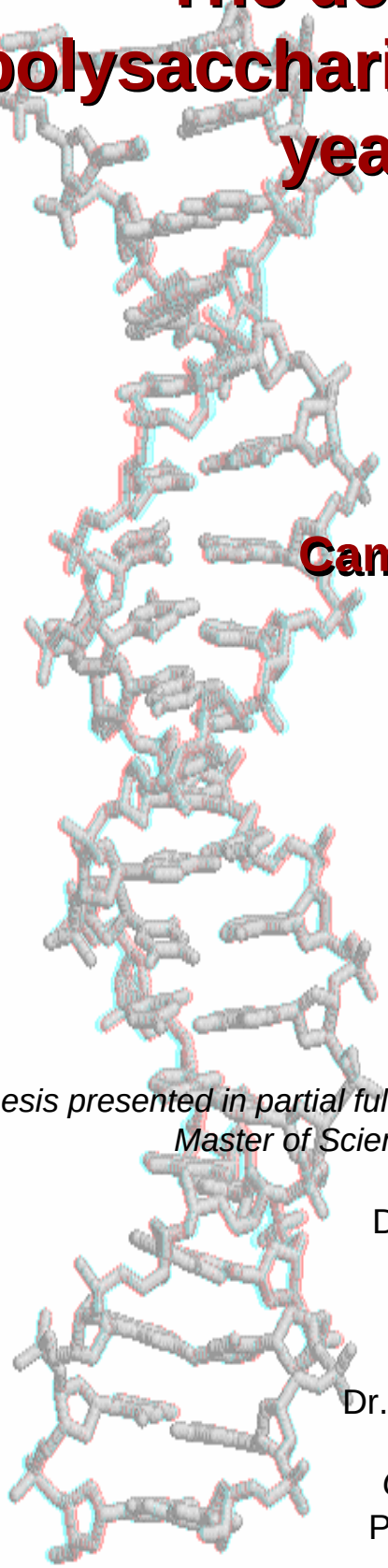
The development of polysaccharide degrading wine yeast strains



by

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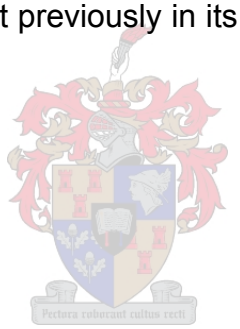
DECLARATION

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



Campbell Louw **Date**

SUMMARY

The polysaccharides that are present in wine originate from the grapes, the fungi that grow on the grapes and from other microorganisms that come into contact with the must during winemaking. The grape-derived polysaccharides of most concern in winemaking are pectin, glucan and xylan that can be enzymatically degraded by pectinases, glucanases and xylanases, respectively. These are the main structural polysaccharides of the cell wall of the grape cell. Degradation of the cell walls will result in the separation and rupture of the grape cells, and cell wall-bound compounds will be released into the must. Treating the must with pectinase and macerating enzyme preparations can result in an increase in free-flow juice, an improvement in must clarification and filtration, and an increased extraction of phenols and tannins. The tannins that are extracted polymerise with anthocyanins in red wine during ageing, resulting in increased colour intensity and stability. Wine aroma is also influenced by enzyme treatment. The degradation of the cell wall contributes to the release of glycosidically-bound terpene or alcohol precursors from the berries. The hydrolysis of these precursors during fermentation can result in an improvement in aroma. It can thus be seen that it is possible to improve wine quality and processing by supplementing the endogenous enzymes that are present in the fermentation with commercial enzyme preparations. Commercial enzymes are typically crude fungal preparations.

The majority of commercial pectinase and glucanase preparations are derived from *Aspergillus* and *Trichoderma,* respectively. Since the endogenous polysaccharase activity of *Saccharomyces cerevisiae* is very limited, the heterologous expression of specific polysaccharase genes in an industrial yeast strain can improve the winemaking process, resulting in a higher quality wine without the addition of expensive commercial enzyme preparations. Since only the desired enzymes are secreted by the recombinant strain, there will be no undesired side-activities, which can be detrimental to wine quality. Several pectinase-, glucanase- and xylanase-encoding genes, cloned from a variety of organisms, have been expressed successfully in laboratory strains of *S. cerevisiae.* Attempts have also been made to construct industrial wine yeast strains that express these polysaccharase genes and secrete the encoded enzymes. Fermentation with some of these strains resulted in a decrease in total phenolics and turbidity, an increase in juice extraction, and alterations in the colour and aromatic profile of the resulting wines.

In this study, four polysaccharide-degrading, recombinant wine yeast strains were constructed. The endo-β-1,4-xylanase gene, *XYN2*, and the endo-β-1,4-glucanase gene, *end1*, were previously cloned from the soft rot fungus *Trichoderma reesei* and the rumen bacterium *Butyrivibrio fibrisolvens,* respectively. These genes were subcloned into different expression cassettes which were used to construct the four integration plasmids. The recombinant plasmids contained the following gene cassettes: *TEF1*$_P$-*XYN2*-ADH2$_T$ (plasmid pDLG29)

ADH1$_P$- *MF*α*1*$_S$ *-end1-TRP5*$_T$ (plasmid pDLG30) *ADH1*$_P$*-MF*α*1*$_S$*-end1-TRP5*$_T$ and *ADH2*$_P$*-XYN2-ADH2*$_T$ (plasmid pDLG33), *ADH1*$_P$*-MF*α*1*$_S$*-end1-TRP5*$_T$ and *YG100*$_P$*-XYN2-ADH2*$_T$ (plasmid pDLG39). These four plasmids were then separately integrated into the *ILV2* locus of the commercial wine yeast strain *S. cerevisiae* VIN13. Wine was made with the four strains constructed in this study, a pectolytic strain, VIN13[pPPK], a glucanase- and xylanase-secreting strain, VIN13[pEX], an untransformed VIN13 strain, and an untransformed strain with the addition of the commercial enzyme preparation Rapidase EX Colour. Microvinification experiments were carried out on Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines. Fermentation with the polysaccharide-degrading strains resulted in significant improvements in juice extraction, colour intensity and stability, and in alterations in the aromatic profiles of the wines produced.

Subject to the approval by the regulatory authorities and eventual consumer acceptance of the use of genetically modified organisms (GMOs) in fermented foods and beverages, it might be required that the GM status of the yeast that is used appears on the label. Currently, there is no robust technique available with which the use of GM yeast can be revealed in a finished wine because the yeast cells and their DNA are removed from or denatured in the wine during filtration and processing. One way with which the undeclared use of a GM yeast in winemaking could be exposed would be to compare the chemical profile of a suspect wine with that of non-GM wine. In order to explore this concept further, a secondary aim of this study was to investigate whether Fourier Transformation Infra Red (FT-IR) spectroscopy coupled with multivariate data analysis could distinguish between wines fermented with transgenic and non-transgenic yeast strains, or between wines fermented with different transgenic strains. The results showed that this method could be used to classify wines fermented with different yeast strains if fermentation with the strain resulted in a unique chemical profile in the resulting wine. This was a preliminary study and these findings were summarised as an addendum to the thesis.

OPSOMMING

Die polisakkariede wat in wyn teenwoordig is, is afkomstig van die druiwe, die swamme wat op die druiwe groei en vanaf ander mikroörganismes wat tydens die wynmaakproses met die mos in aanraking kom. Die belangrikste druifpolisakkariede in wynbereiding is pektien, glukaan en xilaan, wat onderskeidelik deur pektinases, glukanases en xilanases afgebreek kan word. Hierdie is die vernaamste strukturele polisakkariede van ‘n druifsel se selwand. Die afbreking van die selwande veroorsaak dat die druifselle skei en skeur, met die gevolg dat die selwandgebonde verbindings in die mos vrygelaat word. Die behandeling van die mos met pektinase en versappingsensiempreparate kan tot ’n toename in vry-afloopsap lei, sowel as ’n verbetering in mosverheldering en -filtrasie en ’n verhoogde ekstraksie van fenole en tanniene. Die tanniene wat geëkstraheer word, polimeriseer in rooiwyn tydens veroudering, en dit lei tot verhoogde kleurintensiteit en -stabiliteit. Wynaroma word ook deur ensiembehandeling beïnvloed. Die afbreking van die druifselwand dra by tot die vrylating van glikosidiesgebonde terpeen- en alkoholvoorlopers uit die korrels. Die hidrolise van hierdie voorlopers tydens gisting kan lei tot ’n verbetering van die aroma. Dit is dus duidelik dat dit moontlik is om wynkwaliteit en wynbereiding te verbeter deur die endogene ensieme wat in die gisting teenwoordig is met kommersiële ensiempreparate te supplementeer.

Kommersiële ensiempreparate is tipies ongesuiwerde swampreparate. Die meerderheid kommersiële pektinase- en glukanasepreparate word onderskeidelik vanaf *Aspergillus* en *Trichoderma* verkry. Aangesien die endogene polisakkarase-aktiwiteit van *Saccharomyces cerevisiae* baie beperk is, kan die heteroloë uitdrukking van spesifieke polisakkarase-gene in ’n industriële gisras die wynbereidingsproses verbeter en lei tot ’n hoër kwaliteit wyn sonder die byvoeging van duur kommersiële ensiempreparate. Omdat die verkose ensieme deur die rekombinante ras uitgeskei word, sal daar geen ongewenste newe-effekte teenwoordig wees wat ’n nadelige effek op wynkwaliteit kan hê nie. Verskeie mikrobiese gene wat vir pektinases, glukanases en xilanases kodeer, is reeds voorheen uit ‘n wye verskeidenheid van organismes gekloneer en suksesvol in laboratoriumrasse van *S. cerevisiae* uitgedruk. Pogings is ook aangewend om industriële wyngisrasse te konstrueer wat hierdie polisakkarasegene uitdruk en hul enkodeerde ensieme uitskei. Gisting met sommige van hierdie rekombinante gisrasse het gelei tot ’n afname in totale fenoliese verbindings en troebelheid, ’n verhoging in sapekstraksie, en veranderings in die kleur en aromatiese profiel van die gevolglike wyne.

In hierdie studie is vier polisakkaried-afbrekende, rekombinante wyngisrasse gekonstrueer. Die endo-β-1,4-xilanasegeen, *XYN2*, en die endo-β-1,4-glukanasegeen, *end1*, is voorheen reeds onderskeidelik vanaf die sagte vrotswam, *Trichoderma reesei*, en die rumenbakterium, *Butyrivibrio fibrisolvens*, gekloneer. Hierdie gene is in vier integrasieplasmiede in verskillende ekspressiekassette gesubkloneer. Die plasmiede het die volgende geenkassette bevat: $TEF1_P$-*XYN2*-$ADH2_T$ (plasmied pDLG29) $ADH1_P$- $MF\alpha1_S$-*end1*-$TRP5_T$ (plasmied pDLG30) $ADH1_P$-$MF\alpha1_S$-*end1*-$TRP5_T$ and $ADH2_P$-*XYN2*-$ADH2_T$ (plasmied pDLG33), $ADH1_P$-$MF\alpha1_S$-

end1-TRP5$_T$ and *YG100*$_P$*-XYN2-ADH2*$_T$ (plasmied pDLG39). Hierdie vier plasmiede is toe afsonderlik in die *ILV2*-lokus van die kommersiële wyngisras, *S. cerevisiae* VIN 13, geïntegreer. Wyn is met hierdie vier gekonstrueerde gisrasse gemaak, die pektolitiese gisras, VIN13[pPPK], die glukanase- en xilanase-afskeidende gisras, VIN13[pEX], die ongetransformeerde VIN13-ras, en met 'n ongetransformeerde VIN13 gis waarby die kommersiële ensiempreparaat, Rapidase EX Colour, bygevoeg is. Mikro-wynbereidingseksperimente is op Pinot noir-, Ruby Cabernet- en Muscat D'Alexandria wyne uitgevoer. Gisting met die polisakkaried-afbrekende gisrasse het gelei tot 'n noemenswaardige verbetering in sapekstraksie, kleurintensiteit en kleurstabiliteit, asook in veranderinge in die aromatiese profiele van die geproduseerde wyne.

Indien die gebruik van geneties gemodifiseerde organismes (GMOs) in gefermenteerde voedsel en drank deur die reguleringsowerhede goedgekeur en uiteindelik deur die verbruiker aanvaar sou word, sou dit vereis kon word dat die GM-status van die wyngisgis op die etiket van die wynbottel aangebring word. Verpligte etikettering van GM-wyn sal metodes vereis waarmee die 'nalentskap' van GM-gisselle in die finale produk geïdentifiseer en gemoniteer kan word. Tans is daar geen robuuste tegnieke beskikbaar waarmee die gebruik van GM-giste openbaar kan word nie, aangesien die gisselle en hul DNA tydens filtrasie en prosessering verwyder word. Een wyse waarop die onverklaarde gebruik van 'n GM-gis in wynbereiding blootgestel sou kno word, is om die chemiese profiel van die verdagte wyn met dié van 'n nie-GM-wyn te vergelyk. Ten einde hierdie konsep verder te ondersoek was 'n sekondêre doelwit van hierdie studie om te bepaal of FT-IR (Fourier-transformasie-infrarooi) spektroskopie tesame met meervariante data-analise gebruik kan word om te onderskei tussen wyne wat met transgeniese en nie-transgeniese gisrasse gegis is, of tussen wyne wat met verskillende transgeniese rasse gegis is. Die resultate het aangedui dat hierdie metode gebruik kan word om wyne wat met verskillende gisrasse gegis is, te klassifiseer indien die betrokke gisras 'n unieke chemiese profiel in die uiteindelike wyn veroorsaak het. Dit was egter 'n voorlopige ondersoek en is as 'n byvoegsel tot die tesis geskryf.

BIOGRAPHICAL SKETCH

Campbell Louw was born on September 20, 1977 in Cape Town, South Africa. He attended the Primary School, Swartland in Malmesbury and matriculated at High School Swartland in 1995. In 1997 he enrolled for a BSc degree at the University of Port Elizabeth and obtained the degree in 1999, majoring in Microbiology and Biochemistry. In 2000 he obtained a Honours degree in Microbiology from the University of Port Elizabeth. In 2002 he enrolled for an MSc in Wine Biotechnology at the University of Stellenbosch.

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PREFACE

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

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
The influence of the enzymatic degradation of polysaccharides on winemaking

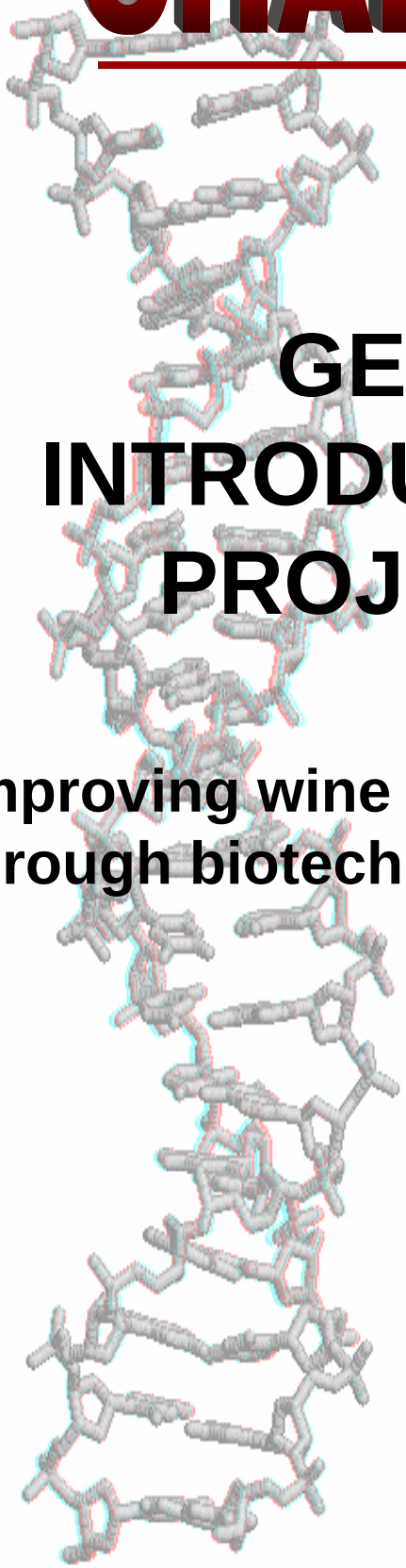
Chapter 3 **Research Results**
The influence of the heterologous expression of polysaccharase genes in *Saccharomyces cerevisiae* on winemaking.

Chapter 4 **Addendum**
Differentiating between wines fermented with transgenic and non-transgenic yeast strains by Fourier transform infrared spectroscopy and multivariate data analysis

Chapter 5 **General Discussion and Conclusions**

CONTENTS

CHAPTER 1



GENERAL INTRODUCTION AND PROJECT AIMS

Improving wine yeast starter cultures through biotechnological innovations

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 GENERAL INTRODUCTION

Wine fermentation is a complex microbiological process that involves the sequential development of various yeast and bacterial strains. In a spontaneous fermentation, several oxidative and apiculate yeasts originating from the grape surface predominate. The final stages of fermentation, however, will be dominated by the alcohol-tolerant *Saccharomyces cerevisiae* strains (Jackson 1994). Winemakers commonly inoculate fermentations with pure wine yeast cultures in the form of active dry yeast. Inoculation with high numbers of an active starter culture improves the reproducibility of the winemaking process (Ribéreau-Gayon 1985). These strains are isolated and selected for their favourable fermentation characteristics. The specific wine yeast strain used as the starter culture plays an important role in the type and style of wine produced. There is an ongoing search for strains with sought after characteristics in order to improve the reliability of fermentation, wine quality and the economics of wine production.

Traditionally, strain development relied exclusively on strain selection. However, *S. cerevisiae* can be genetically manipulated in a variety of ways to produce wine yeast strains with improved or novel oenological properties. Classical genetic techniques, such as hybridisation or mutagenesis, followed by broad trait selection, used to be the preferred way of genetically improving industrial yeast strains (Dequin 2001). With these methods, large genomic regions or entire genomes are rearranged or recombined. The advantages of these classical techniques are that they can be used to improve characteristics under polygenic control and the yeast strains produced are not classified as GMOs (genetically modified organisms) and will thus be immediately viable for industrial use. These techniques, however, cannot be used to alter only a specific characteristic. Genetic engineering is the only reliable method by which a specific, existing property can be altered, a new characteristic can be introduced or an existing one eliminated without affecting any other desirable properties. *S. cerevisiae* is a very important host for the expression of foreign genes. This is possible because it is a eukaryotic organism with associated cellular processes, such as posttranslational processing and secretion, it is single celled and thus easy to culture and manipulate the genetics (Sudberry 1996).

Enzymes that originate from the grape and the microorganisms present during winemaking act as biological catalysts and drive the conversion from grape juice to wine (Van Rensburg and Pretorius 2000). By supplementing the endogenous enzymes of the grapes and the microorganisms present in the fermentation with commercial enzyme preparations, it is possible to improve the processing and quality of the wines produced. These commercial enzyme preparations are obtained by cultivating microorganisms (mostly fungi) that secrete the enzymes under optimum

conditions in bioreactors and harvesting the enzyme. Commercial preparations are not only expensive, but can also contain many unwanted enzymes harvested with the desired enzyme. These enzymes can be responsible for side-activities that are detrimental to wine quality.

Since *S. cerevisiae* can express foreign genes and process and secrete the proteins, it is possible to clone the genes coding for a specific enzyme from the organism of origin and express it in a wine yeast strain. Such a strain will then be able to secrete the desired enzyme while fermenting the must. This allows for more economical production of higher quality wines, without undesired side-activities. Several metabolic pathways in *S. cerevisiae* have also been targeted by gene techniques in order to improve the oenological capabilities of wine yeast strains. Some of the most important areas that have been targeted by the genetic engineering of wine yeasts are improved fermentation performance, processing efficiency, biological control of spoilage organisms, wine wholesomeness and wine flavour and sensory qualities (Pretorius and Bauer 2002).

1.2 AIMS OF THIS STUDY

Glucanase, xylanase and pectinase enzyme preparations are applied to winemaking with the aims of improving the clarification and processing of wine, releasing varietal aromas from precursor compounds, and to increase colour intensity. Genes coding for these enzymes have been cloned from several polysaccharide-degrading organisms. Expressing the genes coding for polysaccharases in *S. cerevisiae* allows for the recombinant strains to degrade problem-causing polysaccharides that traditional commercial yeast strains cannot. The aim of this study was to construct recombinant wine yeast strains that are able to degrade the problem-causing grape polysaccharides glucan and xylan, by separately integrating the *Trichoderma reesei XYN2* xylanase gene and the *Butyrivibrio fibrisolvens end1* glucanase gene into the genome of the commercial wine yeast strain, *S. cerevisiae* VIN13. These genes were to be combined under the control of different promoters in this wine yeast strain. The effects of the newly constructed strains on fermentation will be compared with each other and with polysaccharide-degrading wine yeast strains constructed in previous studies. In order to obtain these goals, the following aims were set:

(i) construction of four integration plasmids containing the endoglucanase and xylanase genes, as well as the *SMR1* (sulfometuron methyl resistance) gene, for the selection of positive transformants;

(ii) construction of recombinant wine yeast strains by separately integrating the integration plasmids into the *ILV2* locus of the *S. cerevisiae* VIN13 strain;

(iii) making wine by fermenting three different grape cultivars with the recombinant strains constructed in this study, a pectolytic and glucanolytic

strain previously constructed, the untransformed strain and the untransformed strain with the addition of a commercial enzyme;

(iv) comparing the effects of the different strains and treatments by doing colour, aroma, chemical and sensory analyses of the wines at different stages during winemaking and ageing; and

(v) comparing the near infrared spectra of the wines fermented with different recombinant wine yeast strains through multivariate data analysis, to determine if FT-IR (Fourier transform infrared spectroscopy) can distinguish between wines fermented with different yeast strains.

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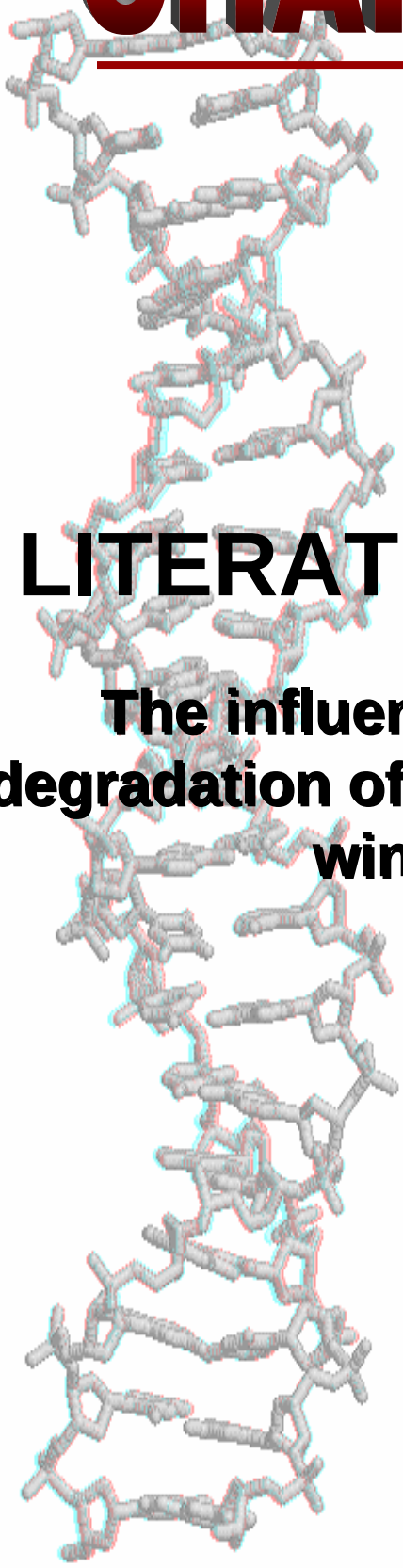
CHAPTER 2



LITERATURE REVIEW

The influence of enzymatic degradation of polysaccharides on winemaking

2. LITERATURE REVIEW

2.1 INTRODUCTION

Polysaccharides that occur in wine originate from the grapes, the fungi on the grapes and microorganisms present in the must. These polysaccharides can cause problems during wine processing. The degradation of grape polysaccharides is very important for juice extraction, wine colour and the aromatic profiles of the wines produced. During winemaking, polysaccharides are degraded through the addition of commercial enzymes. These enzymes are usually produced by bacteria or filamentous fungi (Fernandez *et al* 2000). The yeast *Saccharomyces cerevisiae* plays a dominant role in winemaking and carries out the fermentation of sugars to ethanol, carbon dioxide and a number of by-products. This industrial wine yeast is not only the driving force behind alcoholic fermentation; it is also a very important host for the expression of foreign genes. Genetic engineering has allowed for the expression of multiple foreign genes in laboratory strains of *S. cerevisiae*. An increasing number of these genes are now also being expressed in industrial strains (Pretorius and Bauer 2002). Since the endogenous polysaccharase activity of *S. cerevisiae* is very limited, the heterologous expression of specific polysaccharase genes in an industrial yeast strain can improve the winemaking process and contribute to better quality wines without the addition of industrial enzyme preparations.

2.2 POLYSACCHARIDES FROM GRAPES AND WINE

Polysaccharides are found in wines at levels between 300 and 1000 mg/l, and originate in the grape itself, the fungi on the grape and the microorganisms present during winemaking. The main polysaccharides responsible for turbidity, viscosity and filter stoppages are pectins, glucans (a component of cellulose) and hemicellulose (mainly xylans) (Pretorius 2000).

The grape berry tissue can be divided into the skin (exocarp) and the pulp (endocarp). The skin consists of an epidermis, hypodermis and cuticle (Fig 1). The cells that make up these tissues are insulated from each other by cell walls. The cell walls are mainly responsible for the integrity and texture of tissues and therefore play an important role in fruit processing (Barnavon *et al* 2001).

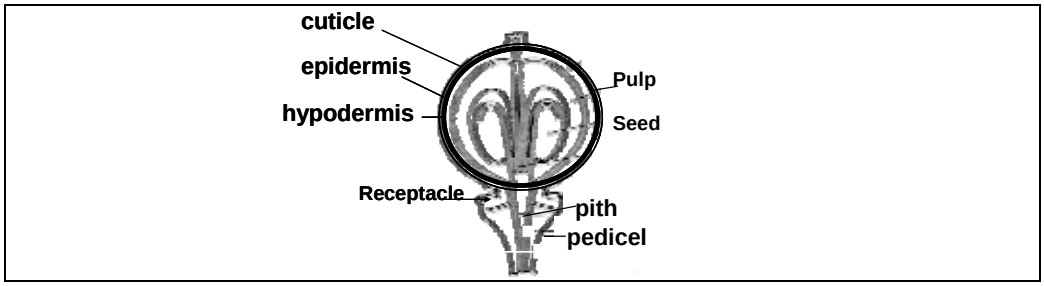


Figure 1 Structure of the grape berry

Grape polysaccharides in wine result from the breakdown and solubilisation of some of the pectic substances in the skin and pulp cell walls of the berry. The pectins are concentrated in the middle lamella, between the cells, and in the primary cell walls. Galacturonic acid is the main component of these polymers and can be highly methylated. Rhamnose, arabinans, galactans and arabinogalactans can also be present in pectic substances. The secondary cell wall consists mostly of cellulose, with some lignin, while the primary cell wall is mostly comprised of cellulose fibres, called microfibrils, in a matrix of pectins, hemicellulose and proteins. Cellulose fibrils are connected to pectins by xyloglucans, and the pectins are connected by arabinogalactans to serine residues in the cell wall. Figure 2 shows the interconnections among the major components of the primary cell walls. Juice extraction will require that the middle lamella walls are degraded to release the cells, and that the cell walls are broken to allow for the vacuole contents to be extracted into the juice or wine (Parley 1997).

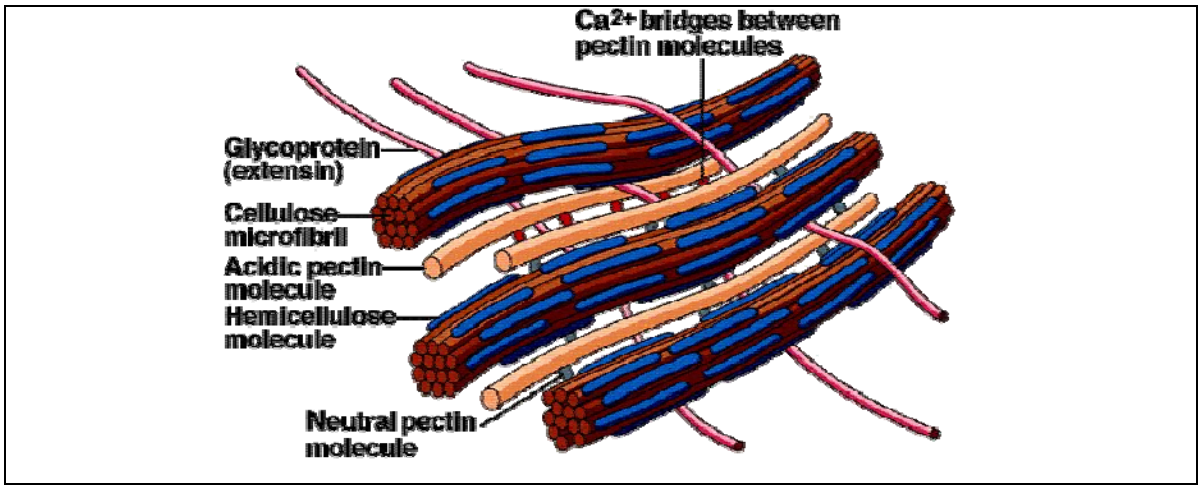


Figure 2 Interconnections among major components of primary cell walls (Moore *et al* 1998)

Yeast is the second major source of polysaccharides in wine. The amount of polysaccharides released by the yeast depends on the strain, as well as on the fermentation and ageing conditions. The yeast will release more polysaccharides at high temperatures in an agitated medium after prolonged ageing on biomass. Yeast polysaccharides are mainly released into dry white wines when aged on the lees, and the release is increased if the lees is stirred into suspension. Polysaccharide complexes are mainly released into red wine after fermentation during high temperature maceration. This only takes place for a short period, because most of the yeast lees is separated from the wine during racking. Two types of exocellular proteins are released by the yeast during fermentation and ageing on the lees, namely mannoproteins and glucomannoproteins. Mannoproteins form 80% of the exocellular polysaccharides and contain 90% mannose and 10% proteins. Glucomannoproteins make up the other 20% of exocellular polysaccharides and contain 25% glucose, 25% mannose and 50% protein. Mannoproteins have an indirect effect on astringency when they combine with phenolic compounds from

grapes or oak. The yeast polysaccharides can also cause filtration problems through membrane fouling. Mannoproteins play a very important role through their stabilising effect during protein precipitation in white wine and tartrate crystallisation in red and white wine (Ribéreau-Gayon *et al* 2000a).

Botrytis cinerea is also a source of polysaccharides in wine. Wines made from botrytised grapes are difficult to clarify and can also pose filtration problems. This is caused by a high molecular weight glucose polymer, β-1,3-1,6-glucan, which is produced by this grey mould. This glucose polymer, which is also known as cinereane, is secreted into the grape juice and is thus later found in the wine. Glucan prevents the sedimentation of cloud particles and this results in clarification problems and is also responsible for filter stoppages (Van Rensburg *et al* 1997). The fouling effect of glucan is increased by the presence of ethanol, which promotes the formation of hydrogen bonds between the chains, creating a three-dimensional lattice structure (Ribéreau-Gayon *et al* 2000a).

2.2.1 PECTIN

Chemically, pectic substances are complex colloidal acid polysaccharides, with a backbone of galacturonic acid residues linked by α (1-4) linkages (Kashyap *et al* 2001). The unsubstituted homopolymeric galacturonan regions are interspersed with rhamnogalacturonan, and the L-rhamnose units are β-1,2- and β-1,4-linked to the 1,4-D-galacturonic acid residues. The rhamnogalacturonan regions are substituted with arabinans, galactans and highly branched arabinogalactans (Nunan *et al* 1997). The carboxyl groups of galacturonic acid are partially esterified by methanol and partially or completely neutralised by sodium, potassium or ammonium ions. On the basis of the type of modification of the backbone chain, pectic substances are classified as protopectin, pectic acid, pectinic acid and pectin (Miller 1986).

