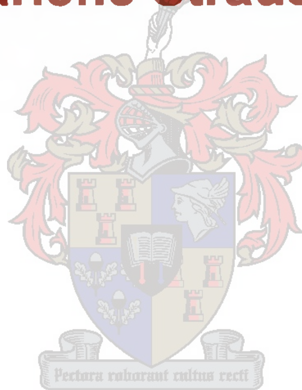
The transformation of wine yeasts with glucanase, xylanase and pectinase genes for improved clarification and filterability of wine

by

Marlene Strauss



Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at Stellenbosch University

March 2003

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DECLARATION

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

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SUMMARY

Cellulose is by far the most abundant carbohydrate available from plant biomass. These biopolymers are therefore an important renewable source of food, fuels and chemicals. Cellulose is embedded in a matrix of hemicellulose, lignin and pectin and is composed of repeating glucose units linked by β-1,4-glycosidic bonds. The individual molecules are held together by hydrogen bonds, forming largely crystalline fibres. The hemicellulose, which is a low molecular weight heteropolysaccharide, coats and binds the cellulose microfibrils, preventing the cellulose from becoming too crystalline. Three predominant types of hemicelluloses are recognised, namely 1,3- and 1,4-β-D-galactans, 1,4-β-D-mannans and 1,4-β-D-xylans, which are named according to the sugar type that forms the polymer backbone. Pectic substances contain rhamnogalacturonan backbones in which 1,4-linked α-D-galacturonan chains are interrupted at intervals with α-L-rhamnopyranosyl residues carrying neutral side chains. Two groups of enzymes, cellulases and pectinases, are required for the microbial utilisation of crystalline cellulose and pectin. Cellulases are multicomponent complexes that are often composed of endoglucanases, exoglucanases and cellobiases. Cellobiose is the major end product of concerted endoglucanase and exoglucanase activity. Cellobiose is then hydrolysed to glucose by β-glucosidases. The enzymatic breakdown of pectic polymers occurs by the de-esterifying action of the saponifying enzymes, pectinesterase, releasing the methyl groups of the pectin molecule, and by hydrolase or lyase action of the depolymerases (pectin lyase, pectate lyase and polygalacturonase), splitting the α-1,4-glycosidic linkages in the polygalacturonate chain.

The yeast *Saccharomyces cerevisiae* has been used extensively in the alcoholic beverage industry for fermentations of wine, beer and other alcoholic beverages for many years. However, it is unable to produce extracellular depolymerising enzymes that can efficiently degrade polysaccharides, which are the main cause of clarification and filtration problems. Enzyme preparations have been used in the alcoholic beverage industries to degrade haze-forming polysaccharides, thereby improving the filterability and quality of products such as beer and wine. An alternative would be to develop *S. cerevisiae* strains that produce extracellular polysaccharidases, enabling the yeast to degrade polysaccharides without the addition of commercial enzyme preparations. These strains can also be very useful in improving the quality of wine, as well as cutting the costs of the winemaking process. The objective of this study was to investigate the effects of two transformed *S. cerevisiae* strains on different wine grape varieties.

The following genes have been cloned and characterised previously: the *Aspergillus niger* endo-β-xylanase gene (*xynC*), the *Butyrivibrio fibrisolvens* endo-β-1,4-glucanase gene (*end1*), the *Erwinia chrysanthemi* pectate lyase gene (*pelE*) and the *Erwinia carotovora* polygalacturonase gene (*peh1*). The yeast alcohol

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dehydrogenase I gene promoter (*ADH1*$_P$), the alcohol dehydrogenase II gene terminator (*ADH2*$_T$), the tryptophan synthase gene terminator (*TRP5*$_T$) and the yeast mating-type pheromone α-factor secretion signal sequence (*MFα1*$_S$) were used to compile the following gene constructs: *ADH1*$_P$-*MFα1*$_S$-*end1*-*TRP5*$_T$ (designated *END1*), *ADH1*$_P$-*xynC*-*ADH2*$_T$ (designated *XYN4*), *ADH1*$_P$-*MFα1*$_S$-*peh1*-*TRP5*$_T$ (designated *PEH1*) and *ADH1*$_P$-*MFα1*$_S$-*pelE*-*TRP5*$_T$ (designated *PELE*).

Two yeast integrating plasmids were constructed, one containing the *END1* and *XYN4* gene cassettes and the other containing the *PEH1-PELE* cassette. These two plasmids were then integrated into the *URA3* locus of two separate industrial wine yeast strains of *S. cerevisiae*. To facilitate selection of the industrial yeast transformants in the absence of auxotrophic markers, the integrating plasmid containing the *END1* and *XYN4* gene cassettes was issued with the dominant selectable Geneticin G418-resistance (*Gt*^{R}) marker. The integrating plasmid harbouring the *PEH1-PELE* gene cassette was issued with the dominant selectable sulphumetronmethyl resistance (*SMR1*) marker. The introduction of these plasmids into commercial wine yeast strains directed the synthesis of *END1, XYN4, PELE* and *PEH1* transcripts and the production of extracellular biologically active endo-β-1,4-glucanase, endo-β-xylanase, pectate lyase and polygalacturonase.

These recombinant yeasts were capable of extracting more colour from grape skins of certain varieties, as well as leading to more freeflow wine as a result of the more effective degradation of glucans, xylans and pectins in the skins. They also led to decreased turbidity in the wine, making it more filterable.

Future work will entail further investigation of the effects of these recombinant yeasts on different white and red wine grape varieties.

Another objective of this study was to screen non-*Saccharomyces* wine yeasts for the production of extracellular hydrolytic enzymes. The reason for this part of the thesis was to determine the types of extracellular hydrolytic enzymes that are produced and to determine which genera produce which kinds of extracellular enzymes. A total of 237 yeast isolates, belonging to the genera *Kloeckera, Candida, Debaryomyces, Rhodotorula, Pichia, Zygosaccharomyces, Hanseniaspora* and *Kluyveromyces*, were screened for the production of extracellular pectinases, proteases, β-glucanases, lichenases, β-glucosidases, cellulases, xylanases, amylases and sulphite reductase activity. These yeasts were all isolated from grapes and clarified grape juice to ensure that they were yeasts found in must during the initial stages of fermentation. This information can be used to pave the way to pinpoint the specific effects in wine of these enzymes produced by the so-called wild yeasts associated with grape must. This information can also be used to transform *Saccharomyces* wine yeasts with some of the genes from these non-*Saccharomyces* yeasts for the production of extracellular hydrolytic enzymes.

However, future research will have to be done to determine the extent of the activity of these enzymes in wine fermentations and to obtain better knowledge of the physiological and metabolical features of non-*Saccharomyces* yeasts.

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OPSOMMING

Sellulose is verreweg die volopste koolhidraat in plantbiomassa. Hierdie biopolimere is dus 'n baie belangrike hernubare bron van voedsel, brandstof en chemikalieë. Sellulose is in 'n matriks van hemisellulose, lignien en pektien gebed en is uit herhaalde glukose eenhede, wat deur middel van β-1,4-glukosidiese bindings geheg is, saamgestel. Die individuele molekules word deur waterstofbindings aan mekaar geheg, wat aanleiding gee tot die vorming van kristallyne vesels. Die hemisellulose, wat 'n lae molekulêre gewig heteropolisakkaried is, bedek en bind die sellulose vesels en verhoed daarmee die vorming van vesels wat te kristallyn is. Drie predominante tipes hemisellulose word herken en sluit 1,3- en 1,4-β-D-galaktane, 1,4-β-D-mannane en 1,4-β-D-xylane in, wat vernoem word volgens die suikereenhede wat die polimeerruggraat vorm. Pektiene bestaan uit 'n rhamnogalakturonaanruggraat waarin 1,4-gekoppelde α-D-galakturonaankettings periodiek met α-L-rhamnopiranosiel residue, bevattende neutrale sykettings, onderbreek word. Twee groepe ensieme, nl. pektinase en sellulase, word deur mikrobes vir die benutting van kristallyne pektinase en sellulase vereis. Sellulase is multikomponent komplekse wat dikwels uit endoglukanase, ekso-glukanase en sellobiase saamgestel is. Sellobiose is die hoof eindproduk van die saamgestelde aktiwiteit tussen endoglukanase en ekso-glukanase en word verder gehidroliseer tot glukose deur β-glukosidases. Die ensimatiese afbraak van pektien polimere vind deur die de-esterifiserings aksie van die versepings ensiem, pektienesterase, plaas. Dit lei tot die vrystelling van die metielgroepe van die pektienmolekuul. Deur die hidrolase of liase aksie van die depolimerase (pektien liase, pektaatliase en poligalakturonase), split die α-1,4-glukosidiese verbindings in die poligalakturonaatketting.

Die gis *Saccharomyces cerevisiae* word al vir jare ekstensief in die alkoholbedryf vir die fermentasie van verskeie produkte, veral druiwe, gebruik. *S. cerevisiae* besit egter nie die vermoë om ekstrasellulêre depolimiserende ensieme wat vir die effektiewe degradasie van polisakkariede verantwoordelik is, te produseer nie, wat die hoof oorsaak van die verhelderings- en filtreringsprobleme in onder andere wyn en bier is. Dit veroorsaak ook dat *S. cerevisiae* nie oor die vermoë beskik om waasvormende polisakkariede in wyn te degradeer nie. Tans word ensiem-preparate in die alkoholiese bedryf vir die degradasie van die probleem polisakkariede gebruik. Sodoende word die filtreerbaarheid en kwaliteit van wyn en bier verbeter. 'n Goeie alternatief is die ontwikkeling van *S. cerevisiae*-rasse wat oor die vermoë beskik om ekstrasellulêre polisakkarase te produseer en dus polisakkariede self sonder die byvoeging van eksterne kommersiële ensiempreparate te degradeer. Hierdie rasse sal baie voordelig wees vir die verbetering van wynkwaliteit, sowel as vir die vermindering van die kostes verbonde aan die wynmaakproses. Die objektief van hierdie studie is dus om die uitwerking

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van twee getransformeerde *S. cerevisiae* rasse, wat ekstrasellulêre polisakkarases produseer, op verskillende wyndruifvariëteite na te vors.

Die volgende gene is reeds voorheen gekloneer en gekarakteriseer: die endo-β-xylanase-geen (*xynC*) van *Aspergillus niger*, die endo-β-1,4-glukanase-geen (*end1*) van *Butyrivibrio fibrisolvens*, die pektaatliase-geen (*pelE*) van *Erwinia chrysanthemi* en die poligalakturonase-geen (*peh1*) van *Erwinia carotovora*. Die alkoholdehidrogenase-geenpromotor ($ADH1_P$), die alkoholdehidrogenase II-geentermineerder ($ADH2_T$), die gistriptofaansintase geen se termineerder ($TRP5_T$) en die sekresiesein van die gisferomoon α-faktor ($MF\alpha1_S$) is gebruik om die volgende geenkonstrukte saam te stel: $ADH1_P$-$MF\alpha1_S$-*end1*-$TRP5_T$ (bekend as *END1*), $ADH1_P$-*xynC*-$ADH2_T$ (bekend as *XYN4*), $ADH1_P$-$MF\alpha1_S$-*peh1*-$TRP5_T$ (bekend as *PEH1*), and $ADH1_P$-$MF\alpha1_S$-*pelE*-$TRP5_T$ (bekend as *PELE*).

Twee gisintegrerings plasmiede is gekonstrueer, een wat die *END1*- en *XYN4*-geenkassette bevat en die ander wat die *PEH1-PELE*-kasset besit. Hierdie twee plasmiede is daarna in twee aparte industriële wyngisrasse van *S. cerevisiae* by die *URA3* lokus geïntegreer. Vir die seleksie van die industriële wyngistransformante in die afwesigheid van ouksotrofiese merkers, is die dominante selekteerbare Geneticin G418 weerstandbiedende (Gt^R) merker in die *END1*- en *XYN4*-geenkasset-bevattende plasmied geïntegreer. Die dominante selekteerbare sulfumetronmetiel-weerstandbiedende (*SMR1*) merker is in die integreringsplasmied, wat die *PEH1-PELE*-geenkasset bevat, geïntegreer vir seleksie. Transformasie van hierdie plasmiede in kommersiële wyngisrasse het tot die direkte sintese van die *END1*-, *XYN4*-, *PELE*- en *PEH1*-transkripte aanleiding gegee, sowel as tot die produksie van die biologies aktiewe ekstrasellulêre endo-β-1,4-glukanase, endo-β-xylanase, pektaatliase en poligalaturonase.

Tydens die wynmaakproses het bogenoemde rekombinante giste aanleiding gegee tot verhoogde kleurekstraksie uit die druifdoppe van sekere variëteite, asook tot verhoogde vryvloei wyn. Dit is verkry deur die effektiewe degradasie van die glukane, xilane en pektiene in die doppe. Die rekombinante giste het ook verlaagde turbiditeit in die wyn tot gevolg gehad, wat die wyne makliker filtreerbaar maak.

Hierdie werk was net die eerste stap. In die toekoms sal verdere navorsing gedoen moet word om die presiese effekte van hierdie rekombinante giste op verskillende rooi en wit druifvariëteite te bepaal.

'n Ander fokus van hierdie tesis was om nie-*Saccharomyces* wyngiste vir die produksie van ekstrasellulêre hidrolitiese ensieme te selekteer. Die rede hiervoor is om te bepaal watter tipes ekstrasellulêre hidrolitiese ensieme geproduseer word, asook watter ensieme deur watter genera geproduseer word. 'n Totaal van 237 gis-isolate wat tot die generas *Kloeckera*, *Candida*, *Debaryomyces*, *Rhodotorula*, *Pichia*, *Zygosaccharomyces*, *Hanseniaspora* en *Kluyveromyces* behoort, is vir die produksie van ekstrasellulêre pektinase, protease, β-glukanase, lichenase, β-glukosidase, sellulase, xilanase, amilase en sulfiet reduktase-aktiwiteit getoets. Hierdie giste is almal vanaf druiwe en druiwesap geïsoleer om te verseker dat dit wel giste is wat

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gedurende die beginfases van fermentasie in die mos teenwoordig is. Hierdie inligting kan nou verder gebruik word om die spesifieke effekte wat hierdie ensieme, wat deur die sogenaamde wilde giste geproduseer word, tydens die beginfases van fermentasies op die mos het, te bepaal. Hierdie inligting kan ook in die toekoms gebruik word om *Saccharomyces*-wyngiste met gene van die nie-*Saccharomyces*-giste te transformeer om ekstrasellulêre hidrolitiese ensieme vir die degradasie van die problematiese polisakkariede in wyn te produseer.

Daar sal egter in die toekoms baie navorsing gedoen moet word om die omvang van hierdie ensiemaktiwiteite in wynfermentasies te bepaal, asook om meer kennis te bekom oor die fisiologiese en metaboliese samestelling van nie-*Saccharomyces* wyngiste.

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BIOGRAPHICAL SKETCH

Marlene Strauss was born on February 5, 1976 in Calvinia, South Africa. She attended the Primary School, Brandvlei and matriculated at High School Calvinia in 1994. In 1995 she enrolled for a BSc degree at the Stellenbosch University and obtained the degree in 1997, majoring in Microbiology and Genetics. In 1998 she obtained the Hons Wine Biotechnology degree whereafter she enrolled for a MSc in Wine Biotechnology in 1999.

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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation and gratitude to the following people for their contributions to the successful completion of my M. Sc. study:

DR. P. VAN RENSBURG, Institute of Wine Biotechnology, who acted as my supervisor, for his advice and encouragement throughout this study, and for critical reading of this thesis;

PROF. M. LAMBRECHTS, Distell, who also acted as my supervisor, for help and reading of this thesis;

PROF. I.S. PRETORIUS, Institute of Wine Biotechnology, who too acted as supervisor, for his advice, guidance and critical reading of this thesis;

DR. R.R. CODERO OTERO, for technical assistance with practical work;

THE STAFF of the Institute of Wine Biotechnology for their assistance;

MY FELLOW STUDENTS, for encouragement, assistance and the many discussions;

ALL MY FRIENDS, for their support en encouragement;

MY PARENTS, for their support and love.

