained from mold-infected grapes, if indigenous microbial growth is not sufficiently suppressed before fermentation or if the juice is clarified or treated with an ion-exchanger. It is reasonable to assume that in such an event, less succinic acid would be produced during alcoholic fermentation, since it is known to be influenced by the size of the viable yeast population (Muratsubaki, 1987; Verduyn *et al*., 1990; Shindo *et al*., 1993; Albers *et al*., 1996). In one of the experiments conducted by Schwartz & Radler (1988) for example, it was shown that fermentative wine yeasts growth, as well as malic acid production was decreased by the absence of divalent zinc ions within the culture medium.

Table 2.7
Most important trace elements required by wine yeasts and their levels within grape juice.

Trace element	Concentration within grape juice (mg/L)	Required Levels (mg/L)
Zn^{2+}	0.17 – 4.8[ab]	0.26 – 0.52[c]
Cu^{2+}	0.2 – 2.93[ab]	0.095[c]
Fe^{3+}	0.3 – 19.3[ab]	0.06 – 0.17[c]
Mn^{2+}	0.66 – 12.9[ab]	0.11 – 0.22[c]

[a]Radvanyi *et al*., 1980; [b]Juhász *et al*., 1987; [c]Walker, 1998a.

2.6.2 VITAMINS

Vitamins can be defined as a class of organic compounds that serve vital metabolic functions as coenzymes or precursors of coenzymes (Horton *et al*., 1996; Walker, 1998a). The vitamins that are commonly required as growth factors by fermenting wine yeasts are listed in **Table 2.8** (Leonian & Lilly, 1942; Walker, 1998a), along with the required levels and the concentrations that can be expected within grape juice. As as is evident from the information provided within **Table 2.8**, grape juice usually contains sufficient amounts of all the required vitamins (Margalit, 1997; Ribéreau-Gayon *et al.*, 2000e). However, it is possible for deficiencies to occur in juice obtained from the grapes with a high incidence of *B. cinerea* (noble rot), in bentonite-fined, clarified, pasteurised or ion-exchanged juice (Rankine, 1998), in juice with excessive bisulphite levels (Leighter & Joslyn, 1969; Boulton *et al*., 1998c) or in juice where indigenous microbial growth were not sufficiently suppressed before innoculation with the selected pure-wine yeast starter culture (Bataillon *et al*., 1996).

Table 2.8
Vitamin requirements of fermenting wine yeasts and their concentrations within grape juice.

Vitamin	Concentration within grape juice (μg/L)	Required levels (μg/L)
Biotin	2–4[b]	0.7–1.3[c]
Pantothenic acid	160–1020[a]	50[d]–80[e]
Thiamine	81–300[a]	250[e]
Nicotinic acid	680–2600[b]	1600–2200[e]
Pyridoxine	327–880[a]	81–91[e]

[a]Juhász *et al*., 1987; [b]Ribéreau-Gayon *et al.*, 2000e; [c]Davenport, 1995; [d]Ough *et al*., 1989; [e]Graham *et al*., 1970.

The potential influence different yeast-vitamins' concentrations might have on succinic acid production during alcoholic fermentation has not been well researched. Vitamins may be added to grape juice as chemical mixtures, autolysed yeast preparations, or both (Eglinton & Henschke, 1993). The addition of commercially available vitamin preparations to highly clarified grape juice had no effect on yeast-biomass production or fermentation rate (Monk, 1982; Monk & Castello, 1984). Since succinic acid production is affected by the size of the viable yeast population during fermentation (Muratsubaki, 1987; Verduyn *et al*., 1990; Shindo *et al*., 1993; Albers *et al*., 1996), it would be natural to assume that an overall increase in vitamin levels will have little or no influence on the levels of succinic acid produced during alcoholic fermentation. However, such an assumption may be entirely incorrect, because it was found that acetic acid production by fermenting wine yeasts is stimulated in a dossage-dependant manner by additions of thiamine (Hannemann, 1985; Eglinton & Henscke, 1993) or nicotinic acid to synthetic grape juice (Monk & Cowley, 1984), which off coarse implies that pyruvate-carbon is directed away from the metabolic pathways that result in the formation of succinic acid within fermenting yeasts. Ribéreau-Gayon and his co-workers (1956) investigated the influence deficiencies in thiamine and pantothenic acid have on the levels of wine yeast fermentation by-products, including that of succinate. Succinic acid production by the wine yeast strain that were selected for experimentation by the before mentioned authors were unaffected by the absence of thiamine within the fermentation medium, but were significantly decreased by the absence of pantothenic acid (Ribéreau-Gayon *et al*., 1956).

2.6.3 LIPIDS

Lipids are structural and functional components of yeast's cellular membranes, which function as selective barriers to the movement of solutes (Boulton & Quain, 2001). The most important lipid classes found within the cellular membranes of wine yeasts are glycerophospholipids, sphingolipids and sterols (van der Rest *et al*., 1995). The simplest glycerophospholipid of wine yeast membranes is called phosphatidic acid, which consists of two fatty acyl groups that is linked to first and second carbons of glycerol-3-phosphate

by means of ester bonds. However, the phosphate group of most glycerophospholipids within wine yeast membranes is also esterified to one of the following polar compounds: *i.e.* ethanolamine; *myo*-inositol; serine; choline or glycerol. The most abundant glycerophospholipids within cellular membranes of wine yeasts are phophatidylinositol, phosphatidylethanolamine and phosphatidylcholine (Patton & Lester, 1991; Ribéreau-Gayon *et al*., 2000a). However, smaller quantities of phophatidylserine, phosphatidic acid and the dimeric form of phosphatidylglycerol (cardiolipin) are also present within wine yeast membranes (Patton & Lester, 1991; van der Rest *et al*., 1995). At second day of anaerobic fermentation within grape must, approximately 80% of the fatty acyl groups within wine yeast membranes' glycerophospholipids are derived from long-chain ($\geq C_{16}$) fatty acids (Valero *et al*., 1998). Almost half (47%) of the long-chain fatty acyl groups from wine yeast membranes' glycerophospholipids were unsaturated and mainly derived from palmitoleic acid ($C_{16:1}$) and oleic acid ($C_{18:1}$) at a ratio of about 2:1. The principal saturated fatty acyl groups of wine yeast membranes' glycerophopholipids are derived from palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) at a ratio of roughly 4:1 (van der Rest *et al*., 1995; Valero *et al*., 1998). Wine yeast membranes' sphingolipids consists of a phosphoryl-ceramide backbone with the phosphate group esterified to inositol, mannosyl inositol or mannosyl di-inositol. The ceramide backbone of wine yeasts' sphingolipids consists of phytosphingosine's (D-erythro-2-amino-octadecane-1,3,4-triol) C_1-phosphorylated form that is N-acylated to an α-hydroxy-C_{26}-fatty acid (Patton & Lester, 1991; Lester & Dickson, 1993; Daum *et al*., 1998). Quantitatively, ergosterol is by far the most important sterol within wine yeast membranes (Zinser *et al*., 1993; van der Rest, 1995; Valero *et al*., 1998).

Ethanolamine; serine; choline and glycerol can all be biosynthesized by fermenting wine yeasts (McMaster & Bell, 1994; Margalit, 1997), but some wine yeast strains have an absolute growth factor requirement for exogenous supplies of *myo*-inositol (Leonian & Lilly, 1942; Walker, 1998a). Normally, such yeasts utilize up to 3 mg/L of an exogenous *myo*-inositol supply during alcoholic fermentation (Graham *et al*., 1970). However, the *myo*-inositol content of grape juice range from 380−710 mg/L (Ribéreau-Gayon *et al*., 2000g), which implies that fermenting wine yeasts are unlikely to suffer from a *myo*-inositol deficiency when fermentation is conducted within grape juice.

Wine yeasts require molecular oxygen to biosynthesize unsaturated long-chain fatty acids (Bloomfield & Bloch, 1960; Wakil *et al*., 1983; Snoek & Steensma, 2007), wine yeast sterols (Parks, 1978; Parks & Casey, 1995; Snoek & Steensma, 2007) and the phytosphingosine moiety of wine yeast spingolipids (Daum *et al*., 1998). The crushing of grapes results in a rapid uptake of oxygen. Grape juice saturated with air at 20°C contains 8−9 mg/L dissolved oxygen (Boulton *et al*., 2000a). However, a substantial part of a grape juice's dissolved oxygen content will become unavailable to yeasts due to oxidation of phenolic compounds and its displacement from the must by added or yeast-produced carbon dioxide. In fact, almost all of the dissolved oxygen will be removed from the must within the first three hours of batch fermentations (Haukeli & Lie, 1979; O'Connor-Cox *et al*., 1996). For the most part in other words, wine yeast ferment in the absence of molecular oxygen under the usual winemaking conditions. Fermenting yeasts therefore

depend on the lipid content of the growth medium for supplies of unsaturated long-chain fatty acid sources, sterols and sphingolipids.

Long-chain fatty acids are not found in the free form within *V. vinifera* L. berries (Roufet *et al*. 1987). In stead, they are present within grapes as part of glycerophospholipids, triacylglycerols (neutral lipids) and glycolipids (Gallander & Peng, 1980; Roufet *et al*., 1986; 1987). Wine yeasts can assimilate and modify these lipids according to their needs (Kohlwein *et al*., 1996). Glycerophospholipids constituted the largest fraction (64−72%) of the extractable lipids from the pulp and skins of *V. vinifera* L. berries. Triacylglycerols made up the next largest group of extractable lipids from the before mentioned *V. vinifera* L. berry parts, whereas the glycolipidic fraction thereof constituted only 8−14% of the total (Roufet *et al*., 1987). Triacylglycerols (oils) can also be extracted from grape seeds when they are damaged during the crushing process (Cao & Ito, 2003). On average, more than 70% of the fatty acyl groups within the lipid fractions from the pulp and skins of *V. vinifera* L. berries are derived from unsaturated long-chain fatty acids (Roufet *et al*., 1987). This is also true of grape-seed oil's fatty acyl groups (Cao & Ito, 2003). More than half of unsaturated fatty acyl groups within the lipid fractions of *V. vinifera* L. berries' pulp and skins are derived from linoleic acid ($C_{18:2}$), while the rest it is derived from more or less equal quantities of oleic- ($C_{18:1}$) and linolenic acid ($C_{18:3}$) in addition to traces (≤ 1.4%) of palmitoleic acid ($C_{16:1}$) (Roufet *et al*., 1987; Guilloux-Benatier *et al*., 1998). The relative distribution of the different unsaturated long-chain fatty acyl groups of *V. vinifera* L lipids are roughly the same for different berry parts and different varietals of *V. vinifera* L (Roufet *et al*., 1987; Cao & Ito, 2003). However, grape skins contain 1.5−3 times more long-chain fatty acids than the pulp (Roufet *et al*., 1987). Contact between the juice and skins of grapes therefore favours the extraction of unsaturated long-chain fatty acids, which can further be increased by recovering juice from grape skins with the aid of a press (Ferreira *et al*., 1995; Guilloux-Benatier *et al*., 1998). For example, the total concentration of linoleic- and linolenic acid from lipids detected in the free-run juice of Chardonnay after contact with the pomace for 16 hours at 20°C was less than 1.5 mg/L, but could be increased to approximately 10 mg/L by the addition of the press-juice (Ferreira *et al*., 1995).

Grape phytosterols are mainly extracted from the cuticular wax (Higgens & Peng, 1976) and skins (Le Fur *et al*., 1994) of berries. β Sitosterol is the main grape phytosterol and accounts for approximately 85−90% of grapes' sterol contents. The remaining phytosterol contents of grapes consist of approximately equal amounts of stigmasterol and campesterol (Dagna *et al*., 1982; Le Fur *et al*., 1994). The cuticular wax of grapes also contains oleanic acid (Radler, 1965; Dagna *et al*., 1982), which performs a similar role than ergosterol in that it also stimulates fermentation by ensuring higher cell numbers throughout fermentation (Brechot *et al*., 1971; Larue *et al*., 1980). It is therefore possible that oleanic acid is converted into ergosterol by wine yeasts according to Valero *et al*. (1998).

Ethanol produced by fermenting yeasts will aid the extraction of lipids from the skins and particulate matter (pomace) of grapes, because it is better solvent for lipids than

water. Grape juice is fermented in contact with the skins and pomace during red wine production. It would therefore be very unlikely for fermenting wine yeasts to become deficient in either unsaturated long-chain fatty acids or sterols under such conditions. However, during white wine production substantial amounts of grape derived lipids will be lost during pre-fermentation clarification (Bertrand & Miele, 1984; Delfini *et al*., 1992; Ferreira *et al*., 1995; Guilloux-Benatier *et al*., 1998) due to the removal of colloidal material to which lipids adsorb. Even greater losses in unsaturated long-chain fatty acids can result from extended contact between the juice and grape skins prior to pre-fermentation clarification if the activity of grape-derived lipoxygenases is not inhibited (Joslin & Ough, 1978; Cayrel *et al.*, 1983; Roufet *et al*., 1986).

2.6.3.1 Usaturated fatty acids and succinic acid production

It has been known for some time now that ethanol-washed and powdered mycelia from *Asperigillus niger* will stimulate alcoholic fermentation, increase the production of succinic acid and greatly diminish the production of acetic acid by fermenting wine yeasts under anaerobic conditions (Ribereau-Gayon *et al*. 1952). The stimulating effect on alcoholic fermentation by wine yeasts is also exhibited when anaerobic fermentations are carried out in the presence of *Aspergillus oryzae* (Hayashida *et al*., 1974). Initial research by Hayashida *et al*. (1974) suggested that the proteolipid fraction isolated from *Aspergillus oryzae*'s mycelia was responsible for the above mentioned phenomenon. Further investigation into this matter revealed that 58.5% of *Aspergillus oryzae*'s proteolipid are phospholipids with 77.5% of their fatty acyl groups being unsaturated (Hayashida *et al*., 1976). Sterols accounted for less than 0.2% of *Aspergillus oryzae*'s proteolipid (Hayashida & Ohta, 1978). All of the above seem to indicate that supplies of unsaturated long-chain fatty acids somehow elevate the production of succinic acid during anaerobic fermentation by wine yeasts.

2.6.3.2 Sterols and succinic acid production

It has been shown that increasing concentrations of grape phytosterols or 15 mg/L ergosterol in combination with 5 mg/L oleic acid elevate the production of succinic acid by fermenting wine yeasts under anoxic conditions (Luparia *et al*., 2004).

The cytoplasm of every yeast is enveloped by a plasma membrane, as well as by an outer cell wall that consists of polysaccharides (β-glucans) and polysaccharide-protein complexes called mannoproteins (Ribéreau-Gayon *et al*., 2000a). The plasma membrane of yeasts on the other hand, is essentially made up out of a bilayer of lipids with polar head groups (glycerophospholipids and phosphatidylsphingolipids). The ethanol produced by fermenting wine yeasts causes an uncontrolled influx of protons into yeasts (Leão & van Uden, 1984a; Cartwright *et al*., 1986), because high concentrations of ethanol destroys the plasma membrane's ability to act as a hydrophobic barrier between the yeast cytoplasm and the aqueous environment in which it normally grows. This can be ascribed to two factors. Increasing concentrations of ethanol within the growth medium will

gradually replace the water molecules (hydration-spheres) that surround the polar head groups of the plasma membrane's phosholipids, which causes the phospolipid polar heads to repel each other more strongly (Hossack & Rose, 1976). Secondly, when ethanol molecules associate with the hydrocarbon tails of the plasma membrane's phospholipids they diminish the hydrophobic properties of this region within the yeast plasma membrane (Leão & van Uden, 1984a). ATP is consumed by the plasma membrane ATPases of the yeast to extrude protons from the cell (Serrano, 1978; Willsky, 1979). A passive influx of protons caused by increasing concentrations of ethanol within the growth medium will therefore elevate the consumption of ATP by plasma membrane ATPases in order to prevent acidification of the yeast cytoplasm (dissipation of the proton motive force across the yeast plasma membrane).

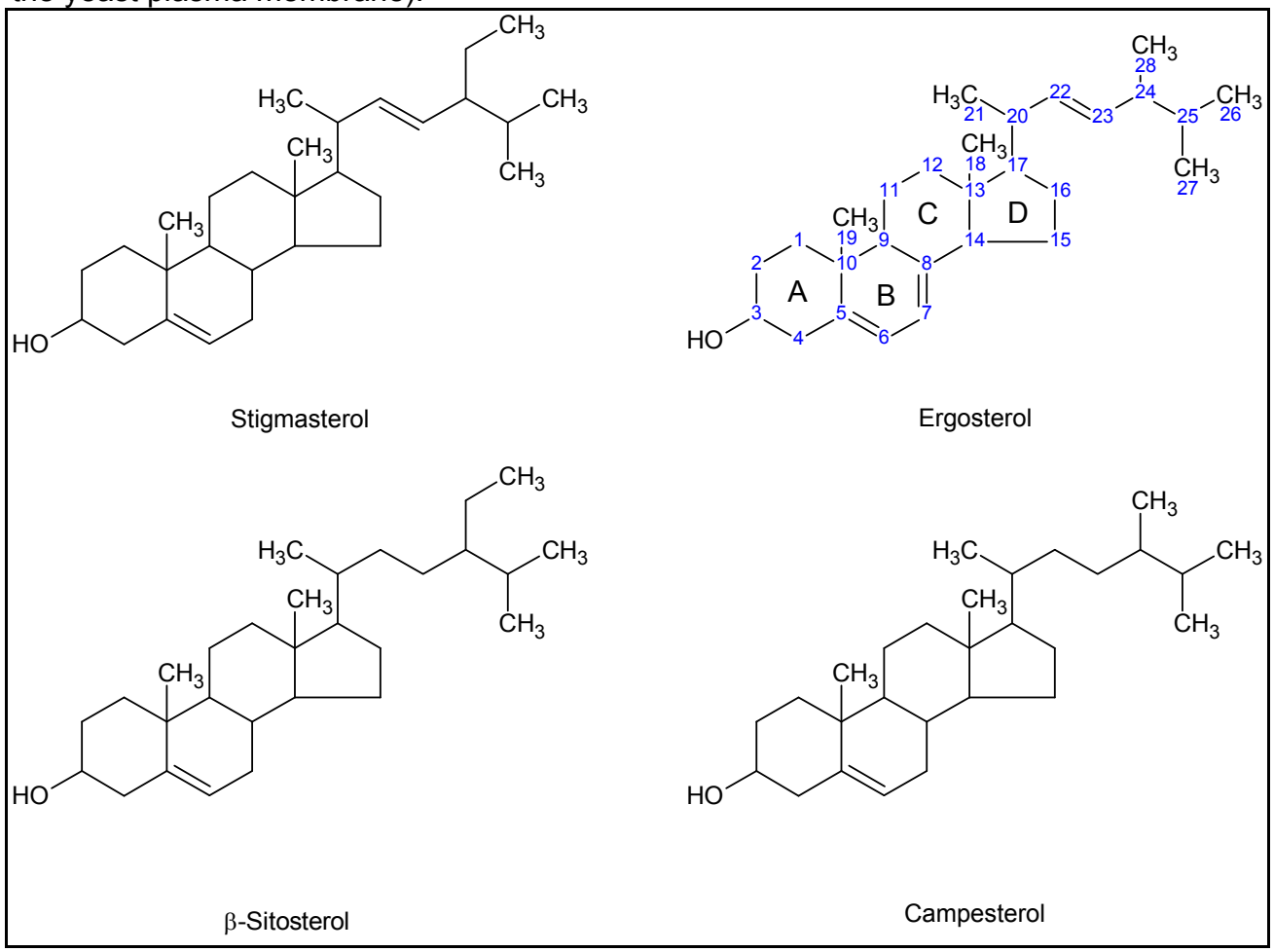

Figure 2.10 Structures of the most important *V. vinifera* phytosterols compared to that of the main sterol within wine yeasts, *i.e.* ergosterol.

Proteins with various functions are embedded within the yeast plasma membrane (van der Rest *et al*., 1995) and their activities are influenced by the type of membrane lipids with which they are direct contact with (Serrano *et al*., 1988; van der Rest *et al*., 1995). When ethanol molecules associate with the hydrocarbon tails of the plasma membrane's phospholipids they can reduce the activity of important plasma membrane proteins by restricting the movement of phospholipids' hydrocarbon

tails or by altering the functional conformation of these proteins. Plasma membrane ATPases (Cartwright *et al*., 1986), hexose permeases (Thomas & Rose, 1979; Leão & van Uden, 1982), ammonium-specific permeases (Leão & van Uden, 1983) and the general amino acid permease (Leão & van Uden, 1984b) are among the yeast plasma membrane proteins that are inhibited by increasing concentrations of ethanol within the growth medium. High concentrations of ethanol within the growth medium weakens the proton-motive force across the yeast plasma membrane due to the passive influx of protons into yeasts in addition to the dimished ability of plasma membrane ATPases to expel protons from the yeast cytoplasm (Cartwright *et al*., 1986). The weakening of the proton-motive force across the yeast plasma membrane by ethanol also reduces the uptake of inorganic phosphate (Borst-Pauwels & Dobbelmann, 1972; Thomas & Rose, 1979), which is needed for the production of ATP. A decrease in hexose permease activity by ethanol (Thomas & Rose, 1979; Leão & van Uden, 1982) can decrease the flux of glycolysis within fermenting wine yeasts, because the activities of the glycolytic pathway's enzymes remain virtually the same throughout fermentation (Larue *et al*., 1984; Dombeck & Ingram, 1987). This implies that ethanol can also reduce the rate of ATP production within fermenting wine yeasts by inhibiting the uptake of hexoses. In other words, the proliferation and viability of anaerobically-grown wine yeasts will be decreased in the presence of relatively high ethanol concentrations due to its inhibiting effect on the uptake of nutrients needed for the production of ATP and protein biosynthesis in combination with an increased consumption of ATP.

When wine yeasts are able to biosynthesize their own sterols, *i.e*. under aerobic conditions, the proportion of ergosterol within their plasma membranes increase upon exposure to relatively high concentrations (> 6% v/v) of ethanol (Del Castillo Agudo, 1992; Novotný *et al*., 1992; Alexandre *et al*., 1994). On the other hand though, exposure to ethanol causes a decrease in the overall sterol content of wine yeasts' plasma membranes (Walker-Caprioglio *et al*., 1990; Alexandre *et al*., 1994). The extent to which ethanol decreases the proliferation and viability of anaerobically grown wine yeasts is considerably lessened by exogenous supplies of ergosterol or stigmasterol in the presence of non-limiting concentrations of unsaturated long-chain fatty acids (Thomas *et al.*, 1978; Mishra & Kaur, 1991). The alkyl chain at C-17 of ergosterol or stigmasterol contains an unsaturated bond between C-22 and C-23 (**Figure 2.10**). Sterols with a saturated C-17 alkyl chain (*e.g*. cholesterol or campesterol) are less capable of protecting anaerobically grown wine yeasts against the toxic effects of ethanol (Thomas *et al.*, 1978). When ergosterol or stigmasterol is inserted into the yeast plasma membrane's outer monolayer, it will compensate for the ethanol-induced, increased repulsion between membrane phospholipids' polar head groups by inserting its Δ^{22}-unsaturated hydrocarbon tail between the fatty acyl groups of membrane phosholipids and by forming a hydrogen bond between its own hydroxyl group and the polar head of a phospholipid (Hossack & Rose, 1976; Bottema *et al*., 1985; Ribéreau-Gayon *et al*., 2000a). In fact, it has been suggested that the presence of the double bond at C-22 in the alkyl chain of ergosterol or stigmasterol stabilizes the distal part of the phospholipid fatty acyl chain with which it is in contact with

(Hossack & Rose, 1976). Hence, it is believed that Δ^{22}-sterols contribute to forming a more effective barrier towards the movement of ethanol molecules into the hydrophobic interior of a yeast's plasma membrane (Thomas *et al*., 1978), thereby conserving the plasma membrane proteins' respective activities and its ability to inhibit a passive influx of protons into yeasts. In other words, the availability of Δ^{22}-sterols for uptake by anaerobically-grown yeasts result in higher intracellular levels of ATP, because plasma membrane ATPases will consume less ATP to extrude protons from cells and the rate of ATP production will be less inhibited by the effects of ethanol on the uptake of hexoses and inorganic phosphate. Exogenous supplies of Δ^{22}-sterols also ensure that the uptake of assimilable nitrogen sources by anaerobically-grown fermenting wine yeasts will be less inhibited by increasing concentrations of ethanol. Higher intracellular ATP and assimilable nitrogen levels explain why supplies Δ^{22}-sterols stimulate growth and enhance the survival of fermenting wine yeast under hypoxic conditions (Larue *et al*., 1980; Mishra & Kaur, 1991). In other words, the addition of Δ^{22}-sterols to the culture medium results in a larger yeast population throughout anaerobic alcoholic fermentation, which provide a partial explanation as to why succinic acid production by fermenting wine yeasts were increased by increasing levels of a grape phytosterol mixture under anoxic conditions (Luparia *et al*., 2004). Higher intracellular ATP levels increase the formation of acetyl-CoA via the pyruvate dehydrogenase bypass within fermenting wine yeasts (Steensma, 1997). Both ATP and acetyl-CoA stimulate pyruvate carboxylase activity within wine yeasts (Ruiz-Amil *et al*., 1965; Miller & Atkinsin, 1972; Myers *et al*., 1983; Pronk *et al*., 1996), which will favour the formation of oxaloacetate and citrate, which are both precursors of succinate within non-respiring wine yeasts.

