

The Breeding of Yeast Strains for Novel Oenological Outcomes

by

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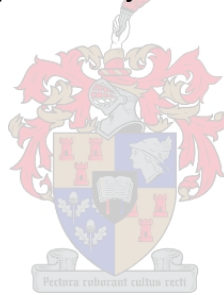
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DECLARATION

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



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SUMMARY

The quality of wine is influenced by a variety of factors, most noticeably the quality of the grapes, winemaking practices and the yeast strains used for alcoholic fermentation. Although several yeast strains are present in the must at the beginning of fermentation, strains of *S. cerevisiae* quickly dominate and survive alcoholic fermentations. This dominance of *S. cerevisiae* prompted research that led to the development of a multitude of industrial yeast starter cultures. Starter cultures are usually capable of quick and complete fermentations, with minimal production of deleterious substances such as volatile acidity, H₂S, SO₂ and ethyl carbamate. Yeast strains should be able to survive the stressful environment created during alcoholic fermentation, whilst possibly offering novel oenological benefits such as pectinolytic activity, killer activity and malic acid degradation. The increased production of volatile esters and higher alcohols may also be desirable, as this will allow the production of wines that are more aromatic.

In this study, VIN13 was crossed with *S. paradoxus* strain RO88 and WE14 by using a micromanipulator. VIN13 was chosen for its fast and complete fermentation ability and moderate aroma production potential. Other factors such as the presence of killer activity and low production of volatile sulphur compounds also favoured the selection of VIN13. *S. paradoxus* strain RO88 was selected for its ability to degrade malic acid and the favourable impact on aroma production during fermentation. Hybrids between these yeasts may have the potential to produce more aromatic wines, with the added bonus of pectinolytic activity and a strong fermentation capacity. The first crossing yielded 5 hybrids between VIN13 and *S. paradoxus* strain RO88. Two of these hybrids stood out in the sense that they were able to degrade more malic acid than VIN13 and they also possessed killer and pectinolytic activity. Cinsaut wine was made and the 2 hybrids were shown to have higher aroma compound capacity than the parental yeasts. This was also confirmed during sensory evaluation. The second crossing between VIN13 and WE14 yielded 10 hybrids with low H₂S production potential and killer activity. WE14 was selected for its ability to produce very aromatic wines and also the slower fermentation capacity. Hybrids between these yeast may have the potential to produce wines with an increased aromatic content and the fermentation rate might be slower, thereby improving the aroma profile of the wine. After microvinification, 5 hybrids were selected on the basis of fermentation rate differing from that of the parental yeasts and favourable oenological traits, such as fast and complete fermentation, high production of glycerol and low production of volatile acidity. Pinotage wine was made and it was shown that some of the hybrids produced more esters and higher alcohols than the parental yeasts. Sensory evaluation also showed the aroma production potential of the hybrids, as some of the hybrids were shown to score higher for banana, cherry and tobacco characteristics.

OPSOMMING

Talle faktore beïnvloed die kwaliteit van wyn, mees noemenswaardig hiervan is die kwaliteit van die druiwe, wynmaak praktyke en die gisrasse wat vir alkoholiese fermentasie gebruik word. Alhoewel daar verskeie gisrasse teenwoordig is in die sap aan die begin van fermentasie, domineer *S. cerevisiae* vinnig en oorleef ook die alkoholiese fermentasies. Hierdie dominansie van *S. cerevisiae* het navorsing aangemoedig wat gelei het tot die ontwikkeling van 'n groot verskeidenheid gisrasse. Hierdie gisrasse is in staat tot vinnige en volledige fermentasies met minimale produksie van nadelige verbindings soos vlugtige suur, H₂S, SO₂ en etiel karbamaat. Die gisrasse is ook in staat om die stresvolle omgewing wat geskep word tydens alkoholiese fermentasie te oorleef. Verkieslik moet hulle ook nuwe oenologiese voordele bied, bv. pektinolitiese en killer aktiwiteit asook die afbraak van appelsuur. Verhoogde produksie van vlugtige esters en hoër alkohole is ook gesogd, aangesien dit die produksie van meer aromatiese wyne sal toelaat.

In hierdie studie is VIN13 met *S. paradoxus* ras RO88 en WE14 gekruis met behulp van 'n mikromanipulator. VIN13 is gekies aangesien dit sap vinnig en volledig fermenteer met positiewe impak op aroma produksie. Faktore soos killer aktiwiteit en lae produksie van vlugtige swawelkomponente het ook die seleksie van VIN13 bevoordeel. *S. paradoxus* strain RO88 is geselekteer want dit kan appelsuur degradeer en die aroma van wyn verbeter. Hibriede tussen die giste mag dus die potensiaal hê om meer aromatiese wyn te produseer en kan ook pektinolitiese aktiwiteit toon. Die eerste kruising tussen VIN13 en *S. paradoxus* ras RO88 het 5 hibriede opgelewer. Twee van hierdie hibriede het uitgestaan in die sin dat hulle meer malaat as VIN13 kon afbreek en dat hulle killer en pektinolitiese aktiwiteit besit. Cinsaut wyn is gemaak en die 2 hibriede het hoër konsentrasies van sekere aroma komponente geproduseer as ouergiste. Hierdie resultate is ook weerspieël in die resultate van die sensoriese analise. Die tweede kruising tussen VIN13 en WE14 het 10 hibriede gelever, waarvan almal lae H₂S produksie potensiaal getoon het en killer aktiwiteit. WE14 is geselekteer aangesien dit die vermoë het om heelwat meer aromatiese wyne te produseer en ook vir die stadiger fermentasie. Hibriede tussen die giste mag ook potensieël wyn produseer met verhoogde aromatiese inhoud en die fermentasie tempo kan stadiger wees, wat kan lei tot 'n verbetering in die aromatiese profiel van die wyn. Nadat wynmaak op kleinskaal afgehandel is, is 5 hibriede geselekteer na gelang van fermentasie tempo wat verskil van die van die ouergiste, vinnige en volledige fermentasie, hoë produksie van gliserol en lae produksie van vlugtige suur. Pinotage wyn is ook gemaak en daar is gewys dat sommige van die giste meer esters en fusel alkohole geproduseer het as die ouergiste. Sensoriese analise het die positiewe aroma produksie potensiaal van sommige van die hibriede getoon, aangesien sommige van die hibriede hoër punte gekry het piesang, kersie en tabak karakter.

This thesis is dedicated to my parents.
Hierdie tesis is opgedra aan my ouers.

BIOGRAPHICAL SKETCH

Bernard Mocke was born in Bellville, South Africa on 19 March 1978. He attended the Lochnerhof Primary School (Strand) and Matriculated at the Strand High School in 1996. Bernard enrolled at the University of Stellenbosch in 1997 and obtained a BscAgric degree in Food Science and Biochemistry in 2000.

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PREFACE

This thesis is presented as a compilation of 5 chapters. Each chapter is introduced separately.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**

The breeding of wine yeasts for novel oenological outcomes

Chapter 3 **Research Results**

The breeding and characterisation of a novel wine yeast

Chapter 4 **Research Results**

The breeding and characterization of a red wine yeast

Chapter 5 **General Discussion and Conclusions**

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1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 GENERAL INTRODUCTION

The fermentation of must, to yield wine as end product, can be considered as the action of yeast species competing with each other in a very specific habitat. In traditional spontaneous fermentations, the microbes present on the surface of the grape skins participate in these natural wine fermentations (Pretorius *et al.*, 1999). While the composition of the microflora varies from case to case, it has been reported that generally in the early and middle stages of fermentation during which the ethanol concentration rises to 3-4%, yeasts of the genera *Kloeckera*, *Hanseniaspora*, *Candida*, *Metschnikowia* and *Pichia* are dominant. The yeasts in the later stages of alcoholic fermentation are alcohol-tolerant species, such as *Saccharomyces cerevisiae*, *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*. Modern wineries, aiming for reliable fermentation and the production of wines with predictable quality, use carefully selected starter cultures of *S. cerevisiae*. The traditional approach to develop wine yeasts was to select wild yeasts from grapes, grape musts, wines and winery equipment (Shinohara *et al.*, 1994). These strains are selected on the basis of the following characteristics: fermentation rate, fermentation at low temperature, sulphur dioxide (SO₂) tolerance, high sugar tolerance, low production of volatile acids, desirable flavour production, killer activity and general wine quality. There are several genetic techniques available to develop new wine yeasts. Gene cloning offers the most accurate way to achieve desired results, but it is not commercially accepted (Davies, 2001). Other, more conventional techniques such as hybridisation, rare mating, spheroplast fusion and mutagenesis are thus used to effect changes and develop new yeast strains (Pretorius, 2000).

Intra-species hybridisation involves the mating of haploids of opposite mating-types to yield a heterozygous diploid. Haploid strains from different parental diploids, possessing different genotypes, can be mated to form new diploid strains. Thus, in theory, crossbreeding can permit the selection of desirable characteristics and the elimination of undesirable ones. The problem arises that many wine yeasts are homothallic and the use of hybridization techniques for the development of new wine yeasts strains has proved difficult. This problem can be overcome by direct spore-cell mating using a micromanipulator. The diploid yeasts (parental yeast strains) are sporulated and tetrad dissection is done with the aid of a micromanipulator. The haploid spores of parental yeast strains with different genotypes are brought into close proximity with each other and are allowed to mate under growth conditions. If mating between the spores does occur, a new, diploid yeast is formed. This diploid strain might have properties different from that of either parental strain. Theoretically speaking, breeding can be used to introduce or select desirable characteristics and eliminate undesirable characteristics (Van der Westhuizen, 1990). Several yeast development projects have

been successful in developing new and novel yeast strains, focusing on wine wholesomeness, improved fermentation performance, processing efficiency, biological control of spoilage organisms and wine aroma (Pretorius, 2000). Yeast breeding projects offer many of the abovementioned possibilities and future work will yield a wide variety of new wine yeasts.

1.2 AIMS OF THIS STUDY

Malic acid, together with tartaric acid, are the dominant fixed acids in grapes, contributing 70-90% or more of the titratable acidity at the beginning of fermentation. Biological deacidification is the process of employing microorganisms to decrease the acidity of a defined medium. Biological deacidification of wine typically reduce the concentration of malic acid, thereby enhancing the flavour of the wine and also reducing the potential for post-fermentation spoilage of the wine by bacterial and yeast contaminants (Thornton and Rodriguez, 1996). Biological deacidification is usually done with the aid of malolactic bacteria starter cultures, but also includes the use of yeasts that are able to degrade malate under conditions similar to those employed in winemaking. Yeasts such as *Schizosaccharomyces pombe* are capable of doing this effectively, but they usually produce off flavours. In comparison, *Saccharomyces paradoxus* strain RO88 was found to degrade malic acid effectively and also produce wine with a good aroma profile (Redzepovic *et al.*, 2003). This yeast is very closely related to *S. cerevisiae* and falls within the *sensu stricto* group. Some authors even postulate that *S. cerevisiae* is a domesticated species originating from its closest relative, *S. paradoxus*, a wild species found all over the world associated with insects, tree exudates and fermenting plant extracts (Naumov, 1996). Yet another positive oenological trait of *S. paradoxus* strain RO88 is its strong pectinolytic activity. Strong fermentation capacity is needed in most industrial yeast strains, necessitating the need for yeasts that are capable of fast and complete fermentations. These industrial strains must also be capable of good aroma production in wine. It is noted, however, that more aromatic wines can be made by a reduction in the fermentation rate (Torija *et al.*, 2003). Other positive oenological traits include low production of H₂S, presence of killer activity and stress resistance. In order to obtain these goals (decrease in fermentation rate, degradation of malic acid, pectinolytic activity, production of more aromatic wines, killer activity and the production of low amounts of volatile sulphides), the following aims were set:

- (i) Construction of novel hybrids between the industrial yeast strains (VIN13 and WE14) and between VIN13 and *S. paradoxus* strain RO88.
- (ii) Phenotypical analysis of all the putative hybrids by means of plate assays.
- (iii) Identification of hybrids by CHEF and PCR.
- (iv) Identification of slower fermenting hybrids by small scale fermentation trials and microvinification with three different grape cultivars and synthetic must with the parental yeasts and hybrids.

- (v) Comparing the different strains on the basis of fermentative capacity and aroma, chemical and sensory analysis of the finished wines during and after ageing.
- (vi) Construction of a hybrid that can degrade significant amounts of malic acid.

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2. THE BREEDING OF WINE YEASTS FOR NOVEL OENOLOGICAL OUTCOMES

2.1 INTRODUCTION

The wide range of quality wines available today can be ascribed to the vast amount of knowledge and insight gained over a period of nearly 7000 years (Pretorius *et al.*, 1999). Numerous scientists tried to understand and explain the process that is known as wine making. Pasteur suggested that grape juice is converted into alcohol and the other constituents of wine by the action of yeast (Drysdale and Fleet, 1988). Pasteur was also first to propose that wine yeasts are present on the surface of grapes in vineyards. This statement however, has been the subject of intense debate, as subsequent studies confirmed this statement and others discredited Pasteur's statement (Mortimer and Polsinelli, 1999).

In traditional spontaneous fermentation, the microbes that were present on the surface of the grape skins participate in natural wine fermentation (Pretorius *et al.*, 1999). In the early and middle stages of fermentation, during which the ethanol concentration rises to 3-4%, yeasts of the genera *Kloeckera*, *Hanseniaspora*, *Candida*, *Metschnikowia* and *Pichia* are dominant. These yeasts are also the dominant species found on grapes. The yeasts in the last stages of alcoholic fermentation are alcohol-tolerant species, such as *Saccharomyces cerevisiae*, *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspota* and *Zygosaccharomyces*. The vast amounts of various yeast species present in the grape must make it impossible to predict the fermentation onset, duration and outcome.

Modern wineries, aiming for reliable fermentation and the production of wines with predictable quality, therefore use carefully selected starter cultures of *S. cerevisiae*. The traditional approach to develop wine yeasts was to select wild yeasts from grapes, grape musts, wines and winery equipment (Shinohara *et al.*, 1994). Modern day strains are carefully selected on the basis of several characteristics, including: fermentation rate, fermentation at low temperature, sulphur dioxide (SO₂) tolerance, high sugar tolerance, low production of volatile acid, desirable flavour production, killer activity and good wine quality. Selected industrial wine yeasts are usually capable of enhanced flavour formation, thus producing more aromatic wines. This might be due to the production of glycerol, organic acids, volatile esters and higher alcohols.

Flocculation is another useful property of industrial *Saccharomyces* strains that can be introduced by hybridisation (Shinohara *et al.*, 1997). During fermentative brewing, wine making, ethanol and biomass production, flocculent yeasts rapidly aggregate and settle in the later stage of fermentation. This helps to separate yeast cells from fermenting liquid.

Apart from yeast breeding projects, genetic engineering also allows scientists to bring forth new and novel wine yeast starter cultures that are optimized for specific

outcomes such as polysaccharide degradation, increased glycerol production or higher ester production (Pretorius and Bauer, 2002). Methods such as variant selection, mutagenesis and hybridization (mating, spore-cell mating, rare mating, cytoduction, and spheroplast fusion) are known as 'shotgun' approaches. Large genomic regions or entire genomes are combined or simply rearranged. A negative aspect is that abovementioned methods are not specific enough to modify a wine yeast for a particular outcome. Some properties of the yeasts might be improved, whereas other properties might be compromised. The specific benefit of these methods are that the resulting yeasts are not classified as GMOs. This benefit is further reinforced by the fact that scientific, technical, economic, marketing, safety, legal and ethical issues resist the use of recombinant wine yeast for the production of wine on a commercial scale.

2.2 HISTORICAL AND TECHNICAL PERSPECTIVE OF COMMERCIAL WINE YEAST

2.2.1 THE ORIGIN OF WINE YEAST

The origin of wine yeast is still under debate, but it is agreed that it originates either from the vineyard or the cellar (Mortimer and Polsinelli, 1999). This uncertainty is derived from the fact that it is near impossible to find or isolate *S. cerevisiae* from grapes. Some investigators have even incorrectly argued that *S. cerevisiae* does not exist in nature at all and that it can only be found in the winery environment. Only one in about one-thousand grape berries actually carries wine yeast. However, it was found that grape berries that are damaged are indeed very rich in micro organisms such as *S. cerevisiae*. Further, one in every four of these berries is *S. cerevisiae* positive. The positive berries have between 100 000 and 1 000 000 wine yeast cells on them. Evidence suggests that these yeasts are clonal. Polsinelli *et al.* (1996) presented evidence for the diversity of wine yeast strains recovered from a single grape vine.

It has been shown that many insects carry microorganisms on their bodies (Mortimer and Polsinelli, 1999) and that *S. cerevisiae* is not an air-borne contaminant. This still leaves the question of the unique source rather than the vector of yeast strains such as *S. cerevisiae* on grapes. Some authors postulate that *S. cerevisiae* is a domesticated species originating from its closest relative *Saccharomyces paradoxus*, a wild species found all over the world associated with insects, tree exudates and fermenting plant extracts (Naumov, 1996). The occurrence of *S. cerevisiae* in vineyards would then be the consequence of back transportation from the cellars to the vineyards by insects.

The historical aspects of wine yeasts provide further useful information concerning the origin and commercialisation of yeast. The word *cerevisiae* is derived from the word, *Cerus*, which means when directly translated 'the goddess of cereal' (<http://listproc.ucdavis.edu/archives/ven3sum2003/log0307/0004.html>). It is well known that strains of *S. cerevisiae* include strains used for beer making.

Historically, wine fermentations were caused by the yeast genera present on the surface of the grapes. Some of these yeasts were able to complete the fermentation and were dubbed “wine yeast”. These wine yeasts started establishing themselves in wineries and on winemaking equipment, eventually leading to the winery becoming the source of the “wine yeast”. It was quickly noted that better wines seemed to come from better wineries with thus better established “wine yeast”. The production of commercial yeast began after Pasteur defined the role of wine yeast. The production of beer yeast paved the way for these first productions. Late in 1800, Bakers Yeast started to be produced commercially. The commercial production of yeast for the wine industry however did not enjoy as much attention and the first wine yeast strains were made commercially available early in 1960. The first commercial production of wine yeast was probably made in Milwaukee .

2.2.2 AVAILABLE INDUSTRIAL YEAST STRAINS

Industrial yeast strains are highly specialized organisms that are capable of surviving under extremely stressful conditions, such as high initial sugar levels at the beginning of fermentation, the presence of SO₂ and high ethanol concentrations at the end of fermentation (Querol *et al.*, 2003). Some of the desirable features, apart from stress resistance, are fermentation at low temperature and production of glycerol (Degre, 1993). Undesirable traits are the production of volatile acidity, SO₂ and hydrogen sulphide, foaming properties and the formation of ethyl carbamate precursors.

The use of dry wine yeast ensures a quicker onset of fermentation and will aid in the production of a wine with uniform quality (Degre, 1993). However, before the technology was available to produce dry wine yeast, liquid cultures had to be used. Liquid yeast cultures were developed in 1930 (Institut Laclaire, France). In the mid 1960s, dry wine yeast was produced for the first time to fulfil the needs of a large Californian winery. The two strains that were produced were Montrachet and Pasteur Champagne. The worldwide distribution of these strains offered only limited success due to negative technical features. For example, strain SB1 was recommended for stuck fermentations despite its sensitivity to killer yeasts. It became evident that these strains were only suitable in very few circumstances and that a selection process had to be devised that would take into account the different regions of the world, the style of wine being made and the grape variety. In the mid 1970s, Lallemand developed new strains of dry wine yeast with technical and oenological properties that allowed more consistent wine quality. Research made the desirable features of these new yeasts and their impact on wine better understood and allowed the subsequent development of yeast improvement and development projects.

2.2.3 INDUSTRIAL YEAST STARTER CULTURE PRODUCTION

The cultivation of yeast starter cultures is a process in which pure yeast cultures are grown under sterile conditions after which active and vigorous cells are selected. Test tubes with the required nutrients are inoculated with the pure cultures

(<http://theartisan.net>). Subsequently, these pure cultures are transferred to larger vessels until the cell numbers reach the level necessary to produce a commercial starter. The optimum conditions in a tank fermenter are established by continuous nutrient addition, sufficient aeration and strict temperature control. Heat sterilized, diluted molasses is used as it is the least expensive source of sucrose, glucose and fructose. The molasses is further supplemented with nitrogen, phosphate, sulphuric acid and sodium carbonate. Small amounts of minerals and trace elements are also added. Nitrogen is added in the form of ammonia or an ammonia salt. Phosphate is supplied as phosphoric acid or di-ammonium phosphate. Sulphuric acid and sodium carbonate are included as processing aids for pH control. Oxygen, calcium, magnesium and trace amounts of iron and zinc are also added to the tank fermenter. When the required amount of yeast have been cultivated, they are separated from the remaining nutrient matter by centrifugation. The cells are washed and centrifuged, yielding a creamy suspension of pure, active yeast, which has a solids content of approximately 15–18%. The wet suspension can be used to inoculate must, but the preferred method is to use active dried yeast.

Active dried yeast is made by passing the yeast cream through a filter press or rotary vacuum filtration unit. Once pressed, the cake is extruded through a rectangular nozzle to form a strand that is cut into the proper length and weight. The cake is then extruded through perforated plates to form thin strands. These strands are cut into elongated pellets and are then passed through a fluid bed drier. The pellets are then ground into small granules. Other conventional, but effective drying methods such as the fluid bed system, rotating drum system, spray drying, vacuum drying and freeze-drying are also used commercially (Cerrutti *et al.*, 2000; Luna-Solano *et al.*, 2004). As the popularity of active dried yeast starter cultures grows, research is increasingly focused on the above mentioned procedures to determine optimal conditions for drying and to increase yeast viability after rehydration (Attfeld, 1987). There is also increasing need to predict and control the stability of dried yeast.

Cerrutti *et al.*, (2000) studied the effects of vacuum-drying and freeze-drying on the cell viability of *S. cerevisiae* with different endogenous concentrations of trehalose. Intracellular trehalose exerts a protective effect on yeasts during stressful and extreme environmental conditions such as desiccation, freezing, osmotic stress and heat shock (Hottiger *et al.*, 1989; Van Laere, 1989; Wiemken, 1990; Van Dijck *et al.*, 1995; Hounsa *et al.*, 1998). Trehalose also provides thermal stability to the cells (Attfeld *et al.*, 1992). These protective effects are linked to the stabilization of membranes and the preservation of enzyme activity. Viability and thermal stability could be improved by the addition of disaccharides, such as trehalose and maltose, to the media in which the cells are to be dried (Cerrutti *et al.*, 2000). Trehalose could act by replacing water molecules involved in the maintenance of the tertiary structure of proteins through multiple external hydrogen bonds. Another hypothesis to explain the protective effect of trehalose is that it forms glassy structures which assure physical stability. The hydrogen-bonding capacity of compounds utilized as protective agents for

phospholipids and membrane proteins and the accessibility of the protective substance to the interior of the cells could be critical factors for determining the survival of cells submitted to different treatments. Cerrutti *et al.* (2000) found that vacuum-drying is more optimal for cell viability than freeze-drying. Internal concentrations of trehalose in the range of 10-20% protected cells in both dehydration procedures and the addition of external trehalose improved the viability of *S. cerevisiae* cells that contained 5% internal trehalose during dehydration.

During the last 15 years, much attention has been given to an alternative preservation method, namely cell immobilization (Turker and Hamamci, 1998; Iconomopoulou *et al.*, 2001). Yeast cells are fixed in gel forming materials such as sodium alginate, agar, k-carrageenan and pectic acid and can be added to must or any other suitable product that must be fermented (Iconomopoulou *et al.*, 2001). Regarding the possible industrializing of this process, the following technical conclusions were made: (i) The technical challenges of this technique and the training in the new technology of immobilized cells are obstacles for industrialization. (ii) A new simpler bioreactor system and (iii) high operational stability of the bioreactor are the new prerequisites for a cost effective application of immobilized cells on an industrial scale. In the light of these conclusions, freeze-dried immobilized cells are proposed. This product will serve as a substitute for free freeze-dried wine yeasts and natural fermentation, should it be found that freeze-dried immobilized cells would improve the rate of fermentation and the quality of the wine. Iconomopoulou *et al.* (2001) showed the feasibility of low temperature wine making using freeze-dried gluten supported biocatalyst. Improved results in terms of fermentation kinetics at low temperatures (5-15°C), volatile compound production and operational stability from batch to batch were shown by freeze-dried immobilised cells on gluten as compared to free freeze-dried cells.

2.2.4 YEAST REHYDRATION

Proper rehydration is the most critical phase in using dried yeast (<http://theartisan.net>). Improper rehydration causes damage to the cell walls which results in the leakage of cytoplasm and thus soluble yeast enzymes that are necessary during fermentation and growth. This increases the risk of sluggish or stuck fermentations. Optimal rehydration is usually done in a water and juice mixture at 40°C as lower temperatures might cause unacceptable losses in soluble yeast cell constituents for certain strains. Yeast nutrients such as GO-FERM, produced by Lallemand, are used during the rehydration of dried yeast to ensure the proper utilization of sugars during fermentation (Loubser, 2003). GO-FERM consists of inactivated yeast cells that contain high levels of essential vitamins (pantothenic acid and biotin), minerals (magnesium, zinc and manganese) and amino acids.

2.3 HYBRIDIZATION OF YEAST AND OTHER GENETIC APPROACHES

2.3.1 HYBRIDIZATION OF HOMOTHALLIC AND HETEROTHALLIC YEASTS

Industrial wine yeast strains are usually diploid or aneuploid, whereas most laboratory strains are either haploid or diploid (Barre *et al.*, 1993). Haploid spores can mate with each other, thereby creating a diploid yeast again. Intra-species hybridisation involves the mating of haploids of opposite mating-types to yield a heterozygous diploid (Hammond, 1996). Progeny are recovered by sporulating the diploid, recovering individual haploid ascospores and repeating the mating/sporulation cycle as desired. Haploid strains from different parental diploids, possessing different genotypes, can be mated to form a new diploid strain. Thus, in theory, crossbreeding can permit the selection of desirable characteristics and the elimination of undesirable ones. The problem arises that many wine yeasts are homothallic and the use of hybridization techniques for the development of new wine yeasts strains has proved difficult (Pretorius and Van der Westhuizen, 1991). This problem can be overcome by direct spore-cell mating using a micromanipulator. The diploid yeasts (parental yeast strains) are sporulated and tetrad dissection is done with the aid of a micromanipulator. The haploid spores of parental yeast strains with different genotypes are brought into close proximity with each other and are allowed to mate under growth conditions.

2.3.2. OTHER GENETIC TECHNIQUES USED IN YEAST IMPROVEMENT PROJECTS

Apart from the abovementioned mating, mutagenesis, rare mating, spheroplast fusion and gene cloning also offer further possibilities for yeast improvement projects.

It was determined that the frequency of spontaneous mutation in *S. cerevisiae* at any given locus is approximately 10^{-6} (Pretorius and Van der Westhuizen, 1991). By using mutagens, the rate of mutations are vastly increased in a yeast culture. Rare mating is a method that can be used to mate yeast strains that do not express a mating type with haploid *MATa* and *MAT α* strains (Hammond, 1996). With this method, new hybrids will be generated and these hybrids can be used in further breeding projects.

Spheroplast fusion is a direct, asexual technique that can be employed in breeding and mating projects (Hammond, 1996). Opposite mating types are not required, thus increasing the number of crosses that can be done. Spheroplast fusion can be exploited to make very unusual crosses and is a valuable tool in strain development projects.

Gene cloning and transformation offer the scientist the possibility to alter wine yeasts on the molecular level with great precision. Certain properties can be modified whereas other unwanted traits can be eliminated. Another possibility is the introduction of a completely new trait. These changes can be made without altering other desirable properties. The scope for the application of recombinant DNA technology is indeed very wide. Possible applications are listed in Table 1. Unfortunately, public resistance hampers the commercial application of gene cloning. The main objection against gene

cloning is by a significant number of educated people finding the transfer of genes from one organism to another unacceptable for various reasons (Davies, 2001). Several issues concern the public: (1) the safety issue, human and environmental and whether or not GMOs are safe in the immediate and long term time-frames; (2) a political issue centring on who will own the technology, how it will be applied and who will benefit from it; (3) scientific and technical issues associated with transferring genes between one host and another.

TABLE 1 Applications of recombinant DNA technology (Adapted from Pretorius, 2000).

<p>(a) amplification of gene expression by maintaining a gene on a multi-copy plasmid, integration of a gene at multiple sites within chromosomal DNA or splicing a structural gene to a highly efficient promoter sequence</p> <p>(b) releasing enzyme synthesis from a particular metabolic control or subjecting it to a new one</p> <p>(c) in-frame splicing of a structural gene to a secretion signal to engineer secretion of a particular gene product into the culture medium</p> <p>(d) developing gene products with modified characteristics by site-directed mutagenesis</p> <p>(e) eliminating specific undesirable strain characteristics by gene disruption</p> <p>(f) incorporation of genetic information from diverse organisms such as fungi, bacteria, animals and plants</p>
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2.4 IDENTIFICATION OF YEASTS

2.4.1 THE YEASTS SPECIES ASSOCIATED WITH WINEMAKING

The yeast species present on grapes can all play a part in wine flavour and can thus influence the eventual quality of the wine (Lambrechts and Pretorius, 2000). To better understand the contribution made by these wine yeasts, much focus has been put into studies of these wine yeasts. Table 2 lists the most common wine-related yeasts and Table 3 illustrates the classification and reclassification of the *Saccharomyces* species that took place since 1952. The predominant microflora of grapes appear to be the low alcohol tolerant strains of *Hanseniaspora*, *Kloeckera* and *Candida* while *S. cerevisiae* appears only in very low numbers (Peynaud and Domercq, 1959; Fleet *et al.*, 1984; Heard and Fleet, 1985; Lema *et al.*, 1996). Some of these wild yeast strains might persist during the fermentation and produce certain volatile compounds that can affect the fermentation bouquet of the wine.

According to Charoenchai *et al.* (1997), the non-*Saccharomyces* yeasts produce and secrete several enzymes such as esterases, glycosidases, lipases, β -glucosidases, proteases and cellulases. Interactions with these enzymes by grape precursor compounds may produce aroma active compounds and thus play an important role in varietal aroma. In the light of the abovementioned, it is realized that not only *Saccharomyces* yeast strains bear important aroma producing potential.

TABLE 2 Wine-related yeasts (Lambrechts and Pretorius, 2000).

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

TABLE 3 Classification and reclassification of *Saccharomyces* species between 1952 and 1998 as depicted in major taxonomic reference works during this period (Pretorius *et al.*, 1999).

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

2.4.2 CONVENTIONAL AND MOLECULAR IDENTIFICATION TECHNIQUES

Most of the wine yeasts are considered physiological strains of *S. cerevisiae*, but this does not imply that all strains of *S. cerevisiae* are equally suited for the different wine-making practices and demands. Therefore the available identification techniques need to be very precise in order to identify and select the most suitable yeast for the specific fermentation conditions. The more traditional strain identification techniques made use of morphological, physiological and biochemical criteria (Van der Westhuizen and Pretorius, 1992). These taxonomic procedures allow for distinction between species, but they are usually time consuming and not as reliable as molecular techniques. Redžepović *et al.* (2002) used physiological and molecular genetic methods to reveal the oenological potential of *S. paradoxus*, which is thought to be the natural parent species of the domesticated species of the *Saccharomyces sensu stricto* group. The basic oenological characteristics such as ethanol and volatile acidity, fermentation vigour, production of killer toxin and production of H₂S were determined. Since the taxon *Saccharomyces sensu stricto* consists of a species complex of closely related yeasts, rather than four distinct species, taxonomic methods and molecular genetic techniques were used to identify wild strains of the *Saccharomyces sensu stricto* complex. Table 4 illustrates some molecular identification methods.

TABLE 4 Molecular methods for wine yeast differentiation (Adapted from Pretorius, 2000).

Method	Description
Chromatography	Pyrolysis-gas chromatography or gas chromatography of long-chain fatty acid methyl esters
Polyacrylamide gel electrophoresis (PAGE)	Total soluble yeast proteins are electrophoresed and banding patterns analyzed by computer
Restriction enzyme analysis (DNA fingerprinting)	Total, ribosomal or mitochondrial DNA is digested with restriction endo-nucleases and specific fragments hybridized after electrophoretic separation with multi-locus DNA probes such as the <i>Ty1</i> retrotransposon; restriction fragment length polymorphisms (RFLPs) are detected
Electrophoretic karyotyping (chromosome fingerprinting)	Whole yeast chromosomes are separated electrophoretically using pulse-field techniques; chromosome length polymorphisms (CLPs)
Polymerase chain reaction (PCR)	Specific DNA sequences are exponentially propagated <i>in vitro</i> and the amplified products are analysed after electrophoretic separation
Genetic tagging	Specific genetic sequences, with selectable markers, are introduced into yeasts to facilitate their recognition

2.5 YEAST IMPROVEMENT PROJECTS

The ultimate goal of all yeast improvement programmes is to create yeasts that conform to the quality expectations of the winemaker or the client. For instance, a fast fermenting yeast offers obvious technical advantages, but not all winemakers or wine styles require a fast fermentation. This implies that there are numerous research projects that are ongoing and striving to achieve specific, but different targets.

The most obvious targets for yeast improvement are resistance to a variety of stress conditions in wine, an increase or decrease in fermentation rate, low or negligible production of undesirable compounds, enhanced production of aroma compounds or compounds that improve the quality of wine and the degradation or synthesis of compounds which will improve fermentation and the processing and quality of wine. The following section will focus mainly on the aspects that were targeted for this yeast breeding project.

2.5.1 STRESS CONDITIONS ASSOCIATED WITH ALCOHOLIC FERMENTATION AND WINEMAKING

During alcoholic fermentation yeast cells are subjected to several stress conditions (Ivorra *et al.*, 1999). Although many different yeast species are involved in the initial stages of alcoholic fermentation, the *Saccharomyces* yeasts quickly replace them in the following stages as they are more tolerant to ethanol (Querol *et al.*, 2003; Zuzuarregui and del Olmo, 2004). The better and faster a yeast strain is able to adapt to stress conditions or changes in the environment, the higher the probability is that this strain will dominate during the wine making process. Such an adaptive strain would of course have potential as an industrial starter culture for wine making as it might offer advantages including: a decrease in the lag phase, significant reduction of the influence of naturally occurring yeast strains, rapid and complete grape must fermentation and thus the possibility for a higher degree of wine reproducibility and quality. The molecular and physiological response of an organism to changes in the environment is referred to as 'stress response' (Ivorra *et al.*, 1999). The stress response is regulated by sensor systems and signal transduction pathways. This results in the activation of the so-called stress response genes. Some of the genes activated by stress are the HSP (heat shock protein) genes, which encode heat shock proteins such as Hsp12p, Hsp82p, Hsp26p or Hsp104p. Hsp104p is responsible for tolerance to most of the stress conditions associated with wine making while Hsp12p protects membranes against desiccation and ethanol-induced stress. The expression profile of the HSP12 gene may be useful as an indicator of yeast strains with problems in stress resistance and thus prone to stuck fermentations (Ivorra *et al.*, 1999). Ivorra *et al.* (1999) and Carrasco *et al.* (2001) listed several stress conditions that affect yeasts during wine production. Heat-shock stress has been widely studied, although this stress condition can easily be eliminated from the fermentation process by modern temperature control systems. Temperature is one of the most important parameters for the development of alcoholic fermentation as it can

affect the duration and rate of fermentation, but more importantly, the final quality of the wine in term of aroma profile (Torija *et al.*, 2003). Low temperature alcoholic fermentations are becoming more frequent due to the demand to produce wines with more pronounced aromatic profiles. The high risk of stuck and sluggish fermentations are the biggest drawback to these types of fermentations, as low temperatures (10-15°C) restrict yeast growth and lengthen fermentations.