Protopectin is a parent pectic substance and, when hydrolysed, yields pectin or pectinic acid. It is insoluble in water, but the pectic substances formed upon hydrolysis are soluble or partially soluble in water. Pectic acids consist of galacturonans with a negligible amount of methylation on the chain (Fig 3). These regions consist of rhamnogalacturonans that are highly substituted with arabinans, galactans and arabinogalactans. Normal or acid salts of pectic acid are called pectates. Pectinic acids are galacturonans with varying amounts of methylation on the chain. Pectinates are normal or acid salts of pectinic acids.

Pectin is a generic name for a mixture of pectic substances with different compositions, but with pectinic acid as the major component (Kashyap *et al* 2001). Grape pectins are highly esterified with methanol at 70-80% (Ribéreau-Gayon *et al* 2000b).

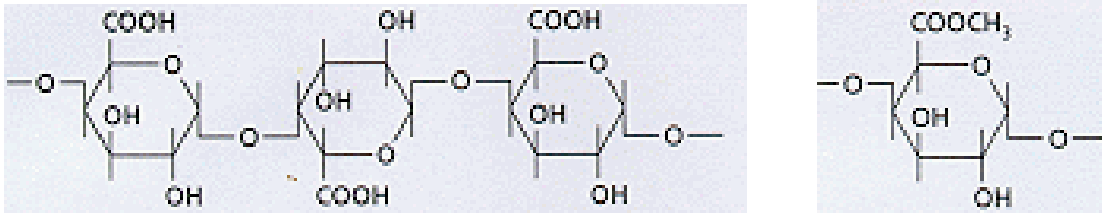


Figure 3 Pectic compounds: pectic acid consists of straight chains of α-1,4 galacturonic acids. Pectinic acid is similar to pectic acid, with some of the residues being methylated (http://helios.bto.ed.ac.uk/bto/microbes/pectin.gif)

2.2.2 CELLULOSE (GLUCAN)

Cellulose is a major polysaccharide constituent of plant cell walls and one of the most abundant organic compounds in the biosphere (Hong *et al* 2001). Glucan is the main component of cellulose (Van Rensburg and Pretorius 2000). The cellulose molecules are unbranched chains of D-glucopyranose residues linked by β-1,4 glycosidic bonds. The length of the polysaccharide chains varies, but can be between 30 and 15 000 D-glucopyranose units (Coughlan 1990). The hydroxyl groups at carbon atoms 2,3 and 6 are unsubstituted. The repeating unit in the cellulose molecule is cellobiose (Goodwin and Mercer 1983). This unit consists of two D-anhydroglucopyranose units linked by β-1,4 glycosidic bonds, rotated 180° to each other (Fig 4).

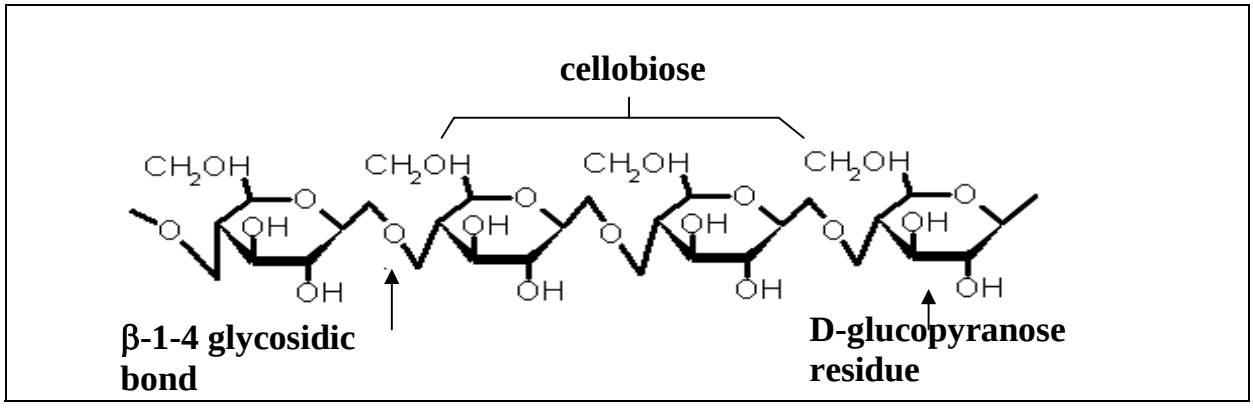


Figure 4 Primary structure of cellulose

A higher level of organisation occurs in cellulose when a large number of polymers twist together into microfibrils (Moore *et al* 1995). A microfibril consists of a bundle of cellulose molecules each arranged with its long axis parallel to the other. These unipolar chains associate through interchain hydrogen bonding and Van der Waals' interactions. The cellulose molecules in the core of the microfibril are arranged in perfect three-dimensional arrays and form a crystal lattice. This crystalline core is surrounded by a paracrystalline cortex, consisting of polysaccharides not arranged in

perfect three-dimensional order (Goodwin and Mercer 1983). In turn, the microfibrils in the cell wall aggregate to form insoluble fibres (Van Rensburg *et al* 1996).

Glucans other than cellulose (β-1,4-glucan) also play an important role in cell wall structure. β-1,3-Glucan plays an important role in the mechanical strength of the cell wall of wine yeasts. This polymer has a shape similar to a flexible wire spring and is very important for cell wall flexibility. β-1,3-Glucan consists of about 1 500 glucose monomers linked by β-1,3 bonds. In the stationary phase, these chains are moderately branched and contain about 3-4% β-1,6-linked glucose residues. The moderate degree of branching prevents crystallisation (Klis *et al* 2002).

β-1,6-Glucan is used in the cell wall to connect cell wall proteins to the β-1,3-glucan network and might also function as an acceptor site for chitin during cell wall stress. In its mature form, β-1,6-glucan is a highly branched, water-soluble polymer, consisting of about 130 β-1,6-linked glucose monomers (Klis *et al* 2002).
The β-1,3-1,6-glucan produced by *B. cinerea* plays an important role in clarification of wine. This molecule consists of a β-1,3-linked main chain of glucose units with branches made up of individual glucose units that are β-1,6-linked. Two out of five units in the main chain are substituted (Ribéreau-Gayon *et al* 2000b).

2.2.3 HEMICELLULOSE (XYLAN)

Hemicelluloses can be subdivided into three groups: xylans, mannans and galactans. Each group contains polysaccharides with considerable structural diversity and is named after the predominant monosaccharide.

Xylans include all the hemicellulose polysaccharides that are rich in D-xylopyranose residues, whether in the main chain or only attached as frequent branches (Goodwin and Mercer 1983).

β-1,4 Xylans are heteropolysaccharides that have a backbone of β-1,4-linked xylopyranosyl residues and constitute 20-35% of the dry weight of monocot and hardwood plants (Zeilinger *et al* 1996, La Grange 1995). The homopolymeric backbone chain can be substituted with O-acetyl, α-1,2-linked glucuronic or 4-O-methylglucuronic acid. However, unsubstituted linear xylans have been isolated from tobacco stalks (Kulkarni *et al* 1999). The most abundant xylan in hardwoods exists as a linear chain of β-1-4-linked D-xylopyranose residues, with seven out of ten xylose residues acetylated, mostly at C-3, but occasionally at C-2. The xylan structure in softwoods is similar, except that α-L-arabinofuranose might be linked to some of the xylose residues by α-1,3 glycosidic linkages (Goodwin and Mercer 1983).

Xylans are categorised according to the common substituents found on the backbone as linear homoxylan, arabinoxylan, glucuronoxylan and glucuronoarabinoxylan. Several links have been reported between xylan and other compounds, such as lignin and hydroxycinnamic acids, most of them through xylan substituents. These side chains determine the reactivity of the xylan molecule with other hemicellulose components and will thus influence solubility and the physical

conformation of xylan, thereby determining the mode and extent of enzymatic cleavage (Kulkarni *et al* 1999).

2.3 ENZYMATIC BREAKDOWN OF POLYSACCHARIDES

The hydrolysis of the aforementioned abundant and widely distributed polysaccharides to assimilable sugars remains a major technological and economical problem in the food and beverage industries. The degradation of polysaccharides can be carried out by acid or alkaline treatment, but this is expensive, time consuming and can generate toxic by-products and waste that are expensive to treat. The enzymatic hydrolysis of polymeric substances by extracellular enzymes, such as amylases, pectinases, cellulases and hemicellulases, is therefore preferred to chemical depolymerisation (Van Zyl and Pretorius 1995).

Enzymes are protein catalysts with great specificity for the biochemical reaction that is catalysed and the molecules that are acted upon. These catalysts increase the reaction rate by lowering the activation energy necessary for the reaction to take place, without undergoing permanent alteration (Prescott *et al* 1996). Most enzymes have clear temperature and pH optima and their action can be inhibited or enhanced by altering these parameters or by the presence of certain other compounds or co-factors. This means that enzymes are effective control points for many biochemical reactions in organisms. Enzymes can be separated and purified for use as processing aids in various industries (Parley 1997).

Enzymes are very important in the winemaking process and wine can be viewed as the product of the enzymatic transformation of grape juice. Throughout the winemaking process, various biochemical reactions are catalysed by enzymes originating from the grapes, yeasts and other microbes associated with the vineyard and winery. By controlling the reaction conditions, enzyme action can be optimised to increase the desired activities. The action of endogenous enzymes can also be extended by the addition of commercial enzyme preparations. Enzyme preparations are used during winemaking in order to improve the clarification and processing of wine, release varietal aromas from precursor compounds, reduce ethyl carbamate formation and lower alcohol levels in the wine (Van Rensburg and Pretorius 2000).

The primary cell walls of grape berries have a common set of structural polysaccharides, with their distribution varying among cultivars (Barnavon *et al* 2001). Since pectin, glucan, and xylan are the major constituents of the grape cell wall, enzymes that catalyse the breakdown of these polysaccharides can help to increase the extraction of juice and other desired compounds from the cells, resulting in a shorter press time. They can also help to clarify the juice by breaking down the large suspended polysaccharide units and, by the same mechanism, decrease the viscosity.

2.3.1 PECTINASES

The enzymes that hydrolyse pectic substances are known as pectic enzymes, pectinases, or pectinolytic enzymes. The pectinases produced by the grape are part of a complex set of cell wall-modifying enzymes. The release and depolymerisation of pectic substances through the action of these enzymes play a very important role in cell elongation, fruit softening and structural changes in the cell wall during grape ripening (Whitaker 1990, Barnavon *et al* 2001).

Pectinases can be divided into two main groups, namely pectin esterases (PE), which de-esterify pectin by removing the methoxyl residues, and depolymerases, which readily split the main chain. Pectin esterases remove methoxy groups from high methoxy pectin to give methanol and low methoxy pectin (Parley 1997). The depolymerising enzymes can be classified according to their preferred substrate, whether cleavage is random or endwise, and if the enzyme acts by trans-elimination or hydrolysis. Polygalacturonases (PG) cleave the glycosidic bonds by hydrolysis, and lyases (PL) break the glycosidic bonds by β elimination. These enzymes can also be classified according to whether they exhibit a preferential hydrolytic power against pectin, pectic acid or oligogalacturonate as the substrate, and whether the mode of action is random (endo-) or terminal (exo-) (Blanco *et al* 1999, Kashyap *et al* 2001). The classification of pectinases is shown in table 1.

Table 1. **Classification of depolymerising pectinases**

Group	Enzyme	Substrate	Action
Polymethylgalacturonases (PMG)		**Pectin**	**Hydrolysis**
	Endo-PMG		Random cleavage of α-1,4 glycosidic bonds
	Exo-PMG		Sequential cleavage of α-1,4 glycosidic bonds from the non-reducing end
Polygalacturonase (PG)		**Pectic acid**	**Hydrolysis**
	Endo-PG		Random hydrolysis of α-1,4 glycosidic linkages
	Exo-PG		Sequential cleavage of α-1,4 glycosidic linkages from the non-reducing end
Polymethyl galacturonate lyases (PMGL)		**Pectin**	**Trans-eliminative cleavage**
	Endo-PMGL		Random cleavage of α-1,4 glycosidic linkages
	Exo-PMGL		Sequential cleavage of α-1,4 glycosidic linkages
Polygalacturonate lyases (PGL)		**Pectic acid**	**Trans-eliminative cleavage**
	Endo-PGL		Random cleavage of α-1,4 glycosidic linkages
	Exo-PGL		Sequential cleavage of α-1,4 glycosidic linkages

2.3.2 CELLULASES (GLUCANASES)

Cellulolytic enzymes, which hydrolyse cellulose to form glucose, can be divided into three types: endo-β-1,4-glucanases, exo-β-1,4-glucanases (cellobiohydrolase), and β-glucosidases (cellobiase). These enzymes are collectively known as cellulases and act in a synergistic manner for the complete cleavage of the cellulose β-1,4 glycosidic bonds (Hong *et al* 2001). Cellulases can be classified into two major groups: one whose members can degrade native crystalline cellulose, and a second that is capable of hydrolysing only soluble substituted cellulose derivatives. Most microbial cellulases can completely degrade crystalline cellulose to glucose, because the microbial cellulase systems are usually multi-enzyme complexes composed of endoglucanase, exoglucanase, cellobiase, xylanases and xylosidases (Levi *et al* 2002).

The effectiveness of the cellulase enzymes is dramatically increased when they work synchronously in an endo-exo synergism. The endo-β-1,4-glucanases randomly attack the inside of the cellulose chain, thereby producing free ends to which the exo-β-1,4-glucanases can bind and remove cellobiose or single glucose units. There is thus an increase in substrate for the exoglucanases due to the cleavage of the glucanase chain by the endoglucanases, and the action of the exoglucanases will result in structural loosening of the cellulose chains, yielding more sites for the endoglucanases to attack (Gan and Taylor 2003, Levi *et al* 2002).
Exoglucanase cleaves cellobiose and D-glucose from the non-reducing end of glucan and cellulose (Bawa and Sandhy 1996).

Cellobiase (β-glucosidase) is a substrate-specific exoglucanase that cleaves the β-1,4 glycosidic bonds between two glucose units of cellobiose (Goodwin and Mercer 1983). Figure 5 demonstrates the enzymatic degradation of cellulose.

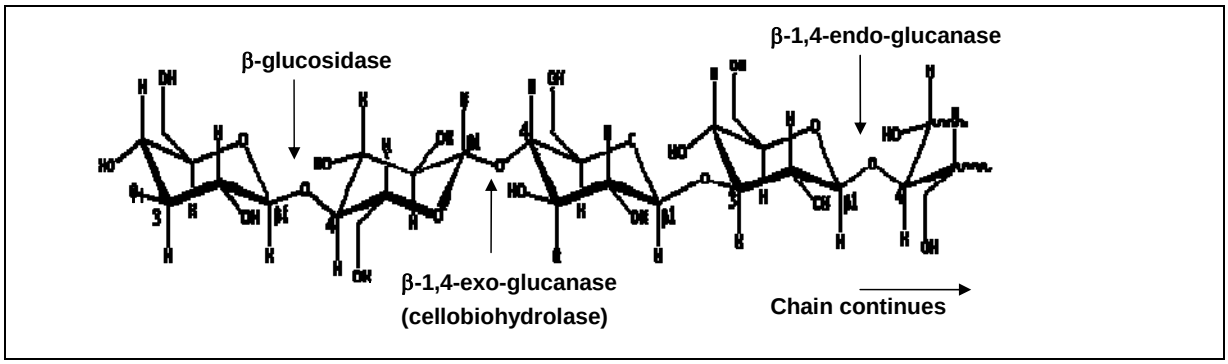


Figure 5 Enzymatic degradation of cellulose

The β-1,4-glucanases retain glycoside hydrolases that cleave the β-1,4 glycosidic bonds between two glucose units in the centre of the polysaccharide chain. Hydrolysis of the glycosidic bonds is catalysed by acid-base catalysis via a double-displacement mechanism, and requires both a proton donor and a nucleophile/base for catalysis. The configuration of the anomeric centre is retained after hydrolysis.

The β-1,4-glucanases generally have three functional units, the catalytic unit, the anchoring unit and the linker unit, which connects the anchoring and catalytic units. In microbial β-1,4-glucanases, the anchoring unit is a cellulose-binding domain (CBD), while in plants it can be a membrane-binding domain or be absent in the secreted enzyme. Several genes have been cloned from bacteria that lack a CBD and consist only of catalytic domains (Levi *et al* 2002).

Most exoglucanases are glycoproteins and exist as single polypeptides with a narrow range of molecular weights. These enzymes are most stable under acidic conditions and prefer low molecular weight cellulose substrates (Van Rensburg and Pretorius 2000).

2.3.3 HEMICELLULASES (XYLANASES)

Hemicellulose has a high degree of polymerisation and is highly branched. The enzymes that catalyse the breakdown of hemicellulose can be divided into the enzymes that degrade the backbone of the chain, and the debranching enzymes, which catalyse the hydrolysis of substituents on the chain. Hemicellulases that hydrolyse the main chain can be classified according to the predominant monosaccharide in the chain and can thus be divided into β-D mannases, β-D galactanases, and β-D xylanases (Thomson 1993).

Xylan degradation is performed by a group of enzymes collectively called xylanases (Chávez *et al* 2002). The endo-β-1,4-xylanases hydrolyse the glycosidic linkages between β-1,4-linked xylopyranosyl groups of the D xylan chain only at unsubstituted regions to produce mixtures of xylo-oligosaccharides (La Grange 1995). The endo-β-1,4-xylanases are single sub-unit proteins, with molecular masses ranging from 8-145 kDa. These enzymes are often glycosylated and this has been linked to the stabilisation of the xylanases in extreme environments (Funaguma *et al* 1991, Kulkarni *et al* 1999). The xylanases vary in substrate specificity towards cellulosic substrates; some are specific only for xylan, while non-specific xylanases will act against carboxymethyl cellulose and xylan. The difference in specificity could be due to differences in the residues involved in the catalytic site of the enzyme (Kulkarni *et al* 1999).

The xylo-oligosaccharides produced by endo-β-1,4-xylanase can be completely hydrolysed to xylose through the action of β-xylosidases. This enzyme is thus necessary for the complete breakdown of xylan to xylose and is found in many bacterial and fungal hemicellulotic systems. β-Xylosidases can be mono- or dimeric proteins, with molecular mass values ranging from 60 to 360 kDa. They are glycosylated, have been shown to have transferase activity, and are competitively inhibited by xylose and show no activity towards xylan (La Grange 1995).

Due to acetylation and substitution on the backbone, the β-xylanases can be prevented from attacking and thus degrading the chain by steric hindrance. These substituents on the chain must thus first be removed by the debranching enzymes before extensive degradation can take place. The synergistic action of the

debranching enzymes, acetylxylan esterases, α-L-arabinofuranosidases and α-glucuronidases, with xylanases is therefore essential for the complete hydrolysis of xylans (Kulkarni *et al* 1999, Van Zyl and Pretorius 1995). Figure 6 shows all the enzymes needed for the complete breakdown of xylan.

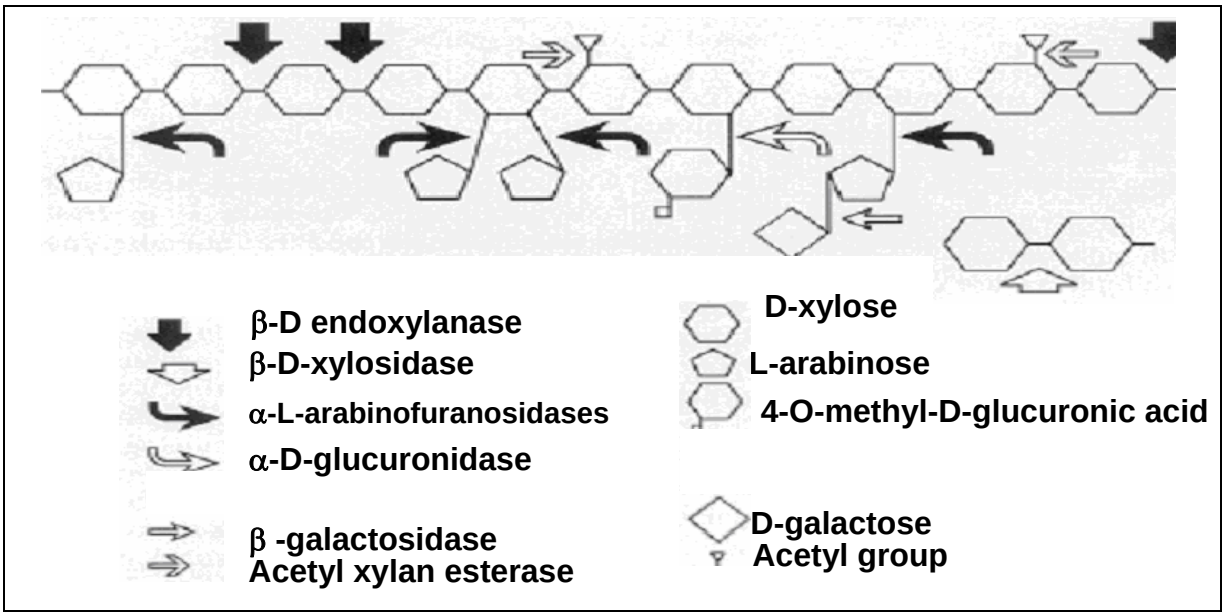


Figure 6 Enzymatic degradation of xylan (Prescott *et al* 2002)

2.4 THE INFLUENCE OF THE ENZYMATIC DEGRADATION OF POLYSACCHARIDES ON WINE PROCESSING

Enzymes are widely used in the juice and wine industries to improve processing and produce clear or stable cloudy juices and concentrates. The addition of enzymes at various stages of the winemaking process can have many positive impacts. It can result in an increased juice yield, improve filterability, increase colour intensity, improve clarity and stability, and result in the extraction of volatile components that can alter the aromatic profile of the wine. Enzyme action can also result in a decrease in viscosity and processing time.

2.4.1 JUICE EXTRACTION

2.4.1.1 Factors influencing juice extraction

When grapes are crushed, the juice in the peripheral cells, which are ruptured slowly, drains from the grape berry. The rate of juice flow depends on the cultivar, the time of draining, restrictions to juice flow, pressure, and the addition of macerating enzymes (Rankine 1998). When making red wine, free-run juice is the juice that flows spontaneously from the pulp due to gravity. If a maceration period is allowed when making white wine, free-run juice can also be collected before pressing. All the wine that remains in the pomace (crushed grapes) after free-run collection constitutes the

press wine. The mechanical operations during pressing lacerate tissues and result in the extraction of certain phenols and tannins, increasing astringency, bitterness and herbaceous characteristics (Ribéreau-Gayon *et al* 2000a). With red wine, the pomace is usually pressed twice, with the juice from the first press still being of a high quality. White cultivars can require more pressing. The free-run and press juice might be blended. Wines made from free-run juice are usually of a higher quality (Rankine 1998).

2.4.1.2 The Influence of polysaccharase action on juice extraction

The addition of pectinases before pressing can result in an increase in free-run juice and yield a pulp with better pressing characteristics, thus resulting in more first-press juice for a better quality wine. Although an increase in free-run juice yield is often seen, the increase in total juice yield is usually less dramatic (Neubec 1975). The enzymes used for juice and colour extraction during maceration are known as macerating enzymes and consist of a mixture of pectinases and cellulases. During maceration, pectinases degrade the middle lamella to form a suspension of intact cells, resulting in thin free-run juice. Cellulases and hemicellulases degrade the cell walls so that the vacuolar content can be released or diffuse into the juice (Parley 1997).

Several studies have shown an increase in free-run juice when pectinases are applied to white must. In a study done on grapes of the Kardaka variety, Canal- showed that the addition of the commercial macerating enzyme, Vinozyme (Novo Nordisk Ferment), at 2 g/hl resulted in an increase of up to 30% in free-run juice (Canal-Llaubères 1989). When a commercial pectinase (Pectinol 60G, Gencor) and macerating enzyme with pectinase, cellulase, and glucanase activity (Macerating Enzyme GC219, Gencor) were compared on *Vitis rotundifolia*, a grape cultivar that is hard to press, the macerating enzyme resulted in a slightly higher juice yield than the pectinase (Sims *et al* 1988). Increases in free-run juice of between 5 and 15% and small increases in total juice yield were also reported upon treatment of must with a macerating enzyme (Plank and Zent 1993). Laboratory and plant trials were conducted to evaluate the efficiency of five commercial enzyme preparations on juice extraction (Haight and Gump 1994). The five commercial macerating enzyme preparations were all tested on a laboratory scale on Ruby red grapes. On the basis of the laboratory trials, the macerating enzyme Cytolase M102 (Genencor International Inc.) was chosen to be evaluated in a commercial plant. Treatment with this enzyme yielded a 19.4% increase in juice yield on a laboratory scale when compared to an untreated control. On a commercial scale, treatment with Cytolase M102 yielded an 8.5% increase in juice yield compared to an untreated control and a 7.9% increase when compared to a commercial pectinase, Pectinol RS (Haight and Gump 1994).

These studies were all done on total and free-run juice yield from must, and not on wine yield, after fermenting on the pomace. Fermenting on the skins could

diminish the yield differences between enzyme- and non-enzyme-treated wine, due to increased skin contact time and the influence of ethanol on extraction. When Pinot noir fermentations were treated with a pectolytic enzyme, Rohapect VR-C (Rohm, Darmstadt, Germany), no increase in free-run or total wine yield was found in comparison to an untreated control (Parley 1997).

2.4.2 MUST CLARIFICATION AND WINE FILTRATION

2.4.2.1 Factors influencing must clarification and wine filtration

New wine has a very high content of suspended particles of multiple origin. These particles make the wine turbid, spoil the appearance, and can affect the flavour as well as the filtration of the wine. Wine can be clarified through gradual settling followed by racking. Polysaccharides can contribute towards turbidity and filtration problems. The main polysaccharides causing these two problems are pectins and the β-1,3-1,6-glucan secreted as a result of grey rot caused by *Botrytis cinerea* (Dubourdieu *et al* 1981). These polysaccharides form protective colloids, causing cloud particles to remain in suspension. These particles cannot be removed through flocculation or aggregation and must be removed through sedimentation. Bentonite fining can be used to remove the cloud-forming particles, but the glucan will remain in suspension and filtration problems will therefore persist. In the presence of alcohol, the β-1,3-1,6-glucan precipitates in a characteristic filament form. This causes extensive filtration problems in the wine after fermentation (Villettaz *et al* 1984, Ribéreau-Gayon *et al* 2000a).

2.4.2.2 The influence of polysaccharase action on must clarification

It has been shown that treating the must with pectolytic enzymes destabilises the colloidal equilibrium, resulting in the aggregation and settling of cloud-forming particles and therefore improving the clarification of the must. The degradation of pectin also results in a distinct improvement in the filtration of pectinase-treated must (Canal-Llaubères 1989). The β-1,3-1,6-glucan can be hydrolysed by commercial macerating enzyme preparations. In a study done by Villettaz et al (1984), it was shown that treating a must containing *B. cinerea* glucan levels of up to 10 mg/L with a commercial enzyme preparation, Glucanex (Novo-Nordisk), consisting of an endo-β-1,3-glucanase and an endo-β-1,6-glucanase, greatly increased the amount of wine that could be filtered per m^2, while reducing the filtration time. Degradation of the *Botrytis* glucan did not alter the organoleptic properties of the wine. Bentonite was used as a fining agent and was applied after glucan hydrolysis, since bentonite binds and inhibits the enzyme (Villettaz *et al* 1984). In another study, two commercial pectinase preparations, Clarex-L and Sparl-L-HPG (Miles Laboratories), were applied to the must of eight different white grape varieties and resulted in an increase in total juice yield, an improvement in clarity and higher quality wines when compared to untreated controls (Brown and Ough 1981).

2.4.3 COLOUR EXTRACTION

2.4.3.1 Factors influencing wine colour

Anthocyanins are flavonoid phenols present in the vacuoles of the skin cells of red grapes. These pigments are responsible for the colour of red wine. They are extracted from the grape skins during maceration and the extraction of phenols from the skin will depend on a variety of factors, such as grape variety, grape maturity, and length and temperature of maceration.

Anthocyanins exist in an equilibrium between five different forms in young red wines: four free forms and one bound to SO_2. The ratio between these forms will determine red wine colour. The red colour comes from the red flavilium form, and when the equilibrium shifts towards this form, it increases colour intensity at a low pH. However, when bound by SO_2, it shifts towards a colourless form, resulting in bleaching.

Anthocyanins often occur as loosely associated complexes with themselves or other compounds. This aggregation can increase the absorption of light and thus the colour intensity. Flavonoid phenols, hydroxycinnamic acid esters and polyphenols are often involved in these complexes (Somers and Westcomb 1982). Heat and alcohol can destabilise the complexes and can thus result in a loss of colour towards the end of fermentation. Flavonoid tannins are also extracted during fermentation. These tannins can polymerise with the free anthocyanins. Polymerisation can be about 25% at the end of fermentation, and continues after fermentation. It can reach levels of up to 40% after a year and can continue to 100% polymerisation after a number of years. Polymerisation is very important for colour stability in red wines, since it protects the anthocyanidin portion of the polymers against oxidation and chemical modification, as well as protecting the anthocyanin molecule against bleaching by SO_2. A larger percentage of anthocyanins are coloured when they are polymerised than when in the free form and this might increase colour intensity, but the red flavilium causes oxidation state changes when it is bound and changes to a yellow-brown colour. The loss of red colour, together with the increase in yellow-brown and the violet colour of the kinoidal form, will cause the wine to progressively take on a brick-like colour (Somers and Westcomb 1982). Polymerisation with tannins will increase at low pH, because of an increase in the flavilium form, and polymerisation is also induced by acetaldehyde (Somers and Evans 1986, Ribéreau-Gayon and Glories 1987). A variation in the content of tannins that are able to react with anthocyanins can be one of the reasons for differences in colour stability between red wines (McCloskey 1974). The extraction and management of anthocyanins in young wines is vital to red wine colour, quality and style, and it has been shown that there is a positive correlation between red wine colour and overall wine quality (Somers 1978).

2.4.3.2 Influence of polysaccharase action on colour extraction

Several studies have shown that the addition of pectolytic or macerating enzymes during maceration increases the extraction of phenols and tannins, which will influence wine colour. However, there are contradicting studies that show that these enzymes do not enhance colour intensity, and might even decrease it. Early studies showed significant decreases in colour intensity in pectinase-treated red wine (Cruess *et al* 1955). These colour decreases were caused by the degradation of anthocyanins by anthocyanases present in these early fungal pectinase preparations (Cruess *et al* 1955, Huang 1955). Ough *et al* (1975) showed that pectinase addition to the pomace can increase the rate of pigment extraction. They showed that the difference in colour intensity between treated and untreated wines was the most significant after 48 hours of skin contact. Longer skin contact diminished the difference in intensity due to the influence of ethanol and other enzymes on extraction. Since pigments are extracted at a higher rate, the pomace can be pressed earlier when it is treated.

The Australian Wine Research Institute tested the ability of a range of commercial pectinases to improve the colour intensity of red wines and their ability to increase the rate of colour extraction from red grape skins. Several macerating, clarifying and colour extraction preparations were compared. The study did not find significant increases in the parameters tested and concluded that pectinase addition for improved colour extraction is unnecessary (Leske 1996).

However, several subsequent studies have shown pectinase and macerating preparations to have a significant impact on colour extraction and stability. Haight and Gump compared the ability of four pectinase preparations with novel side-activities and of a macerating enzyme to improve colour extraction from Barbera grapes during winemaking to that of an untreated control. The pectinases used were Cytolase PLC5 (Gist-brocades), Pectinex BE 3 (Novo Nordisk ferment), Pectinex 5XL (Novo Nordisk ferment), and the macerating enzyme Vinozym EC (Novo Nordisk ferment). All these enzymes showed an increase in colour intensity, with the pectinase preparation Pectinex 5XL showing the greatest increase in colour compared to the untreated wine, followed by Pectinex BE 3 and the macerating enzyme (**http://wwwcaticsufresnoedu**). In trials done by Scottzyme Laboratories in 1996, Pinot noir grapes were treated with two different enzyme preparations, Scottzyme Colour Pro and Colour X, at the highest dosage. The wines that were produced had higher concentrations of total phenols and anthocyanins compared to the untreated controls. This study was expanded in 1997, when five different enzyme preparations were added to Pinot noir grapes at a high and a low dosage. The enzyme preparations were Scottzyme Colour Pro and Colour X, Lallzyme X (Lallemand), Rapidase X Colour (Gist Brocades) and Vinozyme G (Novo Nordisk ferment). All the treatments showed a lower free anthocyanin content after malolactic fermentation, but a higher colour intensity. This was attributed to polymerisation and co-pigmentation of the anthocyanins with the higher phenolic fraction extracted by

the enzyme treatment (Watson *et al* 1999). Parley treated Pinot grapes with a pectinase preparation, Rohapect VR-C (Rohm), and the results were similar to that of the trials done by Scottzyme. The pre-fermentation enzyme maceration did not result in an increase in the extraction of anthocyanins, but the wines produced by enzyme maceration had increased visible colour intensity, which corresponded to that of the more polymeric pigments. The progression from mono- to polymeric pigments was promoted by enzyme extraction, even after clarification, stabilisation and sterile filtration, and Parley attributed this to the breakdown of protective polysaccharide-protein colloids by the enzymes (Parley 1997).

