Factors influencing the fermentation performance of commercial wine yeasts

by

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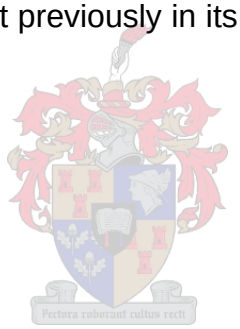
DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.



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Jacques Ferreira **Date**

SUMMARY

The production of quality wine is influenced by numerous factors of which grape quality is one of the most important factors. The production of quality wine, however, is not possible without good winemaking techniques and effective quality control. Critical control points (CCP) during the winemaking process must be identified to ensure optimum wine quality. Grape must is a complex medium that contains different micro-organisms which can be either beneficial or negative to wine quality, depending on the physical and chemical conditions that prevail in the must. Yeasts are responsible for alcoholic fermentation, lactic acid bacteria (LAB) for malolactic fermentation (MLF) and acetic acid bacteria (AAB) for the production acetic acid from ethanol. Yeasts and certain LAB can also produce acetic acid and thereby increasing the volatile acidity (VA) of wine. These micro-organisms can influence each other in complex fashions by competing for growth nutrients and by producing inhibitory substances.

Most winemakers nowadays use commercial yeast strains to inoculate wine fermentations. This, however, does not assure a problem-free fermentation and cases of stuck and sluggish fermentations are annually reported worldwide. In these or most cases fermentation takes longer than 21 days to complete and the wine contains a residual sugar concentration of more than 4 g/L, which can be utilised by wine spoilage micro-organisms such as certain bacteria and other wild yeasts. Stuck and sluggish fermentations also increase the chances of oxidation due to the absence of the protective CO_2 layer on the surface of the wine, which is formed during alcoholic fermentation. Another effect of stuck and sluggish fermentations is that valuable tank space is wasted due to the unexpected time consumption of these fermentation problems. Many factors during the winemaking process can be responsible for stuck and sluggish fermentations. In this thesis the different factors is discussed with the emphasis on the effect of the yeast strain. The way that certain yeast strains influence AAB and LAB numbers during fermentation and MLF through the production of inhibiting by-products such as medium chain fatty acids has not been investigated in detail in the past.

Certain fungicides and pesticides that are used in vineyards to control pests (e.g. mildew) contain copper which can be inhibiting to yeast growth and alcoholic fermentation. Legal limits and withholding periods on these sprays are not always strictly obeyed and can lead to stuck and sluggish fermentations. This motivated us to evaluate the growth and fermentation activities of a selection of commercial wine yeasts in the presence of copper levels in the range of maximum legal limits. The effect of these commercial strains on the LAB and AAB numbers during alcoholic fermentation and MLF were also investigated.

Our results showed that there was no significant difference on numbers of the AAB obtained from fermentations inoculated with different commercial wine yeast

strains. However, with regards to the LAB numbers, one of the strains produced significantly more sulphur dioxide (SO_2), which led to the inhibition of MLF in that wine. Our results further indicated which commercial yeast strains were capable of effectively fermenting high sugar musts and which strains were less effective. From the strains tested VIN13, N96 & L2056 were able to utilize fructose more effectively than NT50, RJ11 & D80. We could further distinguish between yeast strains that produced the lowest (VIN13 & RJ11) and the highest (WE372, NT50 & L2056) VA concentrations in must containing high sugar levels. Strains that were more tolerant against high copper levels were also identified. We tested six yeast strains in must with added copper (0.25 mM cu^{2+}) in the form of $CuSO_4 \cdot H_2O$. Three Cu^{2+}-tolerant (D80, Collection Cepage Cabernet & NT50) yeast strains were distinguished from three less Cu^{2+}-tolerant yeast strains (VIN13, NT112 & RJ11).

This study made a valuable contribution in knowledge gained about commercially available wine yeast strains that can ferment effectively under certain stress conditions. Research such as this, where wine yeasts are evaluated to ferment more effectively during strenuous winemaking conditions, will be very beneficial to winemakers.

OPSOMMING

Die produksie van gehalte wyn word deur verskillende faktore beïnvloed waarvan druifkwaliteit seker die belangrikste is. Die produksie van gehalte wyn is egter nie moontlik sonder goeie wynmaaktegnieke en effektiewe kwaliteitsbeheer nie. Kritieke kontrole punte (KKP) tydens die wynmaakproses moet dus geïdentifiseer word om sodoende ‘n verlaging in wynkwaliteit te vermy. Druiwemos het ‘n komplekse mikrobiologiese samestelling en bestaan uit verskillende mikroörganismes wat voor- en nadelig vir wynkwaliteit kan wees, afhangende van die fisiese en chemiese toestande wat in die mos bestaan. Giste is verantwoordelik vir alkoholiese fermentasie, melksuurbakterieë (MSB) vir appelmelksuurgisting (AMG) en asynsuurbakterieë (ASB) vir die produksie van asynsuur vanaf etanol. Asynsuur word egter ook deur giste en MSB geproduseer en dra so by tot die vlugtige suurheid (VS) van ‘n wyn. Hierdie mikroörganismes kan mekaar op komplekse wyses beïnvloed deur o.a. te kompeteer vir voedingstowwe asook deur die produksie van inhiberende verbindings.

Die meeste wynmakers maak gebruik van kommersiële gisrasse om alkoholiese fermentasies mee uit te voer. Gevalle van sogenaamde slepende en gestaakte alkoholiese fermentasies, waar suiker nie volledig na etanol en CO_2 omgeskakel word nie, kom egter nog gereeld in die wynbedryf voor. In sulke gevalle neem die fermentasie gewoonlik langer as 21 dae om te voltooi met ‘n suiker konsentrasie van meer as 4 g/L wat in die wyn oorbly. Dit is nadelig vir wynkwaliteit aangesien dit nie net die kanse vir bederf deur bakterieë en giste verhoog nie, maar ook die kanse vir oksidasie verhoog a.g.v. die verlies van die beskermende CO_2 lagie bo-oor die wyn. Hoe sekere gisrasse, ASB en MSB getalle gedurende fermentasie en AMG beïnvloed deur die produksie van inhiberende verbindings soos medium ketting vetsure en SO_2, is ook nie baie in die verlede ondersoek nie.

Sommige spuitstowwe wat gebruik word in die bekamping van swamsiektes bevat koper wat inhiberend kan wees vir gisgroei en alkoholiese fermentasie. Wetlike maksimum limiete en onthoudingsperiodes op spuitstofresidue word egter nie altyd gehoorsaam nie en kan lei tot slepende en gestaakte fermentasies. Dit het ons gemotiveer om ‘n seleksie van kommersiële gisrasse te evalueer in terme van gisgroei en fermentasie in die teenwoordigheid van kopervlakke naby die maksimum limiet.

Ons resultate het gewys dat daar nie noemenswaardige verskille in AAB getalle tydens alkoholiese fermentasie tussen behandelings met verskillende kommersiële gisrasse was nie. Een van die gisrasse het wel noemenswaardig meer SO_2 geproduseer wat gelei het tot inhibering van AMG in hierdie wyn. Ons het verder uitgewys watter kommersiële gisrasse instaat is om meer effektief in hoër suiker mos te fermenteer en watter van die rasse minder suksesvol was. Ons het ook rasse geïdentifiseer wat meer weerstandbiedend is teen hoë kopervlakke in mos en sodoende groter kans op ‘n suksesvolle fermentasie sal hê in mos wat koperresidue

bevat wat afkomstig is van sekere spuitstowwe. Die effek van die ASB en MSB getalle gedurende fermentasie en AMG is ook ondersoek. Ons resultate het verder gewys watter kommersiële gisrasse instaat was om mos met hoë suikervlakke meer effektief te fermenteer. Vam die gisrasse wat getoets was het VIN13, N96 & L2056 fruktose meer effektief benut as NT50, RJ11 & D80. Ons kon verder onderskei tussen gisrasse wat die laagste (VIN13 & RJ11) en die hoogste (WE372, NT50 & L2056) vlakke van VS produseer in mos met hoë inisiële suikervlakke. Gisrasse wat meer tolerant was teen koperresidue in mos is ook geidentifiseer. Ons het ses gisrasse getoets in mos met bygevoegde koper (0.25 mM Cu^{2+}) in die vorm van $CuSO_4 \cdot 5H_2O$. Daar is onderskei tussen drie Cu^{2+}-tolerante (D80, Collection Cepage Cabernet & NT50) en drie minder Cu^{2+}-tolerante gisrasse (VIN13, NT112 & RJ11).

Hierdie studie lewer ‘n waardevolle bydrae in die invordering van kennis oor kommersieel beskikbare wyngisrasse wat meer effektief sal fermenteer onder sekere streskondisies wat in mos voorkom. Inligting soos hierdie is belangrik om die wynmaker se keuse uit die reeks bestaande kommersiële gisrasse te vergemaklik.

This thesis is dedicated to my parents, Strauss and Erika, my brother Rikus and my sister Anchen.

Hierdie tesis is opgedra aan my ouers, Strauss en Erika, my broer Rikus en my suster Anchen.

BIOGRAPHICAL SKETCH

Jacques Ferreira was born on 30 June 1978, attended Eikestad Primary School and matriculated at Paul Roos Gymnasium in 1996. Jacques obtained a Bacchelors degree in Agricultural Science (Viticulture and Oenology) in 2001 at the University of Stellenbosch. In 2002 he enrolled for a Master's Degree in Oenology at the same University.

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PREFACE

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately and is written according to the style of the *South African Journal of Enology and Viticulture*.

Chapter 1 **GENERAL INTRODUCTION AND PROJECT AIMS**

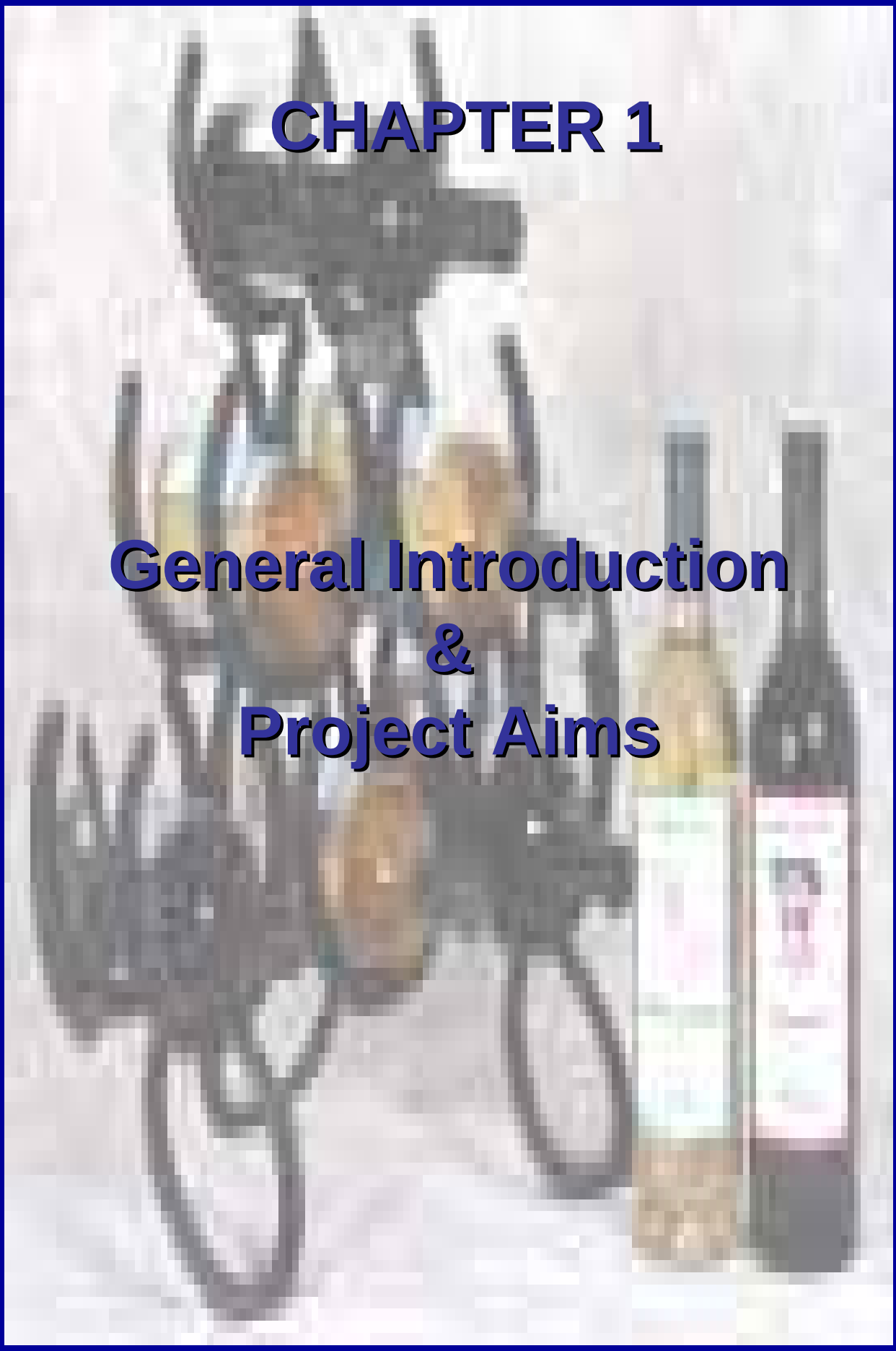
Chapter 2 **LITERATURE REVIEW**
Factors influencing the performance of yeast during winemaking

Chapter 3 **RESEARCH RESULTS**
The effect of commercial yeast strains on acetic acid bacteria and lactic acid bacteria numbers and malolactic fermentation rate during red wine fermentation

Chapter 4 **RESEARCH RESULTS**
The effect of high sugar and Cu^{2+} on the growth, fermentation and volatile acidity production of different commercial wine yeast strains

Chapter 5 **GENERAL DISCUSSION AND CONCLUSIONS**

CONTENTS



CHAPTER 1

General Introduction & Project Aims

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

The art of winemaking has been known for centuries and is believed to initially date back to ancient Mesopotamia. Today, however, winemaking has evolved into a very sophisticated science studied by enologists all over the world. The complexity of wine makes it both a very interesting field of study as well as a challenging one.

This complexity is mainly due to the important role that the biochemistry and interaction of different wine micro-organisms play during the winemaking process (Du Toit & Pretorius, 2000). Micro-organisms that are usually associated with winemaking include yeasts, which are responsible for alcoholic or primary fermentation, lactic acid bacteria (LAB) for malolactic or secondary fermentation (MLF) and acetic acid bacteria (AAB) which produce acetic acid from ethanol. One of the most important tasks of the winemaker is thus to ensure that the desired fermentative yeasts and malolactic bacteria strains predominate in the juice and wine and carry out the fermentations as well as keeping the numbers of undesired micro-organisms, such as AAB, as low as possible. A problem that winemakers and oenologists face is the so-called stuck and sluggish alcoholic fermentations, which have received much attention during the last few years.

This is not surprising, because alcoholic fermentation can be seen as one of the most important, if not the most important, process where alcohol is converted from sugar by yeast (Fleet & Heard, 1993; Ross *et al*., 2002). Sometimes certain factors lead to partial conversion of sugar into alcohol with the effect that sugar remains in the wine after fermentation, which can be utilized by spoilage organisms (Boulton, *et al*., 1995; Henschke, 1997). Incomplete alcoholic fermentations are referred to as stuck, because of a residual sugar content of higher than 4 g/L in the wine after fermentation, where sluggish refer to fermentations that is too slow for practical purposes, and generally takes longer than 21 days to complete (Hencshke, 1997; Bisson, 1999). Numerous studies have been done regarding the causes of stuck and sluggish fermentations and these include unfavourable pH and potassium levels (Boulton *et al*., 1995), high sugar concentrations (Gafner & Schütz, 1996), nitrogen and other nutrient deficiencies (Bisson, 1999), microbial incompatibility (Drysdale & Fleet, 1988), enological practices (Bisson, 1999), inhibition by acetic acid and other fatty acids (Du Toit, 2000) and the presence of certain agrochemical residues (Kundu *et al*., 1981).

Before the introduction of inoculating wine fermentations with pure yeast cultures by Müller-Thurgau in 1980, all wine was made by spontaneous fermentations, due to the presence of the natural grape and cellar microflora. Although spontaneous fermentations are unpredictable and risky due to its dependence on the number and diversity of yeasts present in must, many winemakers today accept this risk to try and produce distinctive wines. A wide variety of commercial wine yeast strains, mainly

Saccharomyces cerevisiae and *Saccharomyces bayanus* are available to the winemaker today. These strains differ in the way that they respond to certain stress conditions in the must, which can be any environmental factor that can have an adverse effect on cell growth, and could thus have a negative effect on fermentation efficiency in certain musts (Ivorra *et al*., 1999; Bauer & Pretorius, 2000). Therefore the primary selection criteria applied to wine yeast strain development programmes are to achieve an efficient conversion of grape sugar to alcohol and carbon dioxide, at a controlled rate without the development of off-flavours (Bauer & Pretorius, 2000).

The yeast strain used can also affect malolactic fermentation (MLF) due to the production of certain medium chain fatty acids (MCFs) and different levels of sulphur dioxide (SO_2). (Henick-Kling & Beelman, 1994; Guilloux-Benatier & Feuillat, 1998).

The importance of the dominating yeast strains during wine fermentations is well understood, but the complex nature of grape juice constantly leaves room for innovative research in all aspects of modern winemaking.

1.2 PROJECT AIMS

This study forms an integral part of a larger research project to determine the causes of stuck and sluggish fermentations in South African wines that is conducted at the Department of Viticulture and Oenology, Stellenbosch University. The specific aims of the study were as follows:

a) to monitor the growth of lactic acid bacteria (LAB) and acetic acid bacteria (AAB) in red wine fermentations inoculated with different commercial yeast strains;
b) to monitor malolactic fermentation (MLF) in red wine fermented with different commercial yeast strains;
c) to study the production of SO_2 and medium chain fatty acids (MCFAs) by different commercial yeast strains;
d) to study the effect of high sugar levels on fermentation capability and volatile acidity production of different yeast strains;
e) to study the effect of copper on the growth and fermentation of commercial yeast strains; and
f) to study the effect of copper on volatile acidity (VA) production of commercial yeast strains.

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CHAPTER 2

Literature review

Factors influencing fermentation efficiency during winemaking

2. FACTORS INFLUENCING FERMENTATION EFFICIENCY DURING WINEMAKING

2.1 INTRODUCTION

Alcoholic fermentation, the conversion of hexoses into ethanol and CO_2, forms the very basis of a successful wine fermentation. The dominating yeasts present in grape must after harvest, either being wild yeast species occurring naturally on the grapes, or from commercial yeast starter cultures are responsible for this. *Saccharomyces cerevisiae* is the most commonly encountered species in wine fermentations for very good reason. Grape must is far from an optimal growth medium for yeast due to several stresses that the yeast is being subjected to during fermentation (Bisson, 1999). *S. cerevisiae* strains have however, evolved to withstand and grow in most of the stress conditions that grape must and wine fermentations offer.

Slow and incomplete sugar utilization during wine fermentations are however, still a chronic problem worldwide, especially in countries with a warm climate. Incomplete fermentations are referred to as stuck, because of a residual sugar content of higher than 2 g/L in the wine after alcoholic fermentation (Bisson, 1999). (Although a residual sugar concentration of less than 5 g/L is often considered by winemakers to be dry, final sugar concentrations are typically below 2 g/L). If the rate of fermentation is too slow for practical purposes it is referred to as a sluggish fermentation (Henschke, 1997), and is generally considered to last longer than 21 days. **Fig. 2.1** illustrates different types of slow and incomplete fermentation profiles. Low final yeast viability is a characteristic of stuck and sluggish fermentations, mainly due to a high ethanol concentration and oxygen deficiency (Blateyron & Sablayrolles, 2001). There are numerous other complex factors leading to problem fermentations that will be discussed in this review. This abnormal fermentation kinetics can lead to unscheduled loss of tank capacity during harvest as well as microbial instability due to residual sugar and loss of the CO_2 blanket (Bisson, 1999; Cramer *et al*., 2001). On the other hand, the fastest possible fermentation rate is not necessarily the preferred goal for the winemaker, because of higher refrigeration requirements and shorter wood contact during barrel fermentations (Bisson & Butzke, 2000).

2.2 GRAPE-RELATED FACTORS THAT INFLUENCE YEAST PERFORMANCE

2.2.1 PHYSICAL PROPERTIES

2.2.1.1 pH and potassium levels

According to Ribéreau-Gayon *et al*. (1998) the pH of must varies between 2.8 and 3.5, but it is well known that in warm climates like South Africa some musts can easily reach pH values of up to 4.3. Such high pH values are not only related to climatic conditions, but also due to hard pressing of the grapes, which releases more potassium from the grape skins (Boulton *et al*., 1995). *Saccharomyces* can readily grow in the juice pH range of 2.8 to 4.2 with inhibition of growth and fermentation below 2.8 (Bisson, 1999). At an external pH of 3.0 the intracellular pH of *S. cerevisiae* is maintained between 5.5 and 5.75 during fermentation conditions. The cells have to pump out protons at the expense of ATP to maintain the intracellular pH within a physiological range optimum for metabolism (Thomas *et al*., 2002). This is why the pH of grape must may be reduced by as much as 0.3 units during fermentation (Bisson, 1999).

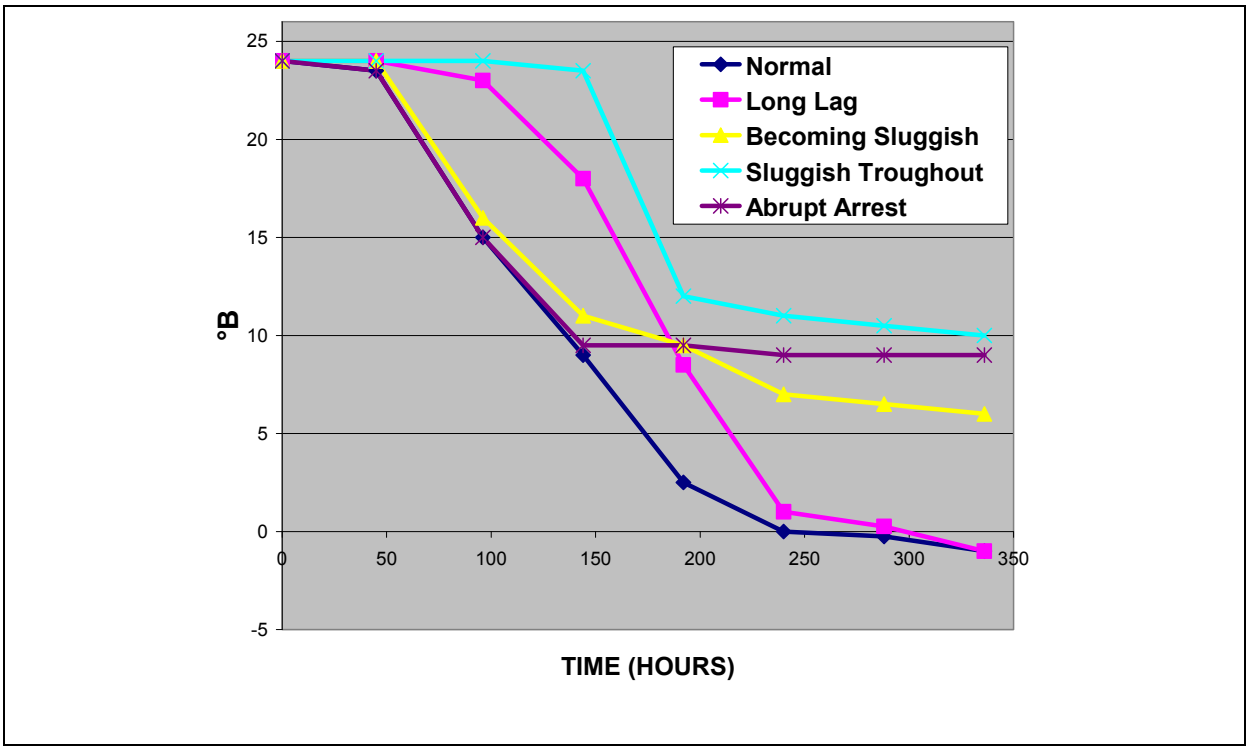

FIGURE 2.1 Example of different types of slow and incomplete fermentation profiles (Bisson & Butzke, 2000).

Studies done by Kudo *et al*. (1998) showed that the potassium concentration of grape juice plays a key role in the pH tolerance of *Saccharomyces*. Potassium does not only accelerate the rate of glucose consumption by the cell, but is also taken up

by the cell in exchange for hydrogen ions to balance cytoplasmic acidification. They found that a molecular K^+/H^+ ratio of 25:1 was the minimum required for the completion of fermentation. In the case of a potassium deficiency fermentation rate may be impacted in one of two ways, either by reducing the fermentation capacity of the individual cells or by reducing the fermentation capacity of the culture through the loss of cell viability. Too high potassium levels relative to the initial pH can cause the medium pH to decrease to such an extent that it becomes inhibitory to fermentation. They also found that potassium additions, if added early in the fermentation, were able to stimulate the fermentation of a potassium-deficient juice (Kudo *et al*., 1998).

2.2.1.2 Sugar levels

Fermentable sugars consist mainly of hexoses and are normally found at varying concentrations in grape juice, depending on the grape variety and growth region. Except for the hexoses, grape juice also contains pentoses at concentrations of 0.1-5 g/L in the form of xyloses, rhamnoses and arabinoses. Although pentoses cannot be utilized by *S. cerevisiae*, it has been found that some non-*Saccharomyces* yeast is able to convert xyloses to ethanol under anaerobic conditions (Ribéreau-Gayon *et al*., 1998).

The fact that the occurrence of problem fermentations is more frequently observed in vintages of well matured grapes containing high sugar concentrations, can be explained by different causes (Gafner & Schütz, 1996). One important reason is that a higher amount of ethanol is produced during alcoholic fermentation which will lead to higher toxicity towards the end of fermentation. The latter is discussed in section 2.3.3.2.