Oxidative stress can also occur during biomass production and yeast drying (Erasmus *et al.*, 2003). Hyperosmolarity is an ever present stress condition for wine yeasts. The high sugar content of must produces osmotic stress in yeast cells which they must resist in order to initiate, carry on and complete the fermentation. A typical must usually contains 160-260 g/L of an equimolar mixture of glucose and fructose and for the production of dessert wines the sugar concentration may be as high as 500 g/L. It was found that high sugar stress up-regulated the glycolytic and pentose phosphate pathway genes. Gene expression profiles indicate how the oxidative and non-oxidative branches of the pentose phosphate pathway were up-regulated and might be used to direct more glucose-6-phosphate and fructose-6-phosphate, respectively, from the glycolytic pathway into the pentose phosphate pathway. The production of acetic and succinic acid were increased due to the upregulation of the specific structural genes. Genes that are involved in the biosynthesis of purines, pyrimidines, histidine and lysine were down-regulated by sugar stress. Osmotic stress can also occur during yeast biomass production, downstream processing and drying (Ivorra *et al.*, 1999). The ethanol concentration of the must also adversely affects the uptake of nitrogen and as can be expected, as ethanol concentrations and nitrogen use increase, nitrogen starvation sets in. Jiranek *et al.* (1995) developed a protocol that uses bismuth-containing indicator media that can be used for the rapid identification of low or non H₂S producing wine yeasts. A high reaction intensity on indicator media does not necessarily signal high H₂S production during fermentation but does however reflect the potential for H₂S production should nitrogen become limited. In the absence of metabolic stress, such as nitrogen starvation or the presence of heavy metals, H₂S is formed in amounts to meet metabolic requirements. Upon nitrogen limiting conditions, H₂S is produced at a rate dependent on the characteristic level of sulfite reductase activity of the strain.

Blateyron *et al.* (2001) identified nitrogen and oxygen deficiencies as major causes of stuck and sluggish fermentations. Other potential mechanisms responsible are thiamin depletion of the must, excessive clarification of juice and inhibition of yeast cell activity by fermentation by-products, pH, killer toxins and pesticides.

Ethanol also imparts chemical stress upon wine yeasts and is often the cause of sluggish or stuck fermentations (Boulton *et al.*, 1996). Excessive amounts of ethanol inhibit the uptake of solutes (sugars and amino acids) and also inhibit yeast growth rate, viability and fermentation capacity. There are several factors that synergistically enhance the inhibitory effects of ethanol (Edwards *et al.*, 1990). These factors include high fermentation temperatures, nutrient limitation and metabolic by-products. Wine yeast strains are generally more resistant to ethanol-induced stress than are non-wine

Saccharomyces strains (Boulton *et al.*, 1996). Furthermore, the physiological response of the wine yeast to ethanol stress is also greater than is the case with non-wine strains. Nevertheless, ethanol stress remains a very important factor during alcoholic fermentation.

2.5.2 TARGETS FOR YEAST IMPROVEMENT PROJECTS

2.5.2.1 FERMENTATION RATE

Many yeast breeding projects focus on fermentation rate, as this parameter indicates the suitability of a yeast for commercial and industrial fermentations. For example, slower fermenting yeasts might be used to make more aromatic white wines, but this decrease in fermentation rate will limit productivity. Wine makers and scientists monitor wine fermentation by studying the fermentation rate (Ribéreau-Gayon *et al.*, 2000). This supervision allows them to observe changes and act quickly should the need arise. Temperature is probably the most important parameter for the development of alcoholic fermentation as it affects the rate of fermentation and the final quality of the wine (Torija *et al.*, 2003). For red wine making, a moderate temperature (18-20°C) favours cell growth and at the end of fermentation a higher temperature (30°C) facilitates the extraction of flavour and colour compounds out of the pomace (Ribéreau-Gayon *et al.*, 2000). By increasing the fermentation temperature, the fermentation rate is increased and therefore the fermentation and ultimately the wine making process is shortened. An increase in temperature might however cause a too vigorous fermentation rate, which could cause foaming which usually results in fermenter overflow.

The reduction of fermentation rate can easily be achieved by reducing the fermentation temperature (Torija *et al.*, 2003). Low temperature (10-15°C) alcoholic fermentation are utilised to produce wines that are more aromatic, but the serious drawback of stuck and sluggish fermentations make these types of fermentations risky. Lengthy fermentations increase the possibility of wine spoilage and decrease the productivity of the wine making process. The yeast strain, wine making style, stress factors, quality expectations and availability of fermentation control mechanisms will ultimately determine the optimum fermentation rate for the specific wine.

2.5.2.2 KILLER ACTIVITY

Some yeast strains secrete extracellular proteinaceous toxins that are lethal to susceptible or sensitive strains of the same species (Van Vuuren and Wingfield, 1986; Bortol *et al.*, 1986; Zagorc *et al.*, 2001). Killer yeasts are immune to their own toxin, but neutral strains exist that do not produce a toxin and are not sensitive to the killer toxins. Killer yeasts possess two major types of double stranded RNA (dsRNA), the L and M genomes, that are separately encapsulated in virus-like particles. The M-genome codes for the toxin and immunity to this polypeptide. The L-genome codes for the major viral coat protein of the viral particles and is also responsible for the polymerase involved in

replication of both the L and M genomes. At least 11 groups of killer yeasts are discerned, K1-11, of which K1-3 are specific to *S. cerevisiae*. The K1 killer yeasts are not important in fermenting grape must as their toxin is inactive at low pH. The K3 toxin has been found to be very similar to the K1 killer toxin. The K2 killer toxin is stable at pH 2.8-4.8 and also at wine making temperatures. Killer toxins K4-11 have been found in the genera *Saccharomyces*, *Candida*, *Cryptococcus*, *Debaromyces*, *Hansenula*, *Kluyveromyces*, *Saccharomyces* (non *cerevisiae*), *Pichia*, *Williopsis*, *Ustilago*, *Torulopsis* and *Zygosaccharomyces*. Van Vuuren and Wingfield (1986) showed that K2 killer yeasts pose a clear threat to the wine industry since their toxins are lethal to sensitive wine yeasts and can cause stuck or sluggish fermentation. In a study of stuck fermentations in a wine cellar, they found that up to 90% of yeast cells in fermenters that exhibited stuck fermentations were dead and that the viable cells in the fermenters were killer yeasts. Another interesting result of this study was that the toxin produced by the killer yeast mediated flocculation of non-flocculent wine yeast strains. This can be of benefit to the winemaker since killer yeasts might be employed to help flocculate non-flocculent yeast strains or yeast strains that flocculate with difficulty. Bortol *et al.* (1986) described the isolation of a wild killer yeast from natural and manufactured food products and the transfer of the killer particle to an industrial yeast by protoplast fusion. The characterised fusion products exhibited killer activity and varying fermentation ability, with some yeasts fermenting more efficiently than the original industrial strains.

Zagorc *et al.* (2001) also stated the enological interest in killer yeast due to their ability to dominate a fermentation. Enological studies indicate that killer activity would allow a yeast species to compete more successfully in a specific habitat by eliminating other yeasts strains. The fermentation characteristics of indigenous killer yeasts were compared with those of commercial starters and it was found that some of the killer strains were as good as the commercial starters. It is suggested that a killer yeast found to have positive enological characteristics, should be used as a starter culture. This allows for an excellent wine to be made and the fermentation process is 'self-protected'. Competing, non-killer yeast strains will be eliminated, creating a more uniform yeast population during the fermentation process.

2.5.2.3 PECTINOLYTIC ACTIVITY

Pectic substances are complex structural polysaccharides that occur mainly in the middle lamella and primary cell wall of higher plants (González-Candelas *et al.*, 1995; Blanco *et al.*, 1999; Kashyap *et al.*, 2001). These substances consist of a main backbone containing a large proportion of partially methyl-esterified galacturonic acid subunits linked by α -1,4 glycosidic linkages. This compound is known as pectin and in the demethylated form pectic or polygalacturonic acid. The enzymes that hydrolyse pectic substances are known as pectic enzymes, pectinases, or pectinolytic enzymes. They are classified into two main groups, namely pectinesterases (PE) (able to de-

esterify pectin by removal of methoxyl residues) and depolymerases (which split the main chain).

Pectinases were some of the first enzymes to be used in homes, where they were added to washing powder (Kashyap *et al.*, 2001). Their first commercial application was in 1930 for the preparation of wines and fruit juices and today pectinases also enjoy applications in the textile and biotechnology industry. Pectic enzymes from fungi such as *Aspergillus niger*, *Penicillium notatum* and *Botrytis cinerea* are useful in wine making as they reduce haze or gelling of grape juice while the grapes are being crushed, before or after the fermentation of the must and at completion of fermentation, when wine is ready for bottling or transfer. The addition of pectinases at the first stage is considered best since it increases the volume of the free-run juice and reduces pressing time. Another advantage of these enzymes is the increased release of anthocyanins of red grapes into the juice. Treatment of the juice at the second stage before or during the fermentation, settles out many suspended particles and often some undesirable microorganisms. Finally, addition of pectic enzymes to the fermented wine increases filtration rate and clarity. The level of enzyme supplemented must however be adjusted to compensate for the inhibitory effect of alcohol on pectinases. The production of pectic enzymes has been widely reported and thoroughly studied in bacteria and filamentous fungi because they play an essential role in phytopathogenesis (Blanco *et al.*, 1999). The pectinase production in yeasts has received less attention and a few yeasts species show this ability. The main problem in using yeast pectolytic enzymes in industrial processes lies in the low yield of these enzymes during fermentation. This can be overcome by cloning and overexpression of the respective structural genes in different genetic backgrounds. For example, the *PGU1* gene has been overexpressed in different strains of *S. cerevisiae*. The *PSE3* gene from *Tichosporon penicillatum* has also been overexpressed in *S. cerevisiae*, with significant increases in yield in comparison with the wild-type. The same approach has been employed for the heterologous cloning of genes from filamentous fungi. Fungal genes encoding pectate or pectin lyases have been cloned and sequenced from *Aspergillus niger*, *Fusarium solani* f. sp. *pisi* and *Glomurella cingulata*. González-Candelas *et al.* (1995) constructed a gene fusion between the *S. cerevisiae* actin gene promoter and the cDNA of the *Fusarium solani* f. sp. *pisi pelA* gene. This expression cassette has been introduced into the industrial yeast strain T₇₃. The resulting recombinant strain was able to secrete active pectate lyase enzyme into the culture medium.

2.5.2.4 BIOLOGICAL DEACIDIFICATION

Malic acid, together with tartaric acid, are the dominant fixed acids in grapes, contributing 70-90% or more of the titratable acidity at the beginning of fermentation (Volschenk, 1996). L-Malic acid is an essential compound, with important cellular functions in metabolic pathways such as the tricarboxylic acid cycle (TCA), glyoxylate cycle and malate-aspartate shuttle and is synthesized from glucose via pyruvate in

grapes. Malic acid concentrations in grapes range between 2.0 and 4.0 g/L, but can reach concentrations as high as 6.0 g/L. Amounts as high as 14 g/L have been measured in the cold viticultural regions. Winemaking usually comprises two main fermentation processes: alcoholic fermentation and malolactic fermentation (MLF) which is performed by various lactic acid bacteria (LAB) (Lonvaud-Funel, 1995). Even though MLF is the second fermentation in winemaking, it is far from being of secondary importance, as it could improve wine quality and stability. The MLF is usually started by inoculating with selected starter cultures. Apart from the bacteria being able to survive in the wine, another test of their efficiency is the strain-specific organoleptic changes that is effected in the wine due to bacterial metabolism. On the other hand, yeast growth and survival in juice is easy to induce (Thornton and Rodriguez, 1996).

Yeasts are more resistant than bacteria to sulphur dioxide, the antioxidant/antiseptic widely used in the wine industry. Yeasts are not susceptible to bacteriophage attack, can grow at the very low pH of high acid juice and have simple growth requirements which are satisfied by the majority of grape juices. *S. cerevisiae*, *Zygosaccharomyces bailii* and *Schizosaccharomyces malidevorans* are yeast species found in wine which can degrade malic acid while growing on sugars. *Schizosaccharomyces pombe* is able to convert all L-malic acid into ethanol and CO₂ (malo-alcoholic fermentation) simultaneously with the utilization of glucose. Microbiological deacidification may include the use of yeasts that are able to degrade malate under conditions similar to those employed in winemaking (Sousa *et al.*, 1995). It was found that ethanol and acetic acid, at concentrations representative of winemaking, inhibited the transport of L-malic acid in *Ss. pombe*. Glucose transport was not significantly affected either by ethanol or by acetic acid. The uptake of labelled acetic acid followed simple diffusion kinetics, indicating that a carrier was not involved in its transport. Therefore, the undissociated acid appears to be the only form that enters the cells and is probably responsible for the toxic effects. In the light of the abovementioned, it was suggested that deacidification by *Ss. pombe* during wine fermentation should take place before, rather than after, the main alcoholic fermentation by *S. cerevisiae*.

Deacidification of must by genetically unaltered *S. cerevisiae* strains remains a difficult task, as initial studies on resting cells of anaerobically grown cells of *S. cerevisiae* indicated that the uptake mechanism of malate is by simple diffusion of the non-dissociated form (Salmon, 1987). Although remarkable differences exist within the *Saccharomyces* species with regards to malic acid degradation during alcoholic fermentation (from 0 to 3 g/L malic acid), strains of *Saccharomyces* are regarded as the most inefficient metabolisers of extracellular malic acid (Redzepovic *et al.*, 2003). In comparison, strains of *Ss. pombe* and *Z. bailii* can degrade high concentrations of malic acid.

The biggest factor influencing the ability of a yeast to degrade extracellular malic acid is the efficient transport of the dicarboxylic acid (Redzepovic *et al.*, 2003). The efficacy of the intracellular malic enzyme should also be taken into account, although it seems to be of lesser importance than the transport mechanism. *Ss. pombe* has an

active transport system for the uptake of malic acid, as well as an intracellular malic enzyme with a very high substrate affinity ($K_m = 3.2$ mM). In contrast, *S. cerevisiae* lacks an active uptake mechanism and has a very low substrate affinity ($K_m = 50$ mM). These differences are illustrated in Figure 1.

Unfortunately, *Ss. pombe* species are not ideally suited for wine fermentation due to their temperature and alcohol sensitivity. The production of undesired fermentation aroma has also been reported. Redzepovic *et al.* (2003) has shown that an indigenous thermotolerant strain, *S. paradoxus* strain RO88 was able to degrade lesser quantities of malic acid than *Ss. pombe* strain F (38% and 90% respectively), but higher quantities than typical wine yeast strains. This strain was also able to produce a wine of good quality.

In order to facilitate even greater and faster degradation of malic acid by wine yeasts, scientists turned to gene cloning. Denayrolles *et al.* (1995) cloned the *mleS* gene of *Lactococcus lactis* encoding malolactic enzyme. The *mleS* gene was cloned in a yeast multicopy vector under a strong promoter and malic acid degradation was tested during alcoholic fermentation in synthetic media and must. Although yeasts expressing the *mleS* gene produced L-lactate from L-malate, malate degradation was far from complete. In the light of abovementioned research, the importance of the intracellular synergism between the *mleS* and the malate permease gene (*mae1*) became evident and scientists opted for the co-expression of these genes in *S. cerevisiae*.

Volschenk *et al.* (1996) constructed malolactic yeasts by co-expressing the malate permease gene (*mae1*) of the fission yeast *Ss. pombe* and the *Lactococcus lactis* malolactic gene (*mleS*) in *S. cerevisiae*. The recombinant strain of *S. cerevisiae* actively transported malate and completely metabolised malate to lactate within three days in Cabernet Sauvignon and Shiraz grape musts at 20°C. The malolactic fermentation in Chardonnay grape must was completed within 7 days at 15°C. These data illustrate the importance of the transport system for malate.

In a subsequent study, Bony *et al.* (1997) achieved complete malolactic fermentation by using *S. cerevisiae* strains coexpressing the genes *mleS* and *mae1* coding for the *L. lactis* malolactic enzyme and the *Ss. pombe* malate permease under the control of yeast promoters. A strain expressing several copies of *mae1* and one copy of *mleS* degraded 3 g/L of malate in 4 days under enological conditions, without metabolic side effects.

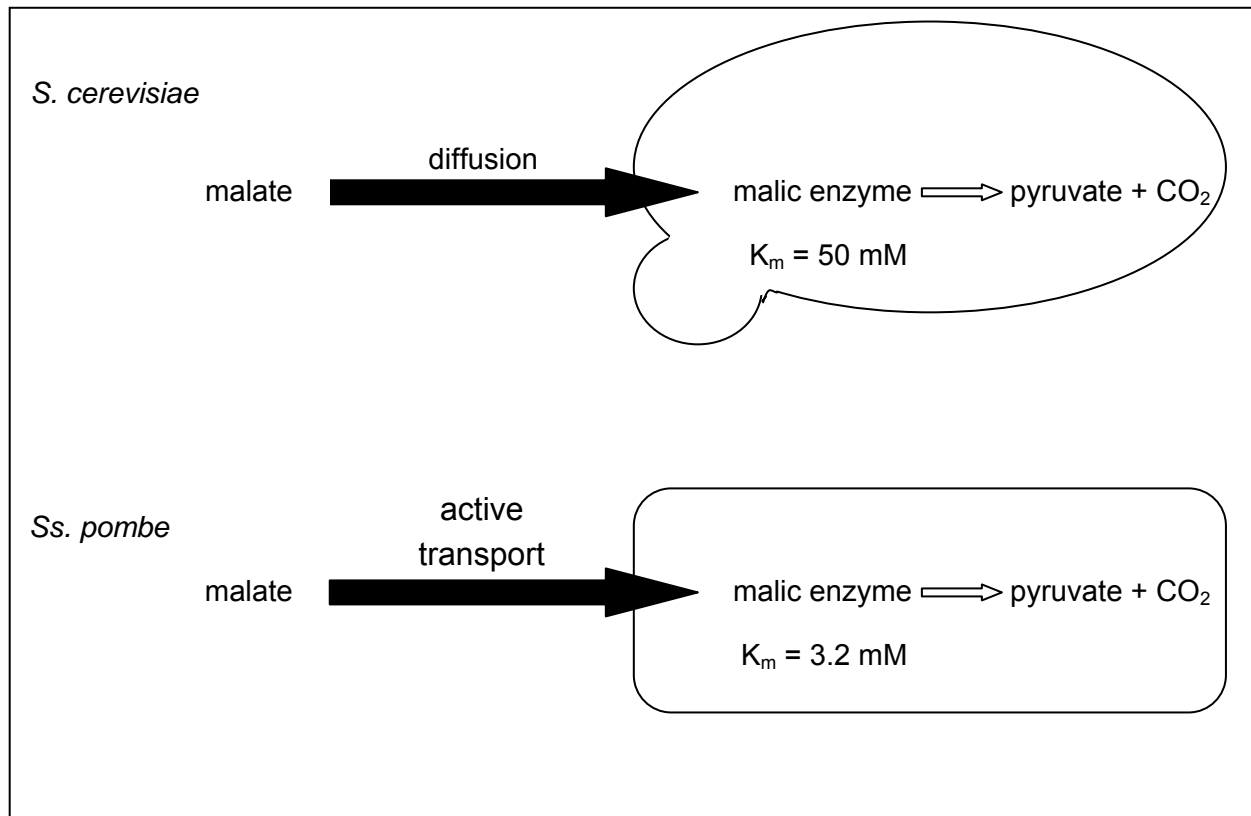


Figure 1 (Volschenk, 1996). The differences between *S. cerevisiae* and *Ss. pombe* in their ability to degrade malate. Both yeasts contain the malic enzyme, but the malic enzyme of *S. pombe* has significantly higher substrate affinity for malate than the malic enzyme of *S. cerevisiae*. Furthermore, malate enters *S. cerevisiae* by simple diffusion as opposed to the active malate transport system in *S. pombe*.

2.5.2.5 AROMATIC PROFILE

Choosing the right yeast or yeast strain for the production of desirable tastes and flavours for wines is very important and significant, as the impact of yeast strain on the aroma and quality of wine has been demonstrated in various studies (Falqué *et al.*, 2001; Patel and Shibamoto, 2003). The preferable flavours of wine depend on a balance of volatile constituents such as acids, alcohols, aldehydes, ketones and esters. The particular importance of each compound on the final aroma depends on the correlation between chemical composition and perception thresholds. Further, the formation of volatile compounds during alcoholic fermentation and the impact they will have on the end product depends not only on the particular yeast species, but also on the particular strain of the species. Numerous compounds in wine are formed during yeast fermentation and a definitive positive correlation was shown between the yeast used and the production of volatile chemicals, including alcohols, esters and acids in fermenting must.

2.5.2.5.1 HIGHER ALCOHOLS

Alcohols possessing more than two carbon atoms are referred to as higher alcohols (Lambrechts and Pretorius, 2000). They also have a higher molecular weight and boiling point than ethanol. Higher alcohols quantitatively represent the largest group of aroma compounds in alcoholic beverages and can have a significant influence on the taste and character of wine. Below 300 mg/L they usually contribute to the desirable complexity of wine, but when their concentrations exceed 400 mg/L, the higher alcohols are regarded as a negative influence on the quality of the wine. Some higher alcohols, their threshold values and the odour they impart on wine are illustrated in Table 5.

Table 5 Some higher alcohols produced by yeast and their concentrations, threshold values and odours in wine (Rankine, 1969; Salo, 1970a; Shinohara and Watanabe, 1976; Baumes *et al.*, 1986; Nykänen, 1986; Renger *et al.*, 1992; Fabre *et al.*, 2000; Lambrechts and Pretorius, 2000; Nurgel *et al.*, 2002; Majdak *et al.*, 2002; Peinado *et al.*, 2003).

Compound	Concentration in wine (mg/L)	Threshold value (mg/L)	Odour
n-Propanol	9 – 68	500	Stupefying
Isobutanol	9 – 28	500	Alcoholic, nail polish
2-Phenylethyl alcohol	10 – 180	25 - 105	Floral, rose, honey
n-Butanol	0.5 – 8.5	5	Pharmaceutical
Hexanol	0.3 – 12	1.1	Herbaceous
Isoamyl alcohol	45 – 490	60 - 180	Marzipan

Mateo *et al.* (2001) compared higher alcohols production between *S. cerevisiae* strains and yeasts responsible for spontaneous fermentation. *S. cerevisiae* strains produced higher levels of higher alcohols and the spontaneous fermentations yielded lower amounts of higher alcohols. Further, an increment in the inoculation concentration lead to an increment in the total higher alcohol concentration. The ratio of the contents of esters to higher alcohols is known to influence the sensory properties of fermented beverages (Mateo *et al.*, 2001; Valero *et al.*, 2002). Wines with increased contents of esters possess an enhanced fruity flavour, that could be improved if the higher alcohol contents were decreased. Taking into account that ester quantity contribute to the fruity, flowery and generally pleasant quality of wine and concentrations of higher alcohols exceeding 400 mg/L are regarded as a negative quality factor, the following relationship was successfully related to the olfactory quality of wine:

$$[\text{long chain esters}] \times \frac{400}{[\text{higher alcohols}]}$$

This relationship defines that the value as a result of multiplication is dimensionless and a lower value merely indicates a lower olfactory wine quality. A higher value would indicate a higher olfactory wine quality.

2.5.2.5.2 VOLATILE ESTERS

Esters are produced by yeasts during alcoholic fermentation as products of sugar metabolism and are considered the most important group influencing aroma in wine. Esters impart the pleasant smell usually associated with wine and are considered the most important flavour compounds produced by yeasts during alcoholic fermentation (Lambrechts and Pretorius, 2000). It is believed that esters make the greatest contribution to the desirable fermentation bouquet of wine (Rojas *et al.*, 2003). The fruity odours of the fermentation bouquet are primarily due to a mixture of the esters hexyl acetate, ethyl caprylate and ethyl caproate. Table 6 illustrates some volatile esters, their threshold values and aromas.

Table 6 Some volatile esters produced by yeast and their concentrations, threshold values and odours in wine (Salo, 1970a; Fabre *et al.*, 2000; Lambrechts and Pretorius, 2000; Nurgel *et al.*, 2002; Majdak *et al.*, 2002; Peinado *et al.*, 2003).

Compound	Concentration in wine (mg/L)	Threshold value (mg/L)	Odour
Ethyl caprylate	0.05-3.8	0.58	Sweet, pear, banana, brandy
Ethyl caproate	Trace-3.4	0.08	Fruity, green apple, banana
Ethyl caprate	Trace-2.1	0.5	Brandy, fruity, grape, floral
Diethyl succinate	-	1.2	Fruity, melon
2-Phenylethyl acetate	0.01-4.5	1.8	Rosy, honey, fruity, flowery
Hexyl acetate	-	0.67	Apple, cherry, pear, flower
Isoamyl acetate	0.03-8.1	0.16	Banana, pear, fruity, sweet
Ethyl lactate	-	150	Fruity, buttery

Ester production during alcoholic fermentation is closely related to the particular yeast species involved. Although *S. cerevisiae* is considered to be the main wine yeast, there are several other non-*Saccharomyces* wine yeasts that can contribute favourably to the flavour of wine. Mixed cultures of wine yeasts in fermentations can be beneficial, as it was shown that in a combined fermentation *S. cerevisiae* and *H. guilliermondii* produced elevated levels of acetate esters such as 2-phenylethyl acetate. Despite the advantages of using pure cultures of *S. cerevisiae* for alcoholic fermentations, many authors claim that the contribution of the indigenous yeasts to the regional features of a given wine should not be overlooked. The potential of spontaneous and mixed culture fermentation should thus be considered. Mateo *et al.* (2001) illustrated this in a study where spontaneous fermentation yielded the highest level of ethyl acetate. Below the perception threshold value of 12 mg/L, it may contribute to the fruity aroma of wine. In another study, it was shown that low concentrations of esters were produced in wines in which spontaneous fermentation took place, particularly ethyl lactate, which can contribute a fruity or buttery aroma (Nurgel *et al.*, 2002). In yet another study, Romano *et al.* (2003) used strains of *S. cerevisiae*, *Hanseniaspora uvarum*, *Candida stellata*, *Zygosaccharomyces fermentati* and *Saccharomycodes ludwigii* to determine the effect of yeast strain on wine aroma. It was concluded that it would be advantageous to use

mixed starter cultures. Non-*Saccharomyces* yeasts survive in fermentations longer than expected and their survival and their growth is significant as they influence the chemical composition of the wine. Further, when *S. cerevisiae* is added to grape must partially fermented by apiculate yeasts, the wine produced is more complex and has better aroma.

2.5.2.5.3 VOLATILE FATTY ACIDS

The volatile fatty acids found in wine are usually saturated, straight-chain fatty acids, with palmitoleic acid considered to be the only important unsaturated fatty acid (Lambrechts and Pretorius, 2000). The volatile content of wine is usually 500-1000 mg/L (10-15% of the total acid content). Normally more than 90% of the volatile acid in wine consists of acetic acid (> 0.2 - < 2 g volatile acidity/L).

Acetic acid becomes objectionable near its perception threshold of 0.7-1.1 g/L and values between 0.2 and 0.7 g/L are considered optimal. By law, the volatile acidity of wines may not be higher than 1.0–1.5 g/L, depending on the country. Yeasts synthesize much the same fatty acids irrespective of the nature of the raw materials used. However, the fatty acid composition of yeasts is highly variable; changes in growth substrate and minor alterations in growth conditions (pH, temperature, presence of nutrients) as well as the growth rate of the organism itself may affect the relative proportions of the individual components. Majdak *et al.* (2002) indicated that *S. paradoxus* strain RO88 has the potential to produce almost equal concentrations of fatty acids as *S. cerevisiae* strain RO64 and indigenous yeasts. This again illustrates the potential of using *S. paradoxus* in wine making, as it offers at least the same aroma potential as commercial wine yeast starter cultures. Table 7 illustrates some volatile fatty acids, their threshold values and aromas. The volatile fatty acids usually impart unpleasant aromas in wine, above their threshold values. Examples of these aromas are rancid and cheesy notes. Below their threshold values, the volatile fatty acids add to the complexity of wine and can impart fruity and citrus aromas.

Table 7 Some volatile fatty acids produced by yeast and their concentrations, threshold values and odours in wine (Salo, 1970a; Baumes *et al.*, 1986; Fabre *et al.*, 2000; Lambrechts and Pretorius, 2000; Nurgel *et al.*, 2002; Peinado *et al.*, 2003).

Compound	Concentration in wine (mg/L)	Threshold value (mg/L)	Odour
Isobutyric acid	Traces	8.1	Pungent
Isovaleric acid	<3	0.7-1.5	Rancid, cheese, putrid
Hexanoic acid	Traces-37	8	Sour, vinegar, fatty, pungent
Octanoic acid	Traces-41	13	Oily, fatty, soapy, faint fruity
n-Butyric acid	Traces	2.2	Pungent
Decanoic acid	Traces-54	10	Fatty, rancid, citrus, phenolic
Propionic acid	Traces	20	Rancid, slightly pungent

2.5.2.6 VOLATILE SULPHIDE PRODUCTION

Volatile sulphur-containing compounds are found in many foods and beverages (Mestres *et al.*, 2002). They usually contribute to unpleasant aromas, although some of them have been reported to play a positive role in the overall aroma profile. In a high mark-up product like wine, this ambiguous role of volatile sulphur compounds needs to be considered. These compounds are usually classified, based on the boiling point of 3-methylthiopropanol (b.p. 90°C), as light sulphur compounds (b.p. < 90°C) and heavy sulphur compounds (b.p. > 90°C). The light sulphur compounds are responsible for bad odours in wine (rotten eggs, cabbage, rubber, garlic, onions, etc.) because of their high volatility and low perception levels. Most of these compounds can be eliminated with wine aeration. The heavy sulphur compounds cannot be eliminated and usually their aroma is unpleasant at even very low concentrations. However, very low levels of volatile sulphur compounds are typical of some varieties and give these wines a distinctive aroma.

Other highly volatile sulphur compounds, such as carbonyl sulphide and dimethyl sulphide, are less significant in reduction defects (Moreira *et al.*, 2002). The latter has been considered by some authors to contribute to the bouquet of wines. Among the less volatile sulphur compounds, 3-(methylthio)-1-propanol (methionol) is present in wines at concentrations of up to 5 mg/L. When this compound occurs at concentrations above its threshold value (1.2 mg/L), it contributes a cauliflower aroma. Other less volatile sulphur compounds are: 2-mercaptoethanol (poultry-like aroma), 2-methyltetrahydrothiophen-3-one (metallic, natural gas odour), 2-methylthioethanol (French bean), ethyl-3-methylthiopropionate (metallic, sulphur aroma), acetic acid-3-(methylthio) propyl ester (cooked potatoes) and 4-methylthiobutanol (chive-garlic aroma).

Abnormally high concentrations of H₂S may be produced during the fermentation of musts with nitrogen deficiencies (Ribéreau-Gayon *et al.*, 2000). Yeast protease activity is stimulated in must with a nitrogen deficiency, causing sulphur amino acids to be released by proteins. The total nitrogen content of grape juices ranges 40-fold from 60 to 2400 mg/L and can therefore be growth-limiting (Pretorius, 2000). Grape juices with nitrogen levels below 150 mg/L have a high potential to cause problem fermentations due to inadequate yeast growth and poor fermentative activity. To remedy this, ammonium sulphate is frequently added to must to prevent H₂S from forming (Ribéreau-Gayon *et al.*, 2000).

2.6 THE APPLICATION OF YEAST BREEDING PROGRAMMES AND HYBRID YEASTS

The use of selected starter cultures has improved the reproducibility and the predictability of the quality wines, especially during the last 30 years (Rainieri and Pretorius, 2000). The improvement of wine made on large scale cannot be denied,

although there is criticism that commercial wine yeast strains possess very ordinary characteristics. The selection of yeasts for winemaking consists of identifying those cultures that can ferment grape juice efficiently and produce good quality wines. The selection is usually carried out within the genus *Saccharomyces* and is aimed at technological characteristics such as ethanol tolerance, fermentation vigour, osmotolerance, resistance to SO₂ and presence of killer factor. Qualitative characteristics include fermentation by-products such as glycerol, acetic acid, acetaldehyde and isoamyl alcohol. The production of H₂S and SO₂ is also closely monitored and the action on malic acid is another important quality factor.

Apart from *S. cerevisiae*, other *Saccharomyces* strains that can easily be isolated from grape juice and wine belong to the group *Saccharomyces uvarum* (Rainieri *et al.*, 1999). Positive traits of these strains is the ability to ferment vigorously at low temperatures (6-10°C) and the production of high concentrations of glycerol and succinic acid (Castellari *et al.*, 1994). A negative trait is the production of high amounts of higher alcohols, which can spoil the aromatic characteristics of wine.

Another option for yeast breeding is hybridisation of yeasts of different species. The possible characteristics of the resultant hybrids are difficult to predict; nevertheless this is one of the ways by which novel traits can be introduced into wine strains (Naumov *et al.*, 1992).

2.6.1 EXAMPLES OF APPLIED YEAST BREEDING AND IMPROVEMENT PROJECTS

Glycerol in wine originates mainly as a by-product during fermentation by yeast and is thought to add to the body and smooth mouth-feel (Eustace and Thornton, 1987). The amount of glycerol produced is also influenced by the *S. cerevisiae* strain used in the fermentation (Rankine and Bridson, 1971). The properties of Chardonnay wine produced with different wine yeasts strains of *S. cerevisiae* and hybrid strains that were bred to produce elevated glycerol concentrations was evaluated (Prior *et al.*, 2000). The hybrid strains of *S. cerevisiae* were bred by back-crossing three times a Premier Cuvée strain with yeast strain Ba25 isolated from a spontaneous wine fermentation as described by Prior *et al.* (1999). The study by Prior *et al.* (2000) showed that the hybrid strains produced higher amounts of glycerol, coupled with higher amounts of acetic acid, volatile acidity, acetoin, acetaldehyde and 2,3-butanediol production than the wine yeast strains. The levels of some of these metabolites were strongly linked to elevated glycerol production. It was also found that the hybrid strains fermented the juice more slowly than the wine yeast strains, although dryness was achieved in most instances. These results suggest that further breeding and selection might yield yeast strains for fermentation that improves the body of wine without impacting on the overall balance of wine. In another study, Lucca *et al.* (2002) made an intergeneric osmotolerant hybrid yeast, PB2, by protoplast fusion between *Torulaspora delbrueckii* and *S. cerevisiae* in order to study glycerol and arabitol production. It was found that the hybrid produced more glycerol and arabitol than both the parental strains.

Flocculation and non-H₂S production are important traits in winemaking (Patrizia *et al.*, 1985). In wine yeasts 1% of yeast strains show flocculation at the highest levels and 1% do not produce H₂S. For this reason it is highly improbable that natural yeast strains possessing both these characteristics can be found. Patrizia *et al.* (1985) utilised spore conjugation to develop new and improved hybrid wine yeasts of *S. cerevisiae*. A highly flocculent yeast strain was crossed with a non-H₂S forming yeast strain to form a new and improved wine yeast. Apart from being highly flocculent and non-H₂S forming, this yeast also had other relevant winemaking characteristics such as a high fermentation rate and high ethanol production. In a related study, Shinohara *et al.* (1997) constructed a wine yeast with moderately flocculating activity. The flocculation property of the strains (ABXL-1D, RIFY 1029, RES-5) was introduced into the non-flocculent wine yeasts (RIFY 1001, IAM 4274) by mating. An improved hybrid WWR-2 was obtained by a back cross ([RIFY 1001 x RES-5] x RIFY 1001) and this hybrid demonstrated a practical flocculation and good fermentation property in experimental winemaking.

Caridi *et al.* (2002) studied the fermentative behaviour of two hybrid wine yeast strains, a first-generation hybrid-strain (obtained by hybridisation of a cryotolerant strain of *S. bayanus* with mesophilic strain of *S. cerevisiae*) and the other obtained by hybridisation of a thermotolerant strain *S. cerevisiae* with a mesophilic strain of *S. cerevisiae*. The fermentative behaviour of a commercial wine yeast strain, *S. cerevisiae* K1, was compared to that of the hybrid yeasts. The goal of the study was to obtain wine with a high content of polyphenols from Gaglioppo grape must with a limited phenolic content. The Folin-Ciocalteu index (indicative of the total polyphenol content) of the wine showed significant differences between the wines made with the different yeast strains. Higher levels of polyphenols were found in the wine made with the hybrid strains and the reference strain produced the lowest amount of polyphenols.

Van der Westhuizen (1990) created VIN13 by hybridising N76 (228) with *S. bayanus*. This hybrid displays characteristics of both parents, such as the killer phenomenon and the assimilation of galactose. It has a fast fermentation rate and it was shown that wine made with this yeast was not compromised in terms of desired oenological characteristics, specifically aroma.

2.7 CONCLUSION

The age-old adage says that “the best wines are made in the vineyard”. Although this is still true today, scientists have come to realise that the yeast strains involved in the winemaking process are also of cardinal importance. Specific starter cultures of *S. cerevisiae* allows for better predictability of the quality of the wine being made and also improves quality. Apart from the ability to ferment must to dryness and the production of a pleasant and becoming bouquet, starter cultures must also have a range of other new or novel qualities to set them apart from the ordinary. A wider range of starter cultures would benefit the marketability of the South African wine industry. Novel

hybrid yeast strains also offer the benefit of immediate consumer acceptance whereas GMO's and the use thereof are often criticised. Hybrid wine yeasts have been successfully introduced into the South African industrial market, allowing winemakers the chance to make better or different styles of wine. More novel hybrids have been constructed recently and they show promising results in terms of fermentation performance and efficiency, viability, biological deacidification and aroma profiles.