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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**
From polysaccharides to fermentable sugars – the alteration

Chapter 3 **Research results**
Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts

Chapter 4 **Research results**
The heterologous expression of polysaccharase genes with oenological relevance in *Saccharomyces cerevisiae*

Chapter 5 **General discussion and conclusions**

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CONTENTS

CHAPTER 1: GENERAL INTRODUCTION AND PROJECT AIMS 1

1.1 THE GENETIC IMPROVEMENT OF WINE YEAST STARTER CULTURES 1

1.2 AIMS OF THIS STUDY 2

1.3 REFERENCES 3

CHAPTER 2: FROM POLYSACCHARIDES TO FERMENTABLE SUGARS – THE ALTERATION 4

2.1 FROM POLYSACCHARIDES TO FERMENTABLE SUGARS – THE ALTERATION 4

2.2 BIO-INTEGRATION OF POLYSACCHARIDES 5

2.3 POLYSACCHARIDES FROM GRAPE BERRIES 7

2.4 BREAKDOWN OF POLYSACCHARIDES 8

2.4.1 Cellulose (Glucan) 9
2.4.1.1 Occurrence and structure 9
2.4.1.2 Hydrolysis 10
2.4.1.3 Cellulolytic microorganisms 13

2.4.2 Hemicellulose (Xylan) 14
2.4.2.1 Occurrence and structure 14
2.4.2.2 Hydrolysis 17
2.4.2.3 Xylanolytic microorganisms 18

2.4.3 Pectins 21
2.4.3.1 Occurrence and structure 21
2.4.3.2 Hydrolysis 22
2.4.3.3 Pectolytic microorganisms 23

2.5 INDUSTRIAL POLYSACCHARASES (ENZYMES) 25

2.6 EXPRESSION OF POLYSACCARASE GENES IN YEASTS 28

2.6.1 Glucanolytic, xylanolytic and pectolytic activity in *S. cerevisiae* 30
2.6.2 Expression of bacterial endoglucanase genes in *S. cerevisiae* 30
2.6.3 Expression of fungal xylanase genes in *S. cerevisiae* 31
2.6.4 Expression of bacterial pectinase genes in *S. cerevisiae* 31

2.7 CONCLUSIONS 32

2.8 REFERENCES 32

CHAPTER 3: SCREENING FOR THE PRODUCTION OF EXTRACELLULAR HYDROLYTIC ENZYMES BY NON-*SACCHAROMYCES* WINE YEASTS 41

3.1 INTRODUCTION 42

3.2 MATERIALS AND METHODS 43

3.2.1 Yeast strains 43

Stellenbosch University http://scholar.sun.ac.za/

3.2.2 Media and screening procedures 43
3.2.2.1 Pectinase activity 44
3.2.2.2 Protease activity 45
3.2.2.3 Glucanase activity 45
3.2.2.4 β-Glucosidase activity 45
3.2.2.5 Cellulase activity 45
3.2.2.6 Xylanase activity 45
3.2.2.7 Starch-degrading activity 45
3.2.2.8 Sulphite reductase 45
3.2.3 Enzyme assays 46

3.3 RESULTS **46**
3.3.1 Yeast strains 46
3.3.2 Pectolytic activity 47
3.3.3 Proteolytic activity 47
3.3.4 Lichenase activity 47
3.3.5 Glucosidase activity 47
3.3.6 Cellulase activity 49
3.3.7 Glucanase activity 49
3.3.8 Xylanase activity 50
3.3.9 Starch hydrolytic enzymes 50
3.3.10 Sulphite reductase 50

3.4 DISCUSSION **50**

3.5 ACKNOWLEDGEMENTS **54**

3.6 REFERENCES **54**

CHAPTER 4: THE HETEROLOGOUS EXPRESSION OF POLYSACCHARASE GENES WITH OENOLOGICAL RELEVANCE IN *SACCHAROMYCES CEREVISIAE* **57**

4.1 INTRODUCTION **58**

4.2 MATERIALS AND METHODS **59**
4.2.1 Microbial strains, plasmids and growth conditions 59
4.2.2 Construction of recombinant *E. coli*-yeast shuttle plasmids 59
4.2.3 Plate assays 60
4.2.4 Microvinification experiments 61
4.2.5 Chemical composition 62
4.2.6 Gas-liquid chromatography 62

4.3 RESULTS **62**
4.3.1 Genetic transformation of the industrial wine yeast strain VIN13 62
4.3.2 Microvinification 63

4.4 DISCUSSION **67**

4.5 LITERATURE CITED **68**

Stellenbosch University http://scholar.sun.ac.za/

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS 70

5.1 PERSPECTIVES 70

5.2 DISCUSSION AND CONCLUSION 70

5.3 REFERENCES 73

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CHAPTER 1

GENERAL INTRODUCTION AND PROJECT AIMS

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GENERAL INTRODUCTION AND PROJECT AIMS

1.1 THE GENETIC IMPROVEMENT OF WINE YEAST STARTER CULTURES

Until the early years of the seventeenth century, wine was considered to be the only wholesome, readily storable product, and this accounted for the rapid global improvement in wine fermentation technology. Today, wine is consumed as *a first-choice lifestyle product of moderation*. Wine has become synonymous with culture and a convivial lifestyle around the world and plays a major role in the economies of many nations, which produce approximately 26 billion litres of wine annually from about 8 million hectares of vineyards across the world. However, the worldwide decline in the consumption of alcoholic beverages resulted in a widening gap between wine production and wine consumption. The current oversupply of 15-20% of the global wine crop (ca. 5500 million litres of wine per annum) has led to fierce competition for market share. There has been a significant shift in consumer preference from basic commodity wines to premium and ultrapremium wines (Pretorius 2000). The wine industry therefore is forced to transform itself from a production-orientated industry to a market-driven enterprise. In this paradigm, wine quality is defined as "*sustainable customer and consumer satisfaction*". These changes have already led to increased diversity and innovation in the wine industry, much to the benefit of the consumer. There is an urgent demand for the further enhancement of wine quality, purity, uniqueness and diversity in an effort to meet the consumer challenge. Fundamental innovations in traditional winemaking practices are currently revolutionising the wine industry, while the forces of *market pull and technology push* continue to challenge the tension between tradition and innovation. Today there is a new, and for the moment controversial, focal point for innovation in winemaking – the genetic improvement of the two main organisms involved, the grape cultivar and the wine yeast (Pretorius 2000). This study will focus on the latter.

Yeasts are predominant in the ancient and complex process of winemaking. In spontaneous fermentations there is a progressive growth pattern of indigenous yeasts, with the final stages invariably being dominated by the alcohol-tolerant strains of *Saccharomyces cerevisiae*. This species in known universally as the *wine yeast* and is widely preferred for initiating wine fermentations. The primary role of wine yeast is to catalyse the rapid, complete and efficient conversion of grape sugars to ethanol, carbon dioxide and other minor, but important, metabolites, without the development of off-flavours. However, due to the demanding nature of modern winemaking practices and sophisticated wine markets, there is an ever-growing quest for specialised wine yeast strains possessing a wide range of optimised, improved or novel oenological properties. Over the last few years, considerable progress has been made in developing new wine yeast strains. However, two decades after the first successful yeast transformation, the wine industry is entering the twenty-first

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century without a transgenic wine yeast being used on a commercial scale to produce wine (Pretorius 2000).

Numerous genes involved in the production of flavour-related esters (e.g. alcohol acetyl transferases) and aroma terpene-liberating enzymes (e.g. β-glucosidases), antimicrobial compounds (e.g. bacteriocins) and enzymes (e.g. lysozyme), health-related antioxidants (e.g. resveratrol-producing stilbene synthases), as well as proteases and polysaccharide-degrading hydrolases (e.g. amylases, pectinases, cellulases and hemicellulases), have been expressed in wine yeast strains. This will possibly result in an increase in the quality of wine and beer, while lowering the manufacturing costs (Thomsen *et al.* 1988; Colagrande *et al.* 1994; Pretorius 1997). The lowering of the manufacturing costs will be due to the fact that less or no industrial enzyme preparations will be needed for filtration and clarification problems, or to get more flavour and colour from the grape skins to name only a few applications.

1.2 AIMS OF THIS STUDY

Saccharomyces cerevisiae has been used for many years both as a model system for unravelling the molecular details of gene expression and the secretion process, and as a host for heterologous proteins of biotechnological interest. The main goal of this study was to introduce an endoglucanase-encoding gene from *Butyrivibrio fibrisolvens* H17c (Berger *et al.* 1989), an endoxylanase-encoding gene from *Aspergillus niger* ATCC 90196 (Luttig *et al.* 1997), a pectate lyase gene from *Erwinia chrysanthemi* EC16 (Keen *et al.* 1984) and a polygalacturonase-encoding gene from *E. carotovora* subsp. *carotovora* (Willis *et al.* 1987) into industrial wine strains of *S. cerevisiae*, as well as to observe the effects of these transformed strains on wine production. We wanted to see what the effect of the expression of these genes would be on the colour, aroma, turbidity en chemical composition of the must and wine. We also wanted to look at the effects of the enzymes produced by the above-mentioned genes on different varieties of wine grapes. To obtain these goals, the following aims and approaches were taken:

(i) Construction of two yeast integrating plasmids, one containing the endoglucanase and xylanase genes, as well as the *SMR1* (sulphumetronmethyl resistance) marker gene for selection of the transformed yeast, and the other containing the two pectinase genes and the *KanMX* (geneticin resistance) gene for positive selection.

(ii) The expression and secretion of these genes in an industrial wine strain of *S. cerevisiae*

(iii) Testings on colour, aroma, tubidity and amount of wine and the chemical analysis to determine the effects of these polysaccharases on the wines, as well as their effects on different wine grape varieties.

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Another objective of this study was to investigate the different types of enzymes that are produced by non-*Saccharomyces* yeasts for possible use in wine yeasts and also their use in wines for specific effects during the initial phase of wine fermentations.

To achieve these goals, the following were done:

(1) Isolation of non-*Saccharomyces* yeasts from grapes in vineyards and cellars, as well as from clarified grape juice.

(2) Identification of the yeasts.

(3) Screening of the yeasts on solid and liquid media for the production of different extracellular enzymes.

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CHAPTER 2

LITERATURE REVIEW

From polysaccharides to fermentable sugars – the alteration

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LITERATURE REVIEW

2.1 FROM POLYSACCHARIDES TO FERMENTABLE SUGARS – THE ALTERATION

According to Katō (1981), plant cell walls contribute to the fundamental aspects of plant biology, which include the morphology, growth and development of the plant cells. The structurally complex polymers, which could either be polysaccharides or glycoproteins, form the walls that surround the growing plant cells. These primary cell walls contribute to a 0.1 – 0.2 μm thick layer composed of approximately 10% protein and 90% polysaccharide (Kretovich 1966; McNeil *et al.* 1984). Just outside the primary cell wall lies the secondary cell wall, which consists of three layers, an outer, middle and inner layer. These layers are made up of various amounts of polymers that interact with each other and are able to change depending on their function (Bacic *et al.* 1988). The space between the secondary and primary cell walls is filled by the middle lamellae (0.2 – 1.0 μm) and serves to bind these two cell walls together (Glazer and Nikaido 1995). In 1993, Wong and Saddler determined that the plant species, tissue and growth conditions (age) determine the occurrence, abundance, distribution and structure of the polymer, as well as its relationship with other cell wall components.

The polysaccharides of the cell wall are chemically divided into pectin, starch, hemicellulose and cellulose, according to Kretovich (1966). The above polymers are found in abundance in nature and constitute the major portion of living matter. Polysaccharides are composed of derived or simple sugars connected by glycosidic bonds between the hydroxyl group (OH) on the anomeric carbon of the one monosaccharide and any hydroxyl group of the other monosaccharide (Stanley and Zubay 1993a).

According to their structure, polysaccharides can be devided into hetero- or homopolymers (Stanley and Zubay 1993a). When the polysaccharide is composed of one type of sugar building blocks, it is called a homopolymer, and when it is composed of more than one type it is a heteropolymer. Furthermore, these homo- and heteropolymers can be divided into branched or linear polymers (Davidson 1967). The degree of polymerisation, which is the number of monosaccharides present in a single complex macromolecule, determines the size of the polymer.

Microorganisms also produce polymers. These microorganisms may produce a multiplicity of enzymes for the efficient degradation of the polysaccharides required for cell growth (Warren 1996). A diverse group of glycoside hydrolases with different modes of action are used for the microbial degradation of polysaccharides. These microbial polysaccharides are used for different purposes, such as to produce emulsions, to stabilise suspensions, to encapsulate materials, to flocculate particles and to modify the flow characteristics of fluids (Glazer and Nikaido 1995).

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A wide range of bacteria and fungi have the ability to decompose pectin, starch, hemicellulose and cellulose for the sole purpose of obtaining carbon and energy. For plants to defend themselves agains fungi and bacteria, they have physical and structural barriers on the surfaces of the epidermal walls that prevent these microbes from penetrating the plant tissues. These barriers are polymers, which vary according to age, location and environment, and also are species specific (Bacic *et al.* 1988; McNeil *et al.* 1984).

2.2 BIO-INTEGRATION OF POLYSACCHARIDES

During photosynthesis, sugars are produced and then converted to different end products. This process also leads to the synthesis of polysaccharides. The primary building blocks of polysaccharides are a large variety of hexoses that are all derivatives of glucose. These hexoses are formed through a serious of conversions that occur at the level of monophosphorylated sugar (glucose–1–phosphate) or nucleoside diphosphate sugar (uridine diphosphate – glucose) (MacGregor and Greenwood 1980).

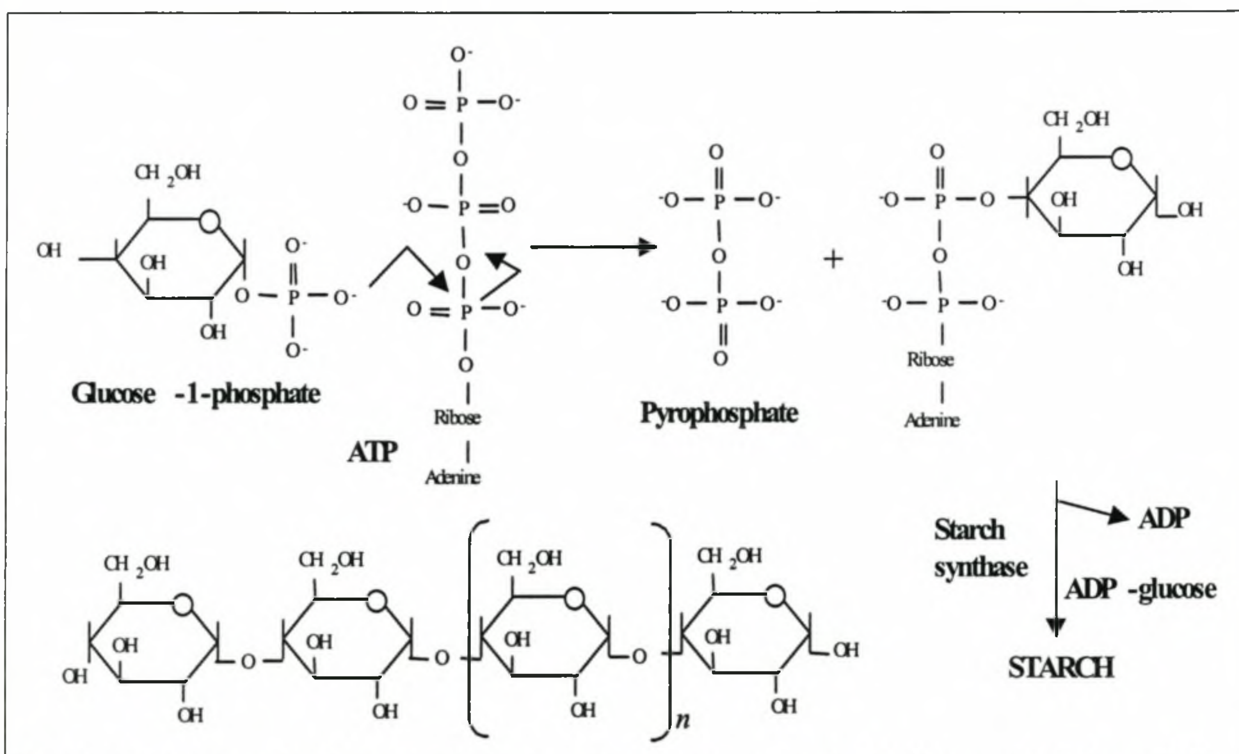

Fig. 1 Polysaccharide biosynthesis, using starch as an example (Atkinson and Zubay 1993; Stanley and Zubay 1993a).

The activated substrate for the formation of polysaccharides is the nucleoside sugar, which is produced by the reaction of a monosaccharide phosphate with a nucleoside triphosphate (Fig. 1) (Atkinson and Zubay 1993). The monosaccharide is transferred from a nucleotide sugar to the non-reducing end of the growing polysaccharide chain.

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Each stage in the pathway of polysaccharide synthesis (Fig. 2) (Fincher and Stone 1981) is catalysed by a specific enzyme and polyaccharides therefore are products of polysaccharide synthetases and glycosyl transferases (Bacic *et al.* 1988). The supply of monosaccharides as precursors, their activation to the nucleotide form, their interconversion, as well as the polymerisation or polysaccharide assembly, control the amount of cell wall polysaccharides that are formed (Fincher and Stone 1981). Branching sugars are further synthesised by their respective branching enzymes, leading to post-polymerisation modifications. These branching enzymes catalyse the breakage of oligosaccharides from the non-reducing end of a polymer chain and their reattachment by a α-1,6-bond (MacGregor and Greenwood 1980). The sugars are then translocated to the sites of cell wall synthesis, where deposition occurs, and converted to UDP-GLc [uridine 5'-(α-D-glucopyranosyl pyrophosphate)]. The nucleoside portions of uridine (U) may be replaced by adenosine (A), cytidine (C), guanosine (G) or thymidine (T) (Atkinson and Zubay 1993).

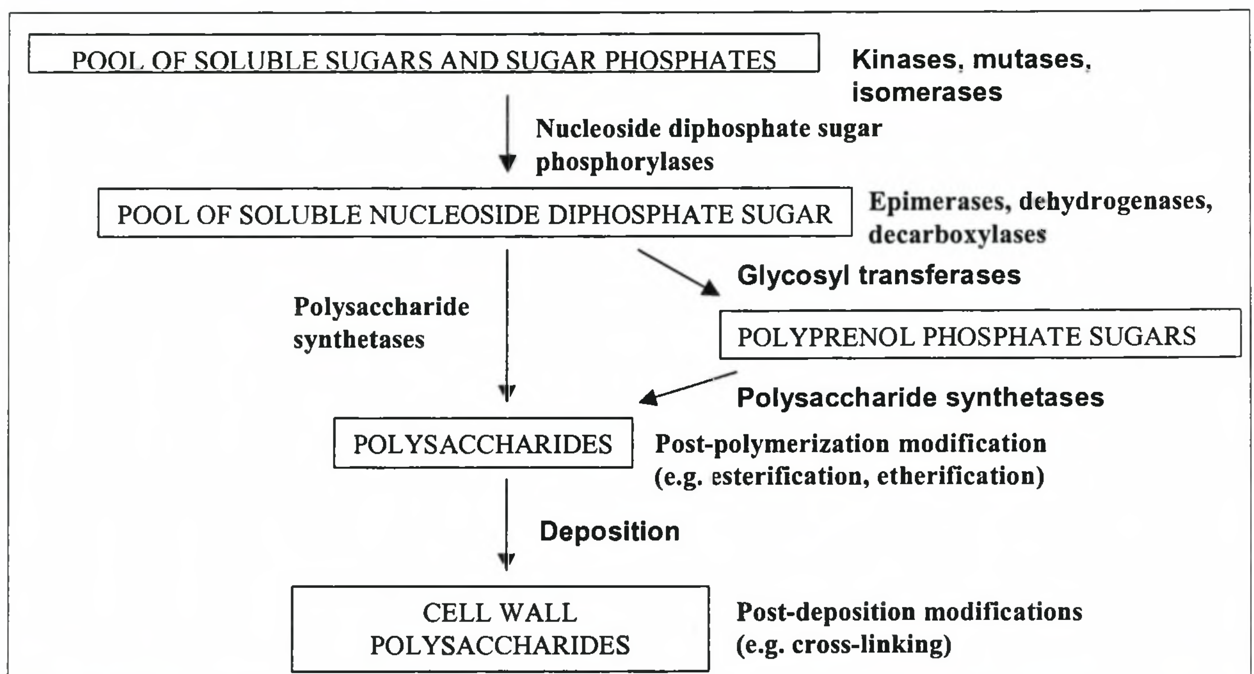

Fig. 2 Different stages of general synthesis and interconversion of monosaccharides for cell wall polysaccharide formation (Fincher and Stone 1981).

Polysaccharides can also be produced through the gluconeogenesis pathway, in which sugars are synthesised from less reduced metabolites, such as the intermediates from the tricarboxylic acid cycle (Peynaud and Ribéreau-Gayon 1971). It has been observed that the sugar content increases and the acidity level decreases during the development and growth of the grapevine, mainly during the ripening of the grape berry (Lavee and Nir 1986). This can be explained by gluconeogenesis and the synthesis of different polysaccharides. For example, malic acid can be converted via several enzymatic reactions to sugars and therefore lead to the synthesis of polysaccharides (Fig. 3).