2.5 THE INFLUENCE OF THE ENZYMATIC DEGRADATION OF POLYSACCHARIDES ON WINE AROMA AND FLAVOUR

Wine flavour is the result of a complex combination of multiple components that gives each wine its distinctive character. The flavour of the wine is dependent on several sensory perceptions sensed by the nose and mouth, and is a combination of the taste, odour and mouth-feel of the wine. Wine aroma only refers to the odorous component of the wine, but aroma and flavour are often used interchangeably. Wine flavour develops during a variety of stages and is influenced by several factors, which contribute to its complexity. The most important factors are grape metabolism, (influenced by grape variety and climatic conditions), biochemical changes occurring prior to fermentation (oxidation and hydrolysis reactions occurring during juice extraction and maceration), fermentation metabolism, and chemical and enzymatic changes that occur after fermentation, during ageing (Ribéreau-Gayon *et al* 2000a).

Wine aromas are made up of several hundreds of volatile compounds belonging to a variety of classes, such as hydrocarbons, alcohols, terpenes, esters, aldehydes, ketones, acids, lactones, and sulfur and nitrogen compounds. The concentrations and olfactory perception thresholds of these compounds vary considerably, and the impact of a compound on the aroma of a wine will thus depend on the type of compound as well as the concentration. The varietal aroma of wine refers to the individual aromatic profile of wines made from each grape variety and is dependent on the concentrations and combinations of the volatile compounds. Table 2 shows some of the volatile compounds and its associated flavour and aromacharacteristics. Wine flavour is also greatly influenced by phenolic compounds. This group of compounds mainly affects the appearance, taste and mouth-feel, but can also affect aroma. The phenols originate mainly from the grape berries and stalks, or are extracted from wood during ageing, and are responsible for most of the flavour differences between red and white wines (Dumon *et al* 1991, Ribéreau-Gayon *et al* 2000a). The most important flavour and aroma compounds are discussed in the following section.

Table 2 Volatile compounds that occur in wine and their associated effects on aroma and flavour (http://www.lal.ufl.edu/).

Component	Aroma descriptor	Thresholds (ppb)	
		Aroma	Taste
Alcohols			
propanol, 2	-	-	-
butanol, 1	-	500	-
hexanol, 1	cut grass, sweet	2500	-
Acids			
octanoic acid	oily, rancid, sweat-like	3000	5300
decanoic acid	unpleasant, sour, fatty	10000	3500
acetic acid	pungent, stinging sour	-	-
Esters			
hexyl acetate	sweet, fruity	2	-
ethyl acetate	pleasant, ethereal, fruity	5-5000	3000-6600
ethyl hexanoate	fruity	1	-
linalyl acetate	sweet, floral, fruity	-	-
phenyl ethyl acetate	rose	-	-
benzyl acetate	apple	-	-
isoamyl acetate	banana	-	-
Terpenes			
geraniol	floral, rose-like	40-75	-
α-terpineol	floral	-	-
citronellol	rose like, fresh	40	-
nerol	sweet, fruity, floral	300	-
linalool	floral, green, citrus	6	-

2.5.1 PHENOLIC COMPOUNDS

2.5.1.1 Influence of phenolic compounds on flavour and aroma

Phenols are compounds with a hydroxyl group directly bonded to an aromatic ring (McMurry 1992). The phenolic compounds in wine can be divided into two groups, flavonoids and non-flavonoids. The most common flavonoids in wine are flavonole and catechin, while anthocyanin and leucoanthocyanins are also found in red wine. The flavonoids can occur as monomers or they can polymerise with other phenols or sugars. When catechins and leucoanthocyanins polymerise, they are known as procyanidins. Flavonols bonded to sugars are known as glucosides, and when linked to non-flavonoids by ester bonds they form acyl derivatives. The non-flavonoids are primarily derivatives of hydroxycinnamic acid and hydroxybenzoic acid. They occur

mainly in the grape cell vacuoles and can be esterified to sugars, organic acids and various alcohols. Flavonoid and non-flavonoid polymers are called tannins, and the occurrence of polymeric tannin is high in grape seeds, moderate in grape skins and absent in clarified juice.

The phenol content of the wine increases during fermentation on the skins, and then decreases as phenols are bound and precipitated by proteins during fining and ageing, although it can increase during wood ageing due to extraction from the wood. The tannin content changes after bottling due to structural modifications that take place. The procyanidin molecules from the grapes can polymerise, condense with anthocyanins and combine with plant polymers, such as proteins and polysaccharides originating from grapes, yeast and fungi. These transformations have a major effect on wine flavour (Ribéreau-Gayon *et al* 2000b, Dumon *et al* 1991, Moore *et al* 1995).

Phenolics and tannins can contribute to several positive and negative characteristics in wine. Some of the positive characteristics due to tannins are body, backbone, structure, fullness and roundness, while bitterness, roughness, harshness, astringency and thinness are faults that can be attributed to phenolic compounds. Tannins react with glycoproteins in saliva and proteins in the mouth wall, and the type and concentration of tannins will determine if the wine is perceived as having a soft and balanced taste or as bitter and astringent. This interaction between the proteins in the mouth and the tannins will depend on the polymerisation of the tannins, with astringency increasing with the degree of polymerisation up to a maximum level, where the polymers become too bulky too interact with the proteins (Arnold *et al* 1980). A maximum level of bitterness is reached at the tetramer level of polymerisation, while the maximum astringency is reach at octamer level. It has been shown that ethanol increases the intensity of bitterness, while it decreases the intensity of astringency (Delcour *et al* 1984). When tannin, procyanidin and anthocyanin fractions were isolated from the grapes and stalks and taste tests were done on the extracts, it was found that catechins with a low level of polymerisation (dimers, trimers) did not contribute much to astringency. Oligomeric and polymerised procyanidins resulted in body, bitterness and astringency. Condensation with polysaccharides resulted in a decrease in astringency, but more fullness and roundness. It was also found that tannins from seeds contribute to wine structure and body, while tannins from the skin affect roundness, fullness and colour. However, excessive seed tannins can result in very high astringency, while high skin tannins can cause bitterness and herbaceous characters (Ribéreau-Gayon and Glories 1987). Although anthocyanins are present in high concentrations in red wines, they do not contribute directly to the flavour of the wine. The anthocyanins combine with the condensed tannins in the must and wines, increasing the amount of polymeric phenols that are retained in the wine. These polymers affect astringency and flavour (Singleton and Trousdale 1992).

2.5.1.2 Influence of polysaccharase action on phenolic compounds

Several studies have shown that treating must with polysaccharases results in an increased extraction of total phenols. In one of the early studies, treatment with pectinase preparations resulted in a small increase in flavonoids in white must, a 12% increase in flavonoids in red must, and an increase in total phenols in both white and red must. No significant quality difference was found between the treated and untreated wines during tasting (Ough and Berg 1974). Zent and Inama showed that the treatment of red Valpolicella grapes with macerating enzymes resulted in an increased extraction of total phenols and tannins compared to untreated controls. When tested through tasting and composition analysis, these wines showed an improvement in taste and quality (Zent and Inama 1992). In the studies done by Scott Laboratories (described under colour extraction, section 2.4.1), it was found that the addition of Scottzyme colour Pro and Colour X resulted in an increased extraction of total phenols and anthocyanins. The Pinot noir wines that were produced had an enhanced aroma and a spicy and fruity flavour intensity, as well as enhanced bitterness and astringency characteristics compared to the untreated controls. When five commercial enzyme preparations were subsequently compared, it was found that treatment with all five preparations resulted in an increase in total phenols, polymeric anthocyanins, polymeric phenols and catechin in the treated wines in comparison to untreated controls. The enzymes were applied at both high and low doses, and it was found that treatment with low dosage rates produced wines with enhanced fruity, floral, spicy and body characteristics. The high dosages showed similar aroma characteristics, but resulted in enhanced acidity, bitterness and astringency characteristics (Watson *et al* 1999). Treatment of Cabernet Sauvignon must with the commercial pectinase, Lallzyme EX-V, resulted in wines with a higher total and polymer phenol content. When tasted directly after bottling, no significant difference was found between the treated and control wines. When tasted after a year, the treated wine was preferred, with more colour and complexity, as well as more noticeable mouth-feel characteristics than the untreated control wine (Guerrand and Gervais 2002).

2.5.2 MONOTERPENES

2.5.2.1 Influence of monoterpenes on flavour and aroma

Terpenes are chemically characterised in terms of their characteristic carbon skeleton, consisting of 5-carbon isoprene units. Depending on the number of isoprene units, they can be classified as mono-, di-, tri- or sesquiterpenes. Monoterpenes are an important group of aromatic compounds that contribute significantly to the fruity and floral aromas of wine. In contrast to many other aromatic components found in wine, monoterpenes originate primarily from the grape. These compounds form the axis of the wine bouquet. Because the bouquet is unique to a variety, monoterpenes can be used analytically for varietal characterisation (Mateo

and Jiménez 2000, Strauss *et al* 1986). Monoterpenes occur in the form of simple hydrocarbons, aldehydes, alcohols, acids and esters. About 40 monoterpenes have been identified in grapes. Among the most odoriferous are the monoterpene alcohols, especially linalool, α-terpineol, nerol, geraniol, citronellol and ho-trienol, which are responsible for a floral or rose-like aroma (Ribéreau-Gayon *et al* 2000b, Strauss *et al* 1986).

The monoterpenes are present as free, volatile forms, and as glycosidically-bound non-volatile precursors (Ganga *et al* 2001). The non-volatile forms are flavourless and occur as disaccharide or monosaccharide glycosides (Ganga *et al* 1999). Glycosidic bonds are formed with the sugars glucose, arabinose, rhamnose and apiose. The glycosides can be enzymatically hydrolysed or they can be degarded chemically by acid hydrolysis.

Enzymatic hydrolysis of the glycosides is carried out by various enzymes, which act sequentially according to two steps. Firstly, the terminal sugar is cleaved by either an α-L-rhamnosidase, an α-L-arabinosidase or a β-D-aposiase, releasing a β-D-glucoside. A β-D-glucosidase then acts on the β-D-glucoside to release an aromatic monoterpene. Grapes contain β-D-glucosidases, but these enzymes are unstable, with low activity at grape juice or wine pH values, and have been shown to be unable to hydrolyse the glycosides of tertiary alcohols such as linalool and α-terpineol. It has been shown that *S. cerevisiae* alters the terpene profile of the wine, but most of the β-glucosidases that are produced are inhibited by ethanol. Fungal glycosidases are often present as contaminants in commercial pectinase preparations made from *A. niger*. These fungal enzymes are not inhibited by wine pH or ethanol, and treating wine with commercial glycosidase preparations or macerating enzymes with these side-activities will bring out the flavour of young wines made from Muscat-flavoured grape varieties (Vázquez *et al* 2002, Mateo and Jiménez 2000).

2.5.2.2 Influence of polysaccharase action on monoterpenes

The largest fraction of bound terpenols is located in the grape skin, with lower amounts being found in the pulp and juice. Treatment of the must with cellulases, hemicellulases and pectinases that degrade the cell wall might contribute to the release of bound precursors from the skin and pulp, and their subsequent hydrolysis during fermentation can lead to an improvement in wine aroma (Zoecklein *et al* 1997). Several studies showed an increase in wine aroma as a result of treatments with commercial enzyme preparations, but it was not possible to know which enzyme actions to attribute these effects to, since there are side-activities (hemicellulolytic, cellulolytic, and glycosidic) in these preparations (Ganga *et al* 2001, Haight and Gump 1994). Studies were done in which purified fungal glucanase and xylanase enzymes, produced by recombinant *S. cerevisiae* strains, were added to the must, or the must was inoculated with the recombinant polysaccharide-degrading strain. These studies showed an increase in aroma precursor levels (including terpene glycosides) compared to wines made from untreated musts. The terpene levels

(aglycon moieties) of these wines were also higher, resulting in more fruity aromas that could be detected sensorially (Ganga *et al* 2001, Gil and Vallés 2001, Ganga *et al* 1999, Pérez-González *et al* 1993). The increases in bound precursors in these wines were as a result of the degradation of the cell walls by the glucanase and xylanase enzymes. Since the aglycon fraction also increased, there must have been subsequent hydrolysis of the precursors, as the glucanase and xylanase enzymes do not posses glycosidic activity and this hydrolysis must have been chemical or due to endogenous glycosidases. If such endogenous glycosidic activities are present, an increase in free volatile terpenes and thus fruitier aroma can be expected if must is treated with glucanases and xylanases, due to the release of more bound precursors and their subsequent hydrolysis (Gil and Vallés 2001).

2.5.3 HIGHER ALCOHOLS

2.5.3.1 Influence of higher alcohols on flavour and aroma

Alcohols with more then two carbon atoms are known as higher alcohols. Higher alcohols, together with esters and acids, are the aroma components present at the highest quantity in wine and are important for the sensory properties and quality of the wine. Small amounts of higher alcohols contribute positively to wine quality, while excessive amounts might detract from quality. With the exception of 2-phenyl ethanol, most higher alcohols smell bad and, at high concentrations, can contribute to heavy solvent-like odours, e.g. isoamyl alcohol. Higher alcohols are also important for ester formation during the ageing of wine (Gil *et al* 1996).

Most higher alcohols are formed as by-products of the yeast during fermentation. They can be formed by the reductive denitrification of amino acids, or can be synthesised from sugars. Higher alcohols can also be formed by spoilage bacteria or yeasts, and wines displaying higher alcohol off-flavours are usually infected by such spoilage organisms. The most important higher alcohols in wine are the straight chain higher alcohols: 1-propanol, 2-methyl-propanol (isobutyl alcohol), 2-methyl-1-butanol (active amyl alcohol), 3-methyl-1-butanol (isoamyl alcohol) and 2-phenyl ethanol. 2-Phenyl ethanol is the most important higher alcohol originating from the grapes and plays an important role in the rose aroma of wine. Other important higher alcohols originating from the grape are 1-hexanol, 3-octanol, 1-octene-3-ol and benzyl alcohol (Sponholtz 1988, Rapp 1986). It has been shown that the formation of each higher alcohol formation is influenced to varying degrees by winemaking practices, fermentation temperature, juice clarification and the ripeness of the fruit (Houtman and Du Plessis 1981, Moreno 1988). The influence of these factors varies between higher alcohols. Clarification of the juice decreases higher alcohol production. Moreno showed that out of six higher alcohols measured at different fermentation temperatures, 2-phenyl ethanol was the only one significantly influenced with the highest production occurring at a fermentation temperature between 20°C-25° (Moreno *et al* 1988).

2.5.3.2 Influence of polysaccharase action on higher alcohols

The higher alcohols originating from the grape can also occur as glycosidically-bound aroma precursors in the grape. Treatment of the must with cellulases, hemicellulases and pectinases that degrade the cell wall might therefore contribute to the release of bound precursors from the skin and pulp. Subsequent hydrolysis of these precursors during fermentation can lead to an improvement in wine aroma. Gil and Vallés showed that treating must with X_{22}, endo-β-1,4-xylanase produced by a recombinant *S. cerevisiae* strain, resulted in increased levels of isobutyl-alcohol, 1-butanol, 1-hexanol, *cis*-3-hexen-1-ol and benzyl alcohol glycosides in the wine that was produced, in comparison to untreated wine. Treatment with EGL1 endo-β-1,4-glucanase produced by a recombinant *S. cerevisiae* strain resulted in wines with increased levels of *cis*-3-hexen-1-ol and isobutyl-alcohol glycosides compared to the control wine. The aglycon levels of these glycosides were also increased (Gil and Vallés 2001). In another study, it was shown that inoculating the must with a recombinant wine yeast strain producing *A. niger* endo-β-1,4-xylanase led to the production of wine with increased levels of 1-butanol, 1-hexanol and 1-pentanol, and very significant increases in 3-methyl-1-butanol, phenyl methanol and 2-phenyl ethanol (Ganga *et al* 1999). Inoculating the must with a recombinant wine yeast strain secreting a *Trichoderma longibrachiatium* endo-β-1,4-glucanase leads to wine being produced with increased levels of 2-butanol, iso-amyl alcohol, 1-hexanol and 2-phenyl ethanol. The increases in higher alcohols are not only due to the release of glycosidically-bound precursors. The enzymatic action of the polysaccharases on the grape skins alters the composition of the must during fermentation, and such changes could influence yeast metabolism and result in increased or decreased levels of higher alcohol production (Gil and Vallés 2001).

2.5.4 ESTERS

2.5.4.1 Influence of esters on flavour and aroma

Esters are formed during a reversible condensation reaction between the carboxyl group of an organic acid and the hydroxyl group of an alcohol or phenol, with the release of water. There are a large number of different alcohols and acids present in wine, therefore the number of possible esters is also very large. Ethyl acetate and isoamyl acetate are the most important esters found in wine (Plata *et al* 2003). Ethyl acetate is the most abundant ester found in wine, since it is formed in a reaction between acetic acid and ethanol. Ethanol occurs at high concentrations in wine and the primary alcohols are the most reactive.

The amounts of esters present in the grapes prior to fermentation are negligible and esters are formed due to the enzymatic action of the yeast. The synthesis of acetate esters is ascribed to at least three acetyltransferase activities: alcohol acetyltransferase (AAT), ethanol acetyltransferase (EAT) and iso-amyl

acetyltransferase (IAT). These are all sulfhydryl enzymes, which react with acetyl coenzyme A and form esters, depending on the degree of affinity with the corresponding higher alcohol (Pretorius 2000). The most important esters that are formed are the ethyl esters formed between ethanol and straight chain, saturated fatty acids and the acetate esters formed between acetic acid and higher alcohols (Ribéreau-Gayon *et al* 2000b, StEvans *et al* 1969). The ethyl esters of fatty acids have pleasant odours of wax and honey and can contribute to the aroma of white wines. The acetic esters of higher alcohols are present in moderate quantities and are responsible for many of the fruity characteristics, e.g. benzyl acetate has an apple-like aroma, phenyl ethyl acetate has a rose aroma and isoamyl acetate (3-methylbutyl acetate) has a banana-like aroma (Bertrand 1983). Isoamyl acetate has been shown to be the impact compound, responsible for the unique fermentation bouquet of Pinotage wine formed during fermentation (Van Wyk *et al* 1979). Ester formation can be influenced by a variety of factors. There is variation in esterase activity between different yeasts. Low fermentation temperatures promote the synthesis of fruity esters, while high temperatures promote ester hydrolysis and juice clarification and low levels of SO_2 promote ester formation and retention (Killian and Ough 1979).

2.5.4.2 Influence of polysaccharase action on ester formation

Higher alcohols are important as precursors for ester formation during ageing (Gil *et al* 1996). The formation of esters depends on the esterase activity of the yeast and the presence of the corresponding precursor higher alcohol in the must. It has been shown that ethyl acetate production by a *Kloeckera apiculata* strain increases after 48 hours, once the ethanol concentration in the must starts to increase. In addition, the isoamyl acetate concentration increases after 72 hours of fermentation, after enough isoamyl alcohol for ester synthesis has accumulated in the must (Plata *et al* 2003). Several studies show that treating the must with polysaccharases results in alterations in the higher alcohol and monoterpene profiles of the wine. These studies also show changes in the production of some of the corresponding esters (Ganga *et al* 1999, Gil and Vallés 2001, Pérez-González *et al* 1993). Ganga *et al* show that endo-β-1,4-xylanase treatment results in increases in 2-phenylethanol and the corresponding ester, 2-phenylethyl acetate. They also show increases in linalool and linalyl acetate, and in 1-hexanol and hexyl-acetate, in wines produced from treated must (Ganga *et al* 1999).

2.6 COMMERCIAL ENZYME PREPARATIONS IN THE WINE INDUSTRY

The industrial use of microbial enzymes in the Western world started more then a hundred years ago, when α-amylase from *Aspergillus oryzae* was patented. Commercial enzyme preparations most commonly consist of the extracellular material produced during controlled fermentation of pure fungal strains. *Aspergillus* is

the major source of enzymes for use in food and beverage manufacturing and *Aspergillus* enzymes are used for such diverse applications as starch processing, cheese manufacturing, juice clarification, brewing, dough conditioning, food preservation, instant tea production and winemaking. *Aspergillus* is, however, not a good source of cellulolytic activity and the fungal strains from *Trichoderma* have taken the lead in the production of these enzymes. There are also some areas in the industry where bacterial enzymes function better than fungal enzymes, for example where pH requirements are alkaline, as in detergents, or where temperature requirements are well above 60°C, as in starch liquefaction (Prescott *et al* 2002).
The use of commercially produced, exogenous enzymes has improved the technology and economy of wine production and has enhanced the quality of wine. Research on the use of enzymes for must clarification started in the 1930s, but pectinolytic enzymes have commonly been used in wine production only since the 1970s and glucanases were introduced to wine manufacturing in the 1980s (Cruess *et al* 1955, Prescott *et al* 2002). These enzyme preparations are used during winemaking in order to improve the clarification and processing of wine, to release varietal aromas from precursor compounds, to reduce ethyl carbamate formation and to lower alcohol levels in wine (Van Rensburg and Pretorius 2000).

2.6.1 COMMERCIAL PECTINASE PREPARATIONS

2.6.1.1 Importance of commercial pectinase preparations in winemaking

Pectinases occur naturally in grapes and are secreted by some organisms of the grape microflora. These endogenous enzymes are, however, inhibited by low fermentation temperatures, SO_2, tannins, bentonite treatment and high alcohol concentration. Due to these inhibiting factors and low enzyme concentrations, the endogenous pectinases are often not sufficient and commercial pectinase preparations are added to improve the clarification and filtration of wine. Experiments on the clarification of wine by pectic enzymes were done by Besone and Cruess as early as in 1936, and trials were conducted on a commercial scale in 1946 (Cruess and Besone 1941, Cruess and Kilbuck 1947). Most commercial pectinase preparations are produced by the *Aspergillus* species, mainly *A. niger.* These preparations have GRAS status (generally regarded as safe) (**http://wwwcaticsufresnoedu**).

The addition of pectinases before pressing can result in an increase in free-run juice and yield a pulp with better pressing characteristics, thus resulting in more first-press juice for a better quality wine (Neubec 1975). It has been shown that treating the must with pectolytic enzymes destabilises the colloidal equilibrium, resulting in the aggregation and settling of cloud-forming particles and thus improving the clarification of the must. Degradation of the pectin also results in a distinct improvement in the filtration of the pectinase-treated must (Canal-Llaubères 1989). Several studies have shown that the addition of pectolytic or macerating enzymes

during maceration increases the extraction of phenols and tannins and will thus influence wine colour (Guerrand and Gervais 2002, Watson *et al* 1999, Zent and Inama 1992).

2.6.1.2 Activity of pectinase preparations

Most pectinase preparations for grape and wine processing contain pectin esterases and endo-polygalacturonases, and might also contain pectin lyase and protopectinase activities (Plank and Zent 1993). The activity of commercial pectinase preparations can be measured in Apple Juice Depectinising Activity (AJDU). These units are based on the reciprocal time required to clarify apple juice at 45°C and a pH of 3.5. Pectinase activity can also be measured in polygalacturonase activity units (PGU). These units are based on the reduction in viscosity of polygalacturonase substrate at 30°C and a pH of 4.2 (Brown and Ough 1981).

Wine pH does not affect the pectinase activity of commercial preparations, but activity can be affected by temperature. Below 10°C pectinase activity in must is negligible, but the activity doubles for every 10°C increase in temperature. Pectinases are proteins and will be inactivated at temperatures above 50-55°C (Hagan 1996). Sulfur dioxide will only start inhibiting pectinase activity above 500 mg/L while ethanol becomes inhibitory above the concentration of 17% (v/v). Bentonite should only be used after the pectinase action is completed (Van Rensburg and Pretorius 2000).

2.6.1.3 Side-activities of commercial pectinases

Commercial enzyme preparations can be rather crude and, besides containing various pectinases, can also contain various other enzymatic activities, such as glycosidases, esterases, proteases, hemicellulases and cellulases, and the action of these enzymes is known as side-activities. The treatment of red grape must with some of the early pectinase preparations resulted in a decrease in colour intensity due to the degradation of pigments by anthocyanases present in the preparation (Cruess *et al* 1955). Decolouration has also been attributed to β-glucosidase side-activities, resulting in the cleavage of glycosidically-bound anthocyanins to form unstable aglycones, which are transformed to a colourless form (Piffaut *et al* 1994).

The presence of pectin-methyl-esterases in pectin preparations causes the release of methanol from pectins. This alcohol remains in the must and is toxic to humans. Treatment with various pectinases results in increases in the methanol level in wine (Brown and Ough 1981, Revilla and González-SanJosé 1998). Methanol levels can also be influenced by a variety of other factors, such as oenological practices, yeast strain and grape cultivar (Revilla and González-SanJosé 1998). Treatment with pectinases can also result in increases in varietal aroma due to the liberation of monoterpenes from glycosidically-bound precursors in the must by β-glucosidase side-activity in the pectinase preparation (Mateo and Jiménez 2000). Improvements in the purity of enzyme preparations can be obtained through genetic

engineering of the fungal strains to enhance or inhibit the expression of genes for certain enzymes, or through improved commercial separation procedures.

2.6.2 COMMERCIAL GLUCANASE PREPARATIONS

2.6.2.1 Importance of commercial glucanase preparations in winemaking

The commercial β-glucanases used in the wine industry are produced by *Trichoderma* species (Bhat 2000). These enzyme preparations consist of purified glucanases, or the glucanases can be part of a macerating enzyme preparation. Macerating enzymes are preparations with pectinase, cellulase and hemicellulase activities. Due to the more complete breakdown of the cell wall, these enzymes can result in improvements in clarity, juice yield and colour extraction in contrast to pure pectinases (Haight and Gump 1994). It has been shown that the treatment of must with macerating enzymes such as Scottzyme Colour Pro, Lallzyme EX and Rapidase EX colour can result in alterations in the colour intensity, aroma, flavour, body and mouth-feel characteristics of the wine (Watson *et al* 1999).

β-Glucanase preparations are used in the industry to improve the filtration of wines produced from *Botrytis cinerea*-infected grapes. This fungus produces a β-1,3-1,6-glucan, which is polymerised by ethanol, resulting in filtration problems. The treatment of infected must with glucanase preparations containing β-1,3 and β-1,6-glucanase activity, such as Glucanex, results in the degradation of glucan and greatly improves the filterability of the wine (Villettaz *et al* 1984). Glucanase preparations have also been shown to facilitate the extraction of cell wall mannoproteins from yeast. This affects the ageing of white wines on lees and, due to the binding of mannoproteins with tannins, can decrease the formation of yellow colour in white wines (Ribéreau-Gayon *et al* 2000a).

2.6.2.2 Activity of glucanase preparations

β-Glucanase activity can be measured in β-glucanase units (BGXU). One β-glucanase unit corresponds to the quantity of enzyme required to produce 1 millimol of reducing sugars per minute from *Botrytis* glucan at 30°C in 10 minutes (Canál-Laubères 1989). Commercial glucanase preparations are normally active between 15 and 50°C at a pH of 3-4. Sulfur dioxide will only start inhibiting pectinase activity above 350 ppm while ethanol becomes inhibitory above 14% (v/v) (Van Rensburg and Pretorius 2000).

2.7 GENETIC IMPROVEMENT OF INDUSTRIAL WINE YEAST STRAINS

Genetic engineering has allowed for the expression of multiple foreign genes in laboratory strains of *S. cerevisiae*. An increasing number of these genes are now also being expressed in industrial strains (Pretorius and Bauer 2002). Laboratory yeast strains are derived from industrial strains. The laboratory strains have special

features which make them very suitable for genetic manipulation. These strains are usually isogenic, haploid, of either a or α mating type, have sporulation competence when diploid and contain multiple auxotrophic mutations. The industrial strains do not have any of these features. They are genetically diverse, diploid or polyploid, with low sporulation competence, and are prototrophic. The gene manipulation techniques developed for laboratory strains can thus not always be applied to industrial yeast strains, but the industrial strains have a superior ability in food and beverage production (Akada 2002).

2.7.1 TECHNIQUES USED FOR THE GENETIC IMPROVEMENT OF YEAST STRAINS

The genetic improvement of industrial yeast strains traditionally relied on classical genetic techniques, such as hybridisation (mating, spore cell mating, rare mating and spheroplast fusion) and mutagenesis. These genetic alterations were then followed by selection for broad traits, such as fermentation capacity, ethanol tolerance, rehydration, flocculation and the absence of off-flavours (Dequin 2001). With these methods, large genomic regions or entire genomes are rearranged or recombined. These techniques are therefore not feasible to alter only a specific characteristic. Modifications might improve some of the properties of the yeast strain, while compromising others. The advantages of these classical techniques are that they can be used to improve characteristics under polygenic control. The yeast strains produced are not classified as genetically modified organisms (GMOs) and will thus be immediately viable for industrial use. Genetic engineering is the only reliable method with which a specific existing property can be altered or a new characteristic can be introduced, without affecting any other desirable properties.

S. cerevisiae is commonly used for the expression of foreign genes due to a combination of several important characteristics: it is single celled and thus easy to culture, and it is easy to manipulate and genetically, it is the best characterised of all eukaryotes and thus acts as model for other systems. It is widely and safely used in the food industry, has GRAS status and it can grow up to high cell densities in controlled fermentations, making it suitable for use in industrial fermentations (Hadfield *et al* 1993, Sudberry 1996, Van Rensburg *et al* 1997).

Some of the important areas targeted by recombinant yeast strain development are the efficiency of the fermentation process, the processing of wine, microbial spoilage, wine wholesomeness and sensory quality (Pretorius and Bauer 2002). Table 3 shows some of the wine properties that can be improved by genetic engineering, the areas focused on and the genes targeted to obtain these improvements.