2.6.4 MOLECULAR OXYGEN

At least 5 mg/L dissolved molecular oxygen is required at the start of alcoholic fermentation to prevent a sluggish or stucked fermentation (Sablayrolles & Barre, 1986). Yeasts require molecular oxygen to biosynthesize ergosterol (Parks, 1978; Snoek & Steensma, 2007) and unsaturated long-chain fatty acids (Bloomfield & Bloch, 1960; Snoek & Steensma, 2007), which are essential for the proliferation of wine yeasts and their ability to endure the toxic effects of ethanol (Thomas *et al*., 1978; Thomas & Rose, 1979; Larue *et al*., 1980; Mishra & Kaur, 1991; Novotný *et al*., 1992). The dissolved molecular oxygen requirement for the biosynthesis of ergosterol range from 0.3−1.2 mg/L, while another 0.9−1.2 mg/L dissolved molecular oxygen is needed for the biosynthesis of unsaturated long-chain fatty acids in order for wine yeasts to complete fermentation of a 15−18°Plato (°Brix) beer-wort (O'Connor-Cox *et al*., 1996). This helps explain why aeration of the culture medium before fermentation stimulates yeast growth (Julien *et al*., 2000). A larger population of wine yeast throughout alcoholic fermentation is likely to result in higher concentrations of succinic acid.

Significant amounts of molecular oxygen can be picked up by grape juice during the crushing of grapes and the recovery of juice from the press. Grape juice saturated with air

contains 8−9 mg/L molecular oxygen at 20°C (Jackson, 1994; Boulton *et al*., 1998f). Due to the oxidation of phenolic compounds and sulphur dioxide however, a lack of available molecular oxygen might occur by the time the juice is innoculated with a pure-yeast starter culture.

High levels of dissolved molecular oxygen during fermentation increase the titratable acidity of saké, partly due to the elevated formation of succinic acid (Nagai *et al*., 1992). Several other reports confirm that aeration during alcoholic fermentation increases the production of succinic acid by wine yeasts (Ribéreau-Gayon *et al*., 1956a; Coote & Kirsop 1973; Magarifuchi *et al*., 1995; Arikawa *et al*., 1999b Arikawa *et al.*, 1999a). Winemakers sometimes deliberately aerate fermenting grape juice when the fermentation becomes sluggish (Boulton *et al*., 1998d). Aeration during alcoholic fermentation also occurs when the must is pumped on top of the grape skin cap in order to extract colour during red wine production (Ribéreau-Gayon *et al.*, 2000f).

Large amounts of proline are very likely to be converted into succinic acid by wine yeasts when they are grown under respiro-fermentative conditions (Duteurtre; 1971; Heerde & Radler, 1978), especially since aeration of the must increases the uptake of amino acids (Jiranek *et al*., 1995). Molecular oxygen can take part in an alternative respiratory pathway within fermenting wine yeasts. This pathway involves the transfer of electrons from ubiquinol to cytochrome b as described by Salmon *et al*. (1998), which helps generate a proton gradient across the inner membrane of yeast promitochondria that can ultimately be harnessed by F_1F_0-ATPases within fermenting yeast's promitochondria (Criddle & Schatz, 1969) to produce ATP from ADP. In other words, another reason for the increased production of succinic acid under respiro-fermentative conditions can potentially be ascribed to higher intracellular levels of ATP, which as explained earlier stimulates the formation of succinic acid precursors by acetyl-CoA synthetase (Steensma, 1997) and pyruvate carboxylase (Miller & Atkinsin, 1972; Myers *et al*., 1983).

2.7 THE INFLUENCE OF PHYSICO-CHEMICAL PARAMETERS

2.7.1 HYDROGEN IONS (pH)

The hydrogen ion content of grape juice is usually reported in terms of pH, which normally range between 2.8 and 4.0. However, grape juices with pH values greater than 4.0 are sometimes obtained from grapes that have have been left for too long on the vine (Boulton *et al*., 1998a).

The production of organic acids by fermenting yeasts is influenced by the pH of the growth medium (Coote *et al.*, 1973). Media with high pH values (*i.e*. low concentrations of hydrogen ions) appear to favour the production of succinic acid by fermenting wine yeasts (Ribéreau-Gayon *et al*., 1956; Thoukis *et al*., 1965; Shimazu & Watanabe, 1981; Fowles, 1992). On the other hand, the results from Fowles' study (1992) indicated that changes to culture medium's pH between values of 2.5 to 5.0, caused no notable increase in succinic acid production by the wine yeast, which conducted the fermentations.

The intracellular pH of wine yeasts are kept at a value of approximately 5.25 (Cimprich *et al*., 1995). The lower the pH of (more acidic) the growth medium, the more readily protons will diffuse into fermenting yeasts when their plasma membranes become less resistent to the toxic effects of increasing ethanol concentrations (Bisson, 1991). Apart from employing active transport systems to extrude protons from their cytoplasms (Serrano, 1978), yeasts also delay intracellular acidification by reducing the uptake of amino acids and ammonium (Casey & Ingledew, 1986; Bisson, 1991; Belviso *et al*., 2004). This implies that yeasts assimilate more amino acids and that spend less ATP (energy) to extrude protons when they conduct alcoholic fermentations in a less acidic environment. In other words, yeasts will grow faster and reach greater cell numbers when they grow in media with pH values closer cytoplasmic optimum of 5.25. A larger yeast population and an increase in the amount of amino acids catabolized to precursors of succinate provide explanation as to why succinic acid production is slightly higher when wine yeasts ferment media with pH values closer to 5.25.

2.7.2 FERMENTATION TEMPERATURE

In the case of mesophilic wine yeasts *sensu* (Castellari *et al*., 1992; 1994; Kishimoto 1994), the production of succinic acid during fermentation increases with increasing temperatures within the range 12−30°C. At temperatures greater than 30°C however, there is a rapid decline in the amount of succinic acid produced by fermenting mesophilic wine yeasts (Shimazu & Watanabe, 1981; Castellari *et al.*, 1995). In contrast to the before mentioned authors' findings though, Ough *et al.* (1969) reported moderate fermentation temperatures within the vicinity of 21°C optimal for the production of succinic acid by mesophilic wine yeasts (*S. cerevisiae* var. *ellipsoideus*). Increases in fermentation temperature between 6 and 18°C also increase succinic acid production by cryotolerant wine yeasts *sensu* (Castellari *et al*., 1992; 1994; Kishimoto 1994), but the effect of temperature changes on the levels of succinic acid produced are more drastic than in the case of mesophilic wine yeasts. The fermentative production of succinic acid by cryotolerant wine yeasts *sensu* (Castellari *et al*., 1992; 1994; Kishimoto 1994) usually start to decline at temperatures greater than 18°C (Castellari *et al.*, 1995; Rainieri *et al*., 1998b).

2.7.2 SULPHUR DIOXIDE

Potassium metabisulphite ($K_2S_2O_5$) or aqueous solutions of sulphur dioxide gas can be added to grape jucie to inhibit unwanted microbial growth and to prevent oxidation of important flavour compounds before alcoholic fermentation. Sulphur dioxide additions usually range between 25 and 100 mg/L (Boulton *et al*., 1998d), depending on the state of the harvested grapes.

Aqueous solutions of sulphur dioxide or potassium metabisulphite can be viewed as sulphurous acid (H_2SO_3), which will be in chemical equilibrium with bisulphite (HSO_3^-) and sulphite (SO_3^{2-}) ions depending on the pH of the medium to which it is added. At the pH of grape juice most of the added sulphur dioxide will be present as bisulphite ions (Boulton *et al*., 1998e). High concentrations of bisulphite ions within the culture medium

can cause an accumulation of NADH within the cytosol of fermenting wine yeasts by inhibiting the formation of ethanol due to the strong binding of intracellular acetaldehyde and the destruction of thiamine and thiamine pyrophosphate (Leichter & Joslyn, 1969; Boulton *et al*., 1998e), which is required as a cofactor of yeast pyruvate decarboxylases. Although the reductive formation of succinic acid will help get rid of excess NADH during alcoholic fermentation, it was found that no additional succinic acid is formed during fermentations in the presence of high bisulphite concentrations (Oura, 1977). In fact, the opposite is true, since even low concentrations of sulphur dioxide (50 mg/L) decreased the production of succinic acid during fermentation of grape juice with 220 g/L fermentable sugar (Shimazu & Watanabe, 1981). This can probably be ascribed to the fact that thiamine pyrophosphate is also required as cofactors of enzymes that are responsible for converting branched-chain amino acids and α-ketoglutarate into precursors of succinic acid. On the other hand though, the addition of a 100 mg/L SO_2 to grape juice with a very high fermentable sugar content (> 400 g/L) increased the production succinic acid and decreased the production of glycerol, as well as acetic acid (Caridi, 2003).

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Chapter 3

RESEARCH RESULTS

Title of scientific article

3. RESEARCH RESULTS

OENOLOGICALLY IMPORTANT FACTORS THAT INFLUENCE THE PRODUCTION OF SUCCINIC ACID BY FERMENTING WINE YEASTS

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Unforeseen increases in titratable acidity during alcoholic fermentation can result in wines that taste too sour. Large amounts of succinic acid produced by fermenting yeasts are believed to be the main cause of titratable acidity increases during alcoholic fermentation. Succinic acid production by nine commercial yeast strains was studied in a chemically defined grape juice-like medium to determine the factors involved in the production of succinic acid during alcoholic fermentation. The main organic acids of grape juice and wine were separated and quantified by means of an improved capillary zone electrophoresis (CZE) method. Between 0.3 and 2.2 g/L succinic acid was produced by fermenting yeasts in synthetic grape juice. The amount of succinic acid produced during alcoholic fermentation depended on the yeast strain, fermentation temperature and chemical composition of the synthetic must. Out of the nine commercial yeast strains selected for this study, Anchor Yeast strain WE372 produced the largest amount of succinic acid in synthetic grape juice at 28°C. The fermentation by-product profile and other physiological attributes of the before mentioned commercial yeast strain indicated that it might be a hybrid between *S. cerevisiae* and *S. uvarum* (*S. bayanus* var. *uvarum*). Succinic acid production by fermenting yeasts will be favoured by moderate to high fermentation temperatures (20°C to 25°C) in grape juice with a nicotinic acid and/ or nicotinamide deficiency, a high sugar content (200 g/L ± 20 g/L), moderate amounts of yeast metabolizable nitrogen (300 ± 50 mg/L YMN), the presence of flavonoids and large supplies of unsaturated long-chain fatty acids. Even higher concentrations of succinic acid can be expected if the fermenting grape juice is aerated. Deficiencies in thiamine, biotin, pyridoxine and pantothenic acid decreased the amount of succinic acid produced during alcoholic fermentation. Manipulation of these factors can be used to control succinic acid production during alcoholic fermentation in order to manage the titratable acidity of wine.

Keywords:

3.1 INTRODUCTION

One of the most striking qualities of wine is its tart, sour taste. The sensory perception of sourness is mainly attributed to the presence of hydrogen ions (protons) at high concentrations (Paul, 1917; Amerine *et al*., 1959; Clarke & Bakker, 2004). Large amounts of weak carboxylic acids (organic acids) are the main sources of hydrogen ions within wine. Once wine enters a person's mouth, the dissociable protons of the organic acids within wine are partially neutralized or, in other words, titrated by the saliva secreted inside one's mouth (Boulton *et al*., 1998a). This explains why the duration and intensity of a wine's sourness is related to its titratable acidity content (Amerine *et al*. 1959, 1965). The sour taste of wine is usually considered as refreshing and it helps balance the other flavours thereof. In fact, wines become watery when its titratable acidity content is too low (Fischer, 2001).

After alcoholic fermentation, the titratable acidity of wine will usually be less than that of the grape juice from which was made due to ethanol-induced precipitation of potassium bitartrate crystals and partial consumption of malic acid by fermenting wine yeasts (Thoukis *et al*., 1965; Rankine, 1966). Occasionally however, increases in titratable acidity are observed during alcoholic fermentation (Ough *et al*., 1969; Vine, 1981; Holgate, 1997; Lamikanra, 1997; Coulter *et al*., 2004). If wine is produced from grape juice with optimum levels of titratable acidity, unforeseen increases in titratable acidity during alcoholic fermentation can be detrimental to the quality of the final product.

According to Holgate (1997), malic acid production by fermenting wine yeasts is responsible for the increases in titratable acidity that were observed within some of the red-grape must fermentations during Australia's 1995 vintage. Under optimal conditions, certain wine yeast strains can produce up to 2 g/L malic acid during alcoholic fermentation (Radler & Lang, 1982; Schwarz & Radler, 1988). However, most wine yeasts consume more malic acid than what they produce during fermentation of grape juice (Rankine, 1966; Fatichenti *et al*., 1984; Delcourt *et al*., 1995).

Although the net production of malic acid by wine yeasts contributes to increases in titratable acidity during grape juice fermentations (Ough *et al.*, 1969), the production of succinic acid is regarded as the primary cause (Thoukis *et al*., 1965; Ough *et al.*, 1969; Lamikanra, 1997). In fact, succinic acid accounts for approximately 90% of the non-volatile acids produced during fermentation of grape juice (Thoukis *et al*., 1965). Between 0.5 and 1.5 g/L succinic acid is normally found within wine (Margalit, 1997; Boulton *et al*., 1998a), but higher concentrations thereof (up to 3.0 g/L) have been detected within certain red wines (Coulter *et al*., 2004).

Acidity adjustments should preferably be carried out before the onset of alcoholic fermentation to allow better integration of the added compound(s) and to ensure that conditions during fermentation favour the quality and microbial stability of the final product. In doing so unfortunately, winemakers run the risk of ending up with wines that may taste too sour if they are unable to accurately predict and take into

consideration the amount of succinic acid produced during alcoholic fermentation. Knowledge with regard to the factors involved in succinic acid's production by fermenting wine yeasts is therefore required in order to manage the titratable acidity of wines more accurately.

Ever since Pasteur (1860) first noticed succinic acid amongst the by-products of alcoholic fermentation, attempts have been made to determine the metabolic pathways and factors involved in its production by fermenting wine yeasts (Genevois, 1936; Kleinzeller, 1941; Ribéreau-Gayon *et al.*, 1956; Stoppani *et al*., 1958; Mayer *et al*., 1964; Thoukis *et al.*, 1965; Heerde & Radler, 1978; Wakai *et al*., 1980; Muratsubaki, 1987; Arikawa *et al*., 1999; Camarasa *et al*., 2003). Up until now however, it remains unclear why wines sometimes end up with exceptionally high levels of succinic acid (Coulter *et al*., 2004).

Therefore, it was decided to investigate the possible causes of very high succinic acid concentrations within wine. Due to complexity of grape juice's chemical composition and the problems associated with sterilizing grape juice, fermentation experiments were conducted within a chemically defined grape juice-like medium. Succinic acid production by nine different industrial wine yeast strains was studied under various conditions with regard to the nutrient status of the synthetic grape juice, temperature and availability of molecular oxygen during alcoholic fermentation.

3.2 MATERIALS AND METHODS

3.2.1 YEAST STRAINS

Nine commercially available wine yeast strains were selected for this study (**Table 3.1**). All of them were obtained in the active dry form and rehydrated without stirring in sterile, 5% (w/v) glucose/ tap-water solution for 15 minutes at 37°C. Active dry wine yeast (ADWY) was weighed off in a flow-laminar cabinet near an open flame in order to avoid contamination by other micro-organisms. The weighing-end of the spatula used for this purpose was dipped in absolute ethanol and flamed prior to weighing.

3.2.2 FERMENTATION MEDIUM

All fermentation experiments were conducted in chemically defined media and the fermentation medium of control experiments had a chemical composition similar to that of Henschke & Jiranek's (1993) grape juice-like medium (**Table 3.2**). Changes to the original medium's yeast assimilable nitrogen (YAN) content are the most noteworthy. Ammonium was omitted and the amino acid profile of the new synthetic grape juice resembled that of Shiraz grapes harvested in 2006 from vineyards near Stellenbosch (South Africa). It is also important to point out that the modified synthetic grape juice contained ten times less nicotinic acid than the original medium

A stock solution of amino acids, twenty times more concentrated than the levels required in the final medium, was prepared by adding potassium hydroxide to help dissolve all of the amino acids in deionised water. The amino acids stock solution was sterilized by means of vacuum filtration through a 0.22 μm cellulose-acetate membrane and divided up into smaller volumes (50 ml) for storage at −20°C until needed.

Table 3.1

Taxonomic classification of the ADWY strains selected for this study according to the information provided by the commercial suppliers thereof.

Strain	Commercial Supplier	Taxonomic Classification
EC1118	Lallemand	*S. cerevisiae* p.r. *bayanus*
DV10	Lallemand	*S. cerevisiae* p.r. *bayanus*
U43	Lallemand	*S. cerevisiae* p.r. *bayanus*
WE372	Anchor Yeast	*S. cerevisiae* p.r. *cerevisiae*
WE14	Anchor Yeast	*S. cerevisiae* p.r. *cerevisiae*
VIN7	Anchor Yeast	*S. cerevisiae* p.r. *cerevisiae*
VIN13	Anchor Yeast	*S. cerevisiae* p.r. *bayanus* × p.r. *cerevisiae*
NT116	Anchor Yeast	*S. cerevisiae* p.r. *bayanus* × p.r. *cerevisiae*
NT112	Anchor Yeast	*S. cerevisiae* p.r. *bayanus* × p.r. *cerevisiae*

[a] Anchor Yeast Bio-technologies (Epping Industria, South Africa); [b] Lallemand Inc. (Montreal, Canada); [c] p.r. = physiological race.

Stocks of all the trace elements were a hundred thousand times more concentrated than the levels thereof within synthetic grape juice. Therefore, the salt of each trace element had to be added separately to the final medium in order to prevent the formation of insoluble complexes and metal oxides. Trace element stocks were autoclaved at 121°C for 10 minutes and stored at 4°C. Vitamin stock solutions were filter sterilized and kept frozen until needed. Tween 80® served as the source of oleic acid within the stock solution of essential lipids, which was prepared in hot, absolute ethanol.

The medium was prepared by adding all the sugars, organic acids and macro-elements into a glass beaker and dissolving them in hot deionised water. After the solution was allowed to cool down to room temperature, amino acids were added and the pH of the solution adjusted to 3.3 with potassium hydroxide. It was then transferred to a volumetric flask and made up to the mark with deionised water after all the vitamins and trace elements were added. The medium was filter sterilized into an autoclaved Schott® bottle, which contained sterile cellulose powder in order to adjust the turbidity thereof to approximately 200 nephelometric turbidity units (Hernández-Orte *et al*., 2006). Since ergosterol precipitated out of the essential lipids stock solution at room temperature, it was heated to 80°C within a water bath, before the required volume was added to the rest of the synthetic grape juice.

Table 3.2

Chemical composition of the synthetic grape juice used in control experiments.

Component	Amount per litre	Component	Amount per litre
Sugars		Amino Acids	
Glucose	100 g	Alanine	150 mg
Fructose	100 g	Valine	145 mg
Organic Acids		Leucine	120 mg
Tartaric acid	2 g	Isoleucine	80 mg
Malic acid	3 g	Glycine	10 mg
Citric acid·H_2O	0.5 g	Serine	150 mg
Macro Elements		Threonine	250 mg
K_2HPO_4	1.14 g	Aspartic acid	100 mg
$MgSO_4 \cdot 7H_2O$	1.23 g	Asparagine	250 mg
$CaCl_2 \cdot 2H_2O$	0.44 g	Glutamic acid	200 mg
Trace Elements		Glutamine	155 mg
$MnCl_2 \cdot 4H_2O$	200 μg	Tryptophan	50 mg
$ZnCl_2$	135 μg	Phenylalanine	50 mg
$FeCl_2$	30 μg	Tyrosine	20 mg
$CuCl_2$	15 μg	Methionine	5 mg
$CoCl_2 \cdot 6H_2O$	30 μg	Lysine	20 mg
$NaMoO_4 \cdot 2H_2O$	25 μg	Histidine	150 mg
H_3BO_3	5 μg	Arginine	500 mg
KIO_3	10 μg	Proline	600 mg
Vitamins		FAN [a]	250 mg
Pyridoxine·HCl	2 mg	YAN [b]	444 mg
½ Ca Pantothenate	1.5 mg	YMN [c]	336 mg
Thiamine·HCl	0.5 mg		
Nicotinic acid	0.2 mg		
Riboflavin	0.2 mg		
p-Aminobenzoic acid	0.2 mg		
Folic acid	0.2 mg		
Biotin	0.125 mg		
myo-Inositol	100 mg		
Essential Lipids			
Oleic acid	120 mg		
Ergosterol	15 mg		
Insoluble material			
Cellulose	0.5 g		

[a] free α-amino nitrogen; [b] yeast assimilable nitrogen; [c] yeast metabolizable nitrogen.

3.2.3 FERMENTATION CONDITIONS

Autoclaved, 250 ml Erlenmeyer flasks served as fermentation vessels. Each flask was filled with 100 ml sterile, synthetic grape juice and inoculated with 0.3 g/L rehydrated active dry wine yeast. Anaerobic fermentation experiments were conducted without agitation of fermenting media and a rubber bung fitted with a water

lock was used to close the opening of each flask. The rubber bungs of fermentation locks were immersed in absolute ethanol and allowed to dry in a flow laminar cabinet before being used. In the case of aerobic fermentation experiments, fermentations were carried out on a rotary shaker and the opening of each flask was plugged with sterile cotton wool, covered by tin foil. Fermentation experiments were carried out at 28°C, except for the few that were conducted at 15°C. The fermented media (wines) were sampled once carbon dioxide formation stopped. Samples were filtered through 0.45 μm cellulose-acetate syringe disk filters (Acrodisc®) and stored at 4°C until they could be analysed.