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3. THE BREEDING AND CHARACTERISATION OF A NOVEL INTERSPECIES WINE YEAST

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3.1 ABSTRACT

Hybridisation of yeast strains allows the incorporation of favourable phenotypical characteristics into a new wine yeast. This circumvents the issue of genetic engineering of wine yeasts. Although the outcome of hybridization cannot be predicted, subsequent selection after mating ensures that the best hybrids can be identified. In this study, 5 interspecies hybrid yeasts were constructed by mating VIN13 and *S. paradoxus* strain RO88 with each other. VIN13 is known for its fast onset and completion of fermentation and good potential for aroma production. *S. paradoxus* strain RO88 has the ability to degrade malic acid and it also displays strong pectinolytic activity. Two of the 5 hybrids displayed unique characteristics. Both are killer positive and have pectinolytic activity. They are also capable to a degree of biological deacidification. The 5 hybrids were also subjected to UV mutagenesis to select strains showing higher degradation of malic acid, but positive results could not be obtained. Clairette blanche and Cinsaut wine was made with the hybrids and yielded positive results in terms of fermentation rate and principal chemical constituents of the wine. Significant alterations in the ester, higher alcohol and volatile fatty acid profiles for the Cinsaut wine was obtained by fermenting with the hybrids. Very positive feedback from a tasting panel also indicated the potential of these two hybrids.

Key words: hybrid, mating, interspecies, fermentation, biological deacidification, malic acid, *S. paradoxus* strain RO88, VIN13, killer activity, pectinolytic activity, aroma

3.2 INTRODUCTION

Malic acid is one of the principal organic acids in grape must and wine and contributes greatly to the acidity of grape must (Redžepović *et al.*, 2003). Together with tartaric acid, malic acid is the dominant fixed acid in grapes, contributing 70-90% or more of the titratable acidity at the beginning of fermentation. The malic acid concentration in grapes usually ranges from 1 to 10 g/L, but in extreme cases up to 16 g/L have been reported. The most important factor influencing malic acid concentration is the viticultural climate. The highest concentration of malic acid is observed in grapes from cool viticultural regions, where the respiration of acids in the grape berry progresses at a slower rate than in the warm climate regions. Not only does malic acid contribute to the acidic taste of wine, it also serves as a substrate for unwanted bacteria after bottling.

The biological deacidification of wine can be described as a process where specific micro organisms such as yeasts and bacteria are utilized to achieve a decrease in wine acidity (Sousa *et al.*, 1995). Malolactic fermentation (MLF) or malo ethanolic fermentation (MEF) is usually employed to accomplish this (Redžepović *et al.*, 2003). During MLF, lactic acid bacteria such as *Oenococcus oeni* convert malic acid into lactic acid and CO₂, whereas yeast species such as *Schizosaccharomyces pombe* and strains of *Saccharomyces* facilitate MEF by converting malic acid into pyruvate via an intracellular malic enzyme. MLF by bacteria can however be less desirable when compared to yeasts that degrade malic acid. Apart from the limited survival capacity of bacteria in the wine, another negative aspect is unfavourable strain-specific organoleptic changes that are effected in the wine due to bacterial metabolism (Lonvaud-Funel, 1995). On the other hand, yeast growth and survival in juice is easy to induce (Thornton and Rodriguez, 1996). Yeasts are more resistant than bacteria to sulphur dioxide, the antioxidant/antiseptic widely used in the wine industry. Yeasts are not susceptible to bacteriophage attack, can grow at the very low pH of high acid juice and have simple growth requirements which are satisfied by the majority of grape juices.

The high substrate affinity and cytosolic location of the *Ss. pombe* malic enzyme enables the yeast to effectively degrade malic acid to ethanol during alcoholic fermentation, but the production of undesired fermentation aroma has been reported. Furthermore, *S. pombe* species are not ideally suited for wine fermentation due to their temperature and alcohol sensitivity. Redžepović *et al.* (2003) have shown that an indigenous thermotolerant strain, *S. paradoxus* RO88 was able to degrade lower quantities of malic acid than *S. pombe* strain F (38% and 90% respectively), but this strain was able to produce a wine of good quality.

In this paper the breeding of 5 hybrid yeast strains with varying ability to degrade malic acid is described. A commercial wine yeast strain, VIN13 (Anchor yeast SA) was crossed with *Saccharomyces paradoxus* strain R088, initially isolated from grapes (Redžepović *et al.*, 2002). *S. paradoxus* R088 was shown to break down significant amounts of malic acid and yield a good quality wine (Redžepović *et al.*, 2003). Wine was made with the two parental strains (VIN13 and *S. paradoxus* R088) and the 5

hybrid strains. Microvinification experiments were carried out on Cinsaut grapes fermented with the above mentioned strains.

3.3 MATERIALS AND METHODS

3.3.1 STRAINS AND CULTURE CONDITIONS

The strains that were used in this study are summarized in Table 1.

For plate assays and microvinification experiments, yeasts were precultured to stationary phase in liquid YPD (1% yeast extract, 2% peptone and 2% glucose) at 30°C on a rotary shaker.

VIN13 and *S. paradoxus* strain RO88 were sporulated on sporulation media by spreading cells onto solid media containing 1% potassium acetate, 0.1% yeast extract, 0.05% glucose and 2% agar (Sherman *et al.*, 1986). These plates were incubated at room temperature for 5 to 7 days.

Hydrogen sulphide (H₂S) production was monitored by using Biggy Agar (Nickerson, 1947). The composition of Biggy Agar is 0.1% yeast extract, 1% glycine, 1% glucose, 0.5% bismuth ammonium citrate, 0.3% sodium sulphite and 1.6% agar. Yeast were screened for the production of H₂S by spotting 5 µL of a liquid YPD culture onto the plates. The intensity of the brown colony colour is directly proportional to the amount of H₂S produced by the yeast.

Yeast were screened for pectinolytic activity by spotting 5 µL of a liquid YPD culture onto pectinolytic screening plates consisting of 1.25% polygalacturonic acid, 0.68% potassium phosphate, 0.67% YNB (without amino acids), 1% glucose, 2 % agar and 0.13% amino acids (McKay, 1988). After 4 days of growth, the colonies were washed off with water and the plates were treated with 1 M HCl. Yeast colonies with pectinolytic activity showed clear zones around the colony.

The ability of yeasts to degrade malic acid was determined by using assay plates consisting of 0.17% YNB (without amino acids and ammonium sulphate), 0.5% ammonium sulphate, 10% glucose, 1% malic acid, 0.01% bromocresol green and 2% agar, to which 500 mL distilled water was added. The pH of this stirred solution was adjusted to 3.3 with KOH and was then autoclaved. The agar was added to 500 mL distilled water and autoclaved separately. After cooling, the two batches were added together, stirred and poured into plates. When the plates were set, 5 µL of a liquid YPD culture was spotted onto the plates. Colonies of yeast that degrade malic acid, turn progressively greener and should be left to grow at least 5 days.

Yeasts were screened for killer activity by spotting 5 µL of a liquid YPD culture onto a mat of killer sensitive *Saccharomyces cerevisiae* (S6) on methylene blue media plates. The mat was prepared by pipetting 150 µL of a YPD culture of *Saccharomyces cerevisiae* (S6) onto the solidified plates and allowing the mat to dry. The preparation of the plate media is as described by Van Vuuren and Wingfield (1986). Yeast colonies

with killer activity were identified by a clear zone around the colony and a blue ring on the periphery of the colony.

Table 1. Yeast strains used in this study

Yeast strain	Description	Source/Reference
VIN13	Commercial diploid strain	Anchor Yeast
<i>S. paradoxus</i> strain RO88	Natural isolated diploid strain Malic acid degradation Pectinolytic activity	Redzepovic <i>et al.</i> , 2002
<i>S. cerevisiae</i> Zim 1859 S6	Killer sensitive	Zagorc <i>et al.</i> , 2001
<i>S. cerevisiae</i> WH300	Whisky yeast strain	La Grange-Nel, <i>et al.</i> , 2004
<i>S. cerevisiae</i> WH314	Whisky yeast strain	La Grange-Nel, <i>et al.</i> , 2004
Hybrid 7	Interspecies hybrid	This study
Hybrid 10	Interspecies hybrid	This study
Hybrid 17	Interspecies hybrid	This study
Hybrid 18	Interspecies hybrid	This study
Hybrid 21	Interspecies hybrid	This study

3.3.2 ASCOSPORE DIGESTION AND HYBRIDISATION OF YEASTS

Ascospores were washed from the surface of the sporulation plates with sterile distilled water and put into an sterile tube (ELKAY). The mixture was centrifuged and the supernatant was removed. The yeast cells were then suspended in 150 μ L Zymolyase (0.5 mg/mL), ICN Immuno Biologicals. This suspension was incubated for 45 minutes at 30°C, after which the suspension was centrifuged. The supernatant was removed and the pellet was resuspended again in sterile distilled water. This suspension of haploid spores was centrifuged once more and yeast was isolated from the pellet and streaked onto YPD broth plates.

The tetrads were dissected on the YPD plates with the use of a Nikon micromanipulator and spore to spore cell mating was employed for hybridization. The yeast were then incubated at 30°C on the YPD plates for 5 days.

3.3.3 CHEF ANALYSIS

Chromosomal DNA samples were prepared according to the embedded-agarose procedure of Carle and Olson (1985). Intact chromosomal DNA were separated using contour clamped homogeneous electric field (CHEF) electrophoresis. The apparatus used was the CHEF MAPPER (Bio-Rad Laboratories, Richmond, USA). All CHEF separations were carried out in a 20 cm square, 6 mm deep, 2% agarose gel made in 0.5 x TBE buffer. Thin sections of the DNA-agarose plugs were loaded into the wells and sealed in with 1% low melting point agarose just prior to the run. The average running temperature of the 0.5 x TBE electrophoresis buffer was maintained at 14°C by

a recirculating water bath. Gels were run for 42 h at a constant voltage of 6.0 V/cm and an angle of 120 degrees. The pulse duration was 60 s for the first 14 h and 90 s for the last 28 h. Gels were stained with ethidium bromide (10 mg/ml) and viewed on a transilluminator.

3.3.4 PCR ANALYSIS

All parental and hybrid yeast strains (as confirmed by CHEF) were subjected to PCR analysis to further confirm that the yeast were indeed hybrids. Genomic DNA was isolated from the yeast strains and used as template (Hoffman and Winston, 1987). DNA was amplified using DNA polymerase (Bioline). The reaction mixture included 500 ng of genomic DNA, 200 μ L of dNTPs, 1mM MgCl, 0.5 μ M of each primer and 5U DNA Taq polymerase. The following Sigma primers were used:

δ 1 (5'-CAAATTCACCTAT^A/_TTCTCA-3') and δ 2 (5'-GTGGATTTTATTCCAACA-3').

The above mentioned primers were used to amplify genomic DNA between the δ sequences (Ness *et al.*, 1993). Denaturing, annealing, extension and storage were carried out as described in Ness *et al.* (1993).

3.3.5 SMALL SCALE FERMENTATION TRIALS AND MICROVINIFICATION

The fermentation performance of the hybrids and parents were compared by inoculating the yeasts into Clairette Blanche juice. The fermentations were done at 25°C in triplicate by inoculating 100 mL juice with every yeast strain at a concentration of 3×10^6 cells/mL. The weight of the samples were taken every day and the accumulated weight loss was determined at the end of fermentation. The chemical composition of the samples was then determined.

The UV mutated hybrids were inoculated into a synthetic media as described in the article by Denayrolles *et al.* (1995). The fermentations were done at 25°C in duplicate by inoculating 2 samples of 100 mL juice with every yeast strain at a concentration of 3×10^6 cells/mL. The weight of the samples were taken every day for a period of 22 days. The malic acid content of the samples was then determined by GC.

Wine was made by using Cinsaut grapes. The grapes were crushed and destemmed and the mixture of juice and skins were divided equally into samples consisting of 8 L juice and 10 kg skins. To each of these samples, 40 ppm SO₂ was added. The fermentations were done in triplicate and each sample was inoculated with a yeast concentration of 3×10^6 cells/mL. The must was then fermented at 25°C. Samples for chemical composition were taken after 6 months of bottle aging.

3.3.6 UV MUTAGENESIS OF HYBRIDS

Hybrids between VIN13 and *S. paradoxus* strain RO88 were grown in YPD broth and equal cell concentrations of the hybrids were mixed. Of the mixture, 1 mL cells was harvested in an eppendorf, washed with distilled water and resuspended in 1 mL distilled water. A 10 times, 100 times, 1000 times and 10 000 times dilution series was made. Of each

dilution, 250 μL was plated on 5 YPG plates (1% yeast extract, 2% peptone, 3% glycerol). An ULTRA LUM UV crosslinker (California, USA) was used for the UV mutagenesis. The UV crosslinker was first sterilized for 3 minutes. The following protocol was used for UV mutagenesis:

Plate series 1: no UV (control)

Plate series 2: 1 mJ

Plate series 3: 3 mJ

Plate series 4: 5 mJ

Plate series 5: 7 mJ

After UV exposure, the plates were immediately wrapped in tin foil and incubated at 30°C for 3 days. Cells were then randomly picked from every plate (approximately 40 colonies), individually grown up in YPD broth and were then spotted on plates that indicated the breakdown of malic acid.

3.3.7 CHEMICAL COMPOSITION

Fourier transform infrared (FT-IR) spectroscopy was used for the determination of ethanol (% v/v), reducing sugar, titratable acidity, volatile acidity, malic acid, lactic acid and glycerol. The instrument used was a WineScan FT120TM spectrometer (Foss Analytical, Denmark) that generates spectra in the wavenumber region 5011-929 cm^{-1} . FT-IR spectroscopy is based on the measurement of the frequencies of the vibrations of chemical bonds in functional groups such as C=O, C-C, C-H, O-H and N-H upon absorption of radiation in the mid infrared region of the electromagnetic spectrum (Smith, 1999). The measured frequencies are processed through a series of mathematical procedures to form FT-IR spectra. To quantify a component of interest using FT-IR spectroscopy, a calibration equation based on the correlation between absorbance (presented by the FT-IR spectra) and the concentrations as measured with the reference method, is established.

3.3.8 GAS-LIQUID CHROMATOGRAPHY

To a sample of 10 mL, 800 μL internal standard (230 ppm 4-methyl-2-pentanol in 12% v/v ethanol) was added. Compounds of interest were extracted for 30 minutes on a rotary mixer with 6.5 mL diethyl ether. The organic phase was recovered and 2 mL transferred to a sample vial.

Analysis were done on an Agilent 6890 series gas chromatograph, equipped with an ALS 7683 liquid sampler, split-splitless injector and FID (flame ionization detector). The GC was fitted with a Lab AllianceTM RH-WAX, 60 mL x 0.32mm ID x 0.5 μL film thickness, capillary column. Hydrogen was used as carrier gas at a flow rate of 3 mL/min, average velocity of 45 cm/sec. 3 μL sample was injected at a split ratio of 15:1, a head pressure of 79 kPa and inlet temperature of 200°C. The column was held at 35°C for 15 min, raised to 230°C at 7°C/min and held at the final temperature for 5 min.

Peak identification was done by comparison with authentic standard retention times. Integration and quantification of peaks were done by Chemstat Rev A.07.01 software using the internal standard calibration method.

Additionally, for the determination of malic acid, the same parameters were used as above, except 2 μL sample was injected and not 3 μL sample. Further, Chemstat Rev A.10.02 software was used for the integration and quantification of peaks.

3.3.9 SENSORY EVALUATION

The sensory evaluation was based on a line scoring system. The tasting panel (consisting of 10 experienced tasters) had to indicate the intensity of a specific flavour by marking the intensity of the flavour. Samples of approximately 50 mL were presented in randomly numbered, clean and clear 125 mL tulip shaped glasses. Samples were evaluated at a room temperature of 23°C. Seven Cinsaut wines fermented with the 2 parental strains and the 5 hybrid strains were evaluated. An additional wine made with one of the parental yeasts (VIN13) was also repeated and included with the wines for every taster, to test the consistency of each of the tasters.

3.4 RESULTS

3.4.1 MOLECULAR IDENTIFICATION OF YEAST STRAINS

The CHEF technique was used as an initial identification method and this was followed up by PCR. In Figure 1a and b, the karyotype of the parental yeasts and the 5 hybrids are illustrated in a CHEF gel. The differences between chromosomal band patterns between the hybrids and the parents can clearly be seen. The identification of UVM hybrids by CHEF are illustrated in Figure 1c.

PCR analysis of the parental yeasts and the 5 hybrid yeasts (as confirmed by the CHEF method) showed very different and distinctive band patterns for VIN13 and *S. paradoxus* strain RO88 (Figure 2a). However, hybrid 10 and 21 had band patterns similar to VIN13, contrary to the CHEF analysis. From Figure 2a, it can also be seen that the band patterns of hybrid 7, 17 and 18 are different from that of both of the parental yeasts, thus confirming that these yeast strains are indeed unique hybrids. In Figure 2b, the parental yeasts strains once again display different band patterns, but the UVM hybrids all seem to have exactly the same band patterns as VIN13.

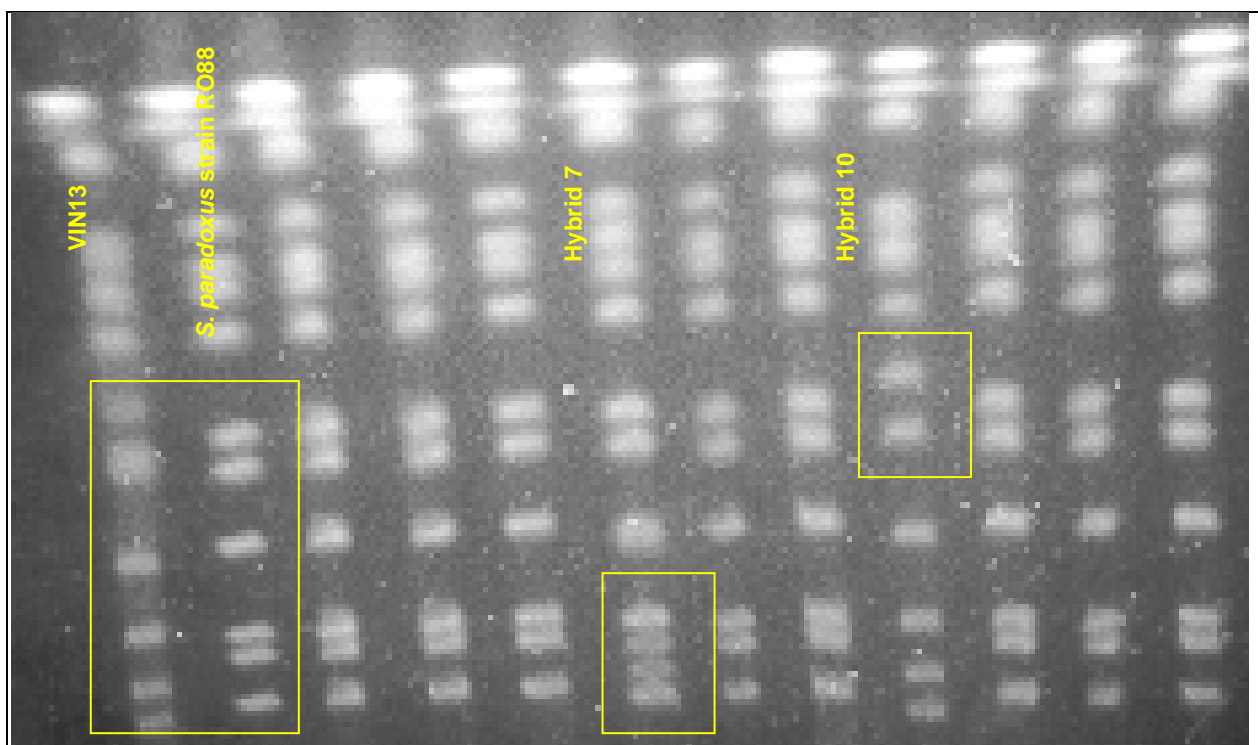


Figure 1a The differences between the parental yeasts, VIN13 and *S. paradoxus* strain RO88, are illustrated. The differences between the karyotypes of the parents and hybrids 7 and 10 are also shown. The arrows and boxes indicate band patterns where the biggest differences in the karyotypes occur.

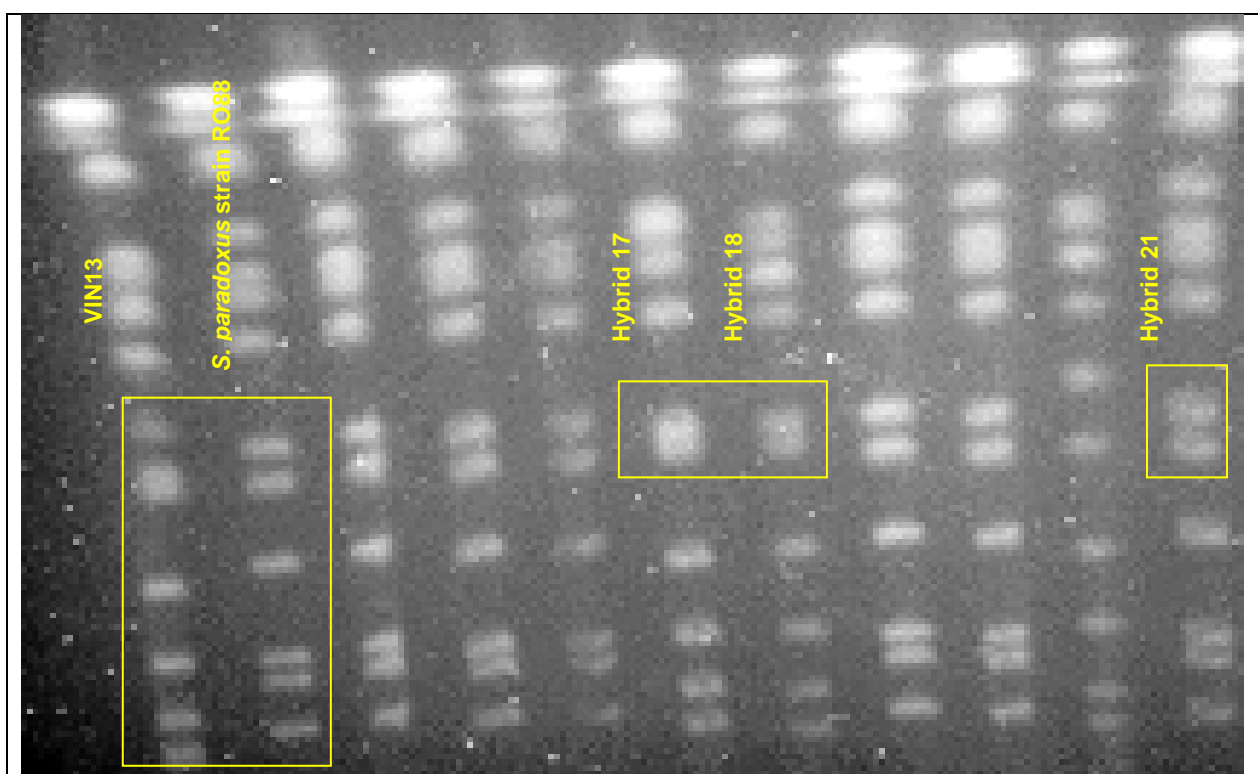


Figure 1b The different karyotypes of the parental (VIN13 and *S. paradoxus* strain RO88) and hybrid yeasts (Hybrid 17, 18 and 21) are illustrated in this photo of a CHEF gel.

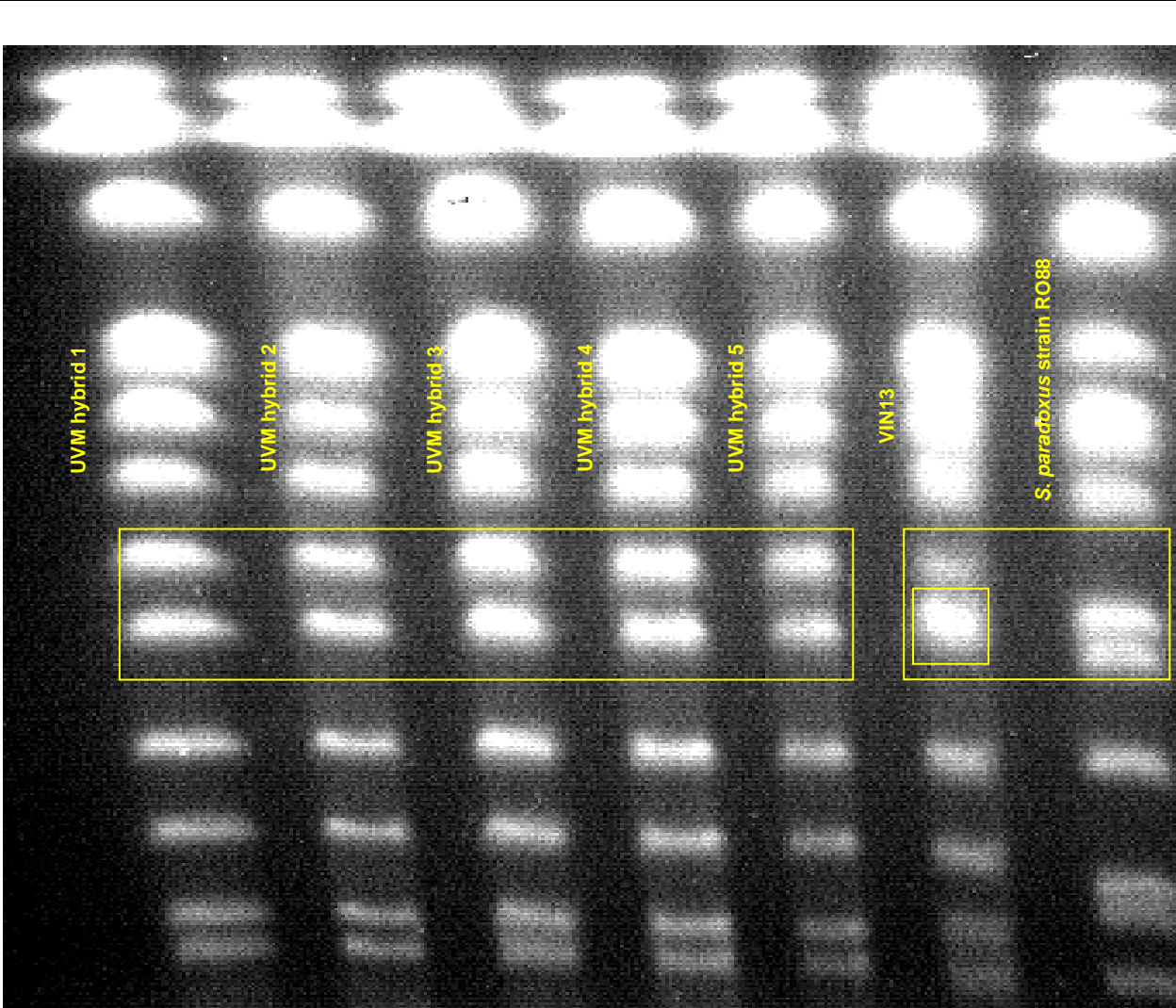


Figure 1c A CHEF gel where the UVM hybrid yeast karyotypes are compared with that of the parental yeasts, VIN13 and *S. paradoxus* strain RO88.

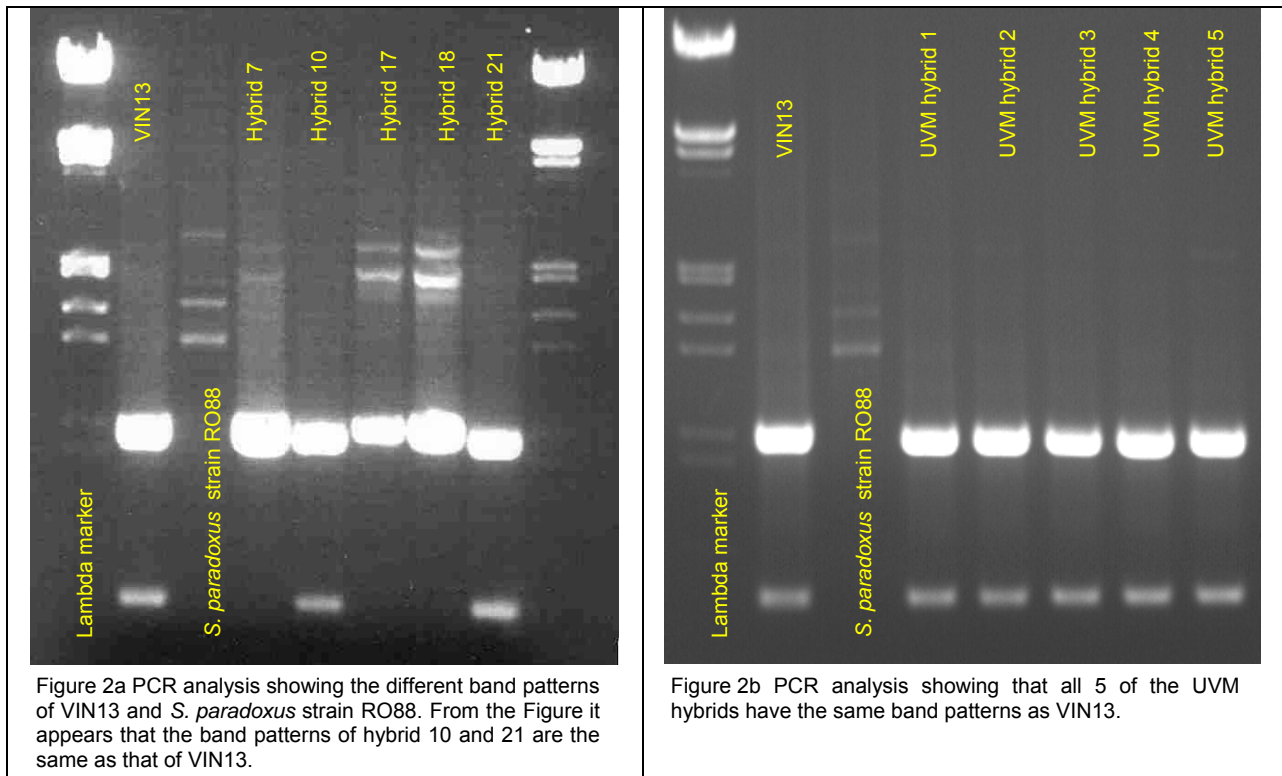
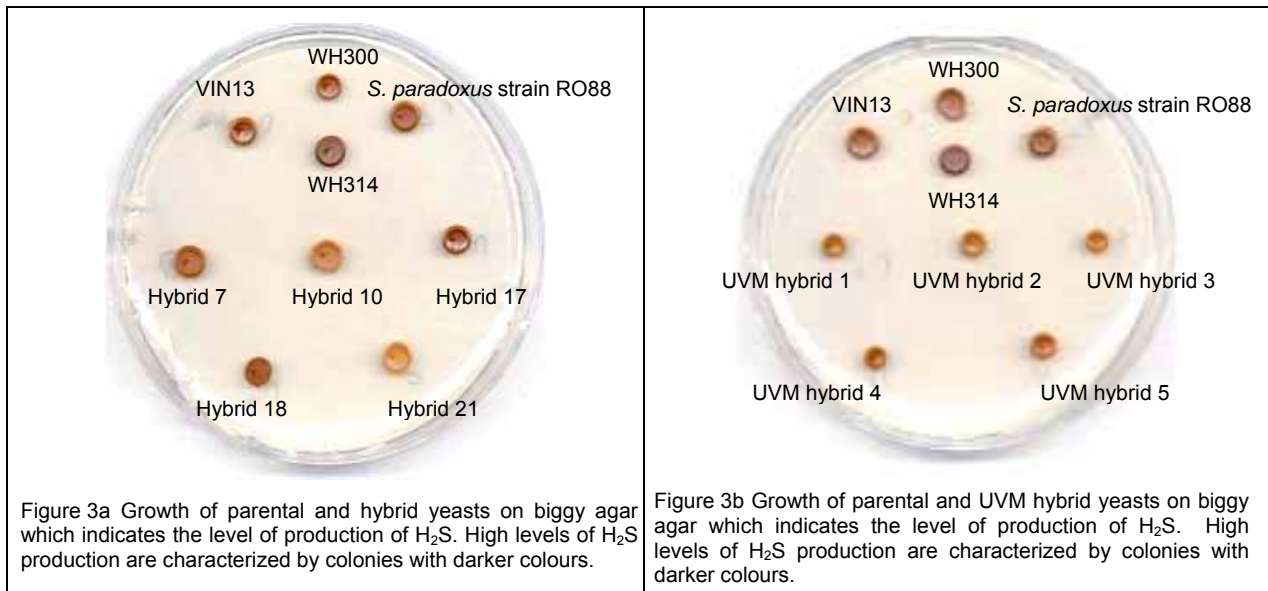


Figure 2a PCR analysis showing the different band patterns of VIN13 and *S. paradoxus* strain RO88. From the Figure it appears that the band patterns of hybrid 10 and 21 are the same as that of VIN13.

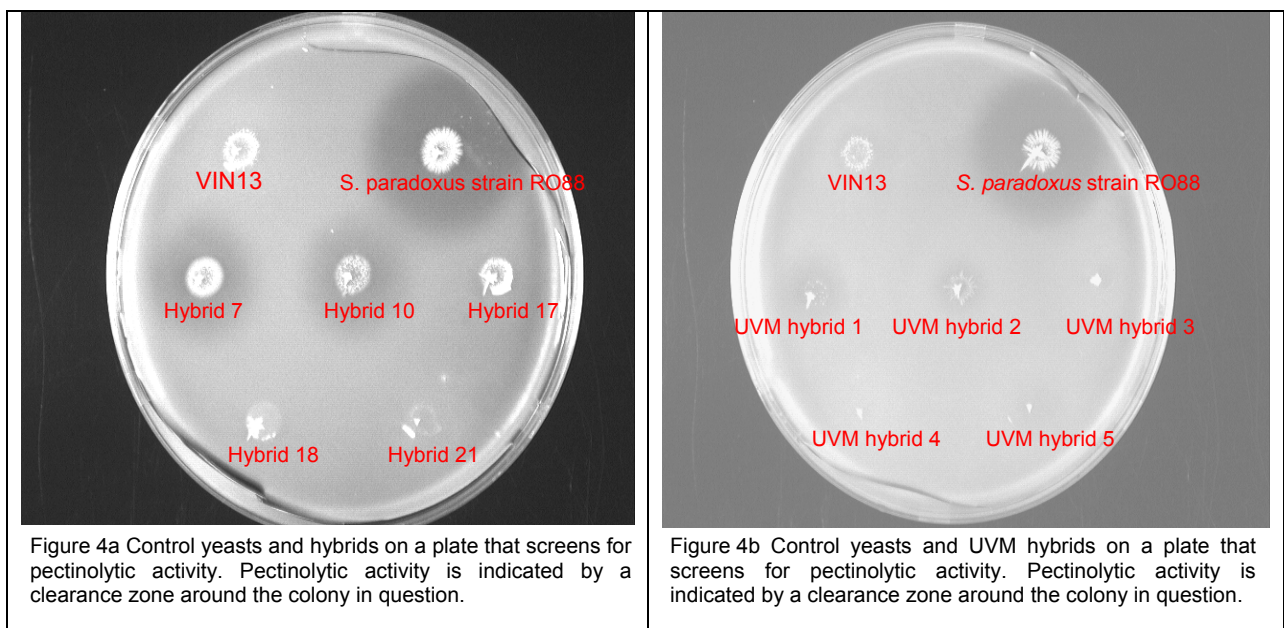
Figure 2b PCR analysis showing that all 5 of the UVM hybrids have the same band patterns as VIN13.

3.4.2 PHENOTYPICAL CHARACTERISATION

The production of hydrogen sulphide (H_2S) by hybrid yeast strains were compared with each other and to the parental yeast and two whisky yeast strains. The whisky yeasts, WH300 and WH314 were included to illustrate high H_2S production, as can be seen by the dark colony colours. The first selection of favourable wine yeasts was done by eliminating yeasts that produced the highest amounts of H_2S . High producers of H_2S are identified by dark brown to almost black colonies and low H_2S producers are white to cream coloured. In Figure 3a and b the yeast colonies are illustrated on Biggy agar. Figure 3a shows the high levels of H_2S produced by the whisky strain, WH314. None of the hybrids produce more H_2S than WH314. Hybrid 17 and 18 produced higher amounts of H_2S when compared to VIN13 and *S. paradoxus* strain RO88. The other 3 hybrids all have light to cream colony colours, which indicate low levels of H_2S production. Figure 3b shows that the UVM hybrids all produce low amounts of H_2S , compared to the control yeasts.