Another reason is that high density musts allow the yeast to experience hypertonic conditions as soon as it is inoculated. These hypertonic conditions lead to an efflux of water from the cell, lowered turgor pressure, reduced water availability and rapid cell shrinkage (Hohmann, 1997). Slaninova *et al*. (2000) showed that yeast cells respond to hyperosmotic shock through the modification of the cell wall and the cytoskeleton. They have demonstrated a disappearance of microtubules and actin microfilaments in the cytoskeleton and invaginations of the plasma membrane. This response can be divided into an immediate response to sudden changes in osmotic pressure and a long term adaptive response to hyperosmotic conditions (Hohmann, 1997; Bauer & Pretorius, 2000). The latter is applicable during winemaking when the cells have to adapt to the high osmotic pressure of the must after inoculation. Two membrane-based sensing mechanisms are involved, which consist of proteins Sln1p and Sho1p, which feed the signal into the high osmolarity glycerol (HOG) pathway (Bauer & Pretorius, 2000). Carrosco *et al*. (2001) have tested different commercial wine yeast strains with regard to osmotic stress and found strains T73 and Lalvin M69 are less affected in terms of viability than three other strains.

The loss of activity of the yeast's sugar transport system is another important cause of slow and incomplete sugar consumption during alcoholic fermentation (Salmon, 1989; Salmon *et al*., 1993; Bisson & Butzke, 2000; Carrosco *et al*., 2001). This inactivation of the hexose transport system is driven by protein synthesis arrest due to exhaustion of assimable nitrogen during fermentation (Salmon, 1996). This inactivation is irreversible and happens through a catabolite inactivation process (Bustana & Lagunas, 1986). A multigene family of transporters, called the *HXT* (hexose transporter) genes, are present in S*accharomyces* and have been extensively investigated (Kruckeberg, 1996; Boles & Hollenberg, 1997). They consist of 18 members ranging from *HXT1* to *HXT17* and *GAL2* which all share the same structural features. Two types of transporter proteins are encoded by different transporter genes, namely high and low affinity proteins, which differ in their affinity for glucose (Lagunas, 1993; Kruckenberg, 1996; Bisson, 1999). The high affinity transporters operate at low substrate concentrations and have more than one substrate binding site. At high sugar concentrations, when multiple substrate molecules try to bind simultaneously, the transporter gets blocked which is referred to as substrate inhibition. Therefore the transporters that are effective at high sugar concentrations have a single substrate binding site (McClellan *et al*., 1989; Bisson, 1999;). High affinity uptake is however, important near the end of fermentation when glucose have been depleted (Schütz & Gafner, 1993). According to Bisson (1999) the transport of glucose and fructose occurs by facilitated diffusion that is only operational over a narrow substrate concentration range. These membrane transport steps regulate the rate of flux through glycolyses due to the toxic effects of free intracellular hexose on the yeast cell (Bisson, 1999).

Another sugar-related cause of stuck and sluggish wine fermentations has arised from the fact that most of these wines contained higher fructose than glucose levels (Schütz & Gafner, 1995; Gafner & Schütz, 1996). Although it is believed that glucose and fructose share the same transporters (Bisson, 1999), the transporters have a lower affinity for fructose because glucose and other sugars are transported in the pyranose rather than the furanose form (Lagunas, 1993; Bisson, 1999). Schütz & Gafner (1995) have tested four *Saccharomyces bayanus* and two *S. cerevisiae* strains for their fructose and glucose uptake capacities. They found that the *S. bayanus* strains clearly exhibit lower fructose uptake than the *S. cerevisiae* strains tested.

2.2.2 YEAST NUTRIENTS AND OTHER FACTORS NECESSARY FOR OPTIMUM YEAST GROWTH AND FERMENTATION

2.2.2.1 Nitrogen sources in must and their uptake by yeast cells

Low initial nitrogen levels in grape must is perhaps the most studied cause of stuck and sluggish fermentations (Bell *et al*., 1979; Ingledew & Kunkee, 1985; Cramer

et al., 2001). Nitrogen is needed by the yeast cell for protein synthesis, such as enzymes for the glycolitic pathway, as well as the permease responsible for transporting amino acids and sugars into the cell (Jiranek *et al*., 1992; Lourens & Reid, 2002). Some researchers have linked low nitrogen to low cellular activity (Bely *et al.*, 1990, 1994) and others have associated it with low resultant biomass (Monteiro & Bisson, 1991). Bely *et al.* (1990) demonstrated the importance of nitrogen and it's effects on fermentation kinetics. An automatic devise for fermentation monitoring was used to find a good correlation between assimable nitrogen content of must and maximum CO_2 production rate.

Yeast assimable nitrogen in grapes consists of ammonia in the form of ammonium ions and amino acids (Jiranek *et al*., 1995). Aspects that influence the composition of these nitrogen sources are vineyard fertilization, berry maturation, vine water status, soil type etc. (Henschke, 1997). Ammonia is the preferred nitrogen source for yeast growth and is the most easily assimilated (Jiranek *et al*., 1995). **Fig. 2.2** shows the central pathways for nitrogen metabolism in yeast cells. The amino acids glutamine and glutamate are preferred for yeast growth (Monteiro & Bisson, 1991; Jiranek *et al*., 1995). These two amino acids serve as nitrogen donors for all the other nitrogen containing components in the cell (Magasanik & Kaiser, 2002). Other amino acids that are also excellent nitrogen sources are asparagine, aspartate, serine and alanine (Cooper, 1982). Monteiro & Bisson (1991) also found that these amino acids supported the most rapid yeast growth rates of all the amino acids that are present in grape must. The amino acid proline on the other hand is not metabolized by yeast under anaerobic conditions and thus not during normal alcoholic fermentation (Ough *et al*., 1991). Different authors have found different ranges of nitrogen concentrations in grape must. Sabbayrolles (1996) described that it ranges from 60 to 400 mg/L and according to Alexandre *et al*. (1994a) from 60 to 2400 mg/L. According to Bely *et al*. (1990) the ammonium nitrogen in grape must range from 17 to 156 mg/L and free alpha amino nitrogen from 28 to 305 mg/L.

The uptake of amino acids happens through a mechanism called active transport. This is the first step in nitrogen utilization via more or less specific permeases (ter Schure *et al*., 2000). Nelissen *et al*. (1997) found that *S. cerevisiae* encodes 19 amino acid permeases that form part of a diverse family of transporters for amino acids, polyamines and choline found in bacteria, fungi and mammalian cells. These amino acid permeases are integral membrane proteins with 12 predicted transmembrane domains, which are delivered to the plasma membrane where they function to take up amino acids for protein synthesis and for use as a nitrogen source (Regenberg *et al*., 1999). The presence of a given nitrogen source does not by itself assure that it will be utilized by the yeast for cellular biomass production. Regenberg *et al*. (1999) further reported that the activity of the general permeases in *Saccharomyces* is decreased approximately 80% by exposure to 5% ethanol. Different nitrogen source transport systems have different ethanol sensitivity levels (Monteiro & Bisson, 1991). Low pH juices also may not be as stimulatory for

ammonium salt uptake by the yeast cell as for higher pH juices (Monteiro & Bisson, 1991). Llauradò *et al*. (2002) suggested that phenolic compounds such as anthocyanins may interact with the assimilation of amino acids.

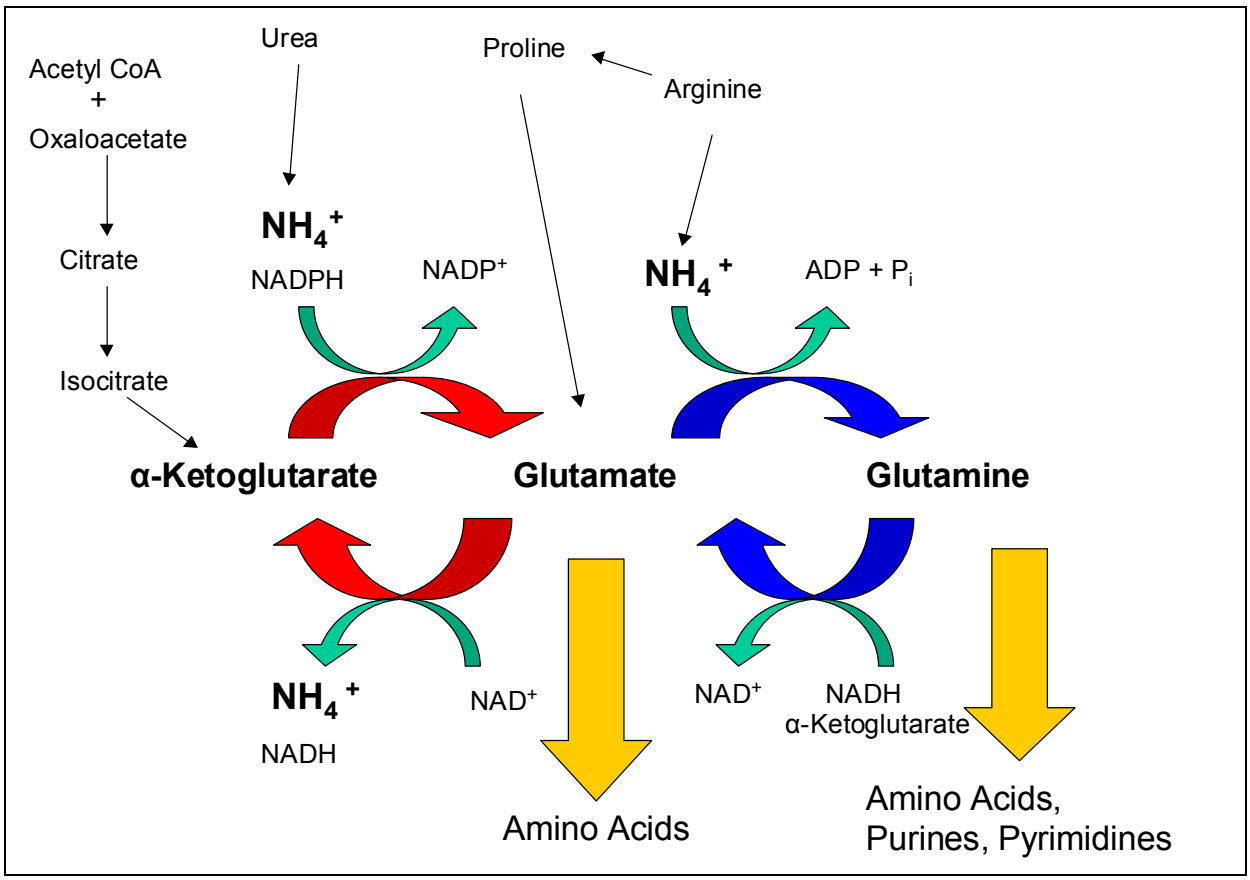

FIGURE 2.2. Central pathways for nitrogen metabolism. The nitrogen compounds in the cell are synthesized from either glutamate or glutamine. The major pathway for glutamate synthesis is through combination of ammonia with α-ketoglutarate, which is synthesized from acetyl CoA and oxaloacetate through the early steps of the citric acid. Glutamine is synthesized by the combination of ammonia with glutamate. The pathways for utilization of a variety of nitrogen sources, including urea, proline and arginine, are shown (Magasanik & Kaiser, 2002).

Differences in nitrogen requirements between enological yeast strains have been studied by Manginot *et al*. (1998). They found that industrial strains have significantly different nitrogen requirements and mostly appear during the stationary phase. Jiranek *et al*. (1992) suggested that yeast nitrogen demand could be another selection criterion for wine yeasts. However, as it has almost no effect on the kinetics at the end of fermentation it will not necessarily solve the problem of stuck or sluggish fermentations (Manginot *et al*., 1998).

2.2.2.2 Vitamins and minerals necessary for optimum fermentation

Natural grape juice are rarely deficient in vitamins (Bisson & Butzke, 2000), and although deficiencies might occur, most *S. cerevisiae* strains are capable of producing all essential vitamins except biotin (Bisson, 1999). Thiamine, biotin and pantothenate are the most important vitamins in alcoholic fermentation where they serve as co-factors in enzymatic conversions (Lourens & Reid, 2002). According to Henschke (1997) vitamin deficiencies may occur due to juices treated with high concentrations of SO_2, pasteurization or ion exchange. The biological activity of thiamine can be destroyed by cleaving the molecule through the disulphite ions originating from sulphur dioxide (Boulton *et al*., 1995). According to Lourens & Reid (2002) the use of mother tanks in the cellar, as well as many kinds of mould infestation on the grapes may also generate certain vitamin deficiencies. Bataillon *et al*. (1996) demonstrated that wild yeasts, especially *Kloeckera apiculata*, is quite efficient in eliminating thiamine from grape juice in a matter of hours.

Bataillon *et al*. (1996) have focused their work on thiamine and studied the effects of this vitamin on biomass production and fermentation kinetics of *S. cerevisiae*. The thiamine concentration in musts ranges from 150 to 750 µg/L (Peynaud & Lafourcade, 1977). *S. cerevisiae* is able to synthesize thiamine *de novo* in a thiamine depleted culture medium, but fermentation is slow and biomass production low and thereby the chances of sluggish or stuck fermentations is enhanced. They also used a thiamin limited synthetic culture medium (MS300) and found that fermentation rates were dramatically decreased.

The minerals PO_4^{2-}, Mg^{2+}, Zn^{2+} and Mn^{2+} are important for yeast growth and alcoholic fermentation (Monk, 1984). Normally grape must contain a sufficient mineral supply to ensure satisfactory fermentation, but mould infested grapes may cause problems (Lourens & Reid, 2002). A deficiency in zinc or magnesium may for instance directly affect sugar metabolism. Magnesium is especially important in stabilizing nucleic acids, proteins, polysaccharides and lipids and plays a key role in metabolic control, growth and cell proliferation (Walker, 1994; Rosslyn & Walker, 2000). Although phosphate limitation has been shown to impact cell growth and biomass yield as well as fermentation rate (Lafon-Lafourcade *et al.*, 1984b; Bisson, 1999), phosphate levels should not be a problem, especially when nitrogen is added in the form of diammonium phosphate (DAP) (Lourens & Reid, 2002).

2.2.2.3 Importance of oxygen, sterols and unsaturated fatty acids

Oxygen is required for the synthesis of cellular compounds, especially sterols, unsattured fatty acids and structural compounds in numerous organic molecules (Julien *et al*., 2000). Under winemaking conditions the yeast does not rely on oxygen for energy production, but for efficient growth a significant amount of free oxygen is required (Ribéreau-Gayon 1999; Bauer & Pretorius, 2000). Sablayrolles (1996) found that the average amount of oxygen needed for yeast cell growth is between 5 and 10

mg/L. A decrease in oxygen availability results in a decrease in biomass production and the rate of glycolyses because of an inhibition of fatty acid and sterol biosynthesis in the yeast (Alexandre & Charpentier, 1998).

These sterols and long chain unsattured fatty acids have been named 'survival factors' (Lafon-Lafourcade *et al.*, 1984b), 'anaerobic growth factors' (Houtman & Du Plessis, 1986; Alexandre *et al.*, 1994a) and 'oxygen substitutes' (Lourens & Reid, 2002). Their role in winemaking is to ensure correct cell membrane integrity and permeability for cellular metabolism and thereby enhancing ethanol tolerance and cell viability towards the end of fermentation.

According to Lourens & Reid (2002) there are sufficient dissolved oxygen in grape must after crushing. Active dried yeast is rich in survival factors due to the highly aerobic conditions under which it is propagated. Certain factors may however, play a role in decreasing the dissolved oxygen in grape must. Grape oxidases and yeast oxidases are able to consume dissolved oxygen during fermentation. The use of small amounts of sulphur dioxide after crush are however, inhibitory to these oxidation enzymes, especially the polyphenol oxidases (Alexandre & Charpentier, 1998). Ascorbic acid (Vitamin C) is sometimes used to prevent oxidation in certain grape cultivars, especially Sauvignon blanc, because of it's oxygen scavenging capability. The use of this acid prevents the synthesis of additional survival factors by the yeast. The end of red wine fermentations have also been reported to be a possible time of risk and that survival factors should be added in such cases (Lourens & Reid, 2002).

2.2.3 MICROFLORA PRESENT IN THE MUST

2.2.3.1 Non-*Saccharomyces* species

Although *S. cerevisiae* is commercially used for inoculating wine fermentations they are rarely found in vineyards with uninfected grapes. It is the non-*Saccharomyces* species or the so-called wild yeasts that are naturally found on grapes with numbers ranging from 10^2 to 10^4 CFU/berry, depending on the climate. Some of these native yeasts include *Kloeckera apiculata*, it's sexual anamorph *Hanseniaspora uvarum*, *Metchnikowia pulcherrima*, it's sexual anamorph *Candida pulcherrima, Pichia anomala* and *P. membranaefaciens* (Fugelsang, 1997; Fleet & Heard, 1993). Some of these non-*Saccharomyces* yeasts may survive normal sulphur dioxide levels in must and grow for the first 3 to 4 days of alcoholic fermentation after which they are killed by the increased ethanol levels (Heard & Fleet, 1988; Fleet & Heard, 1993).

These wild yeasts may use nutrients at the expense of the starter culture, *S. cerevisiae,* by depleting nutrients from the must. The depletion of thiamine from grape juice by *K. apiculata* was demonstrated by Bataillon *et al*. (1996). Another negative effect of these native yeasts is the production of acetic acid. According to

Du Toit (2000) some of these yeasts can produce very high acetic acid levels during fermentation as different authors found for *Brettanomyces*, *Zygosaccharomyces bailii*, *Hanseniaspora uvarum*, *Kloeckera apiculata* and *Candida krusei* (Custer, 1940; Shimazu & Watanabe, 1981).

It is also well known that some yeasts, the so-called killer yeasts, can produce proteinaceous killer toxins, which were first discovered in 1963 by Bevan and Makower. The killer factor exists in three different phenotypes in yeast: killer (K^+R^-), sensitive (K^-R^-), and neutral (K^-R^+). Only the strains with a killer phenotype produce a toxin that is lethal to the sensitive strains (Jacobs & Van Vuuren, 1991). Killer strains have been described in both *Saccharomyces* and non-*Saccharomyces* yeasts. Michalcakova *et al*. (1993) described the production of killer factors by *Hansenula* and *Kluyveromyces* that are active against *Saccharomyces*. Killer strains of *S. cerevisiae* are also well characterized and have been classified into 5 groups (K_1, K_2, K_3, K_{28} and K_3GR_1) (Vadasz *et al*., 2002). It is however, only the K_2 toxin with a pH optimum of between 2.9 and 4.9, which can survive in wine pH, thus posing a threat to wine fermentations when sensitive strains are used as starter cultures. Van Vuuren & Wingfield (1986) isolated a K_2 strain, *S. cerevisiae* T206, which was responsible for stuck fermentations in a South African wine cellar. More recently Zagorc *et al*. (2001) isolated 22 yeast strains from seven Slovenian red wine fermentations that expressed killer activity. These isolates were identified as *S. cerevisiae, Pichia anomala, Pichia kluyveri, Pichia pijperi, H. uvarum* and *Candida rugosa*. They identified *S. cerevisiae* strain S_S 12/10 as the killer strain with the best fermentation properties that produced wine with favourable characteristics. In another study Yap *et al.* (2000) investigated the potential of 14 killer yeasts to antagonize the growth of both *S. cerevisiae* and non-*Saccharomyces* yeast species associated with wine fermentations. Of the killer yeasts studied, *P. anomala* NCYC 434 displayed the broadest killing range at pH 4.5, but with reduced killer-sensitive interactions at a more realistic wine pH of 3.5. They also found that the killer yeast *Williopsis saturnus* var. *mrakii* CBS 1707 remained active against more than 50% of the 26 strains tested, and thus displayed the least change in killer activity at low pH (Yap *et al*., 2000).

The importance of such killer strains lies in the fact that they can dominate spontaneous wine fermentations or fermentations that were initially inoculated with a sensitive yeast strain (Jacobs & Van Vuuren, 1991; Vadasz *et al*., 2002). The fact that the killer factor is proteinaceous makes that it is rapidly bound or inactivated by binding to phenolics or bentonite. The zymocidal toxins are thus not effective, except in clarified juice (Henschke, 1997).

2.2.3.2 Acetic acid bacteria

Acetic acid bacteria (AAB) were first thought to be only associated with aerobic environments, which led to the believe that they did not play any significant role in the

winemaking process (Joyeux *et al*., 1984a). It has however became evident from various studies that AAB are able to survive and even grow in the anaerobic conditions that are normally found during the winemaking process (Drysdale & Fleet, 1985).

AAB belong to the family *Acetobacteriaceae* and are Gram-negative, catalase-positive rods (De Ley *et al*., 1984; Holt *et al*., 1994), although exceptions due to Gram variability and shape are known (Ameyama, 1975; Kittelman *et al*., 1989). AAB are divided into five genera namely: *Acetobacter (A.), Gluconobacter (G.), Gluconacetobacter (Ga.), Acidomonas (Ac.)* and *Asaia (As.)*. Of these, *Acetobacter aceti, Acetobacter pasteurianus, Gluconoacetobacter hansenii*, *Gluconacetobacter liquefasciens* (formerly *A. liquefasciens*) and *Gluconobacter oxydans* have been isolated from grapes and wine so far (Drysdale & Fleet, 1988; Du Toit & Lambrechts, 2002; Trcek & Teuber, 2002). Unspoiled grapes usually contain between 10^2-10^3 cells per ml *G. oxydans*, which are the main species found on grapes (Joyeux *et al*., 1984a; Du Toit & Lambrechts, 2002). This species prefer a sugar-rich environment and usually dies off during alcoholic fermentations. *Acetobacter* and *Gluconacetobacter* on the other hand prefer ethanol as carbon source (De Ley *et al*., 1984) and usually dominate at the end of fermentation (Joyeux *et al*., 1984a; Du Toit & Lambrechts, 2002; Bartowski *et al.,* 2003). One of the factors effecting the number of AAB on grapes is rot or *Botrytis*-infected grapes. AAB counts as high as 10^6 cells per ml in juice from such grapes have been recorded. Usually the dominating genus on infected grapes are *Acetobacter*, probably due to ethanol produced by wild yeasts occurring on the damaged grapes (Joyeux *et al*., 1984a). Other factors that influence the survival of AAB during fermentation are oxygen, temperature, pH, ethanol, sulphur dioxide (SO_2) and other wine micro organisms (Oelofse, 2003). As mentioned earlier it is believed that AAB are not a problem in anearobic wine conditions, but the contrary has been proven since (Drysdale & Fleet, 1989a; Du Toit, 2000; Bartowsky *et al*., 2003). Optimum temperature for *Acetobacter* and *Gluconobacter* were established between 25 to 30^{o}C (Holt *et al*., 1994), although tempratures as high as 35^{o}C has been noted (De Ory *et al*., 1998). Different studies have observed the minimum temperature for the survival of AAB around 10 to 12^{o}C (Joyeux *et al*., 1984a; De Ory *et al*., 1998). Although the optimum pH for growth of AAB is 5.5 to 6.3 (Holt *et al*., 1994), AAB can survive at low pH levels in wine ranging from 3.0 to 4.0 as showed by Drysdale & Fleet (1985). SO_2 definitely contributes to the inhibition of AAB in wine, but does not fully and sufficiently control AAB (Drysdale & Fleet, 1985; Du Toit, 2000).

The presence of spoilage organisms such as AAB has a negative effect on yeast growth and fermentation efficiency by influencing yeast directly or indirectly (Grossman & Becker, 1984; Joyeux *et al*., 1984b). Drysdale & Fleet (1989b) have inoculated yeast and AAB together and found that yeast growth as well as fermentation of glucose and fructose were decreased in the presence of AAB. The reduction in fermentation capability of yeast in the presence of AAB can be attributed

to different causes. The most important cause is the production of acetic acid by AAB which is toxic to the yeast (Eglinton & Hencshke, 1999; Du Toit, 2000). The mechanism of action of acetic acid is discussed in section 2.3.3.2. Studies by Drysdale & Fleet (1989b) showed that *A. aceti* and *A. pasteurianus* increased the acetic acid concentration of grape juice with 10 and 50 mg/L, but that this concentration will however, not affect yeast growth. They further reported a value of 1.64 g/L for *G. oxydans*. Much higher values were obtained in fully aerated wine. Another way that AAB can inhibit yeast performance is by lowering the glucose:fructose ratio, because of their glucophilic nature, and therefore may result in problem fermentations (Joyeux *et al*., 1984b). Du Toit (2000) suggested that other investigations should be done to identify other possible inhibitory mechanisms.

2.2.3.3 Lactic acid bacteria

The occurrence of some lactic acid bacteria (LAB) in grape juice and wine are well known, specifically for their role in malolactic fermentation (MLF). LAB are devided into obligate homofermentative strains like *Pediococcus* and *Lactobacillus* (Lb.), obligate heterofermentative strains like *Leuconostoc (L.)* and *Oenococcus oeni* (formerly known as *Leuconostoc oenos*) (Dicks *et al*., 1995) and *Lactobacillus* and facultative heterofermentative strains like *Lactobacillus* (Van Vuuren & Dicks, 1993: Fugelsang, 1997).

The number of LAB that occur on grapes depends on numerous factors such as climate, region, SO_2 dosage, wine pH etc., but numbers that have been mentioned in previous studies range from 10^2 cells/ml on healthy grapes up to 10^5 to 10^6 cells/ml on spoiled grapes. Winemaking practices like lowering of the pH and sulphur dioxide additions can influence these numbers (Lafon-Lafourcade *et al*., 1983; Fugelsang, 1997).

An increase in the occurrence of stuck fermentations occurred in some Californian wineries when the amount of sulphur dioxide was reduced due to public concerns regarding the health risks associated with this additive (Huang *et al*., 1996). Huang *et al*. (1996) have isolated a wide range of LAB from commercial sluggish fermentation wines from which three were found to retard yeast growth. They have inoculated these three strains in sterile filtered Chardonnay must together with yeast and found that *S. cerevisiae* was inhibited by all three, while *S. bayanus* was only inhibited by one isolate. Two strains were later identified by Edwards *et al*. (1998a) as *Ooenococcus oeni*, while the latter was identified as *Lactobacillus kunkeei* (Edwards *et al*., 1998b). *Lb. kunkeei* is known for it's very high acetic acid productions and is commonly referred to as "ferocious lactobacilli" (Boulton *et al*., 1995). Edwards *et al*. (1999b) showed that this ferocious lactobacilli were able to produce 4 to 5 g/L acetic acid within 9 days in grape must.

2.2.3.4 Moulds

Together with the wide variety of yeast, AAB and LAB, a variety of fungi are also known to occur naturally on grapes. These species include *Aspergillus*, *Botrytis*, *Penicillium*, *Mucor* and *Alternaria*. The most common of these are probably *Botrytis cinerea* which has been suggested to produce toxic substances when present on fruit (Ribèreau-Gayon, 1979). Bisson (1999) further conducted vinifications with fruit heavily infested with mould and could not found a correlation with slow and incomplete fermentations, but further stated that the sensitivity to mycotoxins may be dependent upon the nutritional composition of the medium.