3.2.4 CHEMICAL ANALYSIS OF FERMENTATION BY-PROUCTS

An improved version of Soga & Ross' (1997) capillary electrophoresis (CE) method was used to separate and quantify the most important organic acids of wine. High performance capillary electrophoresis (HPCE) was carried out with Hewlett-Packard's G1600A HP^{3D}CE system (Agilent Technologies, Waldbronn, Germany), which included a build-in photodiode array detector and electrolyte replenishment system. HP^{3D} Chemstation software was used for system control, data acquisition and data analysis. A Hamilton Microlab 500 Series digital diluter (Reno, NV, U.S.A.) was used to dilute filtered wine samples forty times with a 5 mM morpholino ethanesulphonic acid (MES) buffer (pH = 6.20), which also contained 0.5 mM sodium formate as an internal standard and 10 mg/L sodium azide to inhibit microbial growth. The separation electrolyte (pH = 5.60) consisted of 7.5 mM pyridine-2,6-dicarboxylic acid (PDC), 0.5 mM cetyltrimethylammonium bromide (CTAB) and sodium ions as counter ions. Both of the above mentioned solutions were vacuum filtered through 0.45 μm Nylon membrane filters and degassed at the same time. Electrophoresis was conducted at 10°C in a fused-silica capillary column with an internal diameter of 50 μm, an effective length of 72 cm and total length of 80.5 cm (Agilent Technologies, Germany). The column was rinsed and equilibrated with the separation electrolyte between successive runs and a negative electric potential of 25 kV was applied over the capillary ends for a period of 2 minutes prior to sample injection. Approximately 16 nL of sample was injected into the column by applying pressure (50 mbar) at the inlet side of the capillary. A small amount of separation electrolyte (~2 nL) was injected into the column directly afterwards. The electric potential was ramped from zero to −25 kV within half a minute and then kept constant at −25 kV for the remainder of each run. Compounds were detected by means of indirect UV detection at 210 nm Electropherograms were recorded at a detection wavelength of 350 nm and a reference wavelength of 210 nm. Vials containing separation buffer were automatically emptied and refilled with fresh electrolyte after each analysis. A validation report for this method, along with some research notes are provided in **Addendum A**.

Castellari *et al*.'s (2000) high performance liquid chromatography (HPLC) method was used to quantify the levels of glycerol within experimental wines. The original

method was slightly modified by diluting all the samples four times, instead of twenty times with deionised water. Waters' 717 Plus autosampler (Milford, MA, U.S.A.) was used to inject 20 μl of each sample into a 30 cm × 7.8 mm Aminex HPX-87H cation exchange column (Bio-Rad Laboratories, Hercules, CA, U.S.A.). A vacuum filtered solution (0.22 μm cellulose-acetate membrane) of 45 N sulphuric acid and 6% (v/v) acetonitrile served as the mobile phase. The column temperature was maintained at 45°C and compounds were eluted from the column at a constant flow rate of 0.5 ml/minute. Agilent's 1100 Series binary pump and degasser unit was used to degas and pump mobile phase through the HPLC column. The HPLC column was connected to Waters' 996 photodiode array detector, as well as a Waters 410 differential refractometer (Milford, MA, U.S.A.). Organic acids were detected at 210 nm and the refractive index signal was used to detect glucose, fructose, glycerol and ethanol. Chromatographic data were recorded and processed with Waters' Millennium^{32} Chromatography Manager Software (Milford, MA, U.S.A.). Calibrations were performed with external standards.

A gas chromatographic method developed at Stellenbosch University's Department of Oenology and Viticulture was used to separate and quantify the most important higher alcohols, esters and medium-chain fatty acids within wine. One millilitre diethyl ether and a 100 μL internal standard solution (0.5 mg/L 4-methyl-2-pentanol dissolved in ethanol) were added to 5 ml wine within a Pyrex® glass tube and closed with a screw cap. This mixture was sonicated for 5 minutes, then centrifuged for 3 minutes at 4000 rpm and left to separate into two layers. The top layer (organic phase) was recovered and transferred to a GC autosampler vial's insert. A spatula point of sodium sulphate was placed at the bottom of each autosampler vial to remove traces of water from the ether extract. Separation by means of gas chromatography was carried out with Hewlett-Packard's 6890 Series gas chromatograph (Waldbronn, Germany) equipped with a Hewlett-Packard 7683 Series injector, DB-FFAP column (60 m × 0.32 mm I.D. × 0.5 μm film thickness) and flame ionization detector. Three micro litres of ether extract was injected into the chromatographic column in split mode with a split ratio of 15:1 and a split flow rate of 49.5 ml/minute. The temperature at the injection port was set at 200°C and the temperature at the detector was kept at 240°C. Hydrogen served as the mobile phase and its flow through the column was set at a constant rate of 3.3 ml/minute. The oven temperature was raised from 33°C to 240°C at rate of 12°C/minute and then held at 240°C for another 5 minutes. Nitrogen was used as make-up gas (30 ml/minute).

3.2.5 CHEMICAL REAGENTS AND STANDARDS

The chemicals that were used for CE, HPLC and GC analyses were of the highest purity available and purchased from Aldrich Chemical Company (Gillingham, UK), Sigma Chemical Company (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland), Acros Organics (Geel, Belgium), SaarChem (Midrand, South Africa) and Riedel-de-

Haën (Seelze, Germany). Ultra-pure deionised water (18.2 MΩ/cm) was obtained from a Milli-Q water purification system (Millipore, Belford, MA, U.S.A.). Standards were weighed on a Mettler Toledo AB240 analytical balance (Switzerland) and pH measurements were carried out with a WTW 340i pH meter equipped with a WTW SenTiX 41 pH electrode (Weïlheim, Germany). All solutions were prepared in analytical grade glassware at 20°C.

3.2.6 DATA ANALYSIS

Experiments were conducted in triplicate. Student t-tests or one-way analysis of variance (ANOVA) in combination with Fisher Least Square Difference tests were used to determine whether differences between results were statistically significant ($p = 0.05$). This was all carried out with the aid of a computer software package, called STATISTICA 7 (StatSoft Inc., Tulsa, OK, U.S.A.).

3.3 RESULTS AND DISCUSSION

3.3.1 THE INFLUENCE OF WINE YEAST STRAINS' GENETIC MAKE-UP

Lallemand's (Montreal, Canada) wine yeasts EC1118, DV10 and U43 are known within the wine industry as strains of *S. cerevisiae* physiological race *bayanus* (www.brsquard.org; www.scottlab.com). Strains EC1118 and DV10 produced similar amounts of succinic acid during anaerobic fermentation of synthetic grape juice at 28°C. Succinic acid production by strain U43 however, was noticeably less. Although only moderate concentrations of acetic acid (≤ 0.5 g/L) were produced by strains EC1118 and DV10, strain U43 produced exceptionally high levels of acetic acid (> 1.0 g/L) under the above described fermentation conditions. Strains EC1118 and DV10 also corresponded with regard to the levels of glycerol and 2-phenylethanol they produced during fermentation.

Table 3.3

Fermentation by-product profile of the different wine yeasts selected for this study.

Strain	Succinic acid	Acetic acid	Glycerol	2-Phenylethanol
EC1118	0.752 c	0.521 d	6.991 d	14.95 c
	(± 0.036)	(± 0.008)	(± 0.242)	(± 0.16)
DV10	0.813 c	0.413 e	7.280 bcd	14.33 c
	(± 0.016)	(± 0.008)	(± 0.095)	(± 0.60)
U43	0.626 d	1.045 a	7.652 bc	6.91 e
	(± 0.008)	(± 0.002)	(± 0.192)	(± 0.06)
WE372	1.436 a	0.277 g	9.000 a	31.49 a
	(± 0.024)	(± 0.008)	(± 0.132)	(± 0.69)
WE14	0.754 c	0.450 e	7.303 bcd	15.00 c

	(± 0.032)	(± 0.010)	(± 0.201)	(± 0.32)
VIN7	0.525 e	0.905 b	7.131 cd	10.46 d
	(± 0.003)	(± 0.005)	(± 0.056)	(± 0.16)
VIN13	0.990 b	0.220 h	7.305 bcd	20.06 b
	(± 0.039)	(± 0.015)	(± 0.233)	(± 0.18)
NT116	0.988 b	0.330 f	7.712 b	13.90 c
	(± 0.029)	(± 0.005)	(± 0.182)	(± 1.13)
NT112	0.561 de	0.753 c	7.274 bcd	8.15 e
	(± 0.013)	(± 0.010)	(± 0.260)	(± 0.09)

[a] glycerol, succinic- and acetic acid concentrations are reported in g/L; [b] 2-phenylethanol and iso-amylalcohol concentrations are reported in mg/L; [c] letter(s) next to numerical values denote significant differences ($p < 0.05$) within each column; [d] standard error of the mean reported in brackets; [e] fermentation conditions match those of the control experiments.

In comparison to the strains EC1118 and DV10, strain U43 produced slightly more glycerol and significantly less 2-phenylethanol. In other words, strain U43's fermentation by-product profile differ notably from that of the other two *S. cerevisiae* p.r. *bayanus* strains that were studied (**Table 3.3**).

Strains EC1118 and DV10 are mostly used for the production of bottle-fermented sparkling wine (*champagne*), which explains why commercial ADWY producing companies also market them respectively as the French Champagne strains '*Prise de Mousse*' and '*Epernay*' (www.brsquard.org; www.scottlab.com). These wine yeasts are vigorous fermenters and can tolerate high sugar and ethanol concentrations (Henschke, 1997; www.anchorwineyeast.com). *Champagne* wine yeasts were once classified as strains of the formerly known species *Saccharomyces oviformis* (Lodder & Kreger-van Rij, 1952; Pretorius *et al*., 1999; Ribéreau-Gayon *et al*., 2000a), which was incorporated into *Saccharomyces bayanus sensu* (van der Walt, 1970) in 1970 (Pretorius *et al*., 1999; Ribéreau-Gayon *et al*., 2000a). Not long thereafter, *S. bayanus* and sixteen other *Saccharomyces* species *sensu* (van der Walt, 1970) were all reclassified by Yarrow (1984) as *Saccharomyces cerevisiae* (Pretorius *et al*., 1999; Ribéreau-Gayon *et al*., 2000a). *S. cerevisiae* as defined by Yarrow (1984) consequently got divided up into seventeen physiological races with epithets that correspond with the names of the species that got incorporated. In other words, *champagne* yeasts (ex-*S. oviformis*) became known as strains of *S. cerevisiae* p.r. *bayanus* since 1984, which as mentioned above is still used within the wine industry to differentiate them from other wine yeasts. However, today's taxonomists regard ex-*S. oviformis* as part of the *Saccharomyces sensu stricto* species, *S. cerevisiae* (Vaughan-Martini & Martini, 1998). According to micro- and minisatellite based DNA fingerprinting wine yeast strains DV10 and EC1118 are genetically identical (Bradbury *et al*., 2005), which explains the high degree of similarity between their fermentation by-product profiles at 28°C. Wine yeast strain U43's fermentation by-product profile on the other hand, is

surprisingly very different from that of the before mentioned two strains. Although they all belong to the same physiological race of *S. cerevisiae sensu* (Yarrow, 1984), micro- and minisatellite based DNA fingerprinting indicate that only 35% of wine yeast strain U43's genome is related to those of wine yeast strains DV10 and EC1118 (Bradbury *et al*., 2005). In addition, Bradbury *et al*. (2005) pointed out that the size of wine yeast strain U43's genome may be aneuploid, whereas the respective genomes of wine yeast strains EC1118 and DV10 are diploid.

Anchor (Epping Industria, South Africa) wine yeast strains WE372, WE14 and VIN7 are all classified as *S. cerevisiae* p.r. *cerevisiae* (www.anchorwineyeast.com) according to the yeast classification system adopted by the wine industry. However, these wine yeasts differ noticeably from each other with regard to the levels of secondary metabolites each of them produced during fermentation of synthetic grape juice at 28°C (**Table 3.3**). In comparison to the other wine yeasts selected for this study, strain WE372 produced very little acetic acid during alcoholic fermentation and was the largest producer of succinic acid, glycerol and 2-phenylethanol. Unlike the rest of the wine yeast strains in this study, strain WE372 consumed almost no malic acid (< 100 mg/L) during fermentation of synthetic grape juice with low levels of yeast assimilable nitrogen and was the only wine yeast strain that was able to produce more malic acid than what it consumed when either aspartate or glutamate served as the only yeast assimilable nitrogen source within synthetic grape juice (**Table 3.4**).

The fermentation by-product profile of strain WE372 closely resembles that of yeasts, which have been categorized by some as cryotolerant or cryophilic wine yeasts (Castellari *et al*., 1994, 1995; Kishimoto, 1994; Giudici *et al.*, 1995; Bertolini *et al*., 1996; Zambonelli *et al*., 1997; Rainieri *et al*., 1998a, 1998b, 1999a, 1999b; Antonelli, *et al*., 1999; Coloretti *et al*., 2006).

Cryotolerant wine yeasts grow well at temperatures between 6 and 12°C, but struggle to grow at temperatures above 34°C (Walsh & Martin, 1977; Castellari *et al*., 1992; Kishimoto, 1994). Mesophilic wine yeasts on the other hand, find it difficult to grow at temperatures below 12°C and above 40°C (Walsh & Martin, 1977; Castellari *et al*., 1992). Most cryotolerant wine yeasts as defined by Castellari *et al*. (1992) were once classified as *S. cerevisiae* p.r. *uvarum*, while some of them belonged to *S. cerevisiae* p.r. *bayanus* (Castellari *et al*., 1992, 1994). The cold-tolerant ex-*S. carlsbergensis* strains of *S. cerevisiae* p.r. *uvarum* were incorporated into *S. pastorianus sensu stricto*, while the remaining cold-tolerant *S. cerevisiae* p.r. *uvarum* strains were all incorporated into *S. bayanus sensu stricto* (Vaughan-Martini & Martini, 1993, 1998). *S. bayanus sensu stricto* can be divided into two subgroups based on genetic compatibility (hybrid fertility) tests (Rainieri *et al*., 1999a; Naumov, 2000), genetic fingerprinting (Nguyen & Gaillardin, 1997; Nguyen *et al*., 2000; Pulvirenti *et al*., 2000; Nguyen & Gaillardin, 2005; Naumova *et al*., 2005) and electrophoretic karyotyping (Rainieri *et al*., 1999a; Nguyen *et al*., 2000). Rainieri *et al*. (1999a) compared the fermentation by-product

profiles of different *Saccharomyces sensu stricto* species and found that only the strains of *S. bayanus* var. *uvarum* (*S. uvarum*) produced the profiles that typified Castellari *et al*.'s (1994) 'cryotolerant' yeasts.

Table 3.4

The effect of yeast assimilable nitrogen on the malic acid content of wines produced from synthetic grape juice with 3.0 g/L malic acid.

	Malic acid			
	Amino acid mixture		Aspartate	Glutamate
Strain	125 mg FAN/L	250 mg FAN/L	250 mg FAN/L	250 mg FAN/L
EC1118	2.536 c	2.358 b	2.820 cd	2.972 bc
	(± 0.009)	(± 0.012)	(± 0.006)	(± 0.021)
DV10	2.550 c	2.358 b	2.869 bc	3.002 b
	(± 0.004)	(± 0.013)	(± 0.020)	(± 0.009)
U43	2.507 cd	2.402 b	2.575 f	2.590 g
	(± 0.010)	(± 0.010)	(± 0.019)	(± 0.017)
WE372	2.912 a	2.230 d	3.279 a	3.318 a
	(± 0.012)	(± 0.015)	(± 0.020)	(± 0.027)
WE14	2.462 d	2.192 d	2.734 e	2.843 e
	(± 0.038)	(± 0.008)	(± 0.026)	(± 0.039)
VIN7	2.524 c	2.364 b	2.637 f	2.757 f
	(± 0.004)	(± 0.012)	(± 0.016)	(± 0.014)
VIN13	2.511 cd	2.289 c	2.900 b	2.986 bc
	(± 0.012)	(± 0.016)	(± 0.050)	(± 0.012)
NT116	2.519 c	2.191 d	2.789 de	2.887 de
	(± 0.008)	(± 0.025)	(± 0.002)	(± 0.013)
NT112	2.614 b	2.554 a	2.766 de	2.931 cd
	(± 0.026)	(± 0.020)	(± 0.018)	(± 0.020)

[a] values are reported in g/L; [b] standard error of the mean reported in brackets; [c] letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a column; [d] fermented @ 28°C.

Cryotolerant wine yeasts grow well at temperatures between 6 and 12°C, but struggle to grow at temperatures above 34°C (Walsh & Martin, 1977; Castellari *et al*., 1992; Kishimoto, 1994). Mesophilic wine yeasts on the other hand, find it difficult to grow at temperatures below 12°C and above 40°C (Walsh & Martin, 1977; Castellari *et al*., 1992). Most cryotolerant wine yeasts as defined by Castellari *et al*. (1992) were once classified as *S. cerevisiae* p.r. *uvarum*, while some of them belonged to *S. cerevisiae* p.r. *bayanus* (Castellari *et al*., 1992, 1994). The cold-tolerant ex-*S. carlsbergensis* strains of *S. cerevisiae* p.r. *uvarum* were incorporated into *S. pastorianus sensu stricto*, while the remaining cold-tolerant *S. cerevisiae* p.r. *uvarum* strains were all incorporated into *S. bayanus sensu stricto* (Vaughan-Martini & Martini, 1993, 1998). *S. bayanus sensu stricto* can be divided

into two subgroups based on genetic compatibility (hybrid fertility) tests (Rainieri *et al*., 1999a; Naumov, 2000), genetic fingerprinting (Nguyen & Gaillardin, 1997; Nguyen *et al*., 2000; Pulvirenti *et al*., 2000; Nguyen & Gaillardin, 2005; Naumova *et al*., 2005) and electrophoretic karyotyping (Rainieri *et al*., 1999a; Nguyen *et al*., 2000). Rainieri *et al*. (1999a) compared the fermentation by-product profiles of different *Saccharomyces sensu stricto* species and found that only the strains of *S. bayanus* var. *uvarum* (*S. uvarum*) produced the profiles that typified Castellari *et al*.'s (1994) 'cryotolerant' yeasts.

Strain WE372's fermentation by-product profile in synthetic grape juice is in other words similar to the by-product profile associated with *S. uvarum* strains (cryotolerant wine yeasts). However, strain WE372 is not cold tolerant like other *S. uvarum* strains and has a sugar fermentation profile that is characteristic of *S. cerevisiae*'s ex p.r. *cerevisiae* (Anchor Yeast, Industria, South Africa). Our own findings and the information supplied by producers strongly suggest that strain WE372 is an interspecific hybrid between *S. cerevisiae* and *S. uvarum* (*S. bayanus* var. *uvarum*). A number of natural wine isolates, as well as the commercial strain S6U (Lallemand, Montreal, Canada) have also been identified as hybrids between *S. cerevisiae* and *S. uvarum* (Masneuf *et al*., 1998; Naumov *et al*., 2000a; Naumova *et al*., 2005; González *et al*. 2006; Le Jeune *et al.*, 2007; Sipiczki, 2008).

The levels of glycerol, 2-phenylethanol, succinic- and acetic acid that were produced by strain WE14 during fermentation of synthetic grape juice closely matched the production levels of these compounds by the ex-*S. oviformis* strains EC1118 and DV10.
However, the respective fermentation by-product profiles of the ex-*S. oviformis* (*champagne* yeasts) strains in our study (EC1118 and DV10) do not agree with the

In other words, ex-*S. oviformis* (*champagne* yeasts) strains can be classified as cryotolerant wine yeasts according to the original definition thereof (Walsh & Martin, 1977; Castellari *et al*., 1992), because they grow well at temperatures below 12°C (www.brsquard.org; www.scottlab.com) and are not able to grow at temperatures greater than 32°C (Vaughan-Martini & Martini, 1993).

Anchor (Epping Industria, South Africa) wine yeast strain VIN7 is classified within the wine industry as *S. cerevisiae* p.r. *cerevisiae* (www.anchorwineyeast.com), but it is actually a hybrid between two *Saccharomyces sensu stricto* species, *i.e. S. cerevisiae* and *S. kudriavzevii* (Bradbury *et al*., 2005; Sipiczki, 2008). Unfortunately, wine yeast strain VIN7 was the only one of its kind in the present study. Recently however, Gonzàlez *et al.* (2007) compared two such hybrids (Lallemand strains W27 and W46) with the type strain of *S. kudriavzevii* (IFO 1802) and a commercial *S. cerevisiae* strain (Lallamand strain T73). The two *S. cerevisiae* x *S. kudriavzevii* hybrids produced more glycerol than the *S. cerevisiae* strain at low to

moderate fermentation temperatures, but less than *S. kudriavzevii*'s type strain. Similar amounts of glycerol were produced by hybrid strain W27, the type strain of *S. kudriavzevii* and the *S. cerevisiae* strain at 32°C, but hybrid strain W46 formed less than them at this temperature. Glycerol production by the *S. cerevisiae* x *S. kudriavzevii* hybrid in our study, strain VIN7, was similar to that of *S. cerevisiae* strains (EC1118, DV10 and WE14) at 28°C (**Table 3.3**). The *S. cerevisiae* x *S. kudriavzevii* hybrids studied by Gonzàlez *et al*. (2007) formed less acetic acid than the *S. cerevisiae* strain at low to moderate fermentation temperatures, but acetic acid production by hybrids were similar to that of the *S. cerevisiae* strain at 32°C. Strain VIN7 however, was the second largest producer of acetic acid in synthetic grape juice fermented at 28°C (**Table 3.3**). This particular *S. cerevisiae* x *S. kudriavzevii* hybrid find high fermentation temperatures and/or sugar concentrations stressful and can form up to 0.8 g/L acetic acid in grape juice (Lourens & Reid, 2005). Strain VIN7 produced the lowest concentration of succinic acid in synthetic grape juice fermented at 28°C, but the amount produced by strain VIN7 was not significantly different from the amounts produced by strains NT112 and U43.

Strains VIN13, NT116 and NT112 are classified as hybrids between *S. cerevisiae*'s former physiological races *bayanus* and *cerevisiae* (Anchor Yeast, Industria, South Africa). However, all three of these strains possess an enhanced ability to liberate box-tree (broom, blackcurrant), grapefruit (passion fruit) and citrus zest aromas from S-cysteine conjugate precursors (Anchor Yeast, Industria, South Africa), which is characteristic of *S. uvarum* strains (Masneuf *et al*., 2002). The latter mentioned two strains are cold tolerant (Reid & Lourens, 2005) and produced very little acetic acid in synthetic grape juice at 28°C (**Table 3.3**). Strain NT112 on the other hand, is not cold tolerant (Reid & Lourens, 2005) and was the third largest producer of acetic acid in this study. Similar amounts of succinic acid were produced by strains VIN13 and NT116. They produced less succinic acid than strain WE372, but produced noticeably more than the other strains in this study. 2-Phenylethanol production by strain NT116 was similar to that of the champagne strains and strain WE14, but strain VIN13 was the second largest producer of 2-phenylethanol in synthetic grape juice. Strain NT112 produced relatively low amounts of succinic acid, 2-phenylethanol and isoamyl alcohol (**Table 3.3**).

Microsatellite-based DNA fingerprinting indicate that large parts of strain VIN13's genome was derived from a French champagne (ex *S. oviformis*) strain (Bradbury *et al*., 2005). Genetic material from an ex *S. oviformis* strain are probably responsible for strain VIN13's ability to grow at very low temperatures (Vaughan-Martini & Martini, 1993; Reid & Lourens, 2005a) and explains why one of its parents were classified as *S. cerevisiae* p.r. *bayanus*. French champagne strains are heterozygous for homothallism (*HO*/*ho*) (Johnston *et al*., 2000), which probably facilitated its use in cross-breeding programs. Most wild and selected winemaking strains are homozygous for homothallism (*HO*/*HO*) (Mortimer *et al*., 1994; Ribéreau-Gayon

et al., 2000), which makes the isolation of sexually stable haploids difficult. Heterothallic diploids (ho/ho) on the other hand, are rare and incapable of sporulation (Ribéreau-Gayon *et al*., 2000; Sipiczki, 2008). Strain VIN13's enhanced ability to liberate volatile thiols (Anchor Yeast, Industria, South Africa) is characteristic of *S. uvarum* strains (Masneuf *et al*., 2002), but sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA and the sizes (number of nucleotides) of the mating type (*MAT*) alleles indicate that strain VIN13 contains genetic material from only *S. cerevisiae* parents (Bradbury *et al*., 2005). However, it is possible that strain VIN13's *S. cerevisiae* p.r. *cerevisiae* parent is an allodiploid *S. uvarum* x *S. cerevisiae* hybrid. The ITS region of ribosomal DNA from interspecific hybrids is subject to the phenomenon of concerted evolution, in which the ITS sequence from one of the parents can be converted into that of the other parent (Bradbury *et al*., 2005). Large parts of a non-*S. cerevisiae* parent's genome can be lost in interspecific hybrids during successive mitotic and/or meiotic divisions (Sebastiani *et al*., 2002; Antunovics *et al*., 2005b; Sipiczki, 2008), which could account for the absence of *MAT* alleles from *S. uvarum* in strain VIN13.