The pectinolytic activity of the hybrid yeasts and UVM hybrids were compared to that of the parents, VIN13 (no pectinolytic activity) and *S. paradoxus* strain RO88 (strong pectinolytic activity). In Figure 4a, hybrid 7 and hybrid 10 show moderate pectinolytic activity when compared to *S. paradoxus* strain RO88. Invasive growth also seems to be a characteristic of colonies that exhibit pectinolytic activity (Figure 4a and b). Slight pectinolytic activity is observed in UV mutated hybrids 1 and 2 (Figure 4b).



The ability of the hybrids and parents to degrade malic acid is illustrated in Figure 5a and b. The dark colony colours indicate that hybrids 10, 18 and 21 have a greater ability to degrade malic acid than the other hybrids or even the parents, VIN13 and *S. paradoxus* strain RO88. All the UVM hybrids seem to have a greater ability to degrade malic acid than the parental yeasts, which have light green coloured colonies. The UVM hybrids were selected by replica plating colonies (after UV mutation) onto

plates that indicate malic acid degradation (Figure 5c). The colonies with the darker green colour were selected and compared to the parental yeast strains.

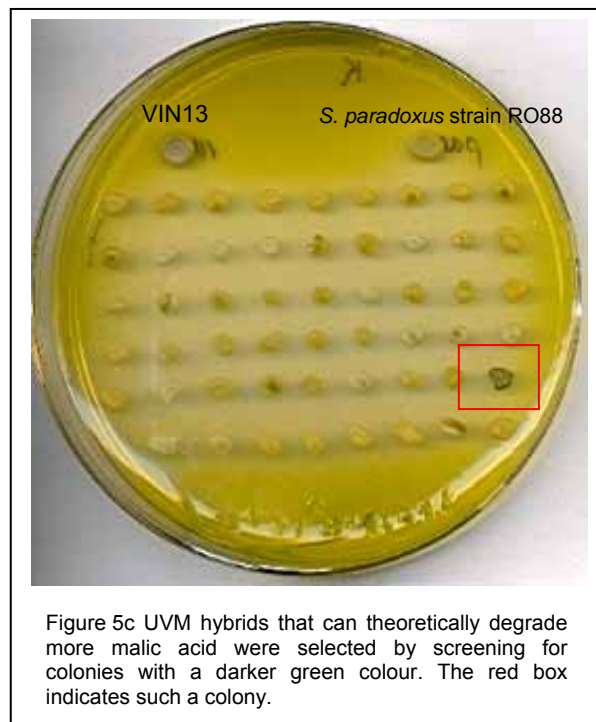
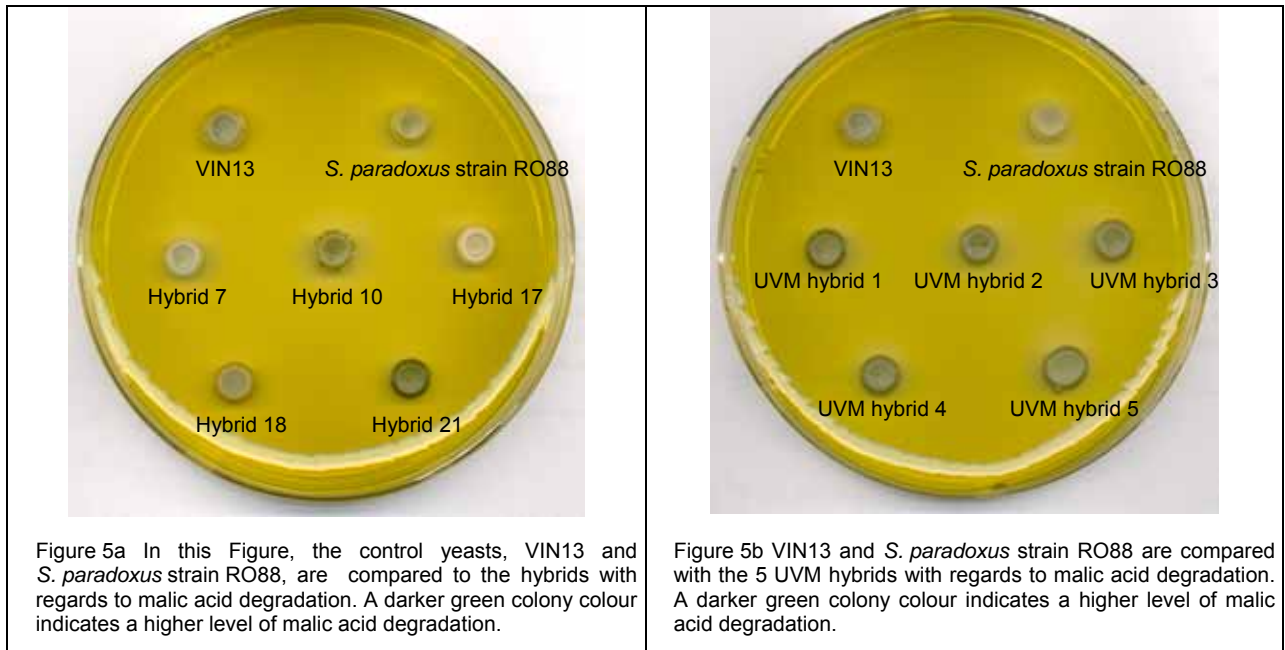
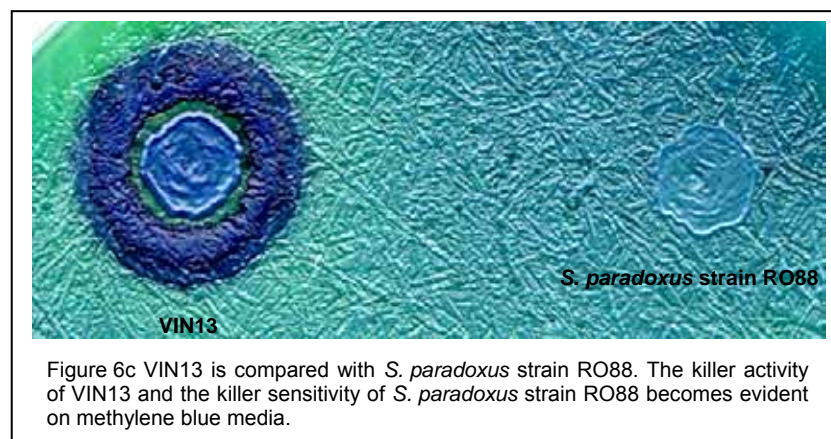
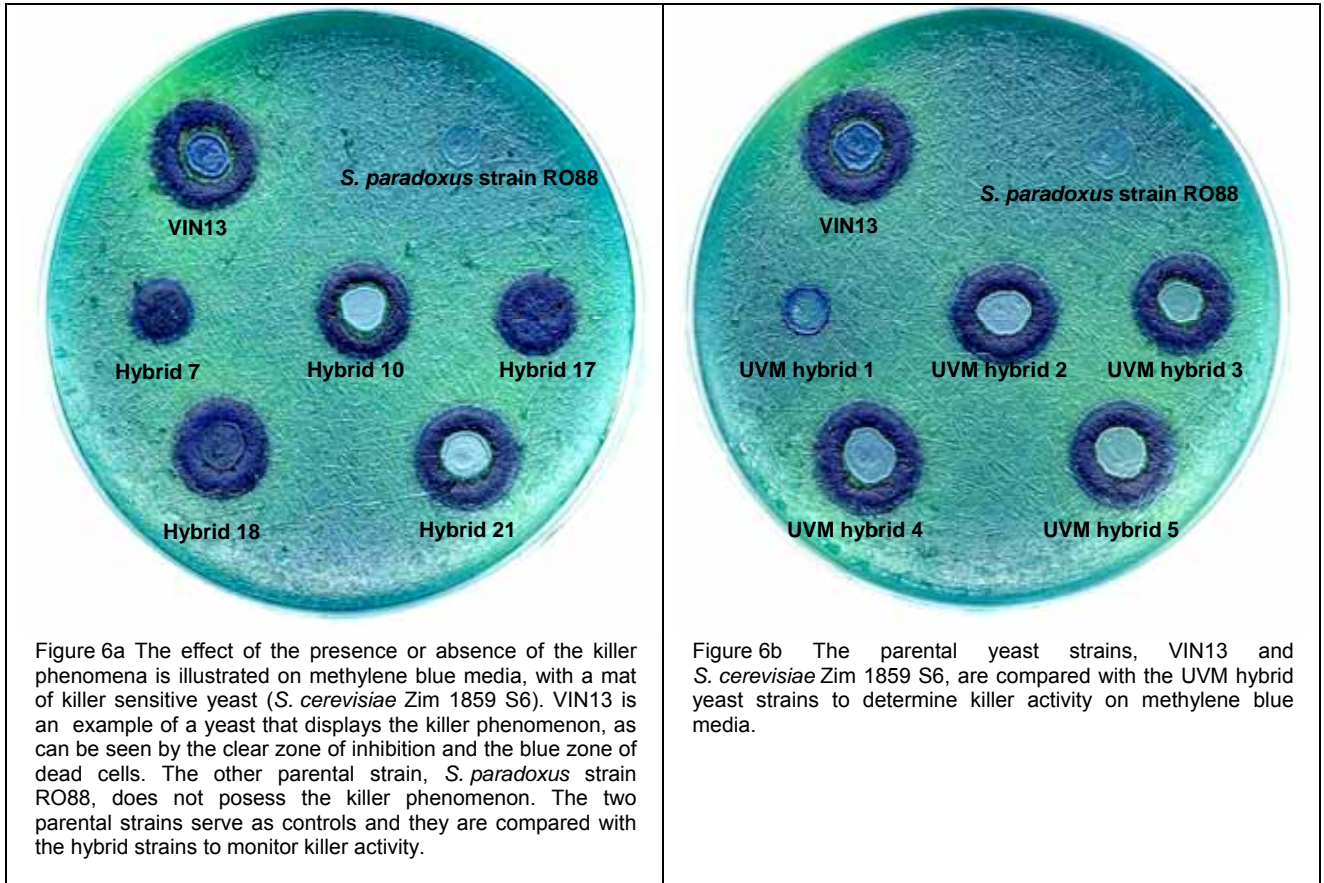


Figure 6a and b illustrates the presence or absence of the killer phenomena in the control and hybrid yeasts on methylene blue media. In Figure 6a the presence of killer activity is confirmed in all the hybrids. The biggest killer activity is invariably seen in VIN13, where the inhibition zone is the largest. In all the other hybrids, the zone of inhibition is smaller. The blue ring around the colonies also indicate killer activity, as this

is a zone of dead cells. From Figure 6b it can be seen that all the UVM hybrids except UVM hybrid 1 possess killer activity. In Figure 6c a typical killer yeast (VIN13) is compared to a killer sensitive yeast (*S. paradoxus* strain RO88). Note the zone of inhibition and the blue zone of dead cells.



3.4.3 SMALE SCALE FERMENTATION TRIALS AND CHEMICAL ANALYSIS

In Figure 7 it can be seen that the hybrids (but for hybrid 18) fermented slightly slower than the parental strains.

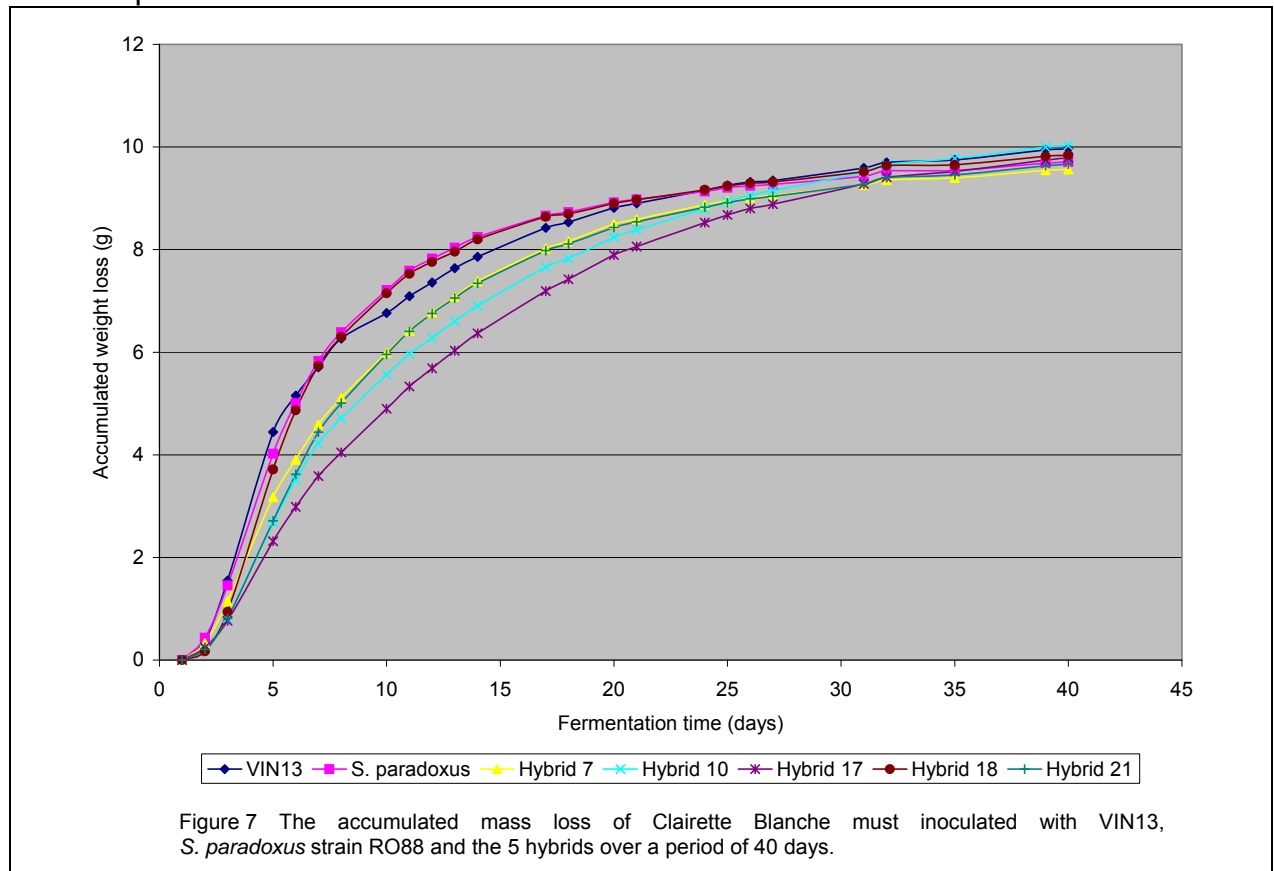
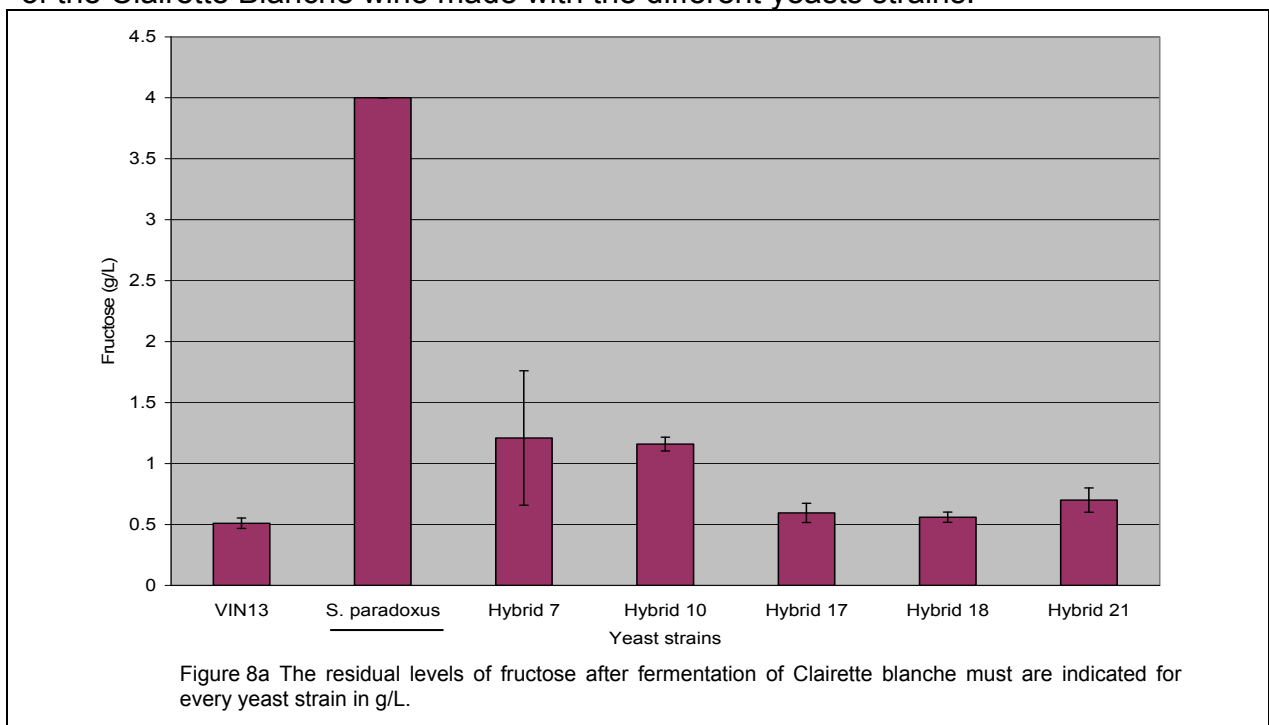
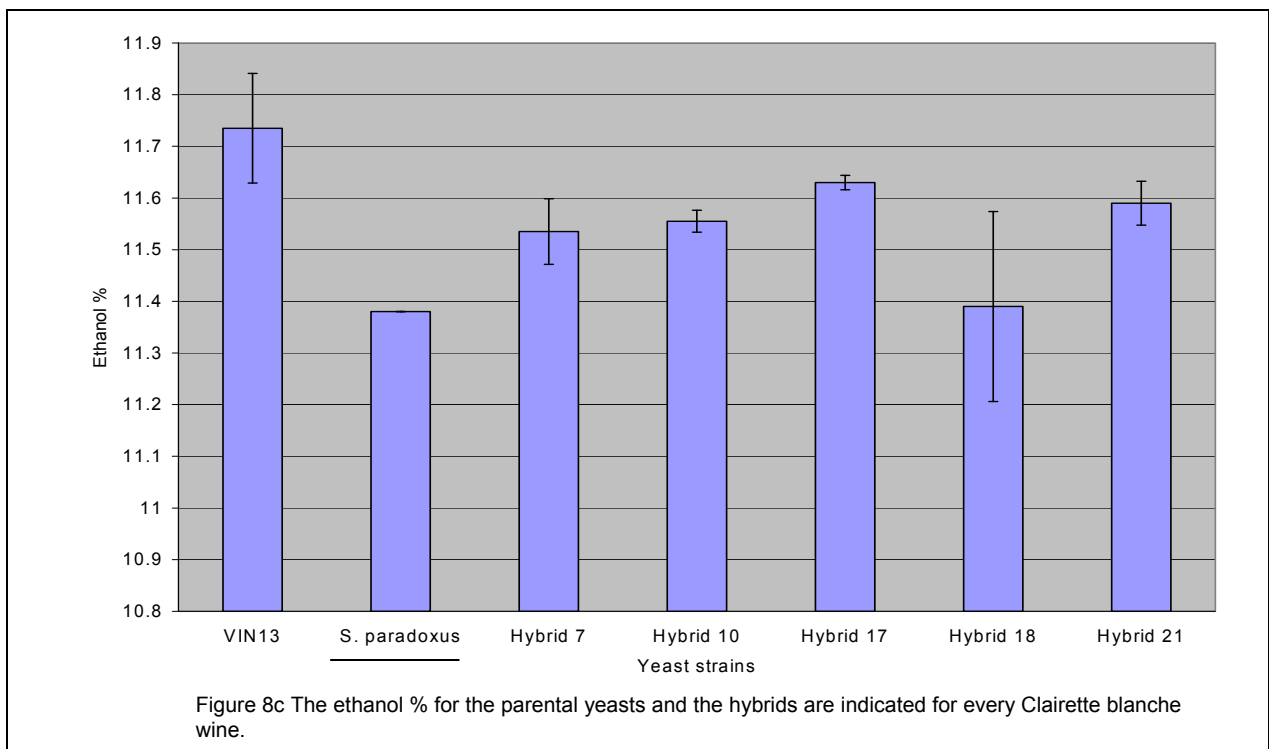
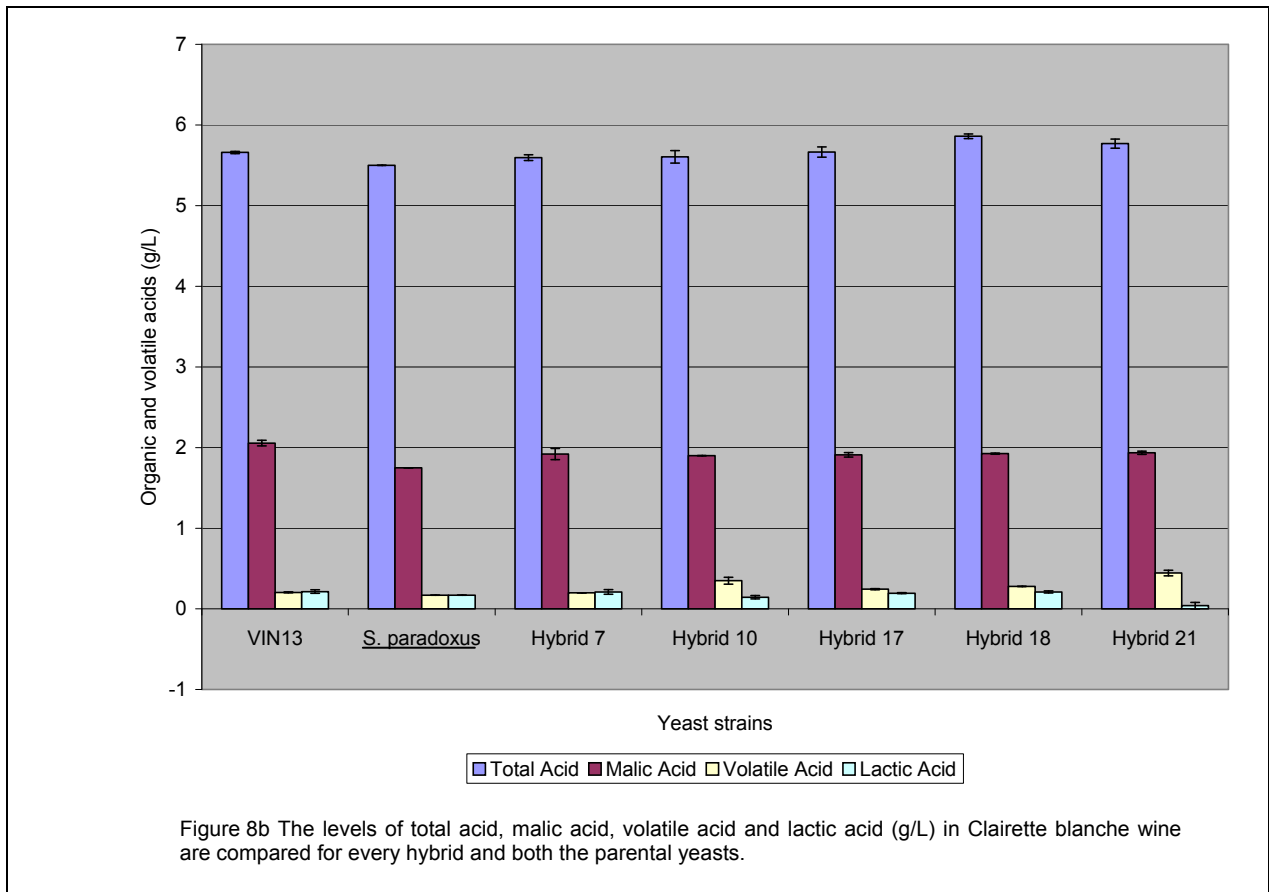
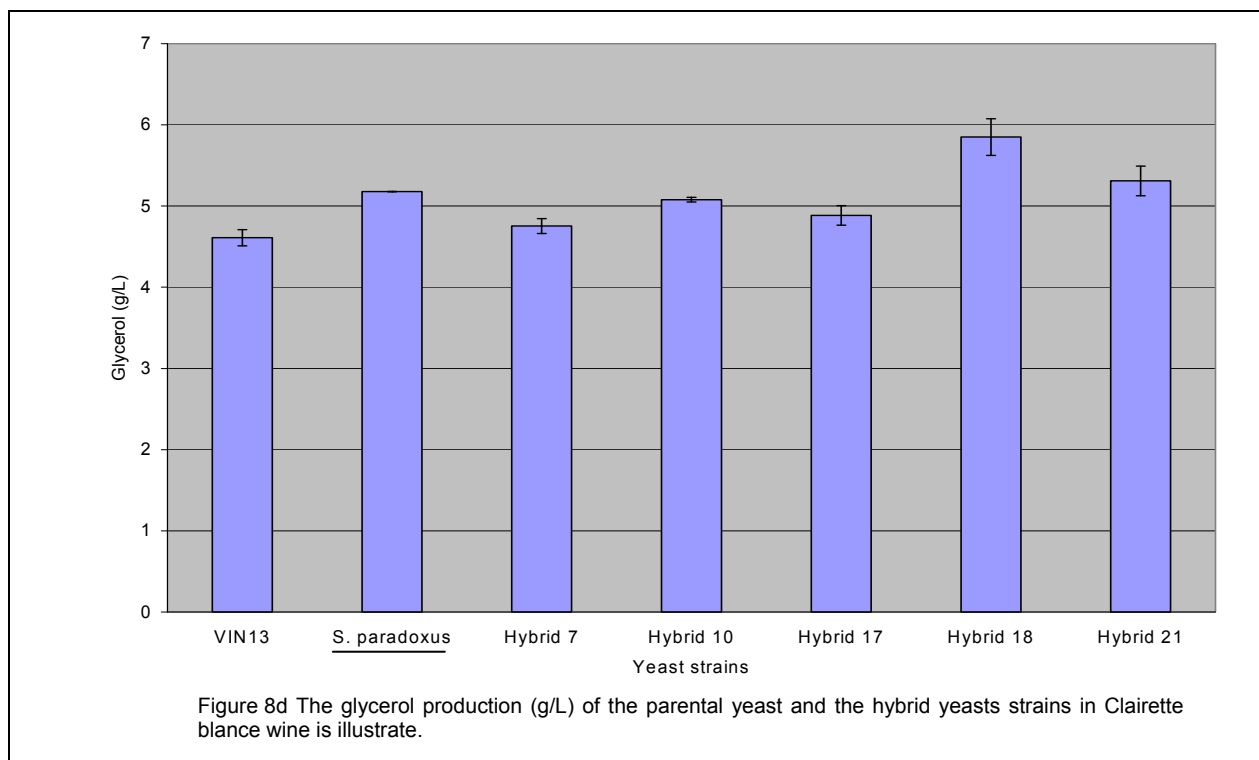


Figure 8a to d shows the glucose, fructose, organic acids, ethanol and glycerol content of the Clairette Blanche wine made with the different yeasts strains.







From Figure 8a it can be seen that fermentation with *S. paradoxus* strain RO88 resulted in wine with the highest level (4 g/L) of residual fructose. Fermentation with VIN13 and the other 3 hybrids yielded similar residual fructose levels in the wine.

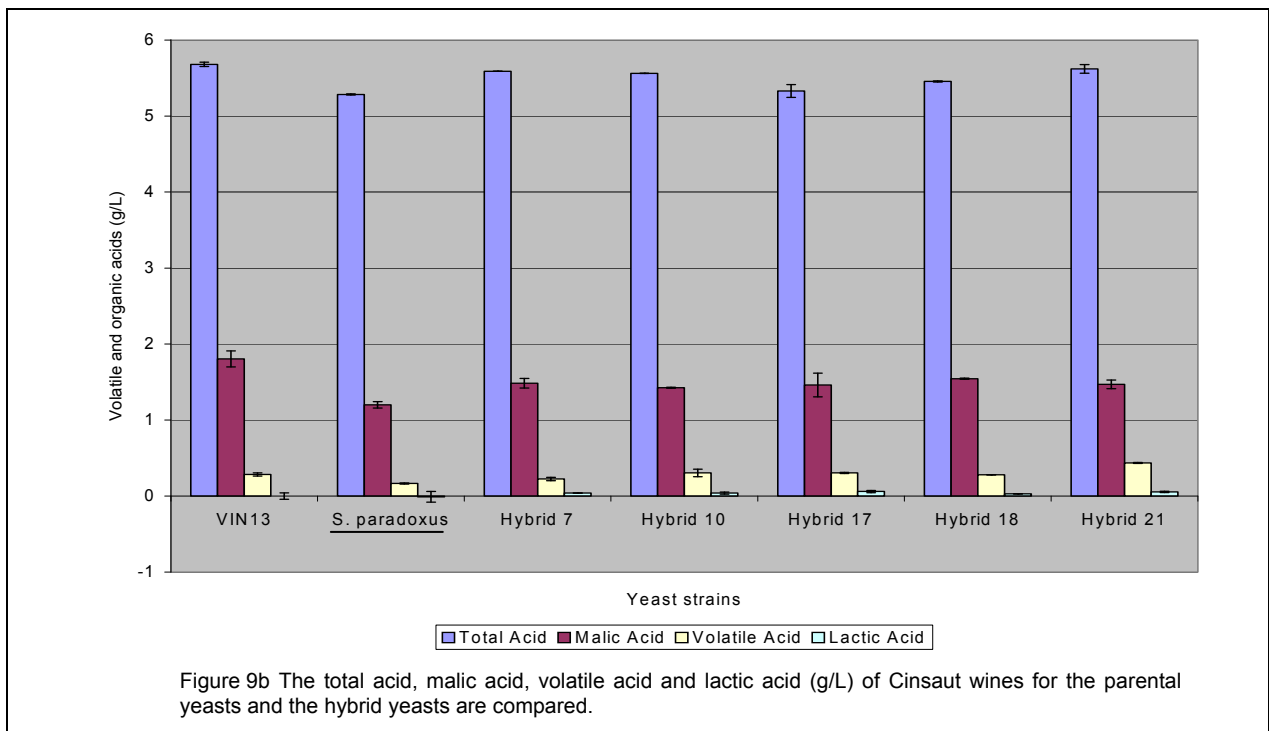
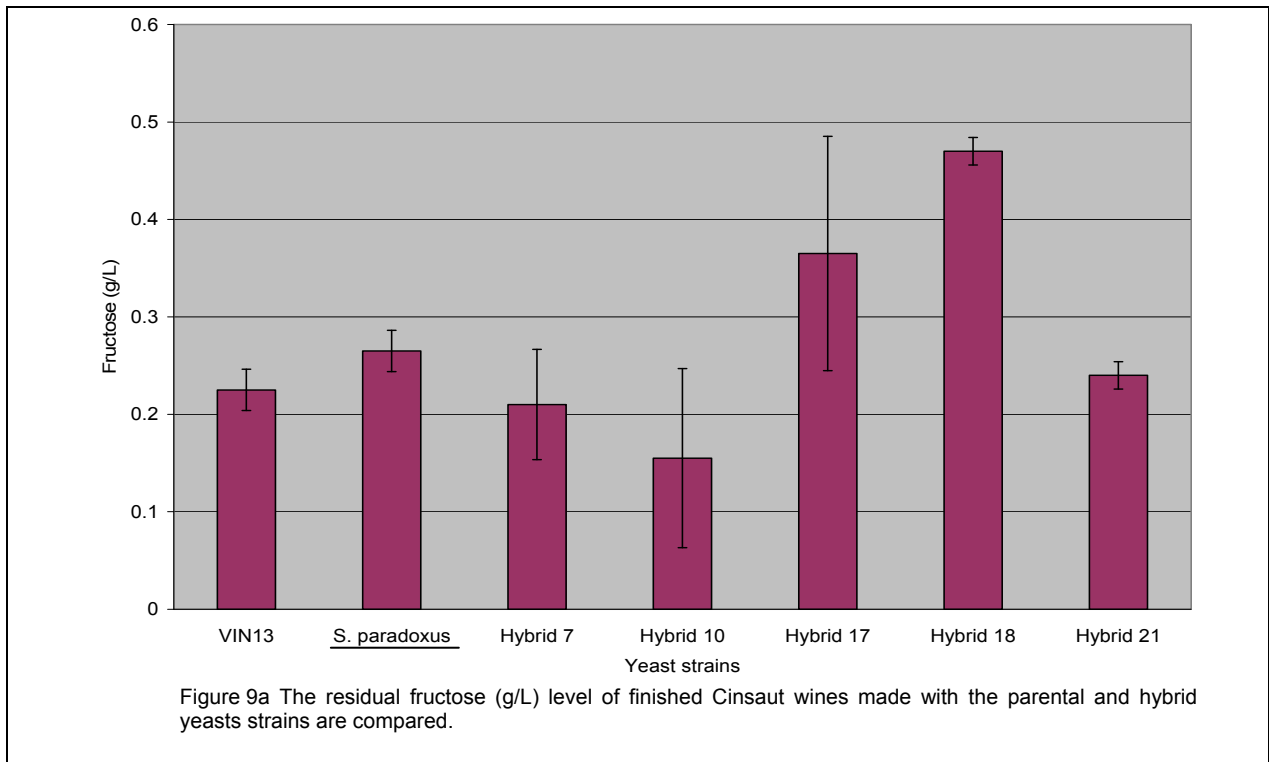
From Figure 8b it can be seen that there were no big differences in the levels of TA (titratable acidity), which ranged from 5.5 to 5.86 g/L. Wine made with *S. paradoxus* strain RO88 resulted in the highest utilization of malic acid, with a final concentration of 1.75 g/L. All the wines made with hybrids had a slightly lower end concentration of malic acid than VIN13. Wine made with hybrid 21 had the highest amount of VA (0.44 g/L) and wine made with *S. paradoxus* strain RO88 had the lowest amount of VA (0.17 g/L).