Another aspect that goes hand in hand with mould infestation is the production of certain compounds in response to infection. These phytoalexins and other enzymes are designed to eliminate the pathogen and make it thus highly likely that yeast growth and fermentation will be affected by their presence in must (Bisson, 1999).

2.2.4 INHIBITORY YEAST METABOLITES AND THEIR MODE OF ACTION

2.2.4.1 Ethanol

The final nail in the coffin of the yeast during winemaking, after having survived several other stress conditions in the must, is toxification due to the accumulation of ethanol as fermentation proceeds. Depending on the ethanol tolerance of a specific yeast starter culture, a high level of ethanol can quickly lead to a stuck or sluggish fermentation. Although *S. cerevisiae* is considered to be an ethanol-tolerant species, the integrity of the cell is affected when concentrations above the ethanol tolerance is reached. One must keep in mind that many other factors work in synergy with ethanol toxicity to create stress conditions for the yeast. Ethanol increases the toxicity of other toxic compounds such as medium chain fatty acids (MCFAs). According to Larue & Lafon-Lafourcade (1989) a MCFA concentration of 3 mg/L can inhibit yeast growth at a 10% (v/v) ethanol concentration. Pampulha & Loureira-Dias (1989) further showed that the effects of ethanol on yeast performance are combined with other factors such as acetic acid, pH and MCFAs. These factors are discussed separately in other parts of this review.

It is well known that this highly toxic product of alcoholic fermentation inhibits yeast growth and cell viability (Thomas, 1979). Casey & Ingledew (1986) found that an ethanol concentration of 3 to 4% (v/v) slowed yeast growth, while growth ceased at a concentration of 8 to 15% (v/v), depending on the yeast strain. The inhibition of the general amino acid permease and the glucose transport system are just some of the effects that ethanol exerts on the yeast (Alexandre & Charpentier, 1998; Bisson, 1999; Bauer & Pretorius, 2000). Some researchers found that an ethanol concentration of up to 8.5% (v/v) does not change the activity of key glycolitic enzymes (Pascual *et al*., 1988), while others found that ethanol inhibits sugar

transport activity (Leao & Van Uden, 1982; Mauricio & Salmon, 1992). Zamora *et al*. (1996) showed that these differences in sugar transport inactivation are because ethanol show different inhibitory patterns depending on whether high or low affinity transport systems are involved.

Some of the more direct effects of ethanol on *S. cerevisiae* include altered organisation and permeability of the plasma membrane. These effects are believed to be due to the increase in polarity of hydrophobic environments, such as the interior of the cell's plasma membrane (Alexandre & Charpentier, 1998; Bisson, 1999). Changes in the lipid composition of the yeast's plasma membrane during ethanol stress have been studied by various authors (Thomas *et al*., 1978; Mishra & Prasad, 1989; Del Castillo Agudo, 1992; Lloyd *et al*., 1993; Alexandre *et al*., 1994a, b). Thomas *et al*. (1978) showed that the enrichment of the plasma membrane with specific fatty acids that increase the unsaturation index correlated well with an increase in ethanol tolerance. According to Bauer & Pretorius (2000) recent data suggests that the ethanol resistance level is rather determined by the ratio of different lipid components in general than by individual lipids. It is further known that the yeast cell responds to ethanol by producing a membrane which is rich in ergosterol and unsatured fatty acids with longer chain lengths and an increased level of desaturation (Alexandre *et al*., 1994a; Bisson, 1999).

Another molecular effect due to the increased permeability of the plasma membrane is the dissipation of the proton motive force which allows the active transport of compounds such as amino acids through proton symport (Bauer & Pretorius, 2000). The cell pumps H^+ ions out of the cytoplasm into the substrate with the help of the stress-regulated enzyme H^+-ATPase (Ambesi *et al*., 2000; Bauer & Pretorius, 2000). In this way the cells maintain its intracellular pH and use the created ion gradient to transport substances against their concentration gradient into the cell. With the increased permeability of the membrane to H^+ ions caused by ethanol, the cytoplasm acidifies, which is particularly the case in a substrate with a low pH such as grape must (Boulton *et al*., 1995).

Another, non-specific, mode of action has been described by different authors due to reduced water activity (a_w) (Jones, 1989; Hallsworth *et al.*, 1998). This reduced a_w, which is a measure of water availability, affects all biological processes in the cell because of their water dependency. According to Hallsworth *et al*. (1998) more than 30% of growth inhibition by ethanol is due to water stress in cells that remain metabolically active at higher ethanol concentrations.

In addition to the above mentioned effects of ethanol on the yeast cell, it has been shown that the cell responds in different ways to ethanol stress. Besides the already-mentioned increase in ergosterol and unsatured fatty acids, two proteins, Hsp30p and Hsp12p, have been shown to play important roles during ethanol stress. Apparently Hsp12p protect membrane integrity (Sales *et al*., 2000), while Hsp30p regulates the plasma membrane H^+-ATPase (Braley & Piper, 1997). These so-called stress proteins are therefore also referred to as heat shock proteins. Ivorra *et al*.

(1999) performed molecular characterizations of wine yeasts in their response to stress conditions, and found that the *HSP12* gene may be useful as an indicator for yeast strains with problems in stress resistance.

2.2.4.2 Acetic acid

It is a well known fact that acetic acid is a normal end-product of fermentation by yeast and because this characteristic is strain dependant, low acetic acid production is one of the criteria for wine yeast selection. The mechanism of acetic acid formation by yeast can be summarized by the Crabtree effect: high sugar and low oxygen conditions in grape must represses oxidative metabolism and induces fermentation by yeast cells by which pyruvate is formed from glucose. The pyruvate can then be decarboxylated to acetaldehyde, which can be secreted, reduced to alcohol or oxidised to acetic acid. In fermenting cells pyruvate is reduced to ethanol and then oxidised by alcohol dehydrogenase to acetaldehyde, which is oxidised to acetic acid by an aldehyde dehydrogenase (Millán & Ortega, 1988; Guidici & Zambonelli, 1992).

One of the most important factors that influence acetic acid production in must by yeast is the yeast strain. According to Du Toit (2000), Hanneman found in 1985 among 100 *S. cerevisiae* strains 13 that produced more than 1 g/L acetic acid. Giudici & Zambonelli (1992) found that from 8 different *S. cerevisiae* strains in synthetic medium, between 52 to 710 mg/L acetic acid were produced. Delfini & Cervetti (1991) classified yeast strains into three groups according to the amount of acetic acid that they formed, namely: low (0.0 to 0.3 g/L), medium (0.31 to 0.6 g/L) and high (more than 0.6 g/L). Other factors that influence acetic acid production by yeast are the presence of certain non-*Saccharomyces* yeasts, nitrogen content, fermentation temperature and excessive clarification of the must. These factors are discussed in the relevant sections of this review.

The effect of different concentrations of acetic acid on biomass production, growth rate and fermentation ability of yeast has been investigated by different authors. A linear decrease in biomass production of *S. cerevisiae* with increasing acetic acid concentrations were observed, namely: a 20% decrease at 1 g/L acetic acid, up to 35% decrease at 2 g/L acetic acid and up to 68% at 6 g/L acetic acid (Pampulha & Loureiro-Dias, 1989; Phowchinda *et al*., 1995; Rasmussen *et al*., 1995;). Growth inhibition of 50% at an acetic acid concentration of 5.27 g/L have been reported, while a decrease of 31% and 74% in ethanol production at 2 g/L and 6 g/L respectively have been reported (Antoce *et al*., 1997).

Acetic acid displays a weak lipophilic character and may accumulate inside yeast cells depending on the relative values of internal and external pH (Pampulha & Loureiro- Dias, 1989). The only way that acetic acid can enter the yeast cell is by simple diffusion of the undissociated form. This is possible due to acetic acid's low pKa of 4.75. The pH of the growth medium determines the rate of acetic acid diffusion into the cell (Arneborg *et al*., 1995; Rasmussen *et al*., 1995; Casal *et al*.,

1996, 1998). According to Casal *et al*. (1996) the internal pH of the cell is normally near neutral and large quantities of the acid have to enter the cell to achieve this equilibrium. Casal *et al*. (1996) found that this system is repressed by high sugar concentrations, as occurs under winemaking conditions. After entering the yeast cell acetic acid releases its proton and affects the internal pH of the cell (Du Toit, 2000).

2.2.4.3 Medium chain fatty acids

It became evident from studies done by Nagodawithana & Steinkrauss (1976) and Novack *et al*. (1981) that ethanol produced during fermentation is more toxic than when the same concentration ethanol is added to yeast, which suggested that other by-products are involved. Studies done by Lafon-Lafourcade *et al*. (1984b) revealed that these inhibiting by-products are fatty acids, in particular octanoic and decanoic acids produced by yeast. Since then numerous studies have been done on these medium chain fatty acids (MCFA) and their role in stuck/sluggish fermentations.

The synthesis of MCFAs, as the synthesis of all fatty acids by yeast, happens in a series of reactions in the cell. Acetyl-CoA is formed through different mechanisms and this is then carboxylated. This reaction is catalysed by a biotin-dependent acetyl CoA carboxylase to form Malonyl-CoA. The acetyl and malonyl groups are then condensated, and this is catalysed by a "condensing enzyme" β-ketoacyl synthase. The β-ketoacyl reductase forms the β-hydroxy homologue, which can be made unsatured and satured by the β-hydroxyacyl-ACP dehydratase and the enoyl-ACP reductase respectively. This activated acyl-CoA group can be extended with two carbons by another malonyl group attaching to it. Fatty acids are formed when these groups are hydrolysed and can also be transformed to the corresponding ester by an alcohol (Ratledge & Evans, 1989). The factors that influence the production of MCFA's by yeast strains during alcoholic fermentation include many winemaking practices such as yeast strain, oxygen addition, fermentation temperature, degree of must clarification etc. (Du Toit, 2000).

The effect of MCFA's on biomass production, growth and fermentation of yeast as well as their mode of action is well characterized. It has been found that octanoic acid and decanoic acid decrease the maximum growth rate of *S. cerevisiae* (Viegas *et al*., 1989, 1998; Viegas & Sá-Correia, 1995a; Alexandre *et al*., 1996). Studies by Viegas *et al*. (1989) showed that octanoic (0 to 16 mg/L) and decanoic acids (0 to 8 mg//l) decreased the maximum specific growth rate of *S. cerevisiae* in the presence of 6% (v/v) ethanol. With 47 mg/L decanoic acid a 50% decrease in the growth activity was observed (Antoce *et al*., 1997). An exponential decrease in biomass production have been reported by several authors. At 50 mg/L octanoic acid decreased this yield from 0.08 to 0.035 dry weights per gram glucose (Viegas *et al*., 1985; Viegas & Sá-Correia, 1995b), while Alexandre *et al*. (1996) found a decrease of more than 50% in cells to which 6 mg/L decanoic acid was added. Except for the decrease in growth rate and biomass an extended lag phase occurred at octanoic acid concentrations between 58 and 70 mg/L. Of these two MCFAs it has been found

that decanoic acid is more toxic than octanoic acid. Viegas *et al*. (1989) found that decanoic acid inhibits yeast growth more than octanoic acid, while Antoce *et al*. (1997) reported a lower minimum concentration of decanoic acid than octanoic acid to inhibit growth completely.

2.2.5 AGROCHEMICAL RESIDUES

The application of chemical fungicides and pesticides in vineyards is common practice to fight a variety of pests. It is often necessary to apply some of these chemicals in the last phase of grape ripening to inhibit pests like mildew and *Botrytis*. This may lead to pesticide residues on the grapes, and therefore maximum residue limits and with-holding periods have been established by different countries (Office International de la Vigne et du Vin, 1994; Nel *et al*., 2003). These agrochemicals should not under normal circumstances influence alcoholic fermentation (Henschke, 1997), but with-holding periods are not always strictly respected and this may lead to sluggish or stuck fermentations (Cassignard, 1975; Kundu *et al*., 1981). Cabras *et al*. (1999) have tested six fungicides (azoxystrobin, cyprodinil, fludioxonil, mepanipyrim, pyrimethanil and tetraconazole) for their influence on the fermentation activity of *S. cerevisiae* and found that they actually stimulated the yeast to some degree to produce more alcohol. On the other hand Doignon & Rozes (1992) described a decrease in some sterols of *S. cerevisiae* in the presence of the triazole fungicide, flusilazole, leading to reduced cell viability. According to Henscke (1997) the active ingredient triadimenol can also inhibit fermentation at a concentration of 1 mg/L. Tromp & De Klerk (1988) performed laboratory trials in which they found that copper concentrations of between 10 mg/L and 15 mg/L slowed alcoholic fermentation. In these experiments however, they only used two different *S. cerevisiae* strains.

2.2.5.1 Copper

Copper is the active compound of various pesticides namely: $CuSO_4.5H_2O$, $CuCl_2.2H_20$ and copper oxychloride (Vidal *et al*., 2001) and it's affects on yeast have been the focuspoint of many studies (Welch *et al*., 1983; Rome & Gadd, 1987; Welch *et al*., 1989; Avery *et al*., 1996; Yu *et al*., 1996; Presta & Stillman, 1997; Azenha *et al*., 2000). Copper is an essential heavy metal to all organisms and is a constituent of enzymes like cytochrome oxydase in the mitochondria, superoxide-dismutase in the cytosol and a ferroxidase in the plasma membrane (Silva & Williams, 1993; Azenha *et al*., 2000). Micronutrients like copper have a very narrow optimum concentration range. De Rome & Gadd (1987) have reported a concentration of 1.5 µM to be optimal for yeast growth but stated that inhibition occurs above 10 µM. According to Azenha *et al*. (2000) the Cu concentration in must usually lies in the 0.010-0.10 mM range. Copper transport in *S. cerevisiae* happens through the high-affinity plasma membrane copper transporters Ctr1p and Ctr3p. The *CTR1* gene is only expressed when copper levels are below 10 µM, but at higher copper

concentrations transport continues through putative low-affinity transporters (Dancis *et al*., 1994). Presta & Stillman (1997) observed a two-stage kinetic mechanism for copper uptake by yeast cells. The first stage of approximately 6 h displays an uptake rate that is dependent on the initial copper concentration, while the second is slower and independent of the initial copper concentration. The mechanism of copper toxicity towards *S. cerevisiae* has been studied intensively. A rapid loss of cellular K^+ levels was observed which explained the permeabilization of the plasma membrane in the presence of elevated Cu^{2+} concentrations (Ohsumi *et al.*, 1988; Gadd, 1993; Avery *et al*., 1996).

2.2.5.2 Genes involved in yeast copper homeostasis

Welch *et al*. (1983) found a direct correlation between the gene copy number at the *CUP1* locus and the resistance level to external copper levels. The *CUP1* locus was discovered in 1955 and is located on chromosome VIII (Hamer *et al*., 1985). A variation in both the size and the copy number of the *CUP1* locus were found. A higher number of the *CUP1* gene led to more chelatin mRNA, which codes for a low-molecular-weight copper binding protein (Welch *et al*., 1983). These copper binding proteins are rich in cysteine and chelate heavy metal ions through thiolate complexes (Hamer *et al*., 1985). The *CUP1* protein has been called either copper chelatin, copper metallothionen (MT), or copperthionein (Hamer *et al*., 1985). Welch *et al*. (1989) further mentioned that together with *CUP1*, *ACE1* (*CUP2*) appears to be the dominating genes in yeast copper resistance. According to Welch *et al*. (1989) *ACE1* is some kind of regulatory gene for expression of the *CUP1 MT* gene product.

Yu *et al*. (1996) identified *SLF1* as a new homeostasis gene. Slf1p plays a role in copper sulfide (CuS) mineralization on the surface of cells cultured in medium containing copper salts. The disruption of *SLF1* led to limited copper sensitivity with resulting cells lacking the normal brownish coloration when grown in $CuSO_4$-containing medium (Yu *et al*., 1996).

Culotta *et al*. (1994) identified the second *S. cerevisiae* MT, containing the *CRS5* locus and demonstrated that this molecule also plays a role in copper ion homeostasis. Jensen *et al*. (1996) showed that a single copy of *CUP1* was far more effective in conferring copper resistance than *CRS5*.

2.3 EFFECTS OF WINEMAKING PRACTICES ON YEAST PERFORMANCE

2.3.1 JUICE CLARIFICATION BEFORE FERMENTATION

It is well known that white wines high in aroma and quality are obtained through the common practice of juice clarification prior to fermentation (Castino *et al*., 1980; Boulton *et al*., 1995). This process is however, not without disadvantages and delayed fermentations and increasing amounts of volatile acidity in clarified juice have been reported by different authors (Delfini & Cervetti, 1987, 1988; Moruno *et al*.,

1993). According to Delfini & Cervetti (1991) high amounts of acetic acid is produced in must that have been clarified excessively.

Moruno *et al*. (1993) have studied the effect of metabolites that are normally present in non-clarified must, but depleted in strongly clarified must, on the production of acetic acid by yeast cells. No correlation was found between the loss of important heavy metals such as Cu, Fe, Zn and Mn, and acetate production. The precipitation of some free amino acids due to clarification also showed no difference in acetate production. The deprivation of unsaturated fatty acids plays an important role in higher acetic acid production in clarified must. Under such conditions the yeast cell has to synthesize them from pyruvate, originating from glycolises, but because the conversion of pyruvate to unsaturated fatty acids is only possible in aerobic conditions, acetate is formed out of acetyl CoA. They further showed that by adding polyphenols, such as anthocyanins and cathechins to synthetic must, a significant decrease in acetic acid concentration was observed (Moruno *et al*., 1993).

In another study, Delfini & Costa (1993) employed different insoluble materials to examine their effects on the production of acetic acid, pyruvic acid and acetaldehyde productions and on the fermentation rate. They found that the grape must clarification deposit had the most consistent and significant effect, while charcoal, bentonite, diatomaceous earth powder, gelatin and silica gel exhibited weak effects on pyruvic acid and acetaldehyde production and consequently on the fermentation rate. They explained this due to the different fatty acid content of different yeast strains together with minor physical effects (Delfini & Costa, 1993).

2.3.2 FERMENTATION TEMPERATURE

Yeast growth and metabolism are severely affected by extreme temperatures. Heat released during fermentation combined with the heat exchange capability of the fermentor can result in fermentations being too hot. Contrary, supercooling of the fermentor will result in too cold fermentations (Boulton *et al*., 1995). Temperature shock is another temperature related problem that might occur during wine fermentations when the temperature of the medium is changed by 5 to 10°C in either direction. During the budding stage the yeast are especially sensitive to this (Henschke, 1997). According to Bisson (1999) one should avoid temperature swings in excess of 5°C. White wine are normally fermented at low temperatures and red wine fermented at higher temperatures and are therefore discussed separately.

2.3.2.1 White wine fermentations

The quality of white wine is directly linked to it's aromatic profile which consists of grape-based aromas and yeast volatile aromas or fermentation bouquet (Boulton *et al.*, 1995). Many winemakers ferment at low temperatures due to the fact that low temperatures, 10 to 15^{o}C, enhance the production and retention of flavour volatiles in white wine. Fermenting at such low temperatures is however, very risky because of

the possibility of stuck and sluggish fermentations. This is due to a reduction in the fluidity of the yeast cell's plasma membrane at low temperatures (Killian & Ough, 1979; Kunkee, 1984; Torija *et al*., 2002). According to Bisson (1999) too much cooling at the latter stages of fermentation can also cause fermentation arrest due to other stress conditions such as higher ethanol levels and MCFAs in the must at that stage.

Torija *et al*. (2002) analyzed the changes in yeast performance and the composition of cell membrane fatty acids during low temperature fermentations (13^{o}C). They further conducted 25°C fermentations with two *S. cerevisiae* strains and a *S. bayanus* strain. As expected, they found that yeast growth was delayed and took longer to reach maximum populations at 13^{o}C. They further found that the concentration of saturated fatty acids (SFA) in the cell membranes were significantly lower at 13^{o}C, of which stearic acid was always lower. The changes in the unsaturation degree of cell membrane fatty acids of *S. cerevisiae* modulated the membrane fluidity at these low temperatures. No change in the unsaturated fatty acid (UFA) percentage was observed for the *S. bayanus* strain at low temperatures, although the concentration of MCFAs was higher.

2.3.2.2 Red wine fermentations

According to Reid & Lourens (2002) high fermentation temperatures are one of the main causes of stuck fermentations in the South African wine industry and that fermentations above 30^{o}C should be avoided. Although modern winemaking minimizes this problem through good cooling systems, the synergistic effect of high sugar musts often place high pressure on these systems (Boulton *et al.*, 1995). Another factor that amplifies the effect of high temperatures is high ethanol levels, especially in the latter stages of fermentation (Piper, 1995). A significant amount of energy is released during fermentation by yeast cells in the form of heat which cause temperature changes and cell stress (Piper, 1997). Henschke (1997) found that small temperature changes of 2 to 3^{o}C can have a negative influence on fermentation efficiency, especially during the yeast's budding stage.

Like all the other stress conditions that occur during the winemaking process, heat-shock stress in yeast cells have been widely studied in laboratory strains. The induction of the so-called heat shock proteins (HSPs) was the first stress response to be studied in detail in *S. cerevisiae*. HSPs is a set of proteins whose synthesis is strongly increased when the yeast are exposed to sudden increases in temperature (Piper, 1997; Morano *et al*., 1998). The most HSPs are however not only induced during heat-shock but also to a number of other stress conditions like ethanol, and are therefore also referred to as stress-protection proteins (Bauer & Pretorius, 2000).

2.3.3 SULPHUR DIOXIDE

The use of sulphur dioxide (SO_2) during winemaking dates back to Egyptian and Roman times, and by now the antiseptic and antioxidant properties of this ancient preservative is well understood and are used by winemakers all over the world (Boulton *et al*., 1995; Ribéreau-Gayon *et al.*, 1998). Of the three forms of sulphur dioxide (SO_2, HSO^-, SO_3^{2-}) that exist in equilibrium in a solution, molecular SO_2, fulfils the antimicrobial action and this depends on pH, temperature and time of exposure. Although SO_2 is highly toxic to bacteria, native yeasts and moulds, commercial wine yeast strains are selected to have a higher tolerance to SO_2. It is well-known that minimal sulphiting (20 to 30 mg/L) before fermentation initially slows fermentation, but eventually fermentation is completed more rapidly. This phenomenon can be attributed to the killing or inhibition of native yeast strains, bacteria and moulds, which enhances the growth conditions for the yeast starter culture (Boulton *et al*., 1995; Ribéreau-Gayon *et al*., 1998). The SO_2 concentration added before fermentation should therefore be tightly controlled not only to inhibit the growth of undesired species, but also to assure sufficient growth of the starter culture (Alexandre & Charpentier, 1998). According to Ribéreau-Gayon *et al*. (1998) Suzzi and Romano found in 1982 that yeasts isolated after fermentation from non-sulphited musts are more sensitive to SO_2 than yeasts from sulphated musts, suggesting that sulphiting before fermentation increases the yeast's resistance to SO_2. Nevertheless, high levels of SO_2 in must could play a part in stuck/sluggish fermentations.

The uptake of SO_2 by *S. cerevisiae* occur by simple diffusion and therefore the rate of SO_2 transport should play an important role in sulphite toxicity (Stratford & Rose, 1986). *S. cerevisiae* accumulates SO_2 very rapidly due to the higher pH inside the cell than in the suspension. Inside the cell SO_2 causes a rapid decrease of intracellular ATP levels which results in cell death. It is also known that SO_2 reacts with NAD^+/NADP, affect enzyme systems as well as cleave thiamine and disulphide bridges of proteins (Alexandre & Charpentier, 1998).

2.3.4 IMPORTANCE OF THE TIMING OF NUTRIENTS AND OXYGEN FOR OPTIMUM FERMENTATIONS

The importance of nitrogen for protein synthesis and glucose uptake by yeast cells (Jiranek *et al*., 1992), as well as the importance of free oxygen are well understood (Ribéreau-Gayon *et al*., 2000; Bauer & Pretorius, 2000). The timing and the amount of nitrogen and oxygen added by the winemaker during fermentation can be even more important to assure problem-free fermentations. Bely *et al*. (1990) showed that the halfway point of fermentation is the best time for nitrogen addition. They showed that nitrogen addition during the second half of the fermentation leads to a longer fermentation in comparison with an early addition, regardless of the temperature and initial nitrogen concentration. An increase in fermentation has been observed within the hour following the addition, regardless of the time of addition. They further stated

that an early addition has the drawback of increasing the maximum instantaneous energy demand of the system. Maximum cell populations have been observed when additions were made during the cell growth period (Bely *et al*., 1990). According to Lourens & Reid (2002) early nitrogen additions in the form of ammonium ions will partly inhibit amino acid uptake, as it is the preferred nitrogen source for yeast cells. They further warn against excessive use of nitrogen at the start of fermentation as this will stimulate yeast growth to such an extent that the overall demand for nitrogen will increase in the later stages of fermentation.

The effectiveness of combined additions of ammonium nitrogen and oxygen have been studied recently. Sablayrolles *et al*. (1996) tested nine combinations by adding 5 mg/L oxygen and 300 mg/L DAP to ten different musts at different stages of the winemaking process namely, before inoculation, at the end of the cell growth phase and at the halfway point of fermentation. They found that for each nitrogen addition stage, oxygen addition at the end of the cell growth phase always led to complete fermentations. During the cell growth phase a large part of oxygen is used for respiration, despite the high sugar concentration, and afterwards for further unsaturated lipid biosynthesis. Nitrogen addition was more effective when it was added at the same time or after oxygen additions. They concluded that the best time for oxygen addition is at the end of the cell growth phase, while nitrogen is most effective when added at the halfway point of fermentation (Sablayrolles *et al*., 1996). Blateyron & Sablayrolles (2001) used one hundred and seventy eight difficult-to-ferment musts, selected by enologists from different regions in France, to perform fermentation trials. They effectively fermented all sluggish and stuck fermentations by adding 7 mg/L oxygen and 300 mg/L DAP at the halfway point of the fermentation process.

