Improving wine yeast for fructose and nitrogen utilization

by

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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SUMMARY

In the wine industry, the importance of selecting an appropriate yeast strain, generally of the species *Saccharomyces cerevisiae*, to ensure reliable fermentation and to achieve a desired level of quality has been well established. As a consequence, the demand for new starter cultures with improved or new oenological characteristics is increasing. Appropriately selected starter cultures can reduce the occurrence of stuck fermentations, impart specific aroma profiles and reduce the development of off-flavours.

Using standard breeding and selection procedures, several wine yeast strains that would be less likely than currently existing strains to experience stuck fermentation have previously been developed at the Institute for Wine Biotechnology. The target of these projects had been to develop strains with improved nitrogen efficiency [defined as the amount of fermented hexoses for a given amount of free amino nitrogen (FAN)], improved fructose utilization and ethanol tolerance. These three parameters are known contributors to stuck fermentation. Two of the strains that had been isolated in these projects, strain 116 for nitrogen efficiency and strain 38-1 for efficient fructose utilization, were chosen as parental strains for the current study. The aim was to further improve and possibly combine these traits in yeast strains by using hybridization followed by various enrichment and directed evolution procedures in a continuous fermentation set-up.

The strategy was to sequentially subject the population of mass-mated hybrids to a number of selective environments for a large number of generations. The yeasts were subjected to a high fructose/glucose ratio for 12 generations, followed by selection in an environment with a limited supply of nitrogen for 54 generations and finally to high ethanol stress. After each round of enrichment, individual strains were analysed to assess the results.

For the hybrid strains selected after enrichment in a medium with a high fructose/glucose ratio, no general improvement could be discerned. However, one of the hybrids, hybrid strain 331, fermented fructose better than the parental strains and other hybrid strains. These results may suggest that the selection pressure was not applied for a sufficient number of generations and may not have been sufficiently strong. In addition, the parental strain may already performing at a rate that may render further improvement more difficult in this genetic background.

The next aim of this study was to enhance fermentation performance of wine yeast hybrid strains in low nitrogen and high sugar conditions. Several hybrid strains 331, RR03 and 05R generated in this study showed improvement in efficiency of nitrogen utilization when compared to the parental strains, indicating a successful selection strategy.

Several strains also showed higher ethanol tolerance, and some strains possessed] combinations of the traits to be improved.

Future research will evaluate these hybrids regarding the production of aromatic compounds and of the sensory profile produced. Such strains would help the wine industry to control the occurrence of stuck fermentations and to produce quality wines.

OPSOMMING

Die belangrikheid daarvan om die korrekte keuse te maak met betrekking tot ‘n gepaste gisras, gewoonlik van die *Saccharomyces cerevisiae*-spesie, om sodoende ‘n betroubare gistingsproses en ‘n bepaalde gehaltevlak te verseker, is reeds deeglik in die wynbedryf gevestig. Gevolglik is daar ‘n toename in die aanvraag na nuwe aanvangskulture met verbeterde of nuwe wynkundige eienskappe. Geskikte aanvangskulture kan die voorkoms van steekfermentasies verminder, spesifieke geurprofiele meebring en die ontwikkeling van wangeure verminder.

Deur die gebruik van standaard teling- en seleksieprosedures is verskeie wyngisrasse deur die Instituut van Wynbiotegnologie ontwikkel wat minder geneig is tot steekfermentasies as bestaande gisrasse. Die doel van hierdie projekte was om gisrasse te ontwikkel met verbeterde stikstofdoeltreffendheid (gedefinieer as die hoeveelheid gefermenteerde heksose vir ’n gegewe hoeveelheid vry aminostikstof (FAN), verbeterde fruktosebenutting en etanoltoleransie. Hierdie drie parameters is bekend daarvoor dat hulle steekfermentasies tot gevolg het. Twee gisrasse wat tydens vorige projekte geïsoleer is, 116 vir stikstofdoeltreffendheid 38-1 vir doeltreffende fruktosebenutting, is as ouerrasse vir hierdie studie geselekteer. Die doel was om hierdie eienskappe verder te verbeter en moontlik te kombineer deur gebruik te maak van hibridisasie gevolg deur verskeie verrykings- en gerigte evolusieprosedures in ‘n chemostaat.

Die strategie was om die populasie hibriede agtereenvolgens in ‘n selektiewe omgewing onder druk te plaas vir ‘n groot aantal genarasies. Die giste was blootgestel ann ‘n hoe fruktose / glukose omgewing geselekteer is. Alhoewel, een van die hibriede, hibried 331 fruktose beter gefermenteer het as die ouerras en as die ander hibriede. Hierdie resultate dui daarop dat ons seleksiedruk dalk nie toegepas is vir ‘n voldoende aantal generasies nie en dat die druk dalk nie sterk genoeg was nie. Dit kan ook wees dat die ouers alreeds op so hoe vlak funksioneer dat dit baie moeilik sal wees om die ouers se vermoë verder te kan verbeter.

Die ander belangrike doel van hierdie studie was om die gistingsvermoë van wyngisrasse in lae stikstof en hoë suikertoestande te verbeter. Die hibriedrasse 331, RR03 en 05R wat tydens die studie ontwikkel is, het beduidende verbetering in doeltreffende stikstofbenutting, en dus gisting oor die algemeen, getoon in vergelyking met hul ouerrasse, wat op ’n suksesvolle verryking strategie aandui.

Verskeie hibriedrasse het ook ’n verbeterde etanoltoleransie getoon. Verder het sommige van hierdie rasse ’n kombinasie van beoogde verbeterde eienskappe besit.

Toekomstige navorsing sal die hibriede beoordeel ten opsigte van die vorming van geurverbindings en sensoriese prfiele. Sulke hibriede kan die wynbedryf help om die voorkoms van steekfermentasies te beheer en hoe kwaliteit wyne te produseer.

This thesis is dedicated to my parents, Melida Manhlapile and Gilbert M Lekau Legodi and my daughter, Kamogelo and my sisters, Dikeledi, Makgwahla, Madira, Makhudu and my brother Lefokana

Hierdie tesis is opgedra aan my ouers, Melinda Manhlapile en Gilbert M. Lekau Legodi, en my dogter Kamogelo, my susters, Dikeledi, Makgwahla, Madira, Makhudu en my broer Lefokana

BIOGRAPHICAL SKETCH

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PREFACE

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

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Chapter 2 **LITERATURE REVIEW**
Understanding the intrinsic factors of grape must and their effect on wine yeast strains during fermentation

Chapter 3 **RESEARCH RESULTS**
Breeding and Chemostat as tools for improving and selecting wine yeast strains of *Saccharomyces cerevisiae* possessing desired oenological traits

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Chapter 1

GENERAL INTRODUCTION AND PROJECT AIMS

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Wine yeast strain improvement is a continuous process aiming at meeting the evolving requests of the winemaking field (Giudici *et al*., 2005). Breeding strategies in agricultural sciences have historically been used to select new, optimised plant varieties or animal breeds. Similar strategies are possible for the genetic improvement of wine yeasts (Marullo *et al*., 2006). Rational genetic improvement programs of yeasts, as for any other organism, must be based on the ability to achieve a precise function or to perform a specific task. Wine yeast strain improvement strategies are numerous and often complementary to each other and the choice among strategies is based on several factors, including the knowledge of the genetic nature of the desired trait (monogenic or polygenic), the knowledge of the genes involved (rational or blind approaches), and the aim of the genetic manipulation (Giudici *et al*., 2005). Classical genetic approaches were first applied to wine yeast strains in the middle of the 1980s, in response to increasing demand for new characteristics (Barre *et al*., 1993). Starter culture strains of *Saccharomyces cerevisiae* should posses a wide range of specialized properties in order to meet the new and challenging demands of the wine producers and consumers (Pretorius, *et al*., 2003). The demand for more specialized wine yeast has continued to grow and the number of commercialized, selected wine yeast strains has increased from about 20 in 1985 to more than 200 today (Barre *et al*., 1993; Pretorius *et al*., 2003).

The principal targets for improvement in wine yeast strains have been divided into the primary and secondary properties. Primary properties are related to central carbon metabolism and fermentative activity, including (1) high fermentation vigour defined as the upper most concentration of ethanol obtainable by fermentation from an excess of sugar, (2) high fermentation purity - expressed as the ratio between volatile acidity (acetic acid, g/l) and % (v/v) ethanol produced at the end of the fermentation process, and (3) high fermentation rate – measured as the ability of a starter culture to bring the fermentative process to fast completion. The secondary properties are defined as those related to the production of compounds that affect other wine quality parameters, such as the body of a wine, its aroma and flavour, including the production of the higher alcohols, esters and monoterpenes or of undesirable off-flavours (Martini, 2003).

Usually, the most important oenological traits, such as fermentative vigour, ethanol yield and tolerance, and growth temperature profile depend on a multitude of loci, qualitative trait loci (QTLs), which are not well characterized, because they are broadly distributed throughout the genome. Monogenic traits are easily extracted by tetrad analysis, because it allows obtaining a monospore culture always expressing the desired phenotype. Polygenic traits are not easily retrievable from the parent genome, because all genes responsible for complex desired phenotype must be co-inherited and present in the resulting hybrids. Moreover, the desired phenotype cannot be always expressed in a single-spore culture (Giudici *et al*., 2005).

Although the classical techniques used in genetic improvement of yeast have been shown to be useful in providing strains with novel characteristics for winemaking, they lack the specificity required to construct a strain with an exact combination of characteristics. In this case, recombinant DNA technology becomes the technique of choice to overcome these limitations. The application or recombinant DNA techniques can go far beyond introducing specific genes into yeasts and this technology has provided some promising results in the improvement of wine yeasts (Rainieri and Pretorius, 2000). However, the successful commercialization of transgenic yeasts for the fermentation industry will depend on a multitude of scientific, technical, economic, marketing, safety, regulatory, legal and ethical issues (Pretorius, *et al*., 2003).

The most tedious part of a breeding project is the selection and testing of hybrids to reduce their numbers for industrial scale testing. Traditional methods had relied on direct plating of the isolates on agar plates. This selection approach is non-targeted and non-specific and a large number of strains needs to be screened to isolate an improved mutant from the mixed population (Parekh *et al*., 2000). The use of chemostat cultures as “evolutionary devices” for selection of mutant micro-organisms was already described in the 1950s and has since been successfully applied to improve numerous physiological traits (Weikert *et al*., 1997; Arensdorf *et al*., 2002). This creates an environment in which the “fitness” of the cells is narrowly dependent upon the rate and /or efficiency with which they metabolise low concentrations of a critical nutrient (Francis and Hensche, 1971). In the case of baker’s yeasts, a strong selection and screening in conjunction with traditional mass mating technique was applied to *S*. *cerevisiae* and improved to efficiently leaven dough (Higgins *et al*., 2001). A similar approach was employed in this study in which chemostat conditions were set to enrich the traits listed under the project aims.

1.2 PROJECT AIMS

Fermentation problems, including sluggish and stuck fermentation, may have serious consequences for wine quality. Besides undesirable high sugar contents in the case that the problem can not be addressed, such fermentations frequently result in the production of off-flavours and high volatile acidity, and may lead to microbial spoilage. The direct consequences are financial losses. For this reason, there is a quest for wine yeast strains that are better able to withstand some of the more common causes of stuck fermentation. In the literature, the most commonly cited causes include insufficient concentrations of available nitrogen, a high fructose over glucose ratio and high level of ethanol. A low concentration of metabolizable nitrogen leads to low biomass formation at the beginning of fermentation, resulting in insufficient fermentation vigour. A high ratio of fructose over glucose is usually observed towards the end of fermentation since fructose is used less efficiently than glucose. A ratio above a certain threshold has been described as inhibitory to wine yeast strains. High ethanol levels also have an inhibitory effect on yeast strains. Two hybrid strains, 116 and 38-1 have been selected in

previous studies at the IWBT. The first strain was selected after enrichment for nitrogen efficient strains, whereas the other strain was selected based on fructose utilization and ethanol tolerance. In this study, we aimed at further improving the genetic makeup for the above traits by hybridization and chemostat enrichment techniques. The approach was to subject the hybrids obtained from mass mating to high sugar levels with variable ratios of glucose and fructose, while at the same time increasing selection pressure by limiting the nitrogen content of the must. In particular, we lowered the concentrations of the amino acids usually preferred as nitrogen sources by *S. cerevisiae*, including leucine, arginine, aspartic acid, glutamic acid and glutamine. The selected hybrids were then evaluated in small-scale fermentation, using media containing variable glucose and fructose ratios, low nitrogen content and high sugar levels.

The specific aims of this study are the following:

1. To improve efficiency of fructose uptake and fermentation in general.
2. To improve fermentation performance at low nitrogen and high sugar concentrations.
3. To improve for ethanol tolerance.

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Chapter 2

LITERATURE REVIEW

Understanding the intrinsic factors of grape must and their effect on wine yeast strains during fermentation

2. LITERATURE REVIEW

2.1 INTRODUCTION

2.1.1 Yeast and winemaking

Winemaking is one of the most ancient technologies of Mankind and is now one of the most commercially prosperous biotechnological processes. Advances in the second half of the 20th century have clearly shown that fermentation of grape must and the production of quality wines is not quite as simple a process as Pasteur, the founder of modern oenology, suggested over a century ago (Moreno-Arribas and Polo, 2005). Traditionally, wines have been produced by natural fermentation due to the development of yeasts originating from the grapes and winery equipment (Esteve-Zarzoso *et al*., 2001). As the importance of *Saccharomyces cerevisiae* in winemaking has long been established, the use of commercial strains of yeast cultures in fermentation is however becoming a common practice and helps to reduce the risk of wine spoilage (Pretorius, 2001; Cappello *et al*., 2004), to prevent stuck fermentation and to improve wine quality (Marullo *et al*., 2004). However, considering the diversity of demands by winemakers and diversity of wines and wine styles, there is no single wine yeast strain that would possess an ideal combination of oenological characteristics. It is clear that the properties of strains should differ with the type and style of wine to be made and the technical requirements of each winery (Snow, 1983; Pretorius, 2001).

While there is a diversity of differing demands regarding wine yeast strain characteristics, there is nevertheless also a clear demand for improvement of some generic features of wine yeast strains. One of these demands concerns yeast that would be less sensitive to conditions that could lead to stuck or sluggish fermentation. Stuck or sluggish fermentation and the frequent development of off-flavour and microbial spoilage under such conditions, are some of the biggest problems that winemakers and the wine industry are facing. Several factors have been investigated and reviewed for their effect on the fermentation process, including temperature, nutrient imbalance (mainly nitrogen sources, minerals and vitamin deficiency), elevated sugar levels, high ethanol content and the type of yeast strain used. Current practices in the industry to overcome these problems include supplementation of the grape must with nitrogenous compounds such as di-ammonium phosphate (DAP), Fermaid and other yeast foods.

This chapter will focus on and emphasize the effects of high sugar concentration on the behaviour of yeast strains (or yeast activity), and on the consequences of nutrient deficiencies in the musts, in particular of nitrogen.

2.1.2 Fermentation of grape must

Fermentation is the key process that transforms grape must into wine. During fermentation the principal grape sugars, glucose and fructose, are converted to ethanol, carbon dioxide and many other constituents (metabolites). The conversion of grape must sugars to ethanol is of central importance in wine production. There are two practices of fermentations, namely spontaneous (un-inoculated) and inducted (inoculated) fermentations.

In a spontaneous fermentation the growth of many microorganisms that are present on grapes or in the cellar, including species such as *Kloeckera apiculata*, *Hanseniaspora uvarum*, *Candida stellata*, *Torulaspora*, *Hansenula*, *Metschnikowia* and *Pichia spp*., predominates in the early phase (Boulton *et al*., 1998; Maráz, 1999). Most of these species are sensitive to increasing levels of ethanol levels, and the majority are completely inhibited when the ethanol content reaches 4% (v/v) (Kunkee and Amerine, 1970). *S*. *cerevisiae* on the other hand, becomes the dominant species when the ethanol content reaches 5% (v/v) (Maráz, 1999). When inoculation with commercially produced active dried wine yeast strain is used, the inoculated strain tends to dominate the fermentation from the beginning. Since the commercial strains are well characterised, such inoculation provides winemakers with a better control of the process, and a better ability to achieve a specific desired outcome. The advantages and disadvantages of the two practices of fermentations have been reviewed in much detail (Pretorius, 2001).

Different commercial strains display markedly different fermentation profiles (Bisson and Butzke, 2000). The activity of such strains depends on grape must composition and oenological practices (Cavazza *et al*., 2004). The variations in the composition of grape must make the fermentation kinetics of wine unpredictable (Sainz *et al*., 2003). The primary objective of making dry table wine is to achieve a complete conversion of grape sugar into alcohol and carbon dioxide at a controlled rate through fermentative activity of wine yeasts (Henschke and Jiranek, 1993), so that the residual sugar in the wine is less than 2-4 g/L (Alexandre and Charpentier, 1998). Under unfavourable conditions, fermentation may be incomplete with high residual sugar, commonly known as stuck fermentation or fermentation proceeds at a rate below average known as sluggish fermentation. Sluggish fermentations present a risk of becoming stuck. Such stuck fermentations occur when nitrogen poor musts or musts with high sugar concentrations are used or when high temperatures are reached during the process (Ivorra *et al*., 1999).

2.2 MAJOR COMPONENTS OF GRAPEMUST AND THEIR IMPACT ON FERMENTATION BEHAVIOUR OF YEASTS

2.2.1 SOLUBLE SOLUTES IN GRAPE MUST

In grapes the main carbohydrates are glucose and fructose, while small amounts of rhamnose, arabinose, xylose, sucrose, and pectin are present (Kliewer, 1967; Margalit, 1997). It is obvious that glucose and fructose, being the main substrates of fermentative growth, impact most directly on the fermentation behaviour of wine yeast strains. Glucose and fructose concentrations affect fermentation in two major ways: The absolute concentration is of importance, since it will directly determine ethanol concentrations and the length of fermentation. The current tendency to harvest fruit at high initial sugar content may result in inhibitory ethanol concentrations and be a contributing factor to the appearance of slow and incomplete fermentation. Indeed, the high sugar requires the expression and maintenance of different enzyme systems that protect the yeast cells from a hyperosmotic and toxic environment (Bisson and Butzke, 2000).