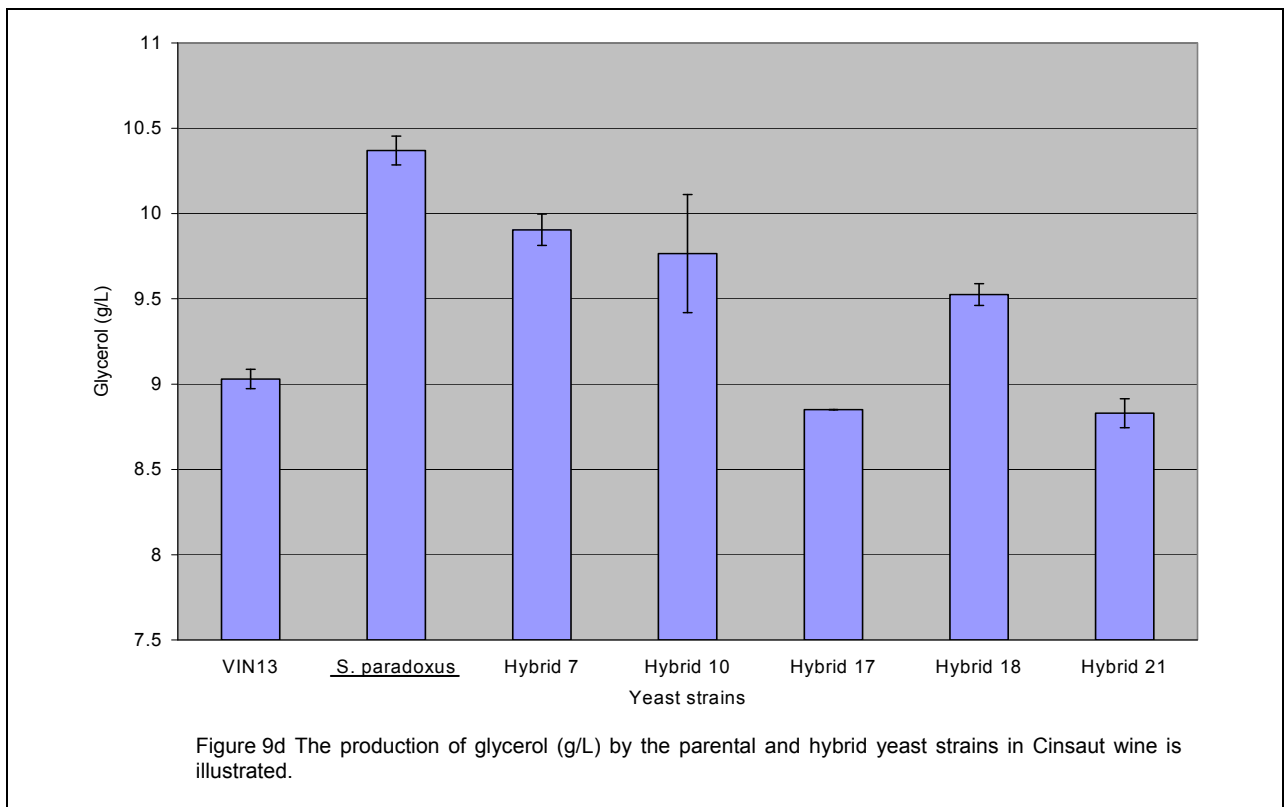
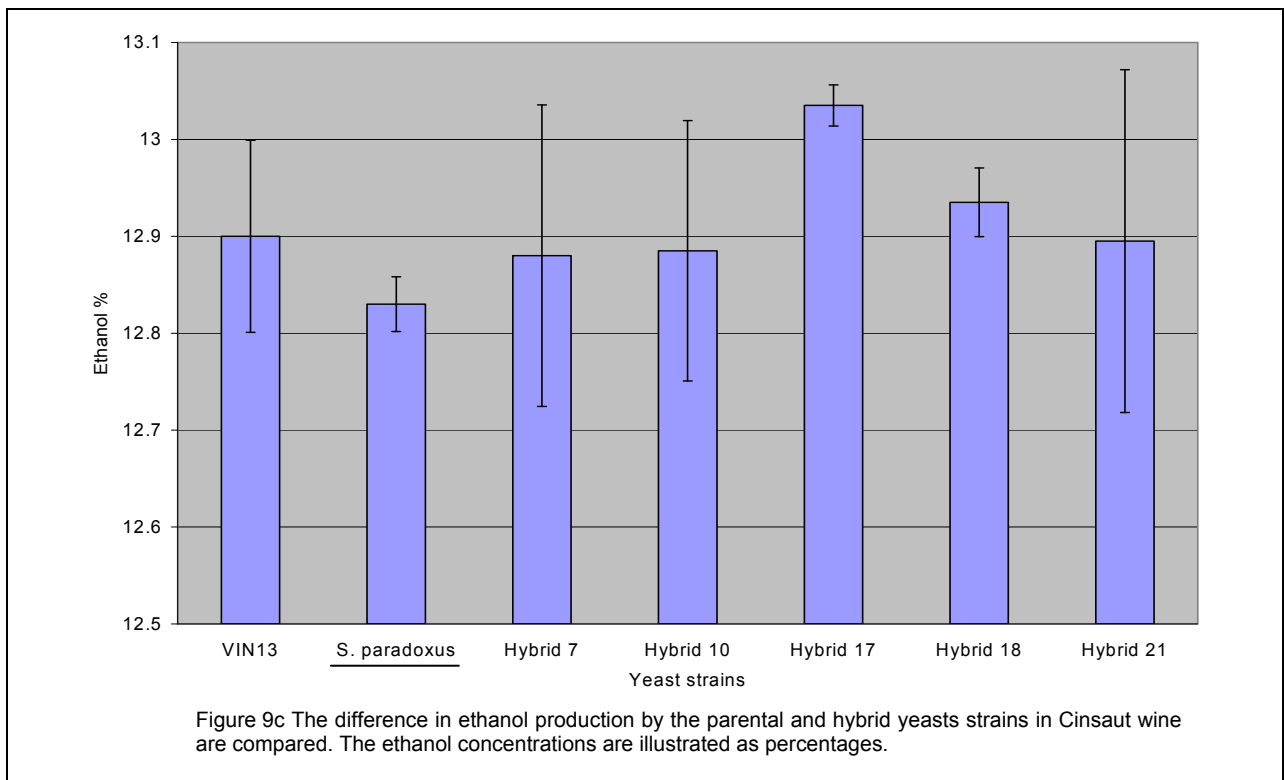
The inoculation and fermentation with the parental strains and the 5 UVM hybrids (described in Materials and methods) lead to no significant reduction in malic acid concentration in neither the parental nor the hybrid yeasts. Subsequently it was decided not to continue with any further experimentation regarding the UVM hybrids.

From Figure 8c it can be seen that VIN13 produced the highest amount of ethanol (11.735%). From Figure 8d it can be seen that hybrids 10, 18 and 21 produced more glycerol than VIN13.

3.4.4 MICROVINIFICATION

Figure 9a to d shows the glucose, fructose, organic acids, ethanol and glycerol content, determined by a FTIR Spectrometry apparatus, of Cinsaut wine made with VIN13, *S. paradoxus* strain RO88 and the 5 hybrids.





From Figure 9a it can be seen that all the glucose was fermented by the different yeast strains and that most of the fructose in the must was also fermented. The malic acid content of wine made with the hybrid yeasts was lower than for wine made with VIN13 (Figure 9b). Wine made with VIN13 had a malic acid content of 1.805 g/L and wine made with *S. paradoxus* strain RO88 had a malic acid content of

1.2 g/L (the most efficient at degrading malic acid). Amongst the hybrids, the most efficient in terms of malic acid degradation was hybrid 10, which produced wine with a malic acid content of 1.425 g/L. Levels of volatile acidity were acceptable for all the yeasts strains, ranging from 0.165 g/L to 0.435 g/L. Volatile acidity of wines are optimal between 0.2 and 0.7 g/L (Lambrechts and Pretorius, 2000). Values above 0.7 g/L become objectionable.

From Figure 9d it can be seen that *S. paradoxus* strain RO88 produced the highest amount of glycerol. VIN13 produced 9.03 g/L glycerol.

The volatile composition of the Cinsaut wines was determined by gas chromatography. The volatile ester, volatile fatty acid and alcohol content can be seen in Figure 10 to 12. Where the concentration of the volatile flavour component is above the specific threshold level, the representative columns are indicated in red (Rankine, 1969; Salo, 1970; Shinohara and Watanabe, 1976; Baumes *et al.*, 1986; Nykänen, 1986; Renger *et al.*, 1992; Fabre *et al.*, 2000; Lambrechts and Pretorius, 2000; Nurgel *et al.*, 2002; Majdak *et al.*, 2002; Peinado *et al.*, 2003).

Figure 10a shows an increase in 2-phenylethyl acetate in the wines made with hybrid 7 and 18. Figure 10b shows that wine made with hybrid 7 resulted in an increase in diethyl succinate. All the yeasts produced diethyl succinate in excess of the perception threshold concentration.

Figure 10d shows that all the yeasts produced ethyl caproate in excess of the perception threshold concentration. Figure 10e shows that *S. paradoxus* strain RO88, hybrid 10 and 21 produced ethyl caprylate in excess of the perception threshold concentration.

From Figure 10f it can be seen that all the hybrids produced levels of isoamyl acetate similar to VIN13. *S. paradoxus* strain RO88 produced the lowest concentration of isoamyl acetate.

From Figure 11a it can be seen that hybrid 7 produced significantly more 2-phenylethyl alcohol than the parental yeasts and the other hybrids. All the yeasts produced 2-phenylethyl alcohol in excess of the perception threshold concentration.

From Figure 11b it can be seen that all the yeasts produced hexanol in excess of the perception threshold concentration. From Figure 11c it can be seen that hybrid 7 produced more isoamyl alcohol than the other hybrids and also both the parental yeasts. All the yeasts produced isoamyl alcohol in excess of the perception threshold concentration. Figure 11g shows that all the yeasts produced methanol concentrations in the normal range of 0 to 0.6 g/L (Rankine, 1998).

Figure 12b shows that hybrid 7 produced the highest amount of isobutyric acid, compared to the other hybrids. In Figure 12c it can be seen that all the hybrids produced isovaleric acid in excess of the perception threshold concentration.

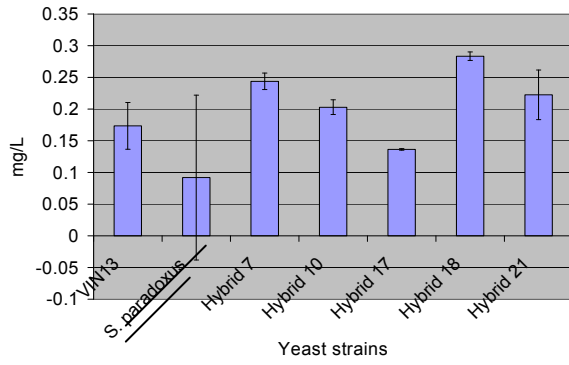


Figure 10a 2-Phenylethyl acetate

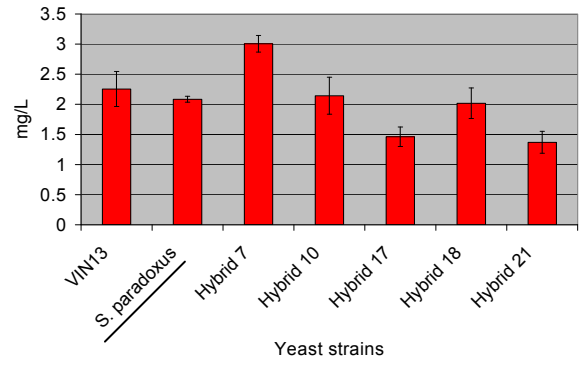


Figure 10b Diethyl succinate

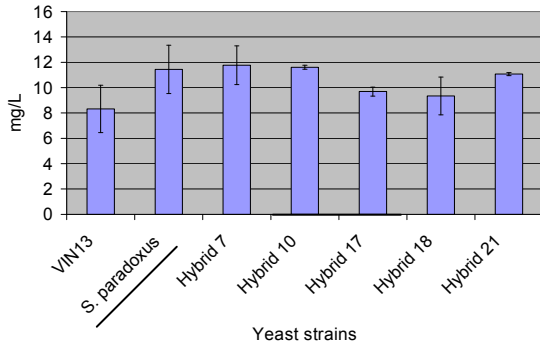


Figure 10c Ethyl lactate

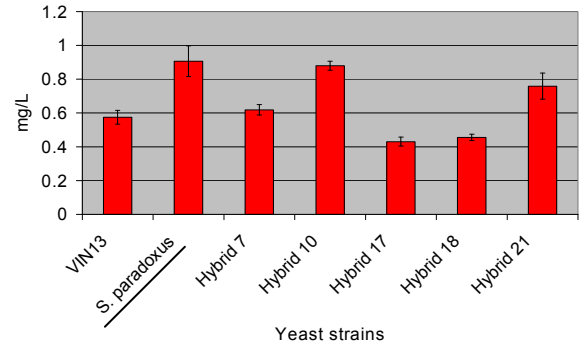


Figure 10d Ethyl caproate

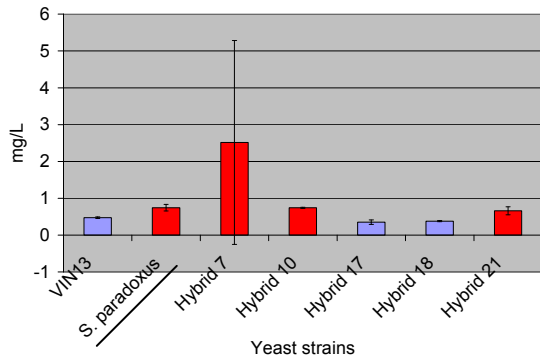


Figure 10e Ethyl caprylate

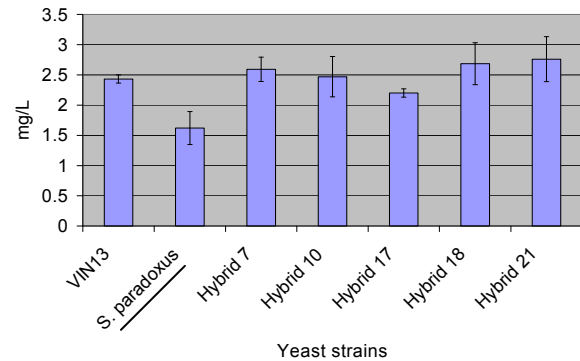


Figure 10f Isoamyl acetate

Figure 10 Concentration of volatile esters in Cinsaut wine fermented with the parental yeasts, VIN13 and *S. paradoxus* strain RO88 and the hybrid yeast strains. Red columns indicate concentrations above the threshold value.

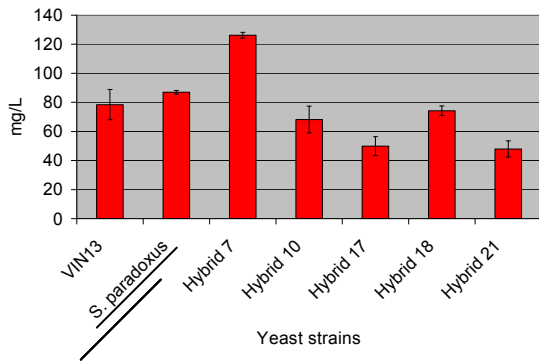


Figure 11a 2-Phenylethyl alcohol

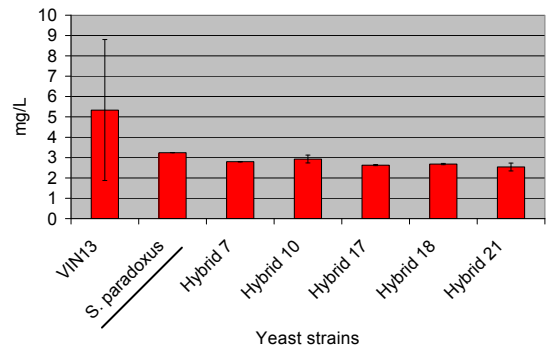


Figure 11b Hexanol

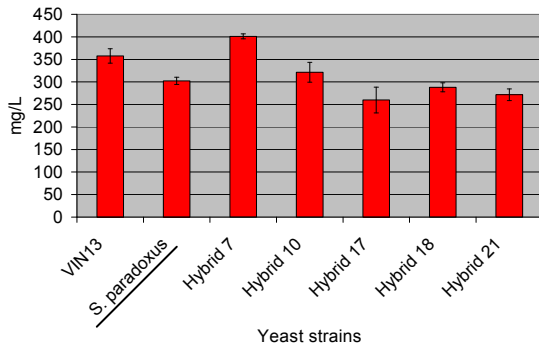


Figure 11c Isoamyl alcohol

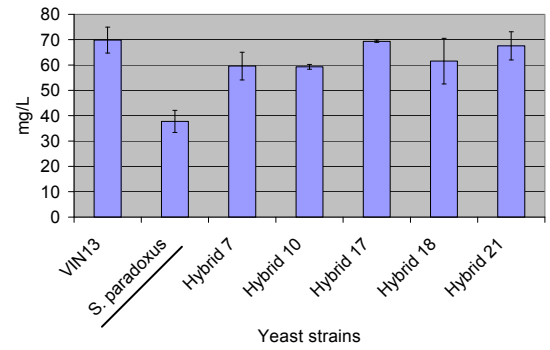


Figure 11d Isobutanol

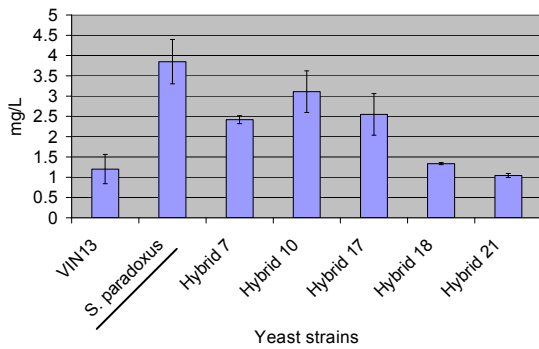


Figure 11e n-Butanol

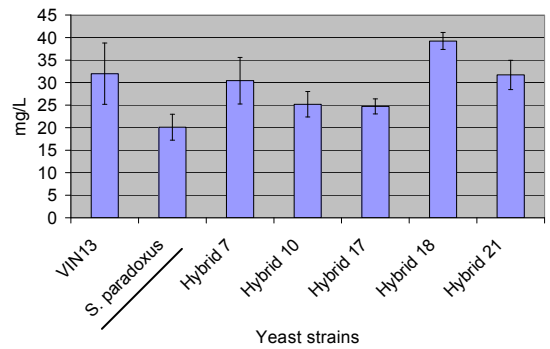


Figure 11f n-Propanol

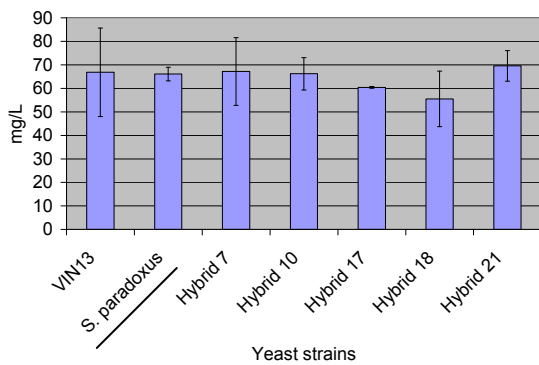


Figure 11g Methanol

Figure 11 Concentration of alcohols in Cinsaut wine fermented with the parental yeasts, VIN13 and *S. paradoxus* strain RO88 and the hybrid yeast strains. Red columns indicate concentrations above the threshold value.

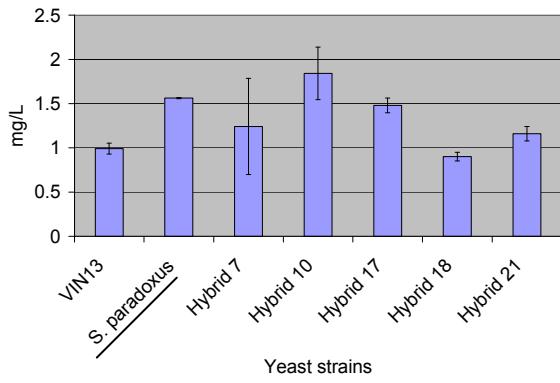


Figure 12a Decanoic acid

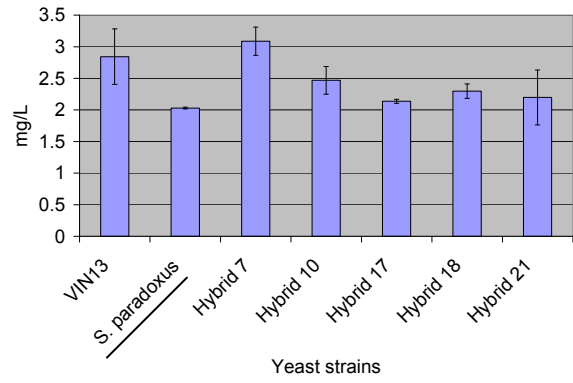


Figure 12b Isobutyric acid

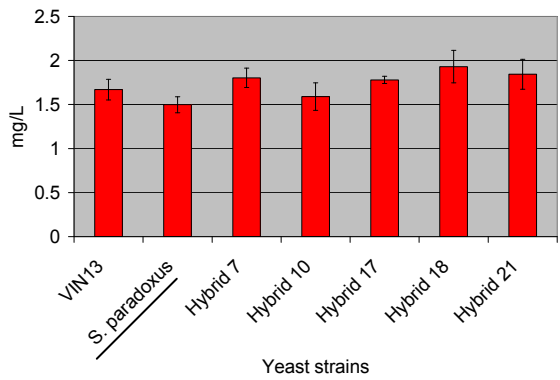


Figure 12c Isovaleric acid

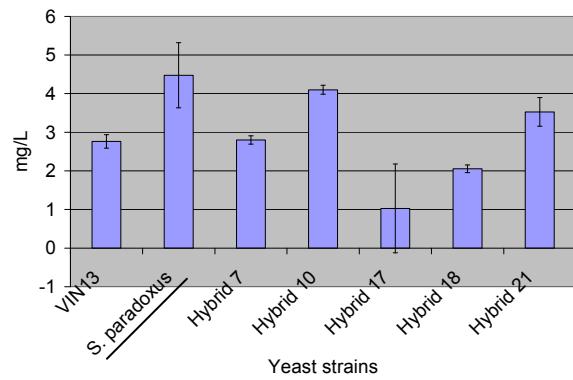


Figure 12d Hexanoic acid

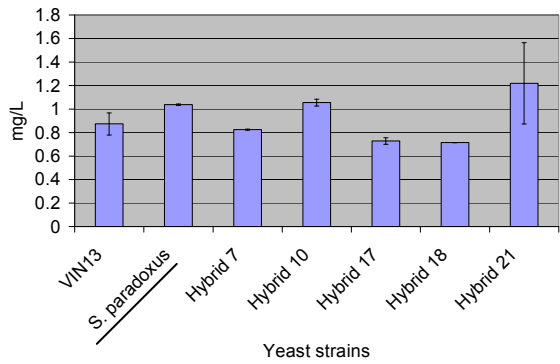


Figure 12e n-Butyric acid

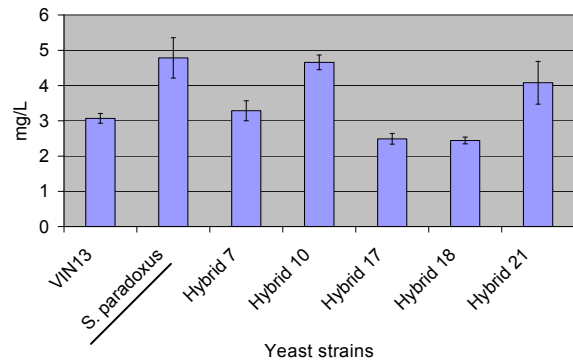


Figure 12f Octanoic acid

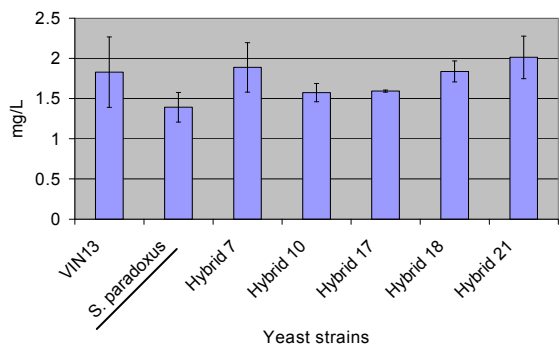


Figure 12g Propionic acid

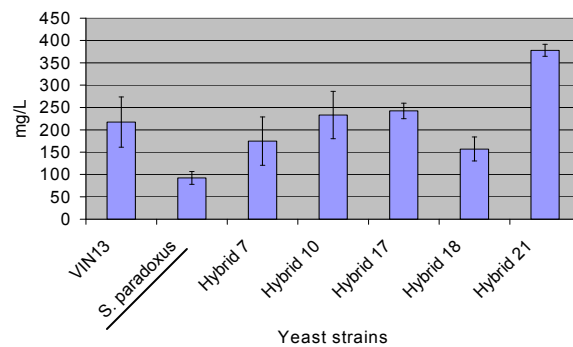


Figure 12h Acetic acid

Figure 12 Concentration of acids in Cinsaut wine fermented with the parental yeasts, VIN13 and *S. paradoxus* strain RO88 and the hybrid yeast strains. Columns indicated in red represent concentrations above the threshold value.

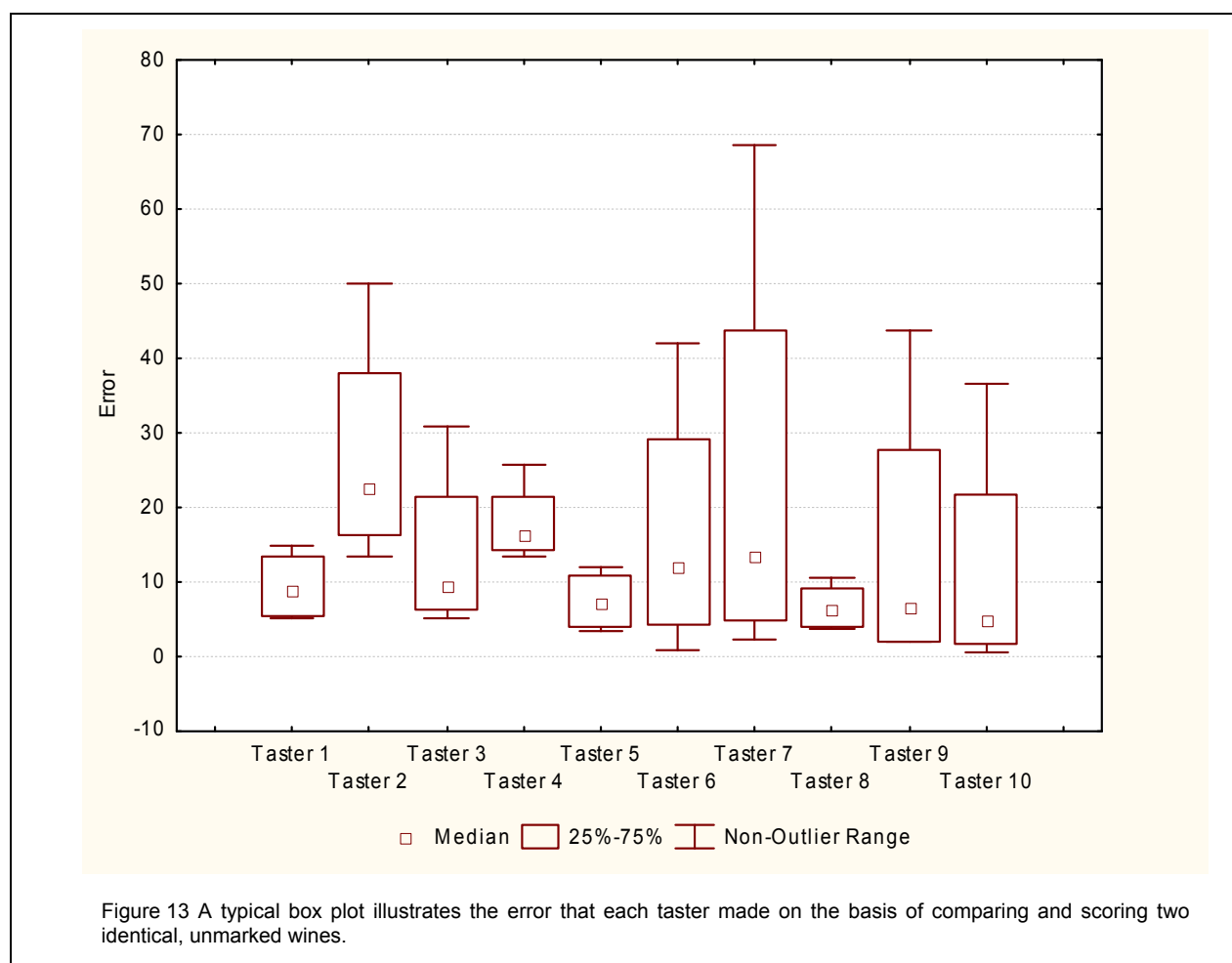
In Figure 12d it can be seen that hybrid 10 produced more hexanoic acid than the other hybrids. Figure 12f shows that *S. paradoxus* strain RO88, hybrid 10 and 21 produced the highest amounts of octanoic acid.

Figure 12h shows that all the yeasts produced levels of acetic acid well below the perception threshold concentration of 0.7-1.1 g/L and that hybrid 21 produced the highest amount of acetic acid. (Corison *et al.*, 1979).

Hybrid 7 thus produced significantly more esters (2-phenylethyl alcohol, diethyl succinate and ethyl caprylate) and higher alcohols (2-phenylethyl alcohol and isoamyl alcohol) than any of the other yeasts. Hybrid 7 also produced the highest amount of isobutyric acid, compared to the other hybrids. Hybrid 10 produced more hexanoic acid than the other hybrids and hybrid 7 and 10 produced more decanoic acid than the parental yeasts.

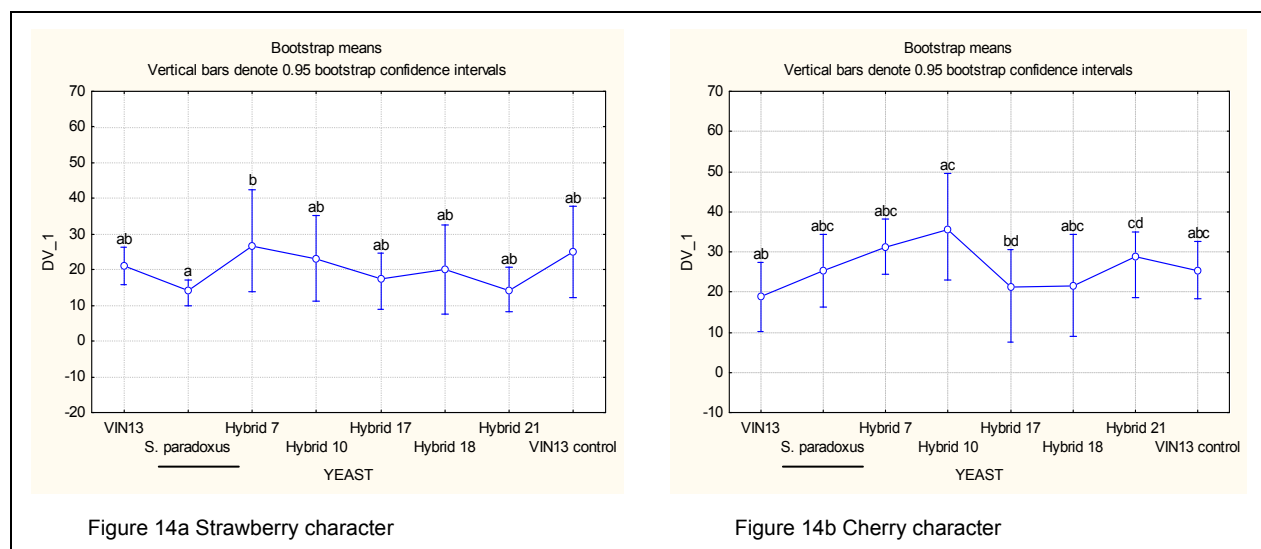
3.4.5 SENSORY EVALUATION

The Cinsaut wine made with VIN13, *S. paradoxus* strain RO88 and the 5 hybrid yeasts were evaluated by a tasting panel consisting of 10 tasters. The consistency of the tasters were tested by doing a box plot of all the scores that each taster gave for the repeated wine (Figure 13).



From the results in Figure 13, it was statistically determined that taster 1, 4, 5 and 8 scored the repeated wines the most consistently and with the smallest margin or error. The other 6 tasters were not included in further results due to their large margins of error and large outer ranges of error in scoring the 2 identical wines.

The bootstrap means for the perception of strawberry, cherry, jam and sulphur compounds are illustrated in Figure 14. The trend for strawberry character (Figure 14a) was the highest with hybrid 7 and 10, which both scored higher than both the parental yeasts. Hybrid 17 scored in between that of the parental strains and hybrid 18 and 21 respectively scored similarly to VIN13 or *S. paradoxus* strain RO88. Hybrid 7 and 10 scored significantly higher for cherry character than both the parental yeasts (Figure 14b) and hybrid 21 scored slightly higher than both the parental strains. Hybrid 17 and 18 scored slightly higher than VIN13. Hybrid 10 and 21 scored significantly higher for jam character than both the parental strains (Figure 14c). The other hybrids scored similar to VIN13 or slightly lower. Hybrid 7 and 10 scored lower than both the parental strains for sulphur compounds (Figure 14d). Hybrid 17, 18 and 21 scored significantly higher than all the other yeasts strains for sulphur compounds.



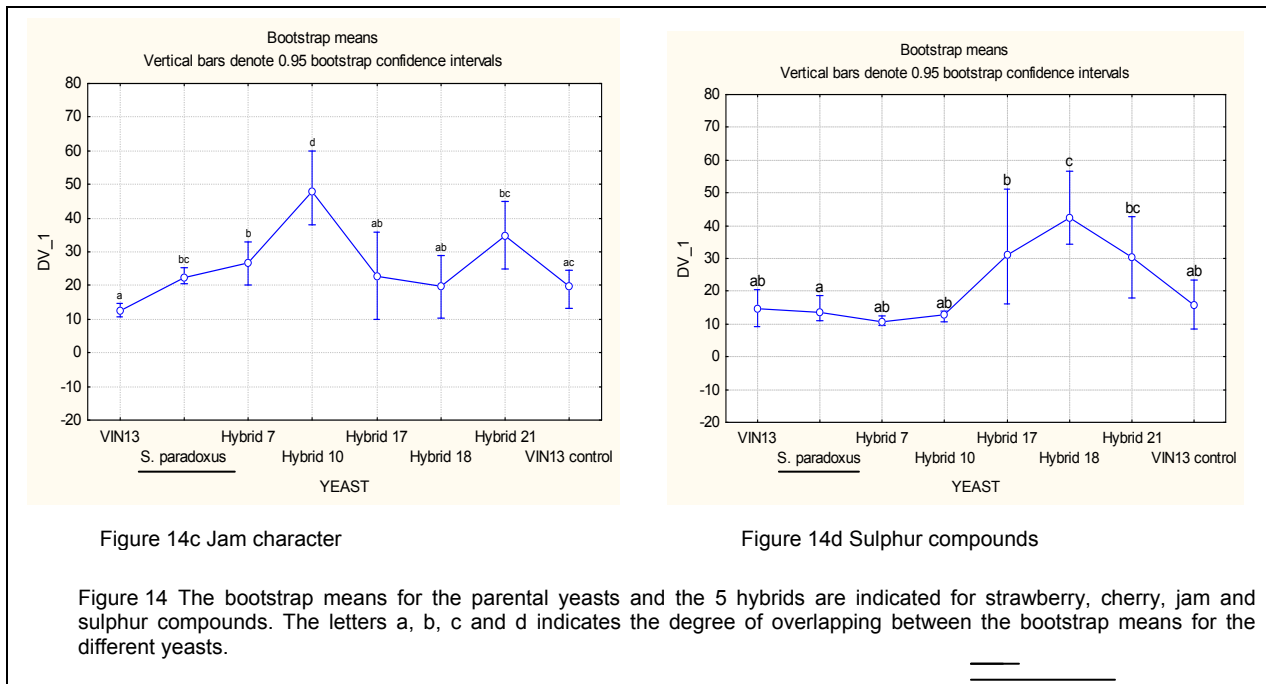
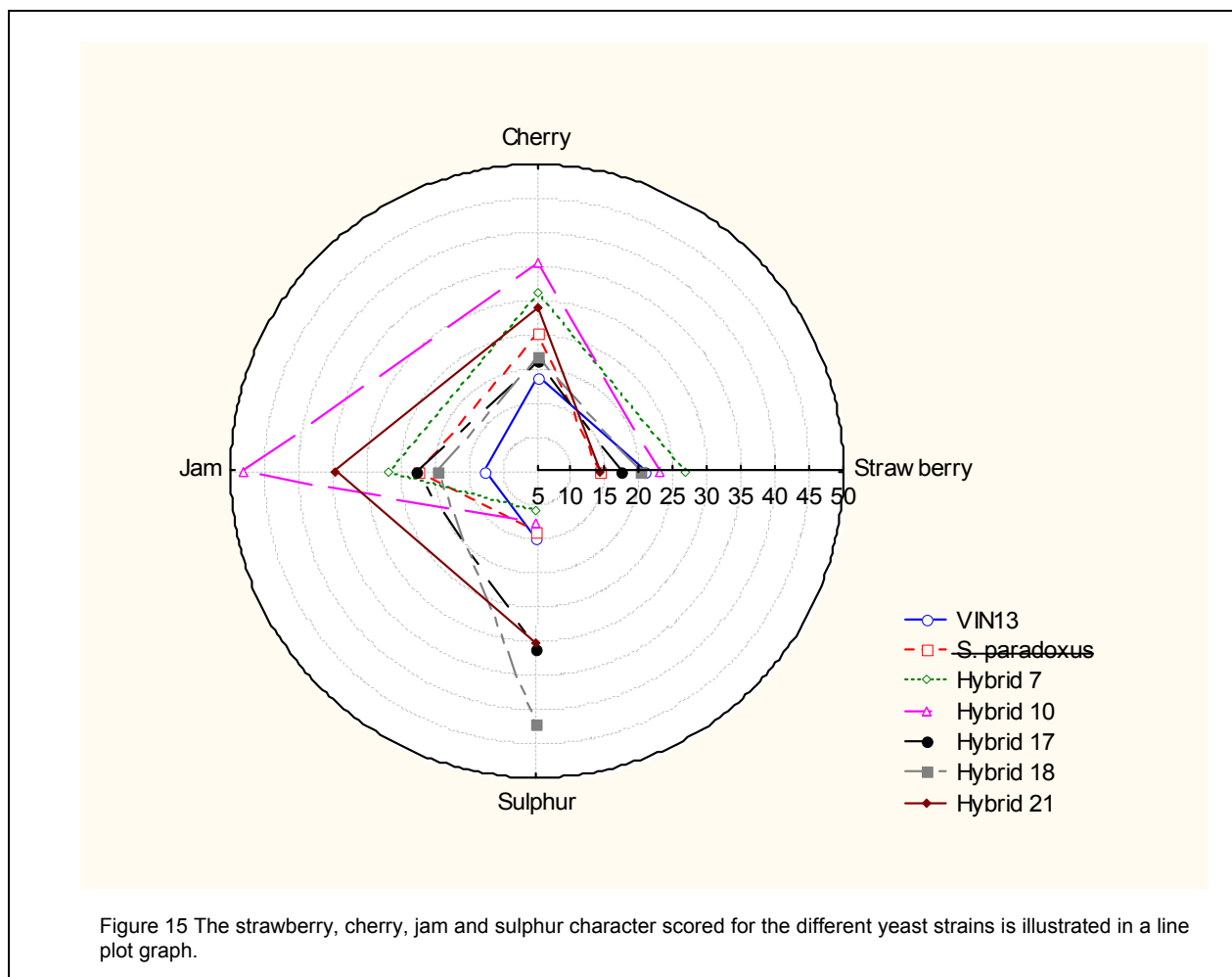


Figure 15 illustrates a line plot of the strawberry, cherry, jam and sulphur character scored for the different yeast strains. Hybrid 7 and 10 scored higher than both the parental strains for strawberry, cherry and jam character and lower than both the parental strains for sulphur compounds. Hybrid 21 also scored higher than both the parental strains for cherry and jam character, but the lowest (as did *S. paradoxus* strain RO88) for strawberry character.