Table 3. Properties and genes targeted by genetic engineering for the improvement of wine yeast strains (Pretorius and Bauer 2002)

Desirable properties	Focus areas	Potential target genes
Improved fermentation performance		
Improved resilience and stress tolerance	Stress response, sterol, glycogen and trehalose accumulation	*GSV1,GSV2, TPS1, TPS2.*
Improved sugar utilisation	Hexose transporters, hexose kinases	*HXT1-HXT8, SNF3, FSY1*
Improved nitrogen assimilation	Improved use of less efficient nitrogen sources	*PUT1, PUT2*
Improved ethanol tolerance	Sterol formation, ATPase activity	*PMA1, PMA2*
Improved tolerance to anti-microbial compounds	Resistance to killer toxins, SO_2, agrochemicals	*KIL1, CUP2*
Reduced foam formation	Cell surface proteins	*FRO1, FRO2*
Improved processing efficiency		
Improved protein clarification	Proteases	*PEP4*
Improved polysaccharide clarification	Glucanases, pectinases, xylanases	*END1, EXG1, CEL1, BGL1, PEL5, PEH1, XYN1-5, ABF2*
Controlled cell sedimentation and flocculation	Flocculins	*FLO1, FLO5, MUC1, FLO11*
Improved biological control of wine spoilage organisms		
Wine yeasts producing anti-microbial enzymes	Lysozyme, glucanases, chitinases	*HEL1, CTS1, EXG1*
Wine yeasts producing anti-microbial peptides	Bacteriocins	*PED1, LCA1*
Wine yeasts producing SO_2	Sulfur metabolism, SO_2 formation	*MET1, MET10, MET16*
Improved wine wholesomeness		
Increased production of resveratrol	Stilbene synthesis	*VST1*
Reduced formation of ethyl carbamate	Amino acid metabolism, urea formation	*CAR1, URE1*
Reduced formation of biogenic amines	Bacteriolytic enzymes, bacteriocins	*HEL1, PED1, LCA1*
Decreased alcohol levels	Carbon flux, glycerol metabolism, glucose oxidation	*GPD1, GPD2, GOX1*
Improved wine flavour and sensory qualities		
Enhanced liberation of grape terpenoids	Glycosidases, glucanases, arabinofuranosidases	*END1, EXG1, CEL1, BGL1, PEL5, PEH1, XYN1-5, ABF2*
Enhanced production of volatile esters	Esterases	*ATF1, IAH1*
Enhanced glycerol production	Glycerol metabolism	*GPD1, GPD2, FPS1, ALD6*
Bio-adjustment of wine acidity	Maloethanolic and malolactic fermentation, lactic acid production	*MAE1, MAE2, mleS, LSH1*
Optimisation of phenolics	Phenolic acid metabolism	*PAD1, pdc, padc*
Reduced sulfite and sulfide production	Sulfur metabolism, H_2S formation	*MET14, MRX1*

2.7.2 IMPORTANT GENETIC TOOLS USED FOR THE PRODUCTION OF HETEROLOGOUS PROTEINS

Gene expression and protein secretion entail a complex multi-step process and barriers can be encountered at various stages, from transcription to protein stability (Romanos *et al* 1992). The choice of the correct vector, promoter and secretion signal is thus crucial for optimal gene expression and protein secretion. Several effective transformation techniques and plasmid vectors, as well as expression and secretion cassettes for the expression of heterologous genes and the secretion of the proteins for which they code, have been developed for *S. cerevisiae,* allowing for broader application and higher specificity during recombinant wine yeast strain development.

Most yeast vectors are hybrids between *Escherichia coli* and yeast-derived DNA. The *E. coli* sequences usually make up the origin of replication and the selective marker of the vector. Multi-copy plasmids have an origin of replication and can replicate autonomously in *E. coli* and *S. cerevisiae,* while integrative vectors do not contain a functional replication origin for yeast and need to integrate into the genome in order to be maintained in a population. Integration occurs exclusively by homologous recombination (Hinnen *et al* 1978, Hinnen *et al* 1994).

Gene transcription depends on the 5' and 3' sequences flanking the protein coding sequence. Constitutive promoters are used for the strong, constant expression of heterologous genes in yeast. Genes placed under the control of these promoters are usually efficiently expressed in the presence of glucose, but can be repressed on non-fermentable carbon sources. Constitutive promoters include the promoters of the genes coding for the glycolytic enzymes 3-phosphoglycerate kinase (*PGK*), glyceraldehydes-3-phosphate isomerase (*GAP/TDH3*), enolase (*ENO*) and triose-phosphate isomerase (*TPI*), and the fermentative alcohol dehydrogenase (*ADH1*) (Smith *et al* 1985, Hinnen *et al* 1994). Regulated expression allows for the growth and expression of the regulated gene to be uncoupled and the regulated gene can thus be expressed later in the growth phase or when induced by certain environmental conditions. This is necessary because certain proteins can interfere with cell growth. These promoters are important when engineering industrial yeast strains, since they allow for a protein to be produced only under certain conditions, which can be induced during the fermentation. The regulated promoters used include those of the genes coding for galactokinase/galactose isomerase (*GAL1, GAL10*), acid phosphatase (*PHO5*), regulated alcohol dehydrogenase (*ADH2*) and the copper resistance gene (*CUP1*). Hybrid promoters are also available that combine elements of a strong constitutive promoter with those of a regulated promoter (Schuster 1985, Hinnen *et al* 1994). Promoters can also allow for a basal level of expression, which can be increased upon induction by environmental factors. An example is the *YG100* promoter of the *S. cerevisiae* heat shock gene *HSP70.* This *YG100* promoter has a strong basal level of expression and is induced by heat shock (Slater and Craig 1987).

The sequence of the foreign gene to be expressed can be very important in determining the translation efficiency of the gene. The untranslated 5' leader sequence of yeast genes is not random and shows a strong bias for A residues. Almost no G residues are found. This is important for the expression of heterologous proteins, since the 5' sequences seem to influence the expression levels of foreign proteins dramatically without affecting the levels of mRNA (Bitter and Egan 1984). The sequence can also influence translation of the foreign genes by codon usage. More then one codon can code for each amino acid and yeast shows a bias towards a certain subset of codons. Translation problems could occur if certain codons are present in the gene to be expressed (Sharp and Cowe 1991).

If a protein that is synthesised is to be secreted, it has an N-terminal extension known as a signal sequence. Signal sequences derived from yeast proteins are often fused to heterologous genes. Frequently-used signal sequences are the ones derived from the yeast invertase (SUC2), acid phosphatase (PHO5), and the yeast α-factor pheromone (MFα1) (Hinnen *et al* 1994).

2.8 DEVELOPMENT OF RECOMBINANT *S. CEREVISIAE* STRAINS EXPRESSING PECTINASE, GLUCANASE AND XYLANASE GENES

2.8.1 DEVELOPMENT OF PECTOLYTIC *S. CEREVISIAE* STRAINS

2.8.1.1 Pectinase-producing organisms

Pectic enzymes are naturally produced by a wide variety of organisms in nature, including bacteria, fungi, yeasts, insects, nematodes and protozoa.

Most commercial preparations of pectic enzymes are obtained from fungal sources. Acidic pectinases used in the fruit juice and wine industry are produced by the *Aspergillus* species, mainly *A. niger,* but also by *A. oryzyzae* and *A. wentii.* The production of pectic enzymes by *A. niger* is inducible by pectic substances in the growth medium (Aguilar and Huitron 1987). Some other fungal sources of pectinases are *Penicillium frequentans*, which secretes an endo-polygalacturonase (endo-PG), *P. italicum*, which secretes a pectin lyase (PL), and *Sclerotim rolfski* and *Mucor pusilus*, which secrete endo-polygalacturonases (Kashyap *et al* 2001).

Pectic enzymes are also produced by a wide variety of bacteria, with the most important being species of *Bacillus, Clostridium, Erwinia, Pseudomonas* and *Xanthomonas* (Rombouts and Pilnik 1980). Most well known pectic enzymes are produced by the genus *Erwinia.* The soft rot-causing species in this genus, *E. carotovora, E. chrysanthemi* and *E. arideaia,* produce large amounts of endo-pectate lyase and endo-polygalacturonase. The *peh1* gene codes for the polygalacturonase produced by *E. carotovora.* The enzyme has a molecular weight of 42 kDa, an optimum activity at pH 5.5 and a temperature range from 35°C-45°C. Calcium is not required for activity (Hinton *et al* 1990, Collmer 1986). The *pel* genes code for the

pectate lyases produced by *E. chrysanthemi,* and it has been shown that the *pelE* gene is highly regulated by catabolite repression, substrate induction and growth phase inhibition in this organism (Chatterjee *et al* 1979, Gold *et al* 1992).
Several yeasts with pectolytic activity have been identified. True polygalacturonase activity has been shown in yeasts that belong to the genera *Candida, Pichia, Saccharomyces* and *Zygosaccharomyces* (Sanchez *et al* 1984). *Rhodotorula* species have been reported to have pectin methyl esterase and PG activity, and *S. fragilis* has been shown to have multiple forms of endo-PGs (Vaughn *et al* 1969, Wimborne and Rickard 1978). It has been reported that several *Saccharomyces* species, including *S. carlbergensis, S. chevalieri, S. oviformis*, *S. uvarum* and *S. cerevisiae*, have polygalacturonase activity (Sanchez *et al* 1984).

Several strains of *S. cerevisiae* have been reported to produce pectolytic enzymes. Blanco *et al* (1998) reported that 75% of oenological strains tested showed limited pectolytic activity. Enzyme synthesis was reported to be constitutive below 2% glucose, while higher concentrations led to total inhibition of enzyme synthesis. A wild type *S. cerevisiae* strain, SCPP, which is used in oenology, has been shown to secrete all forms of pectinases. The *PGL-1* gene coding for the endo-polygalacturonase in *S. cerevisiae* has been cloned (Blanco *et al* 1998). The enzyme named Pgl1P has an M_r of 42kDa and is active from pH 3 to 5.5, with an optimum temperature at 25°C (Gainvors *et al* 2000). This gene is present in a single copy per haploid genome and is detected in all strains, regardless of phenotype. The inability of certain strains to degrade pectins might be due to promoterless *PGL-1* genes, or to non-functional promoters (Blanco *et al* 1998).

2.8.1.2 Heterologous expression of pectinase genes in laboratory strains

The *pelE* pectate lyase gene from *E. chrysanthemi* was the first heterologous pectinase gene to be expressed in *S. cerevisiae.* This gene was inserted into nine different expression-secretion cassettes. The yeast-integrating shuttle plasmids constructed were all transformed and integrated into the genome of the *S. cerevisiae* laboratory strain *ISP52* (Laing and Pretorius 1992). The *peh1* polygalacturonase gene from *E. carotovora* was subsequently inserted into the same expression-secretion cassettes and the plasmids constructed were also transformed and integrated into the genome of the *S. cerevisiae* laboratory strain (Laing and Pretorius 1993a). The promoters used for the activation and initiation of transcription of these pectinase genes were yeast alcohol dehydrogenase ($ADH1_P$), yeast mating pheromone α-factor ($MF\alpha1_P$) and the *Bacillus amyloliquefaciens* α-amylase (AMY_P) promoters. The yeast tryptophan synthase terminator sequence ($TRP5_T$) was used as a signal to terminate transcription and the secretion of the enzymes was directed by the yeast mating pheromone α-factor ($MF\alpha1_S$) secretion sequence. The $ADH1_P$-$MF\alpha1_S$-$TRP5_T$ expression-secretion cassette proved to be the most successful system for the transcription of the *peh1* and *pelE* genes and for the secretion of the polygalacturonase and pectate lyase enzymes in *S. cerevisiae.* Co-expression of two

ADH1$_P$-MFα1$_s$-TRP5$_T$ cassettes, one containing the *peh1* and one containing the *pelE* gene, resulted in both enzymes being secreted. This co-expression synergistically enhanced pectin degradation (Laing and Pretorius 1993b).

The pectin lyase gene (*pnlA*) of *Glomeralla cingulata* has also been expressed in *S. cerevisiae* under the *GAL10* promoter. This led to very low levels of enzyme production, although alterations in the start codon resulted in improved recognition of the promoter and up to ten times improvement in pectolytic activity (Templeton *et al* 1994). Cloning of the *S. cerevisiae PGL1* open reading frame behind the *ADH1* promoter resulted in overexpression of polygalacturonase activity in *S. cerevisiae* (Gainvors *et al* 2000).

2.8.1.3 Development of recombinant pectolytic wine yeast strains

Fermentation with pectolytic wine yeasts might lead to improved clarification and colour extraction and enhanced varietal aroma without the addition of expensive commercial enzymes. In an attempt to construct a pectolytic wine yeast strain, the cDNA *pelA* gene of *Fusarium solani* was expressed under the *S. cerevisiae* actin promoter in an industrial wine yeast strain. The pectate lyase was only secreted during the stationary phase. The delay in secretion could be attributed to problems with protein folding. Wine made with the recombinant strain had the same physico-chemical properties as that made with the untransformed strain (González-Candelas *et al* 1995).

In another attempt to construct a pectolytic wine yeast strain, the pPPK plasmid was constructed by cloning the expression cassettes *ADH1$_P$-MFα1$_s$-peh1-TRP5$_T$* and *ADH2-MFα1$_s$-pelE1-TRP5$_T$* into the integration plasmid Ylp5. This construct was then transformed and integrated into the *URA3* gene of the industrial wine yeast strain VIN13. Wine was made with two cultivars, namely Cinsaut and Pinot noir. Fermentation with the recombinant strain resulted in a decrease in total phenolics, an increase in turbidity and an increase in juice extraction compared to a control fermented with an untransformed *S. cerevisiae* VIN13 strain. Results varied between cultivars (Strauss 2003).

2.8.2 DEVELOPMENT OF GLUCANOLYTIC *S. CEREVISIAE* STRAINS

2.8.2.1 Glucanase-producing organisms

Enzymes that hydrolyse cellulose are produced by a wide variety of bacteria and fungi. In many anaerobic bacteria, the cellulase system consists of high molecular weight exocellular complexes, termed cellulosomes, which comprise a variety of cellulolytic enzymes organised around a non-catalytic, cellulose-binding scaffolding subunit. This structure was first identified in the rumen bacterium *Clostridium thermocellum.* It has been shown that this cellulosome degrades crystalline cellulose efficiently and that the association of cellulolytic enzymes is important for activity (Béguin *et al* 1998).

Aerobic bacteria secrete various extracellular enzymes with different binding domains. These enzymes acts synergistically to hydrolyse cellulose. Bacteria secrete a variety of endoglucanases that show low levels of activity towards crystalline cellulose, and only a few bacterial exoglucanases and cellobiohydrolases have been characterised (Tomme *et al* 1995). Bacterial 1,3-1,4-β-glucanases are usually extracellular enzymes, which indicates that they are involved in the degradation of polysaccharides that can be used as energy source. Genes encoding bacterial 1,3-1,4-β-glucanases have been cloned and sequenced from different *Bacillus subtillus*, *Bacillus fibriosolvens, Fibrobacter succinogenses, Ruminococcus flavofaciens* and *Clostridium thermocelum* strains. All bacterial 1,3- and 1,3-1,4-β-glucanases have a high degree of sequence similarity and have been classified as members of family 16 glycosyl hydrolases. They are mono-domain proteins, with molecular masses in the range of 25-30 kDa, have a wide pH range, a basic pI (8-9) and are quite thermostable compared to plant isozymes (Planas 1998).

Fungi secrete a variety of hydrolytic enzymes, including endoglucanases, β-glucosidases, exoglucanases and cellobiohydrolases. These enzymes act in a synergistic manner in order to degrade crystalline cellulose (Wood 1985, Van Rensburg *et al* 1997). Several fungal cellulases have been characterised and the genes coding for them have been cloned. *A. niger* can produce a variety of cellulases. Hong *et al* (2001) characterised an endo-β-1,4-glucanase that is highly resistant to high temperatures and protease and surfactant treatment. The gene was cloned and designated *eng1.* One of the most studied fungal cellulase systems is produced by *Trichoderma reesei*. This fungus produces a complete set of cellulases which can hydrolyse cellulose to soluble sugars (Karlson *et al* 2002). Six genes encoding two cellobiohydrolases (*CBHI* and *CBHII*), three endoglucanases (*EGLI, EGLII* and *EGLV*) and a β-glucosidase (*BGL1*) have been cloned and sequenced (Takada *et al* 1998).

It has been shown that β-glucanases are secreted by several yeast species. Endo-β-1,3-glucanase and exo-β-1,3-1,6-glucanase are secreted by *Schizosaccharomyces versatilis, Schizosaccharomyces pombe, Candida utilis* and *Pichia polymorpha* (Nombela *et al* 1988). *S. cerevisiae* secretes several endo-β-1,3-glucanases, and it has been reported that the following genes, coding for these enzymes, have been cloned and characterised: *EXG2, BGL1, BGL2* and *SSG1* (Van Rensburg and Pretorius 2000).

2.8.2.2 Heterologous expression of glucanase genes in laboratory strains

The complete hydrolysis of glucans by *S. cerevisiae* would be advantageous to several industries, e.g. brewing, wine and animal feed production. Since *S. cerevisiae* does not produce several important cellulases, e.g. endo-β-1,4-glucanases, which are required for the complete synergistic breakdown of cellulose, several researchers have introduced foreign genes cloned from a variety of cellulolytic organisms into *S. cerevisiae* in order to improve its hydrolytic properties.

The first heterologous glucanase gene expressed in *S. cerevisiae* was the *beg1* gene from *Bacillus subtillus,* coding for an endo-β-1,3-1,4-glucanase. This gene was expressed under its own promoter and the enzyme activity detected was very low (Hinchcliff 1985).

The *end1* gene from *Butyrivibrio fibriosolvens,* coding for an endo-β-1,4-glucanase, was cloned and expressed in *S. cerevisiae* on a multicopy plasmid. Expression was regulated by the *ADH1* promoter and high levels of β-glucanase activity were detected in the medium (Van Rensburg *et al* 1994). Subsequently, the *Phaerochaete chrysosporium* gene *cbh1-4* was cloned and co-expressed with the *end1* gene in *S. cerevisiae*. The following two expression cassettes were constructed and expressed on multicopy plasmids in *S. cerevisiae* Y294 strain: $ADH2_P$-$MF\alpha$-1_S-*end1*-$ADH2_T$ and PGK_P-*cbh1-4*-PGK_T. Enzyme assays showed that co-expression of these cassettes synergistically enhanced cellulose degradation by *S. cerevisiae* (Van Rensburg *et al* 1996).

In another attempt to improve the hydrolytic abilities of *S. cerevisiae,* Van Rensburg *et al (*1997) co-expressed the *B. subtillus beg1* gene with the *Butyrivibrio fibriosolvens end1* gene (encoding an endo-β-1,3-1,4-glucanase) and the *S. cerevisiae EXG1* gene. The *S. cerevisiae EXG1* encodes the main exo-β-1,3-glucanase, and was cloned and overexpressed. The following cassettes were constructed: $ADH2_P$ $MF\alpha$-1_S-*EXG1*-$ADH2_T$, $ADH2_P$-$MF\alpha$-1_S-*beg1*-$ADH2_T$ and $ADH2_P$-$MF\alpha$-1_S-*end1*-$ADH2_T$. and expressed on multi-copy plasmids in *S. cerevisiae* Y294 carrying a *fur1ura3* mutation. Enzyme assays confirmed that complementing the *S. cerevisiae* exo-β-1,3-glucanase activity with the co-expression of the *beg1, end1* and *EXG1* genes enhanced glucan degradation.

Some other cellulose-encoding genes from bacterial origin that has been successfully expressed in *S. cerevisiae* are the *Ruminococcus flaveciens* cellodextrinase gene, *celA* (Van Rensburg *et al* 1995), and the *Cellulomonas fimi* endo-β-1,4-glucanase gene, *cenA* (Skipper *et al* 1985).

Commercial glucanase preparations are produce by fungi, particularly by the *Trichoderma* species. Several cellulase genes from *T. reesei* have been expressed in *S. cerevisiae.* Two endo-β-1,4-glucanase-encoding genes, *egl1* and *egl3*, were cloned and expressed in *S. cerevisiae* under the *PGK1* promoter (Penttilä *et al* 1988) also expressed two cellobiohydrolases originating from *T. reesei* in *S. cerevisiae* under the *PGK1* promoter. Both enzymes were secreted. During processing, the enzymes were overglycosylated, and although this did not significantly alter the specificity of cellobiohydrolase I, the specificity of cellobiohydrolase II decreased compared to the native *T. reesei* enzyme. The *eng1* gene, coding for a highly temperature-resistant endo-β-1,4-glucanase, was cloned from the fungus *A. niger* and was successfully expressed in *S. cerevisiae* on a multicopy plasmid under control of the *GADPH* promoter. The enzyme was secreted, retained its activity and was still stable at temperatures up to 80°C and at pH 3-10 (Hong *et al* 2001).

2.8.2.3 Development of recombinant glucanolytic wine yeast strains

Fermenting wine with glucan-degrading strains can increase the extraction of colour, phenolics and varietal aroma precursors from the grape skin. Glucan degradation can also help to improve filtration and clarification (Watson *et al* 1999, Zent and Inama 1992).

The *Trichoderma longibrachiatum egl1* gene coding for endo-β-1,4-glucanase was cloned into a multicopy plasmid and transformed into the commercial wine yeast strain *S. cerevisiae* T_{73} (CECT1894 Lallemand Inc.). Expression was controlled by the yeast actin promoter. The recombinant strain (designated T_{73}-*EGL1*) secreted the fungal endo-β-1,4-glucanase into the must and an increase in some of the major volatile components was detected by gas chromatography in wines fermented with the recombinant strains when compared to wines fermented with untransformed strains (Pérez-González *et al* 1993).

The fungal endo-β-1,4-glucanase was also harvested after heterologous production by the recombinant *S. cerevisiae* strain T_{73}-*EGL1* in a bioreactor and the addition of the enzyme to the must increased the release of aroma precursors by 12.5% compared to a reference must. Considerable increases were shown in nerol and α-terpineol (Ganga *et al* 2001). In a subsequent study, Bobal grapes were fermented with T_{73} *EGL1* and a xylanase-producing recombinant strain, T_{73}-X_{22}. These fermentations were compared with two fermentations done with untransformed strains to which the endo-β-1,4-glucanase and endo-β-1,4-xylanase, harvested from the recombinant strains in bioreactors, had been added. The experiment included a control to which a commercial enzyme had been added (Lallzyme-Lallemand Inc.), as well as a no-enzyme control. The results showed that inoculation with T_{73}-*EGL1* significantly increased the total phenols, but addition of the endo-β-1,4-glucanase did not. All treatments increased the colour intensity, but there was also a lot of variation between treatments. The volatile components were significantly affected by the various enzyme treatments. Fermentation with T_{73}-*EGL1* resulted in an increase in the alcohols, isobutyl alcohol and 1-butanol, the esters ethyl hexanoate and ethyl-3-hydroxybutanoate, the terpene linalool, and the acids isobutyric acid, hexanoic acid and octanoic acid, when compared to the mean of 16 different fermentations. There was a significant decrease in 3-methyl-1-pentanol, ethyl lactate, isovaleric acid, decanoic acid and lauric acid when compared to the mean. Some results obtained with the recombinant strains were better then with the addition of commercial enzyme. The differences between inoculation and secretion into the media are due to differences in dosage and time of enzyme addition to the must (Gil and Vallés 2001).

2.8.3 DEVELOPMENT OF XYLANOLYTIC *S. CEREVISIAE* STRAINS

2.8.3.1 Xylanase-producing organisms

Several xylanase-producing bacteria and fungi have been characterised. Fungal and bacterial endo-xylanases are almost exclusively single subunit proteins with M_r values ranging from 8.5 to 85 kDa and pI values between 3.6 and 10.3 (Coughlan and Hazelwood 1993).

Bacterial endo-xylanases can be divided into two groups on the basis of their amino acid sequence. The one group is exemplified by *Clostridium thermocellum XynZ* and *Caldocellum saccharolyticum xynA,* and the other by *Bacillus pumillus xynA* and *Bacillus subtillus xynA* (Béguin *et al* 1998). Four xylanase genes have been characterised in the rumen bacterium, *Ruminococcus flaveciens.* One of these gene products (*xynA*) was shown to consist of two domains with independent xylanase activity belonging to two different groups (Flint *et al* 1989, Flint and Zang 1992). Several extremophylic bacteria also secrete xylanases that can be of commercial importance. Alkaliphylic *Bacillus* and *Aeromonas* species produce alkaline xylanases that are important for paper production and in the detergent and fabric industries. Several thermostable xylanases have been isolated from thermophylic bacteria, such as *Thermonospora fusca, Bacillus stearothermophilus* and *Clostridium stercorarium.* Xylanases produced by thermophylic bacteria of the genus *Thermotoga* have been shown to have a temperature optimum at 105°C (Kulkarni *et al* 1999).

Commercially available xylanases are usually produced by the fungi *A. niger* and *T. reesei.* The two most important xylanases secreted by *T. reesei* are the endo-β-1,4-xylanases Xyn1 and Xyn2. The genes *xyn1* and *xyn2* have been cloned and the three-dimensional structure and substrate specificity of the products have been analysed. The pH optimum of Xyn1 is 3.5 to 4.5 and that of Xyn2 is 4.5 to 5.5. Together, these enzyme are responsible for more then 90% of xylan degradation in *Trichoderma reesei* (Saarelainen *et al* 1993, Tenkanin *et al* 1992, Törrönen *et al*). *A. niger* produces three endo-β-1,4-xylanases: $X_{22,}$ X_{24} and X_{34}, which are encoded by *xynA, xynB* and *xynC* respectively. The genes have all been cloned and sequenced (McCabe *et al* 1996, Pérez-González *et al* 1993). Different endo-β-1,4-xylanases from other fungal species have also been characterised. Some of these are *A. tubigensis, A. oryzae* and *A. awamori* (Fernandéz-Espinar *et al* 1994).

2.8.3.2 Heterologous expression of xylanase genes in laboratory strains

S. cerevisiae cannot break down xylan, nor can it utilise the monomeric constituents of the backbone D-xylose as carbon source. In order for *S. cerevisiae* to utilise xylan as carbon source, three genes would have to be introduced by recombinant

techniques: a xylanase, a β-xylosidase and a xylose isomerase, or a reductase/dehydrogenase combination (La Grange 1995). Several recombinant *S. cerevisiae* strains have been constructed that can successfully degrade xylan through the heterologous expression of xylanase genes. Table 4 shows some of the recombinant strains, with genes from fungal origin, that can degrade xylan due to the secretion of endo-β-1,4-xylanases.

Table 4. Heterologous production of fungal endo-β-1,4-xylanases in *S. cerevisiae.*

Organism cloned from	Gene	Promoter	Enzyme	Activity	Optimum pH/Temp	Reference
A. niger ATCC	*xynC*	*ADH2P*	endo-β-1,4-xylanase (Xyn4)	91 nkat ml^{-1}	4 / 60°C	Luttig *et al* 1997
A. niger ATCC	*xlnA*	*ADH2P*	endo-β-1,4-xylanase (Xyn5)	73 nkat ml^{-1}	4 / 60°C	Luttig *et al* 1997
A. niger	*xlnA*	Yeast actin promoter	endo-β-1,4-xylanase (X_{22})	12 U mg^{-1}	6 / 58°C	Ganga *et al* 1998
A. niger	*xlnB*	Yeast actin promoter	endo-β-1,4-xylanase (X_{24})	14 U mg^{-1}	3./ 54°C	Ganga *et al* 1998
A. niger	*xlnC*	Yeast actin promoter	endo-β-1,4-xylanase (X_{34})	12.5 U mg^{-1}	3./ 52°C	Ganga *et al* 1998
T. reesei	*XYN2*	*PGK1P*	endo-β-1,4-xylanase (Xyn2)	1200 nkat ml^{-1}	6 / 60°C	La Grange *et al* 1996
T. reesei	*XYN2*	*ADH2P*	endo-β-1,4-xylanase (Xyn2)	160 nkat ml^{-1}	6 / 60°C	La Grange *et al* 1996

All these fungal xylanases were expressed in *S. cerevisiae* by expressing cDNA copies of the fungal genes in the yeast. *S. cerevisiae* has, however, been transformed with a genomic library of *Penicillium purpurogenum* a fungus that can utilise xylan as carbon source.. An endo-xylanase-producing yeast strain, *S. cerevisiae* 44A, was isolated that secreted endoxylanase A when grown on xylan or xylose, but not on glucose. It was shown that the *P. purpurogenum xynA* gene and its eight introns were successfully inserted into the yeast genome and that the *S. cerevisiae* 44A strain was able to correctly splice the *xynA* introns (Chávez *et al* 2002).

To enable *S. cerevisiae* to degrade xylan to xylose, the endo-xylanase-encoding genes have been co-expressed with xylosidase genes. The xylosidases can degrade the xylobiose, released by endo-β-1,4-xylanase action, to xylose. The *xynB* gene encoding the *Bacillus pumilus* β-xylosidase was expressed on its own as well as with

the *T. reesei XYN2* gene in *S. cerevisiae.* Both genes were placed under control of the *ADH2P*. Co-expression of these genes led to a 25% increase in the release of reducing sugars from Birchwood xylan, compared to the recombinant strain only, which produced Xyn2 endo-β-1,4-xylanase (La Grange *et al* 2000). The *A. niger* β-xylosidase gene *xlnD* has also been co-expressed with the *T. reesei XYN2* gene in *S. cerevisiae.* Co-production of the β-xylosidase and the endo-β-1,4-xylanase allowed for the recombinant *S. cerevisiae* strain to degrade Birchwood xylan to D-xylose (La Grange *et al* 2001).

2.8.3.3 Development of recombinant xylanolytic wine yeast strains

The cellulases and xylanases present in commercial enzyme preparations can improve the extraction of phenolic compounds and contribute to wine aroma by increasing the amount of flavour precursors in the must (Gil and Vallés 2001).
To study the effect of the addition of a pure endo-β-1,4-xylanase on winemaking, the X_{22} endo-β-1,4-xylanase was purified by ultra-centrifugation from a recombinant *S. cerevisiae* strain cultivated in a bioreactor. The X_{22} endo-β-1,4-xylanase was produced by the recombinant yeast strain expressing the *A. niger xlnA* gene and was added to the must during maceration. During colour analysis of the wine, it was found that the absorbance reading at 420nm increased. This wavelength contributes to the yellow colour of wine and the increase was attributed to the release of flavonoid phenols from cellular structures due to xylanase activity. The volatile acidity of the wines also increased, but was still far below the values corresponding to spoiled wine. A qualitative sensory test, conducted by a panel of experts, indicated an increase in fruity aroma in the enzyme-treated wine, compared to that of an untreated reference. There was a decrease in most of the esters and acids formed by the treated wine, although caproic acid and the terpene linalool increased. Both of these compounds contribute significantly to the floral aroma of wine (Ganga *et al* 2001).
Ganga *et al* (1999) also expressed the *A. niger xlnA* gene under the yeast actin promoter in a commercial wine yeast strain, T_{73} (Lallemand Inc.). It was determined that between 89% and 95% of the enzyme produced by the recombinant T_{73}/YepCA1 strain was secreted into the growth medium. An increase in fruity aroma was detected in Chenin blanc wine fermented with the recombinant strain in comparison to a wine fermented with an untransformed strain. Increases were found in many of the esters, higher alcohols, acids and terpenes measured. Significant increases were found in the esters linalyl acetate, phenylethyl acetate and hexyl acetate, as well as in the terpenes linalool, terpineol and geraniol. These compounds all have a low sensory threshold and contribute significantly to the typical floral aroma of wine (Ganga *et al* 1999). When Gil and Válles compared the effects of fermenting Bobal grapes with different recombinant strains and adding the enzymes produced by these strains to the must (refer section 2.8.2.3), the X_{22} endo-β-1,4-xylanase-producing strain resulted in wines with higher concentrations of the alcohols 1-propanol, 1-butanol and 3-methyl-1-pentanol, the esters ethyl lactate, ethyl-hydroxy-

butanoate, and hexanoic acid than any other treatment or strain. When the glycosidically-bound aroma compounds were analysed, a significant decrease in the 1-hexanol glycoside was the only effect observed that could be ascribed to inoculation with T_{73}/YepCA1 (Gil and Válles 2001).

2.9 CONCLUSION

Wine can be viewed as the product of the enzymatic transformation of grape juice. The enzymes catalysing this transformation originate from the grape and yeasts and other microbes (fungi and bacteria) associated with vineyards and wine cellars. Through a better understanding of the enzymatic activities, winemakers have come to learn how to control unwanted enzymes while optimising the desired activities. Industrial wine yeast strains do not secrete all the enzymes needed for the efficient production of quality wines, and endogenous enzymes therefore have to be enhanced through the application of commercial enzyme preparations. Commercial preparations of glucanases, xylanases and pectinases are applied to winemaking with the aim of improving the clarification and processing of wine, releasing varietal aromas from precursor compounds and increasing colour intensity. Several *S. cerevisiae* laboratory strains have successfully been altered by genetic engineering to secrete these enzymes, enabling these strains to break down problem-causing polysaccharides. Recombinant wine yeast strains secreting glucanases, xylanases and pectinases have also been constructed and promising results have been obtained concerning colour extraction, juice extraction and aroma profiles.