2.4 FUTURE PROSPECTS

The occurrence of stuck and sluggish fermentations may be sporadic but are very serious problems world-wide, especially in countries like South Africa that has a warm climate, which give rise to high sugar and alcohol concentrations in grapes and wine respectively. The difficulty to precisely determine the cause of fermentation arrest, especially since factors can interact synergistically in a complex fashion, makes it even more challenging for the winemaker. That is why the winemaker needs to use every tool possible to minimize the chances of problem fermentations. Several studies have been done on the differences between commercial yeast strain starter cultures, but more needs to be done so that the use of a specific commercial yeast strain can be optimized under specific conditions. Some of these parameters include: (1) the nitrogen demand of different yeast strains for successful alcoholic fermentation in high sugar musts as found under South African conditions, and (2) the toxicity levels of fungicides (especially copper) against different yeast strains. The latter will be useful in seasons such as experienced during the 2001 and 2002

seasons in South Africa, when very humid conditions led to high fungal infections and thus needed drastic spraying of fungicides.

All of this information will further help the winemaker to minimize possible interference of normal fermentations and thereby enhance the quality of the wine.

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CHAPTER 3

Research results

The effect of commercial yeast strains on acetic acid bacteria and lactic acid bacteria numbers and malolactic fermentation rate during red wine fermentation

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3. RESEARCH RESULTS

The effect of commercial yeast strains on acetic acid bacteria and lactic acid bacteria numbers and malolactic fermentation rate during red wine fermentation

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Acetic acid bacteria (AAB) numbers and lactic acid bacteria (LAB) numbers were followed over two seasons during alcoholic fermentation in different musts inoculated with different commercial wine yeast strains as well as a non-*Saccharomyces* yeast, *Torulaspora delbrueckii*, and a spontaneous fermentation. Cell numbers were determined before inoculation, in the middle and at the end of alcoholic fermentation. AAB numbers decreased in all the trials during fermentation, except for wine fermented with VIN2000, which contained significantly more AAB at the end of alcoholic fermentation, possibly due to this strain's slower fermentation capability. LAB numbers in the 2002 Cabernet Sauvignon must increased from 8×10^3 cfu/mL before inoculation to 8×10^4 cfu/mL after fermentation in all the strains tested, except for strain NT112 which contained less than 1×10^3 cfu/mL. LAB numbers decreased during alcoholic fermentation for all the other trials, especially for NT112 which showed significantly lower LAB numbers at the end of alcoholic fermentation due to this strain's high sulphur dioxide (SO_2) production. This was further illustrated by the fact that malolactic fermentation (MLF) were not completed in wines fermented with NT112 whereas MLF was completed in all the other wines.

3.1 INTRODUCTION

Since the introduction of all-purpose yeast starter cultures in 1890 by Müller-Thurgau from Geisenheim, several yeast starter cultures are available to the winemaker today. The primary selection criteria for these strains, mainly *Saccharomyces cerevisiae* strains, is to achieve an efficient conversion of glucose and fructose into alcohol and carbon dioxide. Industrial *S. cerevisiae* are highly specialized and capable of concurring severe stress conditions associated with wine fermentations, including high sugar and ethanol levels. However, in spite of their high tolerance to many stress conditions, not all wine fermentations are successful due to the complex nature of grape must (Bisson, 1999; Pretorius, 2000).

One of the most important objectives when making a dry wine is to achieve a residual sugar concentration of less than 5 g/L after alcoholic fermentation. This

enable winemakers to perform certain finishing operations to secure the wine from destructive oxidation reactions (Alexandre & Charpentier, 1998). Complete alcoholic fermentation will further help to prevent spoilage by numerous potential spoilage organisms such as certain yeasts, AAB and LAB (Du Toit & Pretorius, 2000). Sluggish fermentations are generally considered as alcoholic fermentations that last longer than 21 days to reach 5 g/L residual sugar, whereas stuck fermentation refers to an abrupt arrest in alcoholic fermentation (Henschke 1997; Bisson, 1999; Bisson & Butzke, 2000). Although sluggish and stuck wine fermentations are sporadic they can cause serious problems with major economic setbacks for any winery. Numerous causes for these kind of fermentation problems are known and include nutrient limitations and the presence of toxic substances (Bisson, 1999). What makes it even more difficult is that the winemaking process is a very complex ecological niche with interactions between yeasts, bacteria and fungi (Du Toit & Pretorius, 2000).

The presence of spoilage organisms such as (AAB) and some (LAB) has a negative effect on yeast growth and fermentation efficiency directly or indirectly (Grossman & Becker, 1984; Joyeux *et al*., 1984). The most important cause for the reduction in fermentation ability by AAB is the production of acetic acid, which is toxic to the yeast cell (Eglinton & Henschke, 1999; Du Toit, 2000). Although it was first believed that AAB are only associated with aerobic environments, it became evident from various studies that AAB are able to survive and even grow during the relative anaerobic winemaking process (Drysdale & Fleet, 1989; Du Toit & Lambrechts, 2002).

MLF, the decarboxylation of L-malic acid (MA) to L-lactic acid (LA), takes place after alcoholic fermentation and is carried out by lactic acid bacteria (LAB) that belong to the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus* (Van Vuuren & Dicks, 1993). MLF is however, mainly driven by *Oenococus oeni*, which has a high capacity to degrade MA under unfavorable conditions such as low pH, high ethanol concentrations and the presence of other compounds such as SO_2 and certain fatty acids (Carreté *et al*., 2002). SO_2 considerably reduces LAB viability, which may be due to the inhibition of ATPase activity of *O. oeni*. Some LAB such as *Lactobacillus kunkeei* are also known for their ability to produce large amounts of acetic acid, which can lead to yeast inhibition. The production of acetic acid however, is not the only reason for bacterial inhibition of yeast and additional inhibitory mechanisms are probable (Edwards *et al*., 1999).

It is also known that the malolactic fermentability of wines produced from the same must differs according to the yeast strain used in alcoholic fermentation (Lafon-Lafourcade, 1977; Lafon-Lafourcade *et al*., 1983). This can be due to the production of various substances, which differ in concentration between different yeast strains. These inhibitory substances include ethanol, sulphur dioxide (SO_2) and medium chain fatty acids (MCFAs) that play a role in the inhibition of the malolactic activity and growth of *O. oeni* in wine (Lonvaud-Funel *et al*., 1987; Alexandre *et al*., 2004).

The objective of this study was to investigate the effect of different commercial wine yeast strains on the occurrence of AAB and LAB during red wine fermentations. The SO_2 production of the different strains was also compared, as well as the rate of MLF after inoculation with a malolactic starter culture of wines fermented with different yeast strains. The amount of MCFAs produced during fermentation was also determined for the different yeast strains.

3.2 MATERIALS AND METHODS

3.2.1 Alcoholic fermentation conditions

Different commercial wine yeast strains were used to inoculate small-scale red wine fermentations over two seasons (2002, 2003) to monitor the growth of AAB and LAB during these fermentations. All the commercial yeast strains used were *S. cerevisiae*, except for N96 which is *Saccharomyces bayanus*. Rehydration of the yeast strains was carried out according to the yeast supplier's specifications. Before inoculation, the grapes were crushed and destemmed, SO_2 was added depending on the quality of the grapes (**Table 3.1**), and thereafter the pulp was allocated to plastic buckets containing 10 kg pulp each.

The 2002 Pinotage must (**Table 3.1**) was inoculated with 15 different yeast strains (VIN13, VIN2000, WE14, NT112, NT50, N96, RG12, RJ11, VR5, Collection Cépage Cabernet, Collection Cépage Merlot, D80, L2056, L2226 and D254) and *T*. *delbrueckii* in single copy experiments. During the 2002 season eight commercial strains (VIN13, VIN2000, NT112, NT50, N96, RJ11, D80, L2226) as well as a wild yeast strain, *Torulaspora delbrueckii*, were used to inoculate Cabernet Sauvignon must (**Table 3.2**) in duplicate experiments. The 2002 Pinotage experiments served as an initial screening and were therefore not performed in triplicate. Alcoholic fermentation were conducted at 25°C with the skin caps submerged three times daily to ensure sufficient skin contact. Spontaneous fermentations were also conducted for each set of experiments. At the end of alcoholic fermentation the skins were separated from the wine and pressed for each fermentation. The press wine and free running fractions were mixed and allocated into 4,5 L glass bottles sealed with fermentation caps. The pH values and ethanol concentrations are given in **Table 3.3**.

During the 2003 season only six yeast strains (VIN13, VIN2000, NT112, RJ11, L2226 and N96) were selected from the strains used in the 2002 experiments. The same procedures were followed to inoculate Pinotage and Cabernet Sauvignon must. In this case all fermentations were conducted in triplicate.

3.2.2 Sampling stages

For the enumeration of AAB and LAB samples were taken from different stages of the different juices. Samples were taken before yeast inoculation, at the middle of alcoholic fermentation (11-13°B) and at the end of alcoholic fermentation (<0.5°B).

Samples, at the middle and end of fermentation, were taken from the must underneath the skincap. Samples for VA and sugar analysis were taken directly after pressing (see section 3.2.4).

3.2.3 Malolactic fermentation experiments

The same Pinotage wines that were made in the previous experiments were used for the MLF experiments, but only 13 yeast strains (VIN13, VIN2000, WE14, WE372, NT112, NT50, N96, RG12, RJ11, VR5, D80, L2226 and D254) were selected from the original 16 that were used in the AAB and LAB experiments. The 2002 Pinotage wines were made in single copy experiments and was therefore allocated the wine from each 4.5 L bottle into 2 × 2 L glass bottles before inoculation with an *O. oeni* starter culture (Enoferm Alpha, Lallemand) according to the supplier's specifications. The 2003 Pinotage wines were made in triplicate and were thus used as is, before inoculations with the MLF starter culture. We decided on inoculating with bacterial starter cultures due to the low LAB numbers obtained from MRS (De Mann Rogosa Sharp) plate counts. The MLF rate of wines fermented with different commercial wine yeast strains were followed by analyzing L-malic acid L-lactic acid just after press and 30 days after press for the 2002 Pinotage. MA and LA were analyzed just after press and 21 days after press for the 2003 Pinotage (see section 3.2.5 for routine wine analysis).

3.2.4 Enumeration of acetic acid bacteria and lactic acid bacteria

AAB numbers were obtained by plating juice or must onto GYC agar [glucose (5% m/v), yeast extract (1% m/v), $CaCO_3$ (3% m/v) and agar (1.5% m/v)] and Carr agar [ethanol (2% m/v), yeast extract (1% m/v), peptones (0.5% m/v) and agar (1.5% m/v)] and adjusted to pH 5 with concentrated HCl. Yeast and LAB growth were respectively prevented by adding 50 mg/L Actistab (Actistab, Gist-Brocades) dissolved in 1 mL (96% v/v) ethanol and 50 mg/L nisin dissolved in 1 mL methanol to the media. Each dilution was plated out in triplicate, after which it was incubated at 30°C for 5 days, before colony counts were performed.

LAB numbers were obtained by plating juice or must onto MRS agar (5% MRS Agar). Yeast and AAB growth were prevented by adding 50 mg/L pimaricin dissolved in 1 mL (96% v/v) ethanol (Actistab, Gist-Brocades) and 25 mg/L Kanamycin dissolved in 1 mL sterile H_2O, respectively (all nutrient broths and agars used were obtained from Biolab, Merck, South Africa). Plates were incubated anaerobically (xoid, anaerobic system BR 038B) at 30°C for 6 days, after which colony counts were performed.

TABLE 3.1

Composition of the musts used to monitor AAB and LAB numbers during small scale red wine fermentations.

Year and Area	Cultivar	Sugar conc. (°B)	TA[(a)] (g/L TTA)[(b)]	pH	SO_2 dosage (mg/L)
2002 Paarl	Pinotage	27.2	4.60	3.60	30
2002 Franschoek	Cabernet Sauvignon	25.0	6.04	3.87	20
2003 Paarl	Pinotage	23.8	6.54	3.38	20
2003 Paarl	Cabernet Sauvignon	25.0	6.30	3.67	30

[(a)] Titratable acidity

[(b)] Tartaric acid

TABLE 3.2

Commercial yeast strains used in this study.

Species	Strain	Company
Saccharomyces cerevisiae	VIN13, VIN2000, NT50, NT112, WE14 Collection Cépage Cabernet (CC) Collection Cépage Merlot (CM)	Anchor Bio-Technologies
Saccharomyces cerevisiae	D80, L2056 L2226, D254	Lallemand Inc.
Saccharomyces cerevisiae	RJ11, RG12, VR5	Columbit
Saccharomyces bayanus	N96	Anchor Bio-Technologies

3.2.5 Routine wine analysis

The GrapeScan FT 120 instrument (Foss Electric, Denmark) was used to perform routine analysis such as pH, volatile acidity (VA), total acidity (TA), L-malic acid, L-lactic acid, fructose, glucose and ethanol. The instrument utilizes Fourier transform infrared spectroscopy (FTIR). All samples were degassed by filtration before the analysis, using the Filtration Unit (type 70500, Foss Electric, Denmark) with filter paper circles graded at 20-25 μm and with a diameter of 185 mm (Scheicher & Schuell, reference number 10312714). All samples were scanned after press, whereas wines that have been subjected to MLF monitoring were also scanned 21 or 30 days after press. SO_2 analysis were done with the Metrohm titration unit (Metrohm Ltd., Switzerland).

3.2.6 Gas chromatography

MCFA concentrations were tested and analyzed in the 2003 Pinotage and Cabernet Sauvignon after alcoholic fermentation. The method of GC sample preparation was done with a modified version of the method described by Lilly *et* al. (2000). Wine (10 mL) was mixed with 800 μL internal standard (230.2 mg/L 4-methyl-2-penthanol, 12.5% (v/v) ethanol) and volatile compounds extracted with 6.5 mL diethyl ether on a rotary Mixer for 30 min. The organic phase was recovered and 2 mL transferred to a sample vial. Analysis were done on an Agilent 6890 series gas chromatograph, equipped with a ALS 7683 liquid sampler, split-splitless injector and FID (flame ionization detector). The GC was fitted with a Lab Alliance^{TM} RH-WAX, 60 m × 0.32 mm ID × 0.5 μm film thickness, and capillary column. Hydrogen was used as a carrier gas at a flow rate of 3 mL/min, average velocity of 45 cm/sec. 3 μL sample was injected at a split ratio of 15:1, a head pressure of 79 kPa and inlet temperature of 200°C. The detector was kept at 250°C. The column was held at 35°C for 15 min, raised to 230°C at 7°C/min and held at the final temperature for 5 min. Peak identification was done by comparison with authentic standard retention times. Integration and quantification of peaks were done by the Chemstation Rev A.07.01 software using the internal standard calibration method.

3.3 RESULTS AND DISCUSSION

3.3.1 Acetic acid bacteria numbers

Various studies in the past showed that AAB are able to survive and even grow in the relative anaerobic conditions that are normally found in the winemaking process (Drysdale & Fleet, 1985; Du Toit & Lambrechts, 2002). AAB can cause stuck and sluggish fermentations by influencing yeast directly or indirectly. One of these are the production of acetic acid which is toxic to the yeast (Drysdale & Fleet, 1989; Du Toit, 2000). The effect of different commercial wine yeast strains on AAB numbers during

alcoholic fermentation is however, unknown according to our knowledge. It is known that the occurrence of different AAB species differ from one must to the other due to factors such as ethanol levels, pH and acidity, oxygen, sulphur dioxide and temperature (Du Toit & Pretorius, 2002; Oelofse, 2003). In this study however, we only investigated the effect of different yeast strains on the total AAB numbers occurring throughout fermentation, which has not been investigated before in detail. The use of grape musts however, will force certain parameters such as pH, and sugar levels to vary between cultivar and year.

During the 2002 season the growth of AAB numbers were monitored in Pinotage and Cabernet Sauvignon must fermented with different commercial yeast strains. No colony forming units (cfu) were enumerated from the 2002 Pinotage at any stage of fermentation, which indicated that less than10^2 cells/mL were present. For the Cabernet Sauvignon however, both the Carr-media **(Fig. 3.1)** and the GYC media **(Fig. 3.2)** revealed the same trends with regards to the AAB numbers during fermentation for all the yeasts strains used as well as for the wild yeast strain *Torulaspora delbrueckii*, and the spontaneous fermentation. For all the yeast strains used the AAB numbers decreased from 10^5 cfu/mL before fermentation to 10^2 to 10^3 cfu/mL at the end of alcoholic fermentation. During the middle of fermentation, VIN2000 showed a slightly higher AAB count than at the end of alcoholic fermentation. A possible explanation for this is that this strain was the slowest fermenter of all the strains tested in this study. It might thus also be due to a possible substance, produced by VIN2000, which is beneficial for AAB growth. In the middle of fermentation of the 2002 Cabernet Sauvignon with D80, AAB numbers were higher than before fermentation (**Fig. 3.2**).

During the 2003 season growth of AAB were monitored in Pinotage and Cabernet Sauvignon must. For the Pinotage AAB numbers increased from less than 10^4 cfu/mL before alcoholic fermentation to 10^5 to 10^6 cfu/mL towards the middle of fermentation and decreased again to the end of fermentation, probably due to increasing ethanol levels. Only strains VIN2000 and N96 showed slightly higher numbers of AAB at the end of alcoholic fermentation than before fermentation (**Fig. 3.3**). In the case of Cabernet Sauvignon the numbers of AAB decreased from more than 10^4 cfu/mL before fermentation to 10^3 to 10^4 cfu/mL during the middle of fermentation. No colonies could be counted at the end of alcoholic fermentation, which indicated that less than 10^2 cells/mL AAB were present in the wine.

The AAB numbers differed significantly between different yeast strains in all the experiments during the middle of alcoholic fermentation. The reason for this might be that ethanol levels might have differed to a greater extent during the middle of alcoholic fermentation. Sampling at the same ethanol levels during the middle of fermentation is difficult due to the different fermentation rates between different strains. All AAB numbers obtained were the same at the end of alcoholic fermentation, except for VIN2000, which had a higher AAB number. It thus seems that the selected yeast strains does not influence AAB numbers dramatically.

However, a slow fermenter like VIN2000 seems to support the survival of AAB during fermentation better. More research is however, required in this regard.

TABLE 3.3

Ethanol concentrations and pH values in different musts after alcoholic fermentation inoculated with different commercial yeast strains, a *T. delbrueckii* yeast strain and spontaneous fermentation

Yeast	2002		2003			
	Cabernet Sauvignon		Pinotage		Cabernet Sauvignon	
	pH	Ethanol % (v/v)	pH	Ethanol % (v/v)	pH	Ethanol % (v/v)
VIN13	3.99	14.32	4.11	12.76	3.65	14.78
VIN2000	4.04	14.35	4.08	12.66	3.69	14.95
NT112	4.04	14.35	4.09	12.51	3.70	14.93
NT50	4.01	14.32	*	*	*	*
N96	3.98	14.63	3.96	12.67	3.72	15.02
RJ11	4.10	14.58	4.07	12.50	3.66	14.91
D80	4.01	14.34	*	*	*	*
L2226	4.05	14.33	3.89	12.57	3.65	14.74
T. delbrueckii	4.15	14.41	*	*	*	*
Spontaneous	4.04	14.60	4.08	12.64	3.62	14.20

The values for the 2002 Pinotage are not given and ranged between 14.5 to 14.7 % (v/v) ethanol.

* These strains were not used in the 2003 season.

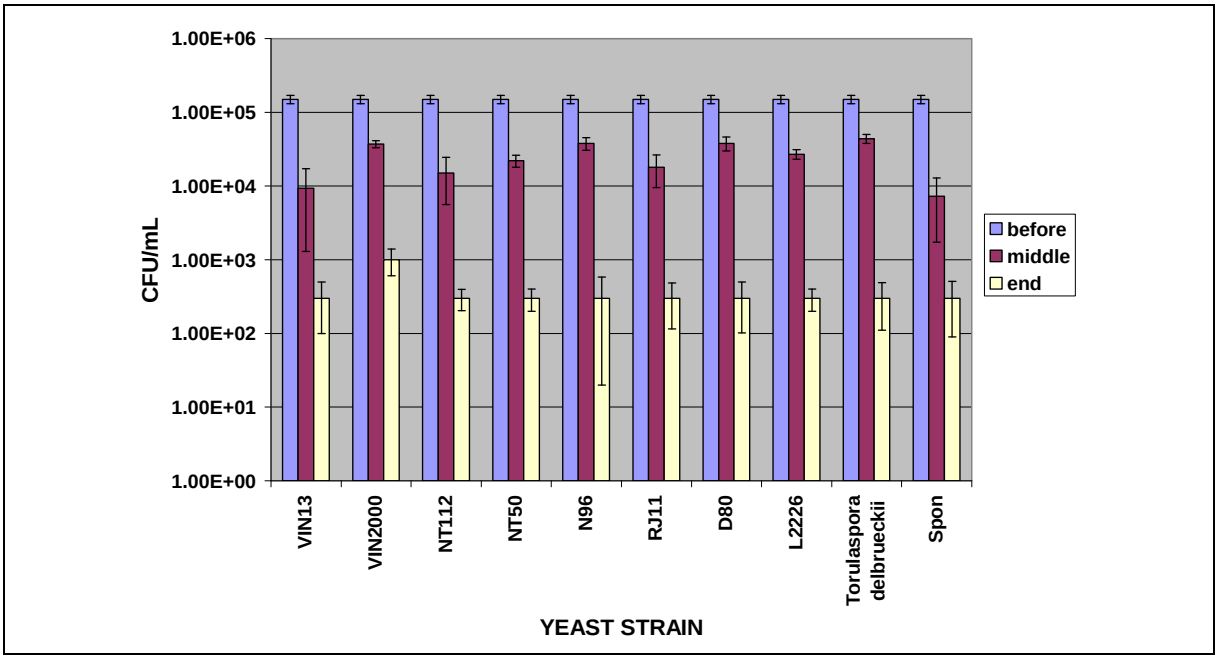

FIGURE 3.1. AAB numbers before, during and at the end of alcoholic fermentation of the 2002 Cabernet Sauvignon determined on Carr media. Error bars indicating the standard deviations for experiments done in triplicate.

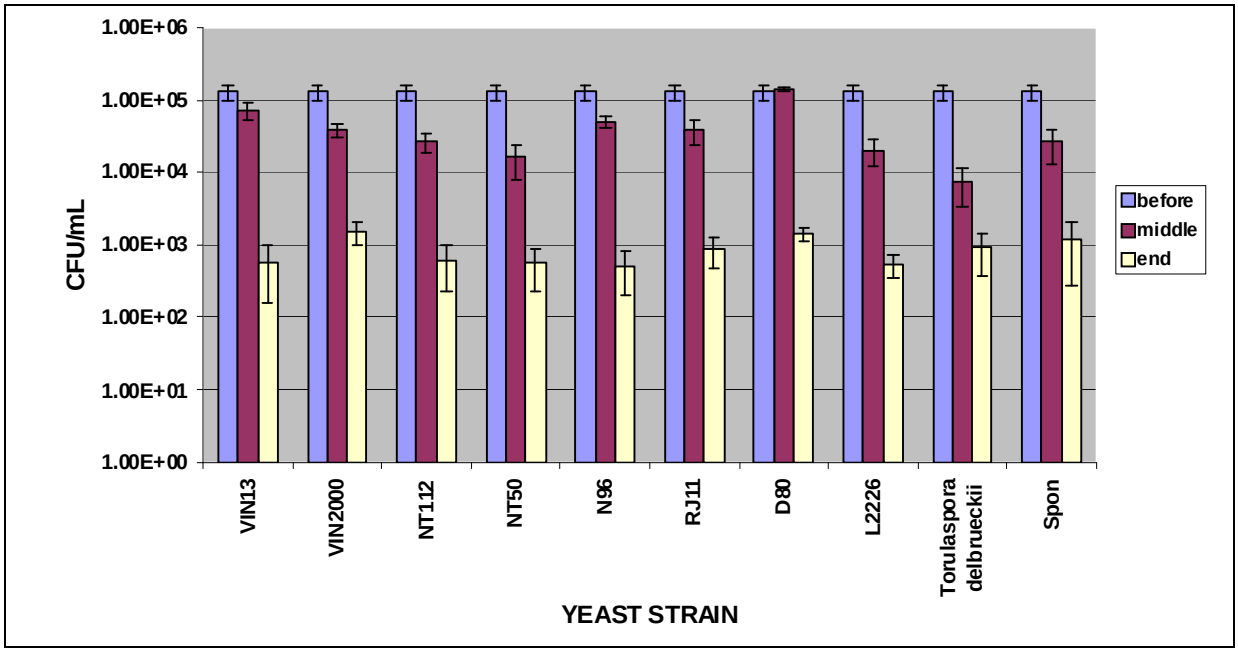

FIGURE 3.2. AAB numbers before, during and at the end of alcoholic fermentation of the 2002 Cabernet Sauvignon isolated with GYC media. Error bars indicating the standard deviations for experiments done in triplicate.

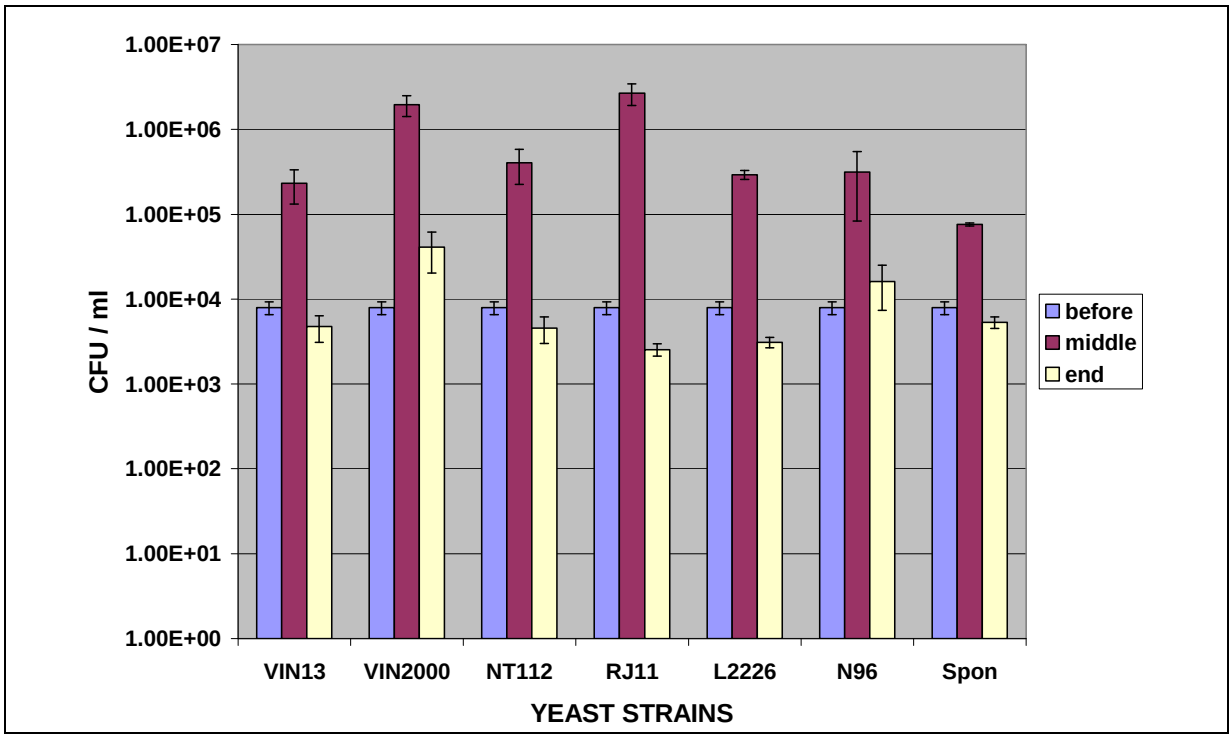

FIGURE 3.3. AAB numbers before, during and at the end of alcoholic fermentation of the 2003 Pinotage isolated with Carr media. Error bars indicating the standard deviations for experiments done in triplicate.