A second important factor is the concentration ratio between those two sugars. The initial ratio of glucose-fructose in grape musts differs from one grape variety to another and is influenced by the harvesting time, regional climatic conditions etc. Kliewer (1967) determined the concentration of glucose, fructose and total soluble solids in the fruits of 28 table varieties, 26 red wine varieties and 24 white wine varieties of *Vitis vinifera* L. The glucose-fructose ratio for the wine grape varieties ranged from 0.74 to 1.05 (Kliewer, 1967). Most research groups found that while glucose predominates in unripe grapes, the glucose-fructose ratio at maturity is about 1 and fructose constitutes the major sugar in overripe grapes (Kliewer, 1967). There is some evidence that a low glucose/fructose ratio may be a contributing factor in many stuck or sluggish fermentations (Berthels *et al*., 2005; 2008).

2.2.1.1 Genes regulating utilization of sugar

Since fermentation is a relatively inefficient way of generating energy, a high glycolytic flux and efficient transportation of sugar molecules, i.e. glucose and fructose is essential. *S. cerevisiae* strains are able to increase their glycolytic capacity by induction of large number of glycolytic genes. For example, glucose utilisation is increased through induction of several hexose transporter (*HXT)* genes (Özcan and Johnston, 1995; Rolland *et al*., 2002). The expression of these specific transporters depends on the concentration of glucose in the medium. High affinity transporters like Hxt6 and Hxt7 are repressed by high concentration of glucose, whereas transporters with low affinity such as Hxt1 and Hxt3 are induced by presence of high concentration of glucose (Rolland *et al*., 2002; Luyten *et al*., 2002). The transporters with intermediate affinity for glucose like Hxt2 and Hxt4 are iduced by low concentration of glucose and repressed

by high concentration of glucose (Rolland *et al*., 2002). Luyten *et al*., (2002) had carried out functional analysis of the *HXT1-7* genes to investigate the role of hexose transporter proteins in synthetic must. The deletion of *HXT2* gene resulted only in a delayed start of fermentation and did not affect fermentation profile. This suggested that Hxt2 transporter protein is involved in lag phase initiation of growth despite high concentration of sugar in the medium, which might be expected to repress expression of the *HXT2* gene (Luyten *et al*., 2002). Mutant carried *HXT3* gene only with other genes deleted were able to grow on glucose containing media regardless of the concentration of glucose (Özcan and Johnston, 1995; Luyten *et al*., 2002). Therefore Hxt3 transporter was thought to play a significant role during fermentation. The Hxt6 and Hxt7 are required at the end of alcoholic fermentation; therefore they must perform efficiently in a medium containing large amounts of ethanol (Luyten *et al*., 2002).

2.2.1.2 Effect of high sugar concentrations on wine yeast strains

The high sugar content in must influences fermentation behaviour in various ways. Upon inoculation, it produces an osmotic stress in yeast cells, which cells must resist in order to carry out the fermentation (Ivorra *et al*., 1999). In response to hyperosmolarity, *Saccharomyces* is able to establish, firstly, immediate cellular changes that occur as a direct consequence of the physico-mechanical forces operating under those conditions. Secondly, primary defence processes are elicited in order to set protection, repair and recovery in motion. Lastly, sustained adaptive events permit restoration of cellular homeostasis under the new circumstances (Mager and Siderius, 2002). Once the cell has adapted, which is a process that demands expensive structural reassignment and maintenance, the cell optimises the growth rate depending on the availability of nutrients (Sainz *et al*., 2003).

If the sugar concentration is raised beyond a strain-dependent limit, for example in late or noble late harvest grapes, the rate of fermentation and maximum amount of alcohol produced decreases (Kunkee and Amerine, 1970). There is also considerable variation depending on the species and strain and the conditioning of the yeast to grow at high sugar concentrations (Kunkee and Amerine, 1970; Reed, 1982). Alcohol production can be lower in a must containing 300 g/L of sugar than in a must containing only 200 g/L of sugar. At ranges beyond 350 g/L of sugar, the concentrated grape must becomes practically non-fermentable. Thus, an elevated amount of sugar hinders yeast growth and decreases the maximum population. Consequently, fermentation slows and can become stuck even before a significant quantity of ethanol is produced (Ribéreau-Gayon *et al*., 2000).

2.2.1.3 Effect of high ethanol concentrations on wine yeast strains

While the efficient conversion of grape sugar to ethanol is of importance in winemaking, it is also vital to secure the availability of wine yeast strains possessing inherent

tolerance toward the ethanol formed. The behaviour of a given yeast cell is dependent on two factors, namely genetic constitution and environmental conditions (Rose, 1987; Ribéreau-Gayon *et al*., 2000).

Ethanol inhibits yeast growth with the yeast cell membrane being the primary target of ethanol toxicity (Ingram and Butzke, 1984). Ethanol indeed permeabilises the cellular membranes. In an acidic wine environment, this will lead to an influx of protons into the cell. To avoid intracellular acidification the cell activates the enzyme ATPase which acts as a proton pump. This energy-intensive mechanism will result in reduced growth and finally growth arrest of cells that remain nevertheless metabolically active and continue to ferment.

The ethanol tolerance of yeast strains to ethanol can be modified by environmental factors, such as aeration or addition of sterols and unsaturated fatty acids or nitrogenous compounds (Rose, 1987; Kunkee and Bisson, 1993). Several hypotheses about ethanol tolerance in yeast have been made, firstly, that incorporation of oleic acid into cell membrane counteracts the fluidizing effects of ethanol and that ethanol inhibits hexose transporters. This is supported by the findings that unsaturated fatty acids composition, particularly oleic acid is the most efficacious in overcoming the toxic effects of ethanol in growing yeast strains (You *et al*., 2003) and some non-*Saccharomyces* yeasts (Pina *et al*., 2004). Sterols and unsaturated fatty acids in particular can not be synthesised under anaerobic conditions such as wine fermentation to provide the yeast cell membrane with more stability, reducing the negative impact of ethanol. The intrinsic resistance to ethanol by various yeast strains has been investigated. However, approaches to determine the ethanol tolerance of yeast are mostly based on growth in the presence of exogenous ethanol (Kalmokoff and Ingledew, 1985; Jiménez and Benítez, 1988; Ansanay-Galeote *et al*, 2001) and consequent measurements of viability (Kalmokoff and Ingledew, 1985). Some researchers however, argue that differences in ethanol tolerance that are found using methods based on growth and viability loss may rather reflect differences in nutritional requirements (Kalmokoff and Ingledew, 1985; Kunkee and Bisson, 1993 and references therein) and that the ethanol tolerance established through such methods may not reflect on fermentation efficiency in wine, sake production and the distilling industries. Santos *et al*., (2008) challenged the current notion that ethanol tolerance expressed in terms of cell viability is a reliable criterion for the selection of yeast strains, particularly to restart stuck fermentations. Instead ethanol tolerance of yeast strains seems to be based on sugar transport proteins and their resistance to ethanol (Santos *et al*., 2008). Earlier, Ansanay-Galeote *et al*., (2001) also reported that decrease of fermentation rate was due to inhibition of hexose transporters by ethanol. This finding favoured the second hypothesis of ethanol.

Ethanol tolerance remains a controversial topic due to the complexity of inhibition mechanism and the lack of universally accepted definition and method to measure ethanol tolerance (D' Amore *et al*., 1990). *S*. *cerevisiae* wine strains show differences in their inherent ability to tolerate ethanol. The genetics of ethanol toxicity is thought to be

polygenic, since of the many research efforts to develop genotypically resistant strains, none has been met with real success (Boulton *et al*., 1998). Aquilera and Benitez (1985) have suggested that about 250 genes might be involved in the control of ethanol tolerance in yeast. The toxicity of ethanol on fermenting yeast leads to stuck fermentation. Therefore, the commercial interest in wine yeast strains that would tolerate high alcohol is increasing.

The genetics of yeast strains regulating its tolerance to high level of ethanol still remains poorly understood. Recently, Hu *et al*., (2007) generated short tandem repeats (STR) maker data and ethanol tolerance (ET) phenotype data of the segregant population (319 segregants) and used it to map QTL underlying phenotypic variation in ethanol tolerance through the composite interval mapping (CIM) analysis. Five QTL displaying significant effects on the trait phenotype was detected and mapped on chromosomes 6, 7, 9, 12, and 16. These QTL detected in the analysis explained a total of 47% of the variation in the ethanol tolerance trait. Mapping on chromosome 9 had the largest additive effect on the trait and it explained up to 25% of phenotypic variation of the trait. According to the CIM analysis five candidate genes fell into the QTL on chromosomes 6, 9, and 16. Chromosome 6 locates gene candidates *HXK1* and *RMD8*. Chromosome 9 locates *PFK26* gene and chromosome 16 harboured *VPS* gene family, namely *VPS16* and *VPS28*.

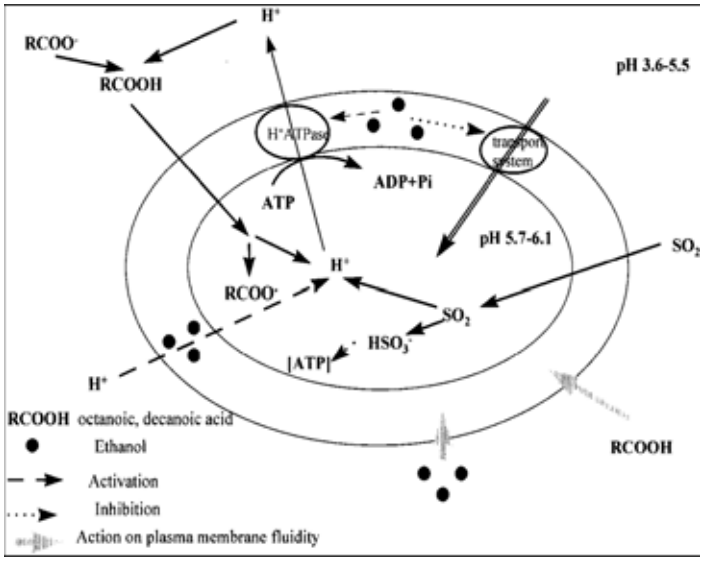

Fig.2.1 Mechanisms of inhibitors action on wine yeast metabolism during winemaking (Alexandre and Charpentier, 1998).

2.2.1.4 Effect of high acetic acid on wine yeast strains

Alexandre and Charpentier (1998) proposed a synergistic mechanism of action for ethanol and organic acid toxicity. The wine environment, because of its low pH, favours the influx of organic acids into cells since only the non-dissociated form can diffuse across the membrane. Once inside the cell, the acid will dissociate, adding to the

amount of protons inside the cell. This intracellular acidification has to be counteracted by the ATPase proton pump, leading to significant requirements for ATP and therefore reduced growth (**Fig. 2.1**). Acetic acid is a by-product of sugar metabolism. During fermentation it can be produced by yeast, fungi and bacteria (Eglinton and Henschke, 2001). A high concentration of acetic acid has been directly linked to stuck fermentations (Eglinton and Henschke, 2001). High permeability of the plasma membrane to not dissociated acetic acid and *S*. *cerevisiae*'s inability to metabolise the acid inside the cell, underlie the yeast's low tolerance to an environment of high ethanol and acetic acid (Casal *et al*., 1998). Acetic acid in high concentration enhances the toxicity of ethanol on yeast's growth, fermentation rate and viability (Rasmussen *et al*., 1995; Alexandre and Charpentier, 1998).

2.2.2 NITROGEN SOURCES AND COMPOSITION IN GRAPE MUST

A wide variety of nitrogen-containing compounds are found in grape must, including ammonia, nitrates, amines, amino acids, peptides, proteins and vitamins (Jackisch, 1985; Margalit, 1997). In terms of the quantitative nutritional requirements of yeast, nitrogen is the second most important nutrient. Amino acids are the most prevalent form of total nitrogen by weight in grape must and wine (Henschke and Jiranek, 1991; Ribéreau-Gayon *et al*., 2000). The grape nitrogen concentration depends on variety, root stock, environment and growing conditioning especially nitrogen fertilisation. Nitrogen content of grapes decreases in the case of over-ripening and rot development and in situations where the vine suffers from drought conditions (Sponholz, 1991; Ribéreau-Gayon *et al*., 2000). In the average grape must, proline and arginine usually represent 30 to 65% of the total amino acid content (Henschke and Jiranek, 1991; Boulton *et al*., 1998), while alanine, glutamate, glutamine, serine and threonine are also major nitrogen sources. Proline accumulation at high levels appears to be associated with grapevine stress, particularly low moisture (Boulton *et al*., 1998). Other nitrogen-containing compounds in musts and wines such as nitrates and nitrites, a variety of amines, vitamins, nucleotides and peptides are found only in small amounts (Jackisch, 1985). Grape musts from the New York area showed total amino acid contents varying from 220 mg/L to 1056 mg/L. The musts in particular contained highly variable amounts of arginine (16 to 136 mg/L), glutamine (13 to 314 mg/L), and asparagine (0 to 15 mg/L), the main amino acids involved in yeast nutrition (Sponholz, 1991). In contrast, German Müller-Thurgau juices showed high amounts of total amino acids ranging from 1217 to 4921 mg/L. Compared to the New York grape musts the arginine contents were found to be high (271 to 1043 mg/L) as were the contents of the amino acid amides (141 to 1246 mg/L). Therefore, concentration of free assimilable nitrogen (FAN) varies with cultivar, season and terroir. In the case of rot development, mainly *Botrytis cinerea*, the amino acid content can be significantly reduced, in one reported case by 41 % of the total amino acids (Sponholz, 1991). The decrease of individual amino acids caused by *Botrytis cinerea* is quite variable, ranging from 7% to 61%. It is of interest that, in

contrast to yeast, this aerobic organism may cause up to 51% decrease in proline (Sponholz, 1991).

2.2.2.1 Transport mechanism of nitrogen compounds in yeast strains

In *S. cerevisiae*, the plasma membrane is not freely permeable to nitrogenous compounds such as amino acids. Therefore, the first step in their utilization is the transport across the plasma membrane (Grenson, 1992). It is now established that *S. cerevisiae* has two classes of mechanism for transporting amino acids across the plasma membrane. There are general amino acid permease (GAP) which can transport all basic and neutral amino acids, but not proline. In addition, *S. cerevisiae* can synthesise a range of at least eleven transport systems each of which is specific for just one or a small number of amino acids (Rose, 1987). The regulation of these transport systems is such that only some are permanently present. These are called constitutive permeases and are ready to transport amino acids for protein synthesis at any time. The additional uptake systems, which are called adaptive or inducible, develop under conditions where they may be both necessary and sufficient for cell growth or survival (Grenson, 1992). Most of the transported amino acids are accumulated inside the yeast cells against a concentration gradient. When amino acids are to be used as a general source of nitrogen, this concentration is crucial because most enzymes which catalyse the first reaction of the catabolic pathways have a low affinity for their substrates (Grenson, 1992; Boulton *et al*., 1998).

Nitrogen control involves activation of the structural genes, which is prevented in the presence of preferred nitrogen sources (Marzluf, 1997). The general amino acid permease, with its broad specificity, its large capacity, and its regulation according to nitrogen availability, is well adapted for taking up any available amino acid as a source of nitrogen. Such characteristics lead to functional specialisation of the amino acid permease. Despite these functional specialisations, there is no exclusive use of a given permease for a specific purpose. For instance, L-arginine can be transported just as efficiently by the specific arginine permease as by the general amino acid permease, either to fulfil a specific arginine requirement in an arginine auxotroph or for use as general source of nitrogen (Grenson, 1992). The genetic diversity in the regulation of nitrogen uptake and its metabolism predicts that yeast strains will vary in their demand for both total and individual nitrogen compounds (Henschke and Jiranek, 1993).

2.2.2.2 Preference of nitrogen sources and their metabolism by yeast strains

The ability of yeast to assimilate various compounds as a source of nitrogen also varies greatly among the yeasts. Certain nitrogenous compounds such as ammonium, glutamine, and glutamate are preferentially used by fungi and yeast; asparagine is also a preferred nitrogen source. Ammonium ions reduce catabolic enzyme levels and transport activities for non-preferred nitrogen sources. This nitrogen catabolite

repression severely impairs the utilization of proline and arginine (Marzluf, 1997; Salmon and Barre, 1998). The utilization of any of the secondary nitrogen sources is highly regulated and requires the synthesis of a set of pathway-specific catabolite enzymes and permeases which are otherwise subjected to nitrogen catabolite repression (Marzluf, 1997).

Nitrogen containing compounds in grape must might be utilised (1) directly in biosynthesis, (2) converted to a related compound and utilised in biosynthesis or (3) degraded thereby releasing nitrogen either as free ammonium ion (NH_4^+) or as bound nitrogen via a transamination reaction (Henschke and Jiranek, 1991; Boulton *et al*., 1998). The biosynthesis of nitrogenous compounds is dependent on the ready availability of precursors in the cellular nitrogen pool. Under conditions where the intracellular supply of NH_4^+ and / or glutamate is limited, a reduced synthesis of nitrogen-containing compounds, including the sulphur containing amino precursors, *O*-acetylserine (OAS) and O-acetylhomoserine (OAH) will result (Henschke and Jiranek, 1991). Most free amino acids are readily assimilated by *S*. *cerevisiae* and are reduced to 10% or less of their original concentration by the end of fermentation. However, a large proportion of free proline remains, indicating that it is not as easily utilised as other amino acids (Van Heeswijck *et al*., 2001; Valero *et al*., 2003).

Arginine, the second most abundant amino acid, is a less readily utilised source of nitrogen. This makes it available for uptake during active fermentation as well as during stationary phase (Bisson and Butzke, 2000). Although arginine is often the most available amino acid in grapes, only three of its four nitrogen atoms are assimilated by *S*. *cerevisiae* during winemaking. The fourth is incorporated into proline, which cannot be used as a nitrogen source in the absence of oxygen (Martin *et al*., 2003). This lack of proline assimilation by yeast during fermentation is thought to be due, firstly, to inhibition of the yeast proline uptake system, proline permease, by other amino acids. Secondly, the enzyme required for proline catabolism in yeast, proline oxidase, requires oxygen for catalytic activity (Van Heeswijck *et al*., 2001; Martin *et al*., 2003).