3.5 DISCUSSION

In this study, 5 hybrids were obtained by classical breeding of haploids. Two of these hybrids (hybrid 7 and 10) seemed to be very unique. An additional UV mutagenesis protocol was followed to create hybrids which displayed increased malic acid degradation, but this protocol did not yield positive results and was abandoned.

During phenotypical analysis, some of the hybrids (including hybrid 7 and 10) were shown to produce levels of H_2S lower than that of the parental yeasts. Excessive levels of H_2S contribute to unpleasant aromas in wine and yeasts with low H_2S production potential should be sought after (Mestres *et al.*, 2002). Hybrid 7 and 10 showed pectinolytic activity, which is advantageous, as such yeasts can reduce haze or gelling during winemaking (Kashyap *et al.*, 2001). Another advantage of using a pectinolytic wine yeast, is the increased release of anthocyanins of red grapes into the juice. All the hybrids tested positive for killer activity. The fact that hybrid 7 and 10 displayed both pectinolytic and killer activity, indicates the incorporation of DNA from both the parental yeasts into the genome of the hybrids. This is an example of the introduction of novel characteristics into a wine yeast (Naumov *et al.*, 1992; Van der Westhuizen, 1990). Most of the hybrids fermented slightly slower than the parental yeasts, indicating that the hybrids may have potential to be used in fermentations where a slower fermentation

rate is sought after. The decrease in fermentation rate alone might however not be enough to effect a positive change in the aroma of wine, as the ester and higher alcohol production of yeasts remains paramount in aroma production (Torija *et al.*, 2003).

The hybrids fermented various musts to dryness and showed minimal and acceptable production of VA (Lambrechts and Pretorius, 2000). In general, the hybrids degraded more malic acid than VIN13, but not as much as *S. paradoxus* strain RO88. The ability of the hybrids to degrade malic acid is positive, but unfortunately the high levels of malic acid degradation as seen in other studies were not achieved (Bony *et al.*, 1997; Volschenk *et al.*, 1997).

Hybrid 7 produced significantly more esters (2-phenylethyl acetate, diethyl succinate and ethyl caprylate) and higher alcohols (2-phenylethyl alcohol and isoamyl alcohol) than any of the other yeasts. It is also important to note that the production of isoamyl acetate was not adversely affected by hybridization, as this is one of the most important esters in wine, implicated in a pleasant fruity aroma (Plata *et al.*, 2003). Fermentation with hybrid 7 and 10 also showed increased levels of volatile fatty acids, such as decanoic acid, isobutyric acid and hexanoic acid. These volatile fatty acids usually impart unpleasant aromas in wine, above their threshold values (Majdak *et al.*, 2002). Below their threshold values, they add to the complexity of wine and can impart fruity and citrus aromas. Sensory evaluation of the wine confirmed the aroma production potential of hybrid 7 and 10, as established by GC analysis.

In conclusion, hybridisation of VIN13 and *S. paradoxus* strain RO88 resulted in two very unique hybrids (out of the 5 hybrids that were obtained), which became evident during phenotypical characterisation. These hybrids both possess the killer phenomenon and pectinolytic activity. They were shown to produce low levels of H₂S, which was confirmed during sensory evaluation. Compared to the parental yeasts, these hybrids degraded more malic acid than VIN13. They also have the ability to ferment white and red must to dryness with acceptable VA production. The increased production of certain aroma compounds was demonstrated by these two hybrids and during sensory evaluation scored the highest for fruity characteristics.

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4. THE BREEDING AND CHARACTERISATION OF A RED WINE YEAST

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4.1 ABSTRACT

There are many targets for yeast strain improvement, such as low sulphur compound production, ethanol tolerance, fermentation vigour, osmotolerance, resistance to SO₂ and presence of killer factor. The proper selection of parental yeasts, the identification of hybrids and selection of positive oenological traits among the hybrids determine the success that will be achieved. In this study, 10 hybrids (out of 44) between two well established commercial wine yeast strains, VIN13 and WE14 were selected on the basis of low H₂S production. VIN13 is known for its fast and robust fermentation capacity and WE14 for its contribution to wine aroma. From these 10 hybrids, 5 hybrids (hybrid 11, 15, 24, 29 and 30) were selected as they showed promising results in terms of fermentation kinetics and phenotypical traits. The final 5 selected hybrids were all found to be killer active and to produce low levels of H₂S in comparison with the parental yeasts. The fermentation rate under differing must compositions aided in the selection of these hybrids. The fermentation rate is important, as it influences the aroma profile of the wine. Slower fermentations result in more aromatic wines, although these lengthier fermentations reduce productivity. Analysis of the principal chemical compounds of Cinsaut and Pinotage wines showed that the hybrids are well suited for fermentation in terms of fermenting must to dryness and producing minimal levels of volatile acidity. Differences in higher alcohol and volatile ester content of the wines also showed the potential of these hybrids. Sensory evaluation indicated that there were also positive differences in terms of tobacco character and astringency between wine made with the parental yeasts and the hybrids. Hybrid 12 (which was one of the 10 selected hybrids) showed the novel characteristic of increased malic acid degradation. It was capable of degrading much more malic acid than the parental yeasts. Cinsaut wine made with hybrid 12 had 50% less malic acid than wine made with VIN13. Hybrid 15 consistently produced levels of glycerol higher than the other hybrids.

Key words: yeast strain improvement, hybrid, breeding, target, fermentation rate, aroma

4.2 INTRODUCTION

In the previous article, the main targets were to develop yeast strains capable of a substantial degree of malate degradation whilst still contributing favourably to wine aroma. In this paper the breeding of 10 hybrid yeast strains and the subsequent selection of 5 of these hybrids is described. The main targets in this study were the development of novel wine yeasts, capable of producing wine with good aroma. These yeasts should also produce as little as possible amounts of compounds that detract from the quality of wine, such as volatile acidity and volatile sulphur compounds. A very successful, commercial wine yeast strain, VIN13 (Anchor yeast SA) was crossed with WE14 (Anchor yeast SA). Wine was made with the two parental strains (VIN13 and WE14) and 10 hybrid strains. Microvinification experiments were carried out on Cinsaut grapes fermented with the above mentioned strains. A second microvinification experiment was carried out with Pinotage grapes, after 5 hybrids were selected from the original 10 hybrids. These 5 hybrids showed promising results in terms of fermentation rate, overall fermentation capacity and aroma production.

4.3 MATERIALS AND METHODS

4.3.1 STRAINS AND CULTURE CONDITIONS

The strains that were used in this study are summarized in Table 1.

For plate assays and microvinification experiments, yeasts were precultured (stationary phase) in liquid YPD (1% yeast extract, 2% peptone and 2% glucose) at 30°C on a rotary shaker.

VIN13 and WE14 were sporulated on sporulation media by spreading cells onto solid media containing 1% potassium acetate, 0.1% yeast extract, 0.05% glucose and 2% agar (Sherman *et al.*, 1986). These plates were incubated at room temperature for 5 to 7 days.

Hydrogen sulphide (H₂S) production was monitored by using Biggy Agar (Nickerson, 1947). The composition of Biggy Agar is 0.1% yeast extract, 1% glycine, 1% glucose, 0.5% bismuth ammonium citrate, 0.3% sodium sulphite and 1.6% agar. Yeast were screened for the production of H₂S by spotting 5 µL of a liquid YPD culture onto the plates. The intensity of the brown colony colour is directly proportional to the amount of H₂S produced by the yeast.

Yeasts were screened for killer activity by spotting 5 µL of a liquid YPD culture onto a mat of killer sensitive *Saccharomyces cerevisiae* (S6) on methylene blue media plates. The mat was prepared by pipetting 150 µL of a YPD culture of *Saccharomyces cerevisiae* (S6) onto the solidified plates and allowing the mat to dry as described by Van Vuuren and Wingfield (1986). Yeast colonies with killer activity were identified by a clear zone around the colony and a blue ring on the periphery of the colony.

Table 1 Yeast strains used in this study

Yeast strain	Description	Source/Reference
VIN13	Commercial diploid strain	Anchor Yeast
WE14	Commercial diploid strain	Anchor Yeast
<i>S. cerevisiae</i> Zim 1859 S6	Killer sensitive	Zagorc <i>et al.</i> , 2001
<i>S. cerevisiae</i> WH300	Whisky yeast strain	La Grange-Nel, <i>et al.</i> , 2004
<i>S. cerevisiae</i> WH314	Whisky yeast strain	La Grange-Nel, <i>et al.</i> , 2004
Hybrid 11	Intraspecies hybrid	This study
Hybrid 12	Intraspecies hybrid	This study
Hybrid 14	Intraspecies hybrid	This study
Hybrid 15	Intraspecies hybrid	This study
Hybrid 22	Intraspecies hybrid	This study
Hybrid 23	Intraspecies hybrid	This study
Hybrid 24	Intraspecies hybrid	This study
Hybrid 26	Intraspecies hybrid	This study
Hybrid 29	Intraspecies hybrid	This study
Hybrid 30	Intraspecies hybrid	This study

4.3.2 ASCOSPORE DIGESTION AND HYBRIDISATION OF YEASTS

Ascospores were washed from the surface of the sporulation plates with sterile distilled water and put into an sterile tube (ELKAY). The mixture was centrifuged and the supernatant was removed. The yeast cells were then suspended in 150 μ L Zymolyase solution (0.5 mg/mL), ICN Immuno Biologicals. This suspension was incubated for 45 minutes at 30°C, after which the suspension was centrifuged. The supernatant was removed and the pellet was resuspended again in sterile distilled water. This suspension of haploid spores was centrifuged once more and yeast was isolated from the pellet and streaked onto YPD broth plates.

The tetrads were dissected on the YPD plates with the use of a Nikon micromanipulator and spore to spore cell mating was employed for hybridization. The yeasts were then incubated at 30°C on the YPD plates for 5 days.

4.3.3 CHEF ANALYSIS

Chromosomal DNA samples were prepared according to the embedded-agarose procedure of Carle and Olson (1985). Intact chromosomal DNA were separated using contour clamped homogeneous electric field (CHEF) electrophoresis. The apparatus used was the CHEF MAPPER (Bio-Rad Laboratories, Richmond, USA). All CHEF separations were carried out in a 20 cm square, 6 mm deep, 2% agarose gel made in 0.5 x TBE buffer. Thin sections of the DNA-agarose plugs were loaded into the wells and sealed in with 1% low melting point agarose just prior to the run. The average running temperature of the 0.5 x TBE electrophoresis buffer was maintained at 14°C by

a recirculating water bath. Gels were run for 42 h at a constant voltage of 6.0 V/cm and an angle of 120 degrees. The pulse duration was 60 s for the first 14 h and 90 s for the last 28 h. Gels were stained with ethidium bromide (10 mg/ml) and viewed on a transilluminator.

4.3.4 PCR ANALYSIS

All parental and hybrid yeast strains (as confirmed by CHEF) were subjected to PCR analysis to further confirm that the yeast were indeed hybrids. Genomic DNA was isolated from the yeast strains and used as template (Hoffman and Winston, 1987). DNA was amplified using DNA polymerase (Bioline). The reaction mixture included 500 ng of genomic DNA, 200 μ L of dNTPs, 1mM MgCl, 0.5 μ M of each primer and 5U DNA Taq polymerase. The following Sigma primers were used:

δ 1 (5'-CAAATTCACCTAT^A/_TTCTCA-3') and δ 2 (5'-GTGGATTTTATTCCAACA-3').

The above mentioned primers were used to amplify genomic DNA between the δ sequences (Ness *et al.*, 1993). Denaturing, annealing, extension and storage were carried out as described in Ness *et al.* (1993).

4.3.5 SMALL SCALE FERMENTATION TRIALS AND MICROVINIFICATION

The fermentation performance of the hybrids and parents were compared by inoculating the yeasts into Clairette Blanche juice. The fermentations were done at 25°C in triplicate by inoculating 100 mL juice with every yeast strain at a concentration of 3×10^6 cells/mL. The weight of the samples were taken every day and the accumulated weight loss was determined at the end of fermentation. The chemical composition of the samples were then determined. The fermentation capacity of the hybrids and parental strains under high balling and low nitrogen conditions were studied by inoculating the yeasts into adapted MS300 (Bely *et al.*, 1990). The high balling synthetic must was prepared by adding equimolar amounts of glucose and fructose to MS300 until a balling of 28° was reached. The low nitrogen synthetic must was prepared by reducing the concentration of all nitrogen containing salts and amino acids by 90%. The fermentations were done at 25°C in triplicate by inoculating 100 mL juice with every yeast strain at a concentration of 3×10^6 cells/mL. The weight of the samples were taken every day and the accumulated weight loss was determined at the end of fermentation. The chemical composition of the samples was then determined.

Wine was made with the parental yeasts and the 10 hybrids by using Cinsaut grapes. The grapes were crushed and destemmed and the mixture of juice and skins were divided equally into samples consisting of 8 L juice and 10 kg skins. To each of these samples, 40 ppm SO₂ was added. The fermentations were done in triplicate and each sample was inoculated with a yeast concentration of 3×10^6 cells/mL. The must was then fermented at 25°C. Samples for chemical composition were taken after 6 months of bottle aging.

Pinotage wine was also made with the parental yeasts and 5 selected hybrids. The grapes were crushed and destemmed and the mixture of juice and skins were divided

equally into samples consisting of 8 L juice and 10 kg skins. To each of these samples, 40 ppm SO₂ was added. The fermentations were done in duplicate and each sample was inoculated with a yeast concentration of 3×10^6 cells/mL. The must was then fermented at 25°C. Samples for chemical composition were taken after 6 months of bottle aging.

4.3.6 CHEMICAL COMPOSITION

Fourier transform infrared (FT-IR) spectroscopy was used for the determination of ethanol (% v/v), reducing sugar, titratable acidity, volatile acidity, malic acid, lactic acid and glycerol. The instrument used was a WineScan FT120TM spectrometer (Foss Analytical, Denmark) that generates spectra in the wavenumber region 5011-929 cm⁻¹. FT-IR spectroscopy is based on the measurement of the frequencies of the vibrations of chemical bonds in functional groups such as C=O, C-C, C-H, O-H and N-H upon absorption of radiation in the mid infrared region of the electromagnetic spectrum (Smith, 1999) The measured frequencies are processed through a series of mathematical procedures to form FT-IR spectra. To quantify a component of interest using FT-IR spectroscopy, a calibration equation based on the correlation between absorbance (presented by the FT-IR spectra) and the concentrations as measured with the reference method, is established.

4.3.7 GAS-LIQUID CHROMATOGRAPHY

To a sample of 10 mL, 800 µL internal standard (230 ppm 4-methyl-2-pentanol in 12% v/v ethanol) was added. Compounds of interest were extracted for 30 minutes on a rotary mixer with 6.5 mL diethyl ether. The organic phase was recovered and 2 mL transferred to a sample vial.

Analysis were done on an Agilent 6890 series gas chromatograph, equipped with an ALS 7683 liquid sampler, split-splitless injector and FID (flame ionization detector). The GC was fitted with a Lab AllianceTM RH-WAX, 60 mL x 0.32mm ID x 0.5 µL film thickness, capillary column. Hydrogen was used as carrier gas at a flow rate of 3 mL/min, average velocity of 45 cm/sec. 3 µL sample was injected at a split ratio of 15:1, a head pressure of 79 kPa and inlet temperature of 200°C. The column was held at 35°C for 15 min, raised to 230°C at 7°C/min and held at the final temperature for 5 min. Peak identification was done by comparison with authentic standard retention times. Integration and quantification of peaks were done by Chemstat Rev A.07.01 software using the internal standard calibration method.

For the determination of malic acid, the same parameters were used as above, except 2 µL sample was injected and not 3 µL sample. Further, Chemstat Rev A.10.02 software was used for the integration and quantification of peaks.

4.3.8 SENSORY EVALUATION

The sensory evaluation was based on a line scoring system. The tasting panel (consisting of 10 experienced tasters) had to indicate the intensity of a specific flavour by marking the intensity of the flavour. Samples of approximately 50 mL were presented in randomly numbered, clean and clear 125 mL tulip shaped glasses. Samples were evaluated at a room temperature of 23°C. Seven pinotage wines fermented with the 2 parental strains and the 5 selected hybrid strains were evaluated. An additional wine made with one of the parental yeasts (VIN13) was also repeated and included with the wines for every taster, to test the consistency of each of the tasters.

4.4 RESULTS

4.4.1 MOLECULAR IDENTIFICATION OF YEAST STRAINS

CHEF was used initially to confirm that hybrid 11, 12, 14, 15, 22, 23, 24, 26, 29 and 30 are indeed hybrids of VIN13 and WE14. The karyotype of the parental yeasts and 5 selected hybrids are illustrated in a CHEF gel (Figure 1). The differences between the chromosomal band patterns between the hybrids and the parents are indicated.

PCR analysis of the parental yeasts showed very different and distinctive band patterns for VIN13 and WE14 (Figure 2). In some cases, the band patterns of the hybrids however, seem similar to those of VIN13, although karyotyping confirmed that the 5 yeast strains are indeed hybrid strains of the parental yeasts.

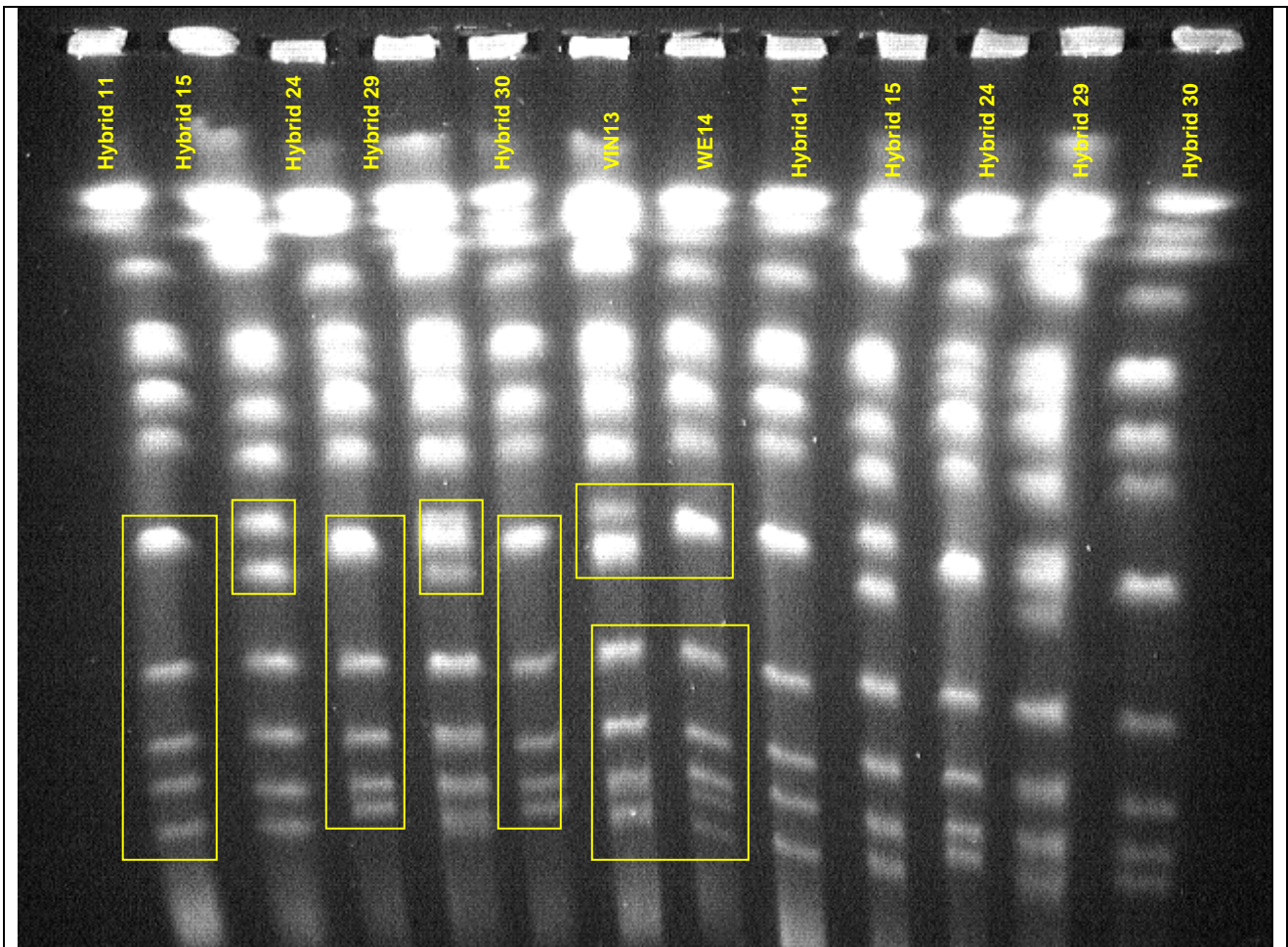


Figure 1 A CHEF gel illustrates the different karyotypes of the different yeast strains. VIN13 and WE14 are compared with hybrid 11, 15, 24, 29 and 30. Areas where the karyotypes differ are highlighted with yellow boxes.

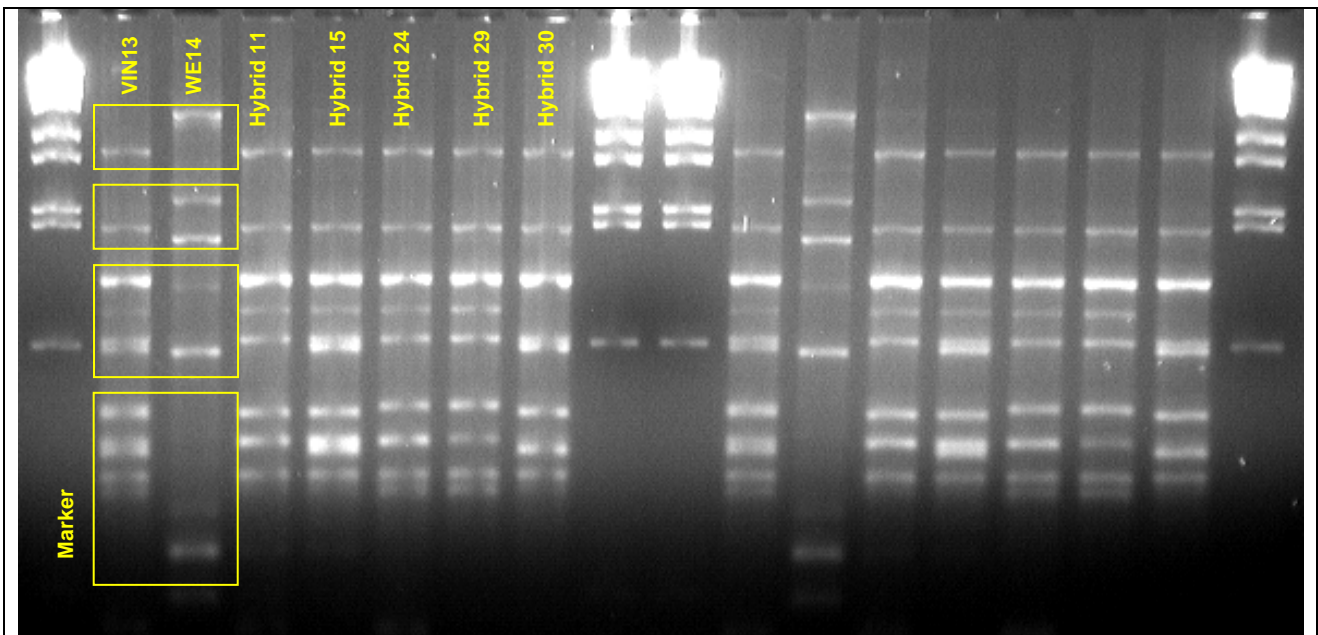


Figure 2 PCR analysis shows the difference in band patterns of VIN13 and WE14. The band patterns of hybrid 11, 15, 24, 29 and 30 are also illustrated.

4.4.2 PHENOTYPICAL CHARACTERISATION

The production of hydrogen sulphide (H_2S) by the hybrid yeast strains were compared with each other and to the the parental yeast and two whisky yeast strains. The first selection of favourable wine yeasts was done by eliminating yeasts that produced the highest amounts of H_2S . High producers of H_2S are identified by dark brown to almost black colonies and low H_2S producers are white to cream coloured. In Figure 1a and 1b the yeast colonies are illustrated on Biggy agar. The yeast strains, WH300 and WH314 were included, as they characteristically produce high levels of H_2S . From Figure 1a it can be seen that hybrid 14 produced the highest amount of H_2S . The colour of the representative colony is lighter than VIN13 and darker than WE14. From Figure 1b it can be seen that all the hybrids produced low levels of H_2S , compared to the parental yeasts and the control yeasts, WH300 and WH314. Hybrid 26 produced slightly more H_2S than the other hybrids. It is also interesting to note that VIN13 produces more H_2S on Biggy agar than WE14. None of the yeast strains produced high levels of H_2S as did WH314 and to a lesser extent, WH300.

Figure 2a and b indicates that all the hybrids are killer positive, as are VIN13 and WE14. The blue zone indicates dead yeast cells and the clear zone (see VIN13 and hybrid 15 in Figure 2a) indicates inhibition of yeast growth. From the Figures it can be seen that the strongest killer activity is displayed by VIN13 and hybrid 15.

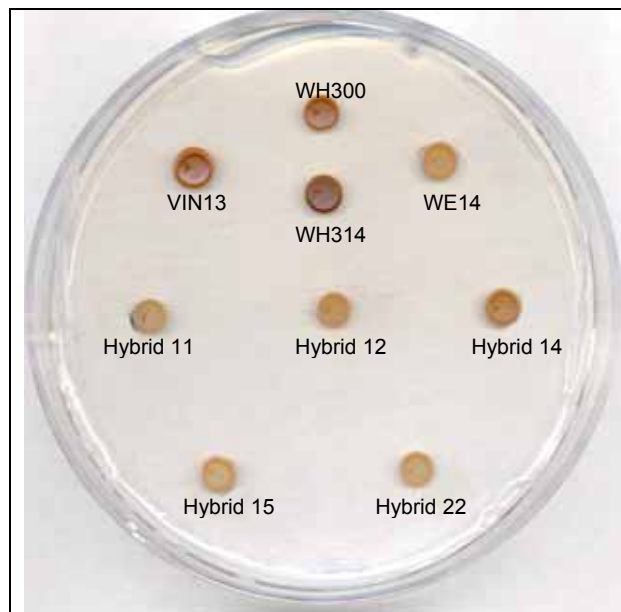


Figure 3a The production of H_2S by different yeast strains on Biggy agar. The control yeasts, VIN13 and WE14 are compared with hybrid 11, 12, 14, 15 and 22. Darker colonies are associated with higher H_2S production.

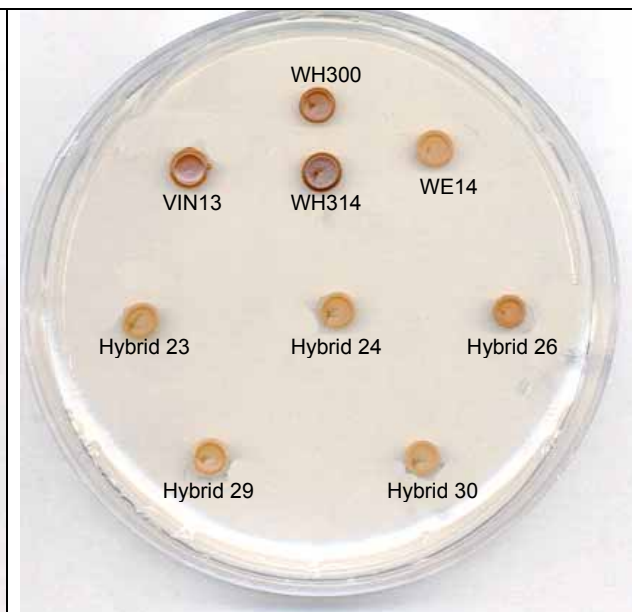


Figure 3b The production of H_2S by different yeast strains on Biggy agar. The control yeasts, VIN13 and WE14 are compared with hybrid 23, 24, 26, 29 and 30. Darker colonies are associated with higher H_2S production.

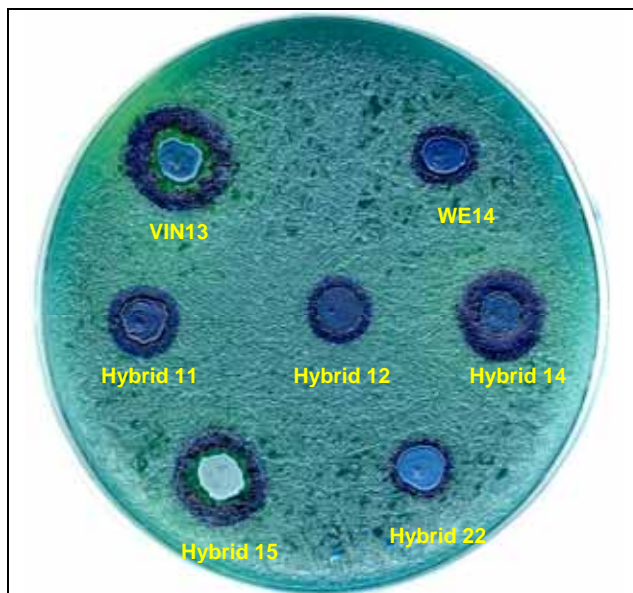


Figure 4a The effect of the presence of the killer phenomena is illustrated on methylene blue media, with a mat of killer sensitive yeast (*S. cerevisiae* Zim 1859 S6). VIN13 and WE14 are examples of yeasts that are killer positive, as can be seen by the clear zone of inhibition and the blue zone of dead cells. The two parental strains serve as controls and are compared with hybrid 11, 12, 14, 15 and 22 to monitor killer activity.

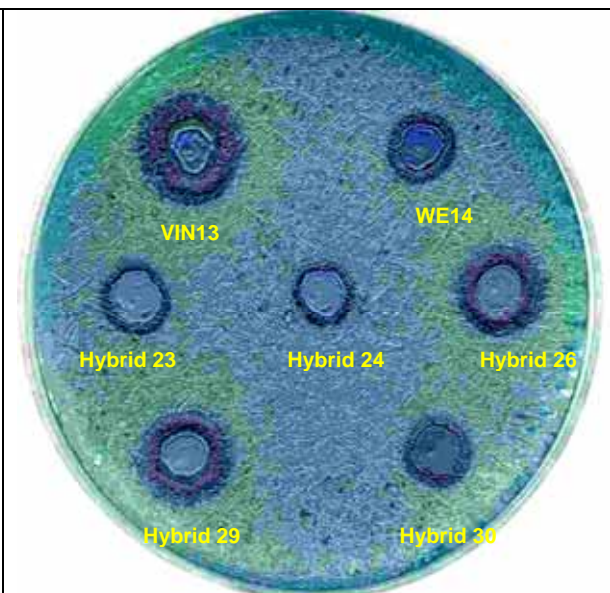


Figure 4b The presence of the killer phenomena is monitored in hybrid 23, 24, 26, 29 and 30.

4.4.3 SMALL SCALE FERMENTATION TRIALS AND CHEMICAL ANALYSIS

The fermentation performance of the parental yeasts and 10 hybrids were evaluated for a period of 18 days in Clairette blanche must (Figure 5a and b).