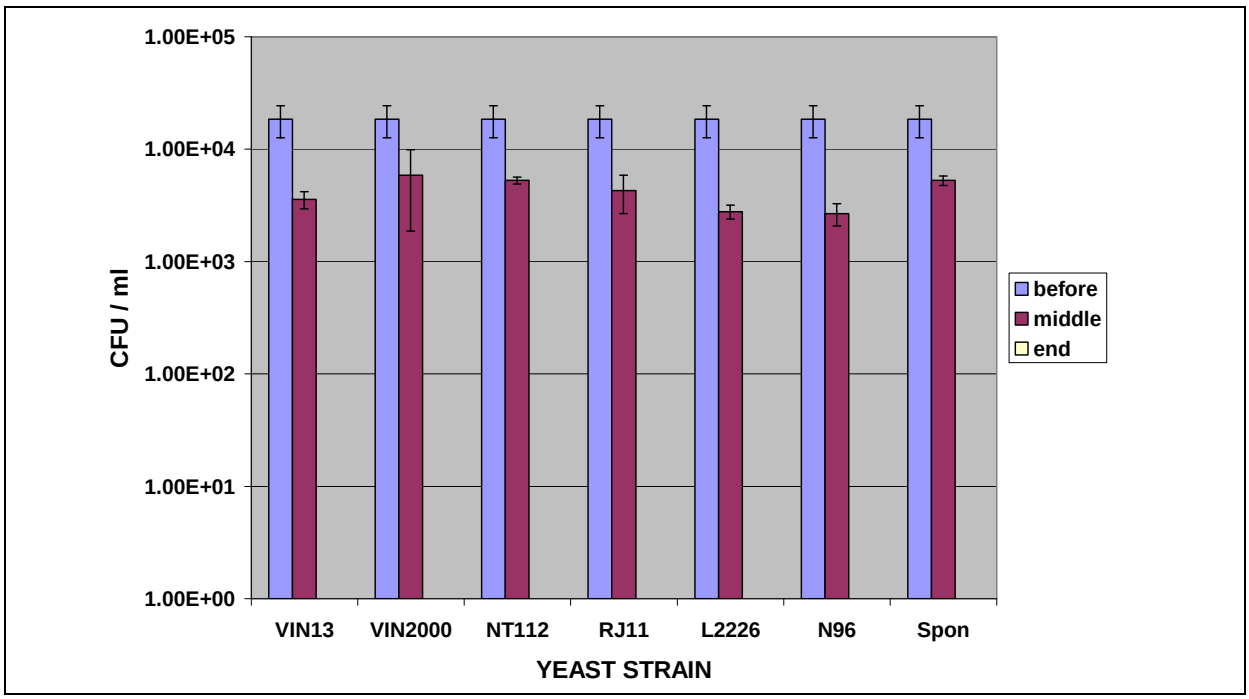

FIGURE 3.4. AAB numbers before, during and at the end of alcoholic fermentation of the 2003 Cabernet Sauvignon isolated with Carr media. No colonies were enumerated at the end of alcoholic fermentation. Error bars indicating the standard deviations for experiments done in triplicate.

3.3.2 Lactic acid bacteria numbers

During alcoholic fermentation of the 2002 Cabernet Sauvignon the LAB numbers increased for all the yeast strains tested and the spontaneous fermentation, except for strain NT112 (**Fig. 3.5**). LAB counts of 7×10^3 cfu/mL before fermentation, and between 5×10^4 and 10^5 cfu/mL at the end of alcoholic fermentation were obtained. However, in the case of fermentation with NT112 LAB counts at the end of alcoholic fermentation were less than 10^3 cfu/mL. We also found no significant differences in the pH values and ethanol concentrations between wine from strain NT112 and the other wines (**Table 3.3**). The significant decrease of LAB numbers in wine fermented with strain NT112 is thus not pH or ethanol related. Other known inhibitors of LAB, besides high ethanol concentrations and low pH values, include SO_2 and MCFAs (Carreté *et al*., 2002). The very high production of SO_2 (see section 3.3.3) by NT112 is thus a good possible explanation for the decrease in LAB. The inhibitory effect of other unknown substances produced by NT112 during alcoholic fermentation is however, also a possibility.

In contrast with these results the LAB cell counts decreased towards the end of alcoholic fermentation for almost all the yeast strains used during the 2003 trials for Pinotage and Cabernet Sauvignon. Although the LAB numbers were very low in the case of the Cabernet Sauvignon, VIN2000 showed an increase in LAB numbers (**Fig. 3.6**). No significant differences in the pH values and ethanol concentrations were

detected amongst the 2003 Cabernet Sauvignon and Pinotage between wines respectively (**Table 3.3**). It is thus clear that pH and ethanol didn't play any role in the higher LAB numbers observed in the same must when fermented with VIN2000. The lower pH values of the 2003 in comparison with the 2002 Cabernet Sauvignon might be responsible for the poor growth of LAB in the 2003 wines. This is however, not due to the fact that more of the molecular form of SO_2 is present at a lower pH, because during alcoholic fermentation all SO_2 is bounded (Ribéreau-Gayon *et al*., 2000).

The increase in LAB numbers towards the end of alcoholic fermentation with VIN2000 can be attributed to this strain's very slow fermentation rate. Slower alcoholic fermentation might thus give other wine microorganisms a better chance of multiplying, due to less competition and slower production of ethanol. VIN2000 showed the same tendency during the 2002 Pinotage trials when the highest number of LAB numbers were present at the end of alcoholic fermentation. LAB numbers at the end of alcoholic fermentation were significantly lower for NT112, which agree with the results obtained from the previous year (**Fig. 3.5**). The LAB numbers dropped from 5×10^3 cfu/mL before fermentation, to less than 10^2 cfu/mL after fermentation when fermented with NT112. One possible explanation for this can be, as in the case of the 2002 wines, due to the high SO_2 production by NT112 (see section 3.3.3).

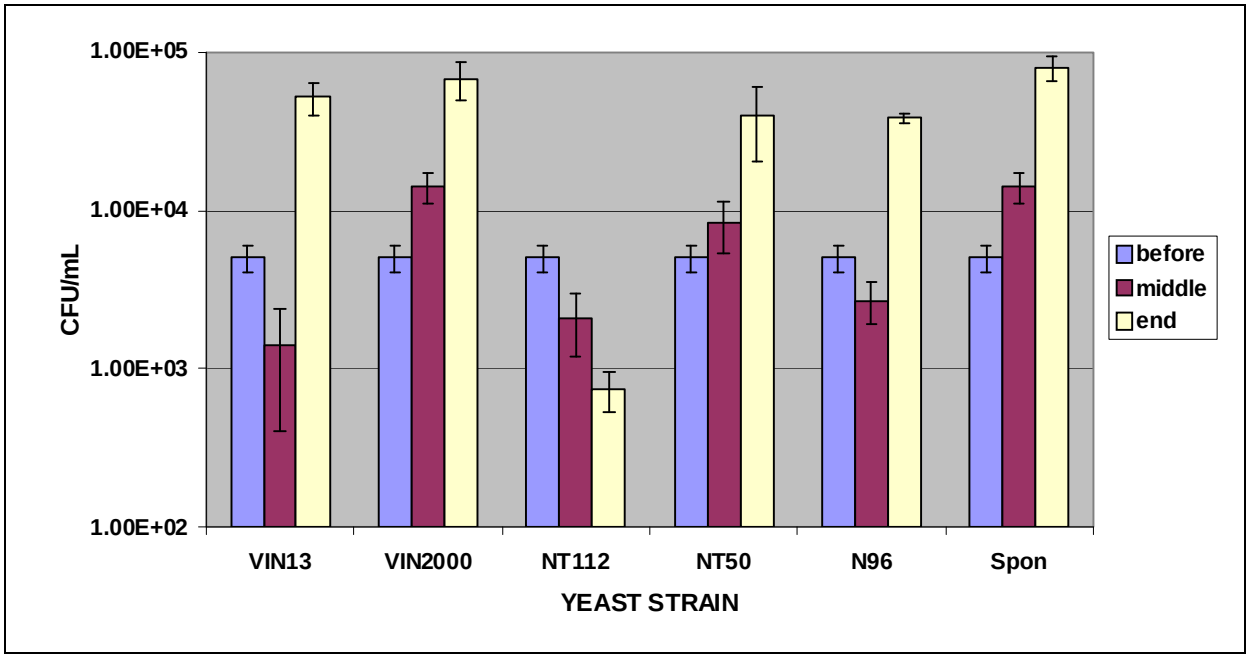

FIGURE 3.5. LAB numbers before, during and at the end of alcoholic fermentation of the 2002 Cabernet Sauvignon isolated with MRS media. Error bars indicating the standard deviations for experiments done in triplicate.

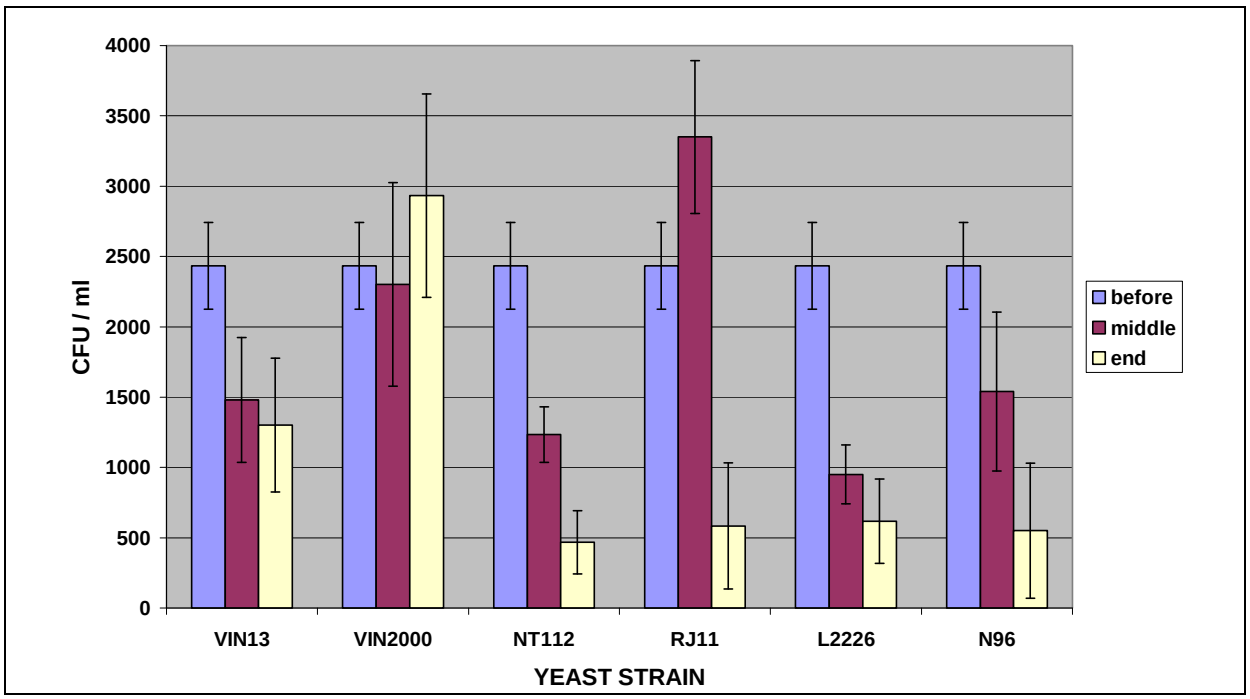

FIGURE 3.6. LAB numbers before, during and at the end of alcoholic fermentation of the 2003 Cabernet Sauvignon isolated with MRS media. No data were available for the spontaneous fermentation. Error bars indicating the standard deviations for experiments done in triplicate.

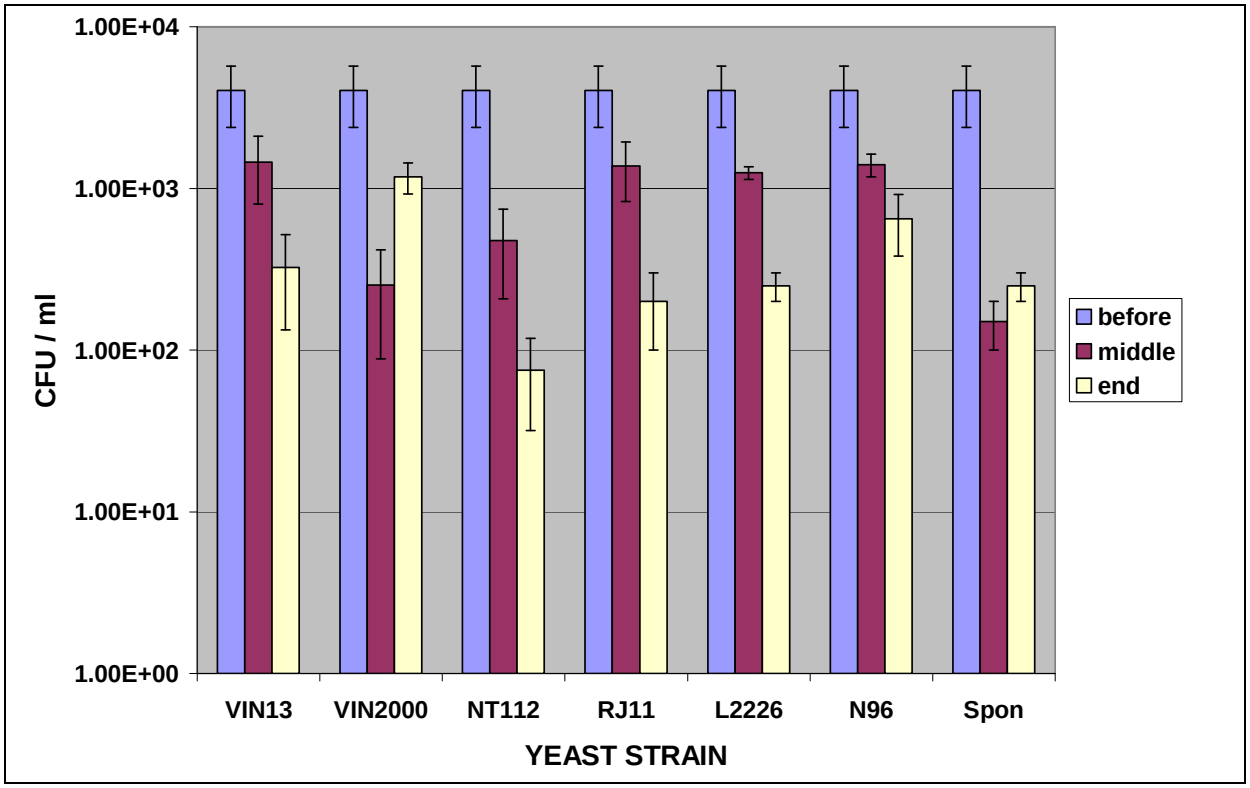

FIGURE 3.7. LAB numbers before, during and at the end of alcoholic fermentation of the 2003 Pinotage isolated with MRS media. Error bars indicating the standard deviations for experiments done in triplicate.

3.3.2 Progression of malolactic fermentation

During MLF, LAB such as *O. oeni* convert MA into LA and CO_2. Some wild yeast species such as *Schizosaccharomyces pombe* and *Saccharomyces paradoxes* can however, also convert MA, but into pyruvate by means of an intracellular malic enzyme (Tortia *et al*., 1993). Biological deacidification can thus be carried out through MLF or maloethanolic fermentation (MEF).

From the presence of LA just after alcoholic fermentation in the 2002 Pinotage (**Fig. 3.8**) it is thus clear that MLF have commenced to some degree during alcoholic fermentation with the different commercial yeast strains. After 30 days however, MLF were only completed in wines fermented with VIN13, VIN2000, WE14, NT50 and RJ11. MLF were almost completed in must samples fermented with WE372 and D254 after 30 days, while MLF were not 50% completed in samples fermented with NT112, N96, RG12, VR5, D80, L2226 and the spontaneous fermentation. This ability of certain wine yeast strains to inhibit MLF has been eported (Fornachon, 1968; Avedovech *et al*., 1992; Henick-Kling & Park, 1994; Gilis *et al*., 1996). Several factors, or their combination, such as the production of bioactive yeast metabolites and competition for nutrients could be involved (Alexandre *et al*., 2004). As far as this study concerns we looked at SO_2 and medium chain fatty acid (MCFA) production between the different yeasts strains (see sections 3.3.3 & 3.3.4).

During the 2003 season 6 yeast strains were used to ferment Pinotage, from which only NT112 did not finish MLF after 21 days (**Fig. 3.9**). The fact that strains (L2226 & N96) which did not finish MLF after 30 days during the 2002 trials, but finished MLF within 21 days during the 2003 trials, might be partly explained due to wine composition differences. The 2002 Pinotage was harvested at 27.2°B and gave ethanol concentrations that ranged from 14.32% to 14.63% alc.(v/v). n comparison the 2003 Pinotage was harvested at 23.8°B and gave ethanol concentrations that ranged from 12.50% to 12.76% alc.(v/v). It has been reported by Henick-Kling (1993) that ethanol concentrations above 14 % alc.(v/v) inhibits growth of *O. oeni*. The higher ethanol concentrations of the 2002 Pinotage wines could also have led to the synergistic inhibition effect of ethanol, MCFAs and SO_2, since these inhibition effects are often observed (Britz & Tracey, 1990; Guerzoni, *et al*., 1995). This synergistic inhibition effect is further enhanced by the fact that higher octanoic acid concentrations were detected in the 2002 Pinotage (see section 3.3.5), as well as the fact that 10 mg/L more SO_2 was added at crush, in comparison with the 2003 Pinotage.

Wine fermented with strain NT112 was however, the only wine which have not completed MLF in the 2003 trials (**Fig. 3.9**). This might be explained due to this strains high SO_2 production (see section 3.3.3). In the spontaneous fermentation samples for the 2003 trials on the other hand, MLF have almost been completed just after alcoholic fermentation. This might be due the fact that inhibition of LAB has been considered to result from the depletion of nutrients by fermenting yeast (Alexandre *et al*., 2004). The spontaneous alcoholic fermentations contained

1×10^6 cells/mL less yeast, because it was not inoculated with a starter culture, and one can therefore accept that natural LAB cultures experienced less competition.

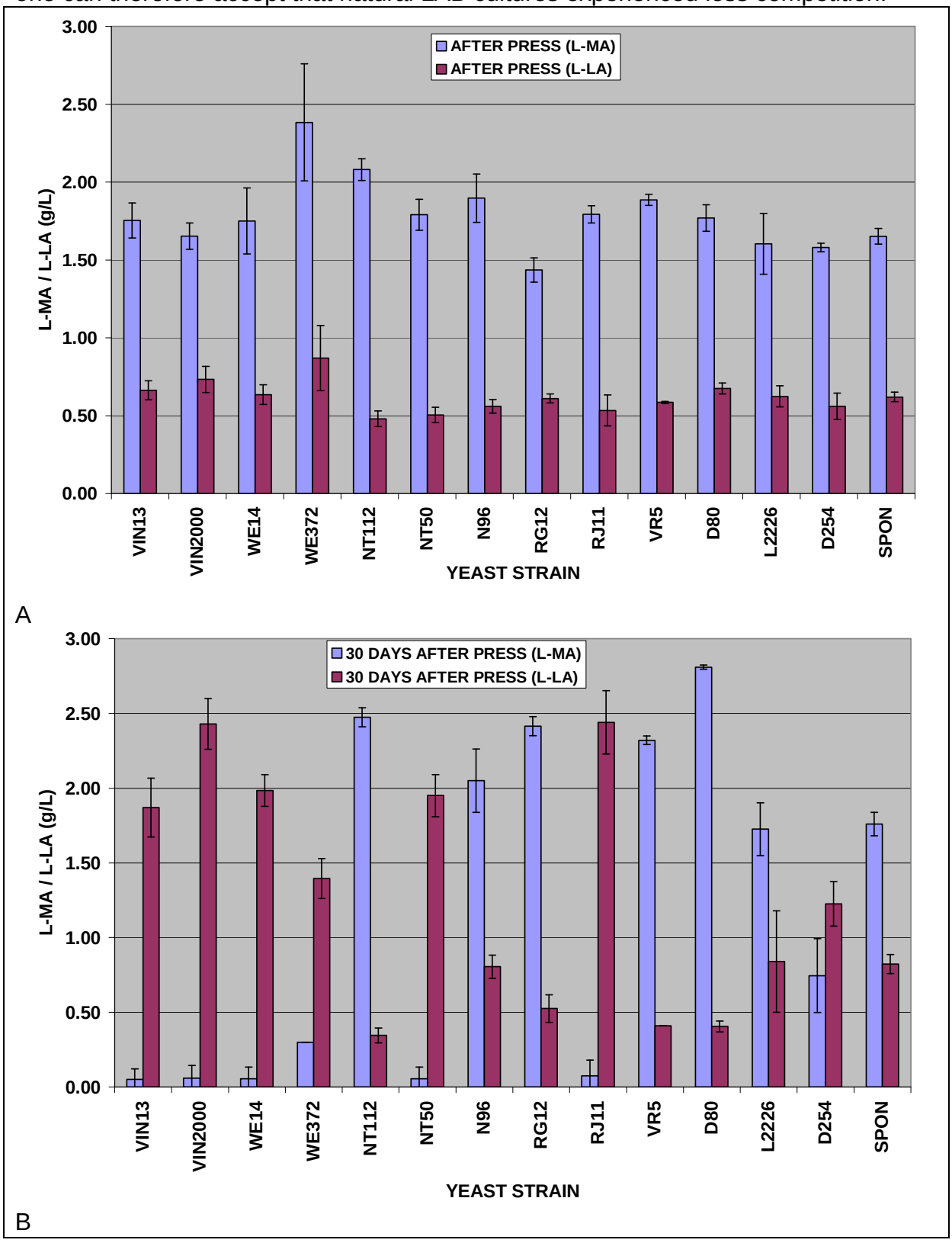

FIGURE 3.8. Malolactic fermentation rate in the 2002 Pinotage fermented with different commercial yeast strains. (A) L-Malic acid (L-MA) and L-lactic acid (L-LA) concentrations just after alcoholic fermentation. (B) L-MA and L-LA 30 days after press.

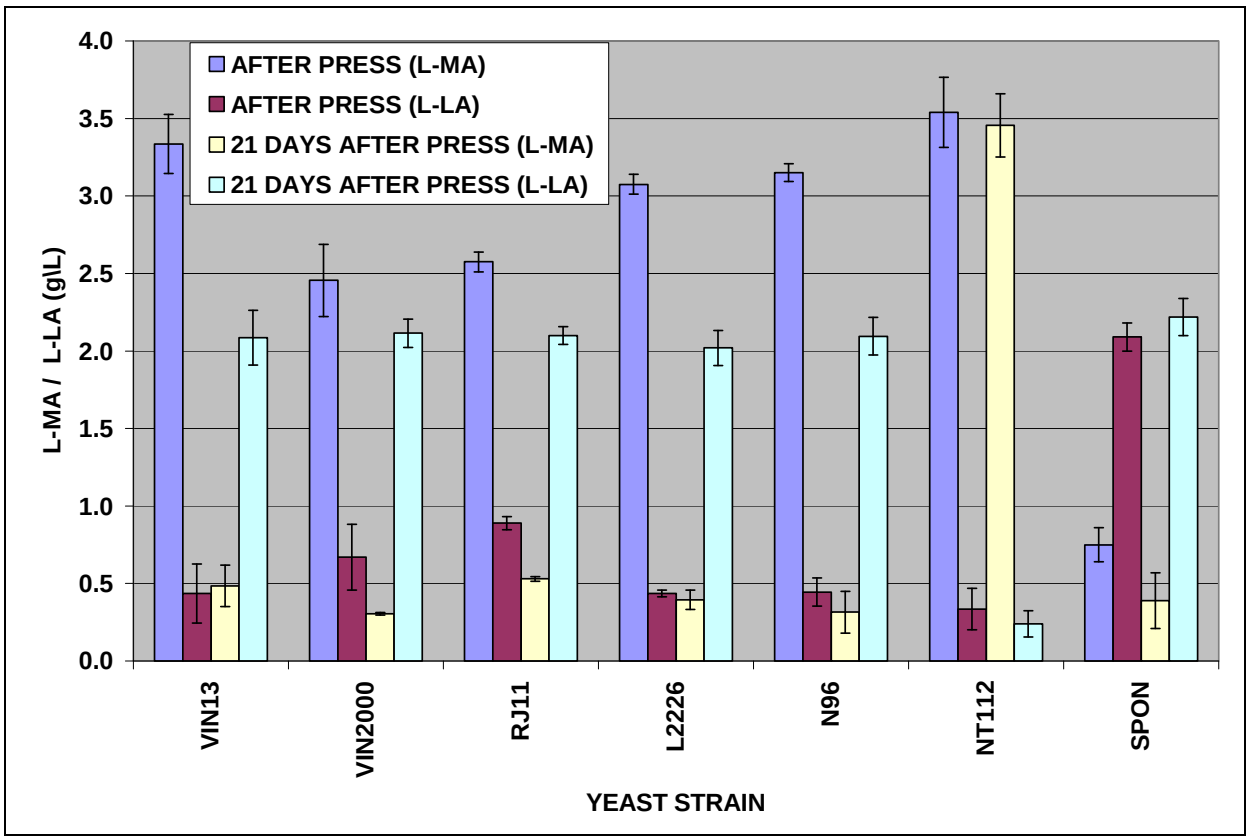

FIGURE 3.9. Malolactic fermentation rate in the 2003 Pinotage fermented with different commercial yeast strains. L-Malic acid (L-MA) and L-lactic acid (L-LA) concentrations just after alcoholic fermentation and press (A), and 21 days after press (Ethanol concentrations ranged from 12.8 to 13 % (v/v) alc. and pH 3.5 to 3.6).