The next preferred group of amino acids includes alanine, serine, threonine, aspartate, asparagines, urea and arginine. Glycine, lysine, histidine and the pyrimidines, thymine and thymidine cannot be utilised by most strains of *Saccharomyces* as a source of nitrogen, but they can readily be taken up directly as biosynthetic precursors (Boulton *et al*., 1998). However, the preference of utilization of nitrogen containing compounds may change depending upon environmental, physiological and strain-specific factors.

2.2.2.3 Effect of nitrogen concentrations on wine yeast strains

The dependence of yeast growth and fermentation activity on the concentration of assimilable nitrogen has led several investigators to define the nitrogen requirement under oenological and brewing conditions. The minimum assimilable nitrogen required for a satisfactory rate of fermentation in clarified juice is considered to be about 140 mg

FAN/L; however, optimum or maximum fermentation rate requires a higher concentration of up to 800-900 mg FAN/L of which only 400-500 mg N/L is assimilated (Henschke and Jiranek, 1993).

Nitrogen concentration (Blateyron and Sablayrolles, 2001) and different combinations of nitrogen sources (Henschke and Jiranek, 1993) influence fermentation kinetics and yeast growth (biomass formation). High nitrogen concentration increased the fermentation rate and decreased the fermentation time (Vilanova *et al*., 2007). During alcoholic fermentation the effect of nitrogen is greater on consumption rate of sugar by fermenting yeast strain than on yeast growth (Taillander *et al*., 2007). Insufficient nitrogen in the grape must diminishes metabolic activity of yeast and biomass yield. However, the effect of nitrogen on fermentation rate and biomass is not clearly distinguished since they are interdependent (Varela, *et al*., 2004). There is strong consensus amongst researchers that low nitrogen grape must affect the yeast ability to ferment sugar optimally. This offers an opportunity and possibility to breed wine yeast strains and select the best strain under nitrogen-limited conditions.

Recently, Mendes-Ferreira *et al*. (2007) used genome-wide of the wine yeast strain *S*. *cerevisiae* PYCC4072 to identify genes that could be potential candidates as biomarkers for predicting sluggish or stuck fermentations in nitrogen deficient and starved nitrogen conditions, irrespective of glucose availability, ethanol production or

Table 2.1 Thirty-six signature genes identified as potential candidates for predicting nitrogen deficiency under winemaking conditions and they overlap with other reported conditions[a] (Mendes-Ferreira *et al*., 2007)

ORF name	Gene name	Association with:					Reference(s)
		Ammonium starvation	Nitrogen limitation	Carbon limitation	ESR	Stationary phase	
YNL270c	*ALP1*			x			4, 21
YMR280c	*CAT8*			x			4, 21
YGL166w	*CUP2*						
YOR180c	*DCI1*			x			4, 21
YKR076w	*ECM4*		x	x	x		4, 6, 21
YPL222w	*FMP40*						
YDL222c	*FMP45*				x	x	6, 12
YMR250w	*GAD1*		x		x	x	6, 12, 21
YDL223c	*HBT1*			x		x	4, 12, 21
YOR391c	*HSP33*	x					26
YLR174w	*IDP2*	x		x		x	4, 12, 21, 26
YML128c	*MSC1*	x		x	x	x	4, 6, 12, 21, 26
YPL134c	*ODC1*	x		x		x	4, 12, 21, 26
YDR313c	*PIB1*						
YDL204w	*RTN2*	x		x	x	x	4, 6, 12, 21, 26
YIL113w	*SDP1*				x		6
YDR238c	*SEC26*						
YMR175w	*SIP18*			x		x	4, 12, 21
YGL208w	*SIP2*			x	x		6, 21
YGR248w	*SOL4*				x		6
YBL106c	*SRO77*						
YLR178c	*TFS1*				x		6
YBR006w	*UGA2*				x		6
YIL101c	*XBP1*				x	x	6, 12
YLR070c	*XYL2*						
YCR061w	*YCR061w*				x		6
YLR272c	*YCS4*						
YDL218w	*YDL218w*	x				x	12, 26
YDR271c	*YDR271c*						
YGR043c	*YGR043c*			x	x	x	4, 6, 12, 21
YLR312c	*YLR312c*	x	x		x	x	6, 12, 21, 26
YMR090w	*YMR090w*		x		x		6, 21
YMR206w	*YMR206w*			x			4
YNL115c	*YNL115c*				x		6
YNL194c	*YNL194c*			x	x		4, 6, 21
YNL195c	*YNL195c*			x	x		4, 6, 21

[a] See included references. x, gene expression is induced.

any other metabolites that can occur during winemaking conditions. Their study discovered 390 genes that were

significantly affected under the conditions cited above. Seventy-two of the above genes showed consistent high expression while 318 had lower expression under all nitrogen deprived conditions relative to the control (reference situation). It was found that 27 of 72 up-regulated genes and 128 of 318 down-regulated genes are among the environmental stress response, *ESR* genes (Mendes-Ferreira *et al*., 2007). This

indicates that lack of nitrogen or insufficient nitrogen under fermentation conditions induces stress on fermenting yeast.

Thirty-six genes were identified as promising candidates for predicting nitrogen deficiency during alcoholic fermentation (Table 2.1), therefore diagnosis of stuck /or sluggish fermentation and the remaining genes could be involved in non-specific responses to nitrogen limitation (Mendes-Ferreira *et al*., 2007).

2.3 COMPOUNDS PRODUCED BY YEAST DURING FERMENTATION

The metabolism of primary carbon and nitrogen-containing compounds yields a few end products of sensory importance for wine quality (Salmon and Barre, 1998). The nature and amount of these compounds influences the spectrum of the end products produced during fermentation (Bisson, 1991). For example, amino acids when deaminated form α-keto acids of higher alcohols (Salmon and Barre, 1998; Ribéreau-Gayon *et al*., 2000). Esters and higher alcohols are the most important secondary products and play a vital role in the aroma and flavour of wines (Lambrechts and Pretorius, 2000). Sensory properties of wines from must supplemented with amino acids depend on the yeast strain (Hernández-Orte *et al*., 2005). The association of specific yeasts with some metabolic characteristics will allow winemakers to produce wines with particular desired style.

2.3.1 Esters

Esters are a large group of volatile compounds and are produced by yeast as secondary products of sugar metabolism during alcoholic fermentation (Lambrechts and Pretorius, 2000). Esters can also be derived from chemical esterification of alcohols and acids during wine aging. Esters contribute to the aroma of wine. The most important acetates of higher alcohols are isoamyl acetate (banana aroma) and phenylethyl acetate (rose aroma) (Ribéreau-Gayon *et al*., 2000; Dequin 2001; Quilter *et al*., 2003). Isoamyl acetate is produced by yeast from isoamyl alcohol, which is itself a by-product of leucine synthesis and phenylethyl acetate (Ribéreau-Gayon *et al*., 2000). The amounts produced are dependent upon yeast species and strains. Other factors such as fermentation temperature, prior clarification and other vinification practices also influence the ester concentration in young wines. During maturation of wines, acetyl esters diminish, while ethyl esters increase in amount (Hühn *et al*., 1999). In the process ethyl esters of medium chain fatty acids which are not influenced by nitrogen are formed. They are formed by condensation of acetyl coenzyme A. These esters have more interesting aromas than others. Hexanoate has a flowery and fruity aroma reminiscent of green apples. Ethyl decanoate has soap-like odours. In white winemaking, the production of these esters can be increased by lowering the fermentation temperature and increasing must clarification (Ribéreau-Gayon *et al*., 2000).

2.3.2. Higher alcohols

Higher alcohols can be considered to exist in two groups; (1) those that are synthesised from the oxidative deamination of an amino acid (catabolically) or involved as an intermediate in the biosynthetic reaction (anabolically) and (2) those that are not directly produced from an amino acid, but from a keto acid that takes part as an intermediate in cell glucose metabolism. The former group includes isoamyl alcohol, isobutyl alcohol and phenyl alcohols, which can be synthesised from leucine (and isoleucine), valine and phenylalanine, respectively, via their ketoacids α-ketoisocaproate (and α-keto-β-methylvalerate), α-ketoisovalerate and phenylpyruvate, the production of which depends on cellular growth and probably, on the presence of oxygen in the medium (Mauricio *et al*., 1997; Giudici and Kunkee, 1994; Ribéreau-Gayon *et al*., 2000). The formation of higher alcohols during yeast fermentation takes place in parallel to ethanol formation (Rapp and Versini, 1991). Higher alcohols are quantitatively the most prevalent aromatic substances. Beltran *et al.* (2005) showed that the anabolic route is of great importance because the increase in isoamyl alcohol and 2-phenyl ethanol was inversely proportional to the consumption of leucine and phenylalanine, respectively. Thus, the closer the nitrogen concentration is to the growth-limiting level, the higher will be the yield of fusel alcohols.

An excess of higher alcohols above 400 mg/L can be regarded as a negative influence on the quality of wine, but at the concentrations generally found in wines, below 300 mg/L, they usually contribute to the desirable complexity of wine (Kunkee and Amerine, 1970; Rapp and Versini, 1991; Lambrechts and Pretorius, 2000).

2.3.3 Hydrogen sulfide

In the production of alcohol and other metabolites by fermentation, hydrogen sulfide (H_2S) liberation is always occurring as a result of the metabolism of *S. cerevisiae* during fermentation. The formation of hydrogen sulfide by yeasts during the fermentation of grape must is a problem as old as the process of winemaking. Hydrogen sulfide is one of the highly undesirable metabolites of alcoholic fermentations. Because of its negative sensory attribute, it is necessary to find yeast strains that are producing low concentration of H_2S (Eschenbruch, 1974; Cappello *et al*., 2004). The formation of hydrogen sulfide in wine has been shown to arise from fermentations of grape musts with low nitrogen level (Eschenbruch, 1974).

Commercial strains of *S. cerevisiae* differ in the production of H_2S during fermentation, which has been attributed to variation in the ability to incorporate reduced sulphur into organic compounds (Spiropoulos and Bisson, 2000). The type of yeast strain also strongly influences the amount of hydrogen sulfide produced. Strains with low or no sulphite reductase activity never produced detectable amounts of this compound (Giudici and Kunkee, 1994; Jiranek *et al*., 1995). In winemaking conditions low pH, i.e. the abundance of hydrogen molecules favours the reaction to create the

volatile H_2S gas (Linderholm and Bisson, 2005), nitrogen composition, and vitamin deficiency (Henschke and Jiranek, 1991). Production of H_2S by *S. cerevisiae* strains ranges from 0 µg/L to 290 µg/L, well above the human detection threshold of 11 ng/L (Linderholm and Bisson, 2005). Assimilable nitrogen regulates H_2S production in fermenting grape must (Henschke and Jiranek, 1991). Addition of DAP at levels of 160 mg N/L and 250 mg N/L was also found to lower the sulfide formation in certain juices, but these led to available nitrogen concentration well above the 140 to 160 mg N/L generally considered adequate for normal fermentation (Boulton *et al*., 1998).

An alternate approach toward eliminating sulfide formation in wine strains is to use genetic analyses to identify the genes that impact sulfide production the most with the aim of altering those genes so that sulfide levels will be reduced (Linderholm and Bisson, 2005). Selection of wine yeast strains with low sulfide reductase activity are required to control H_2S formation and ensure high quality wines. Recently, additional genetic elements that are both increasing and decreasing the level of sulfide formation are defined. This was achieved by systematic analysis of the yeast deletion set of genes that influence formation of sulfide (Linderholm *et al*., 2008). Deletion of *CYS4*, *HOM2*, *HOM6* and *MET17* genes result in accumulation of reduced sulfide, thus explaining the production of high levels of H_2S. Other genes such as *SER33*, *ATP11* and *HHT2* when deleted resulted in production of variable moderate levels of H_2S amongst strains. The mechanism by which loss of these genes affects the formation of H_2S requires a better understanding of their physiological roles in the cell (Linderholm *et al*., 2008).

2.3.4 Acetic acid

Acetic acid is the main component of volatile acidity. Apart from its involvement in stuck fermentations, is also critical for the quality of wines. The concentration of acetic acid in wines is on average around 0.5 g/L and must remain below 0.8 g/L. Yeasts sometimes produce excessive levels of acetic acid, due to either the genetic background of the yeast or the winemaking processes, for example in the case of excessive clarification. (Dequin, 2001). Higher concentrations of acetic acid impart a vinegar taint to wine (Hühn *et al*., 1999). Certain bacterial species can be held responsible for the vinegar taint in wines; these include *Gluconobacter oxydans*, *Acetobacter pasteurianus* and *Acetobacter aceti* (Hühn *et al*., 1999).

2.4 CONCLUSION

In the past, stuck or sluggish fermentations were corrected by blending a stuck wine back into vigorously fermenting must (Henschke and Jiranek, 1993), or by re-inoculation with the active dry yeast (ADY) (Buescher *et al*., 2001; Cavazza *et al*., 2004). This process however may have severe implications on the final quality of the wine. If the alcohol content is already elevated, 10.27% (w/v) [i.e. equivalent to 13% (v/v)], the chances of restarting the fermentation are slim.

In order to control the occurrence of stuck fermentations and off-flavour development, it appears essential that improved *S*. *cerevisiae* wine yeast strains should be used. To date, oenologists have recognised the importance of improved *S*. *cerevisiae* starter cultures that are adapted to the specific type of cultivars and the style of wine they produce (Pretorius, 2000). These improved *S*. *cerevisiae* starter cultures offer many advantages, which may include the quick onset of fermentation, low contamination risk, more rapid and uniform fermentation rate, low levels of residual sugars and the maintenance of flavour properties (Coelho Silva *et al*., 2006). In addition to the above advantages of starter cultures, further improvement and selection for high sugar tolerant strain(s) and hence ethanol tolerant, as well as of nitrogen efficient strains (i.e. strains that ferment optimally in nitrogen deficient must) would be considerably appreciated in the wine industry. A large diversity of new strains would offer winemakers options to manage and control their fermentations by choosing the best adapted strain depending on the type and style of wine to be made.

Such improvements of wine yeast strains are clearly possible since all of the relevant traits are dependent on the genetic potential of individual strains, and all the traits mentioned in this review are polygenic. Since such traits are not easily manipulated, a strategy based on the creation of a large, genetically diverse population of yeast followed by enrichment and direct evolution, appears promising.

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Chapter 3

RESEARCH RESULTS

Breeding and chemostat as tools for improving and selecting wine yeast strains of *Saccharomyces cerevisiae* possessing desired oenological traits

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3. RESEARCH RESULTS

Breeding and chemostat as tools for improving and selecting wine yeast strains of *Saccharomyces cerevisiae* possessing desired oenological traits

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Abstract

The development of new organisms and improving existing organisms that are essential in wine making, in particular wine yeast strains has unlocked the possibilities that can ensure a better control of the process, including helping to prevent stuck fermentation, to improve wine quality and to reduce the risk of wine spoilage. Amongst the main causes of fermentation arrest are nutrient deficiencies, particularly of nitrogen as well as ethanol toxicity. *S. cerevisiae* are glucophillic yeast and utilise glucose more readily than fructose during alcoholic fermentation. This discrepancy can also result in fermentation problems, since fructose is sweeter than glucose and high residual fructose levels may impact wine quality negatively. Wine yeast strains also differ in their ability to produce ethanol during fermentation, and their tolerance to ethanol varies. In this study, we describe the development of new wine yeast hybrid strains with improved nitrogen efficiency through the use of hybridization, enrichment and directed evolution in a chemostat. The study also attempted to improve fructose utilization efficiency and ethanol tolerance of these hybrids. After hybridization of two parental strains that had been selected for their high nitrogen efficiency and high affinity for fructose, respectively, strains underwent enrichment and directed evolution in the chemostat. Several strains were then selected for further analysis. The hybrid nature of the strains was verified by application of PCR and CHEF techniques. The hybrid strains were evaluated in a small-scale fermentation and three out of seven hybrids out-competed the parental strains and completed fermentation in synthetic media containing low α-amino acids (FAN). Only one hybrid strain showed improvement of more than one trait, such as nitrogen and its capacity to utilise fructose at the end of fermentation. Another hybrid strain showed improvement of nitrogen and ability to produce and tolerate high ethanol levels. Such new developed strains would aid the winemakers to control the occurrence of stuck fermentation in late harvested grapes, nitrogen deficient musts and withstand the ethanol formed.

Keywords: Wine yeast, strain improvement, hybridization, nitrogen limitation, ethanol tolerance, stuck fermentation.

3.1 INTRODUCTION

The improvement of wine yeast strains has received significant attention. The aims of improvement are numerous, and include the generation of yeast strains that would be less likely to experience fermentation-associated problems such as sluggish and stuck fermentation or the production of compounds like sulfide production. To reduce the occurrence of sluggish or stuck fermentation, one of the main targets of strain improvement focuses on nitrogen efficiency, defined as the ability to ferment to dryness even in conditions where nitrogen is limiting. Other targets include fructose utilization efficiency and ethanol tolerance.

The factors that influence the occurrence of stuck fermentation include grape must vitamin concentration, magnesium concentration, nitrogen and oxygen availability, as well as ethanol, toxic fatty acids, acetic acid or sulfides. All these factors have been reviewed in detail (Alexandre and Charpentier, 1998). In general, the molecular effects of these factors on yeast include the inhibition of key enzyme activities and the alteration of the plasma membrane. All lead to a decrease in metabolic activity of the yeast, biomass production, cell viability and fermentation rate (Alexandre and Charpentier, 1998).

Many studies have shown that the concentration of assimilable nitrogen in grape must has a decisive influence on the ability of strains to conduct fermentation effectively. Deficiency of nitrogen was found to affect biomass formation and yield, and to lead to a reduction in fermentation rate (Blateyron and Sablayrolles, 2001; Wang *et al*., 2003; Mendes-Ferreira *et al*., 2004; Varela *et al*., 2004). In addition to this general impact of nitrogen concentration on yeast growth and biomass formation, several specific molecular consequences of nitrogen limitation have been described, including the impact on transporter turnover rate and on the expression of at least one hexose transporter-encoding gene, *HXT1* gene (Bisson, 1999). Grape must deficient in nitrogen also frequently leads to the formation of hydrogen sulfide (Giudici and Kunkee, 1994).