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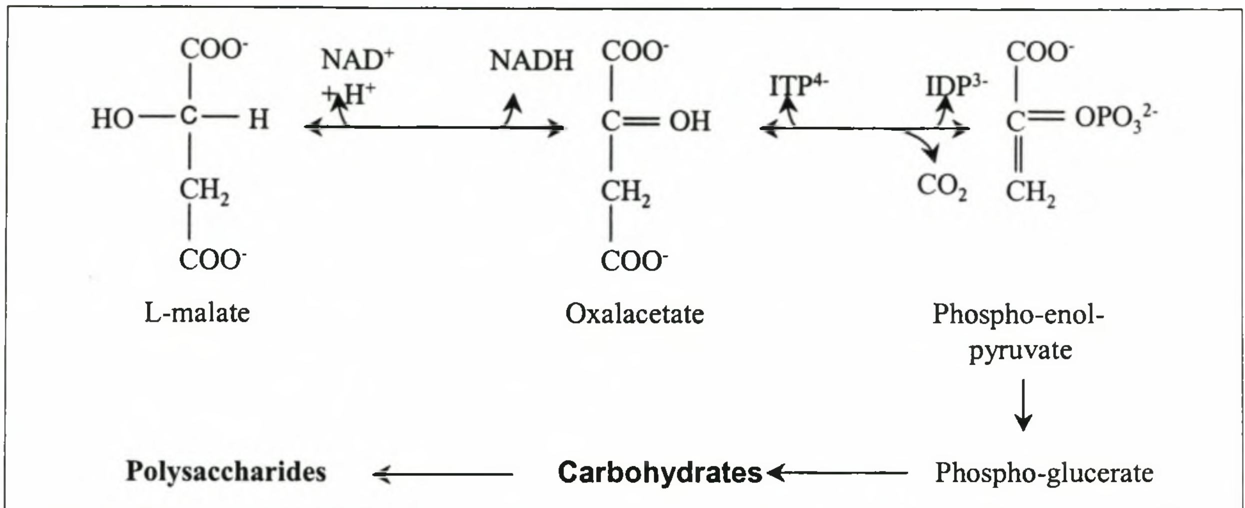

Fig. 3 Carbohydrate synthesis from L-malic acid (Peynaud and Ribéreau-Gayon 1971).

2.3 POLYSACCHARIDES FROM GRAPE BERRIES

The composition and structure of grape berry cell walls are of interest because of their importance in wine production technology.

Each berry consists of a thin, elastic epicarp (the skin), a juicy and fleshy mesocarp (the pulp) and an endocarp, which is indistinguishable from the pulp, that surrounds the carpels containing the seeds (Jackson 1994; Peynaud and Ribéreau-Gayon 1971).

The skin consists of an epiderm and several underlying layers of cells and comprises about 5-12% of the native grape clusters (Winkler *et al.* 1974; Lavee and Nir 1986). It is composed of six to 10 layers of small, thick-walled cells and consists of 78-80% water and 1-2% tannin (Frumkin 1996). The pulp consists of 25-30 layers of cells with large vacuoles containing the cell sap (Lavee and Nir 1986) and accounts for 80-90% of the crushed grape (Weaver 1976; Vedprakash *et al.* 1995). The pulp contains 78-80% water, 10-15% sugar, acids, mineral matter, pectins and heterogenous matter (Frumkin 1996).

The alcohol-insoluble residues obtained from grape berry pulp consist predominantly of cellulose, hemicellulose, xyloglucan and the pectic polysaccharides homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II (Saulnier and Thibault 1987; Nunan *et al.* 1997). The side chains of rhamnogalacturonan I are composed of arabinans, as well as of type I and II arabinogalactans (Saulnier *et al.* 1988). The glycosyl residue composition and alcohol-insoluble residues of pulp and grape berry skins are similar, although polysaccharides have been reported to account for only 50% of the alcohol-insoluble residues of the skin (Lecas and Brillouet 1994).

Buffer-soluble type II arabinogalactan proteins are abundant in grape berry tissue and have been characterised partially (Saulnier and Brillouet 1989). In contrast, the pectic polysaccharide rhamnogalacturonan II accounts for <5% of the pulp and skin cell walls (Lecas and Brillouet 1994; Nunan *et al.* 1997). Nevertheless, rhamnogalacturonan II and arabinogalactan proteins are the quantitatively major

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grape polysaccharides present in wine (Pellerin *et al.* 1995, 1996). Moreover, their presence in wine is believed to affect haze formation and the binding of heavy metals (Szpunar *et al.* 1998).

Vidal *et al.* (2001) determined that there is three-fold more rhamnogalacturonan I and II in the skin tissue than in the pulp tissue. Such results are consistent with the fact that more grape polysaccharides are present in red wine than in white wine. The study by Vidal *et al.* (2001) provided evidence that rhamnogalacturonan II accounts for between 1 and 2% of the berry cell wall, eventhough it is one of the quantitatively major polysaccharides in wine. Rhamnogalacturonan II is also a prominent polysaccharide in juices that are obtained by enzymatic liquefaction of fruits and vegetables (Doco *et al.* 1997). It is possible to obtain about 250mg of rhamnogalacturonan II per kilogram of berries and the rhamnogalacturonan II content of the skin tissue is three-fold higher than in pulp tissue. Red wine usually contains 100 to 150mg/L and white wine 30 to 50mg/L.

Arabinogalactan proteins are a quantitatively major grape polysaccharide in wines. They are released as soon as the grapes are crushed and pressed (Vidal *et al.* 1999). Rhamnogalacturonan I is a quantitatively minor polysaccharide component of wine, even though its concentration in the cell wall is three-fold higher than that of rhamnogalacturonan II. Homogalacturonan, which accounts for 80% of the pectic polysaccharides in grape berry cell walls, has been detected at the initial time of berry processing and its concentration is estimated to be < 100mg/L of the must (Vidal *et al.* 1999). However, no homogalacturonans have been detected in red or white wines. This suggests that homogalacturonans are fragmented by polygalacturonases that come from either the grapes or the yeast used for the fermentations during winemaking.

2.4 BREAKDOWN OF POLYSACCHARIDES

The reduction in the molecular weight of a polymer is called polysaccharide degradation. The degradation of polysaccharides can be induced by four different kinds of mechanisms, according to Gowariker *et al.* 1986. These mechanisms are chemical (acid or alkali), physical (thermal), microbial and enzymatic degradation. All of these mechanisms have their technological and ecological advantages and disadvantages. Hydrolysis by alkalis or acids results in toxic byproducts that are expensive to treat and thus have economic implications. Purified enzymes, on the other hand, are expensive and the stability of the enzymes influences their application. Opposite to the above two mechanisms, hydrolysis by naturally occurring microbial populations is inexpensive, extremely stable, the most important natural process in the environment and does not cause pollution problems (Kubicek *et al.* 1993; Pretorius 1997), making this mechanism the most favourable one for the degradation of polysaccharides. Enzymes produced by these microorganisms can be specific and attack a certain substrate, but they can also be non-specific, attacking

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various substrates (Uhlig 1998). Another advantage is that microorganisms have the ability to carry out single-step or multi-step transformations of organic compounds that are not easily accomplished by conventional chemical methods. These organisms are able to produce useful products from wastes and are therefore quite beneficial to mankind.

The long extended molecules that are closely packed together in the structure give form, support and some protection, contributing to the resistance of polysaccharides to enzymatic hydrolysis. The hydrolytic enzymes have to be secreted or expressed on the cell surface because of the fact that the high molecular weight oligomers are unable to enter microbial cells (Warren 1996). In eukaryotes, the enzymes' secretary pathway starts from the endoplasmic reticulum and moves through the Golgi bodies and vesicles to the membrane (Kubicek *et al.* 1993), where it stays confined to the microbes' cell surface in eukaryotes and prokaryotes or is secreted into the growth media (Biely 1993).

Microorganisms that secrete extracellular enzymes differ in their specificity and properties (Fogarty and Kelly 1990). The substrate and the enzyme involved determine the extent of microbial degradation and the types of degradation products that are formed (Puls and Schuseil 1993). The complete hydrolysis of plant polysaccharides requires several different microbial systems that work together, as organisms differ in their degradation capabilities (Glazer and Nikaido 1995). The potential for a specific substrate to be decomposed by microorganisms is based on its elemental composition, the structure and linkage between repeating units, the nutrients present in the environment, the microbial diversity present and the abiotic conditions, such as pH, oxygen and osmotic pressure (Prescott *et al.* 1993).

2.4.1 CELLULOSE (GLUCAN)

Cellulose is the major polysaccharide in woody and fibrous plants and therefore is the most abundant polymer in the biosphere (Mathew and Van Holde 1990). It forms 40 – 50% of cell wall substances and this percentage is relatively constant between species (Coughlan 1990).

2.4.1.1 Occurrence and structure

Cellulose is a condensation polyalcohol consisting of D-anhydroglucopyranose units linked by β-1,4-glycosidic bonds (Lamed and Bayer 1988). It consists of a linear polymer of glucose units, with each glucose residue rotated 180° with respect to its neighbours along the main axis of the chain (Coughlan 1990). The size of a cellulose molecule can be given as the number of repeating units or the degree of polymerisation (DP). The degree of polymerisation ranges from 30 to 15000 units. The tertiary structure of cellulose is extremely complicated in comparison to the simplicity of the primary structure (Cowling and Kirk 1976; Fig. 4).

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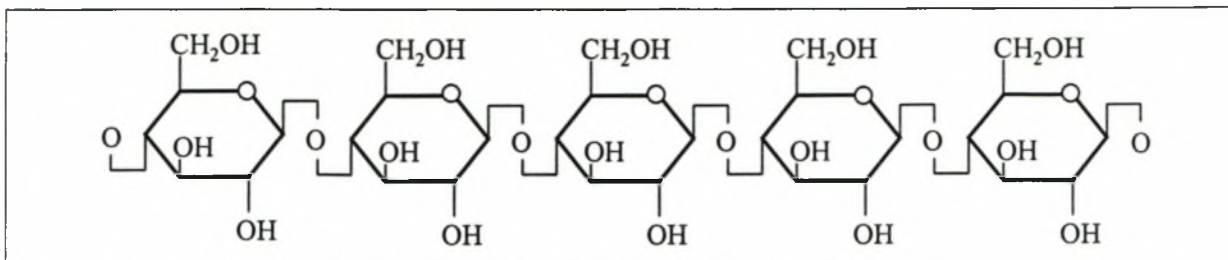

Fig. 4 The primary structure of cellulose (Cowling and Kirk 1976).

Sixty to seventy adjacent unipolar chains associate through interchain hydrogen bonding and Van der Waal's interactions to form ordered crystalline microfibrils. These microfibrils aggregate to form insoluble fibres (Coughlan 1990). There are areas of complete order, i.e. crystalline areas, and also less well-ordered or amorphous regions within the cellulose fibres. It was found that the basis of the water insolubility of cellulose (Bohinski 1987) is the high hydrogen bonding capacity between individual chains. This also gives it a high degree of strength.

Glucans are the major cell wall component in most yeasts, forming more than 50% of the cell wall (Duffus *et al.* 1982). Studies showed that these glucans could be devided into two groups. The first and major group is composed of a linear chain of D-glucose units with β-1,3-linkages containing β-1,6 branchings (Fleet and Phaff 1981). The second and minor group is a β-1,6 glucan with β-1,3 branched lateral chains. These glucans are released into wine during both process fermentation and the cell autolysis, causing filtration and clarification problems.

Another glucan that causes a lot of problems for winemakers is the β-glucan of *Botrytis cinerea*. This β-glucan is composed of a β-D-(1,3)-linked backbone with very short β-D-(1,6)-linked side chains (Fig. 5) (Dubourdieu *et al.* 1981).

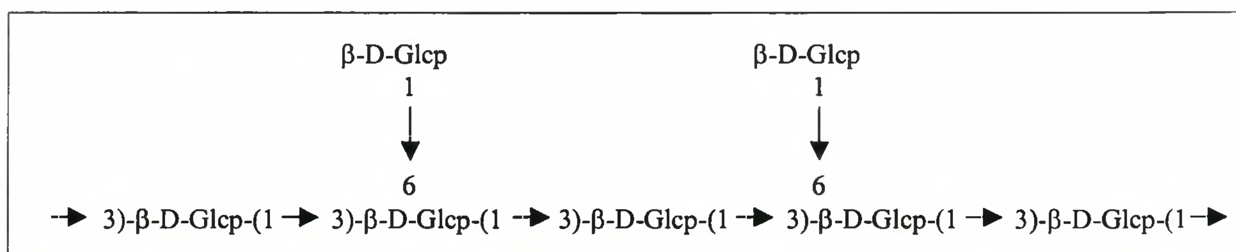

Fig. 5 The structure of the β-glucan of *Botrytis cinerea* (Dubourdieu *et al.* 1981).

This polysaccharide is entirely responsible for the clarification and filtration problems encountered in wines made with botrytised grapes. In wine, these glucans seem to polymerise in the presence of alcohol. It has been reported that the filtration problems follow an exponential curve as soon as the alcohol level rises. This leads to severe problems at the end of alcoholic fermentation.

2.4.1.2 Hydrolysis

Cellulolytic enzymes are produced by a wide range of microorganisms, such as bacteria, actinomycetes and fungi, by higher plants, as well as by invertebrate animals (Finch and Roberts 1985). Vertebrate animals, on the other hand, do not

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have these enzymes and have to rely on cellulolytic microorganisms in their digestive tracts to degrade cellulose.

The degradation of cellulose can be achieved by thermal, chemical or biochemical processes (Alén 1990; Blazej *et al.* 1990). It has been shown that enzymatic hydrolysis of cellulose is environmentally more acceptably than chemical hydrolysis, although it is a slower process.

The crystalline cellulose fibres are embedded in a matrix of hemicellulose, lignin and pectin that is held together by hydrogen bonds. The compactness of the molecules reduces the accessibility of cellulolytic enzymes to cellulose, thereby complicating the enzymatic hydrolysis (Béguin 1990). Firstly, the adsorption of the enzyme to the surface of the cellulose fibre is very important for the enzymatic hydrolysis of cellulose. Temperature and the type of cellulose used influence the adsorption, but it is largely independent of pH. The maximum adsorption occurs at 50°C and a pH of 4.8 (Ghose and Bisaria 1979). Preferential adsorption suggests that endoglucanase is required to initiate degradation. Due to the physical nature of cellulose in wood, the cellulose is not always accessible the adsorption and therefore the rate of hydrolysis is slow. Chemical, mechanical, thermal or microbial pretreatments were found to increase to adsorption rate significantly (Kelsey and Shafizadeh 1980; Ladisch *et al.* 1983).

Reese *et al.* (1950) attempted to explain the enzymatic mechanisms involved in cellulose degradation (Eriksson *et al.* 1990b) by proposing the "C1-Cx" hypothesis. The hypothesis can be formulated as follows:

The hypothesis proposed that crystalline cellulose is modified by the activity of C1 (Reese 1976) and that the modified products are hydrolysed by other enzymes. It was suggested that C1 is a non-hydrolytic chain-separating enzyme that separates the cellulose chains by disrupting the hydrogen bonds (Eriksson *et al.* 1990b). Cx reflects several randomly acting enzymes that can hydrolyse non-crystalline cellulose and β-1,4-oligomers of glucose. The development of more efficient methods of separation has led to the separation of the individual enzymes contained in the cellulase complex. This has contributed to the elucidation of the mechanisms involved in enzymatic cellulose degradation (Bisaria and Ghose 1981; Fig. 6).

Firstly, endoglucanases attack regions of low crystallinity in the cellulose fibre and create free chain ends. These free chain ends are then attacked by exoglucanases that remove the cellobiose. The cellobiose is then finally hydrolysed to glucose by β-glucosidases. The "C1-Cx" concept was later modified and it was postulated that cellulose degradation begins by an initial modification of the crystalline cellulose, followed by synergistic attacks by endoglucanase and exoglucanases (Bisaria and Ghose 1981).

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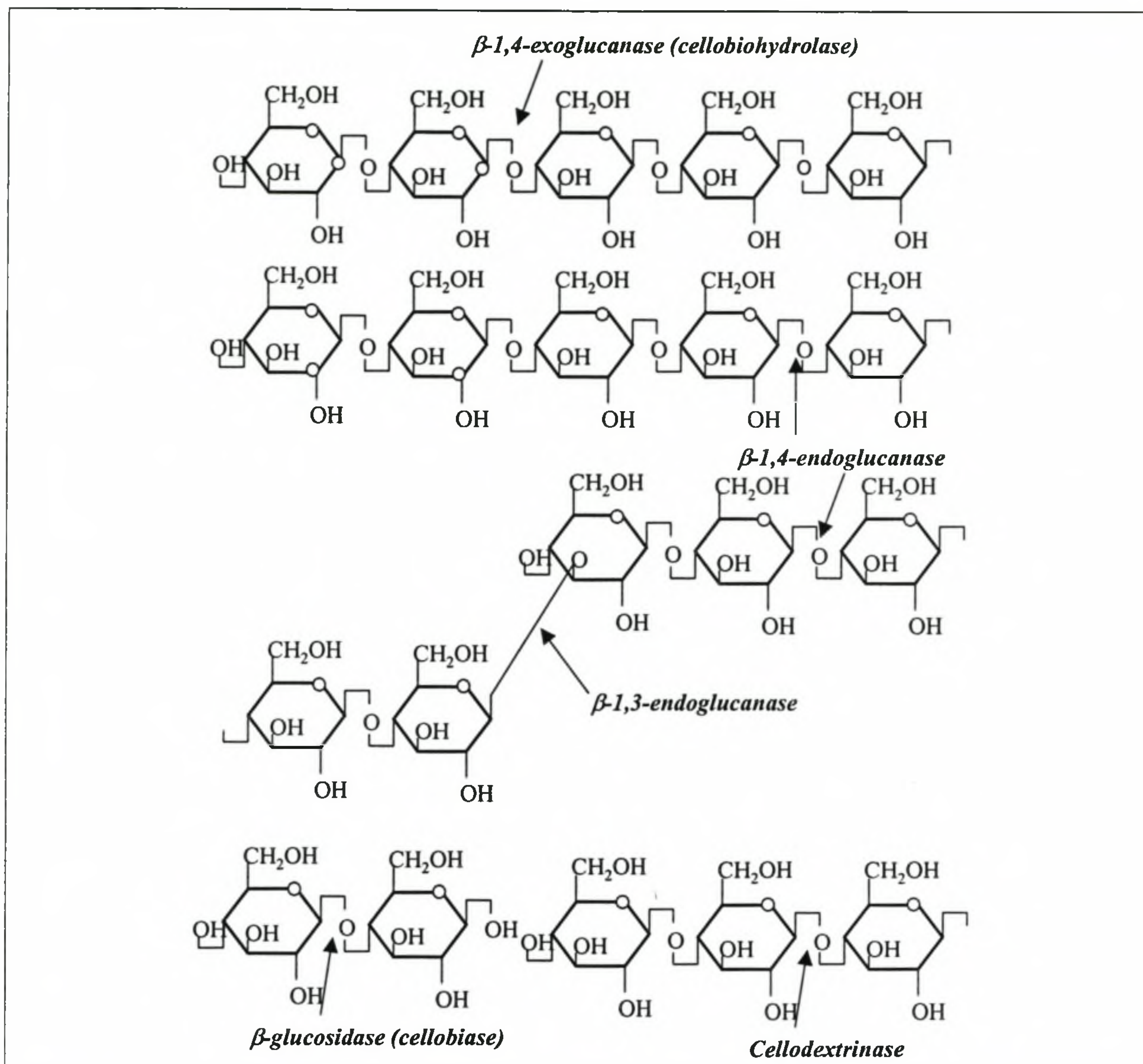

Fig. 6 Schematic representation of the degradation of cellulose (Bisaria and Ghose 1981).