3.3.3 SO_2 production of commercial yeast strains

The highest concentration of free and total SO_2 after fermentation was detected in the 2002 Pinotage fermented with strain NT112, namely 35 mg/L free SO_2 and 94 mg/L total SO_2 (**Fig. 3.10**). Wine fermented with strain L2056 contained the second highest amount of total SO_2, namely 55 mg/L, while strains WE14, RJ11 and D80 all contained less than 30 mg/L SO_2 after fermentation. The free SO_2 analysis after alcoholic fermentation of the 2003 Pinotage also revealed that NT112 produce significantly more SO_2 than any of the other strains tested (**Fig. 3.11**). The free SO_2 of NT112 was 18 mg/L, but were less than 14 mg/L in the samples of all the other strains. Sulphur metabolism of *S. cerevisiae* is very complex, with SO_2 being among numerous sulphur compounds that this yeast is able to produce (Delteil, 2002). The ability of SO_2 production is dependant on various factors, such as strain involvement and medium composition. Most yeast strains produce <30 mg/L SO_2, although some have been reported to produce >100 mg/L SO_2 (Eschenbruch, 1974). For the values obtained in this study one must keep in mind that 30 and 20 mg/L SO_2 was added at crush of the 2002 and 2003 Pinotage musts respectively.

The inability of strain NT112 to allow for completion of MLF in the 2002 and 2003 Pinotage in a timeframe of 30 and 21 days after press, respectively, might thus be

because of the high SO_2 production of this yeast. Other factors that can have a negative effect on MLF include low pH, high ethanol concentrations, fatty acids and copper (Carreté, *et al*., 2002) (see section 3.3.2).

3.3.4 Medium chain fatty acids

Octanoic acid concentrations after fermentation of the 2003 Pinotage ranged from 1 mg/L observed for VIN2000 up to almost 2 mg/L for both VIN13 and N96 (**Figs. 3.12** & **3.13**). Octanoic acid values for the 2003 Cabernet Sauvignon however, showed much lower values for all the yeast strains tested. From these RJ11 and L2226 contained about 0.22 mg/L octanoic acid, while VIN13, NT112 and N96 contained 0.38 mg/L or more octanoic acid after alcoholic fermentation. Levels of MCFAs however, also depend on must composition and winemaking conditions (Houtman *et al*., 1980).

The variation in decanoic acid was less for the yeast strains used in both cultivars in comparison with the octanoic acid (**Figs. 3.12** & **3.13**). After fermentation of the 2003 Pinotage decanoic acid concentrations varied from 0.254 mg/L for wine fermented with VIN2000 to 0.39 mg/L for strain RJ11. For the 2003 Pinotage trials decanoic acid concentrations varied from 0.09 mg/L for VIN13 to 0.19 mg/L for wine fermented with strain L2226.

It is known that octanoic acid only starts affecting yeast growth and fermentation at a concentration of 50 mg/L and decanoic acid at a concentration of 6 mg/L (Viegas & Sá-Correira, 1995). It is thus highly unlikely that the amounts of octanoic and decanoic acid in the above trials would have played any significant role in rendering biomass production, growth and fermentation of the yeast.

It is further known that combinations of MCFA like hexanoic, octanoic and decanoic acid can also inhibit malolactic fermentation (Lonvaud-Funel *et al*., 1987). Houtman *et al*. (1980) reported that 5 to 10 mg/L decanoic acid suppressed the growth of *O. oeni* and that 30 mg/L was lethal to the bacteria. Edwards *et al*. (1990) however, found that decanoic acid and other MCFAs are more inhibitory to yeasts than to LAB, and that the inhibition of MLF is more likely to be caused by other factors. The low concentrations of MCFAs found in our study (**Figs. 3.12** & **3.13**) were thus unlikely to be responsible for any delayed MLF.

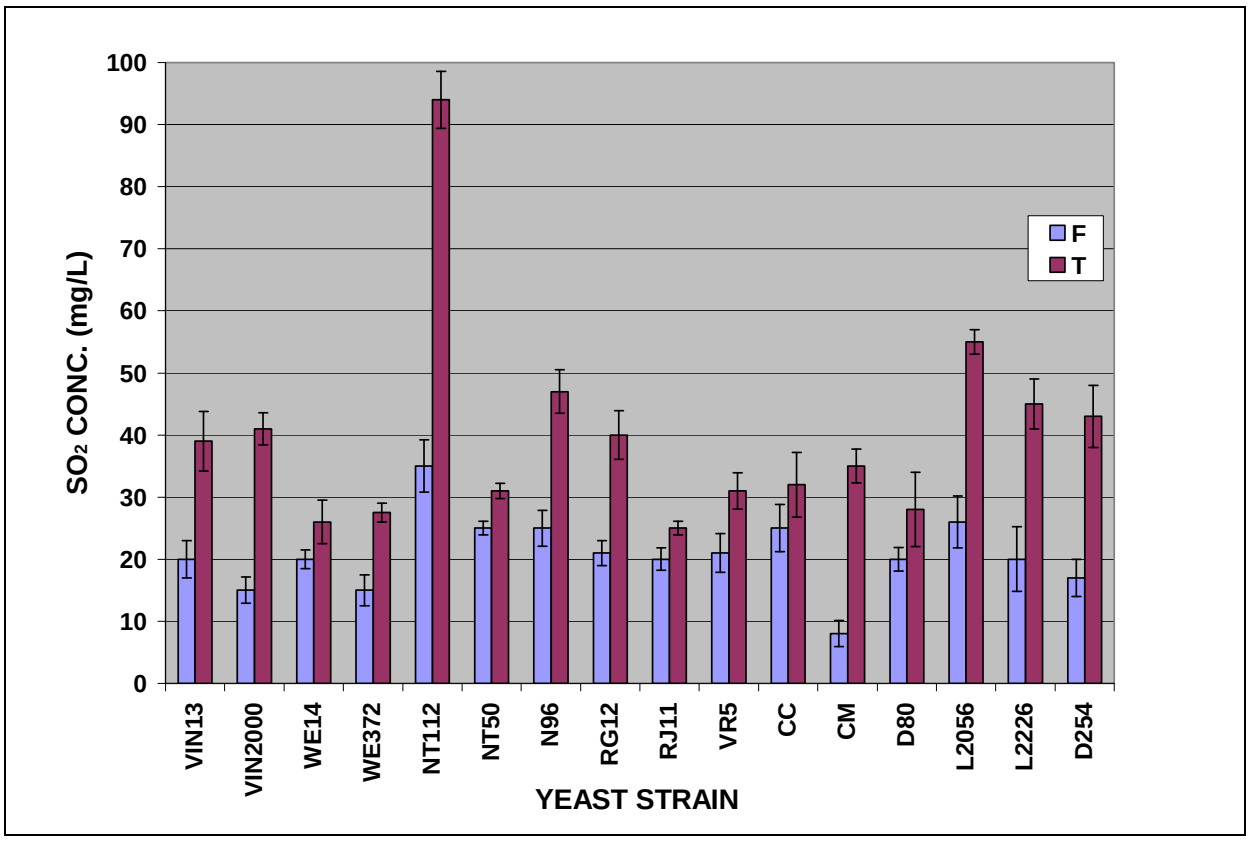

FIGURE 3.10. Amount of free and total SO_2 in Pinotage (2002) after alcoholic fermentation (30 mg/L SO_2 was added after crush).

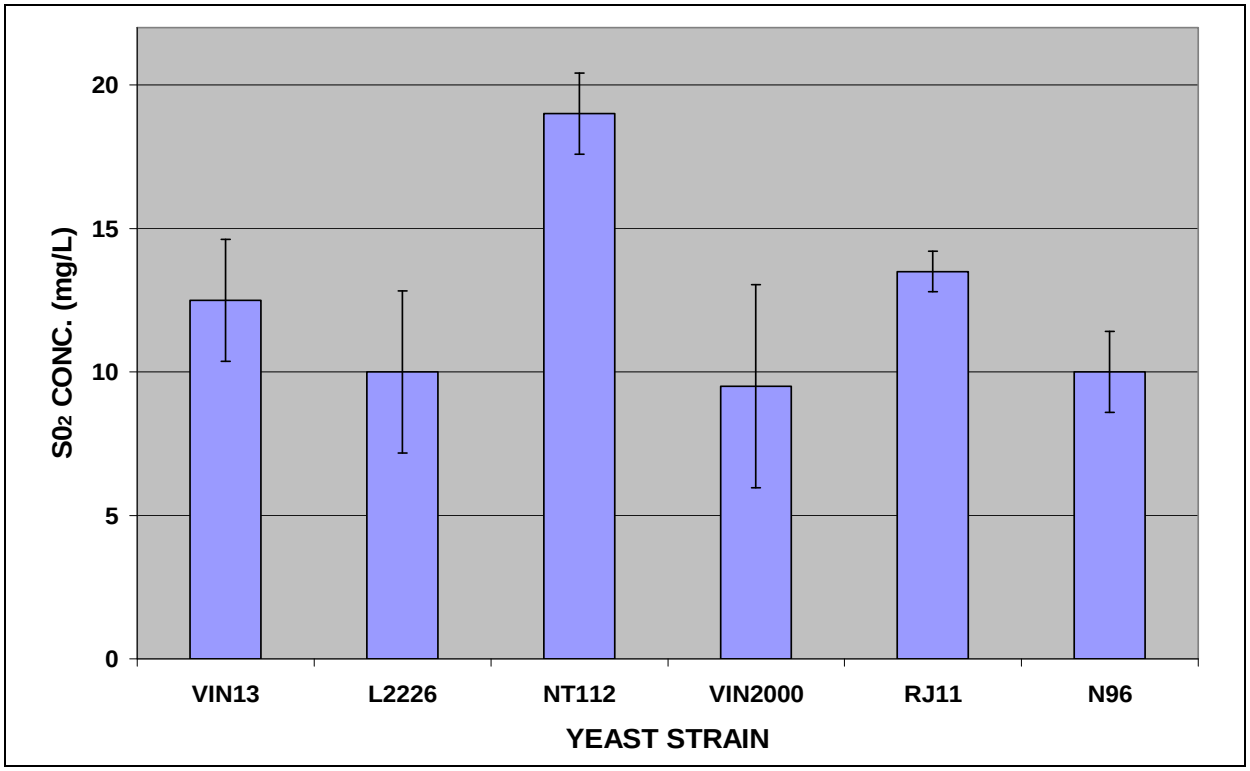

FIGURE 3.11. Amount of free SO_2 in Pinotage (2003) after alcoholic fermentation (20 mg/L SO_2 was added after crush).

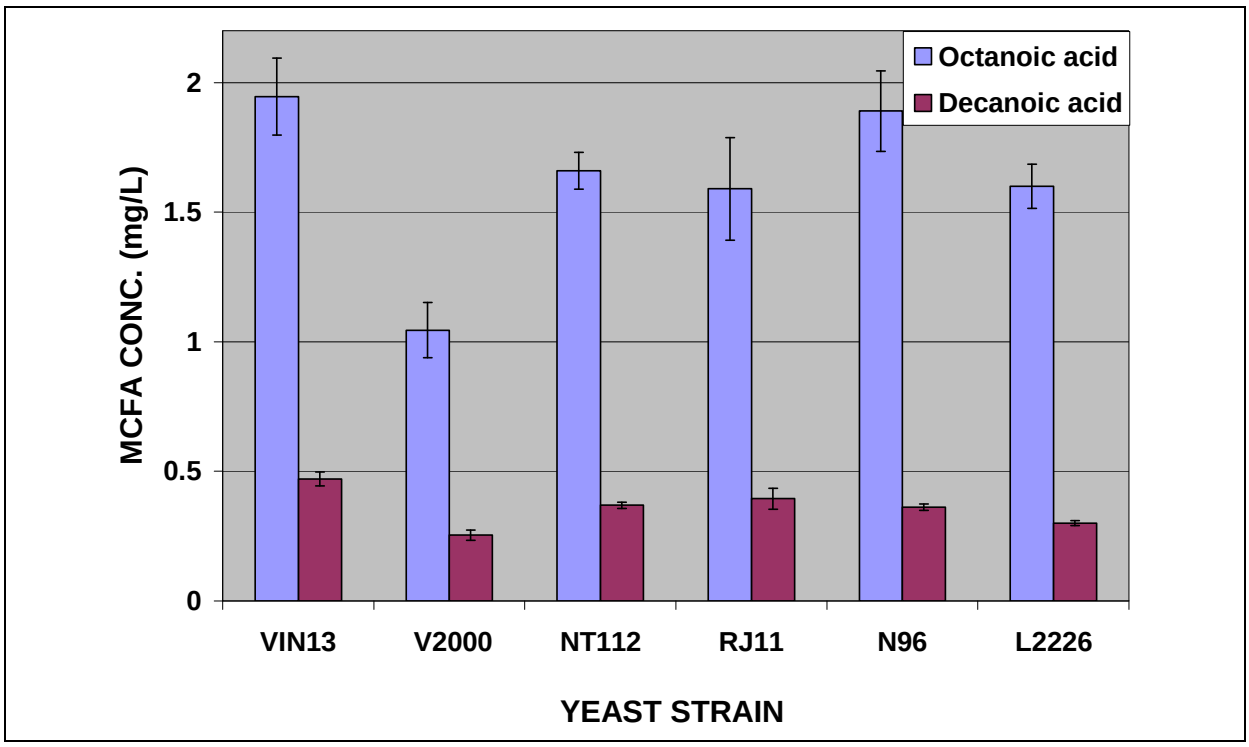

FIGURE 3.12. Octanoic acid and decanoic acid concentrations in Pinotage (2003) after fermentation with different yeast strains.

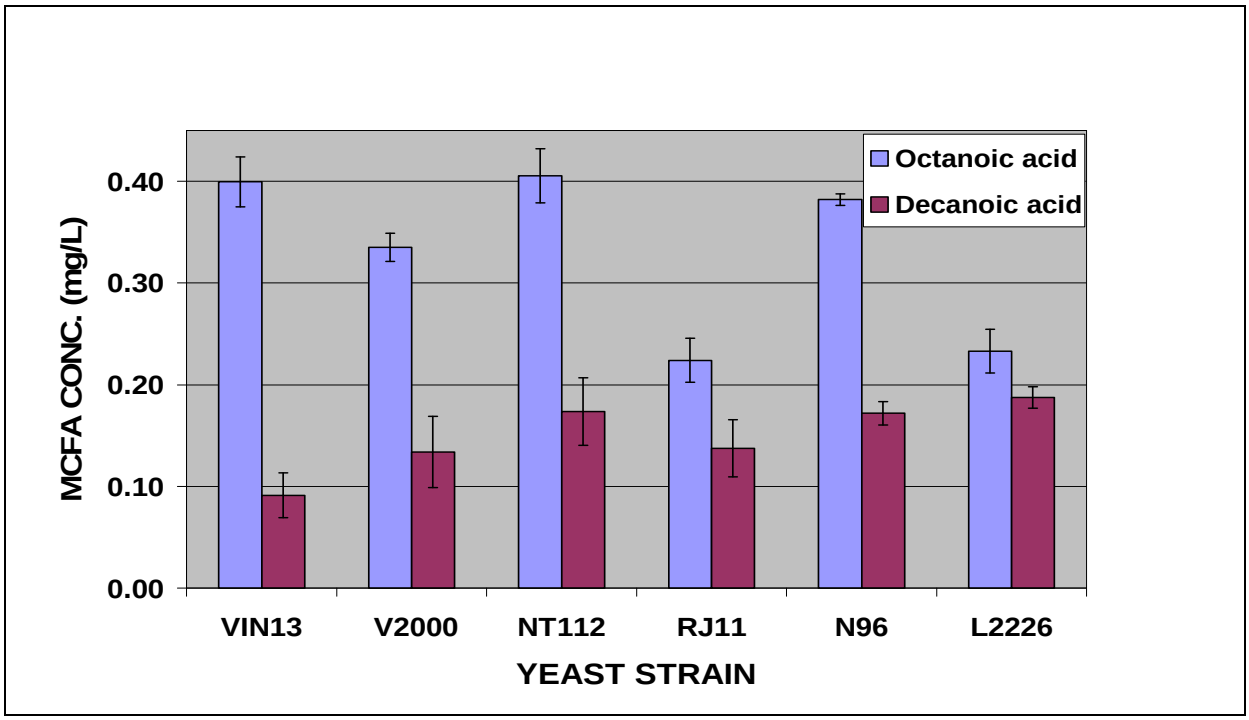

FIGURE 3.13. Octanoic acid and decanoic acid concentrations in Cabernet Sauvignon (2003) after fermentation with different yeast strains.

3.4 CONCLUSIONS

From previous studies it became evident that AAB are able to survive and even grow during relative anaerobic conditions that persist in wine fermentations (Drysdale & Fleet, 1989; Du Toit & Lambrechts, 2002). Little work has been done on the effects of different commercial wine yeast strains on AAB and LAB numbers. From the results obtained in this study it became evident that the yeast strain had very little effect on the AAB numbers during fermentation, except for higher AAB numbers that occurred at the end of alcoholic fermentation with the slow fermenter VIN2000.

LAB numbers were significantly lower only for strain NT112, which was because of it's higher SO_2 production. This was also reflected in the MLF rate, when wine made from NT112 did not finish MLF in any of the trials when most of the others had been finished. Decanoic and octanoic acid levels were also not high enough to inhibit yeast or LAB, but showed variances over two seasons between the different yeast strains.

Although there were not distinguished between different AAB and LAB strains in this study it still serve as an indication of the effect of some commercial yeast strains on bacteria numbers during red wine fermentations. Further trials in future could be aimed at more specific yeast strains and interactions between isolates of different AAB and LAB. The interaction of yeast with wine bacteria should thus also be considered in future for the selection of yeast strains for commercial wine productions.

3.5 ACKNOWLEDGEMENTS

The authors would like to thank Winetech and Anchor Biotechnologies for financial support, as well as Hamilton Russel Vineyards, Paardeberg Winery and Windmeul Cellar for the donation of grapes for these experiments.

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CHAPTER 4

Research results 2

The effect of high sugar and Cu^{2+} on the growth, fermentation and volatile acid production of different commercial wine yeast strains

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4. RESEARCH RESULTS

The effect of high sugar and Cu^{2+} on the growth, fermentation and volatile acidity production of different commercial wine yeast strains

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The effect of must containing high sugar concentrations or copper residues on fermentation efficiency and volatile acid (VA) production were examined with selected commercial wine yeast strains. Accumulated mass loss of strains VIN13, WE14, N96 and RJ11 appeared to be least affected by high sugar concentrations during the first few days of fermentation. It was however, only VIN13, WE372, N96 and L2056 that contained less than 11 g/L fructose after 21 days. VIN13 and RJ11 produced the lowest VA in the 21°B, 25°B and 28°B musts. Fermentation efficiency of six strains was also studied in must containing 0.25 mM Cu^{2+}. We found that strains NT50, Collection Cépage Cabernet (CC) and D80 were not significantly affected whereas VIN13, NT112 and RJ11 contained significantly more glucose and fructose after fermentation in the must containing copper. Copper also increases VA production by yeast strains with RJ11 and NT50 being the most affected.

4.1 INTRODUCTION

Yeast cells differ in the way that they respond to certain stress conditions during wine fermentations. These stress conditions can be any environmental factor which could have an adverse effect on cell growth, which may lead to sluggish or stuck wine fermentations (Ivorra *et al*., 1999). Numerous causes for stuck and sluggish wine fermentations are known. These include more common causes such as high sugar concentrations and less studied factors such as the presence of toxic pesticide residues (Henschke, 1997; Bisson, 1999). Although sluggish and stuck wine fermentations are sporadic they are serious problems which can hold major economic implications for any winery.

The occurrence of stuck and sluggish fermentations is more frequently observed in vintages of well matured grapes with high sugar concentrations (Gafner & Schutz, 1996). Musts containing high sugar levels allow the yeast to experience hypertonic conditions as soon as it is inoculated, which lead to an efflux of water from the cell, lowering turgor pressure, reducing water availability and cell shrinkage (Hohmann,

1997). The ability of yeast cells to start growth and carry out fermentation depends on the degree of osmotic stress that occurs in a specific juice due to its sugar level. Slaninova *et al*. (2000) showed that yeast cells respond to hyperosmotic shock through the modification of the cell wall and the cytoskeleton. This is however, not the only way that high sugar musts affect fermentation. Exhaustion of assimible nitrogen can lead to inactivation of the hexose transport system (Salmon, 1996) and the accumulating effect of ethanol production by yeast lead to higher toxicity towards the end of fermentation (Casey & Ingledew, 1986). High sugar musts can also lead to higher acetic acid production by the yeast cell during fermentation (Monk & Cowley, 1984). Volatile acidity (VA) consists mainly of acetic acid and legal limits on VA concentration are imposed on wine in wine producing countries (Du Toit & Lambrechts, 2002).

Copper is the active compound of various pesticides, namely: $CuSO_4 \cdot 5H_2O$, $CuCl_2 \cdot 2H_2O$ and copper oxychloride (Vidal *et al*., 2001) and it's affects on yeast have been the focuspoint of many studies (Welch *et al*., 1983; Rome & Gadd, 1987; Welch *et al*., 1989; Avery *et al*., 1996; Presta & Stillman, 1997; Azenha *et al*., 2000). Copper is an essential heavy metal to all organisms and has a very narrow optimum concentration range above which yeast inhibition occurs (Azenha *et al*., 2000). Higher than optimal copper levels inhibits the yeast cell due to a rapid loss of cellular K^+ levels, which cause a disruption of plasma membrane integrity and cell death (Avery *et al*., 1996).

These agrochemicals should not under normal circumstances influence alcoholic fermentation (Henschke, 1997), but withholding periods are not always strictly respected and this may lead to sluggish or stuck fermentations (Cassignard, 1975; Sala *et al*., 1996). It is well known that the minimum inhibitory copper concentration differ between yeast strains (Welch *et al*., 1983), although little work has been done thus far on the effect of copper on yeast growth and fermentation efficiency of different commercial wine yeast strains. The maximum legal copper concentration allowed on grapes in South Africa is 20 mg/kg Cu^{2+} (Nel *et al*., 2003), which is equal to 0.3125 mM Cu^{2+}.

In this work we investigated the effect of high sugar must as well as copper residues on the fermentation efficiency and volatile acidity (VA) production of selected commercial wine yeast strains.

4.2 MATERIALS AND METHODS

4.2.1 Yeast strains, media and growth conditions

The commercial yeast strains used in this study are listed in **Table 4.1**. Eight yeast strains (VIN13, WE14, NT50, N96, RJ11, CC, D80 and L2056) were used for the comparative study in musts with different sugar levels, while an extra strain, WE372, was used to compare the effect of different sugar levels on VA production. Six yeast

strains (VIN13, NT112, RJ11, D80, CC and NT50) were used to compare the effect of copper on VA and fermentation performance. All the yeast strains were grown overnight in YPD (1% (w/v) yeast extract, 2% (w/v) bactopeptone and 2% (w/v) glucose), centrifuged at 3 000 rpm and washed with sterile dH_2O before inoculation of the must at 1.0 x 10^6 cells/mL. The different musts that were used are listed in **Table 4.2**. Musts were obtained from the 2003 harvest season and were frozen at -20°C until use. Before fermentation all musts were allowed to settle to remove the excess solids. Diammonium phosphate (DAP) (0.7 g/L) was added to all musts to ensure sufficient nitrogen for optimum fermentations. Glucose and fructose were added in a 50:50 ratio to increase the sugar levels of the Sauvignon blanc must in the range from 21°B to 25°B and 28°B, after which it was filtered through a 0.45 μm filter. Fermentations were carried out at 25°C in triplicate in 200 mL glass bottles filled with 100 mL must, which were sealed with foil. For the copper experiments 0.25 mM Cu^{2+} was added to the Clairette blanche and Colombar juice in the form of $CuSO_4 \cdot 5H_2O$. Clairette blanche juice was used for the trials with strains VIN13, NT112, NT50 and CC. Strains RJ11 and D80 experienced fermentation problems with the Clairette blanche must and Colombar juice was used for these two strains.

TABLE 4.1
Yeast strains used in this study.

Species	Strain	Company
Saccharomyces cerevisiae	VIN13, WE14, NT50, NT112 Collection Cépage Cabernet (CC)	Anchor Bio-Technologies
Saccharomyces cerevisiae	D80, L2056	Lallemand Inc.
Saccharomyces cerevisiae	RJ11	Columbit
Saccharomyces bayanus	N96	Anchor Bio-Technologies

TABLE 4.2
Composition of the musts used in this study.

Cultivar	Initial Sugar conc. ^{o}B	TA[(a)] g/L TTA[(b)]	pH
Sauvignon blanc	21.0	4.29	3.28
Clairette blanche	24.0	4.85	3.33
Colombar	22.5	5.60	3.20

[(a)]Titratable acidity [(b)]Tartaric acid

4.2.2 Monitoring of yeast growth and fermentation

Yeast growth was followed by measuring the absorbance of the fermenting must at 600nm. Fermentation activity was monitored at regular intervals for 21 days by measuring the CO_2 mass loss.

4.2.3 Routine wine analyses

The volatile acidity (VA), glucose and fructose concentrations were analyzed after 21 days of fermentation with the GrapeScan FT 120 instrument (Foss Electric, Denmark). The instrument utilises Fourier transform infrared spectroscopy (FTIR). All samples were degassed by filtration before the analysis, using the Filtration Unit (type 70500, Foss Electric, Denmark) with filter paper circles graded at 20-25 μm and with a diameter of 185 mm (Scheicher & Schuell, reference number 10312714).

4.2.4 Copper analyses

Copper analyses were done by flame atomic absorption spectrometry (FAAS) (Dean, 1960), using a Varian 875 AA spectrometer. The preparation of the standard solution was done by dissolving 1 g of copper metal into a minimum volume of 1:1 nitric acid and diluted to 1 L to give a 1000 μg/mL Cu^{2+} concentration.