There is also growing demand for wine yeast strains with improved fructose utilization efficiency and ethanol tolerance. Sugar content of grapes and musts is highly variable, depending on factors such as grape variety and vintage, and ranges between 180-300 g/L. In grapes, glucose and fructose constitute the vast majority of available hexoses. Glucose predominates in unripe grapes, but the glucose-fructose ratio at fruit maturity is about 1, and fructose constitutes the major sugar in overripe grapes (Kliewer, 1967). Sugar utilization is governed by both genetic capability and regulatory mechanisms. Even closely related *Saccharomyces* strains differ greatly in their ability to utilise sugar (Carlson, 1987). The transport of hexoses is highly regulated to ensure that the yeast receives adequate supply of carbon and energy under various conditions (Luyten *et al*., 2002). During alcoholic fermentation of grape musts, the concentration of glucose and fructose do not decrease equally because wine yeast strains normally convert glucose more efficiently to alcohol and carbon dioxide than fructose (Hopkins and Roberts, 1935; Schuetz and Gafner, 1993). These differences in the rates of

glucose and fructose utilization by *S*. *cerevisiae* demonstrated the key role of hexose transport and the pattern of fructose utilization was directly influenced when the hexose transporter, *HXT3* allele was expressed by the yeast (Guillaume *et al*., 2007).

Efficient sugar utilization by wine yeast strains cannot be discussed without referring to their tolerance to ethanol (Oliver, 1987). The inhibitory effect of ethanol produced during fermentation is complex and is a main contributing factor for slow and incomplete fermentations (Aquilera and Benitez, 1985). Given the high ethanol concentrations reached during vinification, tolerance to ethanol stress is one of the criteria used to select wine yeast strains (Carrasco, *et al*., 2001). Therefore, a better knowledge of the effect of ethanol on yeast would be of interest to improve winemaking fermentations (Ansanay-Galeote *et al*., 2001) and wine yeast strains. Interestingly, although ethanol is an important parameter in alcoholic fermentation, there is no generally used or accepted method to characterise ethanol tolerance (Rose, 1987; Salgueiro *et al*., 1988). Many of available methods used to characterise ethanol tolerance of yeasts differs between research groups.

To ensure a reliable fermentation performance by wine yeast strains without sluggish or stuck fermentation effect, wine yeast strain development (or improvement) is important. The improvements involve the application of one or a combination of strategies that result in the development of new strains with desired phenotypes (Han and Parekh, 2005). Classical genetic approaches were first applied to wine yeast strains in the middle of the 1980s, in response to the increasing demand for new characteristics resulting from the development of pure culture strains (Barre *et al*., 1993). Examples of classical approaches are hybridization, cytoduction, mutagenesis, spheroplast fusion and rare mating (reviewed by Pretorius, 2000). Such hybridization techniques have been successfully used by many research groups to improve wine or beer yeast strains. Shinohara *et al*. (1997) introduced flocculation properties into wine yeast strains of *S*. *cerevisiae*. Hara *et al*. (1980) and Ramírez *et al*., (1998) created killer phenotype hybrids. Sato *et al*., (2002) hybridised a top-fermenting brewer's yeast *S*. *cerevisiae* and a low-temperature fermenting *S*. *bayanus*, and combined the two traits in a single hybrid. In addition to the classical approach, there are targeted approaches for developing improved strains. This includes enrichment methods and genetic engineering (Han and Parekh, 2005). One drawback to the random selection approach is that it is non-targeted and non-specific for the type of mutation, so many strains need to be screened in order to isolate an improved mutant in the mixed population (Parekh *et al*., 2000). Enrichment approaches in a chemostat increase screening efficiency by eliminating large numbers of undesirable mutants from the population. However, it has some limitations as well. Some phenotypes might not be obtained and sometimes false-positive mutants are selected (Han and Parekh, 2005).

In this study we used a strategy combining large-scale random hybridization of spores (mass mating) with uninterrupted enrichment of the fructose and nitrogen efficient hybrids in a chemostat that was in surplus of fructose and limited for nitrogen, respectively. The study used two previously generated wine yeast strains, 116 and 38-

1. These two hybrids had been selected for high nitrogen efficiency and fructose consumption ability, respectively in the breeding program at the Institute of Wine Biotechnology. The aim was to further improve the above traits and to combine the two traits in a single strain in order to reduce the occurrence of stuck fermentations and development of off-flavour compounds. In addition to the above, we attempted to also enhance ethanol tolerance by “semi – continuous” fermentation using a bioreactor.

3.2 MATERIALS AND METHODS

3.2.1 Microorganisms and cultivation conditions

Two wine strains of the yeast *Saccharomyces cerevisiae* 116 and 38-1 were cultured in test tubes containing 5 ml YPD broth media (1 g/L yeast extract, 2 g/L peptone, and 2 g/L dextrose) and incubated for 24 h at 30˚C, 50 rpm.

3.2.2 Induction of sporulation and tetrad formation

Different sporulation media were used to induce formation of spores, potassium acetate medium (10 g/L potassium acetate, 15 g/L agar) and potassium acetate medium supplemented with yeast extract (10 g/L potassium acetate, 25 g/L yeast extract, 20 g/L agar) were used for strain 116 and 38-1, respectively. Pre-cultured cells were harvested, washed with distilled water and plated on sporulation media. Plates were incubated at room temperature and tetrad formation was verified every two days under the microscope for a period of 10 days. To determine the percentage of tetrad forming cells, strains were re-suspended in 2 mL eppendorf tubes containing 1 mL distilled water. Total cell count (unsporulated and sporulated cells) was done using a hemocytometer.

3.2.3. Tetrad digestion

Asci of strains 116 (50 µL) and 38-1 (100 µL), volumes are determined based on the tetrad percentage obtained for each strain and mixed in 2 mL eppendorf tube. Zymolase (1 mg/mL) was added to the mixture to a final concentration of 0.5 mg/mL. The mixture was incubated at room temperature for 1 h, with intermittent vortexing to facilitate cell wall destruction. Complete tetrad digestion was monitored microscopically. After complete digestion was observed, 0.5 mL of distilled water was added to the mixture and left on ice.

3.2.4. Mass mating

The mixture of spores (250 µL per plate) was plated on YPD agar (1 g yeast extract, 2 g peptone, dextrose, 1.5 g agar) and incubated for 3 days at 30˚C. Freeze cultures of genetically diverse population were stored as 40% glycerol (v/v) stocks at -80˚C.

3.2.5. Chemostat conditions

Chemostats were used to select strains attaining the fastest growth rate under the conditions described below. The following components were used to set up a chemostat: a 1 L benchtop fermentor (BioFlo 110 Modular), pump (Minipuls 3GILSON), silicone tubing of different sizes (4x7 mm; 4x8 mm), Hepa-vent In-line filter discs (Whatman, 5.5 cm diameter), sterile syringe (50 mL; Neomedic) attached to a filter for sampling purposes, sterile syringe (5 cc/mL; Lasec S.A) with sterile needle (21g x 1½; Neomedic) for inoculation. To collect samples 50 mL vials or bottles were used. Media used for enrichment was synthetic media (MS x, x denotes the concentration of nitrogen source, pH 3.3) as described by Bely *et al*. (1990) with slight modifications for the purpose of this study. The amino acid stock was dissolved in 2% $NaHCO_3$ (20 g/L) and 13.1 mL of each amino acid stock (300 mg N/L) indicated in table 3.3 was used per litre of synthetic media.

Vitamin stock: Myo-inositol 2000 mg/L; calcium pantothenate 150 mg/L; thiamine hydrochloride 25 mg/L; nicotinic acid 200 mg/L; pyridoxine 25 mg/L; and biotin 0.3 mg/L, all dissolved in water. 10 mL was used per litre of synthetic media.

Trace elements stock: $MnSO_4$. H_2O, 4 g/L; $ZnSO_4$. $7H_2O$, 4 g/L; $CuSO_4$. $5H_2O$, 1 g/L; KI, 1 g/L; $CoCl_2$. $6H_2O$, 0.4 g/L; H_3BO_3, 1 g/L; and $(NH_4)_6Mo_7O_{24}$ 1 g/L, all dissolved in water. 1 mL was used per litre of synthetic media.

Anaerobic factors stock: Ergosterol 1.5 g/L; oleic acid 0.5 g/L, both dissolved in absolute ethanol 99.9% (v/v) added to Tween 80 (50 ml + 70 ml respectively) and dissolved at 65˚C. Sugar: Glucose 125 g/L; fructose 125 g/L. Acids: citric acid 6 g/L; malic acid 6 g/L. Salts: KH_2PO_4 0.75 g/L; K_2SO_4 0.5 g/L; $MgSO_4$. $7H_2O$ 0.25 g/L; $CaCl_2$. $2H_2O$ 0.155 g/L and NaCl 0.2 g/L). Sugars and salts were autoclaved and all the amino acids stocks, vitamins stocks, oligoelements stock, and anaerobic factors (warmed at 65˚C in water bath before use) were sterile filtered (w/0.8/0.2 µm supor® membrane; Acrodisc® Syringe filters) and added into the cooled autoclaved media.

3.2.5.1 Selection of fructose efficient strains

To begin the selection process, the inoculum was prepared in YPD medium and incubated at 30˚C, 180 rpm for 24 h. A description of the synthetic medium (MS, pH 3.3) containing 250 g/L of sugar (i.e. glucose 125 g and fructose 125 g) and yeast assimilable nitrogen (YAN) of 102 mg N/L in terms of nitrogen composition is given in Table 3.1. YAN is expressed as the sum of α-amino aids, free assimilable nitrogen

(FAN) and ammonia. This medium was inoculated with a population of mass mated cells at the initial optical density (OD_{600}) of 0.06 and propagated for about 25 h until OD_{600} reached 12.8. The culture was maintained at 30˚C with agitation speed of 150 rpm, no pH adjustment and a working volume of 800 mL in both batch and continuous culture systems. The bioreactor was not deoxygenated prior to fermentation and later became anaerobic. Feed medium was composed of MS 102 medium with 150 g fructose out of 250 g/L of total sugar. At this stage feed medium was supplied at a dilution rate, *D* (*D* = F/V, where F is flow rate and V is total working volume of the bioreactor) of 0.12/h. The culture was maintained in a steady-state for 12.5 generations. Sampling was done at the end of fermentation and yeast strains were stored at -80˚C in final concentration of 40% glycerol (v/v).

3.2.5.2 Selection of strains with low nitrogen requirements

Studies by Jiranek *et al*. (1995) reported that the kinetics of individual amino acids consumption varied between *S*. *cerevisiae* wine yeast strains. However, it was found that amino acids such as arginine, serine, glutamate, threonine, aspartate, and lysine comprised the bulk of amino acids consumed amongst the strains. In this study to achieve limiting nitrogen concentration, nitrogen sources such as ammonium, aspartic aicd, glutamic acid, arginine, leucine and glutamine were reduced 5 and 10 fold to attain YAN of 102 and 91 mg N/L, respectively. Cells harvested at the end of fructose selection were propagated in MS300 medium (nitrogen content described in Table 3.1) for 25 h until OD_{600} reached approximately 25 under the same physical parameters as in 3.2.5.1. The feed medium contained 250 g/L (i.e. 125 g of glucose and 125 g fructose) of total sugar and 102 (MS102) and 91 (MS91) mg N/L. At these concentrations, nitrogen is the growth limiting factor in the chemostat. Immediately after the OD_{600} of culture reached 25, feed MS102 medium was supplied. After the culture reached a steady state, the conditions were maintained for 31.5 generations at a *D* of 0.173/h. Later the feed was adjusted to MS91 for 23 generation at *D* of 0.08/h. The cumulative number of generations in these selective conditions was 54.5. Sampling was done at the end of fermentation and yeast strains were stored at -80˚C in final concentration of 40% glycerol (v/v).

3.2.5.3 Selection of ethanol tolerant strains

At the end of 54.5 generations in limited nitrogen conditions, the culture was propagated further to obtain cell number, OD_{600} 13.0, and later subjected to increasing amounts of ethanol. To achieve this, a “semi-continuous system” was used. In this process the feed medium comprised of ethanol only was intermittently fed to replace the removed volume of cells and other debris during sampling to equal parts. The initial ethanol concentration of the medium in the bioreactor (working volume of 0.8 L) was measured at 3.52% (w/v) and exogenous ethanol was gradually increased to 7.90% (w/v) in steps of 1.98% (w/v).

The final ethanol measured in the bioreactor was 16.59% (w/v). The OD_{600} and cell viability (in duplicates) were used to estimate yeast growth and survival in these conditions. The overall cultivation period was 18 days and the sample used in this analysis was obtained the last two days.

3.2.6 Differentiation and identification of hybrids

3.2.6.1 CHEF

Strains were pre-cultured in 250 mL Erlenmeyer flasks containing 100 mL YPD broth and incubated for 24 h at 30˚C, 180 rpm. The preparation of chromosomal DNA plugs was done as described by Carle and Olsen (1985). The yeast chromosomes were separated by CHEF MAPPER electrophoresis system (BIO-RAD), following the program used by Raspor *et al*. (2002) with slight modifications using 5.5 V/cm (or 183.3 V) for 15 h with 60 s pulse time, 8 h with 90 s pulse time, and 1 h with 120 s pulse time in 1 % agarose (Seakem® GTG Agarose, Cambrex Bio-Science Rockland, Inc. ME, USA), in 0.5 x TBE buffer cooled at 12˚C. The gel was stained with ethidium bromide (50 µg/mL) and photographed in an UV transilluminator (Alpha Imager Innotech corporate-focus and image instrument).

3.2.6.2 PCR

Strains were grown in test tubes containing 5 mL YPD broth and incubated for 24 h at 30˚C and 50 rpm. Cells were harvested in 2 mL Eppendorf tubes at 3000 rpm (Benchtop centrifuge 5415D) for 3 minutes. Cells were resuspended in 0.5 mL sterile distilled water and centrifuged at 6000 rpm, for 5 minutes. The pellet was resuspended in 400 Ml zymolase buffer (1 mg/mL zymolase in 1 M sorbitol; 100 mM Na_3-citrate; 60 mM EDTA pH 7.0) and incubated for 2 h at 37˚C. 400 µL lysis buffer (2% SDS in 50 mM Tris-HCl; 10 mM EDTA pH 8.0) was added thereafter, carefully shaken and incubated for 10 min at room temperature. Immediately, 200 µL 5M NaCl was added and put on ice for at least 2 h, centrifuged at 13000 x g for 10 min and the pellet was re-suspended in 400 µL TE buffer (10 mM Tris-HCl; 1 mM EDTA pH 8.0). The pellet was dissolved by repeated pipetting up and down. 400 µL PCI (phenol/chlorophorm/isoamyl alcohol at ratio of 25/24/1) was added, vortexed for 5 min and centrifuged at 13000 x g for 5 min. The aqueous layer was transferred to new 2 mL eppendorf tubes, along with two volumes of 100% ethanol solution, incubated for 5 min at -80˚C and centrifuged at 13000 x g for 10 min. The pellet was washed with 70% ethanol and vacuum dried and dissolved in 25-50 µL TE buffer. DNA amplifications were carried out in 25 µL reaction volumes containing 1-2 ng yeast DNA, 2 µM of the primers in Table 3.2, 1 x buffer, 0.2 mM dNTPs, 2.25 mM $MgCl_2$, and 0.1 U Supertherm DNA polymerase enzyme. These primers amplifies the sequences between the delta and sigma sequences.

Table 3.1 Modified nitrogen content in synthetic must (MS) described by Bely *et al*, (1990).

Nitrogen source	Recipe for 300 mg N/L			Recipe for 102 mg N/L			Recipe for 91 mg N/L		
	TNC (mg N/L)	YAN (mg N/L)	Nitrogen (mg/L)	TNC (mg N/L)	YAN (mg N/L)	Nitrogen (mg/L)	TNC (mg N/L)	YAN (mg N/L)	Nitrogen (mg/L)
NH4Cl	120	120	4.6	12	12	0.46	6	6	0.23
Aspartic acid	4.68	4.68	44.51	0.468	0.468	4.45	0.234	0.234	2.23
Glutamic acid	11.47	11.47	120.43	1.147	1.147	12.04	0.574	0.574	6.02
Arginine	30.1	30.1	374.37	3.01	3.01	37.44	1.505	1.505	18.72
Leucine	5.17	5.17	48.43	0.517	0.517	4.84	0.259	0.259	2.42
Glutamine	48.44	48.44	505.27	4.844	4.844	50.53	2.422	2.422	25.26
Tyrosine	1.42	1.42	18.33	1.42	1.42	18.33	1.42	1.42	18.33
Tryptophane	12.3	12.3	179.33	12.3	12.3	179.33	12.3	12.3	179.33
Isoleucine	3.49	3.49	32.73	3.49	3.49	32.73	3.49	3.49	32.73
Threonine	8.93	8.93	75.92	8.93	8.93	75.92	8.93	8.93	75.92
Glycine	3.42	3.42	18.33	3.42	3.42	18.33	3.42	3.42	18.33
Alanine	22.84	22.84	145.3	22.84	22.84	145.30	22.84	22.84	145.30
Valine	5.32	5.32	17.02	5.32	5.32	17.02	5.32	5.32	17.02
Methionine	2.95	2.95	31.42	2.95	2.95	31.42	2.95	2.95	31.42
Phenylalanine	3.22	3.22	37.96	3.22	3.22	37.96	3.22	3.22	37.96
Serine	10.47	10.47	78.54	10.47	10.47	78.54	10.47	10.47	78.54
Histidine	2.95	2.95	32.73	2.95	2.95	32.73	2.95	2.95	32.73
Lysine	1.63	1.63	17.02	1.63	1.63	17.02	1.63	1.63	17.02
Cystein	1.5	1.5	13.09	1.5	1.5	13.09	1.5	1.5	13.09
Proline	**74.55**	**0**	**612.61**	**74.55**	**0**	**612.61**	**74.55**	**0**	**612.61**
Total	**374.85**	**300.30**	**2407.94**	**176.98**	**102.43**	**1420.09**	**165.98**	**91.43**	**1365.21**

[1] Proline is not utilized under anaerobic conditions by yeast strains of *Saccharomyces cerevisiae*

Table 3.1(continues) Modified nitrogen content in synthetic must (MS) described by Bely *et al*, (1990).