3.3.2 FERMENTATION TEMPERATURE

The maximum temperature for the production of red wine lies between 25°C and 30°C to ensure sufficient extraction of phenolic compounds. White wines on the other hand are usually produced at temperatures below 20°C to retain aromas (Ribéreau-Gayon *et a*l., 2000c). Synthetic grape juice was fermented at 15°C and 28°C to investigate the effect of fermentation temperature on succinic acid production (**Table 3.4**).

Temperature's influence on succinic acid production depends on the yeast strain. Temperature increases between 12°C and 30°C stimulate succinic acid production by mesophilic *S. cerevisiae* strains (Ough *et al*., 1969; Shimazu & Watanabe, 1981; Castellari *et al*., 1995). Succinic acid production by *S. uvarum* (*S. bayanus* var. *uvarum*) strains on the other hand, reaches a maximum at about 18°C in the majority of cases (Castellari *et al*., 1995). Temperature's effect on succinic acid production by mesophilic *S. cerevisiae* strains is less striking than in the case of cryotolerant *S. uvarum* strains (Castellari *et al*., 1995). *S. uvarum* strains produce similar concentrations of acetic acid at different temperatures within the range 12°C to 24°C and form only slightly more acetic acid at temperatures greater than 24°C (Castellari *et al*., 1995). Mesophilic *S. cerevisiae* strains on the other hand, produce noticeably more acetic acid at temperatures below 18°C and above 30°C.

Strain WE372's response towards the change in fermentation temperature were similar to that of *S. uvarum* strains in terms of both succinic and acetic acid production. It produced considerably more succinic acid at 28°C than at 15°C and similar amounts of acetic acid at these two temperatures (**Table 3.4**). Strains VIN13, NT116, EC1118, DV10 and WE14 also produced more succinic acid at 28°C than at 15°C (**Table 3.4**), but the change in fermentation temperature had a noticeably lesser

effect on the amount of succinic acid produced by the latter three strains than in the case of strain WE372. Higher concentrations of acetic acid were formed by strains VIN13, NT116 and WE14 at 15°C, but acetic acid production at the two temperatures was not significantly different in cases where fermentation was conducted by strains VIN13 or WE14. Strain EC1118 and DV10 on the other hand, formed markedly less acetic acid at the lower fermentation temperature. Strain WE14's response towards the change in temperature confirms that it is a mesophilic *S. cerevisiae* strain, which differs from the two cryotolerant *S. cerevisiae* strains (EC1118 and DV10).

Wines produced at 15°C had a higher titratable acidity than those produced at 30°C in cases where fermentation was carried out by the *S. cerevisiae* x *S. kudriavzevii* hybrid, strain W27 (Reynolds *et al*., 2001). This implies that strain W27 formed more succinic acid at 15°C than at 30°C, since succinic acid production is the main cause of titratable acidity increases during fermentation (Thoukis *et al*., 1965; Ough *et al*., 1969; Lamikanra, 1997; Coulter *et al*., 2004). Strains U43 and NT112 were the only strains in the present study, which formed more succinic acid at 15°C than at 28°C. The *S. cerevisiae* x *S. kudriavzevii* hybrid in our study, strain VIN7, produced similar amounts of succinic acid at 15°C and 28°C (**Table 3.4**). Less acetic acid were formed by strains VIN7, U43 and NT112 at 15°C than at 28°C. *S. cerevisiae* x *S. kudriavzevii* hybrids produce less acetic acid at low or moderate fermentation temperatures than at high fermentation temperatures (Gonzàlez *et al.*, 2007).

Differences between strains in terms of succinic acid production were less marked at 15°C than at 28°C. Other fermentation experiments were therefore conducted at 28°C in stead of 15°C.

Increase in temperature increases the toxic effects of ethanol (Ribéreau-Gayon *et al.*, 2000a).

3.3.3 FERMENTATBLE SUGAR CONTENT

Agenbach, 1977, Henschke & Jiranek, 1993 – partial inhibition of yeast growth at sugar concentrations above 258 g/L p115 osmotic stress reduced growth rate and cell numbers.(Nishino, 1985)

Mature *Vitis vinifera* grapes contain between 160 and 260 g/L fermentable sugar (Margalit, 1997), but higher concentrations of fermentable sugar can be found in juice obtained from partially dried or botrytisized grapes (Boulton *et al*., 1998; Ribéreau-Gayon et al., 2000d). The fermentable sugar content of the synthetic grape juice was increased to 300 g/L to study the effect of high sugar concentrations on succinic acid production (**Table 3.5**).

Succinic acid production by *S. cerevisiae* strains increase with increasing amounts of fermentable sugar and reaches a maximum at a sugar concentration of about 220 g/L according to results obtained by Shimazu & Watanabe (1981).

Significantly less succinic acid was produced by strains VIN13, NT116, WE372 and WE14 in synthetic grape juice with 300 g/L sugar, compared to the amounts produced in synthetic grape juice with 200 g/L sugar. Strain U43 on the other hand, produced more succinic acid in the must with 300 g/L sugar than in the must with 200 g/L sugar. Succinic acid production in must with 300 g/L was not significantly different from production in must with 200 g/L sugar in cases where fermentation was carried out by strains EC1118, DV10, VIN7 and NT112, but strain NT112 produced more succinic acid in the must with 300 g/L sugar. All the strains studied by us produced significantly more acetic acid in the synthetic grape juice with 300 g/L sugar. Differences between strains in terms of succinic acid production were noticeable in synthetic grape juice with 300 g/L fermentable sugar than in synthetic grape juice with 200 g/L fermentable sugar. Other fermentation experiments were therefore conducted in musts with 200 g/L fermentable sugar.

Gene expression studies indicate that increased sugar concentrations stimulate the uptake of hexoses, the formation of oxaloacetate from pyruvate and the conversion of glutamate into succinate via the formation of γ-aminobutyrate and succinate semialdehyde (Erasmus *et al*., 2003). However, increasing amounts of sugar in the growth medium also elevate the production of glycerol by yeasts (Caridi, 2003; Erasmus *et al*., 2004). Increased glycerol production increases the need for the formation of cytoplasmic NADH (van Dijken & Scheffers, 1986; Nevoigt & Stahl, 1997), which is partially met by increased formation of acetic acid (Michnick *et al*., 1997; Remize *et al*., 1999; Caridi, 2003; Erasmus *et al*., 2004). Glycerol and acetic acid formation directs large amounts of hexose-carbon away from the metabolic pathways involved in succinic acid production.

3.3.4 UTILIZABLE NITROGEN CONTENT

minimal requirement for complete fermentation in the presence of 200 to 260 g/L fermentable sugar = between 120 and 140 mg/L YAN as from ammonium (Agenbach, 1977; Henschke & Jiranek, 1993)
Very high YAN: > 600 mg
Moderate YAN: 300 ppm ± 50 ppm
Very low: <100 ppm (Bell & Henschke, 2005)
Carbon: nitrogen ration effects succinate and acetate production (Albers et al., 1998)

FAN underestimates free amino acids' contribution to the YAN content (51–92%) Bell of grape juice, because some of the amino acid within grape juice and assimilated by yeasts possess amino groups at positions other than alpha (Bisson, 1991; Henschke & Jiranek, 1993). Not all assimible amino acids can be degraded into a carbon-skeleton during fermentation. YAN values do not differentiate between degradable, partially degradable and non-degradable nitrogen sources (Bisson, 1991).

Proline, an imino amino acid, is found in the highest concentrations in most *V. vinifera* juices, but cannot be taken up by yeasts in the absence of molecular oxygen (Duteurtre *et al*., 1971). Proline is therefore not considered part of grape juice's utilizable nitrogen content during alcoholic fermentation. Ammonium salts and free α-amino acids are the most abundant sources of nitrogen in grape juice, utilized by yeasts during alcoholic fermentation, which is why the utilizable nitrogen content of grape juice is often given in terms of free alpha nitrogen (FAN). The FAN content of grape juice does not include nitrogen from amino groups other than those located at the alpha positions (Lie, 1973; Henschke & Jiranek, 1993). However, some α-amino acids contain more than just one utilizable nitrogen atom per molecule and a few of the utilizable amino acids within grape juice contain amino groups at non-alpha positions (Large, 1986). The total amount of nitrogen from utilizable sources in grape juice is known as yeast assimilable nitrogen (YAN) (Bell & Henschke, 2005). All α-amino acids can be incorporated directly into yeast proteins, but not all them can be metabolized by fermenting yeasts (Large, 1986). YAN includes both metabolizable and non-metabolizable α-amino acids (Henschke & Jiranek, 1993). The term metabolically available nitrogen (MAN) will be used to refer to the amount of nitrogen from sources that can be metabolized by yeasts to ammonia under anaerobic conditions. In other words, MAN is the amount of nitrogen that becomes available in a form that can be used by yeasts to biosynthesize new monomeric nitrogen-containing compounds during the biochemical degradation of the original nitrogen source.

The following facts were taken into account to calculate the MAN content of synthetic grape juice. Arginine catabolism in yeasts starts off by being converted into ornithine and urea (Middelhoven, 1964). *S. cerevisiae* degrades urea into two molecules of ammonium and one molecule carbon dioxide (Whitney & Cooper, 1972; Sumrada & Cooper, 1982). One of the two amino groups from ornithine can be transferred to α-ketoglutarate, yielding glutamate and glutamate-5-semialdehyde. Glutamate-5-semialdehyde is then transformed into proline due to spontaneous hydrolysis of glutamate-5-semialdehyde (Boulton *et al*., 1998a). However, proline cannot be degraded in the absence of molecular oxygen (Duteurtre *et al*., 1971). This means that only three of arginine's four nitrogen atoms are metabolically available to yeasts during alcoholic fermentation. Tryptophan, in addition to proline, does not contribute to the MAN content of grape juice due to a lack of molecular oxygen during alcoholic fermentation. Yeasts are also unable to metabolize glycine, histidine and lysine, irrespective of whether oxygen is available or not (Large, 1986).

The synthetic grape juice, in which control experiments were carried out contained 250 mg/L FAN, 439 mg/L YAN and 336 mg/L MAN from a mixture of amino acids. Due to the substantial differences between these amounts, it was decided to replace the mixture of amino acids with ammonium chloride as nitrogen source to investigate the effect of nitrogen concentration on succinic acid production (**Table 3.6**). Ammonium chloride provides the same amount of FAN, YAN and MAN

to yeasts during fermentation and cannot be converted directly into succinic acid like some amino acids (Boulton *et al*., 1998a). Synthetic must with 250 mg/L nitrogen from ammonium represented grape juice with a moderate nitrogen content (Bell & Henschke, 2005). This amount was halved to simulate a must with a low nitrogen content and doubled to simulate a must with a high nitrogen content.

Succinic acid production by the *S. cerevisiae* strain studied by Heerde and Radler (1978) was increased by increasing amounts of FAN up to a concentration of 500 mg/L. However, none of the strains in our study produced more succinic acid in a must with 500 mg/L FAN than in one with 250 mg/L FAN. In fact, increasing amounts of utilizable nitrogen in the must within the range 125 to 500 mg/L decreased succinic acid production by *S. cerevisiae* strains EC1118, DV10 and WE14. This was also true in the case yeast strain NT112. Maximum succinic acid production by the strain now believed to be a hybrid between *S. uvarum* and *S. cerevisiae*, strain WE372, occurred in the must with a moderate nitrogen content. Strain WE372 formed more succinic acid in the must with low nitrogen levels than in the must with high nitrogen levels. Increasing concentrations of utilizable nitrogen in the must affected succinic acid production by yeast strains VIN13, NT116 and the *S. kudrivazevii* x *S. cerevisiae* hybrid, strain VIN7, in the same way as it did strain WE372, but differences between the amounts produced were not always statististically significant. Different concentrations of utilizable nitrogen in the must within the range 125 to 500 mg/L had no significant effect on the amount of succinic acid produced by yeast strain U43.

Previous studies have shown that increasing amounts of utilizable nitrogen in the must up to a concentration of 450 mg/L decreases the production of acetic acid by fermenting yeasts, but increases the production thereof at nitrogen concentrations greater than 450 mg/L (Tromp, 1984; Delfini & Cervetti, 1991). Organic acid production by fermenting yeasts is influenced by the carbon to nitrogen ratio of the growth medium (Larsson *et al*., 1997; Albers *et al*., 1998b). Our own findings are similar to the ones just mentioned, except in cases where fermentation was carried out by strains WE14 and VIN7. Increasing concentrations of utilizable nitrogen in the must within the range 125 to 500 mg/L increased the amount of acetic acid produced by strain WE14. The rest of the strains in the study produced less acetic acid in the must with moderate nitrogen levels than in the must with low nitrogen levels. All the yeast strains, except strain VIN7 produced larger amount of acetic acid in the must with high nitrogen levels than in the must with moderate nitrogen levels. However, differences between the amounts produced in musts with moderate and high nitrogen levels were not statistically significant in cases where fermentation was carried out by strains VIN7, EC1118 and VIN13.

The majority of yeast strains studied by us formed greater amounts of succinic acid and less acetic acid in musts with moderate nitrogen levels than in musts with low nitrogen levels. Moderate concentrations of utilizable nitrogen in the must would have stimulated yeast growth more than low levels thereof (Hernández-Orte *et al*., 2006). This increased the need for TCA cycle intermediates, which are used by cells

to synthesize amino acids, nucleotides and several other important molecules. Pyruvate derived from hexose sugars enter the TCA cycle by first being converted into oxaloacetate and acetyl-coenzyme A (acetyl-CoA). The majority of acetyl-CoA is formed via the formation of acetate during alcoholic fermentation (Steensma, 1997). Increased formation of acetyl-CoA due to stimulated growth is probably the reason why less acetic acid was produced in the must with moderate nitrogen levels than in the must with low nitrogen levels. Glutamate is central to amino acid biosynthesis due to its involvement in transamination reactions (Jones & Fink, 1982), which means that larger quantities of thereof will probably be formed within yeasts as a result of stimulated growth. Transamination reactions involving glutamate generate α-ketoglutarate. Yeasts can get rid of excess α-ketoglutarate during fermentation by forming succinic acid via the remaining reactions of the TCA cycle's oxidative branch (Camarasa *et al*., 2003), which could explain why greater amounts of succinic acid is formed in musts with moderate nitrogen contents than in musts with low nitrogen contents. Glutamate can be stored in vacuoles until needed as nitrogen donors for amino acid biosynthesis (Boulton *et al*., 1998a). However, when glutamate is no longer needed for growth, it can be converted into succinic acid via the formation of γ-aminobutyrate and succinate semialdehyde. Increased cell numbers due to stimulated growth also means that the overall activity of the enzymes involved in succinic acid production are increased.

However, results from this study also indicated that less succinic acid and more acetic acid were produced in musts with high nitrogen levels than in musts with moderate amounts of utilizable nitrogen. Amino acids are transported into yeasts by means of active transport mechanisms coupled to proton symport (Horak, 1986; Henschke & Jiranek, 1993) and

The uptake of ammonium ions (Roon, 1975; Dubois & Grenson, 1979; Peña *et al*., 1987) reduce the proton motive force across the yeast plasma membrane due to the dissociation of one of the protons inside the cytoplasm, causing an increase in the consumption of ATP (Peña *et al*., 1987) by plasma membrane ATPases in order to extrude protons from the cell (Serrano, 1978; Willsky, 1979).

Ammonia is taken up as ammonium ions (Roon, 1975; Dubois & Grenson, 1979; Peña *et al*., 1987), which reduces the proton motive force across the plasma membrane due to dissociation of a proton once inside the cell (Peña *et al*., 1987; Boulton *et al*., 1998) decrease ATP (Peña *et al*., 1987), because ATP is required by plasma membrane ATPases to extrude protons (Serrano, 1978; Willsky, 1979). Many of the amino acid transport systems in yeasts are proton symports and ammonium ions dissociate into ammonia and hydrogen ions once inside the yeast cytoplasm (Boulton *et al*., 1998a). However, protons that enter yeasts must be excreted at some stage to prevent acidification of the cytoplasm and subsequent cell death. Yeasts excrete protons via ATPases located within the plasma membrane, which increases the amount of ATP consumed when nitrogen sources are taken up by yeasts.

Utilizable nitrogen sources are consumed early in the fermentation process and stored within yeast vacuoles due to the inhibitory effect of ethanol on the uptake of these compounds in acidic media (Kitamoto *et al*. 1988; Boulton *et al*., 1998a). This implies that yeasts consume more ATP when grown in musts with high nitrogen levels than in musts with moderate or low nitrogen levels due to the increased uptake of utilizable nitrogen compounds. Acetyl-CoA can be formed from pyruvate by the pyruvate dehydrogenase complex or it can be formed from acetate by acetyl-CoA synthetase during fermentation (Steensma, 1997 Holtzer, 1961; Pronk *et al*., 1994, 1996; Remize *et al*., 2000). Pyruvate decarboxylase competes with the pyruvate dehydrogenase complex for puruvate during fermentation (Holtzer, 1957; Pronk *et al*., 1996), which means that acetyl-CoA formation by the pyruvate dehydrogenase complex will be limited during fermentation due to the very high levels of pyruvate decarboxylase activity in fermenting yeasts (Pronk *et al*., 1996). This implies that most acetyl-CoA is formed via the so-called pyruvate dehydrogenase bypass, which involves the reactions catalyzed by pyruvate decarboxylase, acetaldehyde dehydrogenase and acetyl-CoA synthetase (Pronk *et al*., 1994; Remize *et al*., 2000; Steensma, 1997). Yeasts require ATP to convert acetate derived from pyruvate into acetyl-CoA (Steensma, 1997). ATP is also required by pyruvate carboxylases within yeasts to convert pyruvate into oxaloacetate (Pronk *et al*., 1996; Steensma, 1997). Succinic acid can be formed from oxaloacetate via the reductive branch of the TCA cycle (Camarasa *et al*., 2003), but oxaloacetate can also react with acetyl-CoA to form citrate, which can also be converted into succinic acid via the oxidative branch of the TCA cycle (Camarasa *et al*., 2003) or the GABA bypass (Coleman *et al.*, 2001). Lower levels of ATP caused by the increased uptake of nitrogen sources are probably the main reason why more acetic acid and less succinic acid are produced in musts with high nitrogen levels than in musts with moderate nitrogen levels.

The above experiment was repeated in synthetic grape juice, which contained a mixture of amino acids as nitrogen source (**Table 3.7**). The YAN content of the synthetic grape juice that served as the control in this experiment was slightly less than 500 mg/L, but all the studied strains produced noticeably more succinic acid in this must than in the must with 500 mg/L nitrogen from ammonium chloride. Succinic acid production in the synthetic grape juice that serves as the control corresponded with the amounts produced in synthetic must with 250 mg/L nitrogen from ammonium chloride. The synthetic grape juice with 439 mg/L YAN contained only 336 mg/L metabolically available nitrogen (MAN), which is much closer to 250 mg/L. These findings indicate that succinic acid production is influenced by the metabolizable fraction of YAN during alcoholic fermentations.

Strains of *S. cerevisiae* consume between 3 and 45% of the malic acid that is made available to them during alcoholic fermentation (Rankine, 1966; Radler, 1993), but are also capable of synthesizing malic acid (Schwartz & Radler, 1988; Fatichenti *et al*., 1986) from hexose-derived pyruvate and carbon dioxide during alcoholic

fermentation (Pines *et al*., 1996). This means that a certain percentage of the malic acid found in the wine after fermentation was undoubtedly formed by yeasts. The malic acid content of wines made from synthetic grape juice were less than the original amount present in the juice (**Table 3.8**), which indicates that more malic acid was consumed than produced by fermenting yeasts under the conditions employed in the current study. Note though, that the net consumption of malic acid was noticeably less in synthetic grape juice with low nitrogen levels than in musts with moderate or high nitrogen levels, especially in the case where fermentation was carried out by strain WE372 (**Table 3.8**). Suboptimal nitrogen concentrations of less than 300 mg/L favours the biosynthesis of malic acid by fermenting yeasts (Radler & Lang, 1982; Schwartz & Radler, 1988) and a smaller yeast population caused by low levels of nitrogen probably decreased the total amount of malic acid, which entered yeasts by means of passive diffusion (Radler, 1993; Delcourt *et al*.,1995). Net consumption of malic acid by strain WE372 in synthetic grape juice with low nitrogen levels was considerably less than the amounts consumed by other strains under the same conditions (**Table 3.8**). This finding indicates that strain WE372's ability to synthesize malic acid is greater than that of the other yeast strains in this study and confirmed by results presented in **Table 3.9**. Strain WE372's enhanced ability to synthesize malic acid serve as possible explanation as to why this strain produced noticeably more succinic acid than other commercial yeast strains under certain conditions. Fermenting strains of *S. cerevisiae* can produce succinic acid via the formation of malate according to results obtained by Camarasa *et al*. (2003). Malate has to be converted into fumarate before it can be reduced to succinate within fermenting yeasts, but results obtained by Pines *et al*. (1996) indicated that malate cannot be converted into fumarate within the fermenting *S. cerevisiae* due fumarase's 17 times higher affinity for fumarate. However, the results by obtained by Camarasa *et al*. (2003) proved otherwise. High intracellular levels of malate caused by an enhanced ability to form malate might favour malate's conversion into fumarate within certain yeast strains, thereby stimulating the production of succinic acid. It is not unreasonable to assume that this is in fact the case, since only high extracellular levels of malic acid resulted in increased production of succinic acid during a study conducted by Heerde & Radler (1978).

3.3.5 NICOTINIC ACID (NIACIN)

Nicotinic acid levels in grape juice vary between 0.3 and 9 mg/L (Margalit, 1997). *S. cerevisiae* strains are able to synthesize their own nicotinic acid from tryptophan via the kynurenine metabolic pathway when oxygen is available (Panozzo *et al*., 2002). Dissolved oxygen are available to yeasts during the first few hours after they are introduced to the must (Haukeli & Lie, 1979; O'Connor-Cox *et al.*, 1996) and the synthetic grape juice used in this study contained tryptophan (**Table 3.2**).

Fermentations were carried out in synthetic grape juice with either 0.2 or 2 mg/L nicotinic acid to determine whether succinic acid production is influence by the

nicotinic acid content of the must (**Table 3.10**). The nicotinic acid content of the must had a significant effect on the amount of succinic and acetic acid produced during fermentation, despite the fact that yeasts were able to form their own nicotinic acid. All the yeast strains selected for this study responded in the same way towards higher levels of nicotinic acid in the must. Less succinic acid and more acetic acid were formed by fermenting yeasts in the synthetic juice with 2 mg/L nicotinic acid than in the one with 0.2 mg/L nicotinic acid. Differences between strains in terms of succinic acid production were less noticeable in the synthetic juice with 2 mg/L nicotinic acid than in the one with ten times less nicotinic acid. Other fermentation experiments were therefore conducted in synthetic grape juice with 0.2 mg/L nicotinic acid.

The results presented in **Table 3.10** confirms Monk & Cowley's (1984) findings, which also showed that higher levels of nicotinic acid in grape musts increase the production of acetic acid by fermenting yeasts. Glycerol production on the other hand, will be reduced by higher levels of nicotinic acid in grape juice according to results obtained by Radler & Schütz (1982). Most of the acetic acid produced by yeasts during fermentation is formed by oxidizing acetaldehyde. Acetaldehyde dehydrogenases catalyze the oxidation of acetaldehyde within yeasts and require nicotinamide adenine dinucleotide (NAD^+) or the closely related nicotinamide adenine dinucleotide phosphate ($NADP^+$) as cosubstrate (Steensma, 1997; Remize *et al*., 2000). One of the main physiological roles of glycerol formation within yeasts is to ensure the continuation of glycolysis during alcoholic fermentation by oxidizing excessive levels of NADH back to NAD^+ (van Dijken & Scheffers, 1986; Prior & Hohmann, 1997; Boulton *et al*. 1998). Nicotinic acid is a precursor of both NAD^+ and $NADP^+$ (Horton *et al*., 1996). Judging by our own results and the ones obtained by others, it seems as if the presence of nicotinic acid in musts increases intracellular levels of NAD^+, thus explaining the increased formation of acetic acid and the reduced need for glycerol production. The reduced formation of succinic acid in the must with a higher nicotinic acid content was probably caused by the increased production of acetic acid, which directs carbon away from metabolic pathways that lead to the formation of succinic acid. These same results also indicate that succinic acid production is favoured by lower levels of NAD^+, which implies that considerable amounts of succinic acid are formed via the reductive branch of the TCA cycle.