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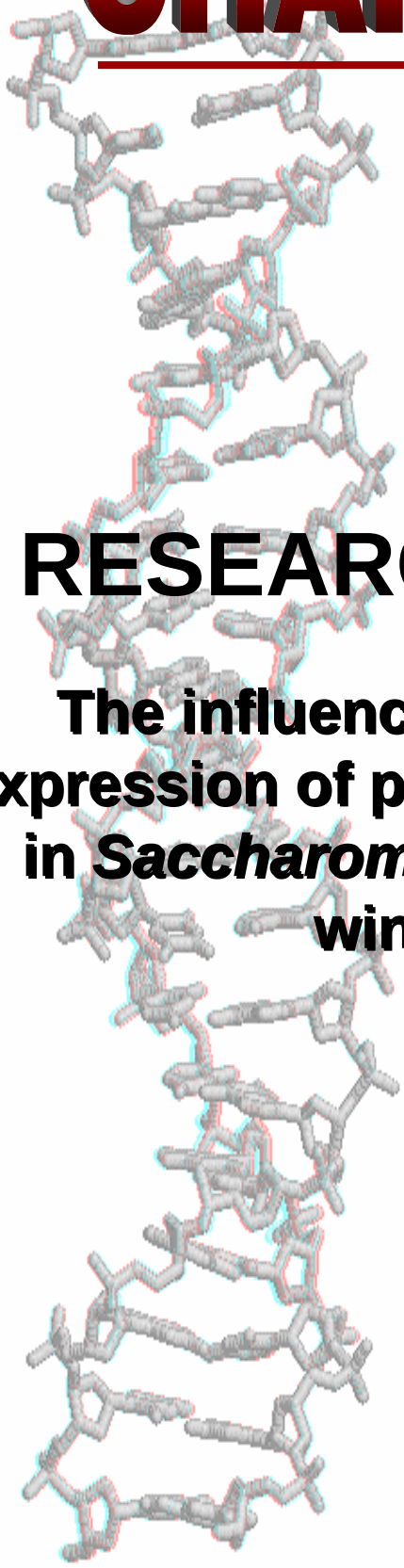
CHAPTER 3



RESEARCH RESULTS

The influence of heterologous expression of polysaccharase genes in *Saccharomyces cerevisiae* on winemaking

3. RESEARCH RESULTS

The influence of the heterologous expression of polysaccharase genes in *Saccharomyces cerevisiae* on winemaking

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Glucanase, xylanase and pectinase enzyme preparations are applied to winemaking with the aims of improving the clarification and processing of wine, releasing varietal aromas from precursor compounds, and increasing colour intensity. Genes coding for these enzymes have been cloned from several polysaccharide-degrading organisms. Expressing these genes in *Saccharomyces cerevisiae* allows for the recombinant strains to degrade polysaccharides that traditional commercial yeast strains cannot. In this study, we constructed recombinant wine yeast strains that are able to degrade the problem-causing grape polysaccharides, glucan and xylan, by separately integrating the *Trichoderma reesei XYN2* xylanase gene and the *Butyrivibrio fibrisolvens end1* glucanase gene into the genome of the commercial wine yeast strain *S. cerevisiae* VIN13. These genes were also combined in *S. cerevisiae* VIN13 under the control of different promoters. The strains that were constructed were compared with each other and with a recombinant wine yeast strain expressing the endo-β-1,4-glucanase gene (*end1*) from *B. fibrisolvens* and the endo-β-1,4-xylanase gene (*XYN4*) from *Aspergillus niger*, a recombinant strain expressing the pectate lyase-encoding gene (*pelE*) from *Erwinia chrysanthemi* and the polygalacturonase-encoding gene (*peh1*) from *Erwinia carotovora* under winemaking conditions. Wine was made with the recombinant strains using Pinot noir, Ruby Cabernet and Muscat d'Alexandria grapes. Wine fermentations of grapes from different cultivars with these recombinant strains resulted in significant increases in free-flow wine, colour intensity and colour stability. Significant alterations in the higher alcohol, acid and ester profiles of these wines were also obtained.

Key words: Recombinant wine yeast, glucanase, xylanase, pectinase, wine processing, wine colour, aroma

Abbreviations: *XYN2* gene, *Trichoderma reesei XYN2* xylanase gene; *end1* gene, *Butyrivibrio fibrisolvens end1* glucanase gene; *XYN4* gene, *Aspergillus niger XYN4* gene; *pelE, Erwinia chrysanthemi* pectate lyase-encoding gene; *peh1, Erwinia carotovora* polygalacturonase-encoding gene

3.1 INTRODUCTION

Throughout the winemaking process, various biochemical reactions are catalysed by enzymes originating from the grapes, yeasts and other microbes associated with the vineyard and winery. The addition of desired enzymes to the fermentation can help to improve wine processing and quality. In wine production, maceration refers to the breakdown of grape solids following grape crushing. The addition of macerating enzymes during this period is common practice (Gil and Vallés 2001). The cell wall of grape berry cells has a common set of structural polysaccharides, with the main constituents being pectin, cellulose and hemicellulose (Barnavon *et al* 2001). The commercial enzyme preparations used during maceration are typically blends of pectinases, cellulases, hemicellulases and other carbohydrase activities (Gump and Haight 1995).

Degradation of the structural polysaccharides by carbohydrases can result in an improvement in juice yield, clarification and filterability during winemaking (Colagrande *et al* 1994, Haight and Gump 1994). The action of these enzymes also facilitates the liberation and solubilisation of phenolic compounds and glycosydic precursors from the cells of the skins, seeds and flesh of the grape berry (Ganga *et al* 2001, Günata *et al* 1985, Gump and Haight 1995, Watson *et al* 1999). Increased extraction of phenolic compounds, such as tannins, leads to more polymeric pigments being formed in aged red wine, resulting in increased colour intensity and stability (Haight and Gump 1994, Watson *et al* 1999). Terpenols are important constituents of the fruity aroma of Muscat grapes (Günata *et al* 1985). The terpenols are present as non-volatile, glycosydically-bound precursors and as free volatile forms (Mateo and Jiménez 2000). The glycosydically-bound terpenols are located mainly in the grape skins (Günata *et al* 1985). Enzymatic degradation of the cell wall contributes to the release of bound precursors from the berries. The hydrolysis of these precursors during fermentation can result in an improvement in wine aroma (Zoecklein *et al* 1997).

The majority of commercial pectinase and glucanase preparations are derived from *Aspergillus* and *Trichoderma* respectively (Canal-Llaubères 1993). Commercial enzymes are typically crude fungal preparations and, besides containing the desired enzymes, impurities such as mucilage, proteins and undesired enzyme side-activities might be present (Wightman *et al* 1997). Since the endogenous polysaccharase activity of *S. cerevisiae* is very limited, the heterologous expression of specific polysaccharase genes in an industrial yeast strain can improve the winemaking process. A higher quality wine can be made without the addition of expensive commercial enzyme preparations. Since only the desired enzymes are secreted by the recombinant strain, there will be no undesired side activities, which can be detrimental to wine quality.

In this paper we describe the construction of four polysaccharide-degrading wine yeast strains, expressing the *Trichoderma reesei* endo-β-1,4-xylanase gene, *XYN2* (La Grange *et al* 1996) and the *Butyrivibrio fibrisolvens* endo-β-1,4-glucanase gene,

END1 (Van Rensburg *et al* 1994). The four recombinant wine yeast strains were constructed by integrating the cassettes into the *ILV2* locus of the commercial wine yeast strain, VIN13 (Anchor Yeast SA). The cassette $TEF1_P$-*XYN2*-$ADH2_T$ was integrated into strain VIN13-DLG29, $ADH1_P$-$MF\alpha1_S$-*END1*-$TRP5_T$ was integrated into VIN13-DLG30, $ADH1_P$- $MF\alpha1_S$-*END1*-$TRP5_T$ and $ADH2_p$-*XYN2*-$ADH2_T$ were integrated into VIN13-DLG33 and $ADH1_p$-$MF\alpha1_S$-*END1*-$TRP5_T$$YG100_P$-*XYN2*-$ADH2_T$ was integrated into VIN13-DLG39. Wine was made with the four constructed strains, as well as with the following strains: A pectolytic strain, VIN13[pPPK] expressing the *Erwinia chrysanthemi* pectate lyase gene *pelE* and the *Erwinia carotovora* polygalactoronase gene *peh1.* A glucanase- and xylanase-secreting strain, VIN13[pEX] expressing the endo-β-1,4-glucanase gene *end1* gene from *Butyrivibrio fibrisolvens* together with the endo-β-1,4-xylanase gene *xyn4* from *Aspergillus niger* (Strauss 2003). An untransformed VIN13 strain and an untransformed strain with the addition of the commercial enzyme preparation Rapidase EX Colour. Microvinification experiments were carried out on Pinot noir, Ruby Cabernet and Muscat d'Alexandria grapes fermented with each of the abovementioned strains.

3.2 MATERIALS AND METHODS

3.2.1 STRAINS AND MEDIA

The genotypes of the microbial strains that were used in this study are summarised in Table 1.

Plasmids were constructed and amplified in *Escherichia coli* DH5α, cultivated at 37°C in Luria Bertani broth (LB) or Luria Bertani agar (Sambrook *et al* 1989), and the media were supplemented with ampicillin at a concentration of 100 μg/ml for the selection of resistant bacteria. *Saccharomyces cerevisiae* VIN13 was grown in liquid YPD (1% yeast extract, 2% peptone and 2% glucose) at 30°C on a rotary shaker at 150 r.p.m. The transformed *S. cerevisiae* strains, VIN13-DLG29, VIN13-DLG30, VIN13-DLG33, VIN13-DLG39 and VIN13-PEX, all contained the *SMR1* (sulfometuron methyl resistance) marker gene and were grown on YPD agar at 30°C with concentrations of sulfometuron methyl (SMM) ranging between 300 and 400 μg/ml. The *S. cerevisiae* VIN13-PPK containing the *KanMX* (geneticin resistance) marker gene was grown in liquid YPD and on YPD agar with concentrations of geneticin ranging between 100 and 400 μg/ml.

Table 1 Microbial strains used in this study

Strain and plasmid	Genotype/Description	Source/Reference
Escherichia coli		
DH5α	*supE*44 *placU169* (ϕ80*lacZ*ρ*M15*) *hsdR17 recA1 gyrA96 thi-1 relA1*	[a]GIBCO/Bethesda
Saccharomyces cerevisiae		
VIN13	commercial diploid strain	Anchor Yeast Technologies (SA)
VIN13-EXS	*URA3::ADH1$_P$- MFα1$_S$ -END1- TRP5$_T$ ADH1$_P$ -XYNC -ADH2$_T$*	Strauss, 2003
VIN13-PPK	*URA3::ADH1$_P$ -MFα1$_S$- PELE- TRP5$_T$ ADH1$_P$- MFα1$_S$ -PEH1- TRP5$_T$*	Strauss, 2003
VIN13-DLG29	*ILV2:: TEF1$_p$-XYN2- ADH2$_T$*	This study
VIN13- DLG30	*ILV2::ADH1$_p$- MFα1$_S$ -END1- TRP5$_T$*	This study
VIN13- DLG33	*ILV2::ADH1$_p$- MFα1$_S$ -END1- TRP5$_T$ ADH2$_p$-XYN2- ADH2$_T$*	This study
VIN13- DLG39	*ILV2::ADH1$_p$- MFα1$_S$ -END1-TRP$_T$ YG100$_p$-XYN2- ADH2$_T$*	This study

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3.2.2 DNA MANIPULATIONS AND PLASMID CONSTRUCTION

Standard methods were followed for manipulating and subcloning DNA fragments, isolating plasmid DNA and transforming *E. coli* DH5α (Sambrook *et al* 1989). Restriction endonuclease-digested DNA was eluted from agarose gels by the freeze-squeeze method (Tautz and Renz 1983). All plasmids used in this study are summarised in Table 2.

Previously, the endo-β-1,4-xylanase gene, *XYN2,* was cloned from the soft rot fungus *Trichoderma reesei* (La Grange *et al* 1996), and the endo-β-1,4-glucanase gene *END1* was cloned from the rumen bacterium *Butyrivibrio fibrisolvens* (Van Rensburg *et al* 1994). For the construction of pDLG30, pAR3 (Van Rensburg *et al* 1994) was digested with *Hind*III and *Sal*I, and a 2.82 kb fragment containing the *ADH1$_p$-MFα-END1-TRP5$_T$* cassette was recovered and subcloned into the corresponding sites of pWX509* (Steyn *et al* 1995).

For the construction of pDLG20, pDLG5 (La Grange *et al* 1996) was digested with *Bam*HI and *Hind*III*,* and a 2.62 kb fragment containing the *ADH2$_P$-XYN2-ADH2$_T$* cassette was recovered and subcloned into the corresponding sites of pWX509*.
For the construction of pDLG33, pAR3 was digested with *Hind*III and *Sal*I, and a 2.82 kb fragment containing the *ADH1$_P$-MFα-END1-TRP5$_T$* cassette was recovered and subcloned into the corresponding sites of pDLG20.

The *SSA1* promoter (*YG100*) was amplified from the genomic DNA of *S. cerevisiae* by polymerase chain reaction (PCR), using two oligodeoxyribonucleotides:
SCSSA1-L 5'-GATCGGATTCCGACAAATTGTTACGTTGT-3' (29 mer; the *Bam*HI restriction site is underlined) and
SCSSA1-R 5'-GTACGAATTCTATTATCTGTTATTTACTTGAATTT-3' (35-mer; the *Eco*RI restriction site is underlined). The sequence for these PCR primers was based on the sequence published by Slater and Craig (1989).

Plasmid pDLG23 was created by subcloning the 0.56 kb *Bam*HI-*Eco*RI $YG100_P$ PCR fragment into the corresponding sites of pDLG5.
For the construction of pDLG24, a 2.20 kb *Bam*HI-*Hind*III fragment containing $YG100_P$-*XYN2*-$ADH2_T$ was isolated from pDLG23 and subcloned into the corresponding sites of pWX509*.

For the construction of pDLG39, the 2.82 kb *Hind*III-*Sal*I fragment from pAR3 containing $ADH1_P$-*MF*α-*END1*-$TRP5_T$ was subcloned into pDLG24.

Table 2 Plasmids used in this study

Plasmid	Genotype/Description	Source/Reference
pAR3	Ap^R GT^R *LEU2* $ADH1_P$- *MF*$\alpha 1_S$ -*END1*-$TRP5_T$	Van Rensburg *et al* 1994
pWX509*	Ap^R SMR^R	Steyn *et al* 1995
pDLG5	Ap^R *URA3* $ADH2_P$-*XYN2*-$ADH2_T$	La Grange *et al* 1996
pDLG29	Ap^R SMR^R $TEF1_P$-*XYN2*-$ADH2_T$	La Grange 1999
pDLG20	Ap^R $ADH2_P$-*XYN2*-$ADH2_T$ *SMR1*	This work
pDLG23	Ap^R $YG100_P$-*XYN2*-$ADH2_T$	This work
pDLG24	Ap^R $YG100_P$-*XYN2*-$ADH2_T$-*SMR1*	This work
pDLG30	$ADH1_P$- *MF*$\alpha 1_S$ -*END1*-$TRP5_T$	This work
pDLG33	Ap^R $ADH1_P$- *MF*$\alpha 1_S$ -*END1*-$TRP5_T$ $ADH2_P$-*XYN2*-$ADH2_T$ *SMR1*	This work
pDLG39	Ap^R $ADH1_P$- *MF*$\alpha 1_S$ -*END1*-$TRP5_T$ $YG100_P$-*XYN2*-$ADH2_T$ *SMR1*	This work

3.2.3 YEAST TRANSFORMATION

The plasmids pDLG29, pDLG30, pDLG33 and pDLG39 were all linearised by digesting the *SMR1* gene with *ApaI.* These integration plasmids were all transformed separately into the *S. cerevisiae* strain VIN13 through electroporation (Ausubel *et al* 1996). Positive transformants were selected by plating out transformed cell suspensions on YPD plates containing concentrations of SMM ranging between 300 and 400 μg/ml.

3.2.4 SCREENING FOR ENZYME ACTIVITY

Transformants were screened for glucanase activity by spotting 10 µl of a liquid YPD culture of each SMM-resistant colony on YPD plates containing 0.3% medium-viscosity carboxymethylcellulose (CMC; Sigma P-C4888:Sigma, St. Louis, Mo., USA). The transformants were grown for two days and the colonies were rinsed off the plates with TE (10 mM Tris-HCl, 1 mM EDTA, pH 7) before staining the plates with 0.1% Congo red, followed by destaining with 1M NaCl (Teather and Wood 1982). CMCase activity was identified by a clear zone around the yeast transformants.

Transformants were screened for xylanase activity by spotting 10 ul of a liquid YPD culture of each SMM-resistant colony on SC^{-Ura} plates containing 0.2% of 4-O-methyl-D-glucurono-D-xylan-remazol brilliant blue R (RBB)-xylan (Sigma) (Biely 1985). Xylanase cleaves RBB-xylan into a colourless product.

Transformants were screened for pectinase activity by spotting 10 µl of a liquid YPD culture onto agarose diffusal plates (0.1 M citrate, 0.2 M Na_2HPO_4, 0.5 g ammonium oxalate, 1 g type II agarose and 0.01 g polygalacturonic acid per 100 ml – pH adjusted to 3.5). The plates were incubated at 25°C overnight, and these were then stained with 0.02% ruthedium red for 60 min at 37°C, after which it was washed off with water. Transformants with pectinase activity showed clear zones around the colony.

3.2.5 PCR ANALYSIS

The transformation of strains showing enzyme activity was confirmed by PCR amplification of the transformed genes. Genomic DNA was isolated from the transformed strains showing clearance zones on selective plates and used as a template (Hoffman and Winston 1987). The DNA was amplified using Expand DNA polymerase (Roche). The reaction mixture included 500 ng of genomic DNA, 200 µM of dNTPs, 0.5 µM of each primer and 1 U of Expand DNA polymerase. The following primers were synthesised by the phosphoramidate method on an Applied Biosystems Model 380A DNA synthesiser:

XYN2 L 5’-GAT CGA ATT CCT CCT ACT AGC CGA AGC AAG-3’ and

XYN2 R 5’-GTA CAG ATC TCT CCC TTT AGC TGA CGG TG-3’ for detection of the *XYN2* gene, and PEND1a-5’-ACG TAA GCT TCT AAA GTT TCT GCC CGC TGG C-3’ and

PEND1-3’ 5’-ACG TAA GCT TGC GGC CGC ATT TAC TGA ACT GAA ATA TTA A-3’ for detection of the *END1* gene. Denaturation, annealing and extension were carried out for 1 min at 94°C, 1 min at 50°C and 1 min at 72°C respectively for the synthesis of the *XYN2* probe, and for 1 min at 94°C, 1 min at 50°C and 2 min at 72°C for the synthesis of the *END1* probe. The cycle was repeated 30 times after an initial denaturation step preceding this cycle at 94°C for five minutes, and a final extension step following the cycle at 72°C for 10 min.

3.2.6 SOUTHERN BLOT ANALYSIS

The transformation and integration of the genes into the *ILV2* locus of *S. cerevisiae* VIN13 was also confirmed by Southern blot analysis. Genomic DNA was isolated from *S. cerevisiae* strains (Hoffman and Winston 1987), digested with *Hind*III, separated on a 1% agarose gel and blotted to a Hybond^{TM} membrane (Amersham International). Southern hybridisations were carried out as described by Sambrook *et al* (1989). Single stranded digoxygenin (DIG)-labelled d-UTP probes were prepared for the detection of the *XYN2* and *end1* genes by polymerase chain reaction (PCR) labelling with the PCR DIG probe synthesis kit (Roche Diagnostics 2000) using the XYN2 L and XYN2 R and PEND1a-5' and PEND1-3' primers respectively

3.2.7 MICROVINIFICATION EXPERIMENTS

Fermentation assays were carried out using the varieties Pinot noir, Ruby Cabernet and Muscat d'Alexandria. Muscat d'Alexandria grapes were crushed and destemmed and then pressed, and 30 ppm of SO_2 was added to the juice. The juice was clarified overnight, after which 6.5 L juice samples were inoculated to a final concentration of 2 x 10^6 cells/ml with the appropriate yeast strains. The red varieties were crushed and destemmed and divided into samples consisting of 8 L of juice and 11 kg of skins each, and 40 ppm of SO_2 was added to each sample. Each sample was inoculated with the appropriate yeast strain to a final concentration of 2 x 10^6 cells/ml. All fermentations were performed in triplicate by inoculating three separate samples of each cultivar with the following yeast strains: VIN13-EXS, VIN13-PPK, VIN13-DLG29, VIN13-DLG30, VIN13-DLG33 and VIN13-DLG39, while VIN13 and VIN13 to which the commercial enzyme preparation Rapidase-X-colour was added (0.3g/l) were inoculated as controls. The must of the red varieties was then fermented at 25°C and that of the white variety at 15°C. Samples (100 ml) were taken before filtration, after filtration and after six months of bottle ageing. These samples were tested for colour, chemical composition and volatile content (acids, higher alcohols and esters).

3.2.8 WINE COLOUR DETERMINATION

Colour measurements were done by spectrophotometer determinations. Three different samples from each wine sample were prepared for colour analysis: wine, wine+SO_2 and wine+HCl. Colour measurements from each sample preparation were taken at 420 nm and 520 nm, from which colour density (A520 wine + A420 wine), colour hue (A420 wine/A520 wine), percentage of red pigment (A520 wine/A520 wine+HCl), SO_2-resistant pigments (A520 wine + SO_2) and total red pigments (A520 wine + HCl) were calculated.
Colour determination was done before filtration, after bottling and after six months of bottle ageing.

3.2.9 CONFIRMING THE DOMINATION OF RECOMBINANT STRAINS

PCR analysis of the genomic DNA of yeast isolated from the lees after fermentation with the primers XYN2 L and XYN2 R and PEND1a-5' and PEND1-3' was used to confirm that the yeast that was inoculated had completed the fermentations.

3.2.10 CHEMICAL COMPOSITION

The levels of ethanol (% v/v), reducing sugar, pH, titratable acidity, volatile acidity, malic acid and lactic acid in the finished wines were determined with the Grapescan 2000 FDIR instrument (FOSS, Denmark).

3.2.11 GAS-LIQUID CHROMATOGRAPHY

To a sample of 10 ml, 800 µl of 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 were transferred to a sample vial.
Analyses were done on an Agilent 6890 series gas chromatograph, equipped with a ALS 7683 liquid sampler, split-splitless injector and FID (flame ionisation detector). The GC was fitted with a Lab Alliance™ RH-WAX, 60mL x 0.32 mm ID x 0.5 µm film thickness capillary column. Hydrogen was used as carrier gas at a flow rate of 3 ml/min and an average velocity of 45 cm/sec. A 3 µl sample was injected at a split ratio of 15:1, a head pressure of 79 kPa and an inlet temperature of 200°C. The detector was kept at 250°C. The column was held at 35°C for 15 min, raised to 230°C at 7°C/min and held at the final temperature for 5 min. Peak identification was done by comparison with authentic standard retention times. Integration and quantification of the peaks were done by the Chemstation Rev A.07.01 software using the internal standard calibration method.

3.2.12 SENSORY EVALUATION

Sensory evaluation of the resultant wines was based on a line scoring system, whereby a panel was asked to indicate if the flavour was acceptable or not and then to mark the intensity of the flavour. The tasting panel consisted of 12 judges trained in wine tasting. The consistency of the tasters was evaluated by comparing the evaluation of two randomly placed replicate wine samples by each taster. Samples of 50 ml were presented in randomly numbered, clear 125 ml tulip-shaped glasses. The samples were evaluated under white light at a room temperature of 22°C ± 1°C. The wines were evaluated in two tasting sessions. Ten Pinot noir wines fermented with different recombinant wine yeast strains and controls were evaluated in the first session. Ten different Pinot noir wines fermented with the same yeast strains were evaluated in the second session one week later.

3.2.13 STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was performed to compare the free-run juice yield, colour parameters and volatile components of Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines at different stages of winemaking and bottle ageing. For significance tests, a critical level of 5% was used and 95% confidence intervals were calculated. This was done using the STATISTICA processing package.

3.3 RESULTS

3.3.1 TRANSFORMATION AND INTEGRATION OF *XYN2* AND *END1* CASSETTES INTO VIN13

The plasmids pDLG29, pDLG30, pDLG33 and pDLG39 were all transformed and integrated separately into the *S. cerevisiae* strain VIN13 through electroporation. Positive transformants were selected on YPD plates containing concentrations of SMM ranging between 300 and 400 μg/ml.

The expression of the various gene cassettes was confirmed by screening on selective agar plates. Positive transformants showed clearance zones around colonies degrading the glucan, pectin or xylan substrate (data not shown). Positive transformants were also confirmed by PCR analysis (Fig 1).

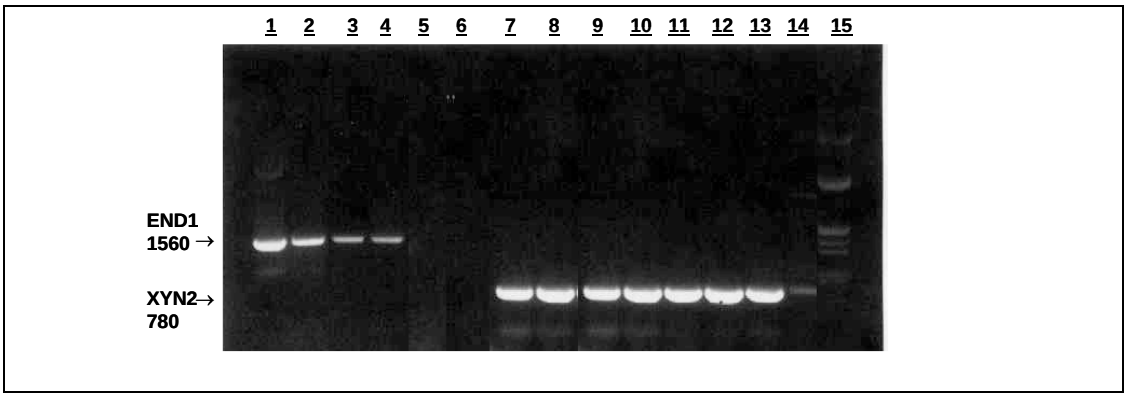

Figure 1 PCR analysis showing the presence of the 1560 bp *END1* and 780 bp *XYN2* genes in positively transformed strains. Lane 1: *S. cerevisiae* VIN13-DLG30, Lane 2: *S. cerevisiae* VIN13-DLG33, Lanes 3 and 4: *S. cerevisiae* VIN13-DLG39, Lane 5: *S. cerevisiae* VIN13 (non-recombinant strain) PCR reaction with *end1* primers, Lane 6: *S. cerevisiae* VIN13 (non-recombinant strain) PCR reaction with *XYN2* primers, Lanes 7 and 8: *S. cerevisiae* VIN13-DLG30, Lanes 9 and 10: *S. cerevisiae* VIN13-DLG33, Lanes 11, 12, 13 and 14: *S. cerevisiae* VIN13-DLG39, Lane 15: λBSTEII marker.

The integration of the *end1* gene into the *ILV2* locus of the *S. cerevisiae* strains VIN13-DLG30, VIN13-DLG33 and VIN13-DLG39, and of the *xyn2* gene into the *SMR1* gene of the strains VIN13-DLG29, VIN13-DLG33 and VIN13-DLG39, was

confirmed through Southern blot analysis by probing with a 1563 bp DIG-labelled DNA fragment of the *B. fibriosolvens end1* gene and a 780 bp DIG-labelled DNA fragment of the *T. reesei xyn2* gene respectively (Fig 2).

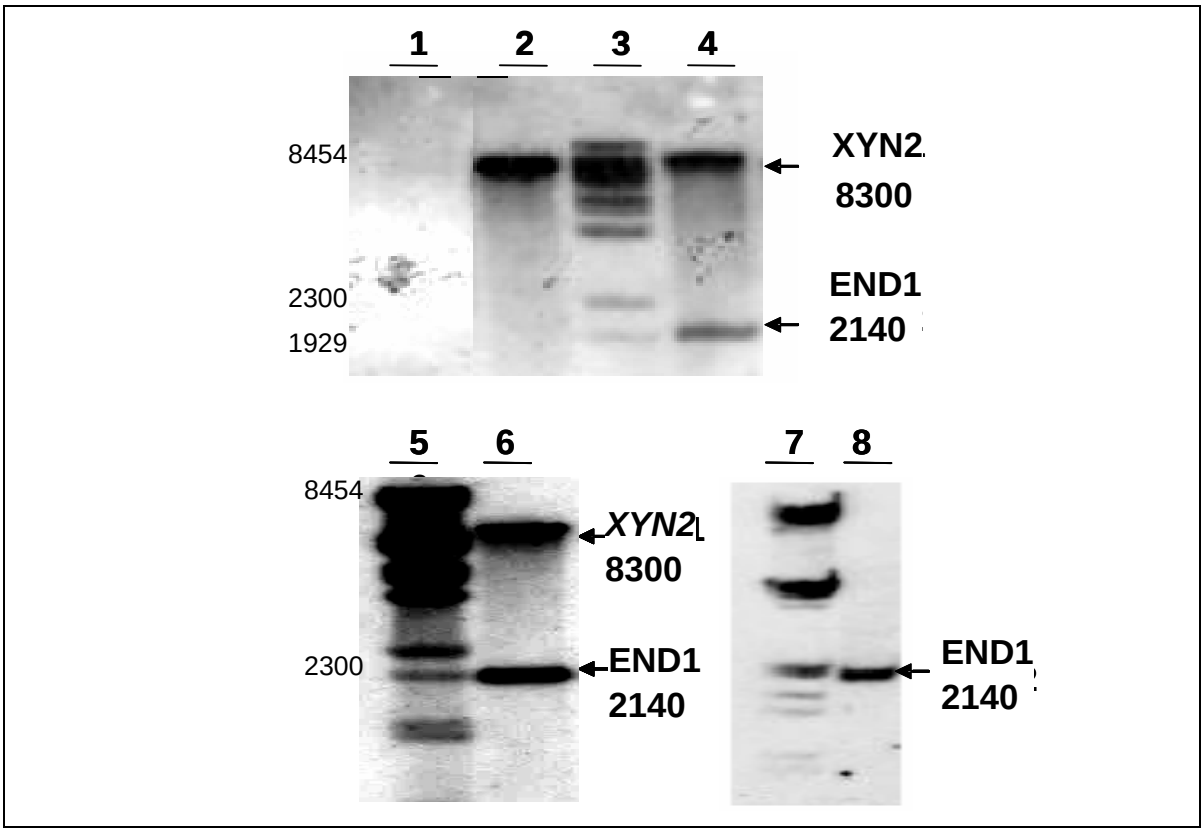

Figure 2 Southern blot analysis showing the integration of the *xyn2* and *end1* genes into the *ILV2* locus of the recombinant *S. cerevisiae* VIN13 strains. The *XYN2* gene can be seen as an 8300 bp fragment and *END1* as a 2140 bp fragment. Lane 1: *S. cerevisiae* VIN13 (non-recombinant strain), Lane 2: *S. cerevisiae* VIN13-DLG29, Lane 3: λ*BSTE*II marker, Lane 4: *S. cerevisiae* VIN13-DLG39, Lane 5: λ *BSTE*II marker, Lane 6: *S. cerevisiae* VIN13-DLG33, Lane7: λ *BSTE*II marker, Lane 8: *S. cerevisiae* VIN13-DLG30.

3.3.2 PRESS YIELDS AND FREE-FLOW WINE

There were no significant differences in total wine yield by Ruby Cabernet or Pinot noir must fermented with the different recombinant wine yeast strains or upon treatment with a commercial pectinase preparation. In this study, free-flow wine was extracted without applying any pressure to the skins. Figure 3 shows the effects of fermenting Ruby Cabernet must with different recombinant strains on the extraction of free-flow wine.