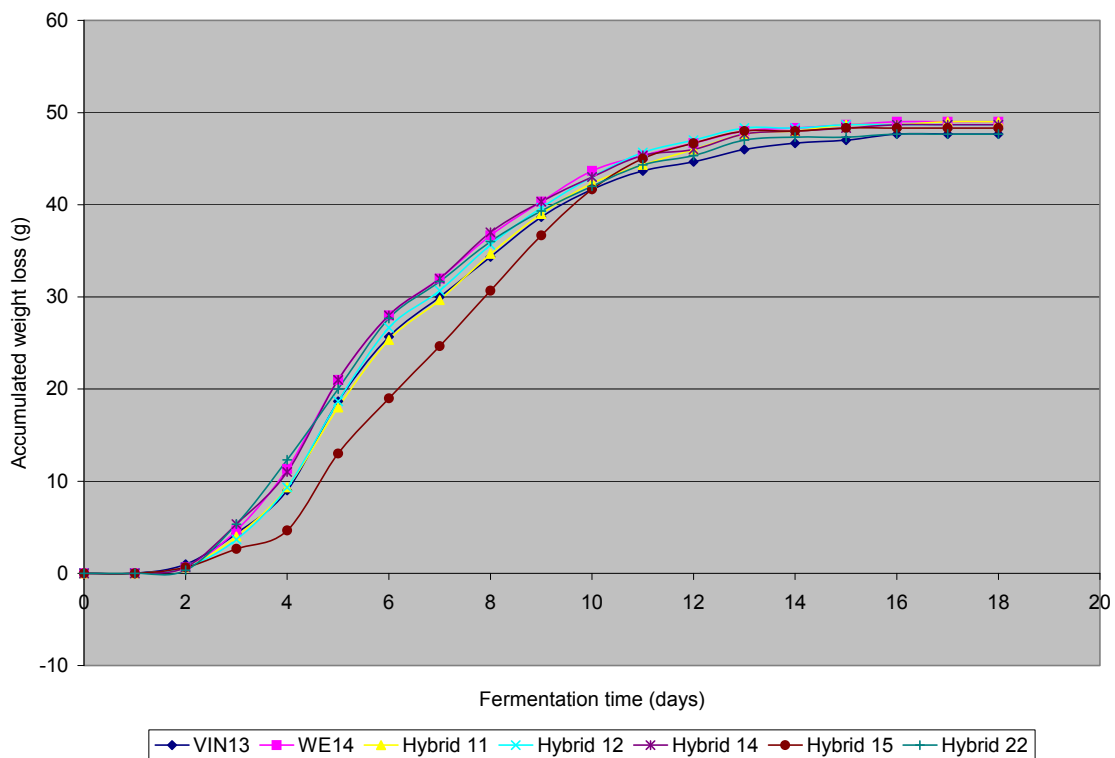


Figure 5a VIN13, WE14, hybrid 11, 12, 14, 15 and 22

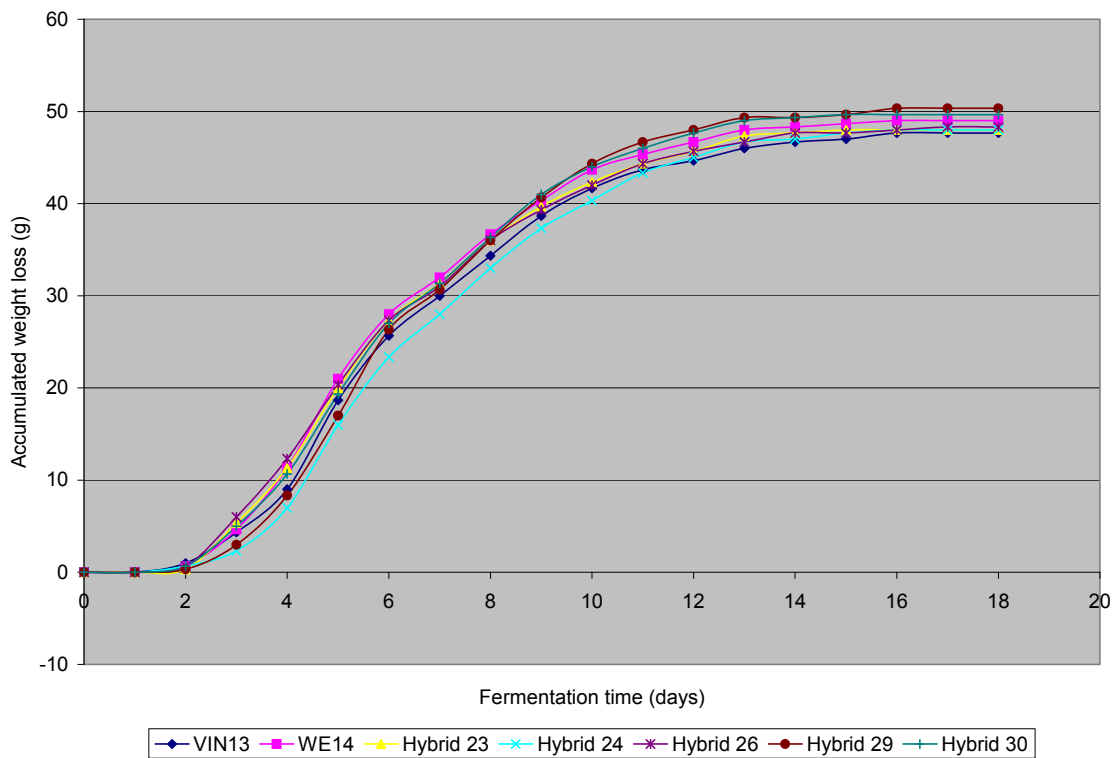
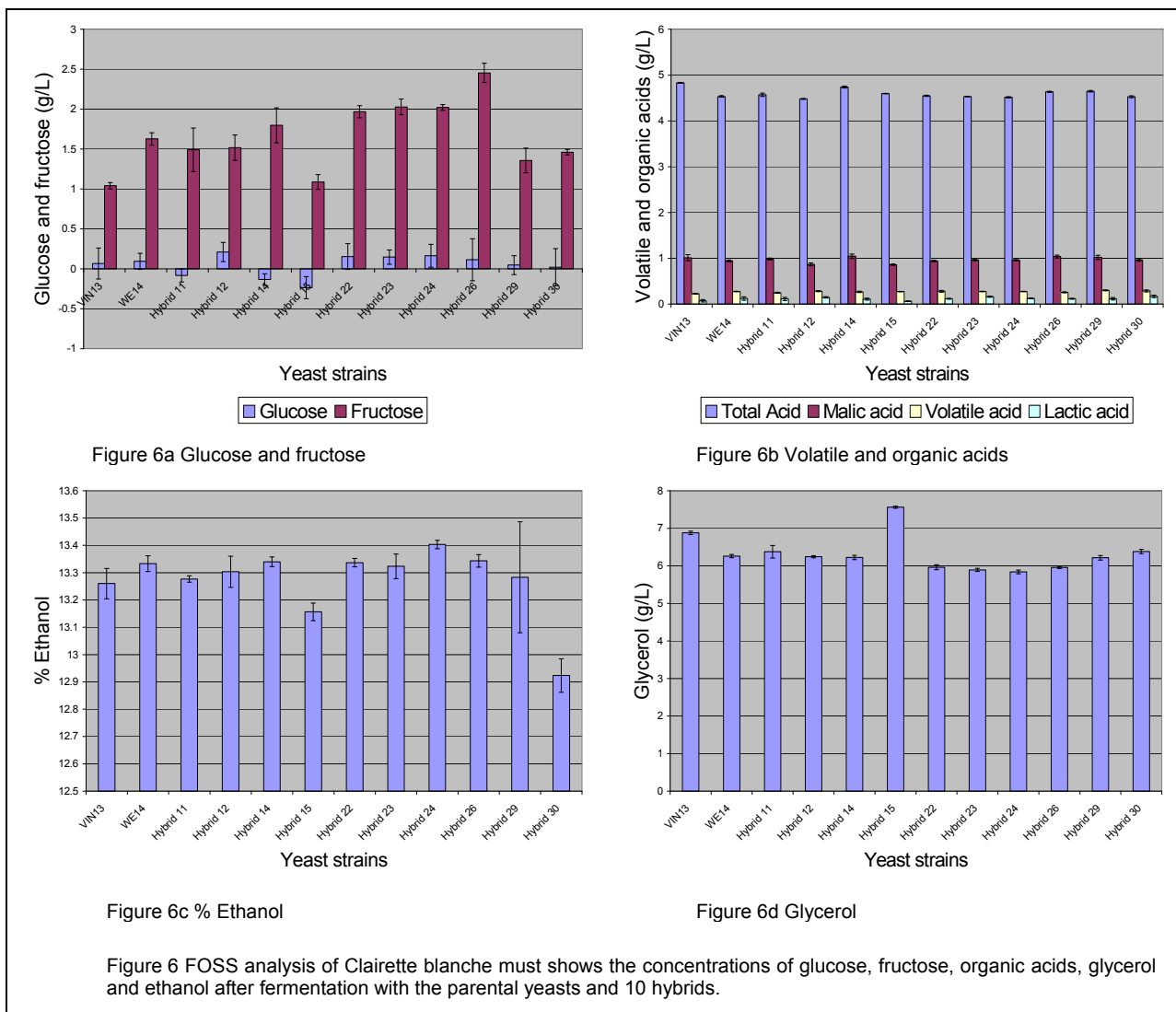


Figure 5b VIN13, WE14, hybrid 23, 24, 26, 29 and 30

Figure 5 The accumulated mass loss of Clairette Blanche must inoculated with VIN13, WE14, and 10 hybrids over an 18 day period.

From the fermentation curves in Figure 5a and b it can be seen that VIN13 and WE14 did not differ significantly. Fermentation with WE14 resulted in slightly more mass loss. The biggest differences were seen with hybrid 15, 24 and 29. These hybrids all fermented slower than the parental yeasts at some stage or most of the time during fermentation. Fermentation with the other hybrids resulted in mass loss curves that were mostly very similar to the parental yeasts.

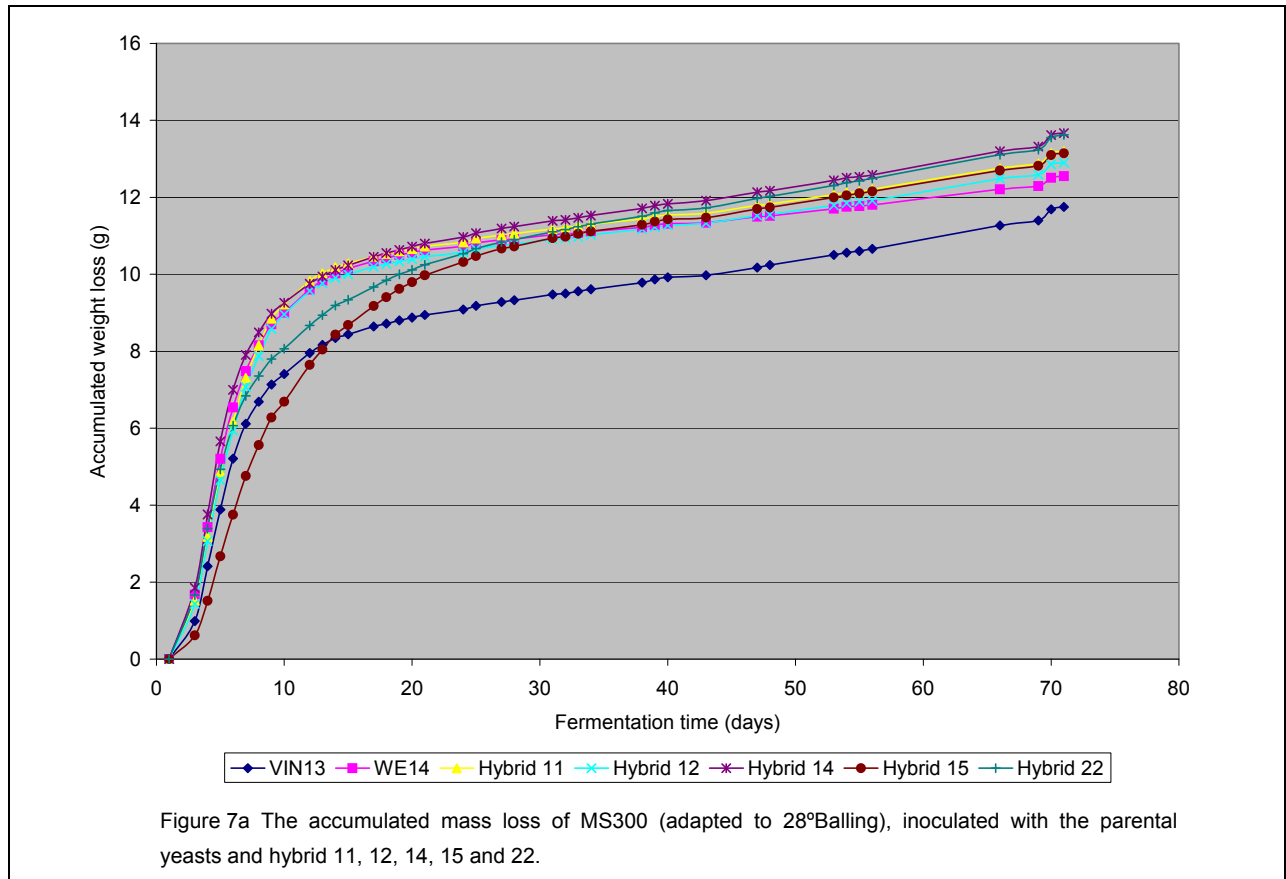
Figure 6a to d shows the glucose, fructose, organic acids, ethanol and glycerol content of the Clairette Blanche wine made with the different yeasts strains. From Figure 6a it can be seen that all the yeasts fermented the must to dryness. There was less than 0.25 g/L glucose left in all of the fermentations and less than 2.5 g/L fructose for all of the fermentations. Fermentation with VIN13 and hybrid 15 resulted in the lowest residual fructose levels. Hybrid 15 produced the highest amount of glycerol and the second highest amount of glycerol was produced by VIN13 (Figure 6d).



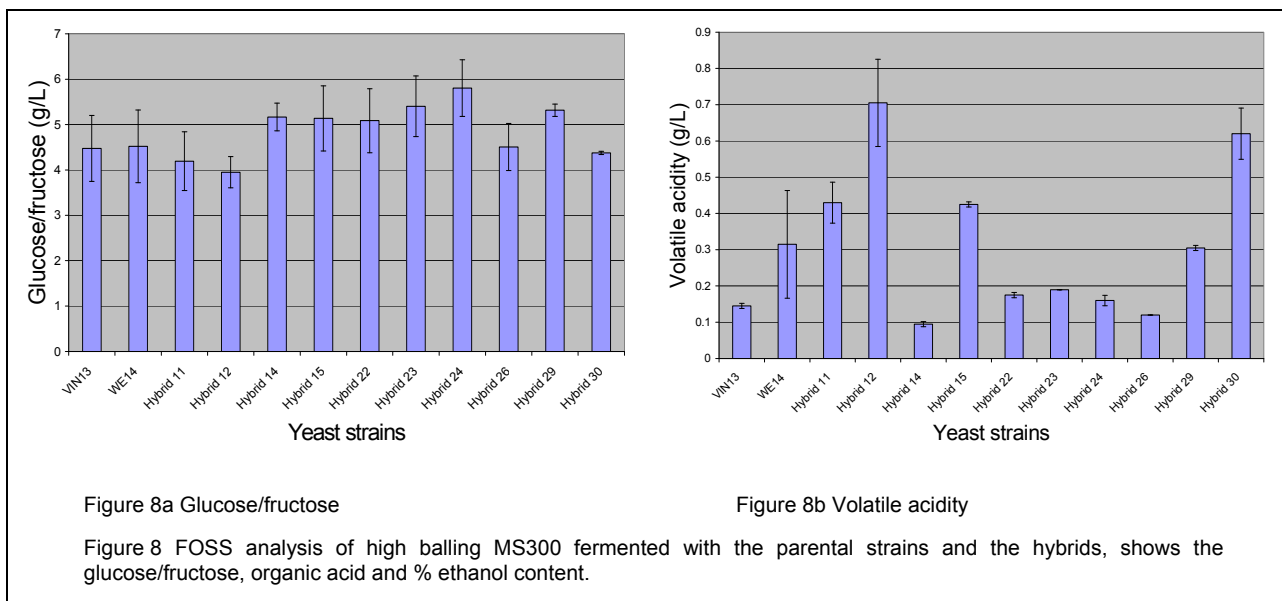
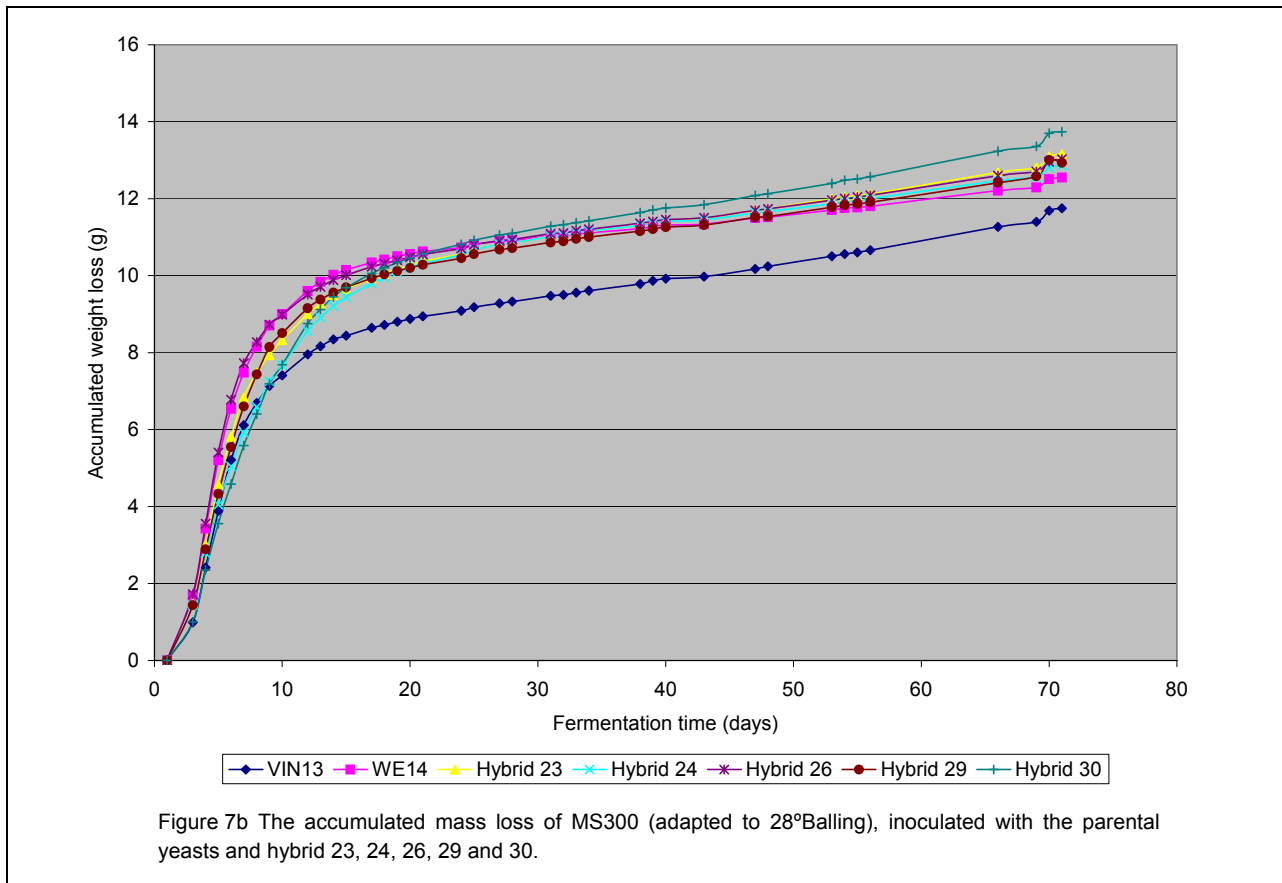
4.4.4 HIGH OSMOTIC AND LOW NITROGEN STRESS SIMULATION EXPERIMENT

4.4.4.1 FERMENTATION IN HIGH BALLING SYNTHETIC MUST

The effect of a high balling synthetic must (MS300) on the fermentation profile and chemical composition of the parental yeasts and the hybrids is illustrated in Figure 7 and 8. The must had a Balling of 28° before fermentation started.



The fermentations with WE14 was faster than with VIN13. Most of the hybrids follow a fermentation profile similar to that of WE14. The biggest difference was seen in fermentations with hybrid 15, that initially fermented slower than the parental yeasts, but fermented faster than both the parental yeast near the end of fermentation.



No significant differences could be seen from the glucose/fructose (glucose + fructose) content in Figure 8a, after fermentation of the high balling must. From Figure 8b it can be seen that hybrid 12 and 30 produced the highest amounts of volatile acidity. Hybrid 14, 22, 23, 24 and 26 produced the highest levels of volatile acidity.

4.4.4.2 FERMENTATION IN LOW NITROGEN SYNTHETIC MUST

The effect of low nitrogen synthetic must on the fermentation profile and chemical composition of the parental yeasts and the hybrids is illustrated in Figure 9 and 10.

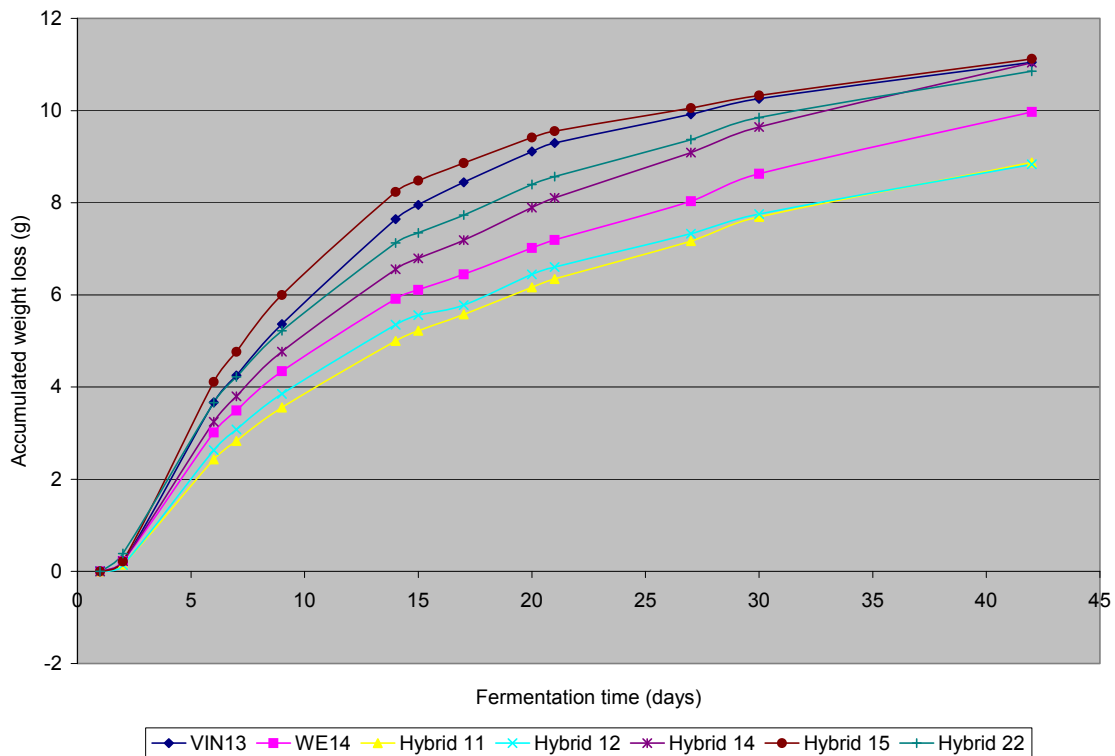


Figure 9a VIN13, WE14, hybrid 11, 12, 14, 15 and 22

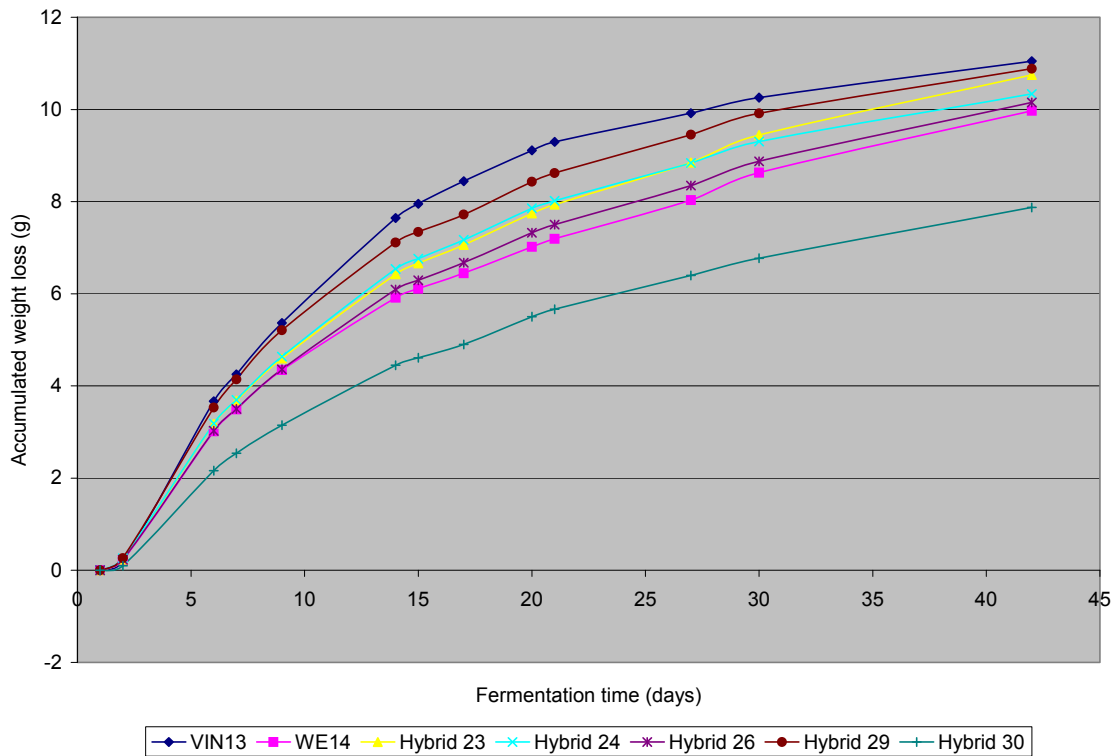
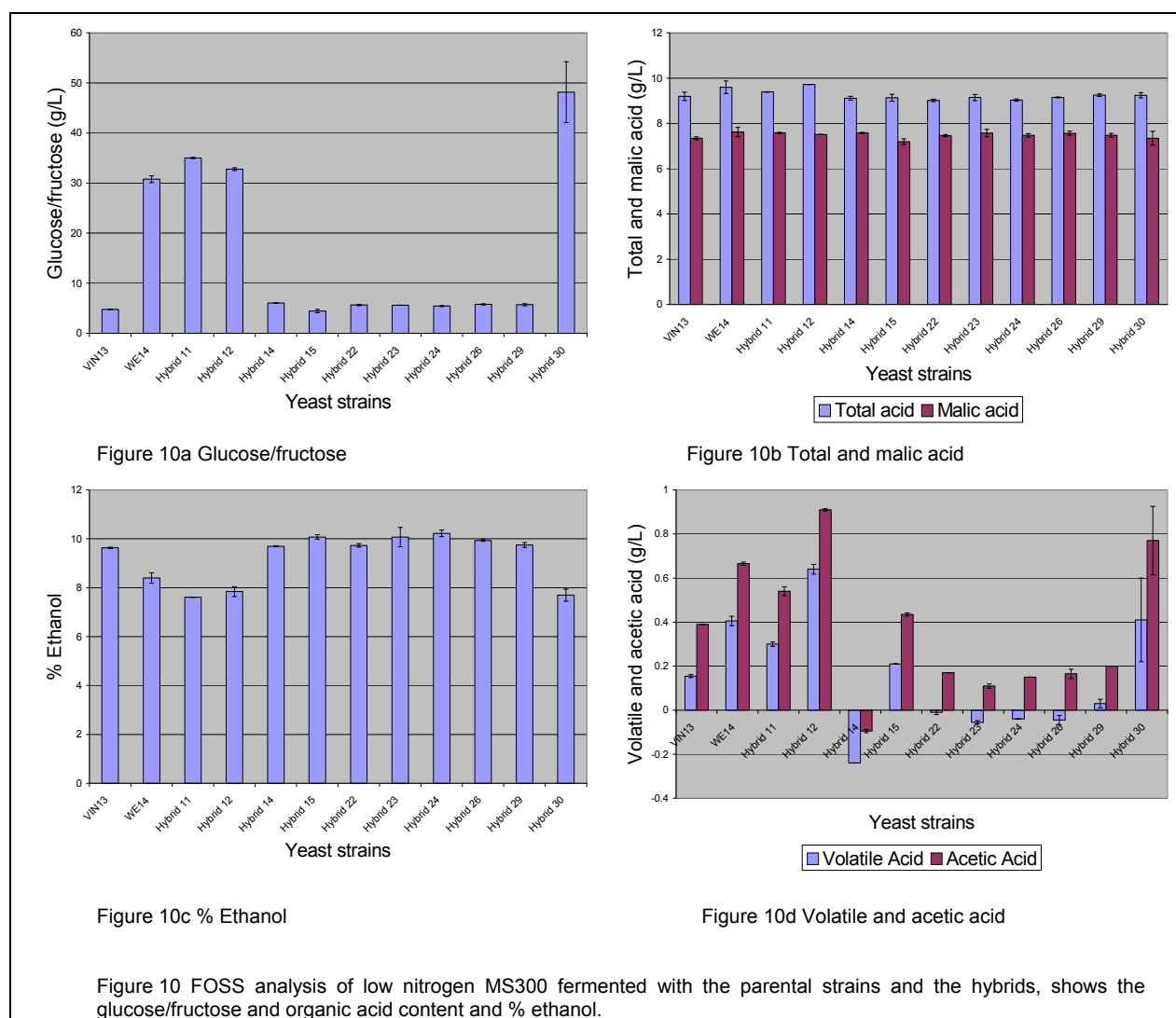


Figure 9b VIN13, WE14, hybrid 23, 24, 26, 29 and 30

Figure 9 The accumulated mass loss of MS300 (low nitrogen adaptation), inoculated with the parental yeasts and the hybrids.

From the onset of fermentation, VIN13 fermented faster than WE14 (Figure 9). Most of the hybrids displayed fermentation rates slower than or in between that of the parental yeasts. Hybrid 15 fermented faster than both the parental yeasts and fermentation with hybrid 15 also resulted in the lowest residual amount of glucose/fructose (glucose + fructose), as seen in Figure 10a. WE14, hybrid 11, 12 and 30 fermented the slowest and also resulted in the highest residual amount of glucose + fructose (Figure 10a). There were no significant differences between the other yeast strains in terms of total and malic acid content (Figure 10b). WE14, hybrid 11, 12 and 30 produced the lowest amounts of ethanol (Figure 10c) and the highest amount of volatile and acetic acid (Figure 10d).



4.4.5 MICROVINIFICATION

Cinsaut wine was made with the parental yeasts and the 10 hybrids. Five of these hybrids were selected (see discussion) and Pinotage wine was made with the 5 selected hybrids and parental yeasts. Figure 11 and 13 shows the malic acid, volatile acidity and glycerol content determined by a FTIR Spectrometry apparatus, of the

Cinsaut and Pinotage wine respectively. Figure 12 shows the levels of decanoic acid, diethyl succinate and hexyl acetate of the cinsaut wine as determined by GC. Figure 14, 15 and 16 shows the the volatile ester, volatile fatty acid and higher alcohol content of the Pinotage wine as determined by GC.

All the yeasts fermented the Cinsaut must to dryness and no significant differences in sugar content was seen between the hybrids. No significant differences were seen in the total acidity of the different wines, but there was a big difference in the malic acid content (Figure 11a) of the wine made with hybrid 12. Wine made with hybrid 12 had almost 50% less malic acid than wine made with VIN13. Fermentation with VIN13 also resulted in the highest amount of malic acid in the finished wine. From Figure 11c it can be seen that VIN13 and hybrid 15 produced the highest amounts of glycerol.

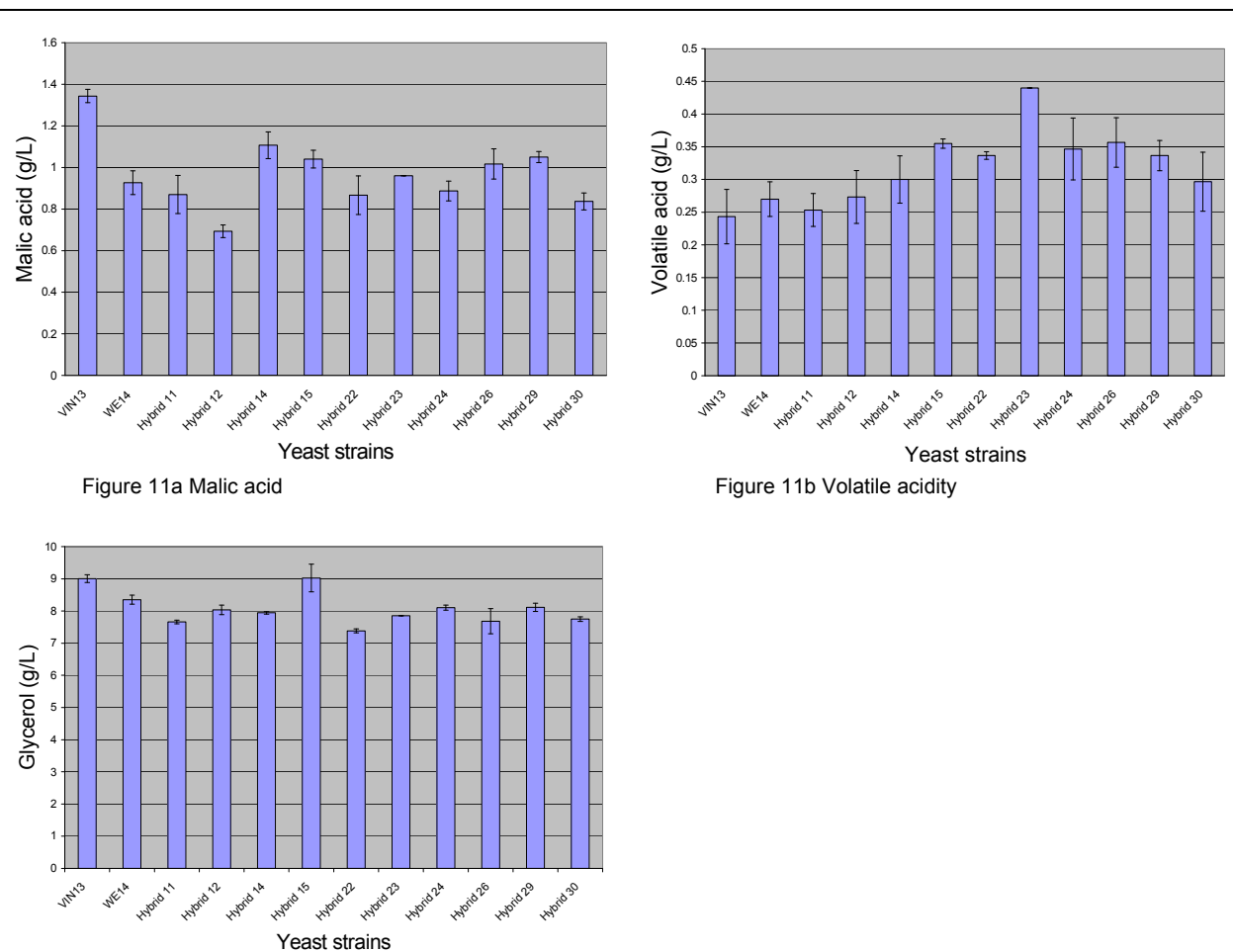
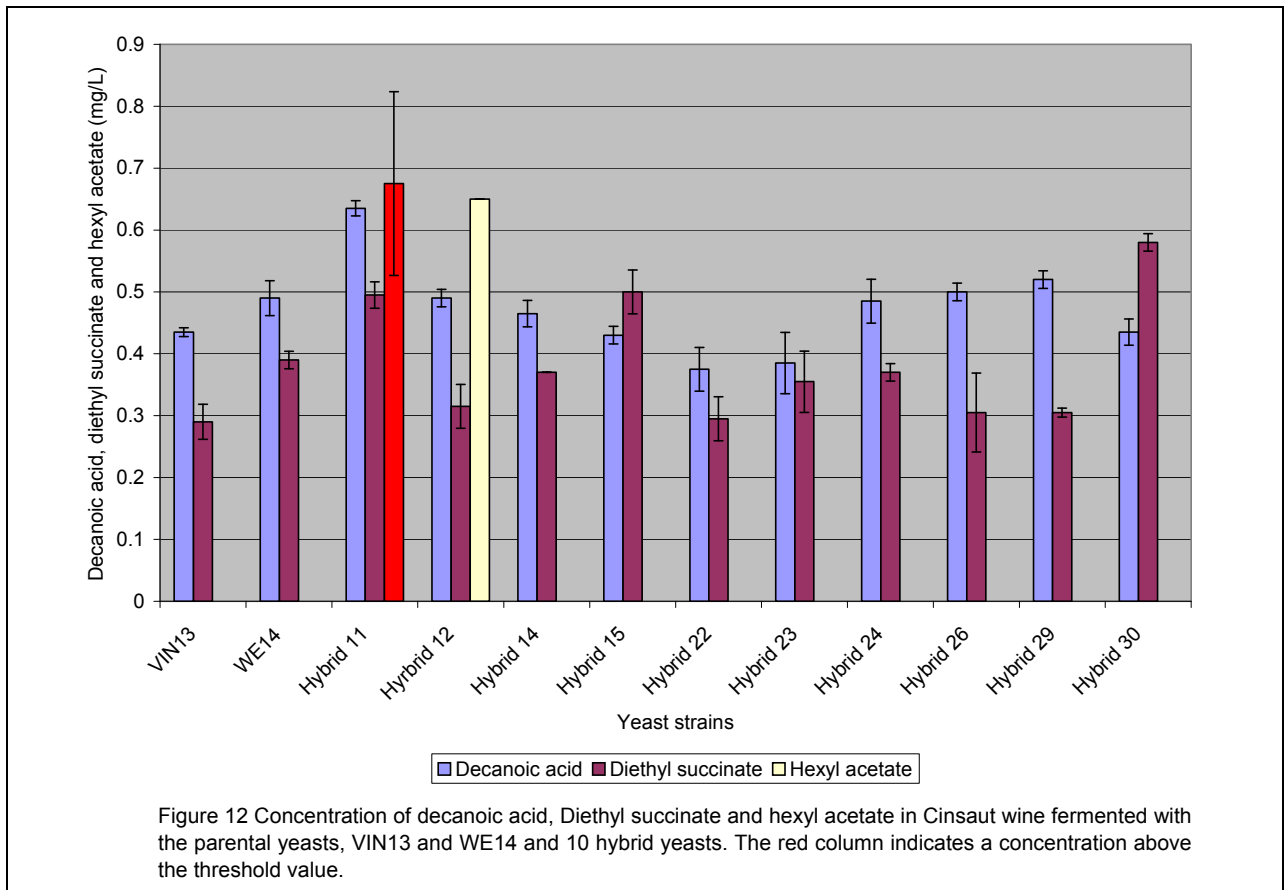
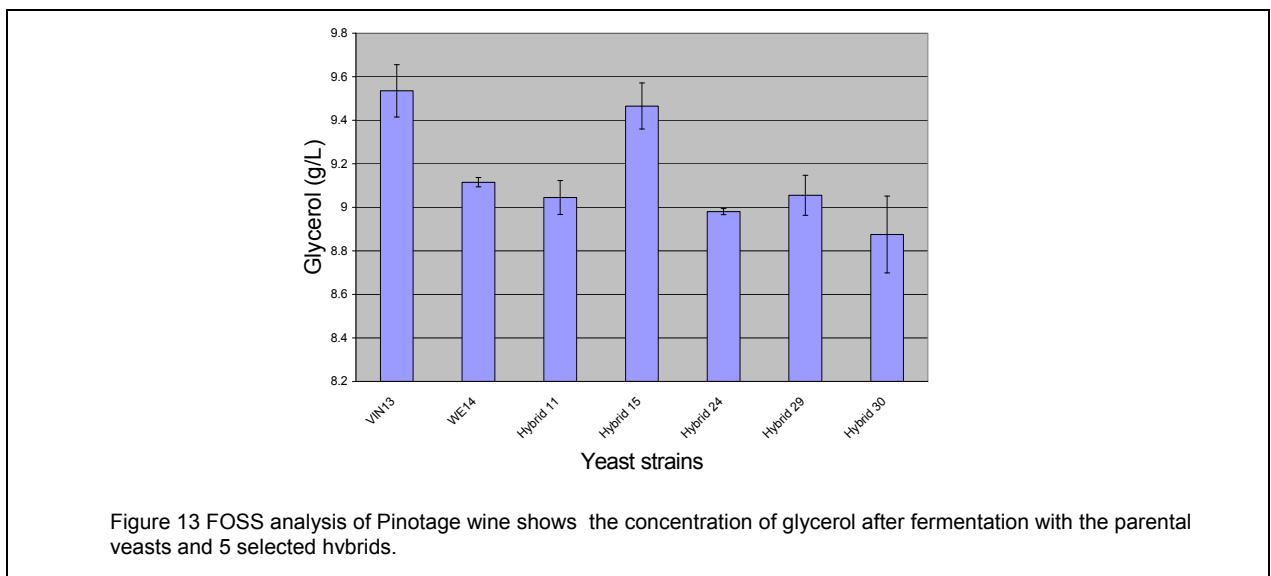


Figure 11c Glycerol

Figure 11 FOSS analysis of Cinsaut wine shows the concentrations of glucose, fructose, organic acids, glycerol and ethanol after fermentation with the parental yeasts and 10 hybrids.