3.3.6 THIAMINE

The thiamine content of grape juice vary between 0.1 and 1 mg/L according to Margalit (1997), but the maximum amount of thiamine detected in Hungarian *V. vinifera* musts was only 0.3 mg/L (Juhász *et al*., 1987). High concentrations of dissolved sulphur dioxide can cause thiamine deficiencies in grape musts due to chemical degradation of thiamine by bisulphite ions (Leighter & Joslyn, 1969; Boulton

et al., 1998d). A lack of thiamine can also caused by indigenous yeast growth prior to inoculation with a pure starter culture.

Thiamine was omitted from synthetic grape juice to study its effect on succinic acid production during alcoholic fermentation (**Table 3.11**). Less succinic acid was formed in the must without thiamine than in the control must. The decrease in succinic acid production caused by the omission of thiamine was statistically significant in all of the cases, except the one where fermentation was carried out by strain NT112. Like most strains in this study though, strain NT112 produced less acetic acid in the must without thiamine than in the control must, which agree with the findings of Ribéreau-Gayon *et al*. (1956). Strain WE372 on the other hand, produced slightly more acetic acid in the absence of thiamine.

Yeasts require thiamine for the biosynthesis of thiamine pyrophosphate (TPP), which is a coenzyme of pyruvate decarboxylases (Hübner *et al*., 1992; Hohmann, 1997). All yeasts are capable of synthesizing thiamine from pyrimidine and thiazole (White & Spenser, 1979). Strains of *S. cerevisiae* synthesize the pyrimidine moiety of thiamine from pyridoxine and histidine, whereas the thiazole unit thereof is synthesized from cysteine, glycine and D-ribulose 5-phosphate or D-xylulose 5-phosphate (White & Spenser, 1979; Nosaka, 2006). .Fermenting yeasts are unable to biosynthesize thiamine (). Lower intracellular levels of TPP will decrease the overall activity of pyruvate decarboxylases within yeasts, which is responsible for the formation of acetaldehyde from pyruvate. Since most acetate is formed from acetaldehyde during fermentation (), the before mentioned chain of events explain why fermenting yeasts produce less acetic acid in synthetic grape juice without thiamine. It is not clear however, why strain WE372 produced more acetic acid under the same conditions. As mentioned before, acetate is the primary source of acetyl-CoA during alcoholic fermentation (Steensma, 1997) and can combine with oxaloacetate to form citrate. Citrate is a precursor of α-ketoglutarate, but further conversion into succinate depends on the activity of α-ketoglutarate dehydrogenase, which is a TPP-dependant enzyme (Horton *et al*., 1996). The decreased activities of pyruvate decarboxylase and α-ketoglutarate dehydrogenase, which are caused by lower intracellular levels of TPP, is in other words responsible for the decreased production of succinic acid observed in media without thiamine.

3.3.7 FLAVONOIDS

Additions of (+)-catechin to synthetic and real grape juice decreased the production of acetic acid (Garcia Moruno *et al*., 1993; Caridi, 2003) and increased the production of succinic acid (Caridi, 2003) by fermenting yeasts under osmotic stressful conditions. (+)-Catechin is a type of phenolic compound, known as a flavan-3-ol (Margalit, 1997; Ribéreau-Gayon *et al*., 2000e). Up to 200 mg/L (+)-catechin can be extracted from red grapes when the juice is fermented in contact with the pomace (Goldberg *et al*., 1998). Much lower concentrations of (+)-catechin will be found in the fermenting juice of white grapes, because flavan-3-ols are mainly found in the seeds

and stems (rachis and pedicle) of grapes. Acetic acid production in synthetic grape juice can also be decreased by the addition of grape anthocyanins (Garcia Moruno *et al*., 1993). However, acetic acid production in synthetic grape juice was less effected by the addition of anthocyanins than by the addition of (+)-catechins, even though the concentration of anthocyanins added was considerably more (Garcia Moruno *et al*., 1993). Anthocyanins and flavan-3-ols belong to the same group of phenolic compounds, known as flavonoids. Other grape flavonoids include flavan-3,4-diols (leucoanthocyanidins) and flavonols (Margalit, 1997; Ribéreau-Gayon *et al*., 2000e).

A 100 mg/L (+)-catechin was added to synthetic grape juice to verify whether succinic acid production is increased by the presence of flavonoids in the growth medium (**Table 3.14**). Catechin additions to synthetic grape juice caused each of the selected yeast strains in our study to produce less acetic acid and more succinic acid. However, its effect on succinic acid production was not statistically significant in cases where fermentation was carried out by strains DV10 and WE372. This was also true with regard to its influence on acetic acid production by strain EC1118.

As mentioned before, most acetic acid is formed from acetaldehyde as a precursor of acetyl-CoA during fermentation (Remize *et al*., 2000). (+)-Catechin can react with acetaldehyde (Timberlake & Bridle, 1976), which could have prevented acetaldehyde, from taking part in other reactions. As in the case of other phenolic compounds, flavonoids are capable of forming complexes with proteins (Asano *et al*., 1982; Clifford, 1986). Acetaldehyde dehydrogenases may be more reactive towards flavonoids than other enzymes and yeast proteins. The formation of bonds between acetaldehyde dehydrogenase and flavonoids can cause conformational changes within the enzyme, which might prevent acetaldehyde from reaching the enzyme's active sites. The decreased formation of acetate will also lead to a decrease acetyl-CoA formation within yeasts. Although a decrease in acetyl-CoA formation will not affect the formation of succinic acid from oxaloacetate via the reductive branch of the TCA cycle, it will definitely decrease the production of succinic acid via the formation of α-ketoglutarate. However, the observed increase in succinic acid production indicates that acetyl-CoA formation was not reduced by the addition of flavonoids to the growth medium. In fact, the decrease in acetic acid production implies that less pyruvate was directed away from the metabolic pathways involved in succinic acid production.

Flavone inhibits mitochondrial NADH dehydrogenase in yeasts which use FAD as prosthetic group (de Vries & Grivell, 1988). Similar inhibition of succinate dehydrogenase by flavonoids a possible cause for the observed increase in succinate production.
Increased yeast growth increases succinic acid production (Ough *et al*., 1969).

Acetate due to limited acetyl Co A synthetase activity (van Urk et al., 1990) and ATP deficiency. Reaction requires ATP.

3.3.8 UNATURATED LONG-CHAIN FATTY ACIDS

These findings were ascribed to an increase in intracellular ATP (Kiessling, 1949) by the before mentioned authors. Higher intracellular ATP levels stimulate the formation of succinate within fermenting wine yeasts by increasing the formation of oxaloacetate and acetyl-CoA.

S. cerevisiae yeasts can synthesize all the fatty acids that they need for growth (Daum *et al*., 1998), but require molecular oxygen to synthesize long-chain unsaturated fatty acids (Bloomfield & Bloch, 1960; Ratledge & Evans 1989; Daum *et al*., 1998). The crushing of grapes results in a rapid uptake of oxygen. Grape juice saturated with air contains between 6 and 8 mg/L dissolved oxygen (Ribéreau-Gayon *et al*., 2000c), but all of it is removed from the must within the first three hours after inoculation with yeast (O'Connor-Cox *et al*., 1996). A substantial part of a grape juice's dissolved oxygen content will be lost to yeasts due to oxidation reactions with phenolic compounds in the juice and entrainment by the carbon dioxide produced by fermenting yeasts. In other words, the largest part of carbon-catabolite repressed yeast growth take place in the absence of molecular oxygen. Fermenting yeasts therefore depend on the lipid content of the growth medium to supply them with necessary fatty acids and sterols.

The synthetic grape juice used in this study provided long-chain unsaturated fatty acids to fermenting yeasts in the form of Tween80®, which is the commercial name for the polyoxyethylene sorbitan mono-oleate (Polysorbitan 80). Tween80® was omitted from the synthetic grape juice to investigate the influence of unsaturated fatty acids on succinic acid production during fermentation (**Table 3.15**). Synthetic grape juice was sterilized by means of vacuum filtration, which probably removed most of its dissolved oxygen content. However, a small amount of oxygen could have been taken up by the medium during the decantation of medium into Erlenmeyer flasks. Fermentation vessels were closed with fermentation locks immediately after the medium was inoculated with yeasts, which prevented any further uptake of oxygen. Ergosterol (15 mg/L) was not omitted from the synthetic grape juice, which did not contain Tween80®, to ensure a better survival rate of the yeast population in the absence of unsaturated fatty acids and an uncertain amount of dissolved oxygen. In media with non-limiting levels of oleic acid (172 mg/L), fermenting yeasts require 7 mg/L ergosterol for optimal growth under completely anaerobic conditions (Andreasen & Stier, 1953; Ribéreau-Gayon *et al*., 2000a). Optimal concentration of oleic acid ranges from 56-113 mg/L for anaerobic fermentation in media with non-limiting levels of ergosterol (20 mg/L) (Andreasen & Stier, 1954). Tween 80 alone does not support growth (Andreasen & Stier, 1953) under anaerobic conditions.

Similar amounts of succinic acid were produced in the control must and in synthetic juice without unsaturated fatty acids in cases where fermentation was carried out by strains EC1118, VIN7, NT116 and WE14. The only strains in this study, which produced more succinic acid in the control must than in the synthetic juice without unsaturated fatty acids, were strains DV10, VIN13 and WE372. Strains U43 and NT112 on the other hand, produced significantly more succinic acid in the absence of unsaturated fatty acid supplies. All the yeast strains selected for the study, except strain WE372, produced noticeably more acetic acid in the control must than in the synthetic juice without unsaturated fatty acids. Although the difference was not statistically significant, strain WE372 produced slightly more acetic acid in the absence of unsaturated fatty acids supplies than in cases where it was added to the synthetic must.

Fatty acid biosynthetic pathways are activated within yeasts when they are grown in media without sources of the necessary fatty acids. Hexadecanoyl-CoA and octadecanoyl-CoA will start to accumulate within fermenting yeasts once the dissolved oxygen content of the must becomes depleted, because molecular oxygen is needed to desaturate the bonds of saturated long-chain fatty acyl-CoA esters (Bloomfield & Bloch, 1960; Ratledge & Evans 1989). High intracellular concentrations of hexadecanoyl-CoA and octadecanoyl-CoA inhibit acetyl-CoA carboxylase, which decreases intracellular malonyl-CoA levels. A shortage of malonyl-CoA prevents further elongation of fatty acyl-CoA esters by the fatty acid synthetase complex (Wakil *et al*., 1983). Medium-chain fatty acyl-CoA esters are released by the fatty acid synthetase complex and then hydrolyzed to recover coenzyme A. This results in the excretion of medium-chain fatty acids (Bardi *et al*., 1999).

Membranes become rigid when its phospholipids lack unsaturated long-chain fatty acids due to the strong hydrophobic interactions between the rectilinear hydrocarbon chains of saturated fatty acids in phospholipids. *Cis* double bonds produces bends in the hydrocarbon tails of fatty acids, since rotation around double bonds are hindered. The bends in the hydrocarbon tail of unsaturated long-chain fatty acids prevent the orderly stacking of phospholipids (Quin *et al*., 1989; Ribéreau-Gayon *et al*., 2000a). Plasma membranes prevent an uncontrolled influx of polar and charged solutes into yeasts by acting as a hydrophobic barrier between the cytoplasm of cells and the outside environment. Polar and charged solutes, such as hexoses and ammonium, are transport of across the plasma membrane by transport proteins (permeases) imbedded within the membrane. The ability of permeases to function properly is influenced by the membrane's fluidity. A lack of unsaturated long-chain fatty acids in the phospholipids of yeast membranes decreases permease activity, which in turn decrease the rate of fermentation (Mauricio & Salmon, 1992) and the production of ATP. Low intracellular levels of ATP will decrease the production of acetyl-CoA from acetate, which leads to a further increase in acetic acid production during fermentation in the absence of unsaturated fatty acids and oxygen (Delfini & Cervetti, 1991; Delfini & Costa, 1993).

However, eight of the nine yeast strains in our study produced less acetic acid during fermentation in the absence of unsaturated fatty acid sources relative to the control. It also did not have any significant effect on the amount of acetic acid produced by the remaining yeast strain, strain WE372. These results indicate that the fermenting yeasts contained adequate levels of ATP during growth in synthetic grape juice without sources of unsaturated fatty acids as long as the medium contained enough sterols. In other words, yeasts membranes were able to function fully as selective barriers when fermenting yeasts were grown in media without sources of unsaturated fatty acids as long as the medium contained adequate supplies of sterols.

Sterols are imbedded within yeast membranes at an angle perpendicular to the membrane's phosholipid bilayer, which prevents the orderly stacking of phospholipids with a low unsaturation index (Ribéreau-Gayon *et al*., 2000a). In other words, the role of sterols in membranes is similar to that of the unsaturated fatty acids of phospholipids. Sterols also ensure that the yeast plasma membrane stays structurally and functionally intact during alcoholic fermentation. Yeasts adapt to the ethanol contents of the fermenting must by increasing the amount of sterols, especially ergosterol, within membranes (Novotny *et al.*, 1992; Arneborg *et al*., 1995, Valero *et al*., 1998). Ethanol is known to fluidize yeasts membranes (Alexandre *et al*., 1994b), but high concentrations of ergosterol and other yeast sterols inhibits this process by forming hydrogen bonds with the polar heads of phospholipids and by inserting its hydrocarbon tail in the hydrophobic region of the membrane's phospholipid bilayer (Hossack & Rose, 1976; Bottema *et al*., 1985; van der Rest *et al*., 1995; Ribéreau-Gayon *et al*., 2000a).

Molecular oxygen is required for the biosynthesis of sterols in yeasts (Parks, 1978; Parks & Casey, 1995). Between 0.3 and 1.2 mg/L dissolved oxygen is needed for sterol biosynthesis and another, 0.9 to 1.2 mg/L dissolved oxygen is needed for unsaturated fatty acid biosynthesis during fermentation in high-gravity wort (O'Connor-Cox *et al*., 1996). Synthetic grape juice with 15 mg/L ergosterol probably contained enough oxygen for fermenting yeasts to synthesize most of the unsaturated and saturated long-chain fatty acids that it required during fermentation, since no oxygen was needed for sterol biosynthesis. Large amounts of acetyl-CoA are required for the biosynthesis of fatty acids and since most acetyl-CoA is formed from acetate during fermentation (Holtzer, 1957, 1961; Pronk *et al*., 1994, 1996; Steensma, 1997; Remize *et al*., 2000), this explains why less acetic acid was produced in synthetic grape juice without sources of unsaturated fatty acids.

Most of the yeast strains in this study produced more succinic acid in the control synthetic grape juice than in the one without Tween 80®. Supplies of unsaturated fatty acids and sterols in the control juice were in excess of what was needed, which meant that oxygen could be used for β-oxidation of oleic acid to acetyl-CoA. Acetyl-CoA hydrolase probably converts some of the excess acetyl-CoA to acetate, which explains why more acetic acid was produced by yeasts grown in media with large

amounts of unsaturated fatty acid supplies (**Table 3.15**). Molecular oxygen is used by FAD-dependant peroxidases or fatty acid oxidases to oxidise fatty acyl-CoA substrates to trans-2-enoyl-CoA in yeast peroxisomes, which is the first step of β-oxidation (Hiltunen *et al*., 2003). Oxygen is also required for the biosynthesis of heme-dependant catalase A (Clarkson *et al*., 1991), which degrade the hydrogen peroxide formed during the before mentioned reaction (Hiltunen *et al*., 2003). Adequate supplies of fatty acids in the control must also reduced the need to biosynthesize acetyl-CoA and malonyl-CoA, which lead to a greater availability of ATP (Horton *et al*., 1996). Adequate supplies of fatty acids in the control juice also reduced fatty acid biosynthesis (Thurston *et al*., 1981) Higher intracellular levels of ATP would have stimulated pyruvate carboxylase activity in yeasts (Miller & Atkinson, 1972), which lead to the increased production of oxaloacetate, as well as citrate. Both oxaloacetate and citrate are precursors of succinic acid during alcoholic fermentation. Sien ieder verduideliking in hoofstuk 4

Yeasts capable of synthesizing their own unsaturated lipids, increase the proportion of phospholipid unsaturated fatty-acyl residues and the ergosterol (and other sterols with a Δ^{22}-unsaturated C-17 side chain) content of their plasma membranes when they are grown in the presence of ethanol (Beavan *et al.*, 1982; Novotný *et al*., 1992; Alexandre *et al*., 1994a). Egosterol help to prevent alcohol-induced fluidization or stretching (Hossack & Rose, 1976; Alexandre *et al*., 1994b) of the plasma membranes by forming hydrogen bonds with the polar heads of phospholipids, as well as by inserting its hydrocarbon tail into the hydrophobic regions of the membrane's phospholipid bilayer (Hossack & Rose, 1976; Bottema *et al*., 1985). A higher proportion of unsaturated fatty-acyl residues in plasma membrane phospholipids compensates for the stretching of the membrane caused by ethanol.

However, yeasts are unable to biosynthesize sterols (Parks, 1978; Parks & Casey, 1995; Daum *et al*., 1998) and unsaturated long-chain fatty acids (Bloomfield & Bloch, 1960; Wakil *et al*., 1983; Daum *et al*., 1998) in the absence of molecular oxygen. Although the fermentation medium will undoubtedly contain a certain amount of dissolved oxygen at the start of alcoholic fermentation, all of it will be removed within the first three hours of fermentation in a static and closed fermentation vessel (Haukeli & Lie, 1979; O'Connor-Cox *et al*., 1996) as in the case of white wine production. Yeasts fermenting under such conditions are, in other words, dependant on the lipid content of the growth medium for supplies of sterols and unsaturated fatty acids. Our fermentation experiments were also conducted in closed, static fermentation vessels, which meant that sterols and/ or unsaturated fatty acids sources had to be added to the synthetic juice so that fermenting yeasts could accumulate and incorporate these lipids into their plasma membranes for protection against ethanol formed during fermentation (Mishra & Kaur, 1991). The synthetic grape juice used by us in control experiments therefore contained both ergosterol

and a source of oleic acid (Chapter 3). In one of our experiments though, the source of oleic acid was omitted to study succinic acid production by yeasts in the absence of unsaturated fatty acid supplies (Chapter 3).

Fluidization of the yeast plasma membrane by ethanol (Alexandre *et al*., 1994b). The resulting passive influx of protons into yeasts (Leão & van Uden, 1984a; Cartwright *et al*., 1986) reduces their intracellular ATP levels, because plasma membrane ATPases consume ATP to extrude protons from the cell (Serrano, 1978; Willsky, 1979) and the weakened proton-motive force across the plasma membrane reduce the uptake of inorganic phosphate (Borst-Pauwels & Dobbelmann, 1972; Thomas & Rose, 1979), which is needed for the formation of ATP. Fluidization of the yeast plasma membrane by ethanol (Alexandre *et al*., 1994b) also alters the environment in which membrane proteins find themselves, which affects their ability to function properly as indicated by the reduced uptake of hexoses (Thomas & Rose, 1979; Leão & van Uden, 1982; Salmon, 1989) by means of facilitated diffusion (Bisson, 1993; Barnett, 1997; Boulton *et al*., 1998g). The inhibiting effect of ethanol on hexose transport is responsible for the decrease in glycolytic flux during alcoholic fermentation even though glycolytic enzyme activities remain virtually the same (Larue *et al*., 1984; Dombeck & Ingram, 1987). In other words, ethanol's ability to inhibit hexose transport reduces the rate of ATP production during fermentation.

Acetic acid production during fermentation is related to the respective activities of acetaldehyde dehydrogenase (Hannemann, 1985; Radler, 1993; Verduyn *et al*., 1990; Remize *et al*., 2000) and acetyl-CoA synthetase (Verduyn *et al*., 1990). The latter mentioned enzyme's activity depends on the intracellular levels of ATP or in other words, the energy status of the yeasts (Steensma, 1997). The amount of acetic acid produced by fermenting yeasts thus indicates how much ATP was available for acetyl-CoA formation during fermentation (Verduyn *et al*., 1990). If the omission of the medium's unsaturated fatty acid source (polyoxyethylene sorbitan mono-oleate) caused yeasts to become less resistant to the toxic effects of ethanol, then these yeasts should have formed more acetic acid (Delfini *et al*., 1992, 1993; Garcia-Moruno *et al*., 1993;) during fermentation than the control's yeasts in response to the reduced levels of ATP in the cell caused by ethanol's toxicity. However, the yeasts that fermented in the absence of unsaturated fatty acids formed significantly less acetic acid, compared to the control (Chapter 3). This finding implies that supplies of ergosterol in synthetic grape juice were sufficient to protect fermenting yeasts against the toxic effects of ethanol. The initial levels of dissolved oxygen in the medium were probably enough for yeast to biosynthesize their own unsaturated fatty acids, because a shortage in unsaturated lipids would have stimulated fatty acid biosynthesis.

The decreased production of acetic acid during fermentation indicated that the omission of the fermentation medium's unsaturated fatty acid source (Tween 80) did not cause yeasts to become less resistant to the toxic effects of ethanol. This finding also seems to suggest that yeasts are able to protect themselves sufficiently against

the toxicity of ethanol during anaerobic fermentation when the growth medium only contains ergosterol as lipid component and when dissolved oxygen is present at the start of fermentation for the biosynthesis of unsaturated fatty acids (Chapter 3).

Confirm Ribereau-Gayon et al. (1956) findings – apergillus niger extract which contains mainly unsaturated fatty acids (Mishra & Kaur, 1991) decrease acetic acid production and stimulates succinic acid production Ribereau-Gayon et al. (1952) reason increased ATP (Kiesling, 1949)

The omission of the synthetic grape juice's source of unsaturated fatty acids caused yeasts to produce significantly less acetic acid during fermentation, which indicated that

Less acetic acid was produced by fermenting yeasts when a source of unsaturated long-chain fatty acids (Tween 80) was omitted from synthetic grape juice (Chapter 3). This finding indicate that some of the unsaturated fatty acids added to synthetic grape juice in control experiments were probably catabolized to acetyl-CoA, which in turn was then hydrolyzed to acetate and coenzyme A. In other words, the synthetic grape juice used in control experiments contained a surplus of unsaturated long-chain fatty acids (Chapter 3). Yeasts probably catabolized all the excess unsaturated fatty acids to acetyl-CoA, which was then hydrolyzed to acetate and coenzyme A.

Fatty acids taken up as CoA esters, broken down to acetyl-CoA and hydrolyzed to recover coenzyme A.

3.3.9 AERATION DURING FERMENTATION

Fermentation vessels were closed with cotton wool plugs and placed on a circular shaker to investigate the influence of must aeration on succinic acid production during alcoholic fermentation. Continues aeration of fermenting musts caused all the yeast strains to produce more than 1 g/L succinic acid during alcoholic fermentation and resulted in the largest amount of succinic acid (~2.2 g/L) produced in synthetic grape juice (**Table 3.17**). This result agrees with the findings of others (Ribéreau-Gayon *et al*., 1956; Coote & Kirsop 1973; Nagai *et al*., 1992; Arikawa *et al.*, 1999b; Chapter 3).