Recipe for 86 mg N/L			Recipe for 80. mg N/L			Recipe for 56.7 mg N/L			Recipe for 45.6 mg N/L		
TNC (mg N/L)	**YAN (mg N/L)**	**Nitrogen (mg/L)**	**TNC (mg N/L)**	**FAN (mg N/L)**	**Nitrogen (mg/L)**	**TNC (mg N/L)**	**FAN (mg N/L)**	**Nitrogen (mg/L)**	**TNC (mg N/L)**	**FAN (mg N/L)**	**Nitrogen (mg/L)**
3	3	0.12	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.117	0.117	1.11	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.28675	0.28675	3.01	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.7525	0.7525	9.36	NA	NA	NA	NA	NA	NA	NA	NA	NA
0.12925	0.12925	1.21	NA	NA	NA	NA	NA	NA	NA	NA	NA
1.211	1.211	12.63	NA	NA	NA	NA	NA	NA	NA	NA	NA
1.42	1.42	18.33	1.42	1.42	18.33	1.00	1.00	12.92	0.81	0.81	10.39
12.3	12.3	179.33	12.3	12.3	179.33	8.67	8.67	126.43	6.97	6.97	101.68
3.49	3.49	32.73	3.49	3.49	32.73	2.46	2.46	23.07	1.98	1.98	18.56
8.93	8.93	75.92	8.93	8.93	75.92	6.30	6.30	53.52	5.06	5.06	43.05
3.42	3.42	18.33	3.42	3.42	18.33	2.41	2.41	12.92	1.94	1.94	10.39
22.84	22.84	145.30	22.84	22.84	145.3	16.10	16.10	102.44	12.95	12.95	82.39
5.32	5.32	17.02	5.32	5.32	17.02	3.75	3.75	12.00	3.02	3.02	9.65
2.95	2.95	31.42	2.95	2.95	31.42	2.08	2.08	22.15	1.67	1.67	17.82
3.22	3.22	37.96	3.22	3.22	37.96	2.27	2.27	26.76	1.83	1.83	21.52
10.47	10.47	78.54	10.47	10.47	78.54	7.38	7.38	55.37	5.94	5.94	44.53
2.95	2.95	32.73	2.95	2.95	32.73	2.08	2.08	23.07	1.67	1.67	18.56
1.63	1.63	17.02	1.63	1.63	17.02	1.15	1.15	12.00	0.92	0.92	9.65
1.5	1.5	13.09	1.5	1.5	13.09	1.06	1.06	9.23	0.85	0.85	7.42
74.55	**0**	**612.61**	**74.55**	**0**	**612.61**	**74.55**	**0**	**431.89**	**74.55**	**0**	**347.35**
160.49	**85.94**	**1337.77**	**154.99**	**80.44**	**1310.33**	**131.26**	**56.71**	**923.78**	**120.16**	**45.61**	**742.96**

[1] Proline is not utilized under anaerobic conditions by yeast strains of *Saccharomyces cerevisiae*
[2] NA means not added

Table 3.2 Primer sets used to identify and differentiate wine yeast hybrid strains.

Primers	Sequences	References
Delta (δ1, forward)	5´-CAAAATTCACCTATATCT-3´	Lavallée *et al*. (1994)
Delta (δ2, reverse)	5´-GTGGATTTTTATTCCAAC-3´	
Delta (δ12, forward)	5´-TCAACAATGGAATCCCAAC-3´	Legras and Karst, (2003)
Delta (δ21, reverse)	5´-CATCTTAACACCGTATATGA-3´	
Sigma specific (σ1, forward)	5´-AGCTCGAGTAATACCGGATGTC-3´	Fingerman *et al*. (2003)
Sigma specific (σ2, reverse)	5´-CATGTATCAAACACGTACGA-3´	

Amplification reactions were performed with HYBAID PCR EXPRESS® thermocycler using the following programme: 3 min at 94˚C followed by 34 cycles of 30 s at 94˚C, 30 s (at 55˚C for δ1 and δ2; at 50˚C for δ12 and δ21; at 45˚C for sigma specific primers) and 2 min at 72˚C and final elongation step of 10 min at 72˚C. Alternative step, hold at 4˚C.

3.2.7 Evaluation of hybrids in small scale fermentation

A laboratory fermentation medium as described by Bely *et al*., (1990), designed to model grape must/must for research fermentations was used. This medium was reformulated in the present study to reflect most nutrients available in grape must such as glucose and fructose, organic acids, anaerobic factors and nitrogen under variable conditions. The medium was adjusted to pH 3.3 with NaOH prior to sterilisation. 13 mL each of amino acids stocks as indicated in table 3.3 was used per litre of synthetic media.

3.2.7.1 Microorganisms and pre-culture conditions

Seven selected yeast hybrid strains (summarised in results section) were plated on YPD agar plate and a single colony was used for further analysis. The colony was inoculated into 100 mL Erlenmeyer flasks containing 50 mL YPD broth and incubated at 30˚C with 180 rpm for 24 hours. Optical density (OD_{600}) of appropriately diluted cells was determined and OD_{600} of 0.1 of each strain was used to inoculate the fermentation flasks.

3.2.7.2 Fermentation

The newly bred hybrids were evaluated against the parent strains 116 and 38-1. All the fermentations were carried out in triplicates in 100 mL MS medium containing 250 g/L of sugar and maintained at 23-24˚C until completion of fermentation without any agitation. The fermentation progress was followed by weight loss as a result of CO_2 evolution. To determine nitrogen efficiency, hybrid strains were evaluated at different levels of nitrogen as referred to in Table 3.1.

To evaluate the fructose efficiency, hybrid strains were subjected to the MS300 medium containing three different fructose levels; 125 g/L, 150 g/L, and 175 g/L (total sugar 250 g/L), respectively. Lastly, ethanol tolerance was assessed in MS medium using different levels of sugar and YAN (nitrogen) in various combinations (Table 3.3).

Table 3.3 Summarises the MS medium composition used to assess ethanol tolerance of hybrid strains

Fermentation trial	Amount of sugar (g/L)	Amount of YAN (mg N/L)
1	300	300
2	300	450
3	350	300
4	350	450

[1] 450 YAN (mg N/L) was obtained by adding 19.7 mL per litre of synthetic media, instead of 13.1 mL of amino acid stock (300 mg N/L)

3.2.6.3 Hydrogen sulfide (H_2S) determination

All hybrid strains, parental strains, commercial wine yeast strains BM 45, VIN 13, and two Whiskey strains WH300 and 3H314 (positive control) were pre-cultured in test tubes containing 5 mL of YPD broth medium for 12 h and incubated at 30˚C, 50 rpm. Cells were appropriately diluted to OD_{600} of 0.1 and inoculated into 5 mL of YPD broth medium and incubated at 30˚C, 50 rpm until OD_{600} of 1 was reached. Cells were harvested in 2 mL eppendorf tubes at 3000 rpm (Benchtop centrifuge 5415D) for 3 minutes. Cells were resuspended in 0.5 mL sterile distilled water and 10 µL for each strain was spotted onto Bismuth sulfite glucose glycine yeast (BIGGY Agar) to qualitatively assess the level of sulfite reductase activity of yeasts. Plates were incubated for three days at 30˚C.

3.2.6.4 Fermentation analysis

Ethanol, glycerol, volatile acidity and residual sugar (glucose and fructose) were analysed using the FTIR-Grape Scan 2000 (FOSS Instruments, Denmark). The ethanol values were expressed in % (v/v) and multiplied by absolute ethanol conversion factor 0.79 g/mL to obtain % (w/v). Data were analysed by taking the mean values of each compound using one-way and two-way analysis of variance (ANOVA). The one-way ANOVA test was used in this study to compare the performance of the hybrid strains against parent strains based on the mean value of the total residual sugar (glucose and fructose) and other products analysed after alcoholic fermentation was completed. To ensure that the differences observed were significant, a two-way ANOVA test was performed. Differences were considered significant when probability value (p) was <0.05.

3.3 RESULTS

3.3.1 Choice of parental strains and hybridization

The parental strains 116 and 38-1 had previously been selected in two separate breeding programs for their ability to ferment optimally in low nitrogen and high fructose conditions, respectively. These strains were then tested for their sporulation efficiency. The percentage of formed tetrads (asci) was 80% and 60% for strains 116 and 38-1, respectively. The results suggested the potential of the two strains to act as parents for breeding program.

3.3.1.1 Selection of wine yeast strains in a chemostat

After sporulation had reached its peak, the sporulated cells were mixed in a 1:2 cell ratio to allow random mating of maximum number of spores. These mass-mated cells were growing at specific growth rates (μ) of 0.165/h, 0.230/h and 0.133/h during cell propagation (batch) in high fructose, nitrogen and ethanol, respectively prior to selection of efficient yeast strains. Selection of fructose efficient strains was carried out in a bioreactor containing medium with low nitrogen concentration and high fructose for 12.5 generations in a steady state, (**Fig. 3.1**). These conditions were set to mimic the fermentation stage at which fructose becomes the predominant sugar, when nitrogen is limited as fermentation progresses.

Selection of nitrogen efficient strains then occurred over a cumulative total of 54.5 generations, determined as the number of times a bioreactor working volume was replaced during steady state (D=μ). During this period, the yeast cells were cultivated for 31.5 and 23 generation in MS102 and MS91, respectively (**Fig. 3.2**). An upsurge in biomass (point B) took place and as a result more stringency was applied (i.e. further lowering nitrogen concentration in the feed medium, however this increase in cell number was considered in the calculation of number of generations. Only samples collected at the end of the process were further analysed in this study.

The improvements for ethanol tolerance enrichment of mass-mated cells were monitored by following cell viability and optical density for 18 days (**Fig. 3.3**). Residual sugar, glycerol and ethanol level were analysed at every point during sampling. After 15 h of batch growth, the amount of ethanol and sugar measured in the bioreactor was 3.52% (w/v) of ethanol and 153.84 g/L of sugar prior to ethanol feed. Immediately after the analysis, 1.98% (w/v) of absolute ethanol was added to the bioreactor as indicated (**Fig. 3.3**). Upon addition of ethanol, a drastic decrease of cell viability and optical density was observed. At time 225 h, the final concentration of about 7.9% (w/v) of absolute ethanol had been added into the bioreactor and 1.6% cell viability. Cell viability gradually decreased to 0.2% of the original cell number at time 447 h (**Fig. 3.3**). At this stage glycerol level had increased to 20.1 g/L (data not shown). The high ethanol

combined with nutrients deficiencies meant that only the vigorous strains could survive. Selection was done at 303 h and at the end of 447 h.

After selection processes for all the traits, about 60 randomly picked colonies were genetically characterised. Sixteen of these colonies could be clearly identified as hybrids. Selective conditions used during enrichment steps of hybrid strains are summarized in **Table 3.4** below.

Table 3.4 Sequential enrichment process of wine yeast hybrid strains in a chemostat.

Strains	Chemostat enrichment conditions			
	150 (g/L) Fructose and 102 mg N/L	102 mg N/L	91 mg N/L	Final ethanol 16.59% (w/v)
331	√	√	√	–
334	√	√	√	–
336	√	√	√	–
337	√	√	√	–
RR03	√	√	√	√
RR04	√	√	√	√
05R	√	√	√	√

[1] (√) refers to enrichment for the particular strains

[2] (−) refers to no enrichment for the particular strains

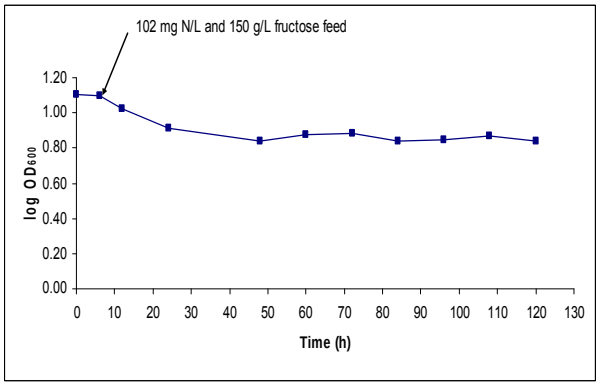

Fig 3.1 Enrichment of hybrid strains in low nitrogen and high fructose conditions using chemostat.

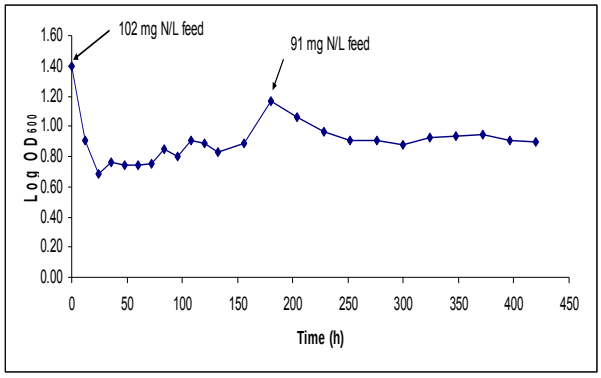

Fig 3.2 Enrichment of hybrid strains in low nitrogen conditions using chemostat.

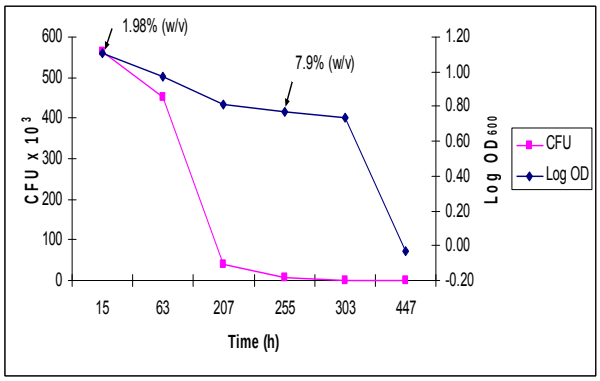

Fig 3.3 Enrichment of hybrid strains in high ethanol high nutrient deficient conditions using chemostat.

3.3.1.2 Strain karyotyping

Identification of strains by separating chromosomes using Pulse-Field Gel Electrophoresis (PFGE) is also known as karyotyping or chromosomal length polymorphism analysis, which differentiates strains based on the number and size of chromosomes. The chromosomal banding pattern obtained for each strain showed differences in terms of number and different mobility of the bands in each strain. Differences appearing in intensities of bands could be as a result of differences in concentration of sample loaded into the wells for electrophoresis (**Fig. 3.4**). Strain RR03 and 05R appear to be hybrids of 116 and 38-1, whereas the hybrid nature of the other strains is not apparent in the karyotype, although there are differences between the strains. These strains could still be of parental genotype and other techniques had to be used to assess their status.

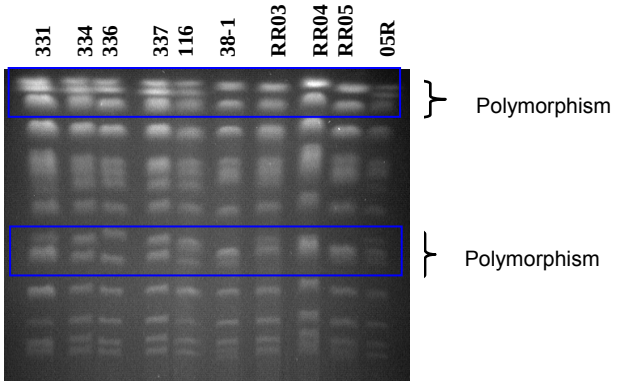

Fig 3.4 CHEF, karyotype analysis of hybrid strains after enrichment.

3.3.1.3 PCR Fingerprinting

To confirm the hybrid identity of the selected strains, we used PCR using δ1 and δ2 and inter- δ12 and δ21 primers and sigma primers. The δ1 and δ2 primers alone revealed the hybrid nature of the strains (**Fig. 3.5a**).

The PCR using these primers amplify the sequences found between the repeat elements and the location of these repeated elements within the genome tend to be different between different strains of *S. cerevisiae*. The hybrid nature was deduced by analysis of bands shared by each hybrid strain and parent strains. The results show that strain 331, 336 and 05R are hybrids of the two parent strains 116 and 38-1.

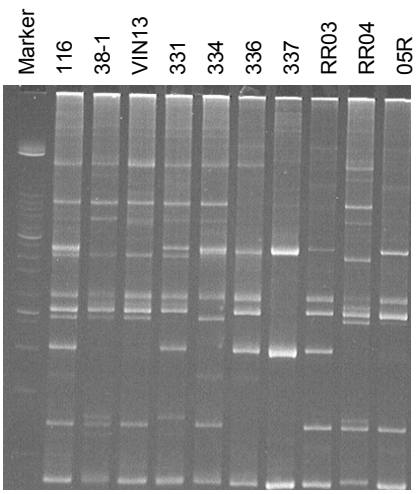

Fig 3.5a Genotype analysis of hybrid strains after enrichment using PCR-δ1 and δ2 primers.

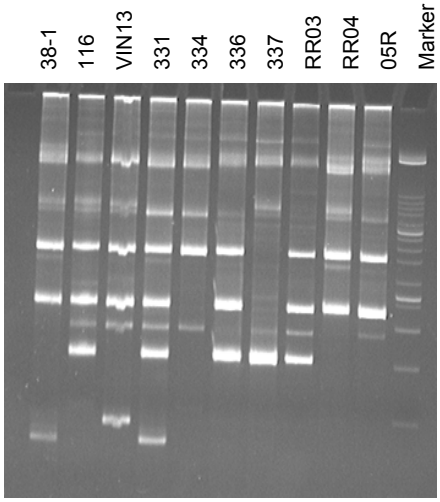

Fig 3.5b Genotype analysis of hybrid strains after enrichment using PCR-δ12 and δ21 primers.

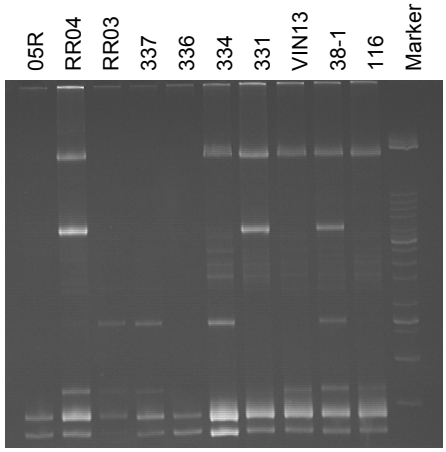

Fig 3.5c Genotype analysis of hybrid strains after enrichment using PCR-σ primers.

To validate this, δ12 and δ21 primers were also used and the results confirmed the hybrid nature of the strains (**Fig. 3.5b**). We also used combination of δ 2 and 12 primers, which confirmed the other strains as hybrids (results not shown).