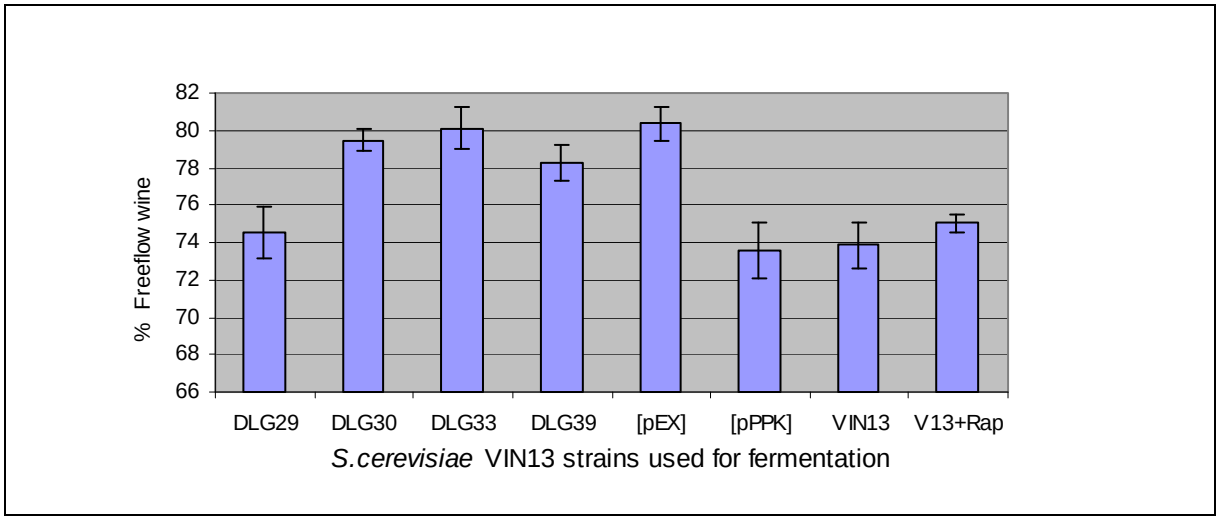

Figure 3 This figure shows the percentage of free-flow wine yielded after fermenting Ruby Cabernet must with different recombinant wine yeast strains (p=0.03, F=3.12). The wine DLG29 was fermented with the recombinant strain VIN13-DLG29, DLG30 with the strain VIN13-DLG30, DLG33 with the strain VIN13-DLG33, DLG39 with the strain VIN13-DLG39, [PEX] with the strain VIN13-PEX, [pPPK] with the strain VIN13-PPK and VIN13+Rap with VIN13 with the addition of 0.3g/l Rapidase-X-colour enzyme.

From Figure 3 it can be seen that the fermentation of Ruby Cabernet must with the recombinant strains secreting glucanase and xylanase resulted in a significant increase in free-flow wine ($p<0.05$). Fermentation with the strains secreting both enzymes, VIN13-DLG33 and VIN13[pEX], resulted in the largest increase in free-flow wine when compared to the untransformed strain. Fermentation with all of the recombinant glucanase-secreting strains resulted in a much larger increase in free-flow wine than treatment with the commercial pectinase preparation.

3.3.3 WINE COLOUR

Colour analysis was done on all the Pinot noir and Ruby Cabernet wines at four different stages: before press, after press, during bottling and after six months of bottle ageing. No significant differences were found in the colour parameters of either of the red cultivars up to bottling ($p>0.05$) (data not shown). Figure 4 (a to d) shows the colour measurements taken for Pinot noir wines fermented with different recombinant wine yeast strains after six months of bottle ageing. Significant differences in colour parameters were obtained after ageing in the fermentations with the different recombinant strains ($p<0.05$).

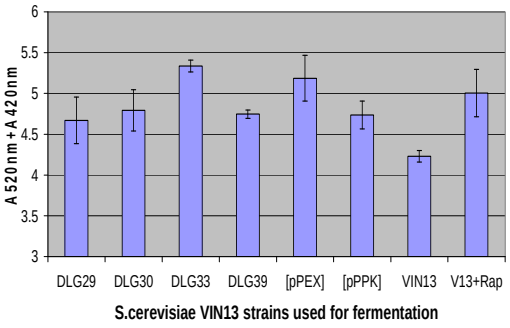

Fig. 4a Colour density (p=0.0035; F=6.658)

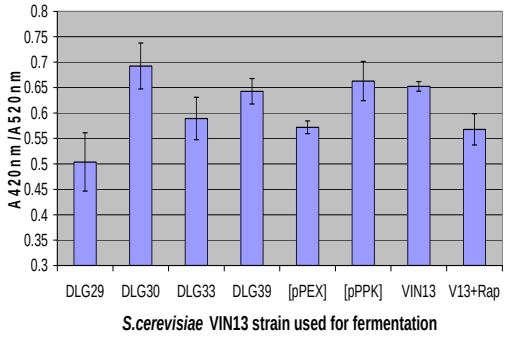

Fig. 4b Colour hue (p=0.0005; F=7.79)

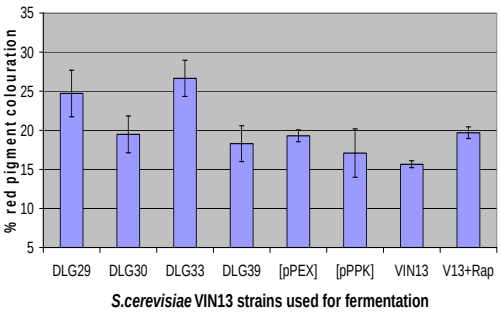

Fig. 4c Percentage of red-coloured pigments (p=0.006; F=7,63)

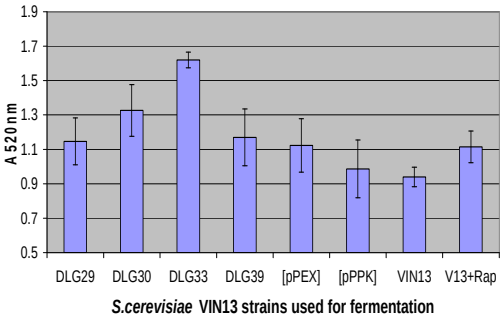

Fig. 4d Concentration of red-coloured pigments resistant to SO_2 bleaching (p=0.001; F=6.92)

Figure 4 This figure shows colour parameters of Pinot noir wines fermented with different recombinant *S. cerevisiae* VIN13 strains after six months of bottle ageing. The wine DLG29 was fermented with the recombinant strain VIN13-DLG29, DLG30 with the strain VIN13-DLG30, DLG33 with the strain VIN13-DLG33, DLG39 with the strain VIN13-DLG39, [PEX] with the strain VIN13-PEX, [pPPK] with the strain VIN13-PPK and VIN13+Rap with VIN13 with the addition of 0.3g/l Rapidase-X-colour enzyme.

From Figure 4a, it can be seen that fermentation of the Pinot noir must with all the recombinant strains resulted in an increase in colour density after six months of bottle ageing when compared to fermentation with the non-recombinant strain. An increase in colour density was also obtained when the fermentation with the non-recombinant strain was treated with the commercial pectinase preparation Rapidase EX Colour (Gist Brocades). The largest increases in colour density were obtained when the Pinot noir must was fermented with the two strains secreting glucanase and xylanase, VIN13-DLG33 and VIN13[pEX].

Figure 5 (a to d) shows the colour measurements taken for Ruby Cabernet wines fermented with different recombinant wine yeast strains after six months of bottle ageing. Significant differences in colour parameters were obtained when fermenting with different recombinant strains ($p<0.05$)

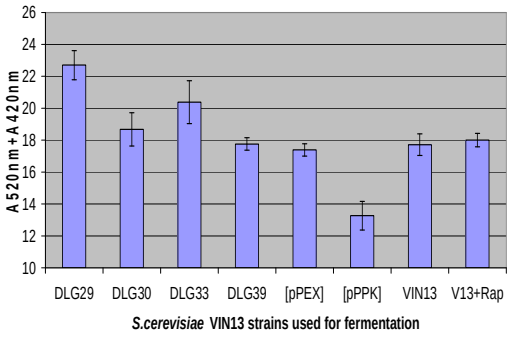

Fig. 5a Colour density (p=0; F=29.45)

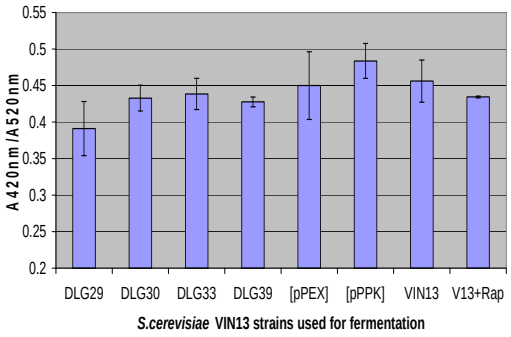

Fig. 5b Colour hue (p=0.05, F=0.0536)

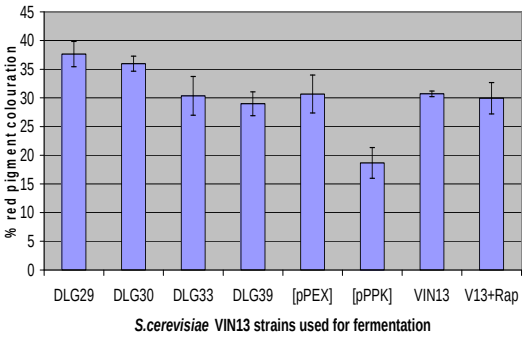

Fig. 5c Percentage of red-coloured pigments (p=0.0001, F=17.147)

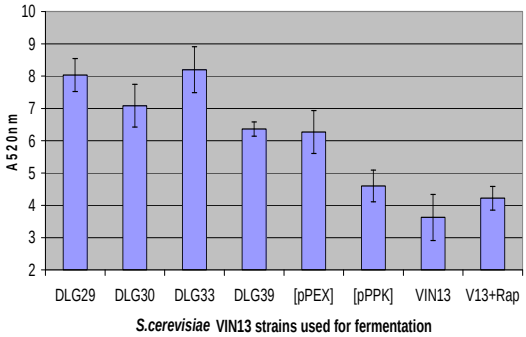

Fig. 5d Concentration of red-coloured pigments resistant to SO_2 bleaching (p=0, F=23.58)

Figure 5 This figure shows the colour parameters of Ruby Cabernet wines fermented with different recombinant *S. cerevisiae* VIN13 strains after six months of bottle ageing. The wine DLG29 was fermented with the recombinant strain VIN13-DLG29, DLG30 with the strain VIN13-DLG30, DLG33 with the strain VIN13-DLG33, DLG39 with the strain VIN13-DLG39, [PEX] with the strain VIN13-PEX, [pPPK] with the strain VIN13-PPK and VIN13+Rap with VIN13 with the addition of 0.3g/l Rapidase-X-colour enzyme.

From Figure 5a it can be seen that fermentation of the Ruby Cabernet must with the glucanase- and xylanase-secreting strain, VIN13-DLG33, and with the xylanolytic strain, VIN13-DLG29, resulted in a significant increase in colour density after six months of bottle ageing when compared to fermentation with the non-recombinant strain. A slight increase in colour density could also be observed upon fermentation with the glucanolytic strain, VIN13-DLG30. Fermentation with the pectolytic strain, VIN13[pPPK], resulted in a large decrease in colour density when compared with the non-recombinant strain.

When the colour hue (Figs 4b and 5b) is compared with the colour density (Figs 4a and 5a), it can be seen that fermentation with the strains that resulted in the highest colour density yielded the lowest colour hue values and these wines therefore were more red than brown. Fermentation with VIN13-DLG29, VIN13-PPK and the addition of enzyme resulted in the higher colour density and lower hue values also resulted in a higher percentage of the pigments being coloured (Figs 4c

and 5c) and more pigments being resistant to discolouration by SO_2(Figs 4d and 5d).
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3.3.4 VOLATILE COMPOSITION

The analysis of the volatile compounds was carried out by gas chromatography. Tables 3, 4 and 5 show the levels of acids, alcohols and esters in the Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines. Only compounds showing significant differences ($p<0.05$) between the fermentations by the different yeast strains, when compared by ANOVA testing, were included.

Table 3 Concentration of volatile components (mg/l) in Pinot noir wines fermented with different recombinant *S. cerevisiae* strains after six months of bottle ageing.

	DLG29	DLG30	DLG33	DLG39	[PEX]	[PPK]	VIN13	V13+Rap
Alcohols								
2-phenylethanol	11.46	12.287	11.037	13.777	14.65	13.027	14.265	14.165
Hexanol	1.31	1.3333	1.8133	1.24	1.8933	1.73	1.68	1.78
i-AmOH	49.533	51.29	65.737	45.557	65.113	60.63	64.275	61.01
i-BuOH	67.513	28.343	37.633	28.737	18.987	18.723	18.92	19.745
MeOH	26.937	34.28	31.18	31.493	40.1	37.087	36.53	48.16
n-PrOH	33.537	35.84	89.067	29.673	51.533	47.68	50.22	42.87
Esters								
2PhenEtAc	0.0667	0.0667	0.0967	0.0733	0.08	0.0767	0.07	0.065
diethyl succinate	0.2067	0.23	0.3233	0.19	0.31	0.2833	0.305	0.275
ethyl acetate	21.867	22.95	31.78	20.787	27.85	26.83	26.86	27.96
ethyl lactate	5.62	4.9633	3.2533	4.06	6.0967	5.71	5.89	5.65
EtC6	0.1367	0.15	0.12	0.1167	0.1667	0.1733	0.175	0.175
EtC10	0.2433	0.2833	0.2967	0.2433	0.37	0.38	0.39	0.39
i-AmAc	1.32	1.2567	1.4667	1.19	1.0933	1.2367	1.325	1.27
Acids								
isobutyric acid	2.7467	0.9933	1.4633	0.97	0.6867	0.5767	0.705	0.665
isovaleric acid	0.6133	0.5733	0.5667	0.5667	0.5867	0.54	0.555	0.565
n-butyric acid	0.2533	0.25	0.2467	0.2233	0.2967	0.25	0.26	0.285
n-valeric acid	0.4667	0.4733	0.6133	0.39	0.4967	0.46	0.46	0.48
octanoic acid	0.4033	0.44	0.3033	0.35	0.5167	0.59	0.59	0.525
propionic acid	15.93	9.21	22.99	8.7367	14.88	14.557	12.47	17.71

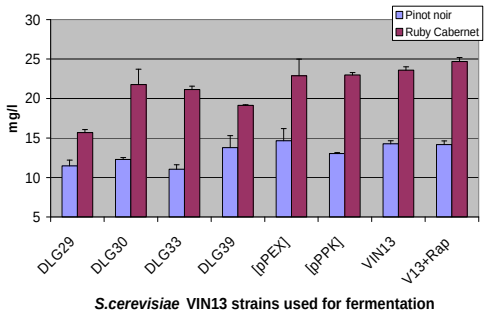
Table 4 Concentration of volatile components (mg/l) in Ruby Cabernet wines fermented with different recombinant *S. cerevisiae* strains after six months of bottle ageing.

	DLG29	DLG30	DLG33	DLG39	[PEX]	[PPK]	VIN13	V13+Rap
Alcohols								
2-phenylethanol	15.68	21.78	21.15	19.13	22.897	22.983	23.59	24.68
hexanol	0.8333	1.03	1.35	1.0333	1.3167	1.3733	1.44	1.405
i-AmOH	27.9	34.727	39.91	31.803	38.863	38.843	41.7	42.41
i-BuOH	27.74	15.85	20.977	18.03	14	12.727	13.02	12.12
MeOH	23.637	34.833	31.893	26.7	35.483	38.84	37.99	59.16
n-PrOH	8.6533	12.737	12.07	9.9867	14.713	15.27	16.34	16.88
Esters								
ethyl acetate	10.453	15.523	32.787	12.153	15.19	19.293	17.485	17.425
ethyl lactate	1.2767	1.6	1.3	1.83	1.7	1.3367	1.95	1.7
i-AmAc	0.1833	0.2033	0.25	0.2	0.1833	0.2167	0.2	0.195
EtC6	0.1433	0.1733	0.15	0.15	0.1767	0.1767	0.175	0.19
EtC8	0.0567	0.08	0.07	0.0667	0.0867	0.08	0.095	0.08
EtC10	0.14	0.2133	0.1933	0.17	0.2167	0.22	0.225	0.24
Acids								
isobutyric acid	1.9167	1.1567	1.3767	1.3067	0.94	0.8467	0.905	0.975
isovaleric acid	1.5033	1.5133	1.31	1.5667	1.4267	1.4	1.5	1.52
octanoic acid	0.2133	0.2867	0.2333	0.25	0.32	0.3033	0.34	0.325

Table 5 Concentration of volatile components (mg/l) in Muscat d'Alexandria wines fermented with different recombinant *S. cerevisiae* strains.

	DLG29	DLG30	DLG33	DLG39	[PEX]	[PPK]	VIN13	V13+Rap
Alcohols								
i-BuOH	84.205	39.907	28.027	34.667	18.277	15.137	17.185	12.065
n-PrOH	31.57	44.073	31.577	27.737	34.747	38.713	62.955	37.22
Esters								
diethyl succinate	1.03	1.51	2.07	0.157	0.886	0.567	0.275	0.07
Acids								
isobutyric acid	2.105	1.2	0.93	0.9333	0.8	0.52	0.825	0.91

Figure 6 (a to d) compares the concentration of alcohols in Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines that showed a significant difference upon fermentation with the different recombinant wine yeast strains ($p<0.05$)



6a 2-phenyl ethanol

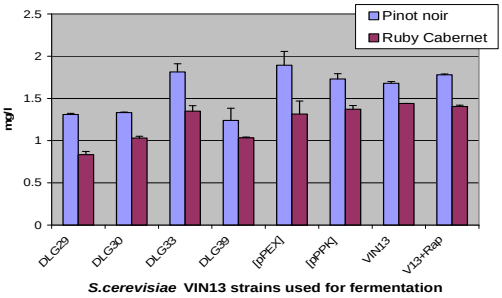


Fig. 6b hexanol

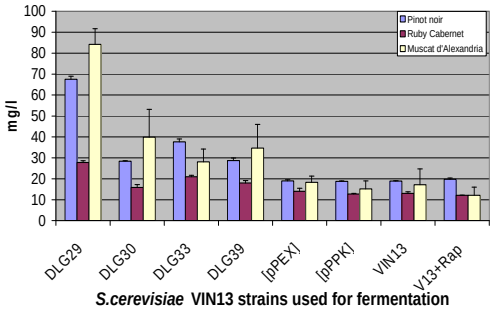


Fig. 6c isobutanol

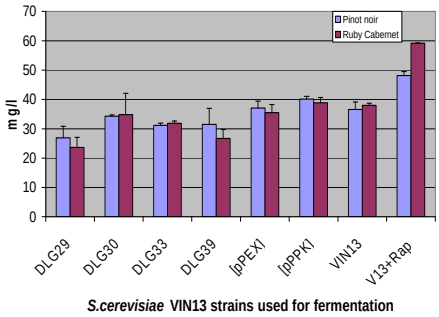


Fig. 6d methanol

Figure 6 Concentration of alcohols in Pinot noir and Ruby Cabernet wines fermented with different recombinant *S. cerevisiae* VIN13 strains ($p<0.05$). The wine DLG29 was fermented with the recombinant strain VIN13-DLG29, DLG30 with the strain VIN13-DLG30, DLG33 with the strain VIN13-DLG33, DLG39 with the strain VIN13-DLG39, [PEX] with the strain VIN13-PEX, [pPPK] with the strain VIN13-PPK and VIN13+Rap with VIN13 with the addition of 0.3g/l Rapidase-X-colour enzyme.

From Figure 6a it can be seen that fermentation with the xylanolytic strain, DLG29, and with the glucanase- and xylanase-secreting strain, DLG33, resulted in a decrease in 2-phenyl ethanol for both red cultivars when compared to fermentation with a non-recombinant strain. Fermentation with DLG39 only resulted in a decrease in 2-phenyl ethanol in the Ruby Cabernet wine. Treatment with the commercial enzyme preparation did not alter the 2-phenyl ethanol level.

Figure 6b shows that fermenting with the strains DLG29, DLG30 and DLG39 resulted in a decrease in the concentration of hexanol for both red cultivars when compared to fermentation with a non-recombinant strain. Treatment with the commercial enzyme preparation did not alter the hexanol concentration.

From Figure 6c it can be seen that fermentation with all the recombinant strains created in this study resulted in an increase in isobutanol for all three cultivars when

compared to fermentation with a non-recombinant strain. A very large increase was obtained for all three cultivars when fermenting with the xylanolytic strain, DLG29.
Figure 6d shows that none of the fermentations with any of the recombinant strains resulted in a large increase in methanol levels in either the Pinot noir or Ruby Cabernet wines, although a slight increase compared to the non-recombinant strain could be seen for both cultivars upon fermentation with the pectolytic strain. Treatment of the must with the commercial pectinase preparation resulted in a significant increase in methanol levels in both the Pinot noir and Ruby Cabernet wines.

Figure 7 compares the concentration of isobutyric acid in Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines fermented with different recombinant wine yeast strains. Fermentation with all the recombinant strains created in this study resulted in a significant increase in isobutyric acid for all three cultivars when compared to fermentation with a non-recombinant strain ($p<0.05$). A very large increase was obtained for all three cultivars when fermenting with the xylanolytic strain, DLG29.

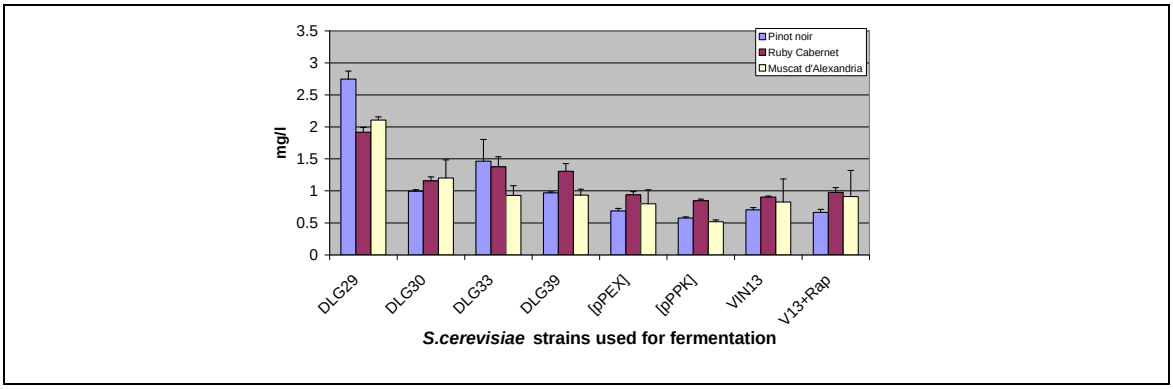


Figure 7 Concentration of isobutyric acid in Pinot noir ($p=0$, $F=51.89$), Ruby Cabernet ($p=0$, $F=30.04$) and Muscat d'Alexandria ($p=0.0002$, $F=14.92$) wines fermented with different recombinant *S. cerevisiae* VIN13 strains.

Figures 8a and b and Figure 9 compares the concentration of some of the esters in Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines fermented with the different recombinant wine yeast strains. Fermentation with the recombinant strains resulted in significant alterations in the ester profiles of the wines ($p<0.05$).

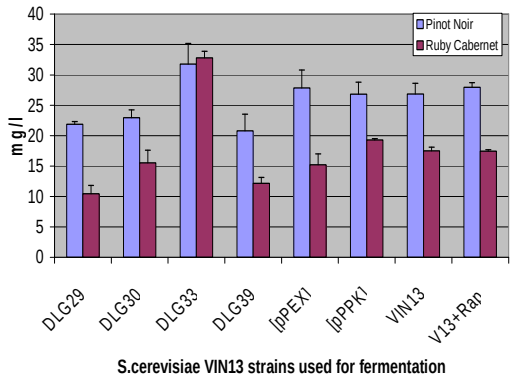


Fig. 8a ethyl acetate

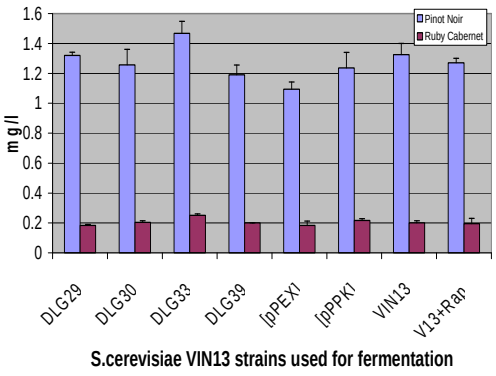


Fig. 8b isoamyl acetate

Figure 8 Concentration of esters in Pinot noir and Ruby Cabernet wines fermented with different recombinant *S. cerevisiae* VIN13 strains ($p<0.05$).

Figure 8a shows that fermentation with the glucanase- and xylanase-secreting strain, DLG33, resulted in a large increase in ethyl acetate in both red cultivars when compared to fermentation with the non-recombinant strain. Figure 8b shows that fermentation with the glucanase- and xylanase-secreting strain, DLG33, resulted in a small increase in isoamyl acetate in both red cultivars when compared to fermentation with the non-recombinant strain.

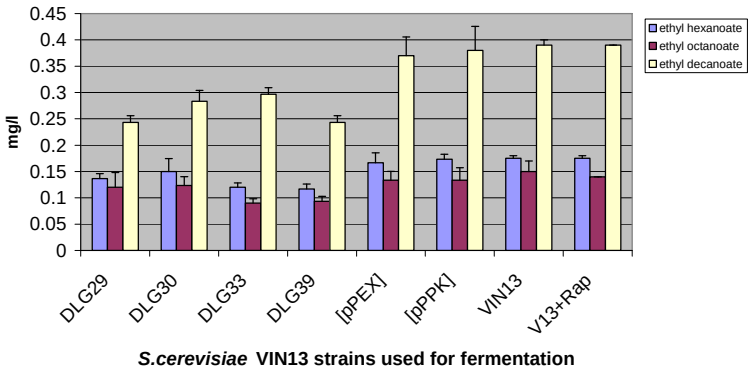


Figure 9 Concentration of the ethyl hexanoate ($p=0.026$, $F=5.81$), ethyl octanoate (0.07, $F=2.41$) and ethyl decanoate ($p=0.00005$, $F=12.428$) esters in Pinot noir wines fermented with different recombinant *S. cerevisiae* VIN13 strains.

From Figure 9 it can be seen that the fermentation of Pinot noir must with the recombinant strains DLG29, DLG30, DLG33 and DLG39 resulted in a decrease in the ethyl hexanoate, ethyl octanoate and ethyl decanoate esters. Fermentation with the different yeast strains resulted in statistically significant differences in ethyl hexanoate and ethyl decanoate ($p<0.05$). Although the differences in ethyl octanoate were not statistically significant between the fermentations ($p=0.07$), the trend observed was similar to that of the other two esters.

3.3.5 SENSORY ANALYSIS

Wines fermented with the same yeast strains received similar scoring in both tasting sessions (data not shown). The Pinot noir wines fermented with the glucanase- and

xylanase-secreting strains, VIN13-DLG33, VIN13-DLG39 and VIN13-PEX, and the wines treated with the commercial enzyme were rated in both tasting sessions as having higher colour intensity and a more intense red shade then wines fermented with VIN13 without enzyme treatment. Wines fermented with VIN13-DLG33 were perceived as having the most fruity and spicy characteristics, as well as the highest colour intensity and red tint out of all the wines evaluated. Figure 10 shows a radar graph comparing the aroma profile, colour parameters and astringency of the wines fermented with VIN13, VIN13 treated with commercial enzyme, VIN13-PEX (Fig 10a) and VIN13-DLG33 (Fig 10b).

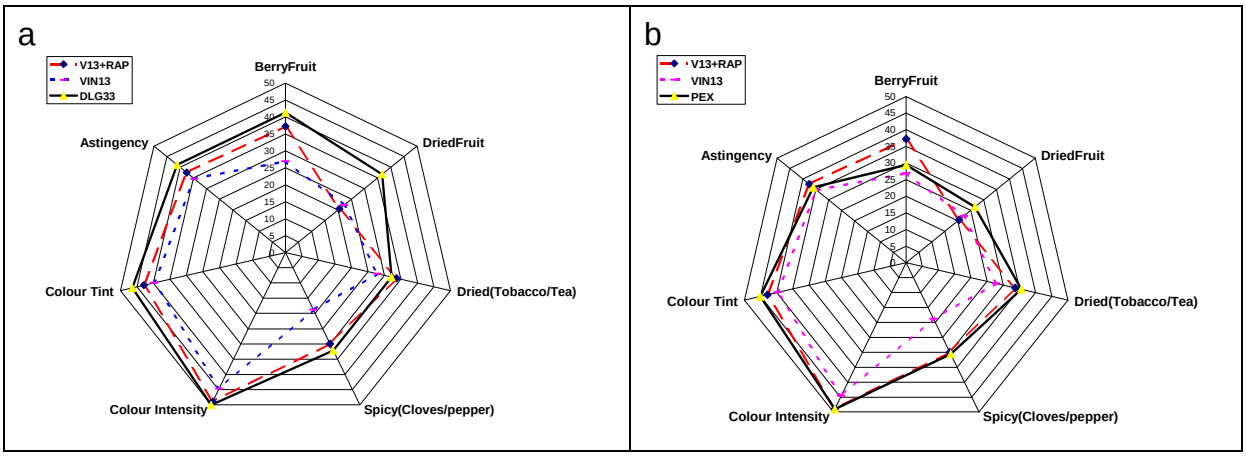


Figure 10 Radar graphs comparing the aroma, flavour, colour and astringency of the Pinot noir wines fermented with VIN13, VIN13 treated with commercial enzyme, VIN13-DLG33 and VIN13-PEX as evaluated by a tasting panel of 12 tasters.

3.4 DISCUSSION

Since the endogenous polysaccharase activity of *Saccharomyces cerevisiae* is very limited, the heterologous expression of specific polysaccharase genes in an industrial yeast strain can improve the winemaking process, resulting in a higher quality wine without the addition of expensive commercial enzyme preparations.

None of the fermentations with any of the recombinant strains resulted in an increase in total wine yield. During fermentation on the skins, the presence of ethanol and SO_2 increases the permeability of the cell walls, diminishing the differences as a result of enzyme treatment of the must when pressure is applied. The fermentation of Ruby Cabernet must with all the glucanase- and xylanase-secreting strains resulted in an increase in free-flow wine (Fig 3) due to the degradation of the cell walls by these enzymes (Haight and Gump 1994). These results are consistent with the information in other papers, which reported improved free-run juice yield upon treatment with commercial macerating enzymes (Haight and Gump 1994, Plank and Zent 1993). Synergistic degradation of the cell walls by the endo-β-1,4-xylanase and the endo-β-1,4-glucanase secreted by the strains VIN13-DLG33 and VIN13[pEX] resulted in the largest increase in free-flow wine. The fact that the free-flow wine yielded upon fermentation with the VIN13-DLG39 strain was less than with VIN13-

DLG33 and VIN13[pEX] might have been due to the lower transcription levels of the xylanase gene under control of the YG100 promoter, which has a low basal level of expression (Slater and Craig 1987).

No significant differences were detected in colour density up to bottling. The anthocyanin content in the different fermentations was probably similar and the enzyme secreted might not have had a significant influence on anthocyanin extraction. However, significant differences in colour intensity and stability were detected after periods of bottle ageing. The increase in colour intensity of the wines fermented with the recombinant wine yeast strains after six months of ageing may be due to increases in the polymeric anthocyanin content or to co-pigmentation effects (Figs 4a and 5a). Cell wall-bound tannins and flavonoid phenols can be released upon enzymatic degradation of the cellulose fibres in the cell wall (Amrani Joutei *et al* 2003). The polymerisation of the anthocyanins with the tannins and phenols that were released could increase light absorption and colour intensity (Somers and Westcomb1982).

Fermentation with polysaccharase-secreting strains resulted in lower hue values and these wines thus had more of a red then a brown shade (Figs 4b and 5b). This might have been due to a higher percentage of the anthocyanins being coloured (Figs 4c and 5c). The increase in coloured pigments can also be attributed to polymerisation, which protects the anthocyanidin portion of the pigments against oxidation, chemical modification and, as can be seen from Figures 4d and 5d, SO_2 bleaching. These results on colour intensity and stability correspond with results reported in studies on the effects of industrial enzyme preparations on colour extraction and stability (Watson *et al* 1999, Gump and Haight 1995). Fermentation with all of the recombinant strains resulted in an increase in colour density when fermenting Pinot noir, while only two strains yielded an increase in colour density when fermenting Ruby Cabernet. This may be because colour extraction from Pinot noir pulp is very difficult and even a small amount of enzyme secreted into the must results in a detectible colour difference, while colour is easily extracted from Ruby Cabernet and more enzyme is needed to give a detectible colour difference. The synergistic action of the enzymes secreted by the VIN13-DLG33 strain proved to be the most efficient in improving colour intensity and stability when fermenting Pinot noir must, producing wines with the highest colour density, best tint and most stable and SO_2-resistant pigments. This strain was also very efficient when fermenting Ruby Cabernet. Fermentation of Ruby Cabernet must with the xylanolytic VIN13-DLG29 strain resulted in large improvements in colour intensity and stability. More xylanase might have been secreted in the must when the *XYN2* gene was regulated by the $TEF1_P$. The degradation of hemicellulases might also play a more important role in colour extraction from the skin cells of this cultivar.