The promitochondria of fermenting yeasts lack an integrated electron-transport chain (Criddle & Schatz, 1969), but alternative respiratory pathways exists in fermenting yeasts when oxygen is available (Salmon *et al*., 1998). In one of these pathways, electrons are transferred from NADH via ubiquinol (reduced form of coenzyme Q) and cytochrome b to oxygen. Molecular oxygen is also used for the biosynthesis of heme-groups (Zitomer and Lowry, 1992) that form part of cytochomes (Horton *et al*., 1996), like the one just mentioned. The transfer of electrons to cytochrome b generates a proton gradient across the promitochondrial inner membrane, which can be used to drive the phosphorylation of ADP. Detectable levels of the ATP-synthase complex's (F_oF_1-ATPase) F_1-structural unit have been found in promitochondrial fractions of non-respiring yeasts (Criddle & Schatz, 1969)

and it would be unlikely for promitochondria not to also contain the F_o-structural unit thereof. This implies that fermenting yeasts are able to generate ATP by means of an adapted respiratory pathway during alcoholic fermentation in the presence of oxygen. ATP supplies energy to many biosynthetic reactions required for yeast growth and is a known activator of pyruvate carboxylases (Miller & Atkinson, 1972) and is acetyl-CoA synthetases (), which are involved in the formation of succinate precursors from pyruvate. ATP is also needed for the extrusion of protons, which are transported across the plasma membrane during the uptake of amino acids (Horák, 1986) and ammonia (Dubois & Grenson, 1979). Amino acids can be degraded to succinate precursors and both types of nitrogen sources are important for yeast growth.

Yeasts also require molecular oxygen for the biosynthesis of sterols (Parks, 1978), unsaturated fatty acids (Bloomfield and Bloch, 1960; Wakil *et al*., 1983; Daum *et al*., 1998), deoxyribonucleotides (Chabes *et al*., 2000) and several vitamins (Hunter White *et al*., 2001; Ishida & Yamada, 2002; Nosaka, 2006; Hall & Dietrich, 2007), which are needed for yeast growth. Sterols and membrane phospholipids with a high degree of unsaturation are needed within yeast plasma membranes to ensure proper functioning of hexose-permeases, thus favouring the uptake of fermentable sugars (Ribéreau-Gayon *et al*., 2000a). Higher cell numbers increase succinate production.

3.4 CONCLUSIONS

The amount of succinic acid produced during alcoholic fermentation depended on the yeast strain, fermentation temperature and chemical composition of the synthetic grape juice. Out of the nine commercial yeast strains selected for this study, strain WE372 produced the largest amount of succinic acid in synthetic grape juice at 28°C. Results obtained during this study indicated that strain WE372 might be a hybrid between *S. cerevisiae* and *S. uvarum* (*S. bayanus* var. *uvarum*). Strain WE372 produced significantly smaller amounts of acetic acid than the other yeast strains of this study and very little acetic acid at 28°C, which indicated that strain WE372, might have had less acetaldehyde dehydroganase activity than the other yeast strains of this study (Hannemann, 1985; Radler, 1993). The effect this has on NAD: NADH balance is the probable cause for its ability to form more glycerol, succinic and malic acid than other strains (Remize *et al*., 2000). Results from our study show that succinic acid production is influenced primarily by the metabolizable fraction of YAN, which we termed metabolically available nitrogen (MAN). Succinic acid production by fermenting yeasts will be favoured by moderate to high fermentation temperatures (20°C to 28°C) in grape juice with a nicotinic acid and/ or nicotinamide deficiency, high sugar content (200 g/L to 240 g/L), moderate amounts of metabolically available nitrogen (300 ± 50 mg/L MAN), the presence of flavonoids and large supplies of unsaturated long-chain fatty acids. Even higher concentrations of succinic acid can be produced if oxygen is made available to fermenting yeasts by aerating the fermenting grape juice. Fermentation temperatures below 18°C, too much

metabolizable nitrogen (> 450 mg/L MAN), very high concentrations of fermentable sugar (> 240 g/L), lipid deficiencies and a lack of pantothenic acid, thiamine, biotin or pyridoxine will decrease the amount of succinic acid produced fermenting yeasts.

Table 3.4

Succinic and acetic acid contents of wines produced at different temperatures.

	Succinic acid		Acetic acid	
Strain	15°C	28°C	15°C	28°C
EC1118	0.582 a (± 0.004)	0.752 b (± 0.036)	0.317 a (± 0.015)	0.521 b (± 0.008)
DV10	0.585 a (± 0.002)	0.813 b (± 0.016)	0.248 a (± 0.014)	0.413 b (± 0.008)
U43	0.726 b (± 0.005)	0.627 a (± 0.008)	0.925 a (± 0.012)	1.045 b (± 0.002)
VIN7	0.523 a (± 0.004)	0.525 a (± 0.003)	0.759 a (± 0.013)	0.905 b (± 0.005)
VIN13	0.597 a (± 0.034)	0.990 b (± 0.039)	0.263 a (± 0.013)	0.215 a (± 0.015)
NT116	0.489 a (± 0.041)	0.988 b (± 0.029)	0.535 b (± 0.033)	0.330 a (± 0.005)
WE372	0.707 a (± 0.040)	1.436 b (± 0.024)	0.314 a (± 0.037)	0.277 a (± 0.008)
WE14	0.556 a (± 0.010)	0.755 b (± 0.032)	0.505 a (± 0.020)	0.450 a (± 0.010)
NT112	0.613 b (± 0.006)	0.561 a (± 0.013)	0.400 a (± 0.023)	0.753 b (± 0.010)

[a]values are reported in g/L; [b]letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [c]standard error of mean; [d]MAN = 336 mg/L; [e]fermentable sugar = 200 g/L.

Table 3.5

The influence of sugar concentration on succinic and acetic acid production.

	Succinic acid		Acetic acid	
Strain	200 g/L	300 g/L	200g/L	300 g/L
EC1118	0.752 a (± 0.036) [c]	0.715 a (± 0.012)	0.521 a (± 0.008)	0.740 b (± 0.014)
DV10	0.813 a (± 0.016)	0.756 a (± 0.025)	0.413 a (± 0.008)	0.658 b (± 0.004)
U43	0.627 a (± 0.008)	0.706 b (± 0.010)	1.045 a (± 0.002)	1.349 b (± 0.008)
VIN7	0.525 a (± 0.003)	0.504 a (± 0.010)	0.905 a (± 0.005)	1.325 b (± 0.014)
VIN13	0.990 b (± 0.039)	0.713 a (± 0.009)	0.215 a (± 0.015)	0.614 b (± 0.003)
NT116	0.988 b (± 0.029)	0.726 a (± 0.013)	0.330 a (± 0.005)	0.519 b (± 0.030)
WE372	1.436 b (± 0.024)	0.785 a (± 0.025)	0.277 a (± 0.008)	1.048 b (± 0.020)
WE14	0.755 b (± 0.032)	0.654 a (± 0.004)	0.450 a (± 0.010)	0.735 b (± 0.006)
NT112	0.561 a (± 0.013)	0.625 a (± 0.035)	0.753 a (± 0.010)	0.928 b (± 0.021)

[a]values are reported in g/L; [b]letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [c]standard error of mean; [d]control; [e]fermented @ 28°C; [f]YMN = 336 mg/L.

Table 3.6

Influence of nitrogen concentration on succinic and acetic acid production when nitrogen is supplied as ammonium chloride.

	Succinic acid			Acetic acid		
Strain	125 mg/L Nitrogen	250 mg/L Nitrogen	500mg/L Nitrogen	125 mg/L Nitrogen	250 mg/L Nitrogen	500mg/L Nitrogen
EC1118	0.894 c (± 0.004) [c]	0.753 b (± 0.020)	0.569 a (± 0.003)	0.506 b (± 0.009)	0.295 a (± 0.026)	0.324 a (± 0.005)
DV10	0.863 c (± 0.004)	0.740 b (± 0.017)	0.550 a (± 0.009)	0.511 c (± 0.008)	0.166 a (± 0.006)	0.280 b (± 0.005)
U43	0.597 a (± 0.018)	0.604 a (± 0.005)	0.570 a (± 0.002)	0.987 c (± 0.007)	0.682 a (± 0.011)	0.767 b (± 0.004)
VIN7	0.446 a (± 0.013)	0.583 b (± 0.011)	0.422 a (± 0.005)	1.206 b (± 0.004)	0.658 a (± 0.017)	0.642 a (± 0.010)
VIN13	0.917 b (± 0.046)	0.994 b (± 0.010)	0.571 a (± 0.015)	0.295 b (± 0.049)	0.117 a (± 0.005)	0.225 ab (± 0.007)
NT116	0.681 a (± 0.016)	1.038 b (± 0.044)	0.566 a (± 0.009)	0.644 c (± 0.005)	0.156 a (± 0.011)	0.240 b (± 0.012)
WE372	1.025 b (± 0.021)	1.203 c (± 0.016)	0.652 a (± 0.011)	0.353 b (± 0.004)	0.221 a (± 0.016)	0.457 c (± 0.010)
WE14	0.937 c (± 0.010)	0.871 b (± 0.011)	0.504 a (± 0.013)	0.240 a (± 0.003)	0.260 b (± 0.003)	0.389 c (± 0.006)
NT112	0.675 c (± 0.003)	0.579 b (± 0.002)	0.484 a (± 0.012)	0.570 b (± 0.011)	0.456 a (± 0.006)	0.569 b (± 0.008)

[a]values are reported in g/L; [b]letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [c]standard error of mean; [d]control; [e]fermented @ 28°C; [f]fermentable sugar = 200 g/L.

Table 3.7

Influence of nitrogen concentration on succinic and acetic acid production when nitrogen is supplied as a mixture of amino acids.

	Succinic acid			Acetic acid		
Strain	220 mg/L YAN	439 mg/L YAN [c]	878 mg/L YAN	220 mg/L YAN	439 mg/L YAN [c]	878 mg/L YAN
EC1118	0.832 b D (± 0.019) [b]	0.752 b (± 0.036)	0.614 a (± 0.011)	0.676 c (± 0.008)	0.521 b (± 0.008)	0.471 a (± 0.012)
DV10	0.738 b D (± 0.016)	0.813 c (± 0.016)	0.592 a (± 0.012)	0.661 b (± 0.019)	0.413 a (± 0.008)	0.417 a (± 0.005)
U43	0.531 a AB (± 0.004)	0.626 b (± 0.008)	0.587 b (± 0.017)	1.271 c (± 0.005)	1.045 b (± 0.002)	0.992 a (± 0.007)
VIN7	0.465 a A (± 0.028)	0.525 a (± 0.003)	0.474 a (± 0.007)	1.306 b (± 0.017)	0.905 a (± 0.005)	0.961 a (± 0.020)
VIN13	0.602 a BC (± 0.012)	0.990 b (± 0.039)	0.661 a (± 0.005)	0.637 c (± 0.003)	0.215 a (± 0.015)	0.392 b (± 0.006)
NT116	0.637 a C (± 0.007)	0.988 c (± 0.029)	0.762 b (± 0.010)	0.737 b (± 0.009)	0.330 a (± 0.005)	0.338 a (± 0.006)
WE372	1.191 b F (± 0.036)	1.436 c (± 0.024)	0.886 a (± 0.007)	0.241 a (± 0.017)	0.277 a (± 0.008)	0.449 b (± 0.008)
WE14	0.937 c E (± 0.009)	0.754 b (± 0.032)	0.638 a (± 0.011)	0.269 a (± 0.007)	0.450 b (± 0.010)	0.417 b (± 0.005)
NT112	0.511 a AB (± 0.013)	0.560 a (± 0.013)	0.541 a (± 0.005)	0.778 b (± 0.008)	0.753 b (± 0.010)	0.697 a (± 0.015)

[a]values are reported in g/L; [b]standard error of mean; [c]control; [d]lowercase letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [e]uppercase letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a column; [f]fermented @ 28°C; [g]fermentable sugar = 200 g/L.

Table 3.10

Influence of nicotinic acid on succinic and acetic acid production.

	Succinic acid		Acetic acid	
Strain	0.2 mg/L Niacin	2.0 mg/L Niacin	0.2 mg/L Niacin	2.0 mg/L Niacin
EC1118	0.752 a (± 0.062) [c]	0.539 b (± 0.032)	0.521 a (± 0.014)	0.582 b (± 0.022)
DV10	0.813 a (± 0.028)	0.595 b (± 0.033)	0.413 a (± 0.015)	0.537 b (± 0.002)
U43	0.627 a (± 0.014)	0.451 b (± 0.020)	1.045 a (± 0.004)	1.167 b (± 0.014)
VIN7	0.525 a (± 0.005)	0.336 b (± 0.022)	0.905 a (± 0.009)	1.071 b (± 0.045)
VIN13	0.990 a (± 0.067)	0.625 b (± 0.043)	0.215 a (± 0.026)	0.501 b (± 0.006)
NT116	0.988 a (± 0.050)	0.778 b (± 0.046)	0.330 a (± 0.008)	0.457 b (± 0.017)
WE372	1.436 a (± 0.042)	0.689 b (± 0.020)	0.277 a (± 0.013)	0.582 b (± 0.017)
WE14	0.755 a (± 0.056)	0.562 b (± 0.020)	0.450 a (± 0.018)	0.562 b (± 0.016)
NT112	0.561 a (± 0.022)	0.444 b (± 0.022)	0.753 a (± 0.018)	0.845 b (± 0.005)

[a]values are reported in g/L; [b]letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [c]standard error of mean; [d]fermented @ 28°C; [e]MAN = 336 mg/L; [f]fermentable sugar = 200 g/L.

Table 3.12

Influence of thiamine on succinic and acetic acid production.

	Succinic acid		Acetic acid	
Strain	Control	Thiamine omitted	Control	Thiamine omitted
EC1118	0.752 b (± 0.036) [c]	0.567 a (± 0.010)	0.521 b (± 0.008)	0.313 a (± 0.007)
DV10	0.813 b (± 0.016)	0.534 a (± 0.017)	0.413 b (± 0.008)	0.295 a (± 0.004)
U43	0.627 b (± 0.008)	0.434 a (± 0.004)	1.045 b (± 0.002)	0.644 a (± 0.010)
VIN7	0.525 b (± 0.003)	0.348 a (± 0.003)	0.905 b (± 0.005)	0.677 a (± 0.016)
VIN13	0.990 b (± 0.039)	0.614 a (± 0.010)	0.215 a (± 0.015)	0.192 a (± 0.005)
NT116	0.988 b (± 0.029)	0.610 a (± 0.004)	0.330 b (± 0.005)	0.240 a (± 0.008)
WE372	1.436 b (± 0.024)	0.783 a (± 0.022)	0.277 a (± 0.008)	0.337 b (± 0.002)
WE14	0.755 b (± 0.032)	0.512 a (± 0.010)	0.450 b (± 0.010)	0.373 a (± 0.008)
NT112	0.561 a (± 0.013)	0.545 a (± 0.008)	0.753 b (± 0.010)	0.659 a (± 0.007)

[a]values are reported in g/L; [b]letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [c]standard error of mean; [d]fermented @ 28°C; [e]MAN = 336 mg/L; [f]fermentable sugar = 200 g/L.

Table 3.15

Influence of flavan-3-ols on succinic and acetic acid production.

Strains	Succinic acid		Acetic acid	
	Control	100 mg/L (+)-Catechin	Control	100 mg/L (+)-Catechin
EC1118	0.752 a (± 0.036)[c]	0.979 b (± 0.015)	0.521 a (± 0.008)	0.487 a (± 0.018)
DV10	0.813 a (± 0.016)	0.854 a (± 0.012)	0.413 b (± 0.008)	0.279 a (± 0.003)
U43	0.627 a (± 0.008)	0.663 b (± 0.008)	1.045 b (± 0.002)	0.947 a (± 0.002)
VIN7	0.525 a (± 0.003)	0.593 b (± 0.011)	0.905 b (± 0.005)	0.821 a (± 0.012)
VIN13	0.990 a (± 0.039)	1.230 b (± 0.036)	0.215 b (± 0.015)	0.095 a (± 0.007)
NT116	0.988 a (± 0.029)	1.194 b (± 0.016)	0.330 b (± 0.005)	0.180 a (± 0.012)
WE372	1.436 a (± 0.024)	1.528 a (± 0.031)	0.277 b (± 0.008)	0.218 a (± 0.011)
WE14	0.755 a (± 0.032)	0.906 b (± 0.001)	0.450 b (± 0.010)	0.359 a (± 0.014)
NT112	0.561 a (± 0.013)	0.747 b (± 0.015)	0.753 b (± 0.010)	0.215 a (± 0.016)

[a]values are reported in g/L; [b]letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [c]standard error of mean; [d]fermented @ 28°C; [e]MAN = 336 mg/L; [f]fermentable sugar = 200 g/L.

Table 3.16

Succinic and acetic acid production in the absence of unsaturated long-chain fatty acids.

Strain	Succinic acid		Acetic acid	
	Control	Tween 80® omitted	Control	Tween 80® omitted
EC1118	0.752 a (± 0.036) [c]	0.737 a (± 0.032)	0.521 b (± 0.008)	0.396 a (± 0.012)
DV10	0.813 b (± 0.016)	0.723 a (± 0.010)	0.413 b (± 0.008)	0.296 a (± 0.006)
U43	0.627 a (± 0.008)	0.724 b (± 0.012)	1.045 b (± 0.002)	0.913 a (± 0.011)
VIN7	0.525 a (± 0.003)	0.537 a (± 0.010)	0.905 b (± 0.005)	0.831 a (± 0.011)
VIN13	0.990 b (± 0.039)	0.822 a (± 0.011)	0.215 b (± 0.015)	0.151 a (± 0.008)
NT116	0.988 a (± 0.029)	0.969 a (± 0.006)	0.330 b (± 0.005)	0.069 a (± 0.004)
WE372	1.436 b (± 0.024)	1.097 a (± 0.003)	0.277 a (± 0.008)	0.298 a (± 0.003)
WE14	0.755 a (± 0.032)	0.714 a (± 0.013)	0.450 b (± 0.010)	0.302 a (± 0.008)
NT112	0.561 a (± 0.013)	0.628 b (± 0.006)	0.753 b (± 0.010)	0.590 a (± 0.006)

[a]values are reported in g/L; [b]letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [c]standard error of mean; [d]fermented @ 28°C; [e]MAN = 336 mg/L; [f]fermentable sugar = 200 g/L.

Table 3.17
The influence of oxygen on succinic acid production by fermenting yeasts.

	Succinic acid	
Strain	Anaerobic	Aerated
EC1118	0.752 a (± 0.036) [c]	1.591 b (± 0.042)
DV10	0.813 a (± 0.016)	1.760 b (± 0.013)
U43	0.627 a (± 0.008)	1.149 b (± 0.012)
VIN7	0.525 a (± 0.003)	1.292 b (± 0.033)
VIN13	0.990 a (± 0.039)	2.137 b (± 0.119)
NT116	0.988 a (± 0.029)	1.402 b (± 0.031)
WE372	1.436 a (± 0.024)	2.171 b (± 0.061)
WE14	0.755 a (± 0.032)	1.274 b (± 0.026)
NT112	0.561 a (± 0.013)	1.434 b (± 0.005)

[a]values are reported in g/L; [b]letter(s) next to numerical values indicate significant differences ($p < 0.05$) within a row; [c]standard error of mean; [d]fermented @ 28°C; [e]MAN = 336 mg/L; [f]fermentable sugar = 200 g/L.

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Chapter 4

GENERAL DISCUSSION AND CONCLUSION

4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 CONCLUDING REMARKS

Low levels of titratable acidity (TA < 6 g/L tartaric acid) accompanied by high pH values (pH > 3.6) are common features of grape juice from the warmer wine producing regions of the world. Warm temperatures increase the respiratory catabolism of malic acid by the vine during the ripening of grapes (Ruffner, 1982; Boulton *et al.*, 1998a). Warmer temperatures also increase transpiration by the vine, thereby causing an increased uptake of potassium ions, which are exchanged with the protons of organic acids stored within the vine's berries and leaves (Boulton *et al.*, 1998a). The main organic acids of grapes may be used to increase the acidity of grape juice or wine (Liquor Products Act, 1989; Randolph, 2001). L-Tartaric acid is preferred over malic and citric acid to acidify grape juice or wine (Boulton *et al.*, 1998b), because it is the strongest organic acid in grape juice (Usseglio-Tomasset, 1978; Boulton *et al*., 1998c), yeasts cannot metabolize tartaric acid and bacteria find it very hard to metabolize tartaric acid at pH values below 4.0 and in the presence of sulphur dioxide (Radler, 1986; Boulton *et al.*, 1998b). The extent to which the addition tartaric acid will affect the pH and titratable acidity of grape juice or wine depends on the amount of acid added, the buffering capacity of the juice or wine and the amount of potassium bitartrate precipitation (Boulton *et al.*, 1998b; Rankine, 1998a). However, tartaric acid additions are expensive and affect the physicochemical stability of wine. Treatment with hydrogenated cation-exchange resins serve as an effective alternative to decrease the pH and increase the titratable acidity of grape juice or wine. Cation-exchange will also help stabilize the wine against potassium bitartrate precipitation (Boulton *et al.*, 1998b; Rankine, 1998a). However, there are several disadvantages to using cation-exchange as a means of acidifying grape juice. Only clarified juice can be treated, the washing and regeneration steps between treatments are time consuming and require the use of expensive and dangerous chemicals, separate ion-exchange columns are needed to treat red and white grape juice, toxic wastewater is generated and vital yeast nutrients such as thiamine, biotin, ammonium, amino acids and metal cations are removed (Boulton *et al.*, 1998b; Rankine, 1998a).

Very high concentrations of succinic acid (> 1.5 g/L) can be produced by yeasts during fermentation of grape juice (Whiting, 1976; Coulter *et al*., 2004). Succinic acid is chemically very stable and resistant to microbial attack under anaerobic conditions (Whiting, 1976; Jackson, 1994). Succinic acid production by yeasts will have little effect on the pH of wine due to succinic acid's weak acidity (Usseglio-Tomasset, 1978; Boulton *et al*., 1998c) and wine's high buffering capacity (Boulton *et al*. 1998c). For example, 1.0 g/L succinic acid will lower the pH of wine by only 0.018 units when it has an ethanol content of 14% v/v, a pH of 3.5 and buffering capacity of 50 mM/ L/ pH (10.95% of succinic acid's first proton dissociated). However, succinic acid production by fermenting yeasts can be manipulated to manage the titratable acidity of wine, which influences the

wine's sour taste (Boulton *et al*., 1998c). A variety of factors can be manipulated to control the production of succinic acid during fermentation of grape juice (Chapter 3), but the most appropriate treatment will be determined by the chemical composition of the juice and the intended style of the wine.

The presence of flavonoids and unsaturated long-chain fatty acids in grape juice stimulate the production of succinic acid by fermenting yeasts (Chapter 3). Unsaturated long-chain fatty acids are derived from the cell membrane lipids of grape skin- and pericarp tissue (Roufet *et al*., 1987; Miele *et al*., 1993), the surface (cuticle) wax of grapes (Radler, 1965) and the oil (triacylglycerols) of grape seeds (Miele *et al*., 1993; Cao & Ito, 2003). Flavonoids are found mainly in the skins, seeds and stems (rachis and pedicel) of grapes (Jackson, 1994; Cheynier, 2006). The solid parts of crushed grapes (pomace) are separated and removed from the juice before alcoholic fermentation to produce white wine, but the juice of red grapes is fermented in contact with the pomace during red wine production. Ethanol, produced during fermentation, facilitate the extraction of flavonoids and lipids from grape tissue, because ethanol destroys cellular membranes and increase the solubility of flavonoids and lipids in grape juice (Rankine, 1998b). In other words, much higher concentrations of flavonoids and lipids will be present in grape must during red wine production than during white wine production (Cheynier, 2006). Winemakers can increase the lipid and flavonoid contents of white grape must by extending the contact period between the juice and pomace before fermentation (Ramey *et al*., 1986; Ferreira *et al*., 1995; Cheynier, 2006). Pre-fermentation contact between the pomace and juice (skin contact) can be carried out for up to 24 hours, as long as the temperature is kept below 20°C and enough sulphur dioxide is added to inhibit microbial growth during this period (Boulton *et al*., 1998b). Sulphur dioxide, pectolytic enzymes, elevated temperatures and pump-overs increase the extraction of flavonoids and lipids from the pomace of grapes in the absence of ethanol (Ramey *et al*., 1986; Boulton *et al*., 1998b; Rankine, 1998b; Cheynier, 2006). Oxidative polymerization of flavonoids (Singleton, 1987; Ribéreau-Gayon *et al*., 2000; Cheynier, 2006) and the oxidative degradation of unsaturated fatty acids (Joslin & Ough, 1978; Cayrel *et al.*, 1983; Roufet *et al*., 1986) can be minimized during pre-fermentation skin contact by the addition of ascorbic acid in combination with sulphur dioxide and/ or by blanketing the juice with carbon dioxide. The press- and free-run juice is usually fermented separately during white wine production due to quality differences between the two juice fractions (Boulton *et al*., 1998b). The press-juice will contain higher concentrations of flavonoids and lipids than the free-run juice (Ferreira *et al*., 1995; Boulton *et al*. 1998b), which means that more succinic acid might be formed during fermentation of press-juice than during fermentation of free-run juice. If this is so, white wine produced from press-juice can be blended with white wine produced from free-run juice to ensure higher levels of titratable acidity in the final product. Grape juice is clarified before fermentation during the production of fruity-style white wines to prevent extraction of astringent phenolic compounds from grape particles during fermentation and to remove most of the oxidative enzymes and esterases associated with particulate matter from grapes (Boulton *et al*. 1998b). Clarification of grape juice not only prevents further

extraction of flavonoids and lipids from grape particles, but also decreases the already extracted lipid content of grape juice (Bertrand & Miele, 1984; Delfini *et al*., 1992; Ferreira *et al*., 1995; Guilloux-Benatier *et al*., 1998). Oleic acid, glycerol-oleic acid esters or commercially available yeast hulls (inactivated yeasts, yeast ghosts) can be used to increase the unsaturated fatty acid and sterol contents (Munoz & Ingledew, 1989; Belviso *et al*., 2004) of clarified grape juice (Liquor Products Act, 1989; Randolph, 2001).