Later, it was shown that a multicomponent enzyme complex (Finch and Roberts 1985) was responsible for the degradation of crystalline cellulose. The individual exoglucanases, endoglucanases and β-glucosidases degrade practically no crystalline cellulose, whereas a mixture of the three enzymes causes extensive hydrolysis (Eriksson and Wood 1985). This showed that the enzymes appear to act in a synergistic manner. One explanation for this is that the substrate concentration for at least one enzyme is rate limiting and that the other enzyme overcomes this limitation for its partner.

Finch and Roberts (1985) suggest that there are two forms of synergism. The first is between the exo- and endo-enzymes. The endoglucanases cleave randomly and provide non-reducing ends that become substrates for the exoglucanases (Ladisch *et al.* 1983). The endo-exo synergism appears only on crystalline substrates and is absent on soluble derivates (Eriksson *et al.* 1990b). The exoglucanase is the limiting enzyme.

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The second form of synergism is where the degradation products are inhibitory to cellulases (e.g. cellobioses) and these products are then removed by hydrolytic and oxidative enzymes. In addition to these two types of synergism, other unusual types have also been observed, including endo-endo and exo-exo synergism (Coughlan 1990; Kubicek *et al.* 1993). Akhtar *et al.* (1988), on the other hand, also suggest multiple enzyme activities of the same type of enzymes (e.g. endoglucanases) for the degradation of the highly heterogeneous cellulose substrate normally encountered in the wild. Endoglucanases attack the cellulose chain randomly and split β-1,4-glycosidic linkages. From there, the polysaccharides are further degraded to glucose by exoglucanases and β-glucosidases.

2.4.1.3 Cellulolytic microorganisms

In 1990, Béguin stated that microorganisms that can degrade crystalline cellulose must secrete more or less complex cellulase systems. These systems consist of a variety of enzymes with different specificities and modes of action that act synergistically to hydrolyse cellulose.

In fungi, it is mostly hydrolytic enzymes that are associated with cellulose degradation. Cellulases from a fungal origin (e.g. *Trichoderma reesei* and *Phanerochaete chrysosporium*) are ordered into three categories: (a) cellobiohydrolases; (b) endoglucanases; and (c) β-glucosidases (Coughlan 1985; Covert *et al.* 1992). It is proposed that the endoglucanases attack the amorphous regions of cellulose fibres, creating sites for cellobiohydrolases, which would then proceed into the crystalline regions of the fibre. Finally, the β-glucosidases will hydrolyse the cellobiose.

For a long time a fundamental difference between fungal and bacterial cellulase systems was thought to be that the former comprised endo- and exoglucanases, and the latter only endoglucanases. Now it is clear that cellulolytic bacteria produce both types of enzymes (Tomme *et al.* 1995). Bacterial systems were also found to produce cellobiohydrolases (Tomme *et al.* 1995; Shen *et al.* 1995; Barr *et al.* 1996), although it previously was thought not to produce these types of enzymes. Aerobic bacteria produce numerous individual, extracellular enzymes with binding modules for different cellulose conformations. Specific enzymes act in synergy to elicit effective hydrolysis. In contrast, anaerobic bacteria possess a unique extracellular multi-enzymes complex, called cellulosome. Binding to a non-catalytic structural protein (scaffoldin) stimulates the activity of the single components towards the crystalline substrate (Schwarz 2001). The most complex and best investigated cellulosome is that of the thermophilic bacterium *Clostridium thermocellum.* The cellulosomes are a discrete, multi-enzyme protein complex adapted for the efficient hydrolysis of cellulosic substrates.

The most well-known cellulolytic microorganisms is the filamentous mesophilic soft-rot fungus *T. reesei* (Eriksson *et al.* 1990a). This organism produces a number of different extracellular hydrolases, such as xylanases and amylolytic enzymes, but

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its production of cellulases has drawn the most attention. *T. reesei's* cellulolytic enzyme system is the best studied among cellulolytic organisms and it also serves as a model system for the enzymatic degradation of cellulose in general (Kubicek *et al.* 1993).

2.4.2 HEMICELLULOSE (XYLAN)

After cellulose, hemicellulose is the most abundant renewable polysaccharide in nature and is found mostly in plant cell walls, which act as the main polysaccharide-containing renewable resource in nature. The major component of plant hemicellulose is xylan.

2.4.2.1 Occurrence and structure

Hemicelluloses are a distinct group of polysaccharides that can by classified according to their chemical composition and structure. Xylans, mannans, galactans and arabinans are the four predominant types (Puls and Schuseil 1993). Xylans, such as 4-O-methylglucuronoxylans and arabino-4-O-methylglucuronoxylans, together with β-mannans, such as glucomannan and galactoglucomannans, are also the major hemicellulose constituents of lignocellulosic wastes (Dekker and Lindner 1979).

Hemicellulose may be linear or branched and it has a degree of polymerisation (DP) of up to 200. The monomers are linked together by β-1,4-glycosidic bonds, but the D-galactopyranose residues are β-1,3-linked and are the exception to the rule. There are two types of hemicellulose, namely homopolysaccharides and heteropolysaccharides. Most hemicellulose in nature occurs as heteroglycans, with the exception of the xylan from the seaweed *Rhodymenia palmata*, which consists solely of β-xylose residues (Barry and Dillon 1940). The heteroglycans in hardwood contain two or more of the following compounds; D-galactose, D-glucose, D-glucuronic acid, 4-O-methylglucuronic acid, D-mannose, D-xylose, L-arabinose and D-galacturonic acid (Coughlan *et al.* 1993). In grasses and cereals, the hemicelluloses are comprised of D-xylose, L-arabinose, D-glucose and D-galactose. Graminaceous hemicelluloses contain a greater variety of linkages and branching types than hardwood, such as ester-linked acetyl, feruloyl and coumaroyl moieties (Puls and Schuseil 1993).

Because of the great extent of different structures and chemical compositions of hemicelluloses in hardwood, softwood and grasses, a unique combination of hemicellulolytic enzymes is needed for effective and total degradation (Puls and Schuseil 1993).

Xylan is composed of β-1,4-linked xylose units, forming a xylan backbone with side chains connected to the backbone (Christov and Prior 1993). In hardwoods and grasses, the main chain contains an O-acetyl group at the C2 and/or C3 positions, whereas in softwoods and annual plants it can be substituted with arabinose at the C3 position. Xylan may further be esterified with phenolic acids in annual plants.

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These phenolic acids facilitate intermolecular cross-linking with xylan, as well as cross-linking between xylan and lignin in the cell wall matrix. Due to the complexity and diversity of xylans from various plant sources, several xylanolytic enzymes are necessary for complete degradation. The synergistic action of these enzymes is a prerequisite, making new sites available on the xylan molecule for enzymatic attack (Coughlan *et al.* 1993).

Regarding the xylans in hardwood, the O-acetyl-4-O-methylglucuronoxylan (glucuronoxylan), the principal xylan of hardwood, consists of a homopolymeric backbone of 1,4-linked β-D-xylopyranose units. Seventy percent of the D-xylose units are acetylated on C-2 and/or C-3 and, on average, every tenth D-xylose is α-1,2-linked to 4-O-methyl-D-glucuronic acid (Eriksson *et al.* 1990b; Puls and Schuseil 1993). Minor amounts of L-rhamnose and D-galacturonic acid are shown to be integral parts of the xylan main chain (Fig. 7) (Atkins 1992).

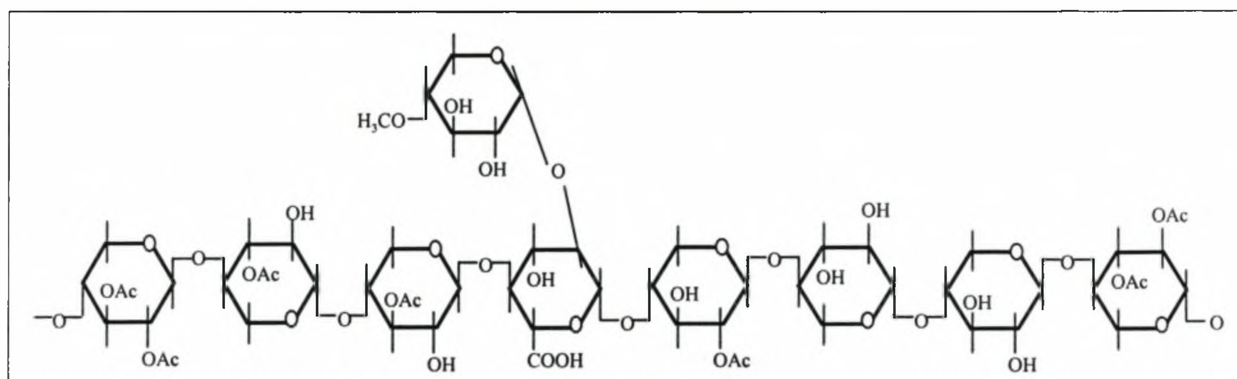

Fig. 7 The structure of O-acetyl-4-O-methylglucuronoxylan from hardwood. Every tenth D-xylose unit carries a α-1,2-linked 4-O-methyl-D-glucuronic acid. Seven out of 10 D-xylose residues contain an O-acetyl group attached at the C-2 or C-3, or at both positions (Puls and Schuseil 1993).

In softwood, on the other hand, the arabino-4-O-methylglucuronoxylans form 7-8% of the dry weight and are comprised of 1,4-linked β-D-xylopyranose units. It was found that seven out of ten residues are substituted with 4-O-methyl-D-glucuronic acid at C-2, and 10-15% by L-arabinofuranose units at C-3 or C-2 (Puls and Schuseil 1993). The xylan in softwood contains no acetyl substituents, as found in hardwood. Every eighth or ninth D-xylose unit also carries one L-arabinose residue (Fig. 8). The DP of arabinoglucuronoxylans is 70-130 (Puls and Schuseil 1993).

The xylans found in grass have the same backbone as the xylans from hard- and softwoods, although a wide variety of xylan structures exist, depending on the species and the tissues within a single species. Characteristic differences are that they contain fewer uronic acids, they are more highly branched and contain large amounts of L-arabinofuranosyl units. These units are mainly linked to the C-2 of xylose. In the internodes of wheat, every seventh D-xylose unit of xylan is substituted by an L-arabinofuranose side chain (Puls and Schuseil 1993). The wheat bran hemicellulose arabino-xylo-oligosaccharide contains a di-substituted xylose unit with arabinose at C-2 and C-3 (Fig. 9) (Puls and Schuseil 1993).

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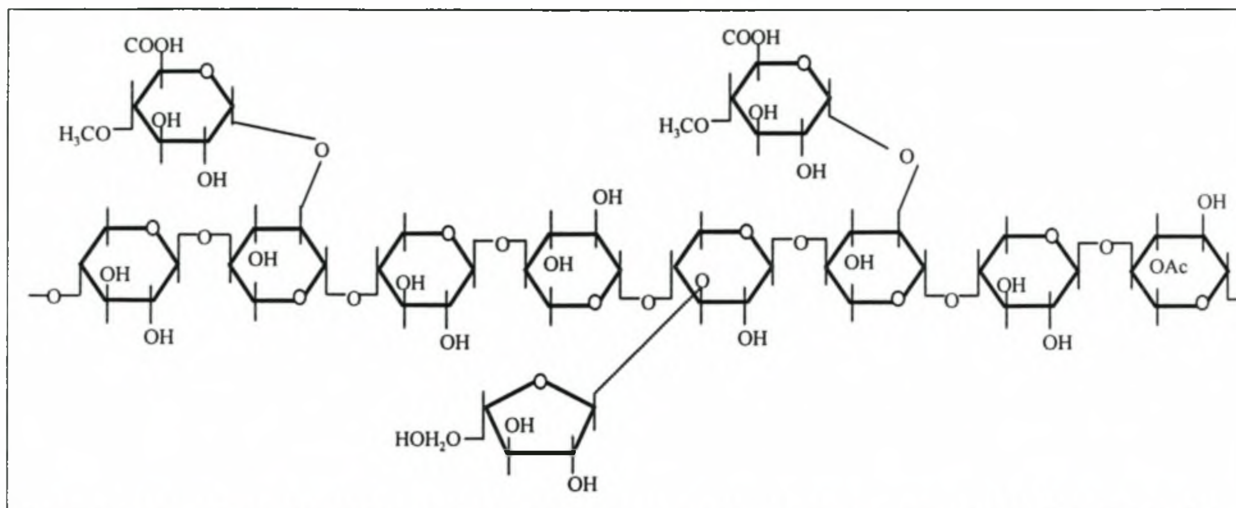

Fig. 8 The softwood arabino-4-O-methylglucuronoxylan structure consists of β-1,4-linked D-xylopyranose residues. The D-xylose units are substituted by terminal 4-O-methylglucuronic residues, α-L-arabinofuranose residues and arabinose (Puls and Schuseil 1993).

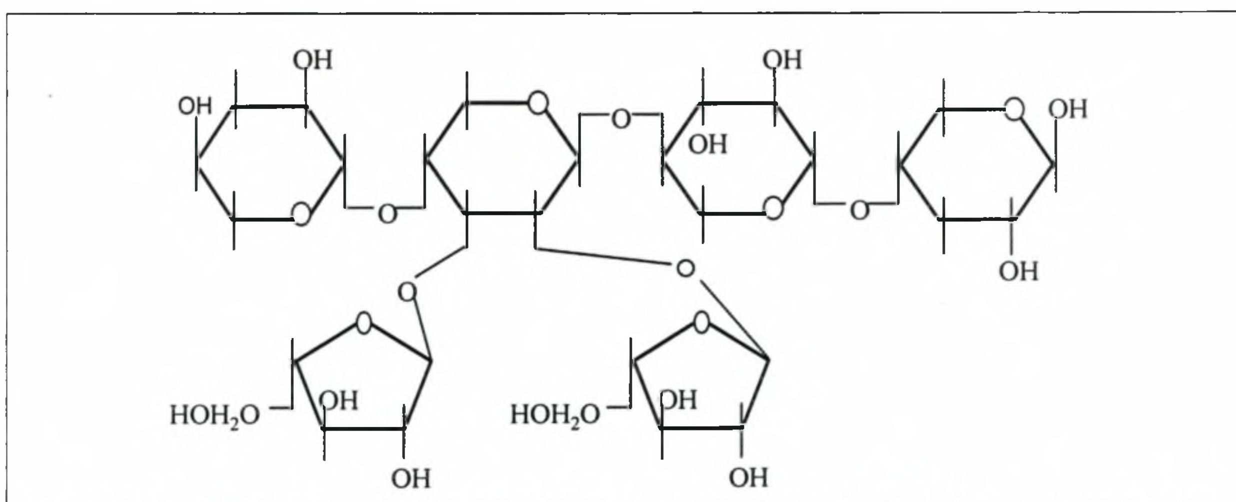

Fig. 9 The arabino-xylo-oligosaccharide from wheat bran hemicellulose with L-arabinose at the C-2 and C-3 positions of a D-xylose unit (Puls and Schuseil 1993).

In graminaceous plants, the xylans contain O-acetyl groups at 1-2% of the dry weight of their cell walls (Bacon *et al.* 1975). Grasses also contain 1-2% phenolic substituents to their arabino-xylan. The phenolic acids, ferulic or coumaric acid residues, are esterified to C-5 of some of the side chain L-arabinosyl residues. It has been estimated that one in every 15 L-arabinose residues of barley straw xylan is esterified with ferulic acid and one in every 31 with p-coumaric acid (Tenkanen *et al.* 1991).

These two acid residues from different heteroxylan molecules may be cross-linked to form diferulic acid or dihydroxytruxillic acid. The cyclo-dimerised ferulic and/or ρ-coumaric acids coupled to arabinoxylan may cross-link xylan intermolecularly and also cross-link xylan with lignin (Christov and Prior 1993).

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2.4.2.2 Hydrolysis

Plant heteroxylan is a complex structure that requires the action of a number of hydrolytic enzymes to obtain complete breakdown. The sites of action of the enzymes involved in the hydrolysis of a hypothetical plant xylan fragment are shown in Figure 10 (Biely 1993).

Endo-1,4-β-xylanase, which will be discussed later, attacks the xylan backbone and generates non-substituted or branched xylo-oligosaccharides. For the complete depolymerisation of xylan to xylose, xylose-producing enzymes are also necessary. Exo-xylanase and 1,4-β-D-xylosidase are able to produce D-xylose through their specific activities (Biely 1993).

The complete depolymerisation of heteroxylan is complicated, however, by the presence of main chain substituents. The action of xylan-degrading and xylose-producing enzymes is hindered by these substituents. Exoglycosidases and esterases liberate the substituents from the xylan backbone as follows: α-L-arabinosyl residues by α-L-arabinofuranosidase; 4-O-methyl-D-glucuronosyl residues or D-glucuronosyl residues by α-glucuronidase; and acetic acid, p-coumaric acid and ferulic acid residues by acetyl xylan esterase, *p*-coumaroyl esterase and feruloyl esterase respectively (Tenkanen and Poutanen 1992).

The action of endo-1,4-β-mannanases, β-mannosidase, α-galactosidase, β-glucosidase and acetylmannan esterase on (acetyl)-galactoglucomannans and glucomannanase in hardwoods and softwoods will not be discussed in this thesis.