The decrease in the levels of isobutanol and 2-phenyl ethanol upon fermentation of the Ruby Cabernet and Pinot noir must was the opposite of what was found in previous studies, which reported an increase in the concentration of these alcohols

when fermenting with glucanase- and xylanase-secreting *S. cerevisiae* strains (Ganga *et al* 1999, Gill and Vallés 2001). These studies attributed the higher levels to the release of more glycosidically-bound precursors from the cell wall as a result of the enzymatic degradation of the cell wall. The increases in this study may be the result of alterations in must composition as a result of enzyme action having an influence on yeast metabolism, resulting in decreased levels of alcohol production.

There was a significant increase in methanol levels in the Ruby Cabernet and Pinot noir wines when the must was treated with a commercial pectinase preparation (Fig 6d). This could have been the result of pectin-methyl-esterases being present in the pectinase preparation. These enzymes release methanol from pectin into the must (Brown and Ough 1981). The concentration was, however, still in the normal range of 0 to 0.6g/l (Rankine 1998). The decrease in methanol concentration when fermenting with the DLG29 strain may be due to must alterations caused by hemicellulose degradation.

The large increases in isobutanol and isobutyric acid concentrations when fermenting Ruby Cabernet, Pinot noir and Muscat d'Alexandria must with the DLG29 strain may be the result of alterations in must composition due to hemicellulose degradation, which will influence yeast metabolism. The isobutanol concentration is still in the normal range of 0 to 0.1g/l. The isobutyric acid levels of all three cultivars fermented with DLG29 are above the maximum of 0.02 g/l, which might cause an off-flavour in the wine (Rankine 1998).

The fermentation of all three cultivars with the glucanase- and xylanase-secreting VIN13-DLG strains resulted in a decrease in the acetate esters (Fig 8). The VIN13-DLG33 strain was an exception. Fermentation of the Pinot noir and Ruby Cabernet musts with this strain resulted in an increase in ethyl acetate and isoamyl acetate. These two esters are the most important esters in wine and are implicated in a pleasant fruity aroma (Plata *et al* 2003). The decrease in ethyl octanoate (Fig 9) corresponds with the decrease in its octanoic acid precursor (Table 4). Since acid production was influenced by polysaccharide degradation in the must, it would also influence the amount of esters produced.

Sensory evaluation of the Pinot noir wines showed that fermentation with the recombinant strains did not only result in differences that could be noticed during the wine analysis, but that these differences could be detected by a tasting panel. Colour evaluation by the tasting panel corresponded with spectrophotometer results and fermentation with the same two strains, VIN13-DLG33 and VIN13-PEX, showed the highest colour intensity and the most red tint (Fig 10). Fermentation with the recombinant strains also resulted in an increase in fruity and spicy characteristics (Fig 10), which could be possible due to the increase in some of the esters (Fig 9).

The wines fermented with the recombinant strains may have made a more significant impact on the wines produced then the addition of commercial enzyme, because the enzyme is produced through out the fermentation.

In conclusion, fermentation with the polysaccharide-degrading strains resulted in significant improvements in juice extraction, colour intensity and stability and in alterations in the aromatic profiles of the wines produced. Fermentation with the recombinant strain VIN13-DLG33, expressing the *T. reesei XYN2* gene under control of the $ADH2_P$ and the *B. fibriosolvens END1* gene under control of the $ADH1_P$ promoter, showed the most significant improvements in all the above mentioned parameters. During the sensory evaluation of the wines, the wine fermented with the aforementioned strain was also evaluated as having the highest colour intensity and red tint and most fruity and spicy characteristics.

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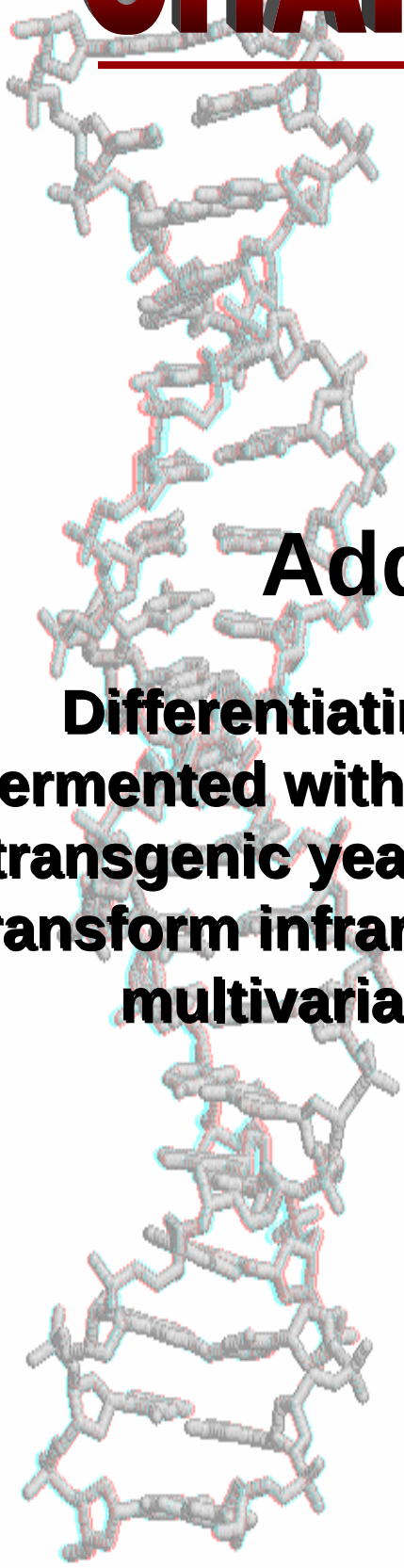
CHAPTER 4



Addendum

Differentiating between wines fermented with transgenic and non-transgenic yeast strains by Fourier transform infrared spectroscopy and multivariate data analysis

4. ADDENDUM

Differentiating between wines fermented with transgenic and non-transgenic yeast strains by Fourier transform infrared spectroscopy and multivariate data analysis

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Considerable progress in the field of genetic engineering has led to the development of a variety of recombinant wine yeast strains. Despite this technology being available and holding definite advantages, no transgenic wine yeast is yet being used on a commercial scale to produce wine. This is not only due to technical and economic limitations, but can mostly be attributed to consumer resistance. Wine trades heavily on its image as a traditional artisan product and this image clashes with the use of GMOs (genetically modified organisms). Consumers are also worried about health and ethical issues in relation to the use of genetically modified products (Lawton 2002).

Because wine is so reliant on its traditional image, authenticity is a very important factor in wine marketing and quality regulation. Authenticity in wine implies, amongst other aspects, that the product is indeed what it claims to be in terms of cultivar, geographic origin, vintage, maturation period and conforming to certain guidelines concerning chemical composition. Authenticity testing in wine is difficult due to the complexity of the product. The number of components that must be tested are continuously increasing and novel analytical approaches are being developed to meet the increasing demands set by the industry (Downey 1998). If transgenic yeast strains are accepted in the wine industry, it may be required that the GMO status of the yeast that has been used appears on the label. This is another factor for which authenticity tests may be necessary.

Major developments in analytical methods and instrumentation have resulted in increasingly sophisticated methods to authenticate wines. Some of the techniques that have been applied in authenticity testing are nuclear magnetic resonance spectroscopy (NMR), spectroscopy, Fourier transform near-infrared spectroscopy and Fourier transform mid-infrared (FT-IR) spectroscopy coupled with multivariate data analysis (Careri *et al* 2002, Downey 1998).

FT-IR is a powerful analytical tool that is used for the quantitative and qualitative analysis of wine in the mid-infrared region (Budinova *et al* 1998). Infrared

spectroscopy involves the interaction of a molecule with electromagnetic radiation. When an organic molecule is irradiated with infrared light, the frequencies of light that are absorbed by the molecule correspond to the amounts of energy needed to increase the amplitude of specific molecular vibrations, such as bond stretching and bending (McMurry 1992). A detector measures the amount of energy that passes through a sample at each frequency and a spectrum can then be created. The IR (infrared) spectrum of a pure substance is unique and can serve as a fingerprint for a component (Behrens 2002). In multiplex instruments, the composite absorbance of all the components in a sample over the complete optical wave number range is collected simultaneously by a detector. This signal is then decoded by a mathematical procedure known as Fourier transformation (Skoog *et al* 1997). As the concentration of a molecule is proportional to the absorption of IR light, it is possible to perform quantitative analysis (Behrens 2002). Because an FT-IR scan of wine contains information on a great variety of components, an extensive calibration process involving multivariate statistical procedures is required for the quantification of individual components (Esbensen 2000).

Qualitative analysis can also be done with FT-IR spectroscopy. Unique regions in the IR spectra of wines are selected, and these regions are compared by multivariate data analysis and are used to classify wines. This discriminative analysis assumes that the spectra of given materials are similar and will differ from spectra of other materials. Mathematical procedures are used to find a formula or model that will minimise within group dispersion, but will maximise the distance between different groups.

Principle component analysis (PCA) is a modelling method that provides an interpretable overview of the main sources of variation in a sample set (Esbensen 2000). The information contained in the original variables (in this study the absorbance at the respective wave numbers), is projected onto a smaller number of underlying or latent variables, called principal components. The first principal component is calculated to explain the biggest variation in the data set, and subsequent components describe, in decreasing order, the remaining variation in the data set. Principal components are calculated to be orthogonal to one another and can therefore be interpreted independently. By plotting the samples (in this study the FT-IR wine spectra) in the space defined by the principal components, the interrelationships between the different samples can be visualised, and sample patterns, groupings, similarities or differences can be detected and interpreted.

Classification tree analysis (CART) is another model that can be used to classify data. In the case of classification trees, the dependent (response) variable is a discrete variable consisting of two or more classes. The concept of entropy (chaos) is used as basis for constructing classification trees. To explain entropy in the framework of classification trees, consider a response variable with two classes namely yes and no. If a data set consists of 50% yes and 50% no responses, then the entropy of that data set is a maximum because the data will have only a 50%

chance of correctly predicting the class of the response variable. The entropy becomes lower as the proportion of one of the classes tends to 100%, and it reaches a minimum when a data set consists of 100% of one class. In this case, the data will have a 100% chance of correctly predicting the class of the response variable.

Entropy can be calculated from a data set using various methods, of which the Gini measure is probably the most common in classification trees. The aim of a classification tree is to divide the data set into subsets in such a way that the subsets have a lower entropy than the full data set. Thus, it strives to group the classes together into subsets, as best as possible, on the basis of the independent or predictor variables. From the tree that is created by the classification trees procedure, rules can be derived that give interpretable descriptions of the data.

The aim of this study was to determine if FT-IR spectroscopy coupled with multivariate data analysis can distinguish between wines fermented with transgenic and non-transgenic yeast strains, or between wines fermented with different transgenic strains. Two methods of multivariate data analysis, CART and PCA analysis, were used to compare these spectra.

Gas chromatographic analysis of the wines that were analysed showed significant differences in the acid, ester and alcohol profiles (chapter 3). Since replicate fermentations with the same strains showed similar wine profiles, it might be possible to identify unique regions in the FT-IR spectra of wines fermented with a specific strain. These regions could serve as a unique fingerprint, allowing for the identification, from a filtered wine sample, of the yeast strain used for fermentation. Genetic techniques cannot be used to identify the strain used, since no yeast DNA is present in the filtered wine sample.

4.1 MATERIALS AND METHODS

4.1.1 MICROVINIFICATION

Wine was made using the grape varieties Pinot noir, Ruby Cabernet, Muscat d'Alexandria and Colombar. Muscat d'Alexandria and Colombar grapes were crushed, destemmed and then pressed, and 30 ppm of SO_2 was added to the juice. The juice was clarified overnight, after which 6.5 L juice samples were inoculated to a final concentration of 2 x 10^6 cells/mL with the appropriate yeast strains. The red varieties were crushed and destemmed and divided into samples consisting of 8 L of juice and 11 kg of skins each, and 40 ppm of SO_2 was added to each sample. Each sample was inoculated with the appropriate yeast strain to a final concentration of 2 x 10^6 cells/mL. Fermentations with Pinot noir, Ruby Cabernet and Muscat d'Alexandria were performed in triplicate, while those with Colombar were performed in duplicate. Separate samples of each cultivar were inoculated with the appropriate yeast strains. The strains that were used for the fermentation of the different cultivars are shown in Table 1. The must of the red varieties was then fermented at 25°C and that of the

white variety at 15°C. Samples (25 mL) were taken six months after bottling for the Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines, and directly after bottling for Colombar. These samples were scanned with the WineScan FT 120 instrument (Foss, Denmark).

Table 1 Wines used for FT-IR spectroscopy

S. cerevisiae strain used for fermentation	Genotype/Description	Source/Reference	Cultivars inoculated
VIN13	commercial diploid strain	Anchor Yeast Technologies (SA)	Pinot noir Ruby Cabernet Muscat d'Alexandria Colombar
VIN13-EXS	*ura3::ADH1$_P$-MFα1$_S$-END1-TRP5$_T$ ADH1$_P$–XYNC-ADH2$_T$*	Strauss 2003	Pinot noir Ruby Cabernet Muscat d'Alexandria
VIN13-PPK	*ura3::ADH1$_P$-MFα1$_S$–PELE-TRP5$_T$-ADH1$_P$-MFα1$_S$-PEH1-TRP5$_T$*	Strauss 2003	Pinot noir Ruby Cabernet Muscat d'Alexandria Colombar
VIN13-DLG29	*ILV2::TEF1$_P$-XYN2-ADH2$_T$*	This study	Pinot noir Ruby Cabernet Muscat d'Alexandria Colombar
VIN13-DLG30	*ILV2::ADH1$_p$-MFα1$_S$-END1-TRP5$_T$*	This study	Pinot noir Ruby Cabernet Muscat d'Alexandria Colombar
VIN13-DLG33	*ILV2::ADH1$_p$-MFα1$_S$-END1-TRP5$_T$ ADH2$_p$-XYN2-ADH2$_T$*	This study	Pinot noir Ruby Cabernet Muscat d'Alexandria Colombar
VIN13-DLG39	*ILV2::ADH1$_p$-MFα1$_S$-END1-TRP$_t$ YG100$_p$-XYN2-ADH2$_T$*	This study	Pinot noir Ruby Cabernet Muscat d'Alexandria Colombar
VIN13-PADC	*PGK1$_P$–PADC-PFKI$_T$*	Smit 2002	Colombar
VIN13-PDC	*PGK1$_P$–PDC-PFKI$_T$*	Smit 2002	Colombar
VIN13-BGLI	*BGLI$_P$-BGLI-BGLI$_T$*	Stidwell 2001	Colombar
VIN13-BGLII	*BGLII$_P$-BGLI-BGLII$_T$*	Stidwell 2001	Colombar
VIN13-ATF1	*PGK1$_P$–ATF1-PFKI$_T$*	Lilly *et al* 2000	Colombar

4.1.2 FT-IR SPECTRAL MEASUREMENTS

All samples were filtered with a Filtration Unit (type 79500, Foss, Denmark), using filter paper circles graded at 20 – 25 µm and with a diameter of 185 mm (Schleicher & Schuell, reference number 10312714), prior to scanning.

FT-IR spectra were generated with a WineScan FT 120 instrument (Foss Electric, Denmark) that employs a Michelson interferometer. Samples were pumped through the CaF_2-lined cuvette with an optical path length of 37 µm housed in the heater unit

of the instrument. Samples were scanned from 5011 to 929 cm^{-1} at 4 cm^{-1} intervals. The frequencies of the IR beam transmitted by a sample were recorded at the detector and used to generate an interferogram. The interferogram was calculated from a total of 10 scans and then processed by Fourier transformation. The background absorbance of water was corrected to generate a single beam transmittance spectrum. The transmittance spectrum for each sample was generated in duplicate in order to calculate the absolute repeatability of the spectral measurements. The calculation of the absolute repeatability has been described (WineScan FT 120 Type 77110 and 77310 Reference Manual, Foss, Denmark, 2001). The transmittance spectra were finally converted into linearised absorbance spectra through a series of mathematical procedures.

4.1.3 MULTIVARIATE DATA ANALYSIS

4.1.3.1 PRINCIPAL COMPONENT ANALYSIS (PCA)

FT-IR spectra were exported to Unscrambler Software (version 6.11, Camo ASA, Trondheim, Norway) for the purpose of PCA. Duplicate spectra were averaged and then autoscaled (mean centred and standardised).

4.1.3.2 CLASSIFICATION TREE (CART) ANALYSIS

CART analysis of the FT-IR spectra of the wines was performed using the STATISTICA software package. The dependent (response) variable γ is a discrete variable consisting of eight classes for Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines and 10 classes for Colombard wines. Rules derived from the classification trees were used to derive interpretable data. The CART analysis model was only used to classify the training set of data.

4.2 RESULTS

4.2.1 VOLATILE COMPOSITION

The analysis of volatile compounds was carried out by gas chromatography. Table 2 shows the levels of acids, alcohols and esters in the Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines that could have contributed to the difference in the FT-IR spectra of the wines fermented with different yeast strains. Only compounds showing significant differences ($p<0.05$) between fermentations by different yeast strains, when compared by ANOVA testing, were included.

Table 2 Concentration of volatile components (mg/L) in Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines fermented with different recombinant *S. cerevisiae* strains after six months of bottle ageing.

	DLG29	DLG30	DLG33	DLG39	[PEX]	[PPK]	VIN13	V13+Rap
Pinot noir								
ethyl acetate	21.867	22.95	31.78	20.787	27.85	26.83	26.86	27.96
i-AmAc	1.32	1.2567	1.4667	1.19	1.0933	1.2367	1.325	1.27
isobutyric acid	2.7467	0.9933	1.4633	0.97	0.6867	0.5767	0.705	0.665
EtC6	0.1367	0.15	0.12	0.1167	0.1667	0.1733	0.175	0.175
EtC10	0.2433	0.2833	0.2967	0.2433	0.37	0.38	0.39	0.39
MeOH	26.937	34.28	31.18	31.493	40.1	37.087	36.53	48.16
Ruby Cabernet								
ethyl acetate	10.453	15.523	32.787	12.153	15.19	19.293	17.485	17.425
i-AmAc	0.1833	0.2033	0.25	0.2	0.1833	0.2167	0.2	0.195
isobutyric acid	1.9167	1.1567	1.3767	1.3067	0.94	0.8467	0.905	0.975
MeOH	23.637	34.833	31.893	26.7	35.483	38.84	37.99	59.16
Muscat d'Alexandria								
i-BuOH	84.205	39.907	28.027	34.667	18.277	15.137	17.185	12.065
isobutyric acid	2.105	1.2	0.93	0.9333	0.8	0.52	0.825	0.91

4.2.2 ANALYSIS OF THE FT-IR SPECTRA

The FT-IR spectrum of a wine provides the collective absorbance of all the IR-active components in the sample. Fig 1 shows the FT-IR spectrum of Colombar wine that was fermented with the recombinant wine yeast strain VIN13-ATF1. Regions a and b could not be used for classification. The regions in Fig 1a showed little repeatability between duplicate spectra (data not shown) and is known to contribute towards noise in the spectrum (Nieuwoudt *et al* 2004). The region in Fig 1b showed little variation in absorbance and could thus also not be used for classification. Prominent peaks were present in the region from 1500 to 929 cm^{-1} (Fig 1c). The latter region is referred to as the fingerprint area and is particularly useful in molecular absorption spectroscopy, since many different IR bands, including those corresponding to the vibrations of the C-O, C-C, C-H and C-N bonds, occur in this region.

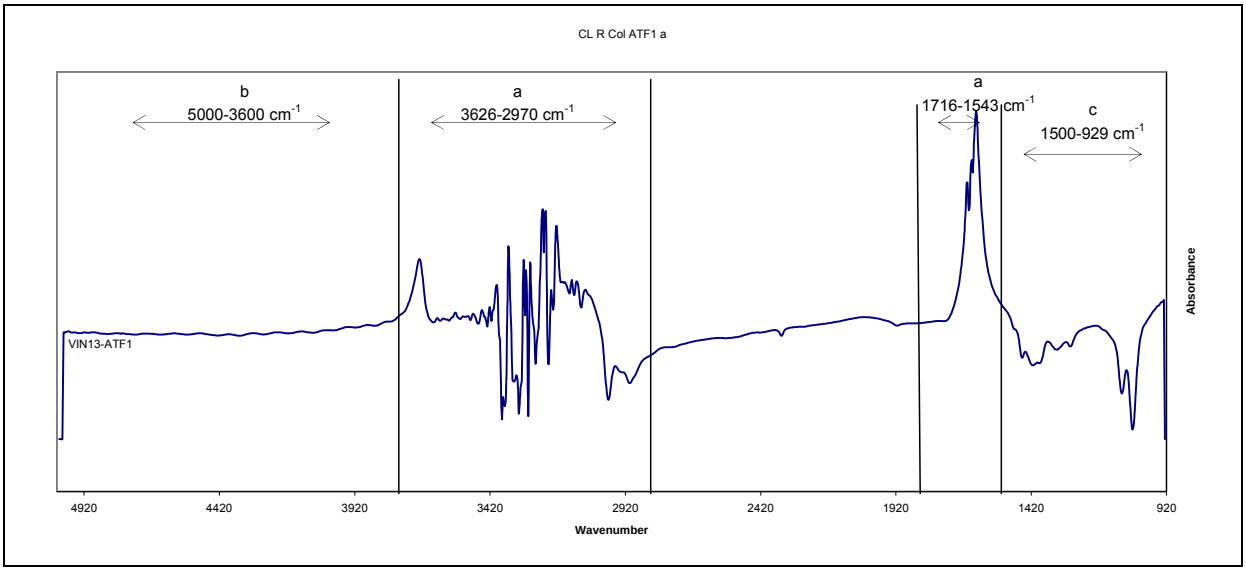

Figure 1 FT-IR spectrum of Colombar wine fermented with the VIN13-ATF1 yeast strains. (a) represents the regions where water contributed considerably to absorbance. (b) represents the region where the scans showed little variation in absorption. (c) represents the fingerprint area used for classification.

4.2.3 PCA MODELLING OF THE WINE SAMPLES

The score plot of PC1 versus PC2 for the Colombar wines shows a clustering of samples related to the yeast strain used for fermentation (Fig 2). Wines fermented with the strains VIN13-DLG39, VIN13-PPK and VIN13-ATF1 were clustered into separate groups. This type of clustering of the wines could also be seen in the score plot for the Pinot noir wines, but no clustering pattern could be recognised for the Ruby Cabernet or Muscat d'Alexandria wines (data not shown).

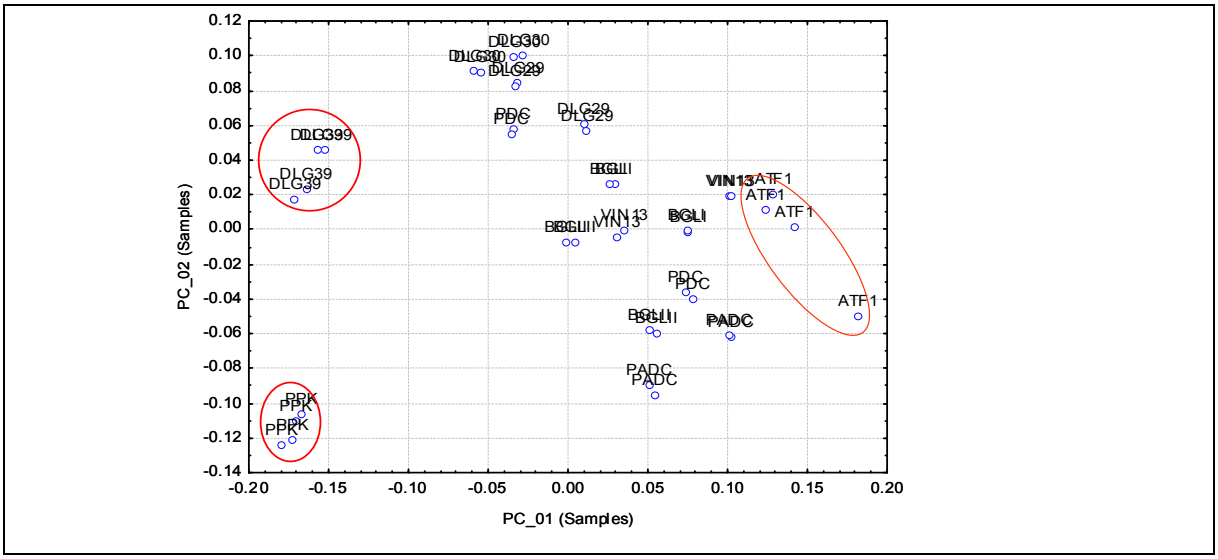

Figure 2 PCA score plot, PC1 vs. PC2, for Colombar wines fermented with different recombinant wine yeast strains.

4.2.4 CLASSIFICATION BY CART ANALYSIS

CART analysis was used to classify wines fermented with different transgenic and non-transgenic yeast strains.

Figure 3 This figure shows the classification of Pinot noir wines fermented with different *S. cerevisiae* VIN13 wine yeast strains by using CART analysis. The wine Gis 29 was fermented with the recombinant strain VIN13-DLG29, Gis 30 with the strain VIN13-DLG30, Gis 33 with the strain VIN13-DLG33, Gis 39 with the strain VIN13-DLG39, Gis PEX with the strain VIN13-PEX, Gis PPK with the strain VIN13-PPK and VIN13+R with VIN13 with the addition of 0.3g/l Rapidase-X-colour enzyme.

From Figure 3 it can be seen that CART analysis was successful in correctly classifying some of the Pinot noir wines fermented with the different recombinant strains. The classification of wines fermented with VIN13-DLG30 (d), VIN13-DLG33 (g), VIN13-DLG39 (c) and VIN13 + commercial enzyme (a) was 100% correct. The model could not, however, distinguish between wines fermented with VIN13 and the pectolytic strain, VIN13-PPK (f). The classification of wines fermented with VIN13-PEX was correct to a level of 86% (b) and that of wines fermented with VIN13-DLG29 75% (e).

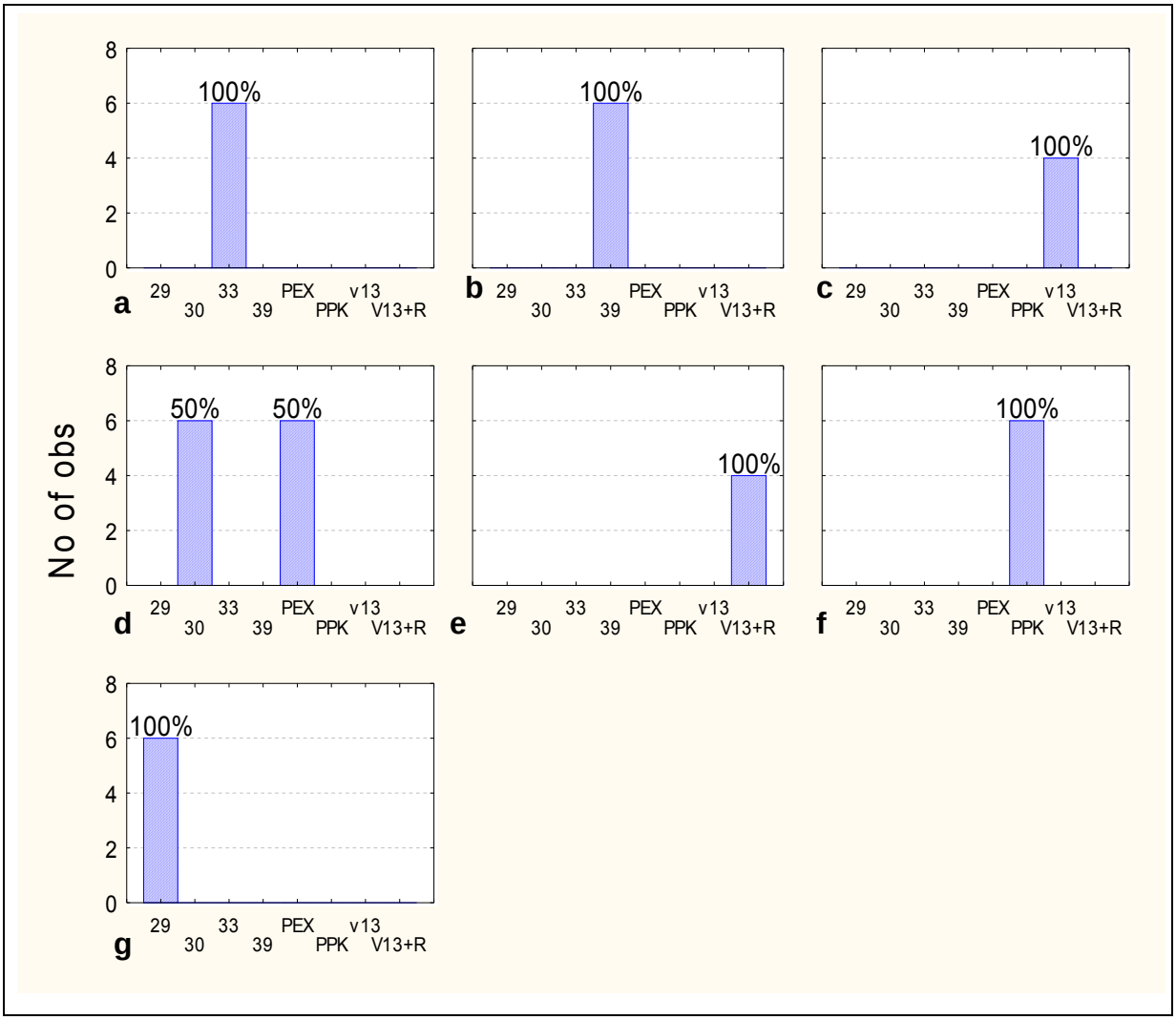

Figure 4 Differentiating between Ruby Cabernet wines fermented with different *S. cerevisiae* VIN13 wine yeast strains by means of discriminative analysis. The wine 29 was fermented with the recombinant strain VIN13-DLG29, 30 with the strain VIN13-DLG30, 33 with the strain VIN13-DLG33, 39 with the strain VIN13-DLG39, PEX with the strain VIN13-PEX, PPK with the strain VIN13-PPK and VIN13+R with VIN13 with the addition of 0.3g/l Rapidase-X-colour enzyme.

Figure 4 shows that the CART analysis correctly classified most of the Ruby Cabernet wines fermented with different recombinant strains. The classification of wines fermented with all the strains, except the glucanolytic strain VIN13-DLG30 and the glucan- and xylan-secreting strain VIN13-PEX (d), was 100% correct. The model could not distinguish between wines fermented with the latter two strains.