4.3 RESULTS AND DISCUSSION

4.3.1 Effect of high sugar levels on fermentation with different yeast strains

The results for the comparison of the fermentation performance of 8 commercial yeast strains are presented in three ways. Firstly, the accumulated mass losses of different yeast strains for a specific must are presented on one graph (**Figs. 4.1 A** & **B**). Secondly, fermentation rates in musts with different sugar concentration for specific yeast strains (**Fig 4.2**), and thirdly, the amount of glucose and fructose present in each wine after fermentation with the different strains (**Figs 4.3** & **4.4**).

Figs. 4.1 A & **B** show the accumulated mass loss of eight commercial yeast strains for a 15 day period in Sauvignon blanc must with sugar levels of 25°B and 28°B, respectively. By comparing these graphs one can distinguish between strong and weaker fermenting strains in the individual musts. Strains NT50, VIN13 and WE14 were the strongest fermenters in the 25°B must, although WE14 started off slower. Compared to the other seven strains tested RJ11 was by far the weakest fermenter in the 25°B must for the first 15 days of fermentation. By comparing **Fig. 4.1 A** & **B** it is clear that strains that fermented faster in the 25°B must did not necessarily ferment the fastest in the 28°B must.

When comparing the different fermentation rates in the 28°B must (**Fig. 4.1 B)** it is clear that two yeast strains stood out, namely VIN13 and D80, with VIN13 being by far the strongest fermenter and D80 the weakest. It is thus clear that VIN13 is potentially more capable of fermenting in high sugar concentration musts compared to the other strains.

By comparing the different sugar levels of the first three days after inoculation of each yeast strain in **Fig. 4.2** one can see that fermentations with strains VIN13, WE14, N96 and RJ11 are less affected by high sugar concentrations than with the other strains. Fermentation with RJ11 actually appeared to be stronger in the higher sugar musts for the first three days. From these three strains VIN13 and RJ11 are the only strains that showed significantly more CO_2 mass loss for the 28°B must in comparison with the 25°B. D80 on the other hand appeared to be the least effective in high sugar must with even less sugar fermented in the 28°B than in 25°B must (**Fig. 4.2**).

After 21 days of fermentation all the glucose has been consumed by all the strains in the 21°B must. In the 25°B must however, N96, RJ11 and CC had more than 1 g/L glucose in comparison with VIN13, WE14, WE372 and NT50, which all contained less than 0.5 g/L glucose after fermentation. No significant differences could be found between the glucose concentrations after fermentation with the different yeast strains of the 28°B Sauvignon blanc must (**Fig. 4.3**). Fructose concentrations varied significantly between the strains after 21 days of fermentation of the 28°B must. VIN13 appeared to utilise fructose the best under high sugar levels with less than 7 g/L fructose remaining after fermentation. The strains with the highest amount of fructose in the 28°B must after fermentation were NT50, RJ11, CC and D80, with more than 15 g/L fructose (**Fig. 4.4**). These yeast can partially lead to a higher risk of sluggish and stuck fermentation due to glucose: fructose imbalance.

Our results agree with the fact that stuck and sluggish fermentations is more frequently observed in vintages of well matured grapes with high sugar concentrations (Gafner & Schütz, 1996). Yeast strains also differ in their ability to ferment higher sugar containing musts. It must however, be kept in mind that the nutrient requirements of yeast strains can differ and the addition of extra nutrients before fermentation might be necessary for certain yeasts strains (P. Loubser, Personal communication).

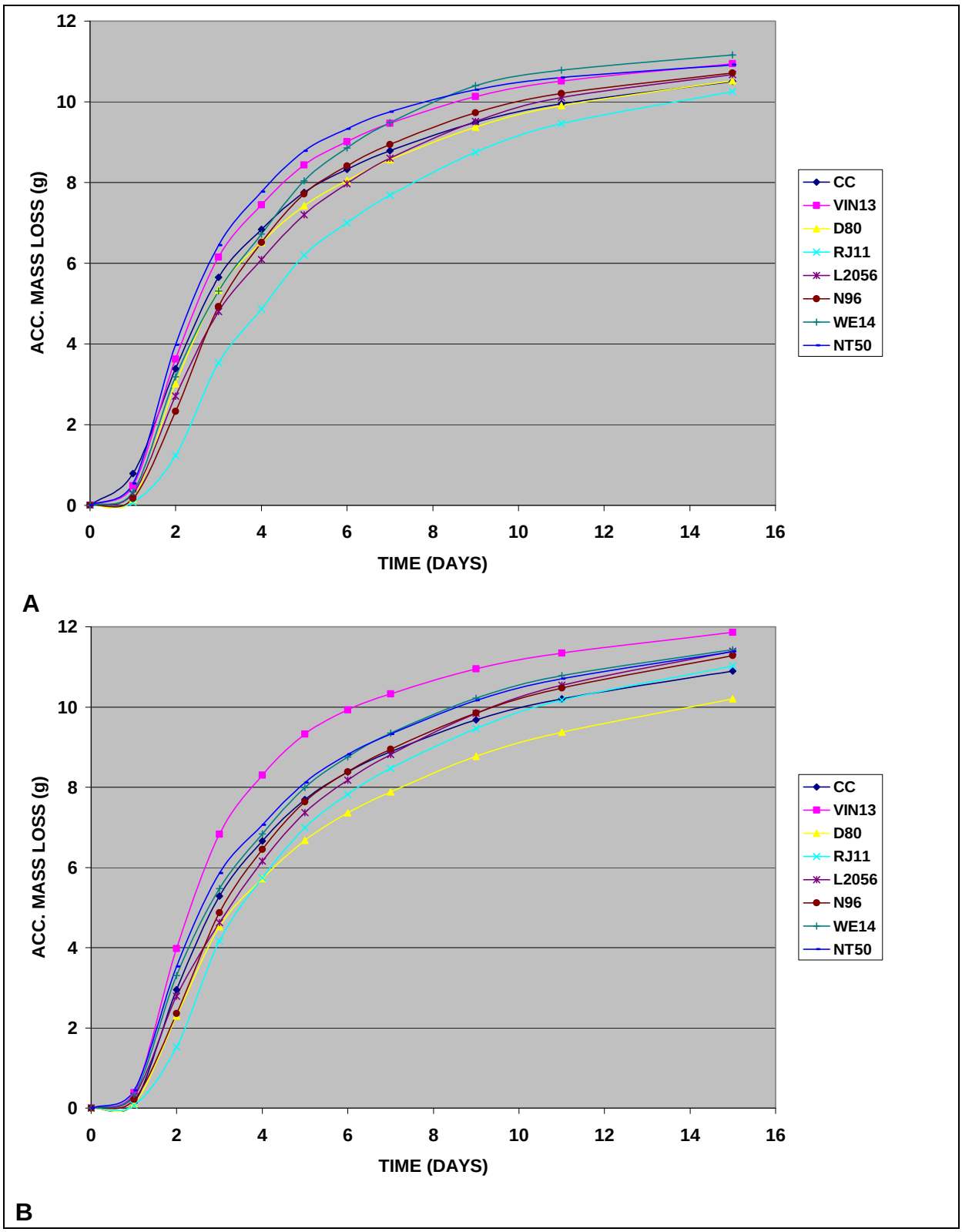

FIGURE 4.1. Fermentation curves of different yeast strains in Sauvignon blanc must with initial sugar levels of (A) 25°B and (B) 28°B. Data shown is the average of the triplicates.

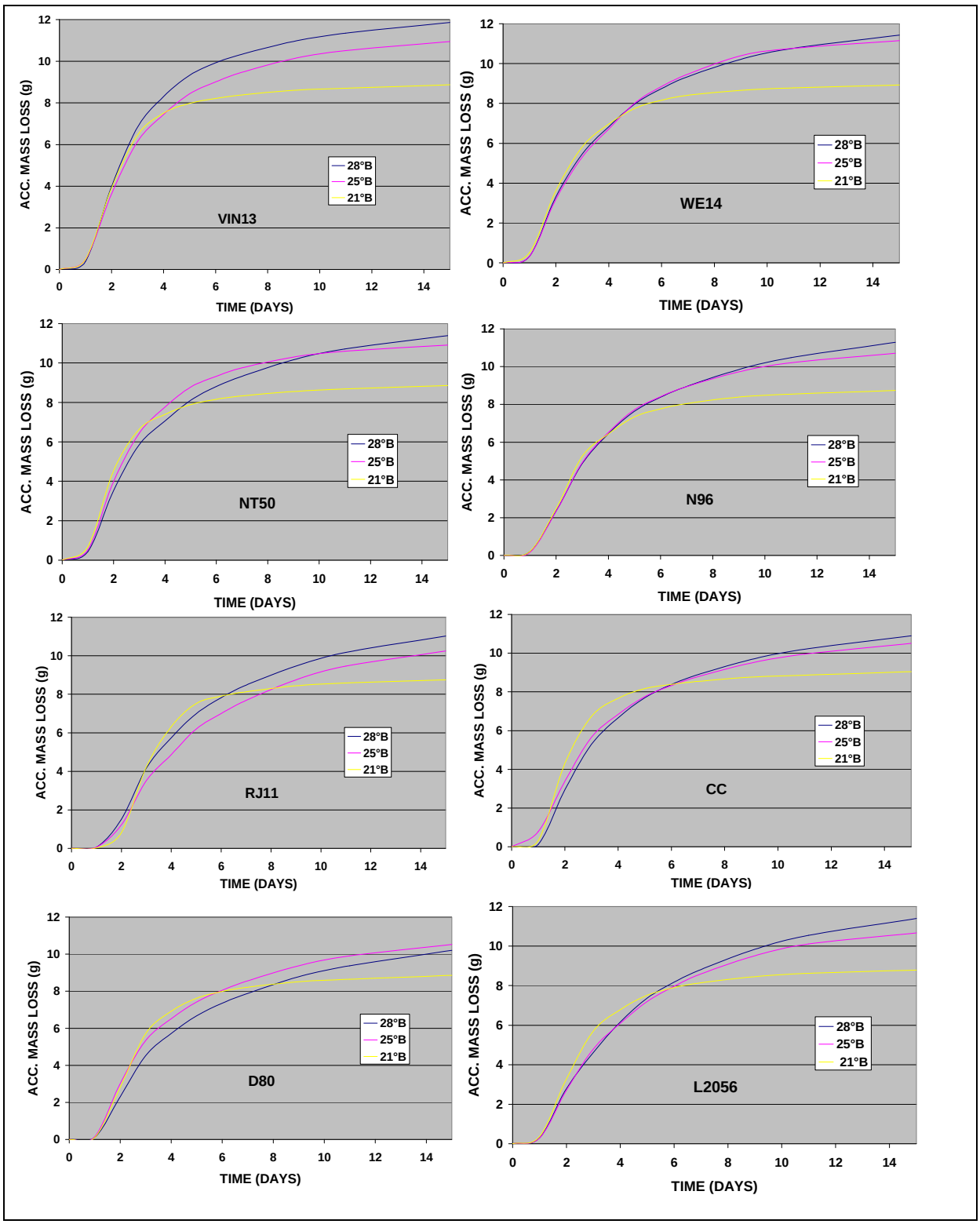

FIGURE 4.2. Fermentation curves of different yeast strains in Sauvignon blanc must with initial sugar levels of 21°B, 25°B and 28°B. Data shown is the average of triplicates.

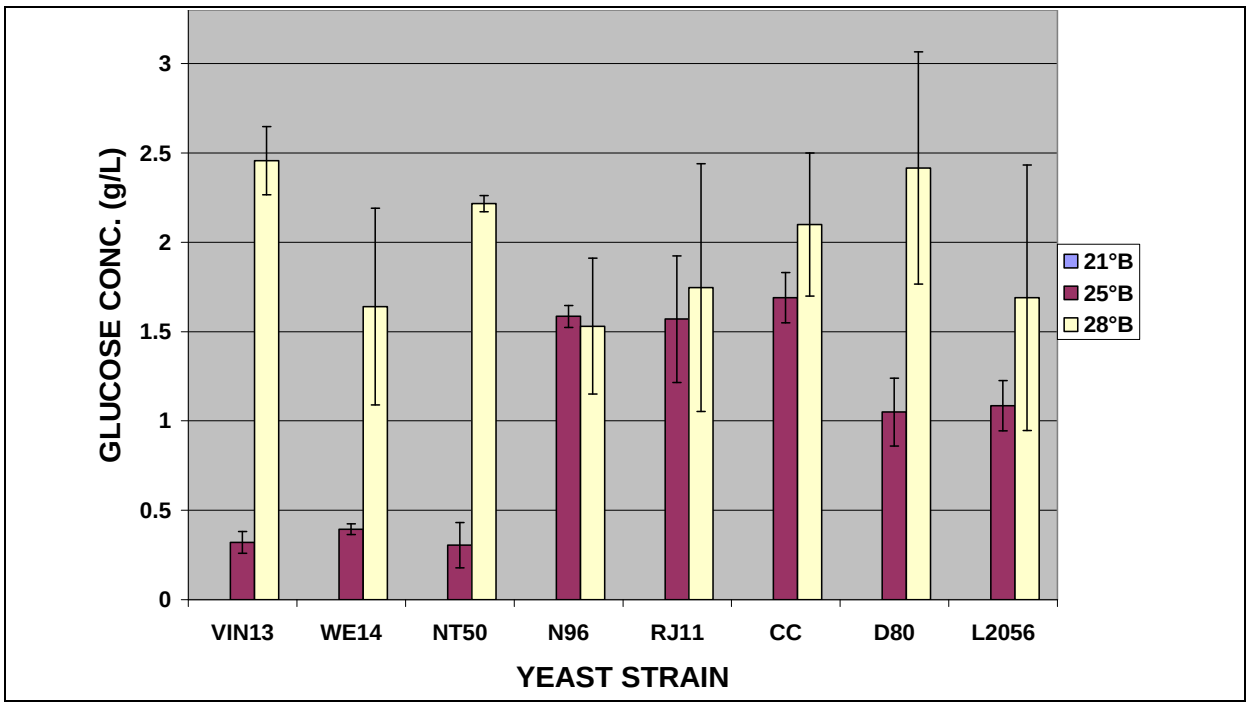

FIGURE 4.3. Glucose concentrations after 21 days of fermentation with different yeast strains in Sauvignon blanc musts of 21°B, 25°B and 28°B. No glucose was detected in the 21°B must.

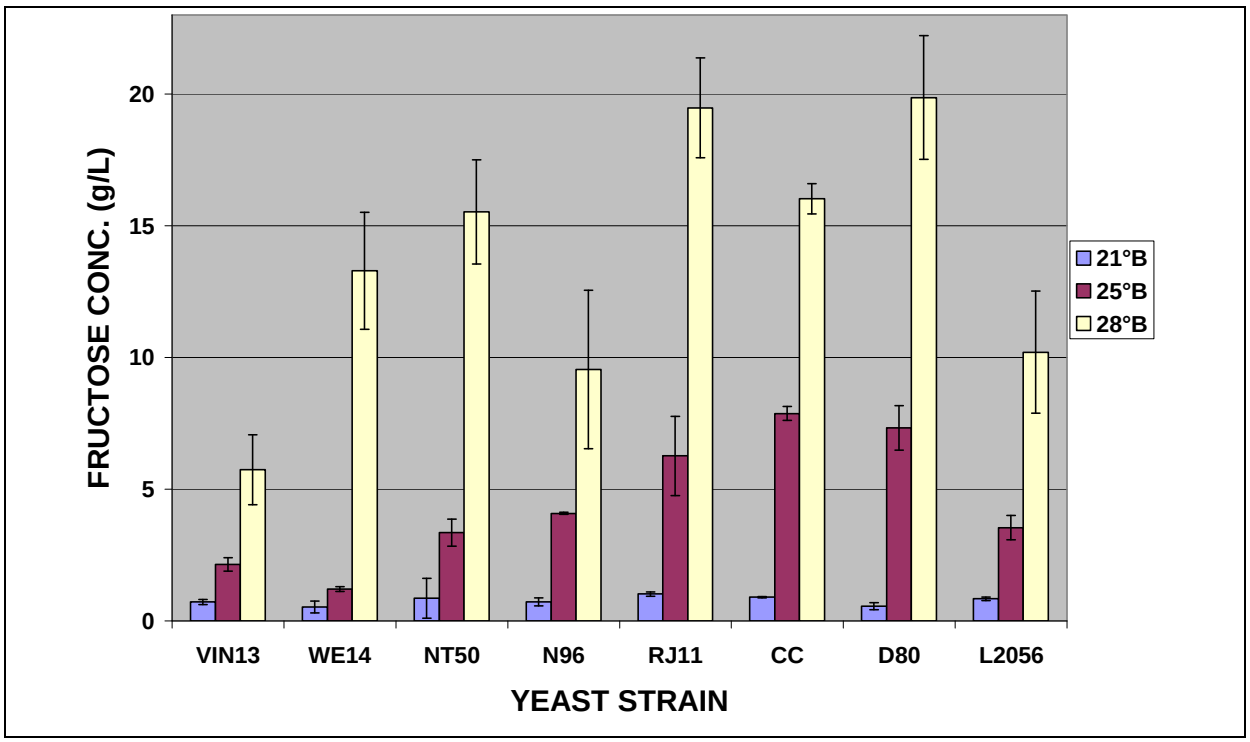

FIGURE 4.4. Fructose concentrations after 21 days of fermentation with different yeast strains in Sauvignon blanc musts of 21°B, 25°B and 28°B.

4.3.2 Effect of high sugar levels on VA production of different yeast strains

The VA concentration in wine consists mainly out of acetic acid, which is a normal end-product of yeast cells during fermentation. The yeast strain is one of the most

important factors affecting acetic acid production during fermentation. Other important factors that increase acetic acid production during fermentation are high initial sugar concentrations and the degree of juice clarification prior to fermentation. Acetic acid bacteria (AAB) and lactic acid bacteria (LAB) can also produce acetic acid and thereby increasing the VA of the wine (Du Toit & Lambrechts, 2002). In this study however, we kept bacteria numbers as low as possible with the use of SO_2 and filtration. The filtration of the Sauvignon blanc must before inoculation could therefore also have influenced the specific VA concentrations obtained, due to the fact that higher amounts of acetic acid is produced in musts that have been clarified excessively (Delfini & Cervetti, 1991). The aim of the trials however, was to compare the VA production of different strains in high sugar concentrations. **Fig. 4.5** shows the VA concentrations after fermentation with different yeast strains in 21°B, 25°B and 28°B must.

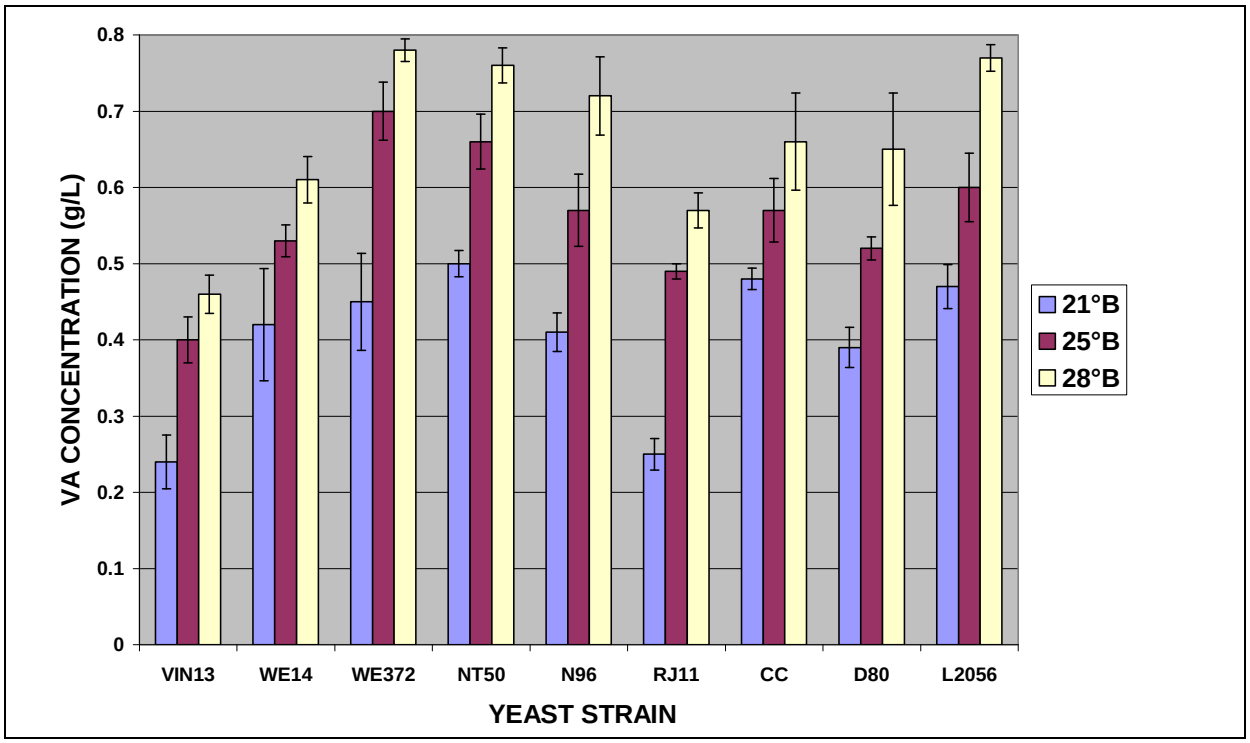

FIGURE 4.5. VA concentrations after fermentation with different yeast strains of Sauvignon blanc musts with different initial sugar concentrations.

VIN13 was the lowest VA producer in all the trials with 0.24 g/L in the 21°B must, 0.40 g/L in the 25°B must and 0.46 g/L in the 28°B must being produced. VIN13 is a killer strain and can be a possible explanation for the lower total VA for this strain due to the influence on VA produced by certain sensitive wild yeasts. However, in this case it is highly unlikely due to the filtration and use of SO_2 before inoculation. RJ11 also produced small amounts of VA at 21°B, but appeared to be more affected by higher sugar levels, although this strains was the second lowest VA producer overall. WE372, NT50, N96 and L2056 produced the most VA (more than 0.7 g/L) in the

28°B must. The higher VA concentrations formed by yeast in higher sugar must conditions (**Fig. 4.5**) agrees with previous work done by other researchers (Rosell *et al*., 1968; Monk & Cowley, 1984; Millán & Ortega, 1988). The legal limit for VA in South African dry wine is 1.2 g/L (Du Toit & Lambrechts, 2002). VA production by yeast strains under high sugar levels is therefore an important criteria for yeast strain selection.

4.3.3 Effect of copper on yeast growth and fermentation

In this study six commercial yeast strains were tested for their tolerance to 0.25 mM Cu^{2+}. In **Fig. 4.6** the growth curves of the different strains for the first 120 to 150 hours are shown in must with no added copper and must containig 0.25 mM Cu^{2+}. From the growth curves one can clearly see that yeast growth was inhibited for the first 100 hours in the must containing copper when fermented with VIN13. Yeast growth of NT50 and RJ11 also appeared to be significantly lower in the must containing 0.25 mM Cu^{2+}. Yeast growth of NT112, CC and D80, on the other hand, was less affected during the first 120 hours in copper containing must.

There was a correlation between inhibition of growth by Cu^{2+} addition and fermentation efficiency (**Fig. 4.7**), with strains being the least affected fermenting the most effectively. Strain NT50 however, seemed to overcome the inhibiting effect of Cu^{2+} in terms of fermentation efficiency, unlike VIN13, NT112 and RJ11. The same trends can be seen with the amounts of glucose and fructose left in the must after 21 days of fermentation (**Figs. 4.8 & 4.9**). Must fermented with VIN13 contained the highest amount of hexose after fermentation in the copper containing must with 6 g/L glucose and 25 g/L fructose. NT112 and RJ11, on the other hand, fermented more glucose in copper containing must, but high levels of fructose were left in these musts after fermentation. No significant differences in glucose and fructose concentrations after 21 days in Cu^{2+}-containing must could be found for D80, CC and NT50 in comparison with the control. Fermentation with the latter strains thus seem to be unaffected by 0.25 mM Cu^{2+}. Yeast strains thus differ in their copper resistance, which was clearly shown in this study. The utilization of fructose is also more affected by copper addition than that of glucose, and this needs further investigation.

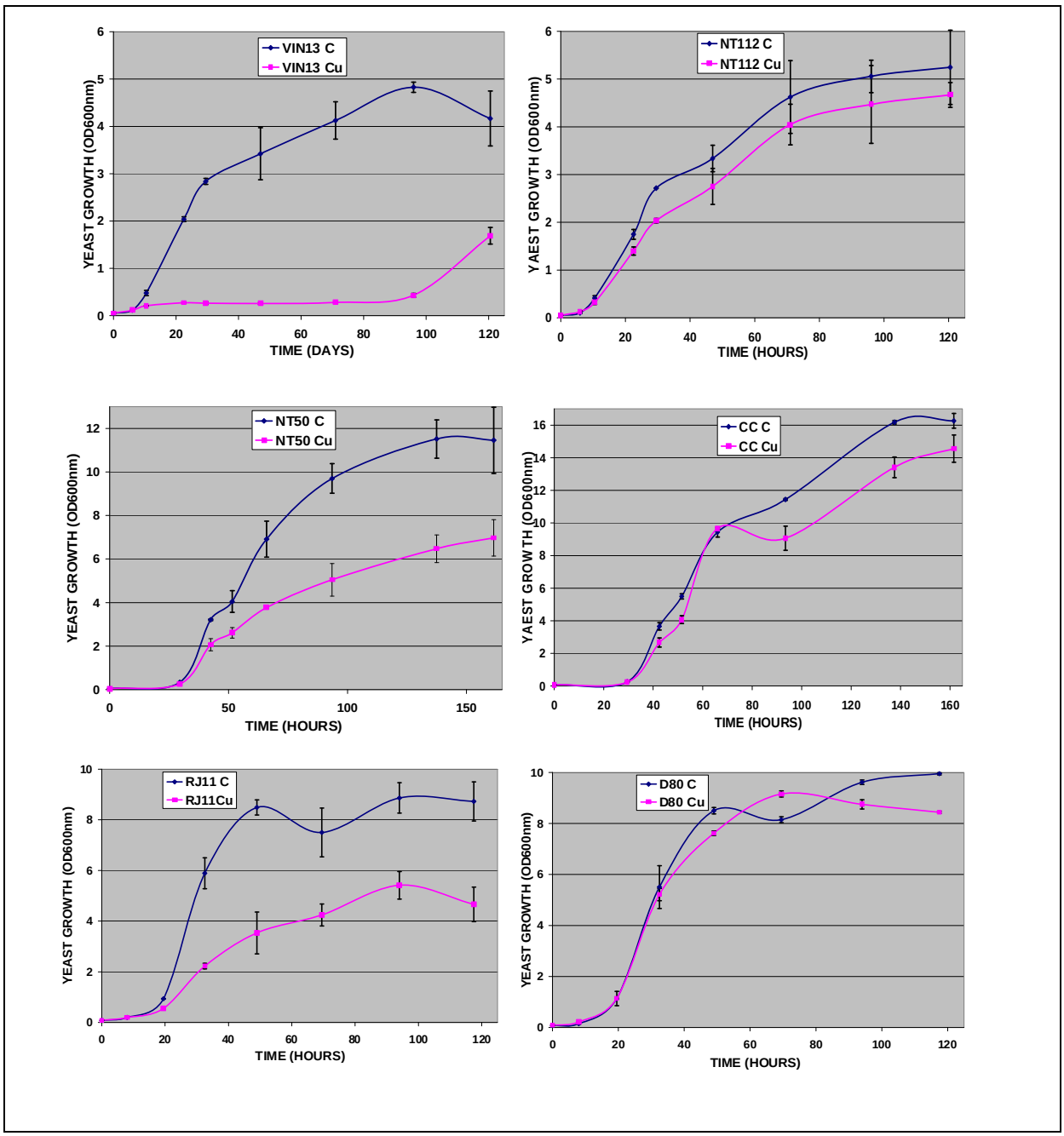

FIGURE 4.6. Yeast growth over time for six commercial wine yeast strains in must containing no Cu^{2+} (C) and in the same must containing 0.25 mM Cu^{2+} (Cu).