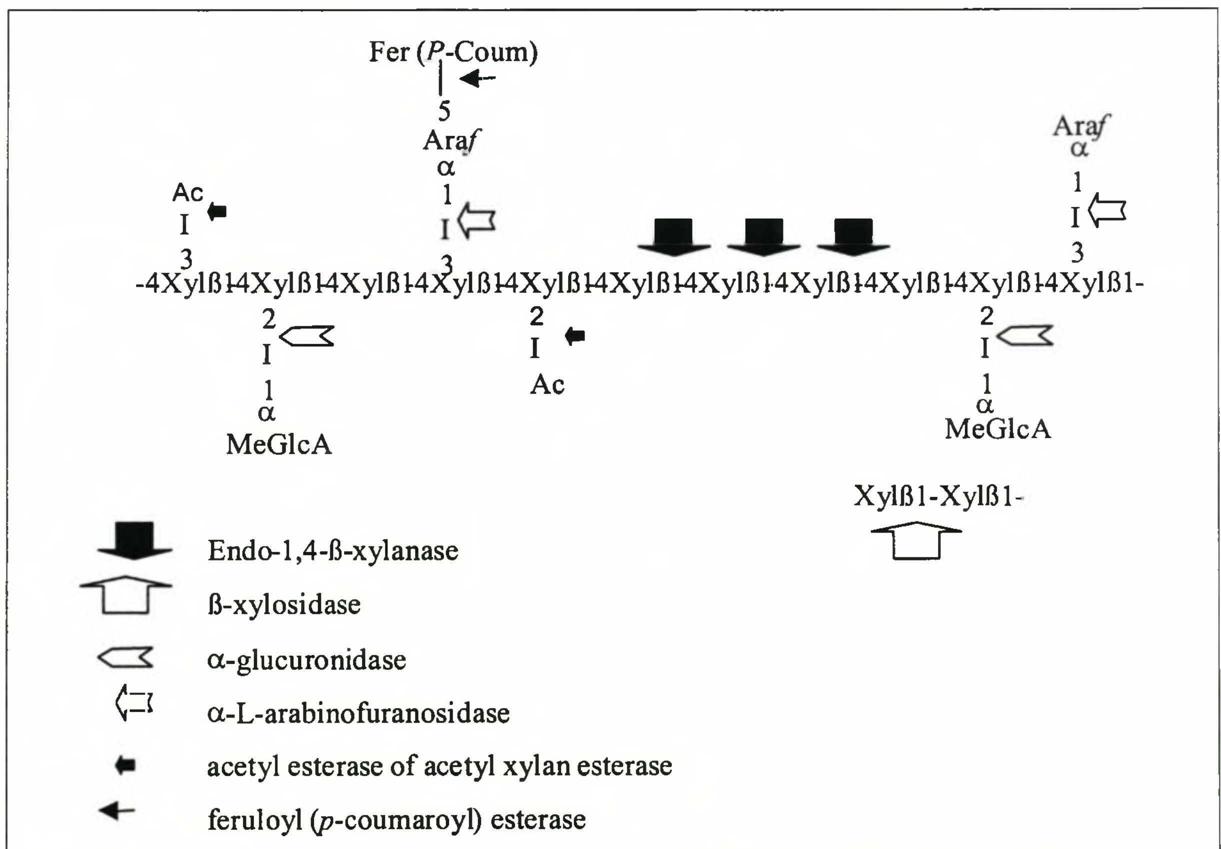

Fig. 10 A hypothetical plant xylan fragment showing the sites of action of the enzymes involved in its hydrolysis (Biely 1993).

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Endoxylanases are classified as debranching and non-debranching enzymes according to their ability to release L-arabinose from arabinoxylans (Dekker 1985). Both types are also capable of attacking glucuronoxylans and unsubstituted 1,4-β-D-xylans. The non-debranching group of xylanases is by far the most common and they degrade heteroxylans randomly. With respect to their physicochemical properties, endoxylanases, with some exceptions, fall into two main classes: those with a molecular weight (Mr) of less than 30 kDa (are usually basic proteins), and those with Mr values in excess of 30 kDa (are usually acidic proteins) (Wong *et al.* 1988).

The enzyme activity of endoxylanases is often determined by measuring the reducing sugars released from solid xylan substrates (Poutanen 1988). Other methods that have been used include viscometry (Kundu *et al.* 1980) and the nephelometric assay (Nummi *et al.* 1983).

Many factors cause variations in the determination of xylanase activity (Khan *et al.* 1986). Reported activities in international units varied by a factor of 3 to 107, depending on the dilution of the enzymes prior to assay. The type of substrate used also determines the xylanase activity, varying from 1.5 to 104 U ml^{-1} among three different xylan preparations from larchwood. The units of enzymatic activity obtained appeared to vary with the availability of easily degradable xylo-oligosaccharides in the substrate. From this, it was clear that defining the composition and the use of standardised methods for the preparation of the xylan substrate would help to minimise variations in the determination of xylanase activity. Enzyme dilution also needed to be highly standardised to minimise variations (Eriksson *et al.* 1990b). Twenty laboratories collaborated in an investigation of assays based on the production of reducing sugars from polymeric 4-O-methyl glucuronoxylan by xylanases. The standard deviation of the results reported was brought down from 108% of the mean to 17% of the mean when all the participants used the same substrate and precise instructions for enzyme dilution for activity determination (Baily *et al.* 1992).

2.4.2.2 Xylanolytic microorganisms

The production of endo-1,4-β-xylanase is found in a variety of fungi and bacteria. The endoxylanases of fungi have been studied more extensively, however, probably because eukaryotic microorganisms such as fungi are better producers of xylanases (Dekker, 1985). The xylanases of *Aspergillus niger* are the best characterised and most studied (Table 1).

Several *Trichoderma* strains also produce endoxylanases that are well characterised, for example *T. reesei, T. harzianum, T. honingii* and *T. lignorum* (Eriksson *et al.* 1990a). Other fungal endoxylanases that have been studied are those of *Coniophora cerebella* (King and Fuller 1968), *Tyromyces palustris* (Ishihara *et al.* 1975) *Poria placenta* (Micales *et al.* 1987), *Polyporus tulipiferae* (Hoebler and Brillouet 1984) and *Chaetomium cellulolyticum* (Dubeau *et al.* 1987), to list a few.

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Table 1 Characteristics of *Aspergillus niger* endoxylanases (Poutanen 1988).

MW (kDa)	pI	pH-optimum	Substrate	Main end products	Remarks
20.8	6.7	4-6	Soluble xylan xylo-oligomers	X_2,X	No activity towards insoluble xylan, arabinose not released
13	8.6	6.0	Soluble xylan		Requires branch points in substrate, arabinose not required
13	9.0	5.5	Insoluble branched xylan	X_3, X_5	
14	4.5	4.9	>X5, soluble xylan	X_2-X_6	Arabinose not released
28	3.7	5.0	>X5, soluble xylan	X_3, X_5	
13	4.0		Xylo-oligosaccharides		Transferase activity No cellulase activity
31	nd*	4.0	Larchwood xylan preferred to oligosaccharides	X, X_2, Ar	Also cellulase activity
50	nd	5.5-6.0			Also cellulase activity
50	nd	4.0-4.5			
33	4.2	4.0	Carboxymethyl xylan, arabino- and glucuronoxylans	$X_2 > X_3 > X_1$ oligomers	No cellulase activity Traces of arabinose liberated No transferase End product inhibition
nd	nd	nd	X_5	X_2, X_3	High transferase activity

* not determined

With regard to bacterial endoxylanases (Table 2), the enzymes of the genera *Bacillus* and *Streptomyces* have been studied the most. Some of the rumen microflora in ruminants also produce xylanases (Brice and Morrison 1982). Other anaerobic bacteria, such as *Clostridium acetobutylicum* (Lee *et al.* 1985), *Clostridium thermocellum* (Wiegel *et al.* 1985) and *Thermoanaerobacter ethanolicus* (Wiegel *et al.* 1983), were also found to produce xylanases.

The production of extracellular xylanases was also found in several yeast genera and the xylanases from *Cryptococcus* were studied extensively (Biely *et al.* 1980; Yasui *et al.* 1984). Xylanases from *Aureobasidium* (Leathers *et al.* 1986) and *Trichosporon* (Stüttgen and Sahm 1982) were also reported.

Practically all cellulase-producing microorganisms also produce xylanases and vice versa. The information found in the literature varies as to whether or not xylanolytic and cellulolytic systems are under separate or common regulatory control. In *Polyporus adustus*, a white-rot fungus, it was found that cellulases, mannanases and xylanases are under the control of a single common regulatory gene (Eriksson and Goodell 1974). It also appears that these enzymes are induced by cellulose and glucomannan. In *T. reesei*, on the other hand, the synthesis of cellulases and

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xylanases is under separate control (Hrmonvá *et al.* 1986). Studies on the regulation of xylanases and cellulases in fungi are often complicated by a simultaneous production of these enzymes and sometimes also by substrate cross-specificity of cellulases and xylanases. Several fungal species produce specific xylanases with little or no cellulases when grown on xylan only, but cellulases are produced together with the xylanases when they are grown on cellulose (Gong *et al.* 1981). Depending on the type of application, preparations consisting of a combination of cellulases and xylanases are used for enzyme preparations with minimal cellulase activity. In order to be commercially feasible, the extracellular levels of these enzymes must be enhanced by either isolating mutant strains with improved productivity or by cloning and increasing the expression of specific genes to increase xylanase production (Berka *et al.* 1992).

Table 2. Characteristics of extracellular bacterial endoxylanases (Poutanen 1988).

Microbial origin	MW (kDa)	pI	pH-optimum	Substrate	Main end products	Remarks
Bacillus circulans	85	4.5	5.5-7	Larchwood xylan	$X - X_4$	
	15	9.1	5.5-7		$X_2 - X_4$	
Bacillus coagulans	22	10	6.0			
Bacillus pumilus	24	nd*	6.5	Larchwood xylan long chains preferred	$X_2 - X_5$	
Bacillus spp.	nd	nd	6 - 7		$X_3 - X_5$	
Streptomyces lividans	43	5.2	6.0	Soluble oat spelts	Mainly X_2	
Streptomyces sp. E-86	40.5	7.3	5.5-6.2	Corn-cob arabino-xylan	X, X_2 xylan	Arabinose not xylan
Streptomyces sp. No3137	50	7.1	5.5-6.5			
	25	10.14	5 – 6	Xylan>X_4>X_3	X, X_2	
	25	10.26	5 – 6			
Streptomyces sp. KT-23	43	6.9	5.5		Mainly X_2 little X	
Costridium acetobutylicum	65	4.45	5.0	Larchwood xylan	$X_5 - X_2$	High activity toward CMC
Costridium acetobutylicum	29	8.50	5.5-6	Larchwood xylan	X_3, X_2	No activity toward CMC

** not determined*

The fungal and bacterial endoxylanases are, in general, almost exclusively single subunit proteins with Mr values ranging from 8.5 to 85 kDa and with pI values ranging from 4.0 to 10.3 (Eriksson *et al.* 1990b). Most xylanases are glycosylated and have acidic pH optima (Coughlan *et al.* 1993), but some xylanases, such as those from an

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alkalophilic thermophilic *Bacillus* sp., are optimally active at alkaline pH values. Hazlewood and Gilbert (1993) did sequence alignments and comparisons of likely secondary structures by means of hydrophobic cluster analysis, which indicated that the catalytic domains of xylanases are conserved between different species, as well as between bacteria and fungi.

2.4.3 PECTINS

Pectins are a major component of the plant cell wall and comprise one of the two major coextensive networks in which cellulose microfibrils are embedded (Carpita and Gibeaut 1993). The term pectic substances is a group designation for those complex colloidal polysaccarides that are rich in α-D-(1,4)-linked chains of galactopyranosiduronic acid residues. Restricted hydrolysis of pectic substances can give rise to pectic acid, pectinic acid and pectin, depending on the degree of esterification and the extent of association with neutral sugar residues (Chesson 1980). Pectic substances are structural heteropolysaccharides and the major constituents of the middle lamellae and primary cell walls of higher plants. They are largely responsible for the integrity and coherence of plant tissue (Rombouts and Pilnik 1978). Pectic polymers have molecular weights ranging between 30 000 and 300 000 (Rombouts and Pilnik 1978). This uniformity does not exist in the pectin or pectate molecule.

2.4.3.1 Occurrence and structure

Most pectin polymers consist of smooth homogalacturonan regions and ramified hairy regions (Van der Vlugt-Bergmans *et al.* 2000). The smooth regions consist of a linear homogalacturonan backbone. The hairy regions, as identified in apples (Schols *et al.* 1996), consist of three different subunits. Subunit I is a xylogalacturonan (xga) (a galacturonan backbone hairily substituted with xylose), subunit II is a short section of a rhamnogalacturonan backbone that has many relatively long arabinan, galactan, and/or arabinogalactan side chains (the hairs), and subunit III is rhamnogalacturonan composed of alternating rhamnose and galacturonic acid residues. Some of the rhamnose residues are substituted with single galactose residues. It is thought that subunit III connects the other two subunits. Isolated hairy regions are referred to as modified hairy regions, since the isolation procedure may alter the sugar composition and degree of methylation of the regions (Schols *et al.* 1990).

The basic linear skeleton is composed of the same repeating building unit, i.e. partially methylated α-D-(1,4)-linked galactopyranosiduronic acid residues (Chesson 1980) Blocks of L-rhamnopyranosyl residues are α-(1,2)-linked to the galacturonan chain at irregular intervals. The fact that there are usually few rhamnose residues present means that long chains of galacturonan are linked together by rhamnose-rich blocks (Pilnik and Voragen 1970). Galacturonosyl residues can be esterified with methanol and/or O-acetylated at C_2 or C_3 (McCready and McComb 1954). The

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neutral sugars, D-galactopyranose and L-arabinofuranose, occur as highly branched arabinogalactans or predominantly linear chains of β-D-(1,4)-linked galactopyranosyl residues, which are associated with some rhamnose-rich regions of the galacturonan chain (Chesson 1980) and linked covalently through a terminal galactopyranosyl residue to the C_4 of rhamnose (Keegstra *et al.* 1973). The proportion and chemical structure of pectic substances may vary considerably, depending on the source, portion and age of the plant material from which it is isolated.

Pectins are generally soluble in water, formamide, dimethyl sulphoxide, dimethylformamide and warm glycerol. In water, they form viscous solutions, depending on the molecular weight and degree of esterification, pH and electrolyte concentration (Deuel and Stutz 1958). The pH of pectic solutions, which is between 2.8 and 3.4, is determined as a function of the degree of esterification. Pectic acids and pectinic acids, which have very low degrees of esterification, are usually only soluble after partial neutralisation (Pilnik and Voragen 1970).

During the processing and storage of fruit products, several enzymatic and chemical changes occur in the pectin content and structure. These involve the degree of esterification, molecular weight, neutral sugar components and acetylation.

2.4.3.2 Hydrolysis

Enzymes are usually characterised on the basis of their action and complex macromolecules, such as the pectic substances, would seem to offer a number of possibilities for enzyme action. Pectic enzymes can be divided into two main groups according to the site attacked: those responsible for saponification of esterified regions of the chain, and the depolymerases responsible for chain cleavage (Collmer and Keen 1986; Kotoujansky 1987). Pectinases such as exo- and endo-polygalacturonases, pectate lyase, pectin methylesterase and β-galactosidase have been proposed to have the capacity to reduce the apparent molecular size of pectic polymers by cleaving neutral side chain residues (De Veau *et al.* 1993; Kristen *et al.* 1998).

Polygalacturonases are the most commonly encountered and widespread of the pectic enzymes and are produced by many fungi, some bacteria and yeasts, as well as higher plants (Rombouts and Pilnik 1978). Yeast polygalacturonase is produced constitutively and cannot catalyse the complete breakdown of pectic acid to galacturonic acid. Polygalacturonases are responsible for the hydrolytic cleavage of the α-D-(1,4)-glycosidic linkages adjacent to a free carboxyl group. Endo-polygalacturonases split the pectic chain at random, resulting in a small increase in reducing end groups, accompanied by a strong reduction in the viscosity of the substrate solution. Exo-polygalacturonases split off monomers and dimers from the non-reducing end of the galacturonan chain, implying a slow decrease in viscosity (Chesson 1980).

Pectin esterases (pectin pectyl hydrolase or pectin methyl esterase) show high specificity for the esterified regions of the galacturonan structure, splitting the methyl

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ester group of polygalacturonic acids (Whitaker 1972). The hydrolysis of these methyl ester groups is thought to proceed in a linear fashion along the galacturonan chain, requiring at least one free carboxyl adjacent to the methyl group under attack (Solms and Deuel 1955).

Initiation of attack occurs at the reducing end of highly esterified pectinic acid, transforming pectin into low methoxyl pectin, pectic acid and methanol (Kotoujansky 1987). When less highly methylated substrates are available, initiation occurs at a number of separate locations (Whitaker 1972). Enzymes from higher plants and bacterial origin exhibit pH optima within the pH range of 7 – 8.5, whereas those produced by fungi fall in the pH range of 4 – 6 (Chesson 1980). The synthesis of pectin esterase is inducible or constitutive (Nasuno and Starr 1967).

The β-eliminative attack of the lyases on the galacturonan chain results in the formation of a double bond between C_4 and C_5 in the terminal residue at the non-reducing end, generating an oligomer with a 4,5-unsaturated galacturonosyl at that end (Kotoujansky 1987). Different lyases can be distinguished on the basis of their preference for highly esterified pectinic acid (pectin lyase) or pectic acid (pectate lyase) and on the degree of randomness in the eliminative depolymerisation and behaviour towards oligomeric substrates (Pilnik and Rombouts 1979).

Pectate lyases are produced predominantly by bacteria, as well as by a few fungi (Fogarty and Kelly 1983). Endopectate lyases are characterised by their alkaline pH optima of pH 8 – 9.6 and their absolute requirement for divalent cations, notably calcium (Pilnik and Rombouts 1979). Enzyme activity is suppressed by chelating agents such as EDTA, but reinstated by the addition of calcium ions (Moran *et al.* 1968; Garibaldi and Bateman 1971; Chesson and Codner 1978). Pectates and low-methoxyl pectins are the best substrates for endopectate lyases.

Exopectate lyase is specific for the penultimate bond at the reducing end of the galacturonan chain, liberating unsaturated digalactosiduronate as the sole end product (Chesson 1980).

2.4.3.3 Pectolytic microorganisms

Besides occurring naturally in plant material, pectic enzymes are also produced by bacteria, fungi, yeasts, insects, nematodes and protozoa.

A wide variety of bacteria are prolific producers of pectic enzymes. The most important genera are *Bacillus, Clostridium, Erwinia, Pseudomonas* and *Xanthomonas* (Rombouts and Pilnik 1980). Certain other genera also produce detectable quantities of pectate lyase and polygalacturonase activies, but these enzymes are not secreted rapidly enough into the culture medium, nor are the levels of enzymes produced as high as those produced by the soft-rot *Erwinia* spp. (Starr *et al.* 1977).