Using the sigma primers (σ) it was possible to reveal the hybrid nature of other strains - RR04, 331 and 334 (**Fig 3.5c**). These primers were initially designed for amplification of *Ty*3-1p, a transposable element in *Saccharomyces paradoxus*, which share 82% nucleotide identity with *S. cerevisiae Ty*3 element (Fingerman *et al*., 2003). The differences observed between the δ and σ amplification results for each strain, suggest that the number of these repeats varies between strains. This work therefore

highlights the importance of different sets of primers to ensure accuracy in strain identification.

3.3.1.4 Hydrogen sulfide (H_2S) analysis

A qualitative method based on BIGGY Agar assay was used to determine H_2S. This medium contains bismuth ions and when H_2S is produced by the strains it reacts with bismuth ions to form bismuth sulfide in a reaction catalysed by yeast sulfide reductase. This reaction leads to yeast colonies turning brown. The browner the colour, the more H_2S was produced by the yeast (**Fig. 3.6**). Scores for H_2S production were determined

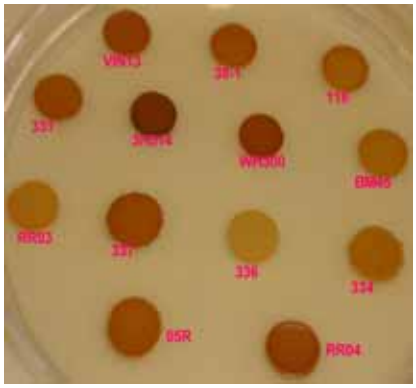

Fig 3.6 BIGGY Agar plate for identification of H_2S producing hybrid strains against parental strains, 116 and 38-1. 3H314 and WH300 are whiskey strains used as positive control.

according to Mortimer *et al*. (1994). The result shows that hybrid strains such as 334, RR04 and 336 have low sulfide reductase activity, and therefore a lower potential to produce H_2S. The hybrid strain 336 appeared to be the lowest H_2S producing strain. These results only provide an indication, since several factors that may induce H_2S production during fermentation are not assessed in this system.

3.3.2 Fermentation kinetics

To assess whether the hybrids show the desired improvements, strains were tested in synthetic media containing varying glucose and fructose concentrations, while the concentration of nitrogen was kept constant (300 mg N/L) in all the trials. In a media containing 150 g/L of fructose and 100 g/L of glucose, the fermentation kinetics for all strains was similar. As the concentration of fructose increased to 175 g/L, the fermentation kinetics of strain RR03 became distinguishable exhibiting a fast fermentation rate, however no significant differences was evident for other strains (**Fig. 3.7**). When the fructose concentration was 200 g/L fermentation progressed similarly in all the strains (data not shown).

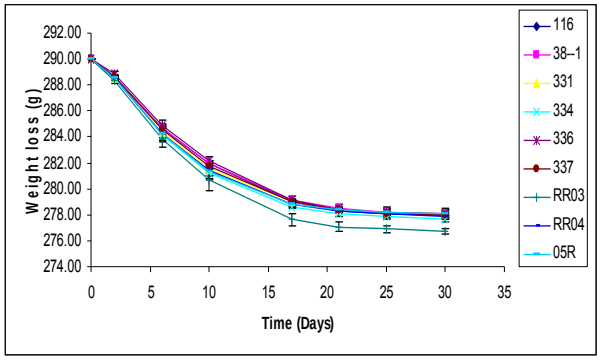

Fig 3.7 Comparison of fermentation kinetics of different enriched hybrid strains against parental strains in synthetic medium containing 175 g/L of fructose.

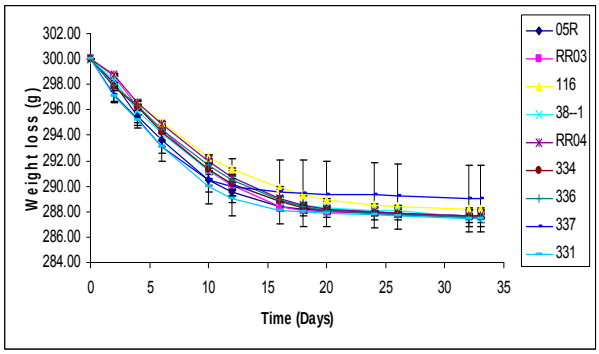

Fig 3.8a Comparison of fermentation kinetics of different enriched hybrid strains against parental strains in synthetic medium containing 300 mg N/L of nitrogen.

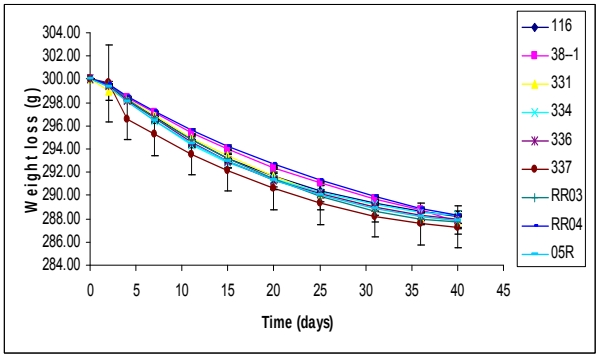

Fig 3.8b Comparison of fermentation kinetics of different enriched hybrid strains against parental strains in synthetic medium containing 45.6 mg N/L of nitrogen.

The fermentation kinetics of the hybrid strains and parental strains were compared under condition of sufficient (300 mg N/L) nitrogen concentration. From the data it is clear that fermentation, for all the strains was completed after 18 days with no significant differences in the fermentation rate (**Fig. 3.8a**). As expected, when fermentation was conducted under limited-nitrogen concentration, the time taken to ferment was longer, but no significant differences were observed between parental and hybrid strains (**Fig. 3.8b**). Conditions of high sugar concentration were applied to evaluate ethanol tolerance, as under winemaking conditions synergism between ethanol (alcohol) and sugar concentration exists. Synthetic must containing high sugar (300-350 g/L) and high YAN (300-450 mg N/L) was used to assess the potential of the hybrid strains to ferment under such conditions. With 300 g/L of sugar, fermentation time was extended significantly. Increasing YAN to 450 mg N/L resulted in reduced fermentation time (**Fig. 3.9a &b**)

When the sugar was 350 g/L, the high concentration of sugar probably affected the fermentation rate of yeast strains. This effect was noted by an increased lag phase and overall time to complete fermentation. Once again a reduction in fermentation time was observed when YAN was 450 mg N/L (**Fig. 3.10a &b**). The observed diauxic-fermentation kinetic pattern of strains in Figure 3.10a was a result of low nitrogen, high sugar and perhaps other metabolites such as alcohol, or acidity of the medium as fermentation progressed. Hybrid strain RR04 shown potentially high fermentation rate,

fermented better than other strains. Increasing the nitrogen concentration relieved the stress imposed on yeast strains and their fermentation kinetics became “normal” with hybrid strains RR04 and 05R fermenting better than parental strains (**Fig. 310b**). It is high substrate concentration (Strehaiano and Goma, 1983) and nutrient limitation (D’ Amore *et al*. 1988a &b), that is responsible for the decrease in growth and fermentation activities of yeast cells at higher osmoticpressure.

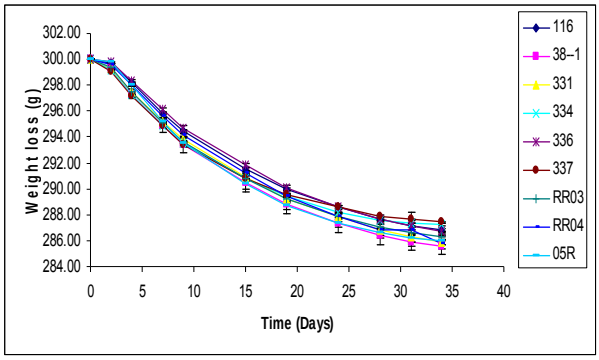

Fig 3.9a Comparison of fermentation kinetics of different enriched hybrid strains against parental strains in synthetic medium containing 300 g/L of sugar and 300 mg N/L of nitrogen.

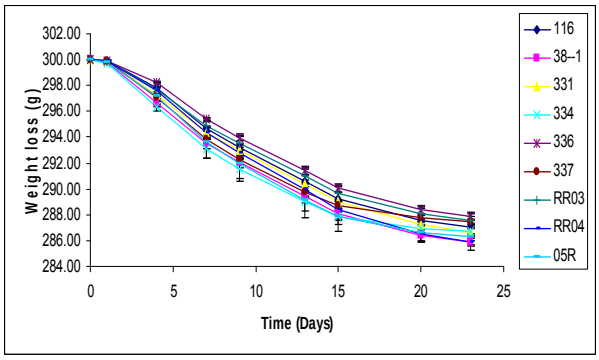

Fig 3.9b Comparison of fermentation kinetics of different enriched hybrid strains against parental strains in synthetic medium containing 300 g/L of sugar and 450 mg N/L of nitrogen.

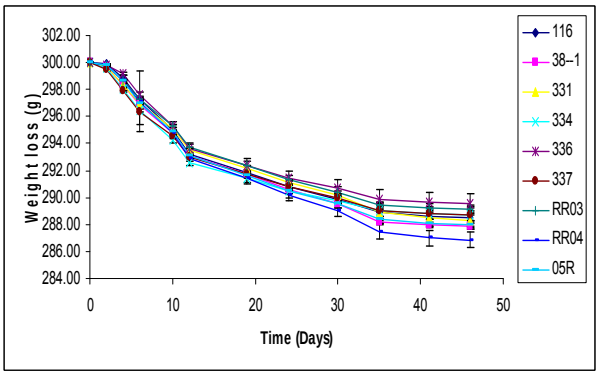

Fig 3.10a Comparison of fermentation kinetics of different enriched hybrid strains against parental strains in synthetic medium containing 350 g/L of sugar and 300 mg N/L of nitrogen.

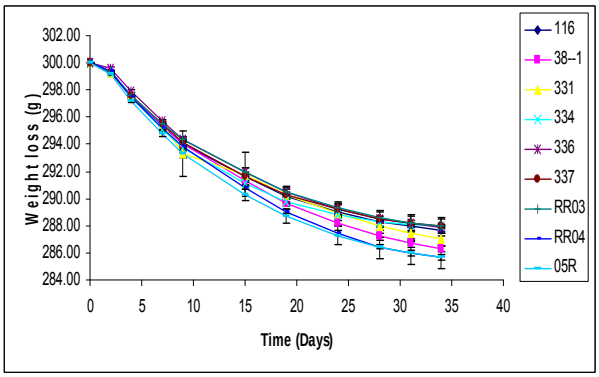

Fig 310b Comparison of fermentation kinetics of different enriched hybrid strains against parental strains in synthetic medium containing 350 g/L of sugar and 450 mg N/L of nitrogen.

3.3.3 Residual sugar

The residual sugar in this case refers to sum of residual glucose and fructose. The analysis was done at the end of fermentation only.

For the fructose experiment, the differences in residual sugar levels between strains was insignificant, ranging from 1.21 g/L for hybrid 336 to 1.55 g/L for hybrid 337 when the synthetic must contained 150 g/L of fructose, to 1.22 g/L for hybrid 336 to 1.62 g/L for parent 38-1 the synthetic must contained 175 g/L fructose. When fructose was increased to 200 g/L, residual sugar ranged from 2.39 g/L for hybrid RR03 to 3.10 g/L for hybrid 336. All these results are illustrated (**Fig. 3.11**). The results indicate no significant difference in terms of fructose utilization in the parental and hybrid strains, with exception for hybrid strain 331, which showed consistently better fructose utilization capacity in all the trials (**Table 3.5**).

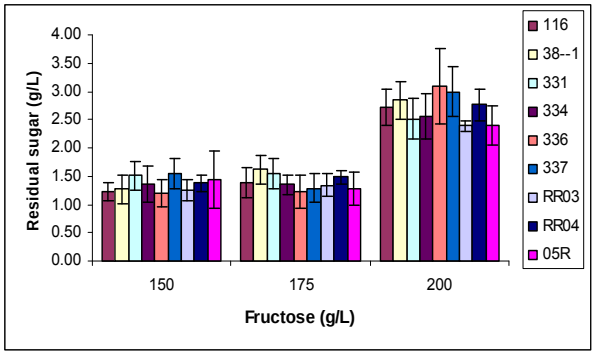

Fig 3.11 Comparison of residual sugar level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing high fructose.

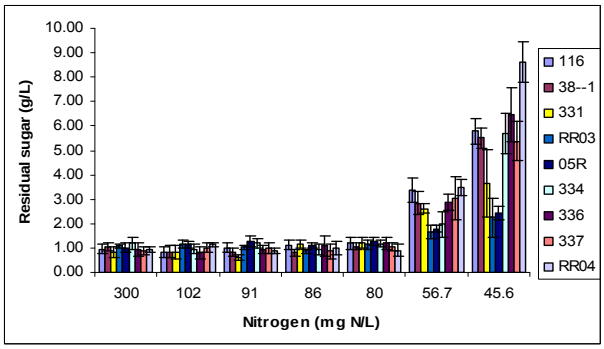

Fig 3.12 Comparison of residual sugar level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing different nitrogen concentration.

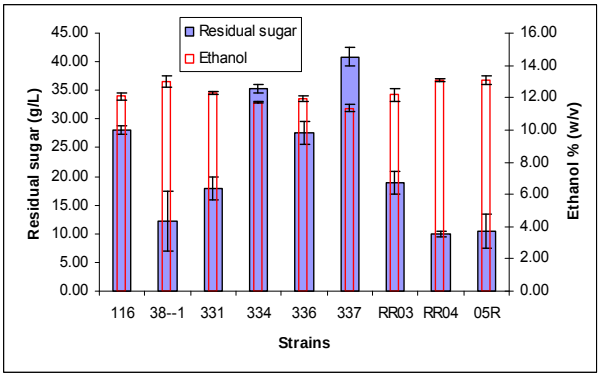

Fig 3.13 Comparison of residual sugar and produced ethanol level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing 300 g/L of sugar and 300 mg N/L of nitrogen.

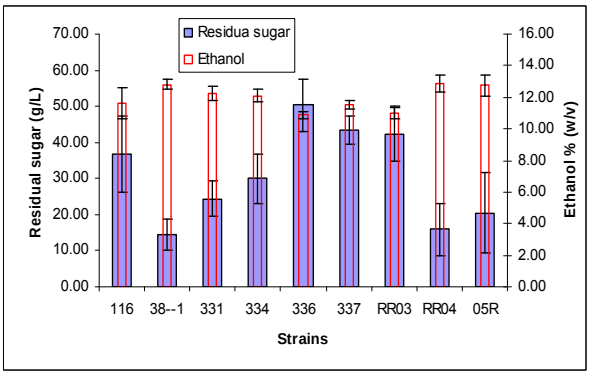

Fig 3.14 Comparison of residual sugar and produced ethanol level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing 300 g/L of sugar and 450 mg N/L of nitrogen.

Table 3.5 Comparison of hybrid strains for efficient utilization of fructose at the end of fermentation

	150 g/L of fructose					175 g/L of fructose					200 g/L of fructose				
Strains	**$Glucose_{av}$**	**Stdev**	**$Fructose_{av}$**	**Stdev**	**G/F ratio**	**$Glucose_{av}$**	**Stdev**	**$Fructose_{av}$**	**Stdev**	**G/F ratio**	**$Glucose_{av}$**	**Stdev**	**$Fructose_{av}$**	**Stdev**	**G/F ratio**
116	0.40	± 0.12	0.83	± 0.07	0.48	0.37	± 0.19	1.02	± 0.10	0.36	0.82	± 0.18	1.91	± 0.16	0.43
38-1	0.40	± 0.23	0.87	± 0.08	0.45	0.58	± 0.18	1.04	± 0.08	0.55	0.63	± 0.09	2.21	± 0.30	0.29
331	0.66	± 0.19	0.86	± 0.13	0.77	0.55	± 0.20	1.00	± 0.09	0.56	0.82	± 0.15	1.69	± 0.23	0.49
334	0.38	± 0.28	0.99	± 0.10	0.38	0.32	± 0.14	1.04	± 0.05	0.30	0.53	± 0.14	2.04	± 0.35	0.26
336	0.35	± 0.11	0.86	± 0.16	0.40	0.20	± 0.21	1.02	± 0.11	0.19	0.62	± 0.15	2.48	± 0.57	0.25
337	0.41	± 0.20	1.13	± 0.10	0.37	0.23	± 0.20	1.06	± 0.06	0.21	0.68	± 0.19	2.32	± 0.43	0.29
RR03	0.34	± 0.16	0.92	± 0.07	0.37	0.27	± 0.17	1.07	± 0.07	0.25	0.70	± 0.12	1.69	± 0.06	0.41
RR04	0.52	± 0.13	0.86	± 0.09	0.60	0.42	± 0.13	1.07	± 0.05	0.39	0.75	± 0.15	2.01	± 0.34	0.37
05R	0.58	± 0.45	0.86	± 0.08	0.68	0.33	± 0.22	0.96	± 0.09	0.34	0.65	± 0.15	1.75	± 0.33	0.37

[1]Experiments were done in independent triplicates. $_{av}$, average of the residual sugars in triplicates and Stdev, for standard deviation

Table 3.6 Comparison of ethanol yield of wine yeast strains under different fermentation conditions at the end of fermentation