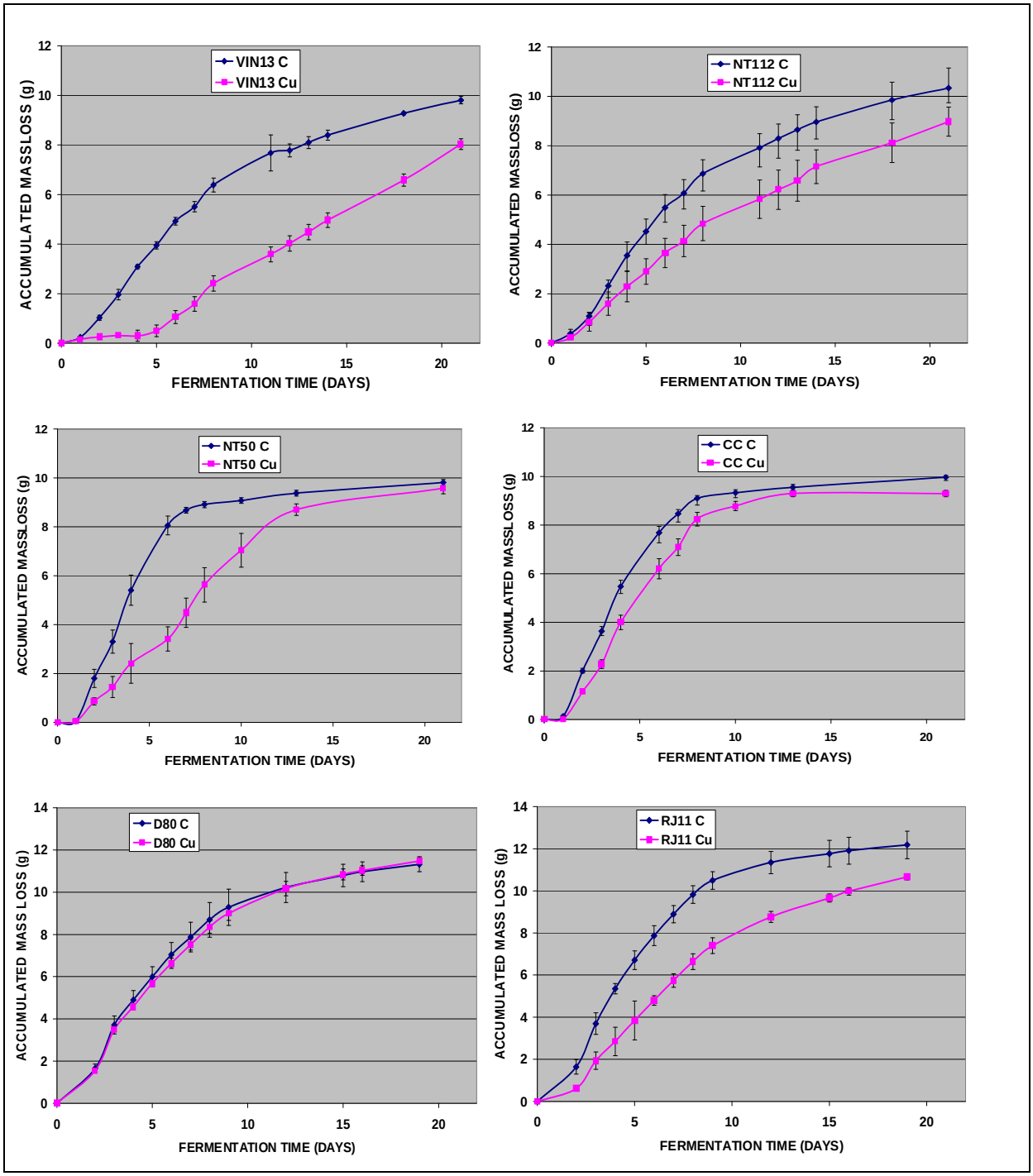

FIGURE 4.7. Fermentation curves, measured as CO_2 mass loss, of six commercial wine yeast strains in must containing no Cu^{2+} (C) and in the same must containing 0.25 mM Cu^{2+} (Cu).

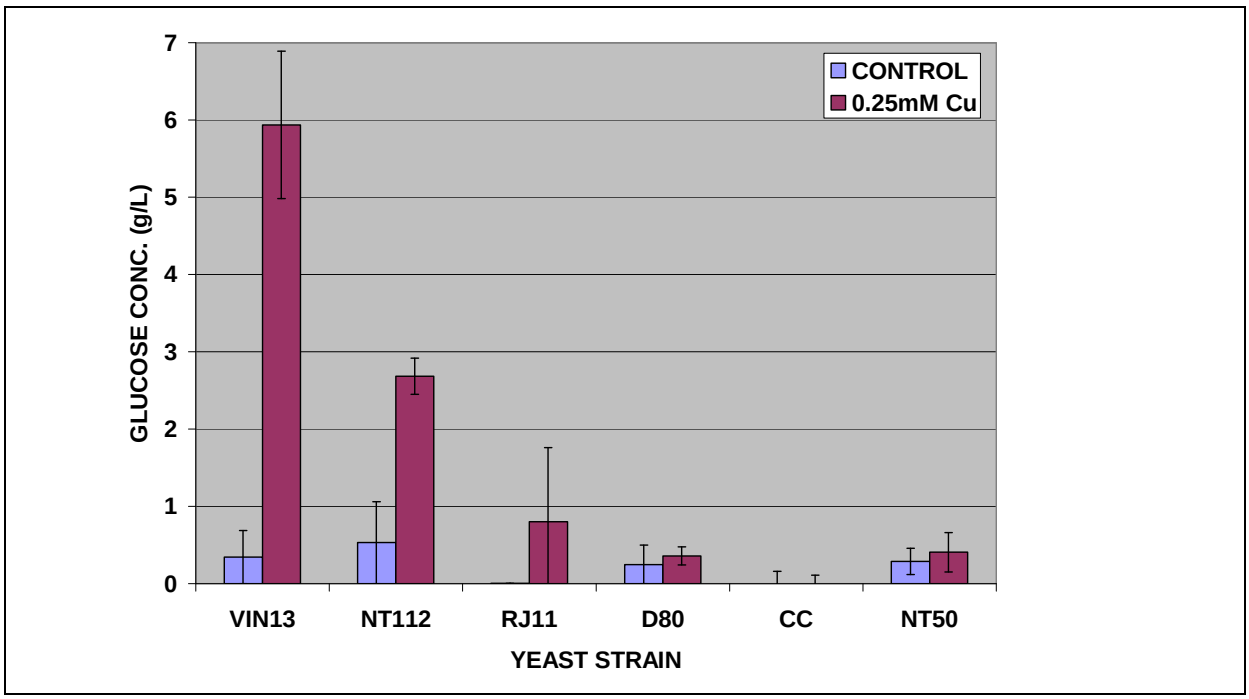

FIGURE 4.8. Glucose concentrations after alcoholic fermentation (21 days) of must fermented with different commercial wine yeast strains with no added Cu^{2+} and of must with 0.25 mM Cu^{2+} added.

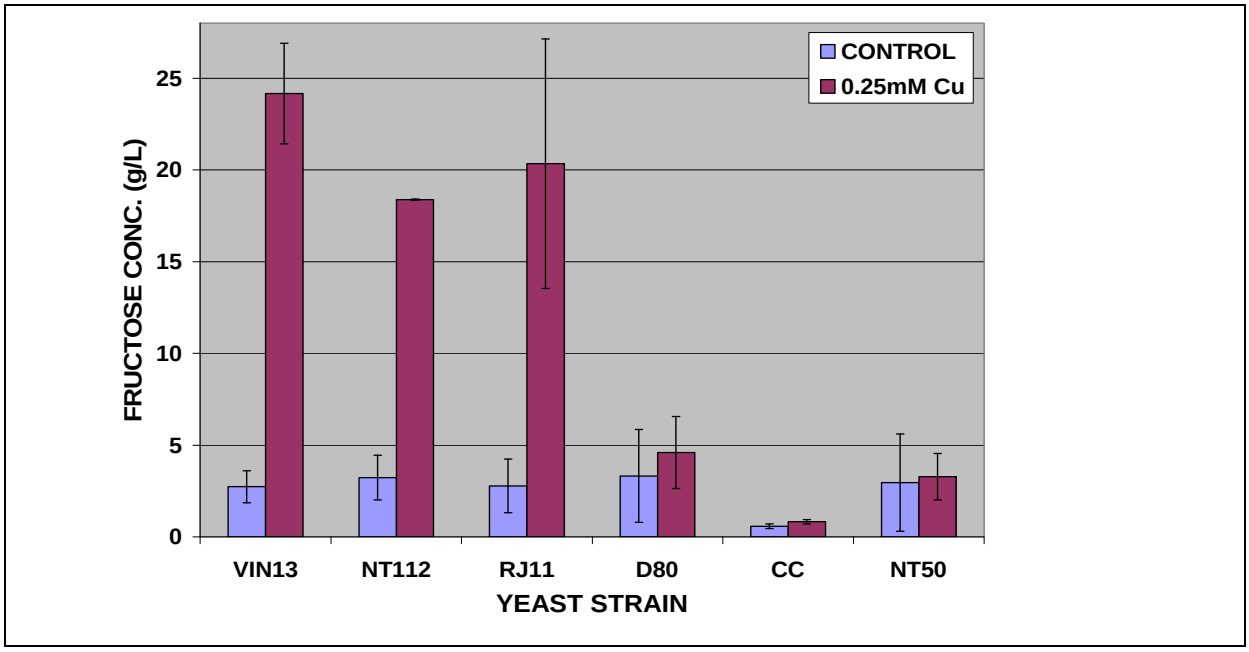

FIGURE 4.9. Fructose concentrations after alcoholic fermentation (21 days) of must fermented with different commercial wine yeast strains with no added Cu^{2+} and of must with 0.25 mM Cu^{2+} added.

4.3.4 Effect of copper on VA production of yeast strains

The effect of copper on VA production by *S. cerevisiae* has not yet been elucidated in detail according to literature. The effect of fermentation with different yeast strains in the presence of 0.25 mM Cu^{2+} on VA production was investigated and is shown in **Fig. 4.10**. VA production by strains D80 and CC was not significantly affected by copper. This correlates with the growth curves and fermentation curves of these yeast strains (**Figs. 4.6** & **4.7**), which showed that copper had no significant effect on their growth and fermentation efficiency. Must fermented with RJ11 and NT50, on the other hand, in the presence of 0.25 mM Cu^{2+}, contained 0.18 and 0.16 g/L more VA than the control. This might indicate that these strains experienced stress conditions in the presence of Cu^{2+} and produced more acetic acid, which correlates well with the growth curves of these yeasts (**Fig. 4.6**).

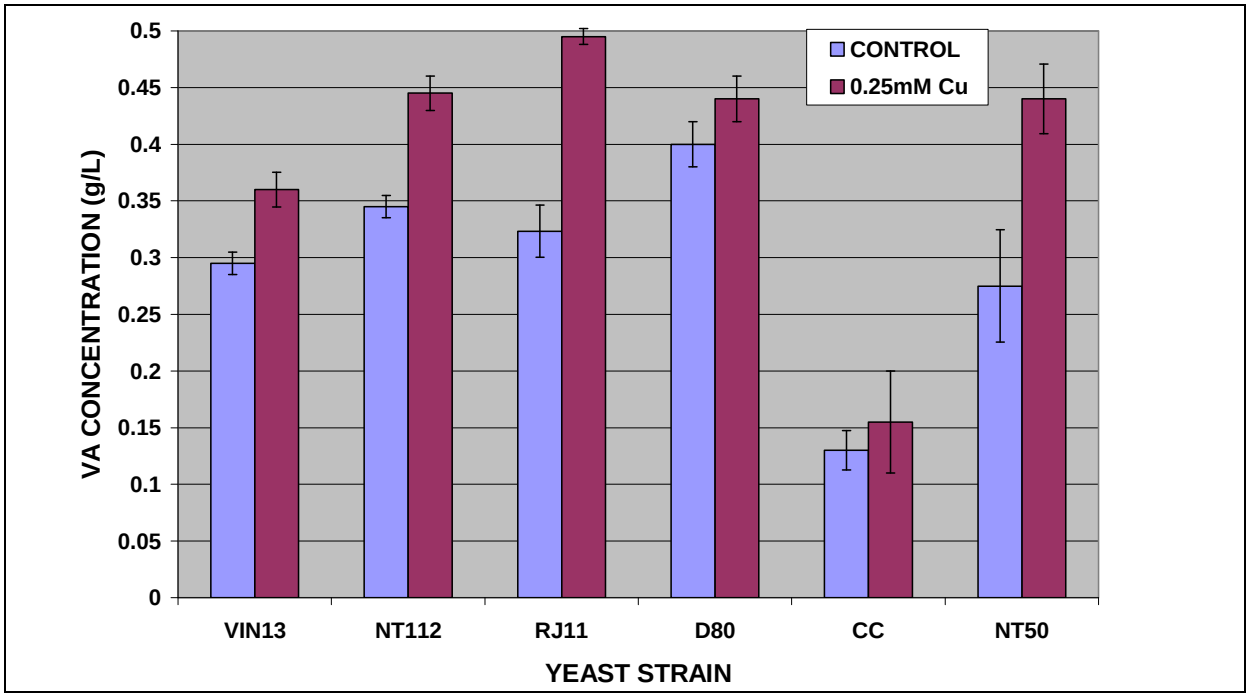

FIGURE 4.10 Volatile acidity concentrations after 21 days of fermentation in must fermented with different commercial wine yeast strains with no added Cu^{2+} and of must with 0.25 mM Cu^{2+} added.

4.3.5 Copper uptake by *S. cerevisiae*

In order to survive, but still protect themselves against metal poisoning, yeast cells requires homeostatic mechanisms to regulate intracellular copper levels (Hamer *et al*., 1985). Copper is transported inside the cell by Ctr1 and Ctr3 proteins and appropriately distributed to Cu-requiring proteins. *CTR1* was shown to encode these high affinity plasma membrane copper transporters (Yu *et al*., 1996). The cells also possess sensors to detect high Cu concentrations which activate protecting mechanisms (metallothionein production) and deactivate Cu transport mechanisms

(Azenha *et al*., 2000). Copper uptake studies in *S. cerevisiae* are however, complicated by the known precipitation of Cu^{2+} ions on membranes as copper sulphide (CuS) mineral lattices (Yu *et al*., 1996).

The copper concentrations of wine after fermentation with different commercial wine yeast strains are shown in **Fig. 4.11**. From this figure it appears as if copper transport into the cells of NT112 and CC was significantly less in comparison with the other four strains. Wine fermented with NT112 contained the most copper after fermentation with more than 7 mg/L Cu^{2+}, while wine fermented with CC contained more than 4 mg/L Cu^{2+} after fermentation.

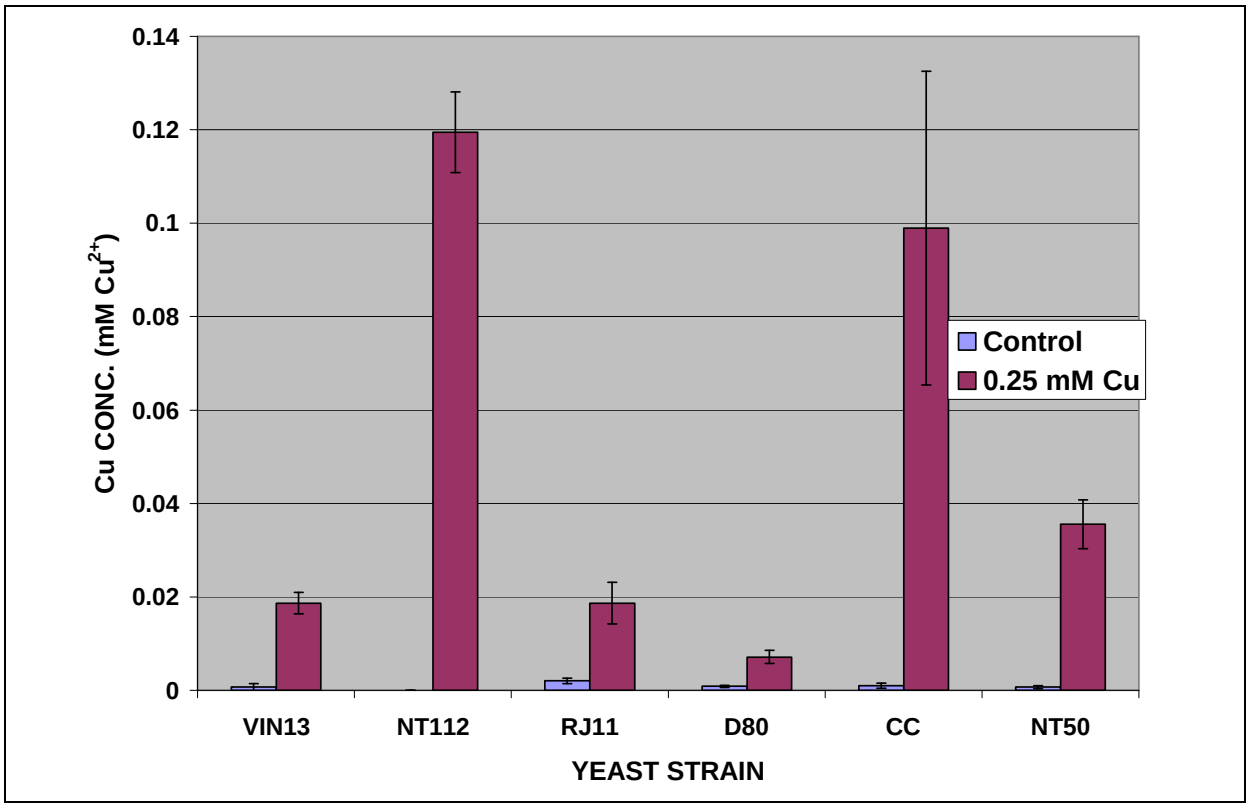

FIGURE 4.11. Cu^{2+} analysis of wine after fermentation with different yeast strains.

4.4 CONCLUSIONS

Fermentation of high sugar must with strains VIN13, WE14, N96 and RJ11 appeared to be least affected during the first few days of fermentation, although it was VIN13, WE372, N96 and L2056 that contained significantly less fructose after fermentation. VIN13 and RJ11 produced the lowest VA of the strains tested, whereas WE372, NT50, N96 and L2056 contained more than 0.7 g/L VA in the 28°B must. It however, appears as if more VA is produced in high sugar musts when fermented with N96 and L2056. It is further clear that fermentation is far less efficient in must containing copper residues when fermented with VIN13, NT112 and RJ11. Fermentation with

D80, CC and NT50 on the other hand is not significantly affected. Another conclusion derived from this study is that the presence of copper stimulates the production of VA by yeast strains.

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CHAPTER 5

General discussion & conclusions

5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 CONCLUDING REMARKS AND OTHER PERSPECTIVES

It is a well known fact that the production of high quality wine is no longer only a form of art, but in fact a science on it's own with factors such as microbial, chemical and physical, working together to form a very complex fermentation substrate. A large amount of research has been done in the last few years on all aspects affecting wine quality. It is however, important that new research is needed to fulfil the demand for knowledge in the ongoing strive for quality wine.

Alcoholic fermentation, the conversion of hexose into ethanol and CO_2, forms the very basis of a successful wine fermentation. This biochemical reaction can be driven by different yeasts, either occurring naturally on the grapes, or by addition of commercial yeast starter cultures to the crushed grapes. The most commonly encountered species is *Saccharomyces cerevisiae*, of which different strains have been selected over the years to fulfil the different needs of different products under different conditions. Incomplete or very slow alcoholic fermentations is called stuck and sluggish fermentations, and can lead to either a complete loss of product or to a diminished quality thereof. Stuck and sluggish fermentations is a chronic problem, but also one of the most challenging in wine production and have received much attention in the past (Henschke, 1997; Alexandre & Charpentier, 1998; Bisson, 1999). With the wide variety of commercial wine yeast strains available today it is necessary that wine producers should be informed about the advantages and disadvantages of these strains, under different stress conditions in must.

Although cutting edge research has been done at the Institute for Wine Biotechnology, Stellenbosch University, to develop genetically improved wine yeast strains, these strains cannot be legally used yet. It is thus important that the available commercial strains should be tested under different stress conditions in order to ensure optimal fermentation performance under specific conditions. These stress conditions can be any grape-related factors that influence yeast performance like: physical properties such as pH, high sugar levels, nitrogen content, and the presence of microflora on the grapes, such as yeast, acetic acid bacteria (AAB) and lactic acid bacteria (LAB) (Bisson, 1999; Bauer & Pretorius, 2000).

Although it was first believed that AAB are only associated with aerobic environments various studies showed that AAB are able to survive and even grow in the relative anaerobic winemaking process (Drysdale & Fleet, 1985; Du Toit, 2000). Factors affecting the growth of AAB in must and wine that have been studied in the past include ethanol, pH and acidity, oxygen, SO_2 and temperature (Du Toit, 2000). The effect of different yeast strains however, on acetic acid bacteria (AAB) numbers during wine fermentations have not been investigated in the past. In this study AAB and LAB numbers were monitored in small scale red wine fermentations inoculated

with different commercial wine yeast strains. On average all the AAB numbers decreased from 10^5 cfu per ml before fermentation to 10^2 - 10^3 cfu per ml after fermentation with no significant difference between the different yeast strains. Although this work served as an initial screening through a shotgun approach, it gave us some insight on the effect of different yeast strains on AAB as a whole. Future work should however, focus on specific AAB strains and thereby try and establish if a direct correlation between certain yeast strains and AAB strains exist.

Interactions between yeast and LAB, in particular *Saccharomyces cerevisiae* and *Oenococcus oeni,* have been studied in much more detail in the past (Fornachon, 1968; Henick-Kling & Park, 1994; Alexandre *et al*., 2004). Studies like this is important to establish a friendlier environment for growth and activity of *O. oeni* in wine to carry out malolactic fermentation (MLF) after alcoholic fermentation. In our study LAB numbers increased in the 2002 season during fermentation for all the yeast strains used, except for NT112 because of this strain's high SO_2 production. The high SO_2 production of this strain also delayed (MLF) in the 2002 and 2003 season. In this study we focused on the production and release of metabolites that are bioactive towards LAB, namely SO_2 and certain medium chain fatty acids (MCFAs). In future work the consequence of competition for and availability of nutrients and growth factors between *S. cerevisiae* and *O. oeni* should also be looked at.

The effect of high sugar levels on fermentation with different commercial yeast strains was also investigated. This is especially important in a warm climate region, like South Africa, where very high sugar levels are reached at harvest. It is thus important that winemakers should know which strains to use in such circumstances, especially when other stress factors also exist in the must. Strains that were identified as strong fermenters in high sugar musts included VIN13, WE372, N96 and L2056, while strains that fermented slower under these high sugar levels included NT50, RJ11, Collection Cépage Cabernet (CC) and D80. Higher sugar concentrations also enhance the production of acetic acid by the yeast (Henschke & Jiranek, 1993). From the commercial strains tested in this study WE372, NT50, N96 and L2056 showed higher VA levels after fermentation, while VIN13 and RJ11 always contained the least VA after fermentation in especially the high sugar musts.

Another factor which can lead to stuck and sluggish alcoholic wine fermentations is the presence of certain pesticides, such as certain copper containing pesticides (Vidal *et al*., 2001). The effect of copper on yeast growth has been the focus point of many studies (Presta & Stillman, 1997; Azenha *et al*., 2000) and the fact that different yeast strains differ in their tolerance to elevated copper levels has been known for a long time (Welch *et al*., 1983, 1989). Our study showed the differences between some commercial yeast strains with respect to copper resistance and also revealed that some strains produce more VA in the presence of copper than other strains. In our trials we found significant differences between the tolerance of commercial yeast strains used by winemakers today in must containing maximum allowable copper

residues. Strains that struggled to finish fermentation in the presence of 0.25mM Cu^{2+} were VIN13, NT112 and RJ11. All three of these strains together with NT50 also produced significantly more VA in the presence of copper in comparison with must containing no copper. D80, CC and NT50 showed no difficulty in fermenting the 0.25 mM Cu^{2+}-must to dryness within 21 days. The difference in copper sensitivity of commercial wine yeast strains can play an important role when the winemaker chooses his/her yeast strains for a certain wine. In future work other pesticides and fungicides should also be considered testing for inhibiting yeast cell growth and fermentation activity.

The complexity and interaction of factors that can influence the performance of commercial wine yeast strains makes the study of stuck and sluggish fermentations very difficult. Every piece of new information increases our knowledge, and this is important to better understand the complexity of wine so that these kind of fermentation problems can be minimized in the future to strive for wines of higher quality.

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