Between 25 and 100 mg/L sulphur dioxide is usually added to grape juice prior to fermentation (Boulton *et al*., 1998b). The greatest part thereof will be present as bisulphite ions at the pH of grape juice (Boulton *et al*., 1998d) and will therefore destroy thiamine (Leichter & Joslyn, 1969; Boulton *et al*., 1998d). The thiamine content of grape juice can also be removed by bentonite (Boulton *et al*., 1998e; Rankine, 1998c), which is sometimes added to clarified grape juice to increase the suspended solids content thereof (Groat & Ough, 1978; Delfini & Costa, 1993; Boulton *et al*., 1998e) and to remove the proteins that is responsible for chill-haze formation in white wine (Boulton *et al*., 1998f; Rankine, 1998c). Suspended solids in grape juice help to ensure a rapid and complete fermentation by keeping fermenting yeasts in suspension and distributed throughout the growth medium (Groat & Ough, 1978; Delfini & Costa, 1993; Boulton *et al*., 1998e). Wine yeasts are unable to synthesize their own thiamine during anaerobic fermentation (Nosaka, 2006; Snoek & Steensma, 2007) and produce less succinic acid during fermentation when thiamine is absent from the growth medium (Chapter 3). Cellulose powder, diatomaceous earth or yeast hulls (Groat & Ough, 1978; Delfini & Costa, 1993; Boulton *et al*., 1998e) can be used in stead of bentonite to increase the suspended solids content of highly clarified grape juice. Sulphur dioxide additions to grape juice can be avoided during the production of red wine and barrel-fermented white wine if the grapes are healthy and if the must is inoculated with a large yeast population shortly after the grapes have been crushed. The addition of sulphur dioxide to grape juice can also be avoided during the production of non-varietal white wine if the clarification process is sped up by means of filtration of centrifugation, because non-varietal white wines do not benefit from pre-fermentation skin contact and can be produced from oxidized juice without any serious quality losses (Boulton *et al*., 1998b). However, it would be near-impossible to avoid the use of sulphur dioxide during the production of aromatic white wine with distinctive varietal character, because oxidation and the growth of spoilage microbes need to be prevented during pre-fermentation skin contact and the cold-settling of juice. Thiamine deficiency in grape juice may be corrected by adding thiamine hydrochloride or Thiazote PH (Laffort, Bordeaux, France) (Liquor Products Act, 1989; Randolph, 2001). However, it is important for winemakers to minimize the thiamine content of grape juice due to the increased production of acetic acid in the presence of increasing thiamine concentrations (Hannemann, 1985; Eglinton & Henscke, 1993). The simplest methods for estimation of grape juice's thiamine content are the European Brewing Convention's standardized thiochrome-fluorescence method (Dawson *et al*., 1951; Šilhánková, 1985) and a microbiological thiamine assay that can be purchased from Roche-biopharmaceuticals (www.rbiopharm.com).

Complex nutrient mixtures like Fermaid K (Lallemand, Montréal, Canada) or autolyzed yeast preparations like Fermaid 2133 (Lallemand, Montréal, Canada) can be used to correct nutrient deficiencies in grape juice. Although the precise composition of the before mentioned products are unknown, they contain several water-soluble vitamins according to the manufacturers (Eglinton & Henschke, 1993; www.scottlab.com). However, the use of these products in grape juice will decrease the production of succinic acid and increase acetic acid production during fermentation due to increased concentrations of thiamine, nicotinic acid and nicotinamide (Monk & Cowley, 1984; Hannemann, 1985; Chapter 3).

Succinic acid production by yeasts increases with increasing fermentable sugar concentrations up to 220 g/L (~24°Brix) (Shimazu & Watanabe, 1981), but starts to decline at higher sugar concentrations due to an increase in acetic acid production (Caridi, 2003; Chapter 3). In South Africa, grape juice may not be diluted with water to lower the sugar concentration thereof (Liquor Products Act, 1989). However, high sulphur dioxide and/ or flavonoid concentrations (≥ 100 mg/L) will help reduce acetic acid production in grape musts with high sugar contents, thereby increasing the production of succinic acid in these musts (Caridi, 2003).

Succinic acid production by yeasts is influenced by the metabolizable portion of the fermentation medium's yeast assimilable nitrogen (YAN) content, which was subsequently termed metabolically available nitrogen (MAN). Yeast metabolizable nitrogen is defined as the total amount of nitrogen that can be converted into ammonium ions when sources thereof are metabolized by yeasts. Moderate concentrations of YMN (300 ± 50 mg/L) was optimal for succinic acid production in synthetic grape juice with 200 g/L fermentable sugar (Chapter 3). The most abundant sources of YMN in grape juice are free α-amino acids and ammonium ions. Relatively inexpensive and simple analytical methods can be used to determine the combined concentration of free α-amino nitrogen (FAN) and ammonium nitrogen in grape juice (Ogorodnik & Merkureua; 1971; Lie, 1973; Ough, 1988; Zoecklein *et al*., 1995a; Gump *et al*., 2002). However, the sum concentration of FAN and ammonium nitrogen greatly underestimates the YMN content of grape juice, because arginine is quantitatively the most important free α-amino acid in grape juice (Henschke & Jiranek, 1993; Margalit, 1997; Bell & Henschke, 2005) and unlike most α-amino acids contains three metabolizable amino groups under anaerobic conditions (Large, 1986; Boulton *et al*., 1998g). The combined concentrations of ammonium nitrogen, FAN and the two additional metabolizable nitrogen atoms of arginine is a good estimate of grape juice's YMN content. Arginine can be quantified by means of a relatively simple and inexpensive spectrophotometric method (Gilboe & Williams, 1956; Ough, 1988). The exact YMN content of grape juice can be calculated from the concentrations of all the contributing amino acids and ammonium. A reverse-phase high performance liquid chromatography (RP-HPLC) or gas chromatography (GC) method can be used to separate and quantify amino acids in grape juice (Amerine & Ough, 1980; Ough, 1988; Lehtonen, 1996; Spayd & Andersen-Bagge, 1996; Hernádez-Orte *et al*., 1997) and the ammonium content of grape juice can be quantified by means of an enzymatic method (Zoecklein *et al*., 1995b; www.rbiopharm.com) or with the use of an ion-selective electrode

(McWilliams & Ough, 1974; Zoecklein *et al*., 1995c). Alternatively, ion-exchange chromatography with post-column derivatization can be used to determine the concentration of each amino acid in grape juice, as well as ammonium (Kluba *et al*, 1978; Amerine & Ough, 1980; Ough, 1988). However, chromatographic methods are time consuming, expensive and require the expertise of an analytical chemist. Diammonium monohydrogen phosphate (DAP) or other salts of ammonium are normally used to increase the nitrogen content of grape juice (Boulton *et al*., 1998b), but autolyzed yeast preparations can be used to increase different amino acids' contribution to grape juice's YMN (YAN) content (Ingledew *et al*., 1986). Between 19% and 29% of the yeast assimilable nitrogen (YAN) in grapes is located in the skins and another 10% to 15% is located in the seeds (Stines *et al*., 2000; Bell & Henschke, 2005). The extraction of free amino acids from these berry parts are enhanced by ethanol and elevated temperatures (Fukui *et al*., 2002). Fermenting yeasts produce notably more acetic acid and less succinic acid in fermentation media with high levels of YMN (≥ 500 mg/L) compared to musts with moderate amounts of YMN (Chapter 3). Therefore, it is important to account for the potential amount of YMN that can be extracted from grape pomace during fermentation of red grape musts. Excessive amounts of YMN in grape juice should preferably be prevented by adapting viticultural practices (Bell & Henscke, 2005), because winemakers can only use bentonite or cation-exchangers to reduce the YMN content of grape juice.

Temperature's influence on succinic acid production will be determined by the type of yeast strain with which the must is inoculated (Castellari *et al*., 1995; Chapter 3). Most *S. uvarum* (*S. bayanus* var. *uvarum*) strains produce the maximum amount of succinic acid at approximately 18°C (Castellari *et al.*, 1995), but mesophilic strains of *S. cerevisiae* usually produce the maximum amount of succinic acid at temperatures close to 24°C (Castellari *et al.*, 1995). Temperature's effect on succinic acid production is less drastic when fermentation is conducted by *S. cerevisiae* yeasts than when it is conducted by *S. uvarum* yeasts (Castellari *et al*., 1995). *S. uvarum* yeasts are capable of producing much more succinic acid than most *S. cerevisiae* yeasts during fermentation of grape juice (Castellari *et al*., 1994; Bertolini *et al*., 1996; Antonelli *et al*., 1999, Coloretti *et al*., 2006). *S. uvarum* yeasts are also capable of producing malic acid during fermentation of grape juice unlike strains of other *Saccharomyces sensu stricto* species, which consume some of the malic acid in grape juice during fermentation (Castellari *et al*., 1994; Giudici *et al*., 1995; Antonelli *et al*., 1999; Coloretti *et al*., 2006). Strains of *S. uvarum* are in other words, ideal to ferment with when increases in titratable acidity are needed. Unfortunately though, *S. uvarum* strains are not commercially available as far as it is known. However, the commercial wine yeast strain Lalvin S6U (Lallemand, Montreal, Canada) is a hybrid between *S. cerevisiae* and *S. uvarum* (Masneuf *et al*., 1998; Naumov *et al*., 2000a; Azumi Goto-Yamamoto, 2001; Naumova *et al*., 2005; González *et al*., 2006). The fermentation by-product profiles of the commercial wine yeast strains Anchor WE372 (Anchor Yeast, Epping Industria, South Africa) and Lalvin W15 indicate that these two strains might also be *S. uvarum* x *S. cerevisiae* hybrids (Chapter 3; www.brsquard.org; www.scottlab.com). Wine yeast strain Anchor WE372 produced more

than double the amount of succinic acid in synthetic grape juice at 28°C compared to the amounts that was produced at 15°C in the same must (Chapter 3). This finding indicated that *S. uvarum* x *S. cerevisiae* hybrids probably produce the maximum amount of succinic acid at temperatures close to the ones that are optimal for the production of succinic acid by mesophilic *S. cerevisiae* yeasts Most fruity white wines are produced at temperatures between 10°C and 16°C (Rankine, 1998d), but most yeasts produce less succinic acid at these temperatures than at higher temperatures (Castellari *et al*., 1995; Chapter 3). Fruity white wines with higher levels of succinic acid can be produced if the juice is fermented with a *S. uvarum* strain at a temperature between 18°C and 20°C. Losses in grape-based aromas and fermentation bouquet will be minimal as long as the fermentation temperature is kept below 20°C (Boulton *et al*., 1998e). In fact, *S. uvarum* yeasts will increase the fruity aromas of white wine, because they produce larger quantities of 2-phenethanol (rose), 2-phenethyl acetate (rose), diethyl succinate (watermelon), 3-mercaptohexanol (passion fruit, grapefruit) and 4-mercapto-4-methylpentan-2-one (box-tree, broom, blackcurrant) than *S. cerevisae* strains (Williams, 1974; Dariet *et al*, 1995; Tominaga *et al*., 1998; Antonelli *et al*., 1999; Jordan *et al*., 2002; Masneuf *et al*., 2002; Coloretti *et al*., 2006). Barrel-fermented white wine is produced at 18-22°C, whereas red grape musts are usually fermented at temperatures between 20°C and 30°C (Boulton *et al*., 1998e). Cryotolerant *S. cerevisiae* (*S. oviformis*, *Champagne*, *Prise de Mousse*) and mesophilic *S. cerevisiae* strains will produce moderate to high levels of succinic acid in fermenting grape juice at temperatures above 18°C (Chapter 3). Very low concentrations of succinic acid can ensured in red wine or barrel-fermented white wine if the must is fermented with a *S. kudriavzevii* x *S. cerevisiae* hybrid at moderate fermentation temperature (Chapter 3). The wine yeast strains Anchor VIN7, Uvaferm CEG, Lalvin W27 and Lavin W46 are examples of commercially available *S. kudriavzevii* x *S. cerevisiae* hybrids (González *et al*., 2006, Bradbury *et al*., 2005; Spiczki, 2008). Fermentations with *S. kudriavzevii* x *S. cerevisiae* hybrids should preferably be conducted at temperatures between 14°C and 24°C, because they produce very high concentrations of acetic acid (> 0.6 g/L) at temperatures outside of this range (González *et al*., 2007; Chapter 3).

Wines with very high concentrations of succinic acid (> 1.5 g/L) can be produced under respiro-fermantative conditions, *i.e.* fermentation in the presence of oxygen (Chapter 3). Grape skins form a cap on top of the fermenting juice during the production of red wine, which must be broken up at regular intervals during fermentation in order to cool the fermenting juice beneath the skin cap and to increase the amount of contact between the skins and fermenting juice so that enough colour and flavour can be extracted. A certain degree of aeration is inevitable when the punch-down or pump-over techniques are used to break the skin cap of fermenting musts (Boulton *et al*., 1998e). In fact, red grape musts are sometimes deliberately aerated during fermentation to assist in the formation of polymerized red pigments, which stabilizes the colour of young red wines (Wildenradt & Singleton, 1974; Pontallier & Ribéreau-Gayon, 1983; Ribéreau-Gayon *et al*., 1983; Singleton, 1987; Singleton & Trousdale, 1987; Bakker *et al*., 1993). Rotating tanks

(Rototanks) or closed tanks with gas build-up/flooding systems can be used to ferment red grape musts under completely anaerobic conditions. Alternatively, carbonic maceration or thermovinification can used to extract red colour from grape skins before alcoholic fermentation (Boulton *et al*., 1998e; Rankine, 1998d) so that fermentation can be conducted anaerobically and in the absence of grape skins. Fruity white wines cannot be produced under respiro-fermentative conditions, because white grape juice does not contain enough phenolic compounds to prevent oxidative browning and oxygen destroys important aroma compounds.

4.2 POSSIBLE FUTURE RESEARCH

The previous section of this chapter dealt with the potential ways in which winemakers can control succinic acid production during alcoholic fermentation of grape juice based on the results of fermentation experiments conducted in synthetic grape juice (Chapter 3). It is important though, to confirm whether the above discussed winemaking procedures influence succinic acid production in the predicted manner. Although we were able to study various oenological important factors' influence on succinic acid production, a number of questions remain unanswered.

The presence of (+)-catechin in synthetic grape juice reduced the production of acetic acid (Garcia Moruno *et al*., 1993; Chapter 3) and increased the production of succinic acid during fermentation (Chapter 3). The (+)-catechin content of synthetic grape juice influenced acetic acid production by fermenting yeasts in a concentration dependant manner (Garcia Moruno *et al*., 1993), which might also be true wit h regard to succinic acid production. (+)-Catechin is classified as a flavan-3-ol, which belong to a group of phenolic compounds called flavonoids (Margalit, 1997; Ribéreau-Gayon *et al*., 2000; Cheynier, 2006). The flavonoid pigments of red grapes, known as anthocyanins (glycosylated and/ or acylated anthocyanidins), possess the same ability than (+)-catechin to inhibit acetic acid production by fermenting yeasts (Garcia Moruno *et al*., 1993). Thus, there is reason to believe that succinic acid production by yeasts might be stimulated by the presence of anthocyanins during fermentation. Thus far however, no one has investigated this possibility. Grapes also contain flavonoids from other flavonoid classes, which might influence acetic- and succinic acid production by fermenting yeasts. The most important types of flavonoids in grapes besides the two already mentioned ones are flavan-2,3-diols (leucoanthocyanidins), flavonols, flavones, flavanones (dihydroflavones) and flavanonols (synonyms: dihydroflavonols, 3-hydroxyflavanones) (Margalit, 1997; Ribéreau-Gayon *et al*., 2000; Cheynier, 2006; Grayer & Veitch, 2006). Reduced acetate formation increases the $NAD(P)^+$: NAD(P)H balance within fermenting yeasts (van Dijken & Scheffers, 1986), which can be restored by increased formation of succinate via the TCA cycle's oxidative branch (Remize *et al*., 2000). However, the mechanism responsible for anthocyanins' or (+)-catechin's ability to inhibit acetate formation in fermenting yeasts has not been resolved yet. Flavanoids can react with acetaldehyde

(Timberlake & Bridle, 1976), which in effect would remove the most abundant and direct precursor of acetate during fermentation (Pronk *et al*., 1996; Remize *et al*., 2000). Acetaldehyde is also the precursor of ethanol. If the removal of acetaldehyde by flavonoids is responsible for the decreased production of acetate during fermentation, a similar decrease in the formation of ethanol is to be expected. However, ethanol production during fermentation remains virtually unchanged in the presence of increasing catechin or anthocyanin concentrations (Garcia Moruno *et al*., 1993). Oxidation of acetaldehyde to acetate is catalyzed by cytosolic acetaldehyde dehydrogenases (Ald6p) in fermenting yeasts (Llorente & Castro, 1977; Dickinson, 1996; Meaden *et al*., 1997; Remize *et al*., 2000). A decrease in acetate formation without a concurrent reduction in ethanol production, indicate that acetaldehyde dehydrogenase activity is somehow inhibited by flavonoids. Flavanoids, procyanidins (condensed tannins, flavonoid polymers) and hydrolysable tannins can interact with proteins (Haslam, 1974; Kumar & Singh, 1984; Clifford, 1986; Dangles & Dufour, 2006) and are therefore capable of inhibiting enzymes (Haslam, 1974; Kumar & Singh, 1984; Beart *et al*., 1985; Kurisawa *et al*., 2003, Kim *et al*., 2004). Flavonoids can block the active site(s) of enzymes by binding in the region of the active site or by causing conformational changes to the protein (Kumar & Singh, 1984; Beart *et al*., 1985). Protein-flavonoid interactions can also reduce the solubility of enzymes, which may cause them to precipitate out of solution and hence prevent them from interacting with their substrates (Kumar & Singh, 1984). Enzymes can also be inhibited by the oxidation products of flavonoids (aryloxyl radicals and quinones), which can react with nucleophilic and oxidisable amino acid residues (tyrosine, tryptophan, and cysteine) of proteins (Dangles & Dufour, 2006). Thus, theoretically it is possible that flavonoids reduce acetic acid production during fermentation by inhibiting the acetaldehyde dehydrogenases within fermenting yeasts. If this is so, the interaction between flavonoids and acetaldehyde dehydrogenases would have to be specific in order to explain why alcohol dehydrogenases, for example, are not also inhibited. Highly specific protein-phenol interactions have been observed between globular proteins with well-defined binding cavities (enzymes and receptors) and properly substituted flavonoids (Zhu *et al*., 1996, Dangles & Dufour, 2006). *S. cerevisiae*'s acetaldehyde dehydrogenases are made up out of multiple subunits (domains) (Saigal *et al*., 1991; Steensma, 1997), which probably have globular three-dimensional structures like the subunits of aldehyde dehydrogenase found in other eukaryotic organisms (Moore *et al*., 1998; Rodriguez-Zavala & Weiner, 2002). Flavonoids might not be the only phenolic compounds with the ability to specifically inhibit acetaldehyde dehydrogenase activity in yeasts. Non-flavonoid phenolic compounds with vicinal hydroxyl groups can be oxidised to aryloxyl radicals and quinones, which in turn can react with nucleophilic or oxidisable amino acid residues within the enzyme's active site(s) (Dangles & Dufour, 2006). The hydrolysable tannins extracted from oak are sources of galic- and ellagic acids, which both contain vicinal hydroxyl groups (Ribéreau-Gayon *et al*., 2000). In other words, less acetic- and more succinic acid might be produced when white grape juice is fermented in oak barrels. In fact, Coulter *et al* (2004) already reported that barrel-fermented white wines

appear to contain higher levels of succinic acid than white wines produced in stainless steel tanks.

Higher concentrations of succinic acid are produced by yeasts in the presence of an unsaturated long-chain fatty acid source than in the absence thereof when fermentations are conducted in synthetic grape juice with $\Delta^{5,7}$-unsaturated sterol supplies (Chapter 3). Results indicated that the synthetic grape juice of control experiments provided a surplus of unsaturated long-chain fatty acids to fermenting yeasts (Chapter 3). Large supplies of unsaturated long-chain fatty acids in synthetic grape juice probably inhibited fatty acid biosynthesis within yeasts, thereby reducing the need for acetyl-CoA biosynthesis. During fermentation, acetyl-CoA is mainly synthesized from acetate and coenzyme A (Holtzer, 1957, 1961; Pronk *et al*., 1996; Remize *et al*., 2000) by means of a reaction that requires ATP (Steensma, 1997). In other words, a decrease in acetyl-CoA formation could have increased the availability of ATP and coenzyme A within yeasts during fermentation, which would have stimulated the pyruvate carboxylase activity within these yeasts (Ruiz-Amil *et al*., 1965; Miller & Atkinson, 1972). An increase in pyruvate carboylase activity directs more pyruvate-carbon towards the two branches of the TCA cycle, which both end up in the formation of succinate during alcoholic fermentation (Camarasa *et al*., 2003). Higher intracellular levels of ATP also stimulate yeast growth, thereby increasing the number of cells that produce succinic acid. Succinic acid production by yeasts should be studied in synthetic grape juices with adequate sterol supplies and increasing concentrations of unsaturated long-chain fatty acid supplies to determine at what concentrations, unsaturated long-chain fatty acids start to stimulate succinic acid production and to determine whether further increases in unsaturated long-chain fatty acids will have any effect on succinic acid production.

Significant increases in titratable acidity is a common occurrence during fermentation of *Vitis rotundifolia* (Muscadine) grape juice (Carrol *et al*., 1975; Vine, 1981) and is caused by the production of high succinic acid concentrations (Lamikanra, 1997). is Arginine is usually the most important free α-amino acid within *V. vinifera* grapes (Henscke & Jiranek, 1993; Margalit, 1997; Bell & Henschke, 2005), but it is seldom the most abundant free amino acid within *V. rotundifolia* grapes (Marcy *et al*., 1981). In fact, the grapes of some *V. rotundifolia* varieties like Noble and Dixie contain very little arginine. *V. rotundifolia* grapes always contain high levels of α-alanine and threonine (Marcy *et al*., 1981). Greater amounts of succinic acid are produced during fermentation when yeasts assimilable nitrogen is provided as alanine or threonine, instead of arginine or ammonium (Heerde & Radler, 1978). It is important, therefore, to investigate whether succinic acid production will be increased if the majority of yeast metabolizable nitrogen in synthetic grape juice is supplied as alanine and/ or threonine, in stead of arginine.