The data presented in Figure 12 indicate that hybrid 11 produced the highest amount of decanoic acid and possibly also hexyl acetate in the Cinsaut wine. Only hybrid 11 and 12 produced hexyl acetate. Hybrid 11, 15 and 30 produced the highest amounts of diethyl succinate.



The Pinotage must was fermented to dryness and no significant differences were seen in the sugar content in the wine. No significant differences were seen in the total

acidity, malic acid or volatile acidity content of the Pinotage wines. From Figure 13 it can be seen that VIN13 and hybrid 15 produced the highest amounts of glycerol.

Hybrid 11 and 15 produced more 2-phenylethyl acetate than any of the other yeasts (Figure 14a) and hybrid 11 produced the highest amount of hexyl acetate (Figure 14b). The amount of hexyl acetate produced by hybrid 11 was in excess of the perception threshold value. Ethyl caproate and isoamyl acetate was also produced in excess of the perception threshold value.

The data in Figure 15a indicate that hybrid 15 produced the highest amount of 2-phenylethyl alcohol and also produced this compound in excess of its perception threshold value. Hybrid 24 and 29 produced more isoamyl alcohol than both the parental yeasts and all the yeasts produced this compound in excess of the perception threshold value.

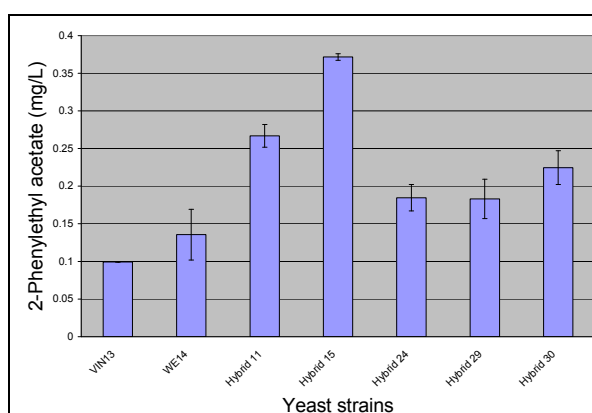


Figure 14a 2-Phenylethyl acetate

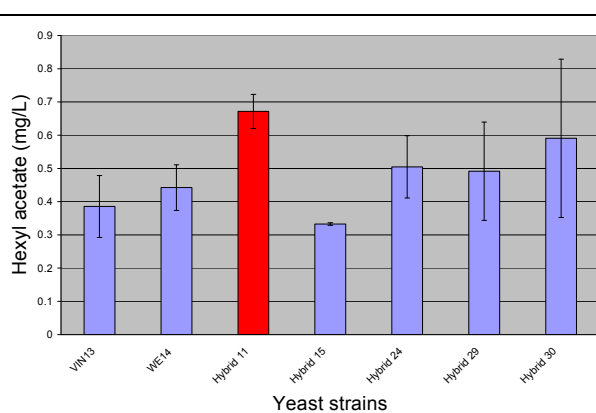


Figure 14b Hexyl acetate

Figure 14 Concentration of volatile esters in Pinotage wine fermented with the parental yeasts, VIN13 and WE14 and 5 hybrid yeast strains. Red columns indicate concentrations above the threshold value.

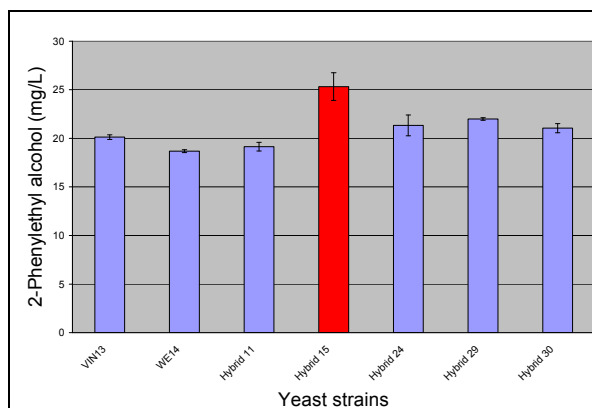


Figure 15a 2-Phenylethyl alcohol

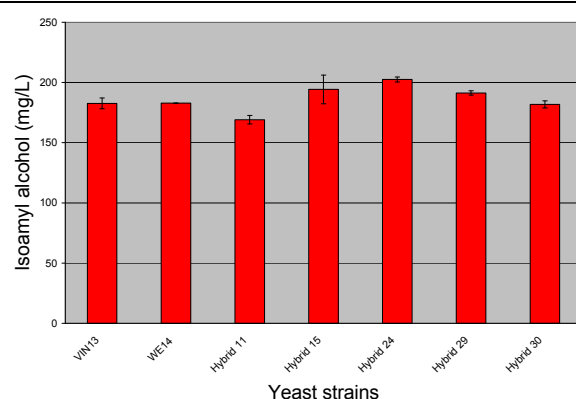
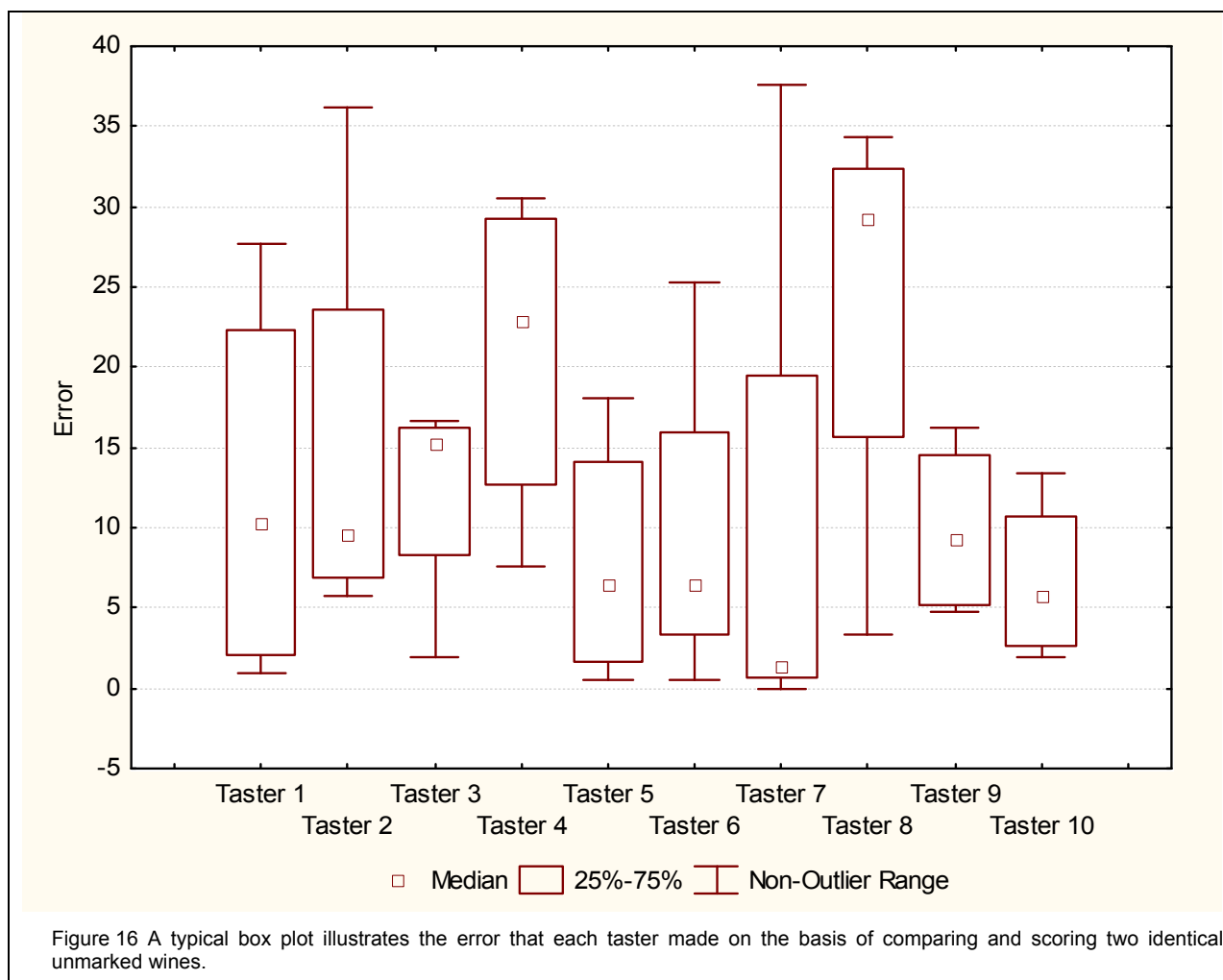


Figure 15b Isoamyl alcohol

Figure 15 Concentration of higher alcohols in Pinotage wine fermented with the parental yeasts, VIN13 and WE14 and 5 hybrid yeast strains. Red columns indicate concentrations above the threshold value

4.4.6 SENSORY EVALUATION

The Pinotage wine made with VIN13, WE14 and the 5 hybrid yeasts were evaluated by a tasting panel consisting of 10 tasters. The consistency of the tasters were tested by doing a box plot of all the scores that each taster gave for the repeated wine (Figure 16).



From the results in Figure 16, it was statistically determined that taster 1, 3, 5 and 9 scored the repeated wines the most consistently and with the smallest margin or error. The other 6 tasters were not included in further results due to their large margins of error and large outer ranges of error in scoring the 2 identical wines. The bootstrap means for the perception of banana, cherry, tobacco and astringency are illustrated in Figure 17.

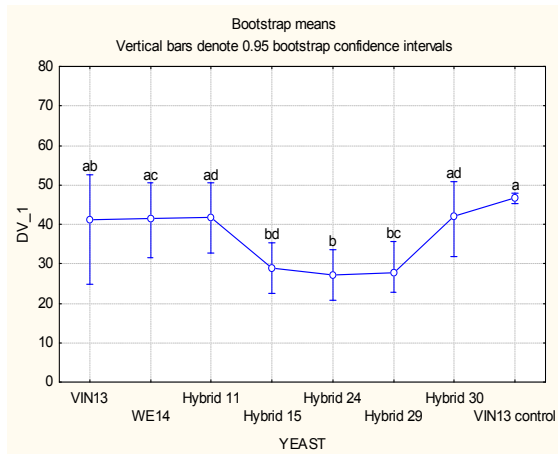


Figure 17a Banana character

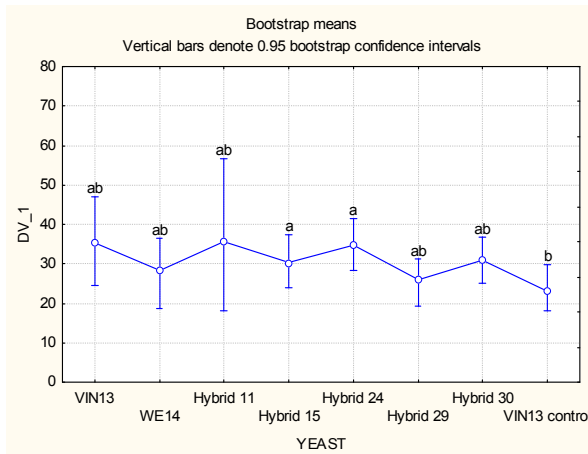


Figure 17b Cherry character

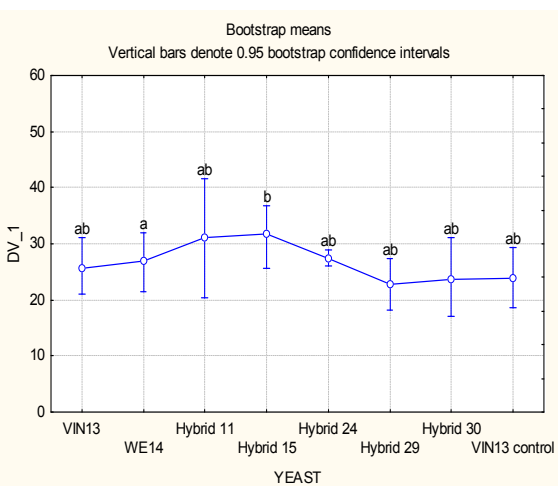


Figure 17c Tobacco character

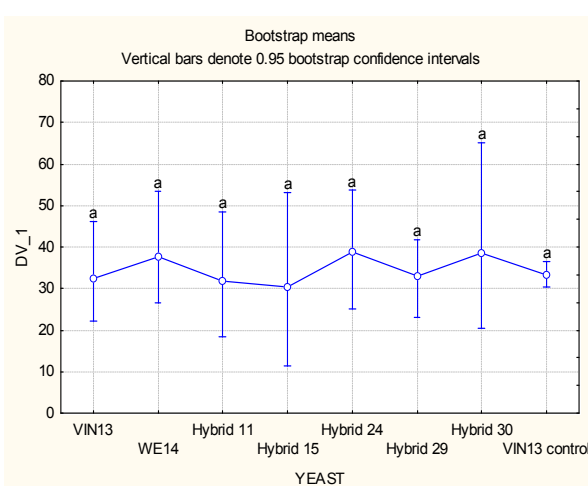
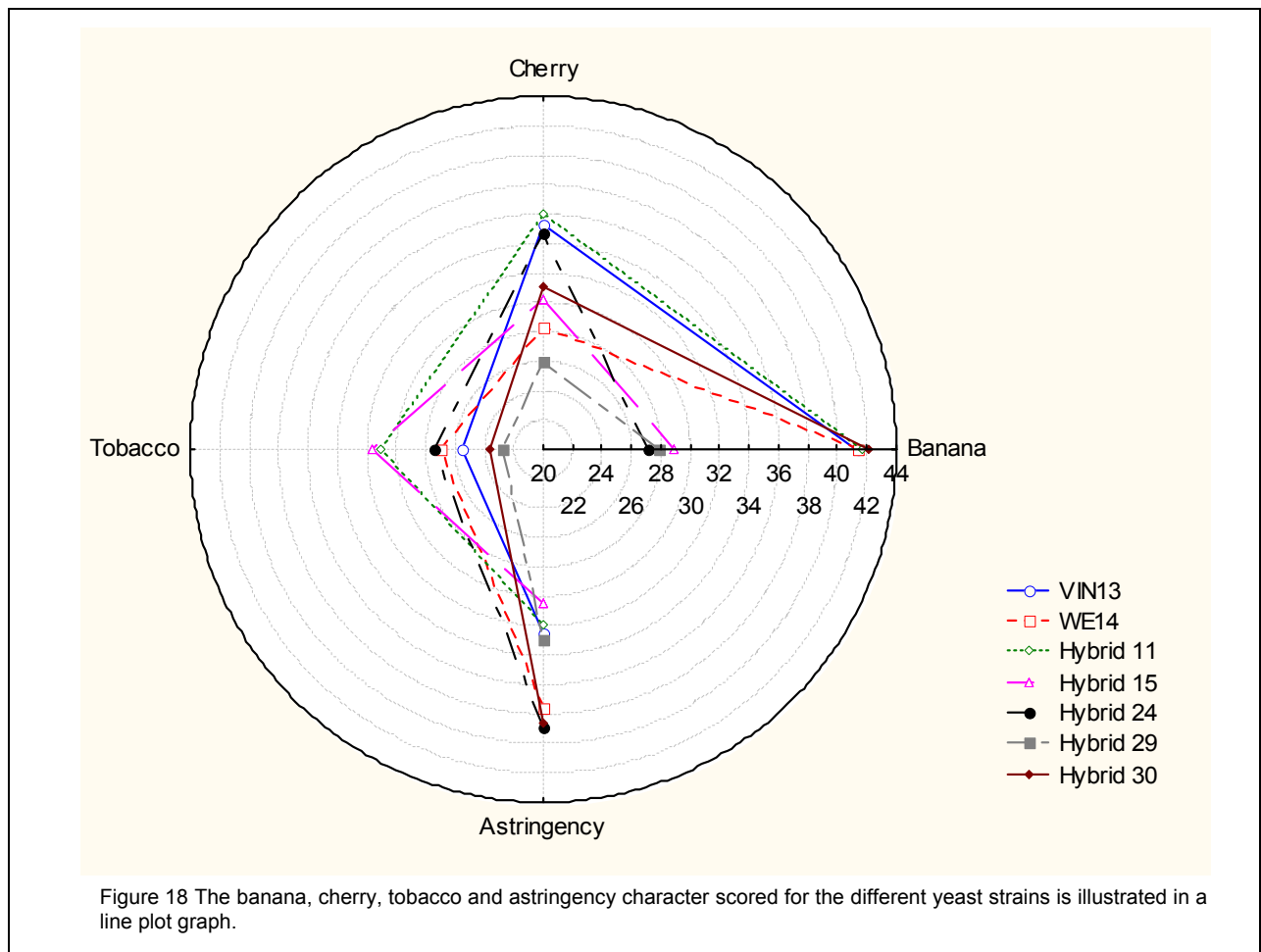


Figure 17d Strawberry

Figure 17 The bootstrap means for the parental yeasts and the 5 hybrids are indicated for banana, cherry, tobacco and astringency. The letters a, b, c and d indicates the degree of overlapping between the bootstrap means for the different yeasts.

The trend for banana character (Figure 18a) of VIN13 and WE14 was higher than all the hybrids, except for hybrid 11 and 30 which scored similar to the parental yeasts. The letters 'ad' indicate the similarity between the confidence intervals of hybrid 11 and 30. The trend for cherry character (Figure 17b) for the hybrids was similar to either VIN13 or WE14, except for hybrid 29 which scored lower than WE14. VIN13 and hybrid 11 had the highest scores. The trend for tobacco character (Figure 17c) was the highest for hybrid 11 and 15. Both these hybrids scored higher than the parental yeasts. Hybrid 24 scored similarly to WE14 and hybrid 29 and 30 got the lowest scores. The trend for astringency (Figure 17d) was very similar for all the yeasts. Figure 18 illustrates a line plot of the banana, cherry, tobacco and astringency character scored for the different yeast strains.



From Figure 18 it can be seen that the highest score for banana character was with hybrid 11 and 30. VIN13 and WE14 scored slightly lower than these two hybrids. Hybrid 15, 24 and 29 scored much lower. Hybrid 11 scored slightly higher than VIN13 for cherry character and hybrid 24 scored slightly lower than VIN13. Hybrid 15 and 30 both scored higher than WE14 for cherry character and hybrid 29 scored the lowest for cherry character. Hybrid 11 and 15 scored significantly higher than all the other yeasts for tobacco character and hybrid 24 also scored slightly higher than both the parental strains. Only hybrid 29 and 30 scored lower than the parental strains. Hybrid 24 and 30 scored the highest and slightly higher than WE14 for astringency. Hybrid 11, 29 and VIN13 scored similarly and hybrid 15 scored the lowest for astringency.

4.5 DISCUSSION

In this study, haploid spores of VIN13 and WE14 were crossed and the potential for 5 selected hybrids as possible commercial starter cultures were assessed. Phenotypical characterisation, small scale fermentation trials and microvinifications all provided valuable insights into the oenological qualities of the hybrids. The effect of hybridisation on aroma profile was also studied by comparing the volatile ester and higher alcohol content of wine made with the parent and hybrid yeasts. The CHEF technique was successfully used to karyotype and identify the different yeast strains (Degré *et al.*, 1989; Vezinhet, 1990; Petersen *et al.*, 1999). Egli *et al.* (1998) reported on the inability of the previously described PCR technique to differentiate between certain closely related yeasts strains. In this study, the hybrids could not be successfully identified by using a PCR method, as some hybrids had similar band patterns to VIN13, although karyotyping confirmed that they were in fact hybrids.

Phenotypical analysis of the hybrids showed that all the hybrids produced relatively low amounts of H₂S. Volatile sulphur compounds in wine usually contribute to unpleasant aromas and the low H₂S production by the hybrids might reflect on this positive characteristic during winemaking conditions (Mestres *et al.*, 2002). Another positive oenological trait is displayed by all the hybrids, namely killer activity. Zagorc *et al.* (2001) stated the enological interest in killer yeast due to their ability to dominate a fermentation. Enological studies indicate that killer activity would allow a yeast species to compete more successfully in a specific habitat by eliminating other yeasts strains. The biggest differences in fermentation dynamics during fermentation of Clairette blanche must was seen with hybrid 15, 24 and 29. The mass loss curve of hybrid 15 differs the most from that of the parents. This is significant as any change or reduction in fermentation rate may lead to more aromatic wines, due to a decrease in the liberation of volatile compounds from the must (Torija *et al.*, 2003).

Wine analysis (using the FOSS apparatus) after a small scale fermentation trial showed that all the yeasts fermented the must to dryness and fermentation with VIN13 and hybrid 15 resulted in the lowest residual fructose levels. Hybrid 15 produced the highest amount of glycerol. The increased production of glycerol by hybrid 15 might explain its lower ethanol production (Remize *et al.*, 1999). Although it has no direct impact on aromatic characteristics, an increase in glycerol production is seen as positive. Sweetness is the main contribution of glycerol to sensory characteristics at levels commonly found in wines. *S. cerevisiae* yeast strains producing large amounts of glycerol would therefore be of considerable value in improving wine quality. Moreover, the overproduction of glycerol at the expense of ethanol might represent an advantageous alternative for the development of beverages with lower ethanol contents versus physical processes which alter the organoleptic properties of the final product. The adaptability and resistance to stress conditions of the parental yeasts and hybrids were evaluated by inoculating them into high balling and low nitrogen synthetic must. During fermentation of high balling must, hybrid 15 differed the most in terms of mass loss, as compared to the parental yeasts. Hybrid 12 (not included in the final selection of

5 hybrids) and 30 produced the highest levels of volatile acidity. This might indicate the unsuitability of these hybrids for high balling fermentations.

Hybrid 15 showed the highest mass loss during fermentation of low nitrogen must. This might indicate that hybrid 15 is more suitable for fermentation under low nitrogen must conditions. Blateyron *et al.* (2001) identified nitrogen and oxygen deficiencies as major causes of stuck and sluggish fermentations. A yeast that is better adapted to ferment under low nitrogen conditions offer obvious advantages to the wine industry. The suitability of hybrid 15, 24, and 29 for fermentation of low nitrogen must is shown by the low (compared to the other yeast strains) glucose/fructose ratios and low levels of volatile and acetic acid after fermentation with these hybrids.

All the hybrids and parental yeasts fermented Cinsaut must to dryness. Wine made with hybrid 12 had almost 50% less malic acid than wine made with VIN13, which had the highest concentration of malic acid. VIN13 and hybrid 15 produced the highest concentrations of glycerol.

The potential of the hybrids to produce higher concentrations of volatile aroma compounds than the parental yeasts became evident when it was seen that hybrid 11 produced the highest amount of decanoic acid and possibly also hexyl acetate in Cinsaut wine (only hybrid 11 and 12 produced hexyl acetate). Hybrid 11, 15 and 30 produced the highest amounts of diethyl succinate. Decanoic acid contributes citrus notes to wine and hexyl acetate, flowery and fruity notes (Lambrechts and Pretorius, 2000). Diethyl succinate contributes tropical fruit notes to aroma (Nurgel *et al.*, 2002; Majdak *et al.*, 2002; Peinado *et al.*, 2003). Some flavour compounds do not exceed the perception threshold level. However, their presence enhances the complexity of the Cinsaut wine (Majdak *et al.*, 2002).

Out of the 10 hybrids, 5 were selected on the basis of novel aspects, factors playing a positive role during wine making or the quality of wine. Hybrid 15 showed novel fermentation kinetics (in comparison with the other yeasts) during fermentation of Clairette blanche, high balling and low nitrogen must. Hybrid 24 and 29 also showed novel fermentation kinetics during fermentation of Clairette blanche must. Furthermore, fermentation with hybrid 15, 24 and 29 was shown to result in the lowest residual amount of glucose/fructose (glucose + fructose), volatile and acetic acid in low nitrogen must. This is significant, as musts with a low nitrogen content is a common problem. Hybrid 11, 15 and 30 were selected, as they were shown to produce elevated levels of certain volatile esters, higher alcohols and fatty acids. These compounds add to the aroma and complexity of wine (Majdak *et al.*, 2002). Hybrid 15 was also shown to produce more glycerol than the other yeasts.

The 5 selected hybrids fermented Pinotage must to dryness. Once again, VIN13 and hybrid 15 produced the highest amount of glycerol. Analysis of the volatile aroma compounds in Pinotage wine showed that hybrid 11 and 15 produced more 2-phenylethyl acetate than any of the other yeasts and hybrid 11 produced the highest amount of hexyl acetate. The amount of hexyl acetate produced by hybrid 11 was in excess of the perception threshold value. Ethyl caproate and isoamyl acetate was also

produced in excess of the perception threshold value by all the yeasts. These volatile esters add rosy, honey, fruity and flowery notes to wine (Nurgel *et al.*, 2002; Majdak *et al.*, 2002; Peinado *et al.*, 2003). Hybrid 15 produced the highest amount of 2-phenylethyl alcohol and also produced this compound in excess of its perception threshold value. Hybrid 24 and 29 produced more isoamyl alcohol than both the parental yeasts and all the yeasts produced this compound in excess of the perception threshold value. These higher alcohols add floral, rose, honey and marzipan notes to wine (Lambrechts and Pretorius, 2000).

Sensory evaluation of the Pinotage wine showed that the increase in some of the esters and higher alcohols were not reflected during the Pinotage wine tasting, as most of the hybrids scored the same as one of the parents for fruity characteristics. Hybrid 11 and 15 scored significantly higher than the other yeasts for tobacco character and also scored the lowest for astringency. These positive changes in tobacco and astringency character are a result of hybridisation and could possibly be attributed to the increase in some of the esters, higher alcohols and volatile fatty acids produced by the hybrids.

In conclusion, hybridisation of VIN13 and WE14 resulted in the selection of 5 hybrids (hybrid 11, 15, 24, 29 and 30) that were shown to produce low levels of H₂S and that have killer activity. Their fermentation kinetics differ under normal and stress conditions, which offers the advantage of novel aspects such as improved stress resistance and moderation of fermentation speed. Hybrid 15 was also repeatedly shown to produce elevated levels of glycerol. Sensory evaluation of the 5 hybrids contrasted with the higher levels of some esters and higher alcohols that were found in Cinsaut and Pinotage wine in that the hybrids scored the same or lower for fruity characteristics.

4.6 LITERATURE CITED

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5. GENERAL DISCUSSION AND CONCLUSIONS

5.1 PERSPECTIVES

The breeding of VIN13 and *S. paradoxus* strain RO88 yielded 5 hybrids. Two of these hybrids were shown to have distinct potential as wine yeasts. Positive phenotypal characteristics include the killer phenomenon, pectinolytic activity and low production of H₂S. Compared to *S. paradoxus* strain RO88, the hybrids did not degrade significant levels of malic acid. UV mutagenesis of hybrids also failed to produce positive results. A mass mating method could offer a solution to obtain hybrids with an enhanced ability to degrade malic acid, although the identification and phenotypal screening of the numerous yeasts might prove to be cumbersome. A very sensitive plate screening method for the detection of malic acid degradation must also be devised in order to narrow down the potential hybrids. Although CHEF was used to identify hybrids, PCR allows for more rapid identification. A reliable PCR method should thus be included in future work, as the method used in this study gave results contrary to results obtained by using the CHEF technique. Hybrids between VIN13 and *S. paradoxus* strain RO88 fermented slower than the parental strains and fermented must to dryness. No negative impact was observed on the principal constituents of wine (such as glycerol and volatile acidity) and the increased production of aroma compounds was shown, which impacts on the quality of wine. But for the low degradation of malic acid by the hybrids, the abovementioned approach succeeded in the goals and yielded at least 2 hybrids that show very good potential as commercial starter cultures.

The breeding of VIN13 and WE14 yielded numerous hybrids. After identification and selection on the basis of positive oenological characteristics, 5 hybrids were selected. Phenotypal characterisation showed that the hybrids all displayed killer activity and that they produced low levels of H₂S. Microvinification under differing must conditions identified at least one of the 5 hybrids as very unique. Must was fermented to dryness, the principal constituents of wine was not adversely affected during winemaking and this hybrid consistently produced elevated levels of glycerol. In general, a reduction in fermentation rate was seen and an increase in some of the volatile esters and higher alcohols. This did not necessarily relate to more aromatic wines, as a tasting panel scored the hybrids and the parental yeasts very similar in terms of fruity notes. Although the fermentation rate was thus altered in some cases, a positive increase in volatile aroma compounds did not result in more aromatic wines. Future work might produce hybrids that have a greater ability to produce more aromatic wines and should include a wider selection of parental yeasts. The potential of non-*cerevisiae* yeasts should not be overlooked, as the potential of *S. paradoxus* strain RO88 was shown in this study.

The biggest limitation on yeast breeding projects is the inability to predict the outcome of mating. The best way to improve the desired outcome of breeding, is the

Careful selection of parental yeasts and the thorough subsequent screening of hybrids and wines in order to eliminate mediocre yeasts. Making wine with a wider variety of grapes, will also create a better impression of the true potential of a wine yeast.

5.2 DISCUSSION AND CONCLUSION

S. cerevisiae is considered the primary industrial wine yeast, for many reasons. The main reason is that it dominates alcoholic fermentations almost from the onset of fermentation (Querol *et al.*, 2003). Advances in science have allowed scientists to understand why *S. cerevisiae* is such an adaptive yeast and also created the possibility of further improving the wine yeast. Traditional or natural fermentations relied on the yeasts strains present in the grape must at the onset of fermentation. The unreliability and unpredictable outcomes of these fermentations prompted the development of starter cultures with positive oenological characteristics such as low production of H₂S and volatile acidity, the killer phenomenon, pectinolytic activity and increased aroma compound production (Pretorius and Bauer, 2002). Conventional yeast improvement methods such as haploid mating and mutagenesis allow for improvement of some yeast properties, whereas other properties might be compromised. Additionally, the resulting yeasts are not classified as genetically modified organism (GMOs). The genetic modification of yeasts strains by gene cloning and transformation introduce new characteristics into yeast strains, thereby improving their performance. Unfortunately, the acceptance of GMOs are hindered by a multitude of scientific, technical, economical, safety and ethical issues (Davies, 2001).

In this study, 3 yeast strains with proven and positive oenological characteristics were selected as parental yeast strains. VIN13 and WE14 were selected and hybridised on the basis of their success as commercial wine yeast starter cultures (Van der Westhuizen, 1990). VIN13 was also hybridised with *S. paradoxus* strain RO88. Redzepovic *et al.* (2002) showed that *S. paradoxus* has the potential to become an industrial yeast strain as it is able to degrade malic acid in synthetic grape and Chardonnay must. A decrease in malic acid concentration in wines with a high total acidity is one of the most important steps in the enhancement of wine quality. Even more important, this study illustrated that *S. paradoxus* strain RO88 produced wine of good quality and aroma. Majdak *et al.* (2002) indicated that *S. paradoxus* strain RO88 has the potential to produce almost equal concentrations of fatty acids as *S. cerevisiae* strain RO64 and indigenous yeasts. Below their threshold values, the volatile fatty acids add to the complexity of wine and can impart fruity and citrus aromas (Nurgel *et al.*, 2002; Peinado *et al.*, 2003). According to Charoenchai *et al.* (1997), yeasts that are not of the *Saccharomyces* genus, produce and secrete several enzymes such as esterases, glycosidases, lipases, β -glucosidases, proteases and cellulases. Interactions with these enzymes by grape precursor compounds may produce aroma active compounds and thus play an important role in varietal aroma. In the light of the abovementioned, it is

realized that not only *Saccharomyces* yeast strains bear important aroma producing potential. The identification and characterisation of all yeast strains with wine-making potential thus remains paramount in yeast improvement projects.

Phenotypical characterisation allowed the selection of hybrids between VIN13 and *S. paradoxus* strain RO88 that produce low levels of H₂S, that are killer positive and show pectinolytic activity. Hybrids between VIN13 and WE14 also produced low levels of H₂S and were killer positive.

During alcoholic fermentation yeast cells are subjected to several stress conditions (Ivorra *et al.*, 1999; Querol *et al.*, 2003; Zuzuarregui *et al.*, 2004). Some yeasts are able to better adapt to stress conditions or changes in the environment. These strains would have potential as an industrial starter cultures for wine making. This study focused on high sugar and low nitrogen induced stress. The high sugar content of must produces osmotic stress in yeast cells which they must resist in order to initiate, carry on and complete the fermentation. The amount of nitrogen that is available for the yeast depends on the assimilable nitrogen sources in the particular must. Blateyron *et al.* (2001) identified nitrogen and oxygen deficiencies as major causes of stuck and sluggish fermentations. By observing fermentation rate it was shown that some hybrids from this study were shown to adapt well to the induced stress conditions and deserve further study. In general, the hybrids fermented slower than the parental yeasts. Slower fermentations were indicated to increase the aromatic content of wines (Torija *et al.*, 2003). Choosing the right yeast or yeast strain for the production of desirable tastes and flavours for wines is very important and significant, as the impact of yeast strain on the aroma and quality of wine has been demonstrated in various studies (Falqué *et al.*, 2001; Patel and Shibamoto, 2003). As most compounds found in wine are formed during yeast fermentation, a definitive positive correlation was shown between the yeast used and the production of volatile chemicals, including alcohols, esters and acids in fermenting must. Higher alcohols quantitatively represent the largest group of aroma compounds in alcoholic beverages and can have a significant influence on the taste and character of wine (Lambrechts and Pretorius, 2000). Higher alcohols such as 2-phenylethyl alcohol, hexanol and isoamyl alcohol impart pleasant aromas on wine such as floral, rose, honey, herbaceous and marzipan (Rankine, 1969; Shinohara and Watanabe, 1976; Fabre *et al.*, 2000; Peinado *et al.*, 2003). Esters impart the pleasant smell usually associated with wine and is considered the most important flavour compounds produced by yeasts during alcoholic fermentation (Lambrechts and Pretorius, 2000). It is believed that esters make the greatest contribution to the desirable fermentation bouquet of wine (Rojas *et al.*, 2003). The fruity odours of the fermentation bouquet are primarily due to a mixture of the esters hexyl acetate, ethyl caprylate and ethyl caproate. These ester imparts flavours such as apple, cherry, pear, flower, banana, brandy, grape and floral (Rankine, 1969; Shinohara and Watanabe, 1976; Fabre *et al.*, 2000; Peinado *et al.*, 2003). Ester production during alcoholic fermentation is also closely related to the particular yeast species involved. In this study it was shown that hybrids between VIN13 and *S. paradoxus* strain RO88 produced

elevated levels of volatile esters, fatty acids and higher alcohols, which resulted in more aromatic wines. This was supported by sensory analysis of Cinsaut wine. Hybrids between VIN13 and WE14 were shown to produce elevated levels of volatile esters and higher alcohols, but this trend was not seen as strongly during sensory analysis of Pinotage wine.

Experimental winemaking trials by Anchor Yeast SA are being conducted as some of the abovementioned hybrids developed in this study show true potential to be used as commercial wine starter cultures.

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