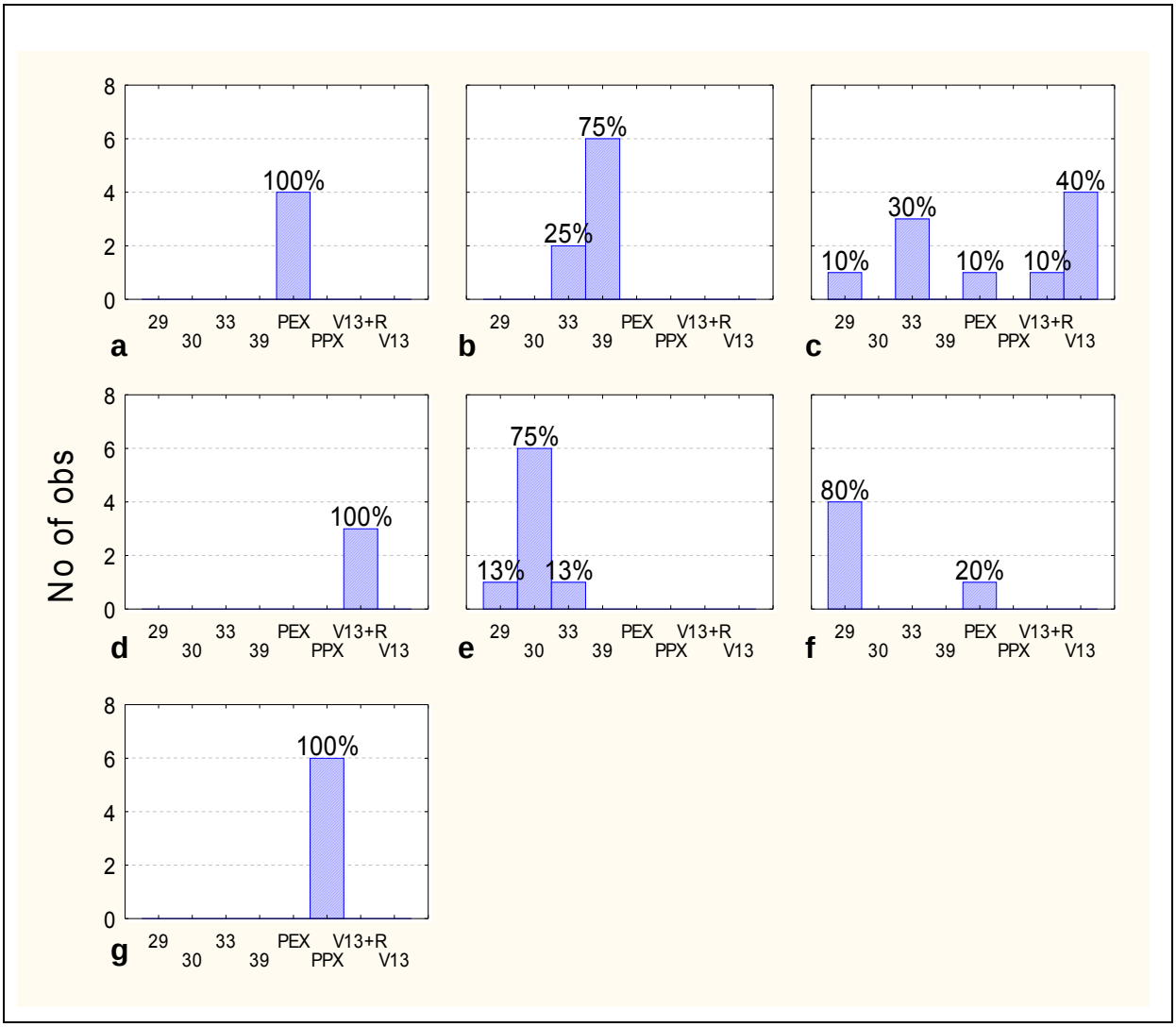

Figure 5 Differentiating between Muscat d'Alexandria wines fermented with different *S. cerevisiae* VIN13 wine yeast strains by means of discriminative analysis. The wine 29 was fermented with the recombinant strain VIN13-DLG29, 30 with the strain VIN13-DLG30, 33 with the strain VIN13-DLG33, 39 with the strain VIN13-DLG39, PEX with the strain VIN13-PEX, PPK with the strain VIN13-PPK and VIN13+R with VIN13 with the addition of 0.3g/l Rapidase-X-colour enzyme.

From Figure 5 it can be seen that, in all cases, CART analysis could not discriminate between Muscat d'Alexandria wines fermented with different transgenic strains. The wines fermented with the pectolytic strain, PPK (g), and with the addition of the commercial pectolytic enzyme, Rapidase X Colour (d), were the only ones that were classified 100% correctly. The model could not distinguish between non-transgenic VIN13 and the other transgenic strains (c). The identification of wines fermented with VIN-DLG39 was 75% correct, but the model could not distinguish 100% between wines fermented with this strain and with the other glucan- and xylan-degrading strain, VIN13-DLG33 (b). The correct identification of wines fermented with VIN-DLG30 was also 75%, but the model could not distinguish 100% between wines fermented with this strain, with VIN13-DLG33, or with the xylan-degrading strain,

VIN13-DLG29 (e). Wines fermented with the xylanolytic strain, VIN13-DLG29, were classified correctly in 80% of the cases, although the model had trouble to distinguish 100% between these wines and wines fermented with the glucan- and xylan-degrading strain, VIN13-PEX (f).

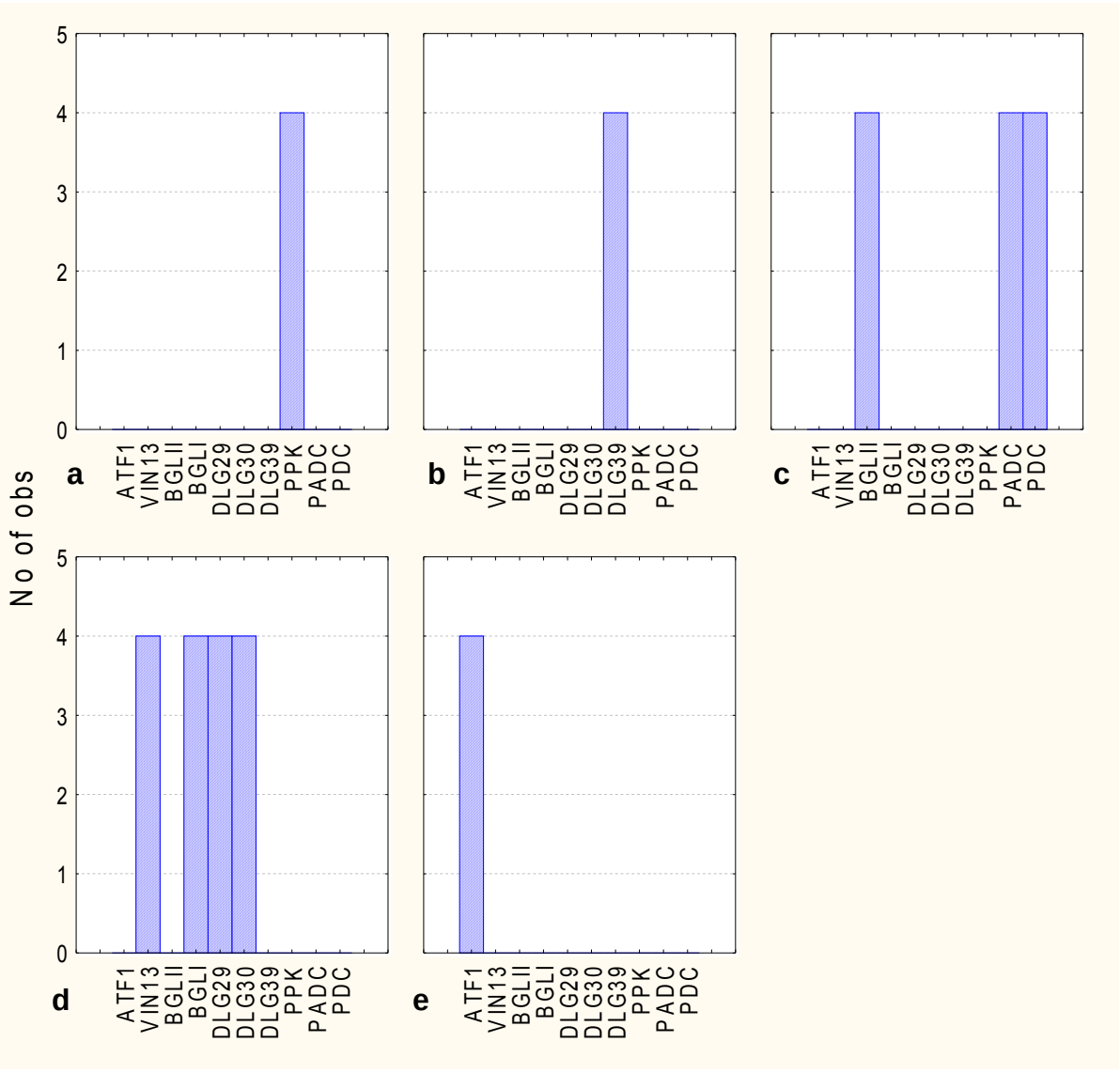

Figure 6 Differentiating between Colombar wines fermented with different *S. cerevisiae* VIN13 wine yeast strains by means of discriminative analysis. The wine ATF1 was fermented with the recombinant strain VIN13-ATF1, BGLII was fermented with the recombinant strain VIN13-BGLII, BGLI was fermented with the recombinant strain VIN13-BGLI, 29 was fermented with the recombinant strain VIN13-DLG29, 30 with the strain VIN13-DLG30, 33 with the strain VIN13-DLG33, 39 with the strain VIN13-DLG39, PEX with the strain VIN13-PEX, PPK with the strain VIN13-PPK, PADC was fermented with the recombinant strain VIN13-PADC, PDC was fermented with the recombinant strain VIN13-PDC and VIN13+R with VIN13 with the addition of 0.3g/l Rapidase-X-colour enzyme.

Figure 6 shows that the classification trees could not always discriminate between the Colombar wines fermented with the different transgenic strains. Wines fermented with the pectolytic strain, VIN13-PPK (a), the glucan- and xylan-degrading strain,

VIN13-DLG39 (a), and the ester-forming strain, VIN13-ATF1 (e), were the only ones that were classified 100% correctly. The model could not distinguish between wines fermented with the β-glycosidase- and cellobiase-secreting strain, VIN13-BGLII, and the decarboxylase-secreting strains, VIN13-PADC1 and VIN13-PDC1 (c). The model could also not distinguish between Colombar wines fermented with non-transgenic VIN13, the glucanolytic strain VIN13-DLG30, the xylanolytic strain VIN13-DLG29 and the β-glycosidase- and cellobiase-secreting strain VIN13-BGLI (d).

4.3 DISCUSSION

FT-IR spectroscopy combined with classification tree or PCA analysis was used to attempt to differentiate between wines fermented with recombinant and non-recombinant, as well as different recombinant *S. cerevisiae* VIN13 wine yeast strains. The analysis of wines fermented with these strains showed differences in the chemical composition of the wines (Table 2). These differences could be expected to be reflected in the FT-IR spectra. This study aimed to determine whether there were differences in the FT-IR spectra that could be used to distinguish between wines fermented with the different strains. An attempt was therefore made to look for regions in the spectra that were uniform for fermentations with the same strain, but differed for fermentations with different strains. These regions could then be used for classification. If must was fermented with a particular recombinant strain and the heterologous enzyme secretion was at too low a concentration, or the enzyme had no impact on the wine, the chemical composition would not be altered and this technique would not be able to distinguish between this wine and wine fermented with a non-recombinant strain.

Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines fermented with the addition of the commercial pectinase, Rapidase X-colour, were classified 100% correctly by CART analysis (Figs 3a, 4e and 5d). The colour parameters of these wines were not unique when compared with those of all the other wines analysed (Chapter 3). Methanol was the only volatile component in these wines that showed levels that differed significantly from all the other wines (Table 2). This could have been due to pectin methyl esterase side-activity being present in the enzyme preparation. The unique region in the absorption spectra of these wines could thus be attributed to the high methanol levels or to a combination of small differences in chemical composition that were similar in all the wines to which the pectinase preparation was added during fermentation. It is also possible that the sugars, salts and preservatives in the commercial preparations could have contributed to unique differences in the FT-IR spectra of these wines.

For the red cultivars, wines fermented with the glucanase- and xylanase-secreting strains VIN13-DLG33 and VIN13-DLG39 were classified 100% correctly. Wines fermented with VIN13-DLG33 showed the most significant improvements in colour intensity and stable pigments (chapter 3). Fermentation with the latter strain also

resulted in high levels of isoamyl acetate, ethyl acetate and isobutyric acid (Table 2). Pinot noir wines fermented with VIN13-DLG39 showed low levels of the esters ethyl hexanoate, octanoate and decanoate (Table 2). These differences could have resulted in differences in the FT-IR spectra of these wines when they were classified by the CART model.

In all cases, however, the model could not distinguish between Muscat d'Alexandria wines fermented with VIN13-DLG39 and VIN13-DLG33. The enzymes secreted by these strains are very important in tannin and phenol extraction when fermenting red must on the pulp. Since white must is not fermented on the pulp, the slight changes in xylanase concentration during the fermentation of these two musts did not result in a sufficient chemical difference to distinguish between these Muscat d'Alexandria wines. All the wines fermented with the pectolytic strain, VIN13-PPK, except Pinot noir, were classified 100% correctly for all the cultivars fermented with this strain. Fermentation with this strain resulted in low levels of isobutanol and isobutyric acid (Table 2). The pectolytic enzymes that were produced altered the chemical composition of both white musts and of Ruby Cabernet sufficiently to classify these wines 100% correctly by FT-IR spectroscopy. Pectinases were either inactivated or produced in too low quantities to affect the composition of the Pinot noir wines.

Ruby Cabernet wines fermented with the xylanolytic strain, VIN13-DLG29, were all classified correctly. The fermentation of Ruby Cabernet must with this strain resulted in a large increase in colour, while the fermentation of Pinot noir must did not. This may be why the Ruby Cabernet wines fermented with this strain were correctly classified 100% and the Pinot noir wines were not.

The differences between the FT-IR spectra of Colombar wines fermented with VIN13-DLG29, VIN13-DLG30 and non-recombinant VIN13 were not enough for the model to distinguish between wines fermented with these strains. The xylanase and glucanase produced by these strains respectively did therefore not result in significant must alterations. However, when the Colombar must was fermented with the VIN13-DLG39 strain producing both these enzymes, the resulting wine was classified 100% accurately. The synergistic action of both these enzymes on the polysaccharides present in the must resulted in alterations in the wines produced that could be used for classifying these wines.

The model could not distinguish between the wines fermented with the two decarboxylase-secreting strains, VIN13-PDC and VIN13-PADC. The decarboxylase enzymes secreted by these strains decarboxylate phenolic acids to substituted phenyl propionic acids, which are then reduced to 4-ethyl derivatives. Previous studies of these strains showed that, if the phenolic acid concentration in the must is very high, the volatile phenols produced are not significantly altered by the additional decarboxylase enzymes (Smit 2002). The presence of very high or very low substrate concentrations in the must might thus have resulted in insufficient alteration of the volatile profiles of the wines to distinguish between them using FT-IR spectroscopy.

When the Colombar must was fermented with the VIN13-ATF1 strain, the resulting wine was classified with 100% accuracy. Previous studies have shown that the fermentation of Colombar must with this strain results in an increase in acetate esters and a decrease in acetic acid due to the overexpressed acetyltransferase activity (Lilly *et al* 2000). CART analysis could thus classify these wines on the basis of their altered chemical profile. Colombar wines fermented with the β-glucosidase-secreting strains, VIN13-BGLI and VIN13-BLGII, could not be identified by FT-IR spectroscopy. Since Colombar is a very neutral cultivar in terms of character, there were probably not enough grape glycoside precursors in the must for fermentation with these strains to result in a unique wine profile.

The classification of the wines in this study was done with a training set of data using a limited sample size. Once more data is available, the model must also be tested using a test set of data. In order to use this technique to confirm that wine was fermented with a specific strain, a large database consisting of FT-IR scans from a variety of wines from the same cultivar and fermented with this specific strain would have to be constructed. Another problem for the determination of authenticity is that the enzymes secreted by the recombinant strains are the same enzymes used in many commercial enzyme preparations. This technique would thus not always be able to distinguish between the profiles of wines fermented with the recombinant strains or with the addition of commercial enzyme.

If the appropriate database of wild and commercial yeast fermentations is available, this technique could also be used to identify, from a small sample of bottled wine, which yeasts were dominant in spontaneous fermentation. This information could be used to replicate a fermentation to produce a desired wine style.

4.4 CONCLUSION

Molecular techniques cannot be used to identify the yeast strain that a finished wine was fermented with, since the yeast, and consequently the DNA, are removed from or denatured in the wine during filtration and processing. In order to identify the strain used for fermentation, it would be possible to use unique chemical differences in the wine resulting from fermentation with a specific strain.

From this study, it could be seen that FT-IR spectroscopy, combined with multivariate data analysis, shows potential to be used to classify wines fermented with different yeast strains, provided that fermentation with a specific strain resulted in a unique chemical profile for the resulting wine.

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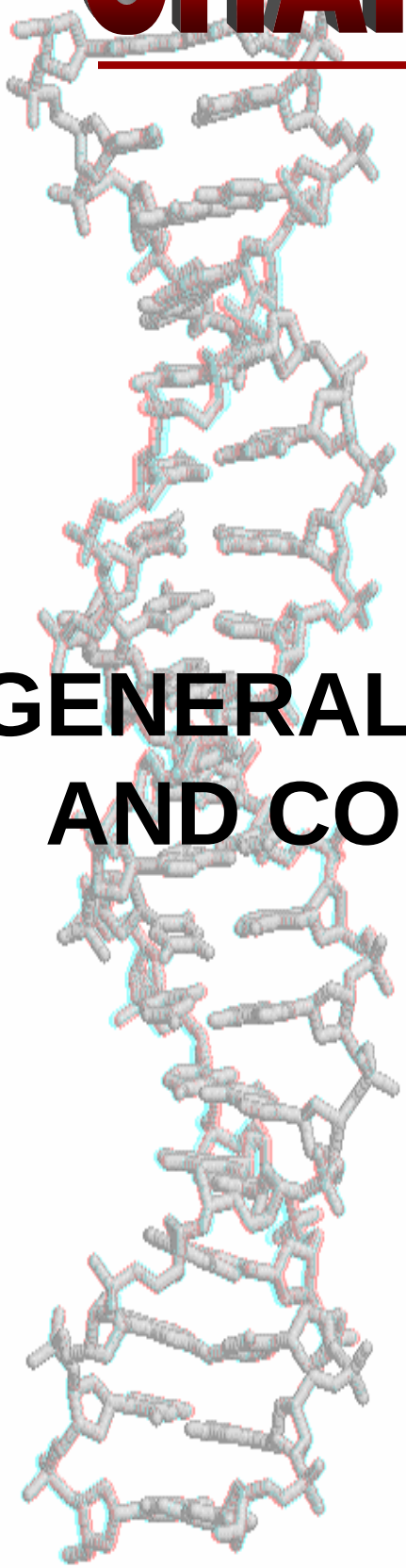
CHAPTER 5



GENERAL DISCUSSION AND CONCLUSIONS

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5.1 PERSPECTIVES

During the winemaking process, enzymes that originate from the grape and the microorganisms present in the must act as biological catalysts and drive the conversion from grape juice to wine. By supplementing the endogenous enzymes present in the fermentation with commercial enzyme preparations, it is possible to improve both wine processing and the end product.

Saccharomyces cerevisiae plays a dominant role in the complex process of winemaking and the primary fermentation of sugars to ethanol, carbon dioxide and a number of by-products is produced by this wine yeast. *S. cerevisiae* is not only widely used for fermentation in various industries, but it is also a very important host for the expression of foreign genes. The majority of commercial pectinase and glucanase preparations used in the wine industry are derived from *Aspergillus* and *Trichoderma* respectively (Canal-Llauberes 1993). Commercial enzymes are typically crude fungal preparations and, besides containing the desired enzymes, it is also possible that impurities such as mucilage, proteins and undesired enzyme side-activities might be present (Wightman *et al* 1997).

Since the endogenous polysaccharase activity of *S. cerevisiae* is very limited, the heterologous expression of specific polysaccharase genes in an industrial yeast strain can improve the winemaking process. It is also possible to make a higher quality wine without the addition of expensive commercial enzyme preparations. Since only the desired enzymes are secreted by the recombinant strain, there will be no undesired side-activities, which can be detrimental to the quality of the wine.

5.2 DISCUSSION AND CONCLUSION

Chapter 1 of this thesis describes the important role that *S. cerevisiae* plays in wine fermentation and explains why it is an ideal host for the expression of foreign genes. Most of the enzyme preparations used for the improvement of wine processing are produced by fungal strains. The heterologous expression of the genes coding for these enzymes by an industrial wine yeast strain can thus result in higher quality of wine being made without the addition of expensive commercial preparations.

Chapter 2 focuses firstly on the different polysaccharides in wine, their role and origin and how they are enzymatically degraded. The polysaccharides in wine originate from the grape itself, from the fungi on the grape and from the microorganisms present during winemaking. The main polysaccharides responsible for turbidity, viscosity and filter stoppages are pectins, glucans and xylans (Pretorius 2000). These polysaccharides are degraded by pectinases, glucanases and xylanases respectively. Section 2.3 discusses the classification, substrate and mode of action of these enzymes.

The addition of these enzymes at various stages of the winemaking process can have many positive impacts. Section 2.4 focuses on the influence of the enzymatic degradation of polysaccharides on juice yield, clarification, filterability, wine colour and the aromatic and flavour profile of the wines produced. When making red wine, free-run wine is the wine that flows spontaneously from the pulp due to gravity. Wines made from free-run juice are usually of a higher quality (Rankine 1998). Several of the studies that are discussed showed an increase in free-run juice when pectinases or macerating enzymes with pectinase, glucanase and xylanase activity were applied to the must (Canal-Llaubéres 1989).

Polysaccharides can contribute towards turbidity and filtration problems because they form protective colloids, causing cloud particles to remain in suspension. It has been shown that treating the must with pectolytic enzymes destabilises the colloidal equilibrium, resulting in the aggregation and settling of cloud-forming particles and thus improving the clarification of the must. Degradation of the pectin also results in a distinct improvement in the filtration of the pectinase-treated must (Canal-Llaubères 1989). In section 2.4.3 the influence of enzymes on wine colour and pigment modification is discussed. Anthocyanins are pigments occurring in the vacuoles of the skin cells of red grapes and are responsible for the colour of red wine. The polymerisation of anthocyanins with other grape compounds is very important for colour stability in red wines. It protects the anthocyanidin portion of the polymers against oxidation and chemical modification, and also protects the molecule against bleaching by SO_2. The studies discussed in this chapter show that the enzymatic degradation of polysaccharides leads to increased extraction of phenolic compounds, such as tannins. This extraction results in more polymeric pigments being formed in aged red wine, and therefore in increased colour intensity and stability (Haight and Gump 1994, Watson *et al* 1999).

Wine aroma is also influenced by enzyme treatment. Several hundred volatile components belonging to a variety of classes contribute to aroma. Section 2.5 discusses the influence of phenols, monoterpenes, higher alcohols and esters on wine aroma and flavour. It then looks at the influence of the degradation of the structural polysaccharides on these aromatic compounds. The action of polysaccharide-degrading enzymes facilitates the liberation and solubilisation of phenolic compounds and glycosydic precursors from the cells of the skins, seeds and flesh of the grape berry (Gump and Haight 1995, Watson *et al* 1999). The phenolics and tannins that are released contribute to several wine characteristics. Some of the positive characteristics due to tannins are body, backbone, structure, fullness and roundness, while bitterness, roughness, harshness, astringency and thinness are faults that can be attributed to phenolic compounds. Enzymatic degradation of the cell wall contributes to the release of glycosidically-bound terpene or alcohol precursors from the berries. The hydrolysis of these precursors during fermentation can result in an improvement in wine aroma (Zoecklein *et al* 1997). Terpenes liberated from the precursors contribute to the fruity aroma of wine. Small amounts of

higher alcohols contribute positively to wine quality, while excessive amounts may detract from quality. The increase in higher alcohols is not only due to the release of glycosidically-bound precursors. The enzymatic action of the polysaccharases on the grape skins alters the composition of the must during fermentation, and these changes could influence yeast metabolism and result in increased or decreased levels of higher alcohol production (Gill and Vallés 2001). Esters are formed during a reversible condensation reaction between the carboxyl group of an organic acid and the hydroxyl group of an alcohol or phenol, with the release of water. Several studies have shown that treating the must with polysaccharases results in alterations in the higher alcohol and monoterpene profiles of the wine. These studies also show changes in the production of some of the corresponding esters (Ganga *et al* 1999, Gill and Vallés 2001, Pérez-Gonzáles *et al* 1993).

Following the discussion of the influence of polysaccharase action on winemaking, this chapter looks at commercial enzyme preparations used in the wine industry. The majority of commercial pectinase and glucanase preparations are derived from *Aspergillus* and *Trichoderma* respectively (Canal-Llauberes 1993).

Section 2.7 of this chapter focuses on the genetic improvement of industrial yeast strains. Previously, classical genetic techniques were the preferred way of genetically improving industrial yeast strains. With these methods, large genomic regions or entire genomes are rearranged or recombined. These techniques cannot be used to alter only a specific characteristic. Genetic engineering is the only reliable method with which a specific, existing property can be altered, a new characteristic can be introduced or an existing one eliminated without affecting any other desirable properties. *S. cerevisiae* is a very important host for the expression of foreign genes. Several important tools used for the production of heterologous proteins in *S. cerevisiae* are discussed. This section also looks at the choice of the correct vector, promoter and secretion signal for optimal gene expression and protein secretion.

The final section of chapter 2 (section 2.8) focuses on the development of recombinant *S. cerevisiae* strains expressing pectinases, glucanases and xylanases genes. Each polysaccharase is discussed by first looking at organisms that produce pectinase, glucanase and xylanase in nature and can thus serve as gene sources for genetic engineering. There is also a discussion on the heterologous expression of genes in laboratory strains and, finally, a focus on the recombinant wine yeast strains that have been developed. Pectic enzymes are produced naturally by a wide variety of organisms in nature, including bacteria, fungi, yeasts, insects, nematodes and protozoa. The *pelE* gene of *E. chrysanthemi,* the *peh1* gene of *E. carotovora* and the *pnlA* gene of *G. cingulata* have been expressed in *S. cerevisiae* laboratory strains under the control of a variety of promoters (Laing and Pretorius 1992, Templeton *et al* 1994). These strains showed improved pectolytic activity. Attempts have also been made to construct pectolytic wine yeast strains. The *F. solani pelA* gene has been expressed under the control of the yeast actin promoter in an industrial *S. cerevisiae* strain (González-Candelas *et al* 1995), but secretion problems were experienced with

this strain during fermentation. The *pelE* gene of *E. chrysanthemi* and the *peh1* gene of *E. carotovora* have been integrated into the *URA3* gene of the industrial *S. cerevisiae* strain VIN13. Fermentation resulted in wines with a decrease in total phenolics, an increase in turbidity and an increase in juice extraction compared to a control fermented with an untransformed *S. cerevisiae* VIN13 strain (Strauss 2003).
Enzymes that hydrolyse cellulose are produced by a wide variety of bacteria and fungi. Bacteria secrete various extracellular enzymes with different binding domains and all bacterial 1,3- and 1,3-1,4-β-glucanases have a high degree of sequence similarity. Fungi secrete a variety of hydrolytic enzymes, including endoglucanases, β-glucosidases, exoglucanases and cellobiohydrolases. These enzymes act in a synergistic manner in order to degrade crystalline cellulose (Van Rensburg *et al* 1997, Wood 1985). Several cellulases produced by *T. reesei* and *A. niger* have been characterised and the genes coding for them have been cloned. *S. cerevisiae* does not produce the endo-β-1,4-glucanases required for the complete synergistic breakdown of cellulose. Several foreign genes cloned from a variety of cellulolytic organisms have been introduced into *S. cerevisiae* in order to improve its hydrolytic properties. The *end1* gene from *B. fibriosolvens* (Van Rensburg *et al* 1994), the *cbh1-4* gene from *P. chrysosporium* (Van Rensburg *et al* 1996), the *beg 1* gene from *B. subtillus* (Hinchcliff 1985), the *cel A* gene from *R. flavefaciens* (Van Rensburg *et al* 1995) and the *cenA* gene from *C. fimi* (Skipper et al. 1985) are some of the bacterial cellulolytic genes that have been successfully expressed in *S. cerevisiae* laboratory strains. Endo-β-1,4-glucanase-encoding genes, originating from *A. niger* and *T. reesei*, have also been expressed in *S. cerevisiae* (Hong *et al* 2001, Pentillä *et al* 1987). Attempts have also been made to construct glucanolytic and xylanolytic industrial wine yeast strains. These included the *S. cerevisiae* T_{73} strain expressing the *T. longibrachiatum egl1* gene and the same industrial strain expressing the *xlnA* gene from *A. niger.* Significant alterations were found in the higher alcohol and ester profiles of the wines that were produced by these strains. Sensory evaluation also indicated that these wines had an increased fruity aroma compared to must fermented with non-recombinant strains (Gil and Váles 2001).
It can thus be seen that the addition of polysaccharide-degrading enzymes during fermentation produces many advantages during wine processing and contributes to the production of a better end product. Similar advantages have been shown with regard to juice extraction, colour extraction and aroma profiles when must was fermented with polysaccharase-secreting wine yeast strains.

The first part of chapter 3 describes the construction of four recombinant wine yeast strains. The endo-β-1,4-xylanase gene, *XYN2*, and the endo-β-1,4-glucanase gene, *END1*, have previously been cloned from the soft rot fungus, *T. reesei* (La Grange *et al* 1996), and the rumen bacterium, *Butyvibrio fibriosolvens* (Van Rensburg *et al* 1994), respectively. These genes were subcloned into different yeast-integrating plasmids. The four recombinant wine yeast strains were constructed by integrating the plasmids containing expression cassettes into the *ILV2* locus of the commercial

wine yeast strain, VIN13 (Anchor Yeast SA). The *TEF1$_P$-XYN2-ADH2$_T$* cassette was integrated into the strain VIN13-DLG29, *ADH1$_P$-MFα1$_S$-END1-TRP5$_T$* was integrated into VIN13-DLG30, *ADH1$_P$-MFα1$_S$-END1-TRP$_T$* and *ADH2$_P$-XYN2-ADH2$_T$* were integrated into VIN13-DLG33 and *ADH1$_P$-MFα1$_S$-END1-TRP$_T$YG100$_P$-XYN2-ADH2$_T$* was integrated into VIN13-DLG39.

The next part of the chapter looks at winemaking with different recombinant yeast strains and the analysis of the wines. Wine was made with the four strains that were constructed, as well as with a pectolytic strain, VIN13[pPPK], a glucanase- and xylanase-secreting strain, VIN13[pEX] (Strauss 2003), an untransformed VIN13 strain and must treated with the commercial enzyme preparation, Rapidase EX Colour. Microvinification experiments were carried out on Pinot noir, Ruby Cabernet and Muscat d'Alexandria wines fermented with each of the abovementioned strains. The fermentation of Ruby Cabernet must with all the glucanase- and xylanase-secreting strains resulted in an increase in free-flow wine due to the degradation of the cell walls by these enzymes. The fermentation of the red musts with the recombinant strains resulted in an increase in colour intensity. This colour increase may be due to increases in polymeric anthocyanin content or to co-pigmentation effects. The best results were obtained during fermentation with the strain VIN13-DLG33. This was probably due to the synergistic degradation of the grape polysaccharides by the endo-β-1,4-xylanase and the endo-β-1,4-glucanase secreted by this strain. Fermentation with the recombinant strains also resulted in significant alterations in the aromatic profiles of the wines that were made.

Sensory evaluation of the Pinot noir wines showed that fermentation with the recombinant strains did not only result in differences that could be seen during wine analysis, but that these differences could be detected by a tasting panel. The colour evaluation by the tasting panel corresponded with spectrophotometer results and fermentation with the same two strains, VIN13-DLG33 and VIN13-PEX, showed the highest colour intensity and the most red tint. Fermentation with the recombinant strains also resulted in an increase in fruity and spicy characteristics, which could be due to the increase in some of the esters.

In conclusion, fermentation with the polysaccharide-degrading strains resulted in significant improvements in juice extraction, colour intensity and stability and in alterations in the aromatic profiles of the wines produced. The results were similar to studies in which commercial enzyme preparations were added during winemaking. It is thus possible to produce wines by fermenting with these strains and to obtain similar results without the addition of industrially produced enzymes. Before these strains can be used in the industry, however, further studies need to be done on the regulation of transcription and secretion of heterologous enzymes during wine fermentation. This will allow for better control over the time and dosage of the enzymes secreted during fermentation. It will thus be possible to construct strains that are tailor made to produce enzymes demanded by the winemaker to suit specific cultivars and wine styles.

If transgenic yeast strains are accepted for use in the wine industry, it may be required that the GMO status of the yeast that has been used appear on the label. Molecular techniques cannot be used to identify the yeast strain with which a finished wine was fermented, since the yeast and thus the DNA are removed or denatured in the wine during filtration and processing. In order to identify the strain used for fermentation, unique chemical differences in the wine resulting from fermentation with a specific strain can be used. The aim of the study conducted in chapter 4 was to determine if FT-IR spectroscopy coupled with multivariate data analysis can distinguish between wines fermented with transgenic and non-transgenic yeast strains or between wines fermented with different transgenic strains. Results showed that this method could be used to classify wines fermented with different yeast strains, if fermentation with the strain resulted in a unique chemical profile for the resulting wine. This was a preliminary study and was written as an addendum to the thesis.

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