All known pectic enzymes can be found either simultaneously or in specific combinations within the genus *Erwinia*. Members of the soft-rot-causing section of the genus, i.e. *E. carotovora, E. chrysanthemi* and *E. aroideae*, produce large amounts of pectic enzymes, notably endopectate lyase and endo-polygalacturonase.

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Endo-polygalacturonase is generally not present in pectolytic bacteria, but Nasuno and Starr (1966a, b) purified an endo-polygalacturonase from *Pseudomonas marginalis*, as well as from *E. carotovora*.

Polygalacturonase from *E. carotovora* has a molecular weight of 42 000 or 45 000 (Hinton *et al.* 1990; Lei *et al.* 1992) and activity between pH 4.0 and 6.5, with a pH optimum of 5.5 (Willis *et al.* 1987; Hinton *et al.* 1990; Saarilahti *et al.* 1990; Lei *et al.* 1992). It has a temperature optimum in the range of 35 – 45°C and does not require Ca^{2+} for activity. The polygalacturonase produced by some species has a very high alkaline pi (Collmer 1986).

The regulation of expression of the polygalacturonase (*peh)* genes is not nearly as thoroughly studied as the regulatory system of the pectate lyase (*pel)* genes. Since the expression of *peh* was constitutive in *E. carotovora* strain SCRI193 (Chatterjee *et al.* 1981), the absence of a Catabolite Activator Protein (CAP) binding site in *peh193* was expected (Hinton *et al.* 1990). It therefore can be concluded that polygalacturonase (*PG*) is uninduced by pectate and unaffected by catabolite repression. However, the presence of a putative kdgR-binding site upstream of the *peh* coding sequence might suggest that PG synthesis is inducible by pectate under certain conditions (Hinton *et al.* 1990).

There are three major differences between *Erwinias* and other pectolytic bacteria with regard to their regulation of pectate lyase synthesis: (i) *Erwinias* produce higher levels of pectate lyase (basal or induced); (ii) pectate lyase synthesis is rapidly induced by exogenous pectic substances; and (iii) pectate lyase is efficiently secreted into the medium (Chatterjee *et al.* 1979; Collmer *et al.* 1982). However, within the genus *Erwinia*, there is substantial variation in the levels of pectate lyase synthesis and export (Chatterjee *et al.* 1979).

Induction of pectate lyase (*PL*) synthesis in culture has been studied extensively in soft-rot *Erwinias* (Collmer *et al.* 1982; Collmer *et al.* 1985; Collmer and Keen 1986). The induction of the *pel* genes in *E. chrysanthemi* is generally caused by polygalacturonate and its metabolic intermediates (Garibaldi and Bateman 1971; Pupillo *et al.* 1976; Tsuyumu 1977), with the true inducers being 2-keto-3-deoxy-gluconate (KDG), 2,5-diketo-3-deoxygluconate (DKII) and 5-keto-4-deoxyuronate (DKI) (Kotoujansky 1987) and some plant extracts (Tomizawa *et al.* 1970).

The *pel* regulatory regions are AT rich in contrast to the GC-rich coding region. They also have a CAP-binding sequence. Results obtained indicated that the pelE promoter is not just regulated by catabolite repression and substrate induction, but that it also is subjected to growth phase inhibition, implying that significant expression only occurs relatively late in the growth phase (Chatterjee *et al.* 1981; Hugouvieux-Cotte-Pattat *et al.* 1986; Gold *et al.* 1992). PL synthesis is also sensitive to temperature (Perombelon 1990), the presence of oxygen (Ji *et al.* 1987), ion concentration (Sauvage *et al.* 1991), or the nitrogen source (Hugouvieux-Cotte-Pattat and Robert-Baudouy 1992).

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The *pel* genes in *E. chrysanthemi* are regulated at different levels, i.e. catabolite repression, negative control by the *kdg*R, *gpi*R and *cri* genes and positive control by the *pecA* gene. PL synthesis is subject to glucose (catabolite) repression (Hugouvieux-Cotte-Pattat *et al.* 1986), as well as "self-catabolite repression" by high concentrations of the true inducer, 4,5-unsaturated digalacturonate (Tsuyumu 1979), but is reversed by exogenous addition of cAMP in *E. carotovora* (Mount *et al.* 1979).

Filamentous fungi are renowned for the secretion of polygalacturonic acid-degrading enzymes. In *Aspergillus niger*, these enzymes are inducible by pectic substances (Aguilar and Huitron 1987), whereas in the yeasts *Kluyveromyces fragilis* and *Rhodotorula*, they are produced constitutively (Bell and Etchells 1956; Rombouts and Pilnik 1972). Commercial pectinases are all derived from *Aspergillus* spp., mainly *A. niger, A oryzae* and *A. wentii.*

Few yeasts are known to be pectolytic. In 1929, Pittman and Cruess concluded that *S. cerevisiae, S. ellipsoideus* and *Mycoderma* spp. had no noticeable effect on pectin. Luh and Phaff (1951) identified significant pectolytic activity (endo-polygalacturonase) only in *Kluyveromyces fragilis* and *Candida pseudotropicalis.* The two above mentioned yeasts produce multiple forms of polygalacturonase that differ in molecular weight and pi (Sakai *et al.* 1984). Other yeast strains found to produce weaker pectolytic activities have since been renamed to the two above strains, or are biochemically very similar to these strains. This emphasises the fact that pectolytic activity is a relatively rare property amongst yeasts and is restricted to only a few species. Several polygalacturonases are produced by the genera *Candida, Debaryomyces, Endomycopsis, Geotrichum, Hansenula, Pichia, Rhodotorula, Trichosporum* and *Zygopichia* (Call and Emais 1978; Sanchez *et al.* 1984; Ravelomanana *et al.* 1986). PG activity has been described in *S. fragilis, S. cerevisiae* and *S. oviformis* (Bell and Etchells 1956; Kotomina and Pisarnitskii 1974), although Luh and Phaff (1954) considered *S. cerevisiae* to be non-pectolytic.

2.5 INDUSTRIAL POLYSACCHARASES (ENZYMES)

The cell walls of plant material are composed of cellulose fibres to which strands of hemicellulose are attached. The fibres are embedded in a matrix of pectic substances linked to structural proteins. The content of cell wall polysaccharides in different plant material varies in composition and quantity, and therefore the composition of enzyme complexes used in industrial applications must be optimised according to the kind of material to be treated. Conventional enzyme preparations capable of breaking down plant cell walls usually contain different activities, such as pectinase, hemicellulase and cellulase activities. However, these products are unable to completely degrade the heteropolysaccharide structure of pectic substances. The latest generation of pectinases has a wider range of activities, enabling them to degrade the so-called hairy parts of the molecular structure of pectin much better than conventional pectinases.

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The enzymes of grapes consist mainly of pectin esterase and polygalacturonase, but they are often insufficient to break down pectic substances and have no effect on the complex polysaccharides found in the cell wall. Since the introduction of pectinases into the wine industry in the 1970s, the development of specific cell wall-degrading enzymes offers winemakers the opportunity to improve wine quality and increase production flexibility. Enzyme preparations are used in a number of applications: for maceration (mash treatment), to release colour and aroma compounds, as well as juice; for clarification (must treatment), to speed up settling and for wine maturation – aroma liberation, wine stabilisation and filtration.

Most commercial preparations of pectic enzymes are obtained from fungal sources (Alkorta *et al.* 1994). It is speculated that all producer strains of commercial pectic enzyme preparations are *Aspergillus* species. However it is difficult to obtain reliable information from the industry about this, for obvious economical reasons (Sakai *et al.* 1993). In 1980, Rombouts and Pilnik found that pectic enzymes account for one quarter of the world's food enzyme production.

Pectin esterases (de-esterifying enzymes) are produced by fungi, bacteria, yeasts and plants and are present in practically all commercial enzyme preparations (Alkorta *et al.* 1994). These enzymes are involved in the changes in the pectic substances of fruits and vegetables during ripening, storing and processing by the de-estrification of the methoxyl group of pectin-forming pectic acid (Sakai *et al.* 1993).

In the fruit juice industry pectic enzymes are used for the clarification of juices (Rombouts and Pilnik 1980) through the reduction of viscosity of the juice as well as the flocculation of the micelles that allows particles to separate by sedimentation or filtration. Another application is the role pectic enzymes play in fruit juice extraction. Because pectic enzymes degrade the pectins in the fruit, it facilitates pressing and ensures high yields (Sakai *et al.* 1993). Other applications of pectic enzymes in the fruit juice industry are in the maceration and solubilisation of fruit tissues to retain integrity of the cell wall in the liquefaciton of tissues, and also the selective treatment of citrus concentrates with endopolygalacturonase to provide limited hydrolysis of pectins to reduce viscosity (Whitaker 1990).

In the wine industry, pectic enzymes have been used as far back as 1947 to increase juice yields, filtration rates, rates of settling and the clarity of wines. The reason pectic enzyme preparations are used for the above reasons is because of the degradation of structural polysaccharides, which interferes with juice extraction, the release of colour and flavour compounds trapped in the grape skins, as well as with the clarification and filtration of wine (Van Rensburg and Pretorius 2000).

Commercially, the activity of pectinase preparations is usually reported as apple juice depectinising activity (AJDU), based on the reciprocal time required to clarify frech apple juice at pH 3.5 and 45°C, and polygalacturonase activity (PGU), based on the reduction in viscosity of polygalacuronate substrate at pH 4.2 and 30°C (Brown and Ough 1981).

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Results obtained after tests done on musts and wines showed an increase in total juice yield, clarity of the wine, filterablility, methanol production, wine quality, browning capacity and the amount of solids that settled in wines treated with the pectinase preparations (Brown and Ough 1981). The addition of pectinases lowers the viscosity of grape juice and causes cloud particles to aggregate to larger units, which sediment out, leading to better settling. It can also improve juice and colour yields if added to a pulp. The pectinases degrade the pectins, leading to thin free-run juice and pulp that is more easily pressed (Van Rensburg and Pretorius 2000). The effects of pectinases in conjunction with cellulases and hemicellulases will be discussed later.

In 1975, Ough *et al.* found that the treatment of red grapes with pectic enzymes might slow down the fermentation rate, but that it sped up pigment extraction and, to a lesser extent, phenolic extraction. The only significant effect on the wine quality therefore was an increase in the intensity of wine colour. The use of pectic enzymes on red grapes led to shorter skin contact, because enzyme-treated wine with a skin contact time of 48 hours was as dark as the control sample with a 72 hour contact time. The result is wines of equal colour but with a lower tannin content because of the shorter time of skin contact. In contradiction to Ough, Wightman *et al.* found in 1997 that some pectinase preparations can reduce the colour of red wine through pigment modification and subsequent degradation.

The commercial β-glucanase preparations authorised for use in winemaking are produced by species of *Trichoderma* (e.g. *T. harzianum*). Commercialised glucanases are normally active between 15-50°C at a pH of 3 to 4. Alcohol concentrations of up to 14% do not influence the activity of these preparations (Van Rensburg and Pretorius 2000).

Glucanases are used in the wine industry for clarification and filtration problems. An industrial glucanase preparation containing an exo-β-1,3-glucanase, an endo-β-1,3-glucanase, an exo-β-1,6-glucanase and unspecific beta glucosidase activity is used to make wine, especially *Botrytis* wines, easier to filter. This preparation permits substantially more wine to be filtered during a certain time without blocking the filters as a result of the degradation of the glucans in the wine. Another advantage of this preparation is that it does not influence the chemical composition of the wine and therefore does not change the wine organoleptically (Villettaz *et al.* 1984). The only change was a higher residual sugar level due to the enzymatic hydrolysis of the *Botrytis* glucan to glucose (about 50 mg/L).

Another industrial enzyme preparation containing a mixture of pectinases (22 000 APU), cellulases (300 carboxymethyl cellulose units) and hemicellulases (405 locust bean gum units) are used to increase the amount of total and free-run juice. This is due to the degradation of the material of the cell wall and the middle lamella by these enzymes (Sims *et al.* 1988). This preparation also results in better settling of the juice. The exact mechanism behind this is not yet totally known, but it is speculated that the cellulases and hemicellulases degrade the cell wall and middle lamella

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material, resulting in a more complete release of the pectic substances and therefore better exposure of the pectic material to the pectinolytic enzymes in the preparation (Robertson *et al.* 1980).

2.6 EXPRESSION OF POLYSACCHARASE GENES IN YEASTS

S. cerevisiae can be genetically manipulated in many ways. Some techniques alter limited regions of the genome, while others are used to recombine or rearrange the entire genome. Techniques having the greatest potential in the genetic programming of wine yeast strains are cloned selection of variants, mutation and selection, hybridisation, rare mating, spheroplast fusion, as well as gene cloning and transformation. The combined use of classical genetic techniques and recombinant DNA methods has dramatically increased the genetic diversity that can be introduced into yeast cells (Pretorius 2000).

Due to technical difficulties and the fact that the requirements of the wine industry have not been defined in genetic terms, little genetic research has been undertaken on *S. cerevisiae* by this industry when compared to that done by the baking and brewing industries. Historically, the development of wine yeast strains has almost exclusively relied on strain selection. Changes in winemaking technology to improve the reliability of fermentation, wine quality and the economics of production have placed new demands on the performance of selected wine yeast strains.

Our laboratory focuses on a number of targets for yeast strain development, which are listed in Table 3. The efficiency of fermentation would be markedly improved by better sugar utilisation, increased tolerance to ethanol, resistance to zymocins and heavy metals, reduced formation of foam, induced flocculation at the end of fermentation and the production of extracellular enzymes. Moreover, wine yeast strains with improved nitrogen and sulphur metabolism have the potential to reduce or eliminate the need for ammonium salts and copper salts for the removal of H_2S. Yeast strains secreting pectinases, glucanases, xylanases and proteases are being proposed to improve wine clarity and filtration efficiency. The secretion of glucanases and glucosidases may also enhance wine flavour by the hydrolysis of flavour precursor glycosides. The overexpression of the yeast's own alcohol acetyltransferase has been shown to be the first step towards enhanced ester production, thereby adjusting the aroma profile of wine considerably. Furthermore, genetically modified wine yeasts have the potential to correct wine acidity by the metabolism of malic acid. Rapid autolytic characteristics of strains used for the production of sparkling wines can affect the time and cost of wine production (Pretorius 2000).

We are also investigating the possibility of developing wine yeast strains with antimicrobial activity, which have been suggested as a partial replacement for the bio-inhibitory properties of sulphur dioxide. External preservatives can be significantly reduced if wine yeast strains secrete natural antimicrobial peptides (such

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as bacteriocins) during fermentation, thereby playing an autosterilising role. To this end, the pediocin and leucocin genes from lactic acid bacteria and the lysozyme gene from chicken egg white were successfully expressed in *S. cerevisiae*. Preliminary results indicate that it is indeed possible to develop bactericidal wine yeast strains that could be useful in the production of wine with reduced levels of potentially harmful chemical preservatives.

Table 3. Targets for, and aims of, strain development (Pretorius 2000).

Targets and aims
Improved quality control and strain handling
Strain maintenance
Molecular marking
Improvement of fermentation performance
Improved viability and vitality of active dried wine yeast starter cultures
Efficient sugar utilisation
Improved nitrogen assimilation
Improved ethanol tolerance
Increased tolerance to antimicrobial compounds
Reduced foam formation
Improvement of processing efficiency
Improved protein and polysaccharide clarification
Controlled cell sedimentation and flocculation
Controlled cell flotation and flor formation
Improvement of wine flavour and other sensory qualities
Enhanced liberation of grape terpenoids
Enhanced production of desirable volatile esters
Optimised fusel oil production
Enhanced glycerol production
Bio-adjustment of wine acidity
Elimination of phenolic off-flavours
Reduced sulphite and sulphide production
Improvement of wine wholesomeness
Resveratrol production
Reduced formation of ethyl carbamate
Improved biological control of wine spoilage microorganisms
Wine yeasts producing antimicrobial enzymes
Wine yeasts producing antimicrobial peptides
Generate new knowledge about the molecular functioning within yeast cells

Eventually, we will be able to introduce the different cassettes needed for the degradation of different substrates in grapes, musts and wines into *S. cerevisiae*. This will make the clarification and filterability of wines a lot easier and quicker, without the addition of undefined cocktails of industrial enzyme preparations. Even

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though the public have a very negative perception of genetically engineered microorganisms, it now is inevitable that carefully constructed recombinant strains of *S. cerevisiae* will play a very important role in industrial processes, especially in the wine industry, in the future.

2.6.1 GLUCANOLYTIC, XYLANOLYTIC AND PECTOLYTIC ACTIVITY IN *S. CEREVISIAE*

The endogenous pectinase, glucanase and xylanase activities of grapes and yeasts are often neither efficient nor sufficient under winemaking conditions to prevent polysaccharide hazes and filter stoppages (Canal-Llauberes 1993). Through the years, industrial enzyme preparations have been developed to supplement these polysaccharide-degrading activities (Colagrande *et al.* 1994). Because commercial enzyme preparations have become expensive, attention has shifted to the native pectinases and glucanases of *S. cerevisiae*. Certain strains of *S. cerevisiae* were reported to produce pectin esterase, polygalacturonase and pectin lyase (Gainvors *et al.* 1994), while all strains of *S. cerevisiae* show some form of glucanase activity (Pretorius 1997). However it was found that these endogenous pectinolytic and glucanolytic activities of *S. cerevisiae* are not sufficient to avoid clarification and filtration problems. A reason for this could be that these enzymes are inactivated by the winemaking conditions.

S. cerevisiae produces several glucanases that differ in structural properties, substrate specificities and appearance through the cell cycle (Pretorius 1997). A property of all these glucanases is that they only have β-1,3-activity and therefore are unable to degrade the β-glucans found in grape skins, as well as the β-glucans produced by *Botrytis cinerea*, which are the cause of many filtration problems. A few of these β-1,3-glucanases are *EXG1 (BGL1), EXG2, BGL2* and *SSG1(SPR1)*, which were cloned and characterised by Pretorius (1997).

S. cerevisiae was also found to be unable to utilise xylose, even if engineered to degrade xylan to xylose (La Grange 1995), because it does not have any xylanolytic activity.

Where pectolytic activity is concerned, it was first assumed that *S. cerevisiae* does not have any pectolytic activity (Bell and Etchells 1956; Luh and Phaff 1951), but later information indicated that some strains of *S. cerevisiae* do possess the ability to degrade pectin (McKay 1990; Blanco *et al.* 1994; Gainvors *et al.* 1994). In 1994, Blanco *et al.* reported that at least 75% of oenological strains, isolated in spontaneous fermentations, that were tested showed pectolytic activity. The SCPP strain of *S. cerevisiae*, however, is the only strain that is known to be capable of expressing pectin lyase and pectin esterase and that therefore is capable of degrading pectins with varying degrees of esterification (Gainvors and Belarbi 1995).