The YAN (YMN) content of *V. vinifera* grapes decreases during the late stages of ripening due to an increase in proline at the expense of arginine (Kliewer, 1968, 1970). As a result, winemakers often need to increase the YAN (YMN) content of grape juice so that yeasts are able to grow to the numbers needed for a complete fermentation (residual sugar < 4 g/L). Salts of ammonium are normally used to adjust the YAN (YMN)

content of grape juice (Boulton *et al*., 1998b). The presence of ammonium in the growth medium reduces the uptake of amino acids by yeasts (Roon *et al.*, 1975; Jiranek *et al*. 1995), because ammonium ions repress dicarboxylic amino acid permease 1, the general amino acid permease, the GABA permease and the proline-imino acid permease (Henscke & Jiranek, 1993). The presence of ammonium also represses the activities of NAD^+-dependant glutamate dehydrogenase (Roon & Even, 1973), arginase and urea amidolyase (Dubois *et al*., 1974, 1977) in yeasts. α-Keto acids, acetyl-CoA, propionyl-CoA and succinyl-CoA are degradation products of amino acids in yeasts (Large, 1986; Boulton *et al*., 1998g) and can all be converted into succinate during fermentation via one or both TCA cycle branches, the GABA bypass or the MCA cycle (Chapter 2). In other words, high concentrations of ammonium in the grape juice might decrease the production of succinic acid from amino acids. To determine whether the ammonium content of grape juice will influence succinic acid production during fermentation, succinic acid production should be studied in synthetic grape juices with the same total YMN content, but different concentrations of ammonium.

Most commercial wineries make use of pure yeast starter cultures to ferment grape juice. A population size of 1-3 x 10^6 cells/ ml is recommended as yeast inoculum to ensure a rapid and complete fermentation, dominated by the selected yeast strain (Fugelsang, 1997). Shindo *et al*. (1993) found that immobilized yeasts produced higher concentrations of succinic acid than a suspension of free yeast cells, but the population size of the immobilized yeast starter culture was much larger than that of the free yeast starter culture. Similar findings were reported by Ciani & Ferraro, 1996 & Ferraro *et al*., 2000, but the immobilized yeasts belonged to different specie (*C. stellata*) than the free yeast suspension (*S. cerevisiae*). In contrast with the above mentioned findings, Kleinzeller (1941) found that slightly more succinic acid was produced in cases where the initial yeast population was less dense. More carefully planned experimentation is needed to verify whether the size of the yeast inoculum influence the total amount of succinic acid produced during fermentation.

Heerde & Ralder (1978) and Shimazu & Watanabe (1981) did not find any significant difference between *S. cerevisiae* and other *Saccharomyces* species in terms of succinic acid production. However, the taxonomic identities of the strains that were studied by them were not based on the genetic makeup of the yeasts. Genetically distinct *Saccharomyces sensu stricto* species differ with regard to the amount of succinic acid they can produce during fermentation (Rainieri *et al*., 1999). However, not all the *Saccharomyces* species encountered within the wine industry were included in Rainieri *et al*.'s study (1999) and fermentation conditions were not optimal for succinic acid production. It is necessary to determine whether the succinic acid production capability of different yeasts is a species or stain related trait, because such information will help narrow the search for new commercial wine yeast strains or strains that can be used for breeding or cross-breeding purposes. Succinic acid production ought to be studied in synthetic grape juice with a composition optimal for the production of succinic acid and should preferably be conducted at a low, moderate and high fermentation temperature.

Only fertile diploid yeast strains should be selected for such a study and each of the following species, varieties and physiological races ought to be represented by at least three strains: *S. bayanus* var. *uvarum*, *S. bayanus* var. *bayanus*, *S. kudriavzevii*, *S. paradoxus*, cryotolerant *S. cerevisiae* (*S. oviformis*) and *S. cerevisiae*'s physiological races *cerevisiae*, *chevalieri*, *italicus* (*steinieri*), *capensis and coreanus*. All of the above mentioned *Saccharomyces* species, varieties and physiological races have been isolated from grapes, fermenting grape juice or wine (Vaughan-Martini & Kurtzman, 1985; Boulton *et al*., 1998g; Naumov *et al*., 2000b; Redžepović *et al*., 2002; Demuyter *et al*., 2004; Sipiczki *et al*., 2004; Antonovics *et al*., 2005; Bradbury *et al.*, 2005; González *et al*., 2006; Lopandic *et al*., 2007; Masneuf-Pomarède *et al*., 2007).

Succinic acid's taste has been described as unpleasant compared to tartaric acid's taste (Coulter *et al*., 2004) due to its salty, bitter taste (Amerine & Cruess, 1960; Coulter *et al*., 2004). The sensory threshold of succinic acid ranges from 34-35 mg/L when it is dissolved in water (Berg *et al*., 1955; Amerine *et al*, 1959). However, wines with very high levels of succinic acid were not identifiably salty or bitter according to the results of an informal tasting held at the AWRI (Coulter *et al*., 2004). This is to be expected, because ethanol increases the taste thresholds of organic acids in aqueous solution and tannins increase the minimum organic acid concentration that brings about a detectable difference in taste (Hinreiner *et al*., 1955; Amerine *et al*, 1959). The sourness difference threshold of an organic acid is minimized by the presence of sugar in the solution, but sugar increases ethanol's effect on the taste thresholds of organic acids (Hinreiner *et al*., 1955; Amerine *et al*, 1959). Sensory analysis of synthetic 'red' (tannin) and 'white' wines should be conducted with a trained panel of tasters to obtain a better estimate of succinic acid's threshold concentration in real wine. Wines with the same pH and titratable acidity, but different concentrations of succinic acid should then be rated by a consumer tasting panel to determine whether succinic acid has any negative influence on wine quality.

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ADDENDUM A.

IMPROVED CAPILLARY ELECTROPHORESIS METHOD FOR THE SEPARATION AND QUANTIFICATION OF WINE'S MAIN ORGANIC ACIDS

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A.1 MATERIALS AND METHODS

A.1.1 CHEMICALS

Pyridine-2,6-dicarboxylic acid (PDC), cetyltrimethylammonium bromide (CTAB), ethylenediamine tetra-acetic acid disodium salt dihydrate (EDTA 2Na·2H_2O), succinic acid, L-malic acid, sodium lactate, sodium acetate and sodium azide were purchased from Fluka (Buchs, Switzerland). Citric acid and L-tartaric acid were obtained from Aldrich Chemical Company (Gillingham, UK), sodium formate solution (0.01M) was from Riedel-de-Haën (Seelze, Germany) and 2-[N-morpholino] ethanesulfonic acid (MES) was from Acros Organics (Geel, Belgium). All of the above mentioned chemicals were of analytical grade, but CE-grade 0.1M and 1M sodium hydroxide solutions from Fluka (Buchs, Switzerland) were used to adjust the pH of CE-buffers. Ultra-pure deionised water (18.2 MΩ/cm) was obtained from a Milli-Q purification system (Millipore, Belford, MA, U.S.A.).

A.1.2 INSTRUMENTATION

Capillary electrophoresis was carried out with Hewlett-Packard's G1600A ^{3D}CE system (Agilent Technologies, Waldbronn, Germany), which included a built-in photodiode array detector and electrolyte replenishment system. HP^{3D} Chemstation software was used for system control, data acquisition and data analysis. Electrophoresis was conducted in bare fused-silica capillaries with an internal diameter of 50 μm, an effective length of 72 cm and a total length of 80.5 cm (Agilent Technologies, Waldbronn, Germany).

Chemicals were weighed off with a Mettler Toledo AB240 analytical balance (Switzerland) and acidity measurements were conducted with a WTW 340i pH meter equipped with a WTW SenTix 41 pH electrode (Wissenschaftlich-Technische Werkstätten, Weïlheim, Germany). Calibration standards and wine samples were diluted within CE microvials, using a Hamilton Microlab 500 Series digital diluter (Reno, NV, U.S.A.).

A.1.3 SAMPLE PREPARATION

Calibration standards and wine samples were filtered through 0.45 μm cellulose-acetate syringe disk filters and then diluted 40 times with a filtered (0.45 μm Nylon membrane) 5 mM MES buffer (pH = 6.20), which contained 0.5 mM sodium formate as an internal standard and 10 mg/L sodium azide as anti-microbial agent.

A.1.4 ELECTROPHORETIC CONDITIONS

A solution of 7.5 mM PDC and 0.5 mM CTAB with the pH adjusted to 5.60 served as the separation electrolyte. It was filtered and degassed by means of vacuum filtration through a 0.45 μm Nylon membrane. The column was equilibrated with separation electrolyte at the beginning of each analysis and a negative electric potential of 25 kV was applied over the capillary ends for 2 minutes prior to sample injection. Samples were injected into the capillary column with 50 mbar pressure applied for 15 seconds at the inlet side of the capillary. This was followed up by the injection of a short buffer plug (50 mBar for 2 seconds). The separation voltage was ramped from zero to -25 kV within half a minute and then kept constant at -25 kV for the remainder of the run. Electrophoresis was carried out at 10°C and electropherograms were recorded at a detection wavelength of 350 nm and reference wavelength of 210 nm. The vials with separation electrolyte were automatically emptied and refilled at the end of each analysis.

A.2 VALIDATION

A.2.1 SELECTIVITY

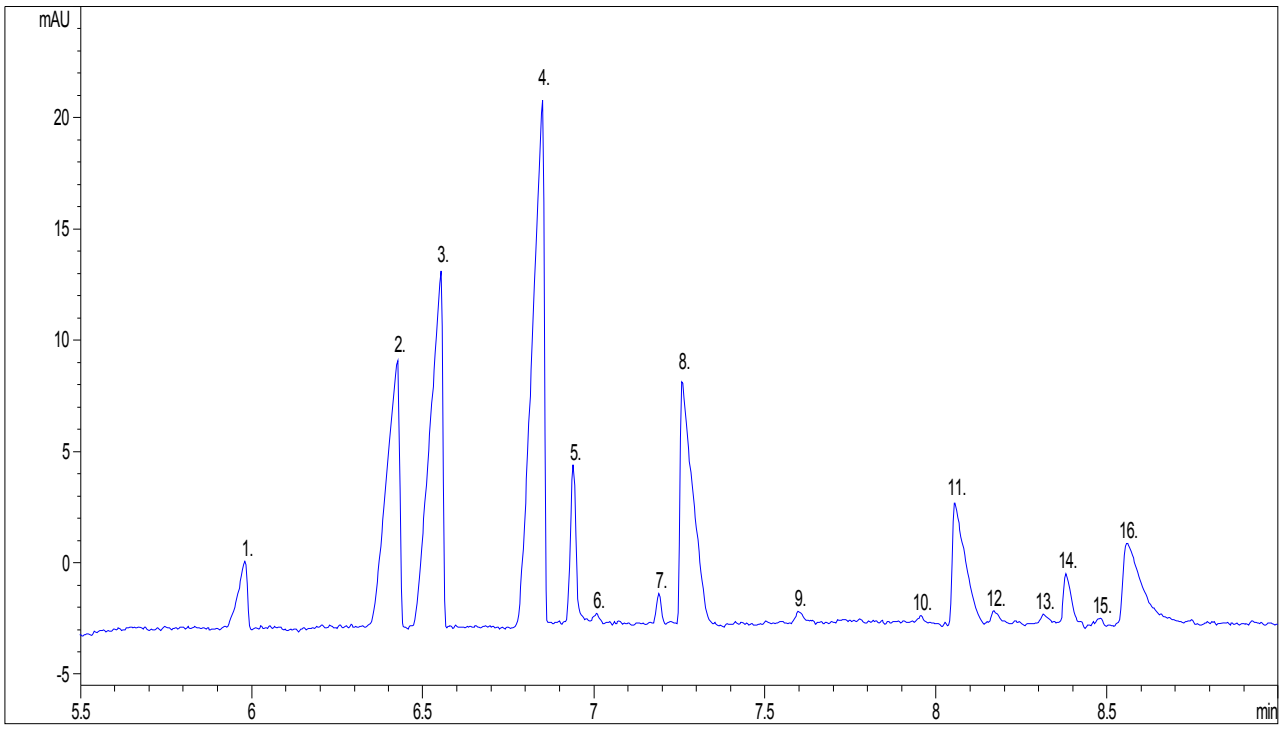

Figure A.1 Electropherogram of organic acids within white wine. Peak numbers correspond with the ones in **Table A.1**.

Table A.1
Selectivity of CE method for a white wine sample.

Peak number	Analyte	Migration time [b]	Resolution
1.	Azide	5.980	10.64
2.	Formate [a]	6.426	7.32
3.	Tartrate	6.553	1.78
4.	Malate	6.849	4.45
5.	Citrate	6.940	1.57
6.	Sulphite	7.008	2.09
7.	Unknown	7.191	5.88
8.	Succinate	7.259	1.46
9.	Pyruvate	7.598	6.45
10.	Glyoxylate	7.957	9.15
11.	Acetate	8.055	1.81
12.	Unknown	8.170	2.21
13.	Unknown	8.314	4.10
14.	Lactate	8.381	1.73
15.	Unknown	8.480	2.24
16.	Phosphate	8.559	1.13

[a]internal standard; [b]values reported in minutes

A.2.2 LINEARITY

Table A.2
Linearity of calibration curves.

Analyte	Linear range [a]	Slope	y-intercept	Upper CL y-intercept [b]	Lower CL y-intercept [b]	r (n = 7)	R^2 (n = 7)
Tartrate	6 - 50	0.160	0.029	0.089	-0.030	0.9999	0.9999
Malate	6 - 50	0.181	-0.062	0.002	-0.125	0.9999	0.9999
Succinate	6 - 50	0.196	-0.033	0.045	-0.111	0.9999	0.9998
Acetate	6 - 50	0.258	-0.038	0.007	-0.082	0.9999	1.0000
Lactate	6 - 50	0.192	-0.058	0.005	-0.120	0.9999	0.9999

[a]mg/L; [b]confidence limits (CL) determined at a confidence level of 95% (p = 0.05)

A.2.3 SENSITIVITY

Table A.3
Limits of detection and quantification

Analyte	LOD_{old} (mg/L) [a]	LOD_{new} (mg/L) [b]	LOQ_{old} (mg/L) [a]	LOQ_{new} (mg/L) [b]
Tartrate	4.0	2.5	13.6	8.4
Malate	3.3	1.9	11.0	6.4
Succinate	4.1	2.6	13.8	8.5
Acetate	3.9	2.8	13.0	9.4
Lactate	4.9	2.9	16.3	9.6

[a] de Villiers *et al*.'s method (2003): ~12 nL sample injected into a 75μm I.D. capillary with total length of 112.5 cm; [b] new method: ~16 nL sample into a 50 μm I.D capillary with a total length of 80.5 cm; [c] LOD: limit of detection at a signal-to-noise ratio of 3:1; [d] LOQ: limit of quantification at a signal-to-noise ratio of 10:1.

A.2.4 REPEATABILITY AND ACCURACY

Table A.4
Repeatability and accuracy of method for white wine samples.

Analyte	% RSD MT (n = 6) [a]	% RSD PA (n = 6) [b]	% RSD CA (n = 6) [c]	% Recovery [a] (n = 3)
Formate (I.S.)	0.139	0.837	0.799	
Tartrate	0.216	0.626	0.459	99
Malate	0.123	0.286	0.229	99
Succinate	0.263	0.994	0.937	97
Acetate	0.160	1.016	0.998	101
Lactate	0.194	1.615	1.395	99

[a]MT = Migration Time; [b]PA = Peak Area; [c]CA = Corrected Peak Area

A.3 CALCULATIONS

Standard deviation of the detector's response to a field blank (water) = average baseline noise of field blank's electropherogram. The average baseline noise of the field blank's electropherogram was 0.4579 mAU.

The standards that were used to construct peak height calibration curves contained 10 mg/L, 8 mg/L, 6 mg/L, 5 mg/L and 4 mg/L of each analyte. Electrophoretic peaks are skewed depending on the difference in mobility between the analyte and background electrolyte (PDC). The highest data point of each peak was therefore taken as peak height. The standards that were used to construct peak area calibration curves contained 50 mg/L, 40 mg/L, 30 mg/L, 20 mg/L, 10 mg/L, 8 mg/L and 6 mg/L of each analyte.

The confidence limits for the y-intercept of each calibration curve are used to determine whether the y-intercept differs significantly from zero. R^2 values indicate how well the calibration data points fit the non-weighted linear regression equations of

calibration curves. These two statistics along with Pearson's correlation coefficient (r value) indicate how linear the detector's response is in relation to the analyte's concentration (Miller, 1991).

$$LOD = \frac{3 \times \sigma_{Blank}(mAU)}{Slope_{PeakHeight}(mAU)}$$ (Miller, 1991; Harris, 2000; www.labcompliance.com)

$$LOQ = \frac{10 \times \sigma_{Blank}(mAU)}{Slope_{PeakHeight}(mAU)}$$ (Miller, 1991; Harris, 2000; www.labcompliance.com)

$$CorrectedPeakArea = \frac{Area_i(mAU \times \min) \times Length_{Total}(cm)}{Time_{Migration}(\min)}$$ (Landers, 1997)

A.4 RESEARCH NOTES

Severe tailing of the citrate peak is observed when electrophoresis is carried out in a 75 μm I.D. fused-silica capillary filled with 7.5 mM PDC/ 0.5 mM CTAB buffer at a pH of 5.60 (de Villiers *et al*., 2003). However, the tailing of the citrate peak is significantly reduced if electrophoresis is conducted in a 50 μm I.D. capillary instead of the 75 μm I.D. capillary. Tailing of the citrate peak is caused by the formation of complexes between citrate and the transition-metal cations (Fe^{3+}; Al^{3+}, Cr^{2+} and Mg^{2+}) that are found within the fused-silica glass of capillary columns (Soga & Ross, 1997). The pyridine-2,6-dicarboxylate ions of the separation buffer is unable to prevent interaction between citrate and the inner wall of the capillary, because citrate is able to form stronger complexes with transition metal cations than PDC (Soga & Ross, 1997). The tailing of the citrate peak can be prevented by including 0.5 mM EDTA (ethylenediamine tetra-acetate) in the separation buffer, thereby increasing the method's sensitivity for the detection of citrate (de Villiers *et al*., 2003).

Succinate and acetate peaks were split into two at high analyte concentrations when pressure was used to inject samples into the column (de Villiers *et al*., 2003). According to these authors this could be prevented by adjusting the sample pH to that of the separation buffer or by using electrokinetic injection to introduce samples to the column after being diluted 80 times with an unbuffered internal standard solution. However, in our opinion electrokinetic injection cannot be used to solve this problem, because the amount of internal standard (formic acid) injected this way depends on the degree to which it is ionized, which off coarse will vary according to the pH of the sample. This will become very apparent during calibration as shown in **Figure A.2**, where it can be seen how the peak area of formate (internal standard) increases as the concentrations organic acids in the unbuffered calibration standards decreases, *i.e.* as the pH increases. This meant that sample pH had to be adjusted to the pH of the separation buffer so that pressure could be used as the method of injection (de Villiers *et al.*, 2003). Wines have strong buffering capacities (Boulton *et al*., 1998) with pH values considerably lower that of the separation electrolyte (pH = 5.60), which implies that a substantial amount of even a strong alkaline solution would be needed to adjust the pH of an undiluted wine sample. This complicates matters for the analyst, because the volume of added base would have to be recorded in order to rework the data so that the concentration of each analyte in the original wine

sample can be determined. Adequate dilution with an appropriate buffer solution serves as a much more suitable alternative for adjusting the pH of each wine sample.

Morpholino ethanesulfonic acid (MES) was chosen to buffer the wine samples at a slightly higher pH that of the separation electrolyte, which is equal to the value of MES's second pK_a value at 20°C. The first pK_a of MES is 1.99 at 20°C, while its second pK_a equals 6.21 at the same temperature (Chen *et al*., 2006). At a pH of about 4.095 (MES's isoelectric point), all of the MES in solution exist as bipolar ions (zwitterions) and therefore carries no net electric charge at this pH. The first pK_a value of MES is that of its sulphonic acid functional group, whereas the second pK_a value is that of the morpholine ring's protonated nitrogen atom. Almost all of the MES in solution (99.9%) will carry a negative charge at pH 6.21, whereas half thereof will still carry a positive charge at the same pH. This implies that half of MES will be present as zwitter ions at the pH that was chosen for the diluent solution, which contained the internal standard. Zwitter-ions do not move in an electric field and therefore the MES buffer did not increase the conductivity of the diluted sample as much as the same concentration of other buffer compounds would. This is important, because the conductivity of the diluted sample should be less than that of the separation electrolyte in order for stacking of the analytes to occur at the boundary between the sample and buffer zones when voltage is applied at the start of an electrophoretic run. In fact, this is why samples are normally diluted in deionised water (Landers, 1997).

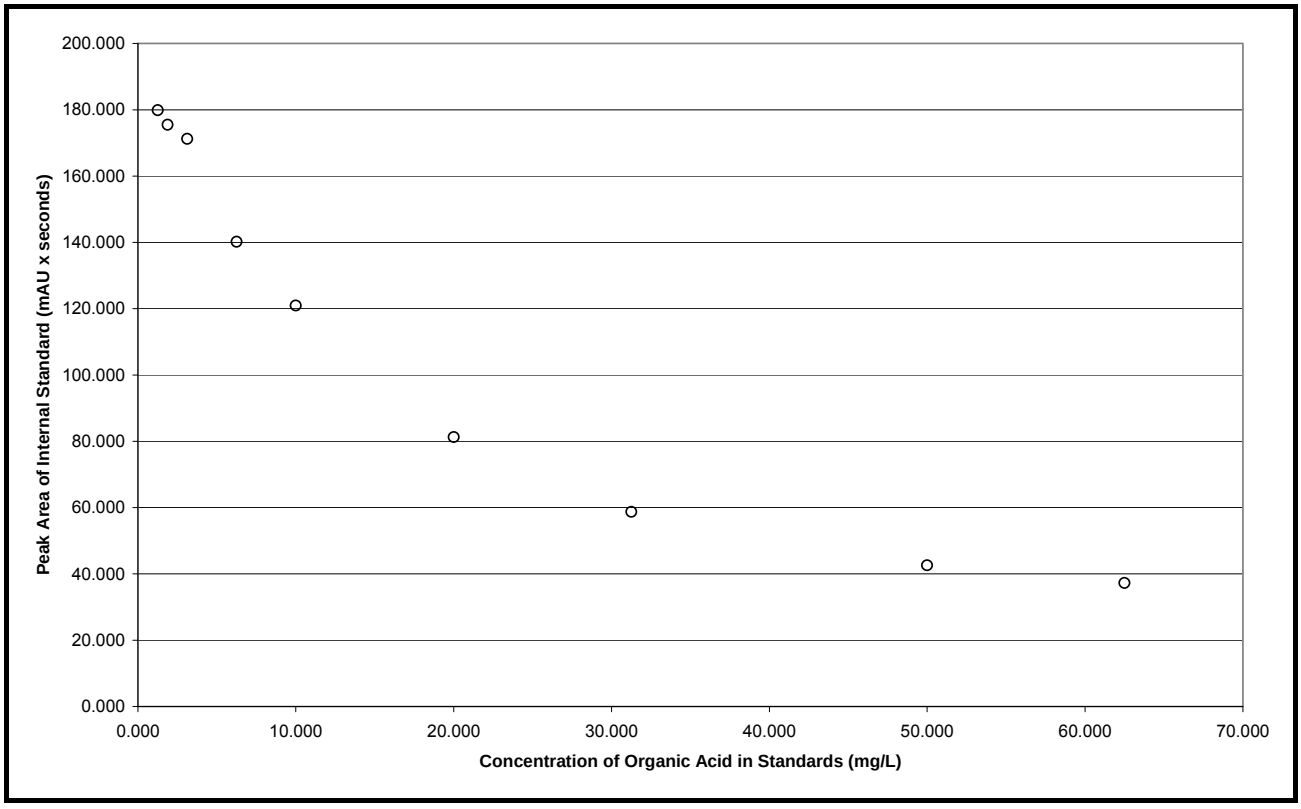

Figure A.2 Peak area of internal standard (formic acid) as a function of the organic acid concentrations in calibration standards when samples are diluted 80 x with a unbuffered internal standard solution and injected electrokinetically (2 seconds at −10kV).

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