	300 g/L sugar and 300 mg N/L			300 g/L sugar and 450 mg N/L			350 g/L sugar and 300 mg N/L			350 g/L sugar and 450 mg N/L		
Strains	**Cs**	**PE**	**$Y_{p/s}$**	**Cs**	**PE**	**$Y_{p/s}$**	**Cs**	**PE**	**$Y_{p/s}$**	**Cs**	**PE**	**$Y_{p/s}$**
116	271.88 ± 0.68*	120.70 ± 2.21*	0.44	263.17 ± 10.58*	115.92 ± 9.71*	0.44	239.90 ± 5.29*	99.91 ± 3.00*	0.42	266.94 ± 3.84*	114.37 ± 2.49*	0.43
38-1	287.92 ± 5.22*	129.88 ± 3.67*	0.45	285.42 ± 4.36*	128.19 ± 2.96*	0.45	252.85 ± 2.25*	108.32 ± 2.19*	0.43	288.16 ± 3.14*	124.25 ± 1.45*	0.43
331	282.11 ± 2.09*	123.03 ± 0.66*	0.44	275.57 ± 4.88*	122.63 ± 4.72*	0.45	238.60 ± 4.27*	100.00 ± 1.66*	0.42	275.13 ± 3.95*	117.50 ± 1.24*	0.43
334	264.62 ± 0.74*	116.93 ± 0.36*	0.44	270.08 ± 6.87*	121.07 ± 4.14*	0.45	227.22 ± 0.88*	94.76 ± 0.68*	0.42	254.06 ± 0.94*	107.98 ± 1.51*	0.43
336	272.45 ± 1.93*	119.26 ± 1.84*	0.44	249.72 ± 7.16*	108.65 ± 2.10*	0.44	212.03 ± 8.42*	84.95 ± 5.82*	0.40	251.74 ± 1.20*	105.99 ± 1.00*	0.42
337	259.15 ± 1.60*	113.52 ± 2.27*	0.44	256.60 ± 4.07*	115.35 ± 2.70*	0.45	224.30 ± 4.93*	93.74 ± 2.51*	0.42	253.91 ± 2.86*	105.58 ± 2.27*	0.42
RR03	281.21 ± 1.98*	121.66 ± 3.79*	0.43	257.64 ± 7.61*	109.73 ± 3.41*	0.43	229.67 ± 2.52*	95.06 ± 0.72*	0.41	262.53 ± 4.91*	105.48 ± 2.58*	0.40
RR04	290.03 ± 0.48*	130.56 ± 0.81*	0.45	284.15 ± 7.25*	128.77 ± 5.24*	0.45	265.16 ± 3.17*	114.76 ± 1.25*	0.43	300.26 ± 0.44*	130.53 ± 1.30*	0.43
05R	289.52 ± 2.99*	130.68 ± 2.84*	0.45	279.5 ± 11.18*	127.41 ± 6.41*	0.46	195.42 ± 6.48*	93.33 ± 3.00*	0.48	284.43 ± 1.57*	122.34 ± 1.07*	0.43

[1]Experiments were done in independent triplicates

[2]Cs, consumed sugar; PE, produced ethanol and Yp/s yield based on product/substrate

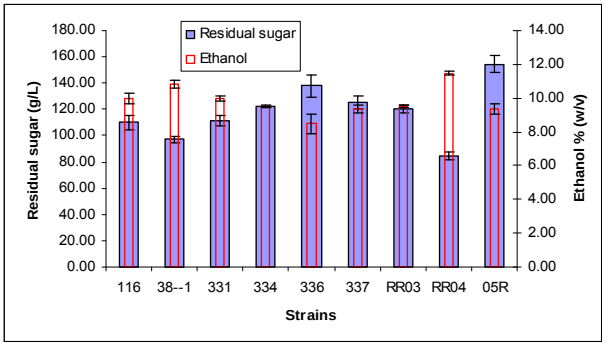

Fig 3.15 Comparison of residual sugar and produced ethanol level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing 350 g/L of sugar and 300 mg N/L of nitrogen.

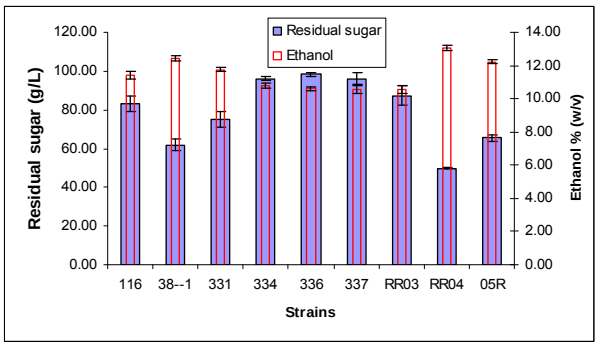

Fig 3.16 Comparison of residual sugar and produced ethanol level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing 350 g/L of sugar and 450 mg N/L of nitrogen.

When nitrogen efficiency was assessed, the residual sugar levels varied between strains ranging from 0.83 g/L for hybrid 331 to 1.20 g/L for hybrid 334 in synthetic must containing YAN of 300 mg N/L. The residual sugar levels remained low, with values of 0.80 g/L for parent 38-1 to 1.13 g/L and 1.14 g/L for parent 116 and hybrid 331, respectively, as the amount of YAN was gradually decreased to 86 mg N/L. Although all strains could still ferment the synthetic must to dryness, a built-up in residual sugar levels and significant differences between strains was observed when nitrogen composition changed to FAN at concentration of 56.7 mg N/L. The residual sugar levels ranged from 1.66 g/L for hybrid RR03 to 3.48 g/L for hybrid RR04. However, as the nitrogen concentration was lowered further to 45.6 mg N/L a distinct selective performance of the strains was evident. The residual sugar levels between strains ranged from 2.25 g/L for hybrid RR03 to 8.62 g/L for hybrid RR04 under these conditions (**Fig. 3.12**). Hybrid strains 331, RR03 and 05R showed significant improvement and completed fermentation in low nitrogen.

When ethanol tolerance was assessed, the fermentation was stuck with residual sugar concentration in the range of 9.97 g/L for hybrid RR04 to 40.86 g/L for hybrid 337 when sugar concentration was 300 g/L and the nitrogen concentration of 300 mg N/L (**Fig. 3.13**). Increasing the nitrogen concentration to 450 mg N/L did not improve the consumption of sugar in most strains, residual sugar ranging from 14.58 g/L for parent 38-1 to 50.29 g/L for hybrid 336. Hybrid strain 334 was the exception and showed improved sugar utilization by 5.45 g/L (**Fig. 3.14**). When the sugar concentration was 350 g/L, the residual sugar remained high ranging from 84.84 g/L for hybrid RR04 to 154.58 g/L for hybrid 05R (**Fig. 3.15**). However, increasing nitrogen concentration resulted in increased consumption of sugar with reduced residual sugar levels ranging from 49.74 g/L for hybrid RR04 to 98.27 g/L for hybrid 336 (**Fig. 3.16**). Although the fermentation was stuck, hybrid strain RR04, 05R and parent 38-1 fermented sugar significantly better than the parental strain 116 and other hybrid strains in all the trials

3.3.4 Ethanol production

Ethanol production ability of the strains was measured at the end of fermentation. One-way test showed that during fermentation of high fructose synthetic must the amounts of ethanol produced in all the trials were not significantly different between most of the strains. These amounts are ranged from 10.85% (w/v) for hybrid 336 to 11.9% (w/v) for hybrid RR04 in fermented synthetic must containing 150 g of fructose of the total sugar. As for high fructose (i.e. 175 g and 200 g of fructose), the amounts ranged from 10.67% (w/v) for hybrid 336 to 11.16% (w/v) for parent 38-1 and from 10.85% (w/v) for hybrid 05R to 11.23% (w/v) for parent 116. These results as illustrated in **Fig. 3.17**.

In the limiting nitrogen experiment, ethanol produced at the end of fermentation varied from one strain to another and data was not always consistent between experiments. Some strains had produced significantly lower amount in one experiment and higher in the others (**Fig. 3.18**). The amount ranged from 10.44% (w/v) for hybrid 337 to 11.32% (w/v) for hybrid 334; 10.78% (w/v) for 331 to 11.35% (w/v) for hybrid 334; 10.40% (w/v) for hybrid 331 to 11.38% (w/v) for parent 116; 10.53% (w/v) for hybrid 337 to 11.26% (w/v) for hybrid 05R; 10.78% (w/v) for hybrid RR04 to 11.24% (w/v); 11.24% (w/v) for hybrid RR04 to 11.46% (w/v) for hybrid 336; 10.28% (w/v) for parent 38-1 to 11.42% (w/v) for hybrid 331 as nitrogen concentration (mg N/L) was varied as follows 300; 102; 91; 86; 80; 56.7; and 45.6, respectively.

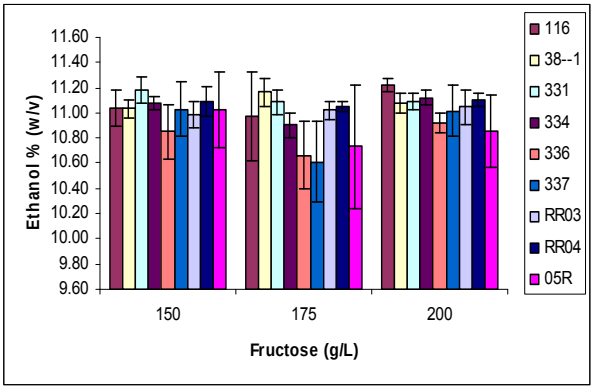

Fig 3.17 Comparison of produced ethanol level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing high fructose

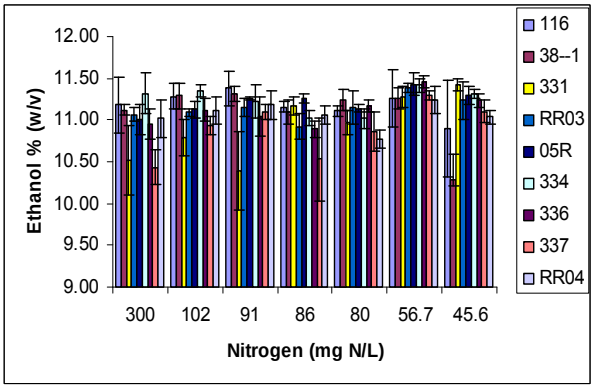

Fig 3.18 Comparison of produced ethanol level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing different nitrogen concentration.

Hybrid strain 337 produced less ethanol (10.44% (w/v)) in the conditions of high nitrogen concentration without stuck fermentation. Parental strain 38-1 produced less ethanol (10.28% (w/v)) under low nitrogen concentration and stuck with 5.85 g/L of sugar. Other strains including parental strain 116, which did not complete fermentation in low nitrogen concentration, produced high ethanol levels. These variations in amount of ethanol produced by the strains between experiments make it uneasy to identify potential lower ethanol producers.

The ability of hybrid stains to produce ethanol, while withstanding high amount of ethanol was investigated in synthetic must containing high sugar and variable nitrogen concentration. The amount ethanol produced varied significantly between strains when

300 g/L of sugar was fermented and ranged from 11.35% (w/v) for hybrid 337 to 13.06% (w/v), 13.07% (w/v) for hybrid RR04 and 05R, respectively (**Fig. 3.13**). The 300 g/L of sugar in the must with nitrogen concentration increased to 450 mg N/L resulted in a decrease amount of ethanol been produced for most of the strains, while only hybrid strains 334 and 337 showed small increase in amount of ethanol by 0.42% (w/v) and 0.19% (w/v), respectively. The amounts ranged from 10.82% (w/v) for hybrid 336 to 12.88% (w/v) for hybrid RR04 (**Fig. 3.14**). We further tested these strains in medium containing 350 g/L of sugar and 300 mg N/L of nitrogen. The results showed that the amount of ethanol produced was lower than in medium containing 300 g/L of sugar. The ethanol ranged from 9.33% (w/v) for hybrid 05R to 11.48% (w/v) for hybrid RR04 (**Fig. 3.15**). However, by increasing nitrogen concentration to 450 mg N/L significant increase of ethanol between 1 and 3% (w/v) was obtained. The ethanol produced was in the range of 10.55% (w/v), 10.56% (w/v) for hybrid strains RR03 and 337, respectively and 13.05% (w/v) for hybrid RR04 (**Fig. 3.16**). Since all the sugar was not fermented to dryness we have decided to determine the ethanol yield of each strain. The ethanol yield ($Y_{p/s}=^{Product}/_{Substrate}$), defined as the amount of ethanol produced over a consumed amount of sugar. This was used as an important measure of how efficiently yeast strains convert sugar (both glucose and fructose) into ethanol. The strains that produced more ethanol were stuck with less residual sugar than other strains. $Y_{p/s}$ was only calculated at the end of fermentation. We found that $Y_{p/s}$ for most strains decreased at high concentration of sugar in the must. Hybrid 05R was the exception with increasing $Y_{p/s}$ (0.48) and produced relatively more ethanol from small amount of consumed sugar (**Table 3.6**).

3.3.5 Glycerol production

The production of glycerol by most strains in high fructose synthetic must was ranged from 3.92 g/L for hybrid 336 to 4.99 g/L for parental strain 38-1.

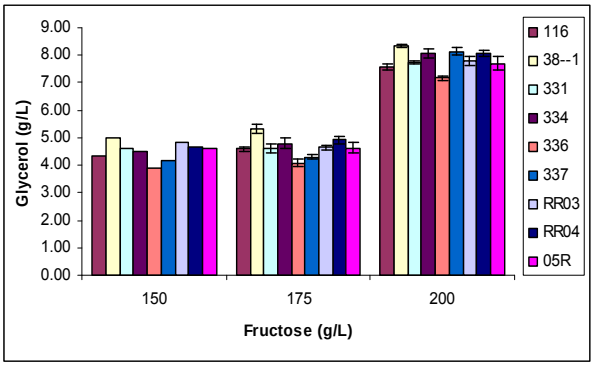

Fig 3.19 Comparison of produced glycerol level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing high fructose

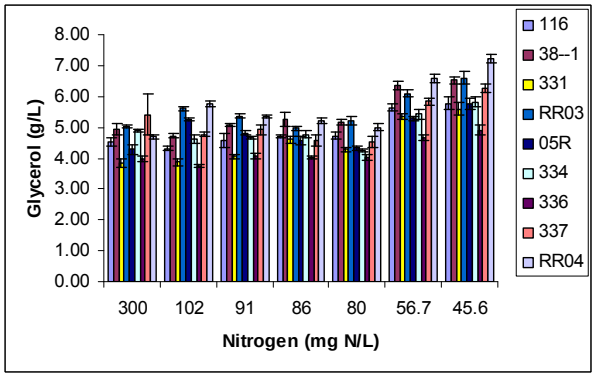

Fig 3.20 Comparison of produced glycerol level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing different nitrogen concentration.

The amount of glycerol increased with increase in fructose concentration in the must. The highest range of glycerol produced in higher fructose concentration was 7.16 g/L for hybrid 336 and 8.34 g/L for parental strain 38-1 (**Fig. 3.19**). The glycerol production

levels in low nitrogen synthetic must ranged from 3.76 g/L for hybrid 336 to 7.22 g/L for hybrid RR04 across all the fermentation trials with slightly different patterns depending on the yeast strain. All the strains showed an increase of glycerol production with decrease of nitrogen concentration. Hybrid strain 336 had always maintained the lowest level of glycerol in all the fermentation trials (**Fig. 3.20**) and therefore is a potential candidate for low glycerol producing strain.

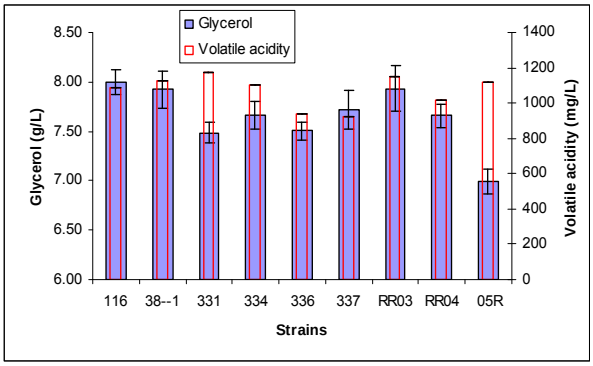

Fig 3.21 Comparison of produced glycerol and volatile acidity level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing 300 g/L of sugar and 300 mg N/L of nitrogen.

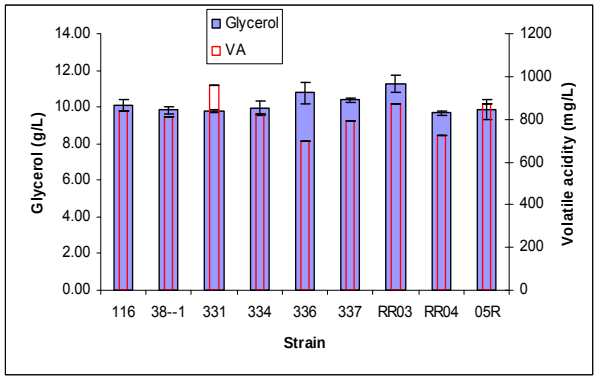

Fig 3.22 Comparison of produced glycerol and volatile acidity level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing 300 g/L of sugar and 450 mg N/L of nitrogen.

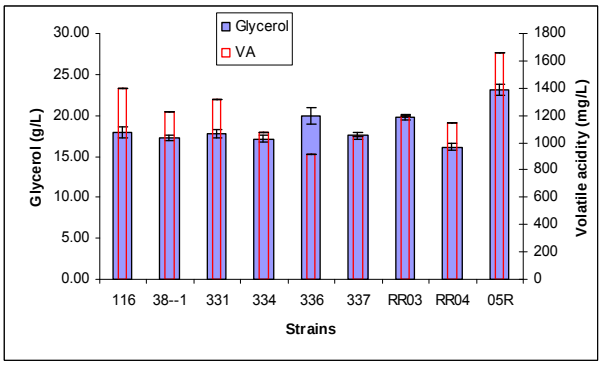

Fig 3.23 Comparison of produced glycerol and volatile acidity level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing 350 g/L of sugar and 300 mg N/L of nitrogen.

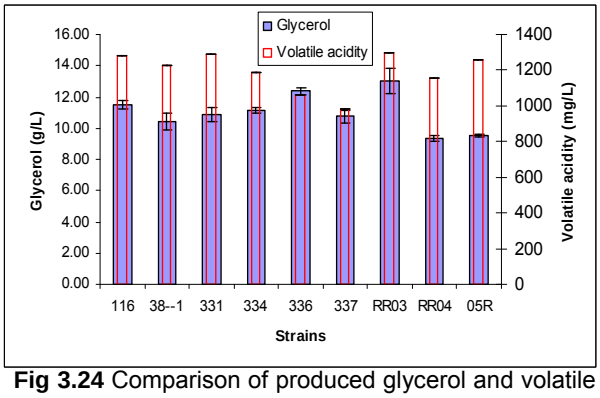

Fig 3.24 Comparison of produced glycerol and volatile acidity level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing 350 g/L of sugar and 450 mg N/L of nitrogen.