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2.6.2 EXPRESSION OF BACTERIAL GLUCANASE GENES IN *S. CEREVESIAE*

The endo-β-1,4-glucanase-encoding gene (*end1*) from *Butyrovibrio fibrosolvens* was expressed in *S. cerevisiae* after its insertion between a yeast alcohol dehydrogenase promoter, $ADH1_P$, and a yeast tryptophan synthase gene terminator, $TRP5_T$. This led to the formation of the $ADH1_P$-$MF\alpha1_S$-*end1*-$TRP5_T$ cassette. This cassette was integrated in a laboratory yeast of *S. cerevisiae*, together with other gene cassettes (Van Rensburg *et al.* 1994). The endo-β-1,3-1,4-glucanase gene (*beg1*) from *Bacillus subtilis* was the first heterologous glucanase gene to be introduced in yeast and it was expressed under the control of its own promoter and signal sequences (Hinchliffe 1985). Little activity was observed, however, and high levels of β-glucanase were observed only after it was expressed under the control of the $ADH2_p$-$MF\alpha1_s$ expression-secretion cassette (Van Rensburg *et al.* 1996). An endo-β-D-1,4-glucanase (*cenA*) from the cellulolytic bacterium *Cellulomonas fimi* was also cloned and expressed in *S. cerevisiae* (Skipper *et al.* 1985). Secretion of active endo-β-1,4-glucanase by the transformed yeast cells was increased when the leader sequence of a secreted yeast protein, the K_1 killer toxin, was inserted in-frame immediately upstream of the bacterial cellulase sequence. In 1993, Nakajima *et al.* fused a DNA segment encoding a signal peptide from yeast invertase (*SUC2*) in-frame to the *Bacillus circulans* β-1,3-glucanase gene (*bglH*) and it was expressed in *S. cerevisiae* under the control of the yeast galactokinase gene (*GAL1*) promoter. However glucanase inhibited the growth of the yeast cell by eroding the yeast cell wall. Other glucanase genes that have been effectively expressed in *S. cerevisiae* are the *Ruminococcus flavefaciens* cellodextrinase gene (*CEL1*), the *Phanerochaete chrysosporium* cellobiohydrolase gene (*CBH1*) and the *Saccharomycopsis fibuligera* cellobiase gene (*BGL1*) (Van Rensburg *et al.* 1994, 1995, 1996, 1997, 1998). After the transformation of *S. cerevisiae* with these gene cassettes, the transformants were able to degrade glucans efficiently and therefore play a positive role in the clarification and filtration problems of wines, especially *Botrytis* wines.

2.6.3 EXPRESSION OF FUNGAL XYLANASE GENES IN *S. CEREVISIAE*

The endo-β-1,4-xylanase-encoding gene (*xyn4*) was previously cloned from *Aspergillus niger* and characterised. *Xyn4* was inserted between the *ADH1* promoter and *TRP5* terminator. The $ADH1_P$-$MF\alpha1_S$-*xyn4*-$TRP5_T$ cassette was formed and integrated into a laboratory strain of *S. cerevisiae* (Luttig *et al.* 1997). Other fungal xylanase genes that were expressed in *S. cerevisiae* are the endo-β-xylanase gene from *Aspergillus kawachii* (*XYN1*), *Aspergillus* (*XYN5*) and *Trichoderma reesei* (*XYN2*). Other genes expressed were the *Bacillus pumilus* xylosidase (*XLO1*) and the *xlnA* and *xlnB* gene cassettes from *Aspergillus nidulans* (Crous 1995; La Grange *et al.* 1996; La Grange *et al.* 1997; Luttig *et al.* 1997; Moes *et al.* 1996; Pérez-González *et al.* 1996). The efficiency of these xylanolytic yeast strains has not yet been tested under winemaking conditions.

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2.6.4 EXPRESSION OF BACTERIAL PECTINASE GENES IN *S. CEREVISIAE*

The first heterologous pectinase genes expressed in *S. cerevisiae* were derived from the soft-rot-causing plant pathologenic bacteria, *Erwinia carotovora*. The pectate lyase-encoding gene (*pelE*) from *E. chrysanthemi* and the polygalacturonase-encoding gene (*peh1*) from *E. carotovora* were inserted into different expression-secretion cassettes and different combinations of yeast and bacterial promoters, secretions signal sequences and gene terminators, and expressed in *S. cerevisiae* (Laing and Pretorius 1992; 1993a). The $ADH2_p$-$MF\alpha1_s$ expression-secretion cassette proved to be the most efficient control cassette for the expression of these two genes, as well as for the secretion of the enzymes in *S. cerevisiae.* This led to the $ADH1_P$-$MF\alpha1_S$-$pelE$-$TRP5_T$ (*PEL5*) and $ADH1_P$-$MF\alpha1_S$-$peh1$-$TRP5_T$ (*PEH1*) cassettes. The two pectinase cassettes, $ADH1_P$-$MF\alpha1_S$-$pelE$-$TRP5_T$ and $ADH1_P$-$MF\alpha1_S$-$peh1$-$TRP5_T$, were expressed in a laboratory strain of *S. cerevisiae* and two commercial wine yeast strains (Laing and Pretorius 1993b). Co-expression of *PELE* and *PEH1* in *S. cerevisiae* synergistically enhanced pectate degradation.

In 1994, Templeton *et al.* expressed the pectin lyase gene (*pnlA*) of *Glomeralla cingulata* in yeast under the control of the *GAL10* promoter. Expression was very low, however, and only after an alteration to the sequence surrounding the start codon, CACC<u>*AUG*</u> to CAAA<u>*AUG*</u>, was the expression improved to a six- to 10-fold increase in pectolytic activity in *S. cerevisiae*.

It is hoped that a pectolytic wine yeast strain would have an advantage toward the clarification of must and wine without the addition of expensive commercial enzyme preparations. These strains might also be able to enhance the colour and flavour of wine.

2.7 CONCLUSIONS

Daunting challenges will have to be overcome during the next few years. But, given the availability of the complete genome sequence as well as methods for the analysis of the transcriptome, proteome and metabolome of *S. cerevisiae*, there is no doubt of new and innovative developments that will be of great benefit to both the winemaker and the wine consumer.

With their broad experience in yeast-based fermentations, winemakers are well placed to explore the rich diversity of yeasts associated with winemaking (whose oenological potential is still largely untapped) and the new opportunities offered by an integrated and holistic approach towards "cell engineering", "metabolic engineering" and "genetic engineering". The tailoring of wine yeast strains by expressing novel designer genes will help the wine industry meet the technical and consumer challenges of the third millennium (Pretorius 2000).

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CHAPTER 3

RESEARCH RESULTS

Screening for the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts

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Screening for the Production of Extracellular Hydrolytic Enzymes by non-*Saccharomyces* Wine Yeasts

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Aims: The objective of this study was to investigate what types of enzymes are being produced by non-*Saccharomyces* yeasts isolated from grapes in South African vineyards and from clarified grape juice. These enzyme profiles could pave the way for attributing specific effects in wine to some of these enzymes produced by so-called wild yeasts associated with grape must.

Methods & Results: In this study, 237 yeast isolates, belonging to the genera *Kloeckera*, *Candida*, *Debaryomyces*, *Rhodotorula*, *Pichia*, *Zygosaccharomyces*, *Hanseniaspora* and *Kluyveromyces*, were screened for the production of extracellular pectinases, proteases, β-glucanases, lichenases, β-glucosidases, cellulases, xylanases, amylases and sulphite reductase activity. These yeasts, representing 21 species, were previously isolated from grapes and clarified grape juice. The production of all extracellular hydrolytic enzymes that were screened for was observed, except for β-glucosidase activity. The amount and range of enzymes produced varied among different isolates of the same species.

Conclusions: This study clearly revealed the potential of non-*Saccharomyces* wine yeasts to produce a wide range of useful extracellular enzymes during the initial phase of wine fermentation.

Significance and Impact of the Study: Enzymes produced by indigenous yeasts associated with grapes and juice might be harnessed to catalyse the desired biotransformations during wine fermentation.

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3.1 INTRODUCTION

The microflora of grapes are highly variable, with a predominance of the low ethanol-tolerant strains of *Hanseniaspora*, *Kloeckera* and various species of *Candida*. The more ethanol-tolerant *Saccharomyces cerevisiae* is present only at low numbers (Peynaud and Domercq 1959; Fleet *et al.* 1984; Heard and Fleet 1985; Lema *et al.* 1996). The influence that all of these yeasts will have on the flavour of wine depends on several factors, such as the method of grape harvest, transport, vinification factors such as temperature, the extent of juice clarification, the use of sulphur dioxide etc. The yeast cell concentration in freshly prepared must is typically 10^3 to 10^5 cfu/ml, but this may vary from near sterility to $>10^6$ yeast cfu/ml after processing. This highly variable content of yeast populations may contribute to the chemical and flavour changes that accompany fermentation. The early stages of most wine fermentations, whether they develop naturally or after inoculation with *S. cerevisiae*, are characterised by the significant growth of indigenous species of non-*Saccharomyces* yeast (Fleet 1992). Prominent among these are species of *Kloeckera*, *Cryptococcus*, *Hanseniaspora*, *Candida*, *Pichia* and *Hansenula* (Fleet *et al.* 1984). While the controlled growth of these indigenous species may, in some circumstances, enhance the chemical complexity and sensory quality of wines, there are other circumstances in which their contribution may result in spoilage (Fleet 1992). Several authors have shown that using different starter cultures and indigenous yeast produces wines with significant differences in chemical composition (for a review see Lambrechts and Pretorius 2000). Recently, three studies illustrated the impact that the growth of non-*Saccharomyces* yeast can have on the sensory character of wines (Egli *et al.* 1998; Henick-Kling *et al.* 1998; Soden *et al.* 2000). Some of these differences could be ascribed to the fact, shown by several researchers, that, in contrast to *Saccharomyces* species, the non-*Saccharomyces* yeasts produce and secrete several enzymes (esterases, glycosidases, lipases, β-glucosidases, proteases, cellulases etc.) into the periplasmic space and the medium, where they may interact with grape precursor compounds to produce aroma active compounds and thus play an important role in varietal aroma (Charoenchai *et al.* 1997).

Enzymes play a definitive role in the production of wine, which could be seen as the product of enzymatic transformation of the grape juice (for a review see Van Rensburg and Pretorius 2000). The enzyme activities do not only originate from the grape itself, but also from yeast and other microorganisms. The winemaker now reinforces and extends the action of these endogenous enzymes through the use of exogenous, industrial enzyme preparations (Fleet 1993). The production of extracellular hydrolytic enzymes by indigenous yeast could be significant and needs to be understood and managed to the benefit of wine production. Moreover, wine yeast could be potential sources for the commercial production of enzymes to be used in the process of winemaking (Charoenchai *et al.* 1997). *Saccharomyces*

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cerevisiae, the principal wine yeast, is not recognised as a significant producer of extracellular enzymes, although a few strains have recently been reported to degrade polygalacturonate (McKay 1990). There is little information on the production of extracellular enzymes by non-*Saccharomyces* wine yeast, although some strains of *Kloeckera apiculata* show extracellular protease activity (Lagace and Bisson 1990; Dizy and Bisson 2000). Various authors have reported glycosidase production by *S. cerevisiae* and the potential for these enzymes to enhance wine flavour (Delcroix *et al.* 1994). Glycosidase activity has been reported in strains of *Candida*, *Pichia* and *Hanseniaspora* (Vasserot *et al.* 1989). A number of sources have been tested for glycosidic activity that may be used under the limiting conditions offered by wine. These sources include plants, fungi, bacteria and yeasts. As these glycosidases are to be exploited to release potential aroma, they must satisfy a few prerequisites. These are specificity, pH optimum, and glucose and ethanol tolerance.

Due to the importance of yeast biodiversity to the wine industry, a comprehensive long-term research programme has been launched by several researchers at the Wine and Fermentation Technology Division of the ARC Infruitec-Nietvoorbij Research Institute and the Institute for Wine Biotechnology at the University of Stellenbosch (for a review see Pretorius *et al.* 1999). As part of this programme, the natural distribution of non-*Saccharomyces* strains was determined in the vineyards of the Western Cape in South Africa. To further characterise the isolated yeast strains, the aims of this paper were to determine the ability of these yeasts to produce extracellular pectinases, proteases, β-glucanases, β-glucosidases, cellulases, hemicellulases, cellobiases as well as enzymes involved in the degradation of starch. The ability of the indigenous wine yeast to produce H_2S was also investigated.

3.2 MATERIALS AND METHODS

3.2.1 YEAST STRAINS

The 237 wine yeast strains used in this study (Table 1) were obtained from the Wine and Fermentation Technology Division, ARC Infruitec-Nietvoorbij. The yeast strains are indigenous strains isolated from four wine production regions of the Western Cape, South Africa, i.e. Constantia (isolates C), Robertson (isolates M), Slanghoek (isolates O) and Stellenbosch (isolates R). The yeast strains are further subdivided into those isolated from vineyards (group 1) and those from clarified grape juice prior to fermentation (group 2).

3.2.2 MEDIA AND SCREENING PROCEDURES

All yeast isolates were grown on YPD plates (containing 1% yeast extract, 2% peptone, 2% glucose and 2% agar) and then replica plated to the media described below to test for extracellular enzyme acitivity.

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Table 1 Yeast isolates used in this study

Yeast	Constantia isolates	Robertson isolates	Slanghoek isolates	Stellenbosch isolates	Total isolates
Kloeckera apiculata	$26_V + 22_C$		$19_V + 12_C$	$18_V + 13_C$	**110**
Kloeckera apis		1_C			**1**
Candida stellata	$4_V + 4_C$	15_C		$9_C + 1_V$	**33**
Candida pulcherrima	2_C	28_V	$9_V + 14_C$	1_V	**54**
Candida colliculosa	2_C	1_C	1_C		**4**
Candida guilliermondii		3_V			**3**
Candida hellenica		3_C	1_C	$2_V + 4_C$	**10**
Candida sorbosa		6_C			**6**
Candida lambica		1_C			**1**
Candida albicans		1_C			**1**
Candida oleophila			2_V	$3_V + 1_C$	**6**
Candida valida			2_C	1_C	**3**
Candida pelliculosa				2_V	**2**
Debaryomyces hansenii		2_C			**2**
Rhodotorula glutinis				1_V	**1**
Rhodotorula mucilaginosa					**1**
Pichia fabrinosa				2_V	**2**
Pichia kluyveri				2_C	**2**
Zygosaccharomyces bailii					**1**
Hanseniaspora guilliermondii					**1**
Kluyveromyces thermotolerans					**1**
TOTAL					**237**

C denotes clarified grape juice and v denotes vineyard

3.2.2.1 Pectinase activity

Yeasts were screened for polygalacturonase production by the method described by McKay (1988), with some modifications. Yeasts were replica plated onto polygalacturonate agar medium containing 1.25% polygalacturonic acid (Sigma), 0.68% potassium phosphate (pH 3.5), 0.67% yeast nitrogen base (YNB, Difco), 1% glucose and 2% agar. The plates were incubated for five days at 30°C. The colonies were rinsed off the plates with distilled water before staining the plates with 0.1% Ruthenium Red. Colonies showing a purple halo were identified as positive.

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3.2.2.2 Protease activity

Extracellular protease production was determined by replica plating yeast colonies onto YPD plates containing 2% casein (BDH laboratories). The plates were incubated for five days at 30°C. A clear zone around the colony identified protease activity.

3.2.2.3 Glucanase activity

Production of β-glucanase activity was determined by replica plating the yeast onto YPD plates containing 0.2% barley β-glucan (Sigma) and YPD plates containing 0.2% lichenan (Sigma). The plates were incubated for five days at 30°C. Colonies were rinsed off the plates with distilled water before staining the plates with 0.03% Congo Red (Teather and Wood 1982). A clear zone around the colony identified glucanase activity.

3.2.2.4 β-Glucosidase activity

β-Glucosidase activity was determined by replica plating the yeast onto selective medium (SC) containing 0.67% yeast nitrogen base (YNB, Difco), 0.5% arbutin (ICN) and 2% agar. The pH of the media was adjusted to 5 before autoclaving. Two millilitres of a filter sterilised 1% ammonium ferric citrate solution was added to 100 ml of medium before pouring the plates. The plates were incubated for five days at 30°C. Colonies showing activity were identified by the discolouration of the media to a brown colour.

3.2.2.5 Cellulase activity

Cellulase production was determined by replica plating the yeast onto YPD plates containing 0.4% carboxymethylcellulose (CMC, Sigma) and YPGE plates (containing 1% yeast extract, 2% peptone, 3% glycerol and 2% ethanol) containing 0.4% CMC. The plates were incubated for 5 days at 30°C. The colonies were rinsed off the plates with distilled water before staining the plates with 0.03% Congo Red, followed by destaining with 1 M NaCl (Teather and Wood 1982).

3.2.2.6 Xylanase activity

Yeasts were screened for hemicellulase activity by replica plating onto SC plates containing 0.2% Remazol Brilliant Blue Xylan (RBB-Xylan, Sigma) and YPGE plates containing 0.2% RBB-xylan. The plates were incubated for five days at 30°C. Colonies showing activity were identified by a clear zone around the colony. Production of cellobiase was assessed on SC plates containing 2% cellobiose as the only carbon source. Growth on the plates indicated cellobiase activity.

3.2.2.7 Starch-degrading activity

Amylolytic activity was determined on SC plates containing 2% starch (Saarchem). After the plates were incubated for six days at 30°C, they were placed at 4°C for precipitation. A clear zone around the colony identified yeast containing amylolytic

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activity. Production of α-amylase was assessed by plating the yeast onto SC containing 40 phadebas (Pharmacia and Upjohn) pills per litre. The plates were incubated for six days at 30°C and yeast showing activity was identified by a clear zone around the colony.

3.2.2.8 Sulphite reductase

The H_2S-production potential of the yeasts was determined by plating the yeasts onto a solid grape juice indicator agar (GJIA) (Jiranek *et al.* 1995) with some modifications. The media contained per litre: 250 ml grape juice, 15 ml succinate (pH 5.1), 735 ml distilled water, 11 g bismuth citrate (Sigma) and 3% agar. The plates were incubated for six days at 30°C. A low H_2S-producing colony was identified by its white colour, whereas a high H_2S-producing colony had a black colour.