Glycerol levels produced in stuck fermented synthetic must containing 300 g/L of sugar ranged from 6.99 g/L for hybrid 05R to 8.0 g/L for parental strain 116 (**Fig. 3.21**). When nitrogen concentration was increased to 450 mg N/L, the profile of most strains changed. Almost all the strains showed increased glycerol level with the exception of hybrid strains RR04 and 05R. These glycerol levels ranged from 9.67 g/L for hybrid RR04 to 11.25 g/L for hybrid RR03 (**Fig. 3.22**). High sugar (350 g/L) when fermented resulted with higher glycerol level in the range from 16.14 g/L for hybrid RR04 to 23.15 g/L for hybrid 05R (**Fig. 3.23**). However, increasing nitrogen concentration did not increase the glycerol level in all the strains. The produced glycerol levels ranged from 9.33 g/L for hybrid RR04 to 13.05 g/L for hybrid RR03 (**Fig. 3.24**).

3.3.6 Volatile acidity production

Volatile acids (VA) can be formed by yeast activity during fermentation and by spoilage bacteria during fermentation or ageing. The term VA is used to encompass all volatile acids in wine with acetic acid being the main component. The difference in the formation of VA was small from one trial to another however greater significant levels existed amongst the fermenting strains. The strains produced VA in the range between 585 mg/L for hybrid 337 and 815 mg/L for hybrid 331. A concentration of 175 g/L fructose in the synthetic must caused insignificant reduction in VA level for all the strains. However, 200 g/L fructose in the must showed significant increase of VA level. Hybrid 337 (735 mg/L) produced higher VA levels. Whereas other strains such as 05R, 38-1, 334, 336 and RR04 showed slight decrease in VA level (**Fig. 3.25**).

VA production decreased with decrease in nitrogen concentration during fermentation of synthetic must. The VA levels were high at 800 mg/L for hybrid RR03 and low for hybrid 337 (240 mg/L) across all the fermentation trials. The reduction of VA levels was consistent for all the strains and glycerol increased dramatically at low nitrogen. Under these conditions, hybrid strain 337 consistently maintained low VA level in all the trials. Nevertheless, hybrid 331 showed an increase of VA in the medium without ammonium and non-preferred amino acids as the nitrogen concentration was reduced its VA level was still lower than in high nitrogen concentration (**Fig. 3.26**).

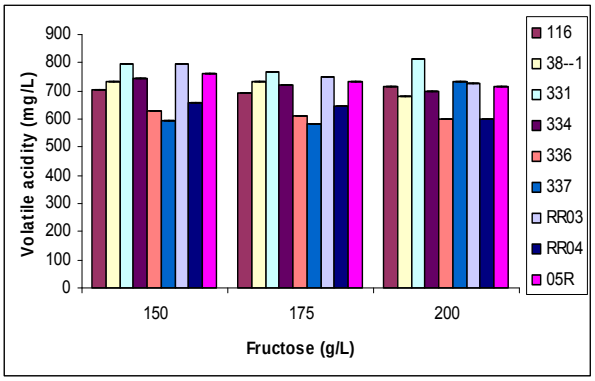

Fig 3.25 Comparison of produced volatile acidity level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing high fructose

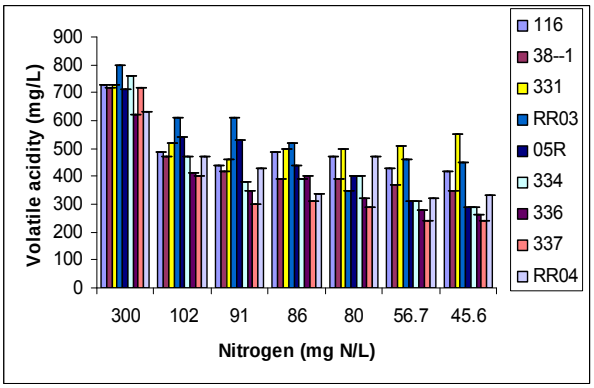

Fig 3.26 Comparison of produced volatile acidity level of different enriched hybrid strains against parental strains at the end of fermentation in synthetic medium containing different nitrogen concentration.

Synthetic must containing high sugar (and 300 mg N/L) stimulated fermenting yeast strains to produce more VA. VA levels produced ranged from 927 mg/L for hybrid 337 to 1177 mg/L for hybrid 331 (**Fig. 3.21**). Increasing the nitrogen concentration to 450 mg N/L had resulted in significant decrease of VA for all the strains. This had ranged from 698 mg/L for hybrid 336 to 960 mg/L for hybrid 331 (**Fig. 3.22**). When the sugar was 350 g/L (and 300 mg N/L) a large increase of VA showing different pattern for the strains was noticed. The VA production ranged from 920 mg/L for hybrid 336 to 1662 mg/L for hybrid 05R (**Fig. 3.23**). Increasing nitrogen concentration had slightly increased

VA in some strains, while other strains produced reduced VA level. The VA production ranged from 980 mg/L for hybrid 337 to 1300 mg/L for hybrid RR03 (**Fig. 3.24**).

Table 3.7 Residual sugar (g/L) of various strains under different nitrogen conditions at the end of fermentations

Strains	300 mg N/L			102 mg N/L			91 mg N/L		
	Total RS	**Glucose**	**Fructose**	**Total RS**	**Glucose**	**Fructose**	**Total RS**	**Glucose**	**Fructose**
116	1.07	0.44 ± 0.21*	0.63 ± 0.05*	1.11	0.60 ± 0.22*	0.51 ± 0.13*	1.08	0.51 ± 0.14*	0.57 ± 0.16*
38--1	1.17	0.45 ± 0.27*	0.72 ± 0.08*	1.07	0.62 ± 0.21*	0.45 ± 0.14*	0.92	0.41 ± 0.14*	0.51 ± 0.10*
331	0.83	0.17 ± 0.70*	0.66 ± 0.10*	0.83	0.40 ± 0.25*	0.43 ± 0.03*	0.61	0.22 ± 0.02*	0.39 ± 0.07*
334	1.21	0.27 ± 0.08*	0.94 ± 0.04*	0.97	0.38 ± 0.06*	0.59 ± 0.06*	1.19	0.60 ± 0.60*	0.59 ± 0.10*
336	0.94	0.21 ± 0.26*	0.73 ± 0.06*	0.8	0.30 ± 0.00*	0.50 ± 0.00*	0.95	0.46 ± 0.10*	0.49 ± 0.03*
337	0.96	0.01 ± 0.02*	0.95 ± 0.06*	1.01	0.34 ± 0.11*	0.67 ± 0.01*	1	0.43 ± 0.06*	0.57 ± 0.14*
RR03	1.03	0.33 ± 0.08*	0.77 ± 0.02*	1.17	0.67 ± 0.06*	0.50 ± 0.05*	1.04	0.60 ± 0.05*	0.44 ± 0.02*
RR04	0.95	0.28 ± 0.05*	0.67 ± 0.04*	1.14	0.75 ± 0.40*	0.39 ± 0.01*	0.89	0.55 ± 0.07*	0.34 ± 0.03*
05R	1.01	0.32 ± 0.09*	0.69 ± 0.04*	1.13	0.78 ± 0.10*	0.35 ± 0.07*	1.19	0.80 ± 005*	0.39 ± 0.04*

[1]Experiments were done in independent triplicates

[2]* values for standard deviations, RS, residual sugar

Table 3.7 (Continues) Residual sugar (g/L) of various strains under different nitrogen conditions at the end of fermentations

Strains	86 mg N/L			80. mg N/L			56.7 mg N/L		
	Total RS	**Glucose**	**Fructose**	**Total RS**	**Glucose**	**Fructose**	**Total RS**	**Glucose**	**Fructose**
116	1.13	0.75 ± 0.06*	0.38 ± 0.04*	1.22	0.77 ± 0.12*	0.45 ± 0.02*	3.35	1.22 ± 0.22*	2.13 ± 0.31*
38--1	0.76	0.53 ± 0.13*	0.23 ± 0.21*	1	0.64 ± 0.21*	0.36 ± 0.09*	2.84	0.97 ± 0.15*	1.87 ± 0.40*
331	1.14	0.82 ± 0.02*	0.32 ± 0.04*	1.23	0.80 ± 0.15*	0.43 ± 0.12*	2.63	1.14 ± 0.05*	1.49 ± 0.19*
334	0.94	0.56 ± 0.09*	0.38 ± 0.10*	1.18	0.64 ± 0.02*	0.54 ± 0.05*	1.96	0.92 ± 0.16*	1.04 ± 0.46*
336	1.08	0.63 ± 0.03*	0.45 ± 0.23*	1.19	0.74 ± 0.10*	0.45 ± 0.03*	2.88	1.2 ± 0.05*	1.68 ± 0.34*
337	0.86	0.42 ± 0.32*	0.44 ± 0.05*	1.03	0.57 ± 0.08*	0.46 ± 0.05*	3.02	1.12 ± 0.22*	1.90 ± 0.78*
RR03	0.86	0.38 ± 0.04*	0.48 ± 0.02*	0.91	0.44 ± 0.11*	0.47 ± 0.08*	1.66	0.88 ± 0.10*	0.78 ± 0.08*
RR04	1	0.68 ± 0.06*	0.32 ± 0.01*	1.13	0.69 ± 0.15*	0.44 ± 0.02*	3.47	0.97 ± 0.10*	2.50 ± 0.35*
05R	1.11	0.74 ± 0.07*	0.37 ± 0.03*	1.26	0.82 ± 0.06*	0.44 ± 0.04*	1.77	1.04 ± 0.07*	0.73 ± 0.05*

[1]Experiments were done in independent triplicates

[2]* values for standard deviations, RS, residual sugar

3.4 DISCUSSION

One of the challenges of any breeding strategy is the careful selection of parental strains. We have carefully selected two strains, namely 116 and 38-1, which are hybrid strains obtained from previous breeding programs. These strains have already been improved for winemaking purposes based on their nitrogen and fructose efficiently. Our aim here was to further improve one of these two traits through breeding and enrichment (chemostat) strategy.

The conditions used for enrichment (see materials and methods) were designed to select those strains that displayed increased genetic "fitness" in high fructose and limited nitrogen environments. The enrichment approach eliminates large numbers of undesirable mutants or strains from the population. This approach has some inherent limitations. Some phenotypes might not be obtained and sometimes false-positive mutants are selected (Han and Parekh, 2005). Another strategy was "semi-continuous" and was used to enrich for high ethanol tolerant strains. This strategy ensured that only those strains that withstand high ethanol and nutritional deficiency would be selected.

Identification of the selected strains from the bioreactor was made possible by use of PCR and strain karyotyping. Karyotype analysis using CHEF was able to reveal differences between the strains (**Fig. 3.4**) and those differences could be attributed to mutations and recombination (crossover) that occured during sporulation and mating and also to note that chemostat selection induces mutations. PCR results confirmed strain 331, 336 and 05R as hybrids, whereas with other strains PCR using σ primers revealed that another strain RR04 as a hybrid. This means that no single PCR method was successful in identifications, and that the use of several PCR methods is essential to complete and authenticate the nature of the strain (**Fig. 3.5a-c**).

The attempt to improve efficiency of fructose utilization by newly generated hybrid strains was successful (**Table 3.5**). Wine yeasts strains showing efficient utilization of fructose would maintain high fermentation rate at the end of alcoholic fermentation (Guillaume *et al*., 2007). Hybrid strains RR03 and 05R showed better fructose utilization in low nitrogen concentration than the parental strains (**Table 3.7**). However, differences in fructose utilization were observed, they depended on the nitrogen content of the synthetic must. The mechanism by which nitrogen concentration may regulate hexose utilization is unclear. Guillaume *et al*. (2007) reported that molecular characterisation of wine yeast strain that had high capacity or high rate for fructose utilization harboured mutations in *HXT3* transporter.

The development of more nitrogen efficient strains was also successful. Three of the developed hybrid strains completed fermentation to dryness at a concentration of 45.6 mg N/L; however the overall period to ferment was protracted. The above nitrogen concentration became limiting for other strains as illustrated in **Fig. 3.12** with residual sugar exceeded 5 g/L. Limiting concentration of nitrogen could be defined as the concentration which is sufficient to lead fermentation to premature cessation. Some researchers have noted that the concentration of nitrogen which is limiting is also

influenced, in synthetic media, by the conditions applied. For instance, EC 1118, a wine strain commercially available worldwide was found to have a limiting concentration of 60 mg N/L (Wang *et al*., 2003) and 50 mg N/L (Varela *et al*., 2004) in similar synthetic media containing 240 g/L of sugar.

Ethanol tolerant strain selection seemed to have been a successful strategy. The reason for this is that two of the selected hybrid strains RR04 and 05R have produced 13.07% (w/v) ethanol (**Fig. 3.13 – 3.16**). These strains are potentially more robust than the parental strains under the conditions used (see materials and methods). Other laboratory tests for ethanol tolerance do not represent or rather mimic the conditions the fermenting yeast strains are subjected to during winemaking. These include addition of exogenous absolute ethanol into the medium, or in agar plates to assess growth and viability.

All the strains completed fermentation in high fructose synthetic must, whereas in low nitrogen concentration synthetic must three of the hybrid strains completed without occurrence of stuck fermentation. High fructose increased the production of glycerol and VA. The proportional increase of glycerol with fructose has been linked to the activation of the pentose phosphate pathway. Yalçin and Özbaş (2005) had obtained high glycerol and specific glycerol production rate in media of high fructose fermented by *S. cerevisiae* Kalecik 1. VA in some strains was also found to increase with fructose. Low concentration of nitrogen increased glycerol levels while VA levels decreased in all the strains

However, in high sugar with glucose-fructose ratio of 1 a sluggish and stuck fermentation occurred. This may be attributed to the small inoculum size, high sugar toxicity, or alcohol toxicity. It is recommended that medium with such high sugar be inoculated with 1×10^6 yeast cells to counteract sugar toxicity (Zoecklein, 2007).

High nitrogen content in synthetic must containing high sugar, 300 g/L increased glycerol level while decreasing VA level without significant improvement of sugar utilization and lower ethanol levels was production. When the sugar was 350 g/L, high nitrogen concentration facilitated sugar utilization, reduced glycerol formation and VA and increased ethanol production. Bely *et al*. (2003) suggested that nitrogen in addition to stimulating growth, it also provide NADH in the redox-equilibrating process which reduces VA formation. High sugar in synthetic must imposed a limit to fermenting wine yeast strains ability to utilise sugar, consequently low ethanol was produced and it also increased formation of glycerol and VA. Regarding fermentation kinetics, high nitrogen concentration in a high sugar synthetic must resulted in a decreased overall fermentation period.

3.5 CONCLUSION

In conclusion, the combination of hybridization and enrichment technique was successful to improve fructose efficiency, nitrogen efficiency and ethanol tolerance of the wine yeast strains and some of the strain(s) produced low H_2S. Some hybrid strains showed a combination of improved traits. Hybrid 331 was found to be a fructose efficient and nitrogen efficient strain. Hybrid 05R acquired nitrogen efficiency and ethanol tolerance traits. Hybrid RR03 is a nitrogen efficient strain, while hybrid RR04 found to be ethanol tolerant strain. These selected strains show greater fermentation efficiency under different conditions and may offer the winemaker a further option in the control related fermentation faults. Future work will focus on testing these strains under winemaking conditions using different cultivars.

3.6 ACKNOWLEDGEMENT

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Chapter 4

GENERAL DISCUSSION AND CONCLUSION

4. GENERAL DISCUSSION AND CONCLUSION

4.1 GENERAL DISCUSSION

Over the past few decades, the trend in the global wine industry has been to shift from spontaneous fermentation of grape must to inoculation by commercial yeast starter cultures. This practice has resulted in a sustained demand for new strains of *Saccharomyces cerevisiae* with desirable oenological characteristics. This study aimed at developing new yeast strains that would be better adapted to extreme oenological conditions. Such developed strains should be able to reduce the occurrence of stuck fermentations and perhaps minimise the chance of off-flavour development. Two parent strains, 116 and 38-1, that had previously been selected and tested in both laboratory small-scale fermentation and winemaking conditions to be nitrogen efficient and fructose efficient, respectively, were used as parental strains for the breeding program.

Hybridization tends to result in many more strains that are detrimentally affected for some generic feature than in improved strains. As our principal aim was to increase the efficiency of two traits and to combine these traits in single new strain(s), we employed hybridization and chemostat enrichment as tools. The results we have obtained indicated that carefully chosen and controlled enrichment conditions could select for those hybrids strains with improved genetic fitness. However, because of risks of contamination involved in the enrichment steps, it is essential to periodically safeguard samples to avoid disastrous experiment.

Some of the principal causes of sluggish fermentation are nitrogen deficiencies, high sugar in grape must and the inhibitory effect of ethanol. The nitrogen efficiency of strains differs from one strain to another. We have generated new wine yeast hybrid strains that are more nitrogen efficient than the parental strains. These strains were able to complete fermentation in nitrogen limited musts in which the parental strains got stuck with high residual sugar (> 5 g/L). One hybrid strain also proved to have potentially high capacity for fructose utilization in conditions of high fructose synthetic must. Ethanol tolerance is a major problem in the wine industry. Because of the variations in the amount of sugar present during grape harvest, many of the *S. cerevisiae* wine yeast strains struggle to complete fermentation in very high sugar must. High sugar must is normally managed by increasing inoculum size and nitrogen supplements. We have generated a hybrid wine yeast strains that withstood significantly higher levels of ethanol than at least one of the parental strains. The increase of anaerobic factors and nitrogen in MS media containing 350 g/L of sugar did not result in significant increase of ethanol production (Data not shown) for almost all the strains.

In summary, several desirable characteristics were improved in the hybrid strains developed during this study. However, these strains require further assessment in real wine making conditions before they can be considered for commercialisation.

Many of the improvements achieved in this study, while significant, other trait(s) in some strains were not possible to improve. This could be attributed to the following reasons:

1. The parental strains had already been selected for these specific traits and we may have reached some inherent metabolic limits of *S. cerevisiae*.
2. The selective pressure and number of generations may have been insufficient.

4.2 CONCLUSION

The aims of the study were achieved. We were able to generate hybrid strains with combined oenological characteristics. And some of the hybrid strains with high sugar and high ethanol tolerance might be a useful tool in the wine industry for the fermentation of specific grape musts.

4.3 FUTURE WORK

All the hybrid strains tested in this study require a final oenological characterisation regarding their ability to produce aromatic compounds and their ability to ferment different musts derived from various grape cultivars. The sensory profile produced by each strain in real musts will also need to be assessed.

The molecular characterisation of those hybrid strains showing improvement requires further attention and will remain a focus of future research.