3.2.3 ENZYME ASSAYS

The amylolytic activity was determined by growing the yeast overnight in 5 ml of YPD broth at 30°C. The cultures were centrifuged (6 K for 2 min.) and the supernatant was used for the assay. Samples of culture supernatants (600 μl) were mixed with 200 μl of 0.5 M sodium-acetate buffer (pH 3.2) and 200 μl of the substrate (0.5% starch solution; Sigma) and incubated for 60 min at 30°C. The reaction was stopped by boiling the mixture for 2 min. After cooling on ice, the liberated glucose was determined with a Trinder assay kit, as recommended by the supplier (Sigma). The units were defined as mmol/L/g dry cells.

Xylanase activity was determined by growing the yeast overnight in 5 ml YPD broth at 30°C. Samples of culture supernatant (100 μl) were mixed with 900 μl of the substrate [1% xylan (Sigma) dissolved in 0.5 M-sodium-acetate buffer (pH 3.2)] and incubated for 60 min at 30°C. The liberation of reducing groups was determined by adding dinitrosalicylic acid (DNS) to the mixture (Miller *et al.* 1960) and boiled for 15 min. The sample was cooled and the absorption was measured at 540 nm. The units were defined as nkat units/g dry cells.

Glucanase activity was tested in 0.1% barley β-glucan (Sigma) and in 0.2% laminarin (Sigma) solutions. Both solutions were made with 0.5 M odium-acetate buffer at pH 3.2. The assays were performed in two parts. One part was used to determine the liberation of glucose with the Trinder kit and the other part was used to determine the liberation of reducing groups by the DNS method.

3.3 RESULTS

3.3.1 YEAST STRAINS

The isolates investigated were mostly strains of *K. apiculata*. The second largest group, *Candida*, contained the most species - eleven compared to the two of *Kloeckera*. Of the 237 isolates, 121 were isolated from the vineyard and 116 from

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the clarified grape juice. The 237 isolates represented 21 different species of yeast. Of the 21 species, nine were isolated from the vineyard and 13 from the clarified grape juice. These isolates were obtained as representatives of non-*Saccharomyces* strains found in a single vintage (1997). However, it should be noted that, depending on climate and viticultural practices, the population dynamics differ from year to year, thereby influencing the composition of the non-*Saccharomyces* population.

3.3.2 PECTOLYTIC ACTIVITY

Only nine isolates, represented by *Candida stellata*, *Candida oleophila*, *Candida pulcherrima*, *Candida valida* and *K. apiculata*, showed pectolytic activity on plates in our study (Table 2). McKay (1990) reported that the secretion of polygalacturonases by some strains of *S. cerevisiae* was constitutive and that the medium must be supplemented with 1% glucose for enzyme production. For this reason, glucose was included in the composition of the polygalacturonate agar used in the present study. However, it is possible that the presence of glucose in the medium could inhibit the production of these enzymes in some of the isolates.

3.3.3 PROTEOLYTIC ACTIVITY

The results in Table 2 indicate that 10 isolates of *C. stellata*, *C. pulcherrima*, *K. apiculata* and one isolate of *Debaryomyces hansenii* showed proteolytic activity in our study. Charoenchai *et al.* (1997) demonstrated that the presence of a readily utilisable nitrogen source repressed extracellular proteases, but, in stark contrast, these isolates showed activity on YPD-based plates that contained a readily utilisable nitrogen source. However, it is possible that, if our medium had lacked amino acids and ammonium sulphate, our results could have been different.

3.3.4 LICHENASE ACTIVITY

Lichenan is a linear, 1,3-1,4-β-D-glucan with a structure similar to that of barley and oat β-D-glucan. Lichenan has a much higher proportion of 1,3- to 1,4-β-D-linkages than the other two glucans. The ratio of 1,4- to 1,3-β-D-linkages is approximately 2:1. Only *C. stellata*, *C. pulcherrima* and *K. apiculata* isolates showed lichenase activity (Table 2).

3.3.5 GLUCOSIDASE ACTIVITY

β-Glucosidase activity can be measured by using β-glucoside analogues, 4-methylumbelliferyl-β-D-glucopyranoside (4-MUG) or ρ-nitrophenyl-β-D-glucopyranoside (p-NPG). However, these substrates are unreliable because 1,3-glucanase activity (which is contained by most yeasts) can also hydrolise these substrates. In this case it is more reliable to use a substrate like arbutin. Colonies showing activity can be identified by the discolouration of the media to a brown colour. In a preliminary study using p-NPG, it appeared that most of the strains

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contained glucosidase activity (data not shown), but when the assay was repeated with arbutin, none of the strains showed glucosidase activity under our test conditions. It was speculated that the presence of glucose could have a considerable effect on the production of β-glucosidases. However, even when omitting glucose, only some weak β-glucosidase activity was detected.

Table 2 Production of extracellular enzymes and H_2S by species of non-*Saccharomyces* wine yeasts

Yeast	Strain	Pectinase	Protease	Glucanase	Lichenase	Glucosidase	Cellulase (YPD)	Cellulase (YPGE)	Xylanase (SC)	Xylanase (YPGE)	Amylase	Bismuth
Candida stellata	C1: 10	-	++	-	-	-	-	-	-	-	-	4
	C1: 14	+++	++	++	++	-	-	++	-	-	+++	1
	C1: 16	+++	++	++	++	-	++	++	-	-	++	2
	M2: 2	-	++	-	-	-	-	-	-	-	-	w
	M2: 12	-	++	-	-	-	-	-	-	-	-	4
	M2: 19	-	-	+	-	-	-	-	-	-	-	5
	R2: 12	-	-	+++	-	W	-	-	-	-	-	3
	R2: 13	-	-	+++	-	W	-	-	-	-	-	4
	R2: 15	-	-	++	-	-	-	-	-	-	-	3
	R2: 16	-	-	++	-	W	-	-	-	-	-	3
	R2: 17	-	-	++	-	W	-	-	-	-	-	2
	R2: 18	-	-	++	-	-	-	-	-	++	-	3
	R2: 19	-	-	++	-	-	-	-	-	-	-	3
C. hellenica	M2: 1	-	-	++	-	-	-	-	-	-	-	4
	M2: 18	-	-	+	-	W	-	-	-	-	-	5
	R2: 22	-	-	+	-	-	-	-	-	-	-	3
	R2: 23	-	-	+	-	W	-	-	-	-	-	4
	R2: 24	-	-	+++	-	-	-	-	-	-	-	4
C. sorbosa	M2: 11	-	-	++	-	-	-	-	-	-	-	4
C. lambica	M2: 4	-	-	++	-	-	-	-	-	-	-	1
C. oleophila	O1: 22	-	-	-	-	-	-	-	-	+	-	W
	O1: 29	++	-	-	-	-	-	-	-	-	-	4
	R1: 13	-	-	-	-	-	-	-	+	+	-	3
C. pulcherrima	M1: 2	-	++	-	+++	-	+	-	-	-	+++	3
	M1: 3	-	-	-	-	-	+	-	-	-	-	4
	M1: 4	-	++	-	+++	-	+	-	-	-	+++	4
	M1: 9	-	-	-	+++	-	-	-	-	-	++	4
	O1: 13	-	-	+++	+++	-	++	-	-	-	+++	3
	O1: 25	-	-	-	-	-	-	-	-	+	-	4
	R1: 21	+	-	+	+	-	-	-	-	-	-	2

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Yeast	Strain	Pectinase	Protease	Glucanase	Lichenase	Glucosidase	Cellulase (YPD)	Cellulase (YPGE)	Xylanase (SC)	Xylanase (YPGE)	Amylase	Bismuth
C. valida	O2: 16	++	-	-	-	-	-	-	-	-	-	2
C. pelliculosa	R1: 17	-	-	-	-	-	-	-	++	++	-	2
Kloeckera apiculata	C1: 1	-	-	-	-	-	++	++	-	-	-	4
	C1: 8	-	++	+++	++	-	++	++	-	-	+++	1
	C1: 9	+++	++	-	++	-	++	++	-	-	+++	1
	C1: 24	+++	-	+++	+	-	-	-	-	-	++	2
	C2: 17	+	++	-	+++	-	++	+	-	-	-	0
	O1: 18	-	-	+++	++	-	+	-	-	-	-	3
	O1: 28	++	-	-	-	-	-	-	-	-	-	4
	R1: 8	-	-	-	-	W	-	-	+	-	-	4
	R2: 3	-	-	+++	-	-	-	-	-	-	-	4
	R2: 7	-	-	+++	-	-	-	-	-	-	-	4
	R2: 21	-	-	+	-	W	-	-	-	-	-	3
	R2: 29	-	-	++	-	-	-	-	-	-	-	3
Debaryomyces hansenii	M2: 5	-	++	-	-	-	-	-	-	-	-	4

+ = POSITIVE RESULT: A CLEAR ZONE (+ = small zones, ++ = bigger zones, +++ = very big zones); - = NEGATIVE RESULT: NO CLEAR ZONE; W = WEAK PRODUCTION; [a]0 = NO H_2S PRODUCTION; 5 = HIGH PRODUCTION

3.3.6 CELLULASE ACTIVITY

Only eleven isolates of *C. stellata*, *C. pulcherrima* and *K. apiculata* showed some cellulase activity. However, it is interesting to note that *C. pulcherrima* showed activity only on YPD-based plates that contained glucose (Table 2). On the YPGE-based plates, no activity was observed for *C. pulcherrima*.

3.3.7 GLUCANASE ACTIVITY

Most isolates of C. *stellata*, *C. hellenica* and *K. apiculata* showed activity on plates, but only a few isolates of *C. sorbosa*, *C. lambica*, *C. pulcherrima* showed activity on plate assays (Table 2). In contrast, more *Candida* species, *Pichia farinosa* and *P. kluyveri* were able to degrade glucan in liquid assays. *C. stellata* (18 028 units) and *K. apiculata* (23 153 units) were the two highest glucose producers. When we tested for the production of reducing groups, *C. stellata* produced 190 nkat units and *K. apiculata* produced 306 nkat units.

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3.3.8 XYLANASE ACTIVITY

One isolate of *C. stellata* showed strong activity only on YPGE-based plates, while one isolate of *C. pulcherrima* showed only little activity on YPGE-based plates. Two isolates of *C. oleophila* showed little activity on YPGE, while one of these isolates also showed activity on YPD plates. One isolate of *K. apiculata* showed little activity only on YPD-based plates. In liquid assays, *K. apiculata* (1 550 units) and *C. stellata* (510 units) produced the highest xylanase activity.

3.3.9 STARCH HYDROLYTIC ENZYMES

Although the degradation of starch is not important from an oenological perspective, most of the isolates were able to degrade starch. It must be noted that the amylolytic activity observed was not α-amylase activity, as no activity was observed on phadebas plates, which are specific for α-amylase activity (data not shown). This ability may be more important for whisky fermentations. In contrast, Charoenchai *et al.* (1997) did not find any amylolytic wine yeast in their study. It is tempting to speculate that this can be due to the low percentage of substrate (0.1 g/L) used in their plates.

3.3.10 SULPHITE REDUCTASE

Although most strains produced high amounts of H_2S, a few did not produce any H_2S (Table 2). Since the production of H_2S by wine yeasts leads to off-odours in the wine, the H_2S-producing potential of the non-*Saccharomyces* yeasts was also tested under oenological conditions. Media containing bismuth citrate have proven to be effective for the selective cultivation and differentiation of *Candida* spp. from pathological samples (Nickerson 1953). Growth on indicator media provides a visual measure of the genetically determined maximal activity of sulphite reductase in any given strain and, consequently, of its potential to produce H_2S (Jiranek *et al.* 1995). Colonies that produce hydrogen sulphide become discoloured in a concentration-dependent manner, ranging from white, through brown, to black. The simplicity of hydrogen sulphide detection inherent in this media has resulted in the adoption of this technique by scientists for the rapid presumptive identification of low or non-H_2S-producing wine yeast (Rupela and Tauro 1984).

3.4 DISCUSSION

Although our study clearly indicated that non-*Saccharomyces* strains are capable of producing pectolytic activity, none of the 23 strains tested showed any pectolytic activity in the study by Charoenchai *et al.* (1997). Pectic enzymes are produced mainly by moulds and bacteria, but are also produced by some yeasts. Significant pectolytic activity was found in *Saccharomyces fragilis* (*Kluyveromyces fragilis*) and *Candida tropicalis*, whereas *Saccharomyces thermantitonum*, *Torulopsis kefyr* and

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Torulopsis lactosa have weaker activity (Luh and Phaff 1951). Pectin esterases were detected in *Debaryomyces membranaefaciens* var. *hollandius*, *Endomycopsis olmeri* var. *minor*, *Candida krusei*, *Hansenula*, *Rhodotorula* and *Zygopichia* (Bell and Etchells 1956). Polygalacturonase activity was found in *Candida silvae*, *Candida norvegensis*, *Geotrichum candidum*, *Pichia guilliermondii*, *Pichia membranaefaciens*, *Torulopsis candida* and *Trichosporum cutaneum* (Call and Emeis, 1978; Sanchez *et al.* 1984; Ravelomanana *et al.* 1986). Furthermore, several *Saccharomyces* species were also reported to have polygalacturonase activity, including *S. carlsbergensis*, *S. chevalieri*, *S. cerevisiae*, *S. oviformis*, *S. uvarum* and *S. vini* (Kotomina and Pisarnitskii 1974; Sanchez *et al.* 1984). Bell and Etchells (1956) reported weak pectolytic activity for *S. cerevisiae*, whereas Luh and Phaff (1951) reported that the *S. cerevisiae* cultures tested by them had no noticeable effect on pectin.

It was later claimed that certain strains of *S. cerevisiae* have the ability to degrade polygalacturonic acid in the presence of glucose (McKay 1990). Recently, a single culture of *S. cerevisiae* was isolated that supposedly produces pectin esterase, polygalacturonase and pectin lyase (Gainvors *et al.* 1994). None of these enzymes has been purified, nor have any of the genes been cloned. Blanco *et al.* (1994) reported that at least 75% of oenological strains tested showed limited pectolytic activity. Endopectate-degrading enzymes occurred primarily in the growth medium, as is the case in most other yeast species. The synthesis of pectic enzymes was reported to be constitutive, providing the glucose concentration in the medium did not exceed 2%. A higher concentration of glucose led to the total inhibition of these pectolytic activities. Interestingly enough, the pectolytic activity was found to be significantly lower with growth on glucose as a carbon source than with galactose. Subsequently, Blanco *et al.* (1998) speculated that all *S. cerevisiae* strains contain a promoterless polygalacturonase gene or else a non-functional one. This structural polygalacturonase-encoding gene (*PGU1*) from *S. cerevisiae* IM1-8b was eventually cloned and sequenced. The predicted protein comprises 361 amino acids, with a signal peptide between residues 1 and 18 and two potential glycosylation points in residues 318 and 330. The putative active site is a conserved histidine in position 222. This *S. cerevisiae* polygalacturonase shows 54% homology with the fungal polygalacturonases and only 24% homology with its plant and bacterial counterparts. *PGU1* is present in a single gene copy per haploid genome and it is detected in all strains, regardless of their phenotype. The expression of the *PGU1* gene in several strains of *S. cerevisiae* revealed that the polygalacturonase activity depended on the plasmid used and also on the genetic background of each strain, but that the enzymatic activity increased in all cases.

The proteolytic activity of yeasts has been studied in relation to protein haze reduction in beer and wine. Our study has confirmed that several species of indigenous wine yeasts can produce proteolytic activity. The vacuolar protease A plays an important role during the autolysis process, which occurs in wines kept on the yeast lees during ageing. However, because of the particular conditions found in

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wine, only a few proteases are active (Lurton 1987). Eight yeast strains were identified that consistently exhibited proteolytic activity in model wine solutions. These were *Candida olea*, *C. flavus*, *K. apiculata*, *Metschnikowia pulcherrima*, *Pichia pinus*, *Torulopsis magnolia*, *Torulopsis monosa* and *Yarrowia lipolytica*. Protease activity was maintained in actual wine solutions, but at somewhat reduced levels. The incubation of a protease concentrate from *K. apiculata* with Chenin blanc and Chardonnay wines showed some degradation of wine proteins. Lagace and Bisson (1990) demonstrated that extracellular proteolytic activities produced by *C. olea*, *C. lipolytica*, *Cryptococcus flavus*, *K. apiculata* and *C. pulcherrima* could be correlated with their ability to reduce wine haze. In a recent study, Dizy and Bisson (2000) demonstrated that strains of *Kloeckera* and *Hanseniaspora* produced the most proteolytic activity in grape juice and affected the protein profile of the finished wines. The protease activity produced did not significantly reduce the heat/chill haze-forming potential of the wines, however. In some cases, the haze level formed was greater in the fermentations with high proteolytic activity.

The significance of β-glucosidases for the wine industry lies in their potential for releasing flavour compounds from glycosidically-bound non-volatile precursors in wine. A few papers have reported that non-*Saccharomyces* wine yeasts contain glucosidase activity, but these results are questionable because of the possibility that the yeast tested may have had no glucosidase activity, but rather that 1,3-glucanases were able to hydrolyse the β-glucoside analogues used in most studies. In contrast to grape glycosidase, yeast glucosidases are not inhibited by glucose. It was reported that, although the β-glucosidase from *Hansenula* sp. isolated from fermenting must was capable of liberating aroma substances in wine, it was less effective in must (Grossmann *et al.* 1987). An intracellular β-glucosidase from *Debaryomyces hansenii* Y-44 was purified and used in the fermentation of Muscat juices (Yanai and Sato 1999). A wine was produced that had a considerable increase in the concentration of monoterpenols. The linalool and nerol contents increased considerably, by 90% and 116% respectively. According to some reports, certain strains of *S. cerevisiae* also possess a β-glucosidase that is located in the periplasmic space of the yeast cell (Darriet *et al.*, 1988; Dubourdieu *et al.* 1988). This activity appears to be very limited and recent studies therefore rather have focused on non-*Saccharomyces* yeasts, such as *Hanseniaspora vinea* (Vasserot *et al.* 1989) and *Candida* species (Günata *et al.* 1990). Results obtained so far from studies on yeast glycosidases indeed suggest that specific yeast strains can affect the varietal aroma of wines (Laffort *et al.* 1989). In a recent study by McMahon *et al.* (1999), it was determined that the β-glucosidase production took place in 32 strains of the genera *Aureobasidium*, *Candida*, *Cryptococcus*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Torulaspora* and *Brettanomyces*. Only one *Saccharomyces* strain exhibited activity, but several of the non-*Saccharomyces* yeast species showed activity, especially *Brettanomyces* (eight of the ten strains tested). However, the majority of the β-glucosidase activity was located in the whole cell fraction, with

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smaller amounts found in permeabilised cells and released into the growth medium. Only *Aureobasidium pullulans* hydrolysed the glycosides found in grapes.

Hemicellulose constitutes a group of polysaccharides that is associated with cellulose in plant cell walls. These complex carbohydrate polymers contain xylan as their main component. β-1,4-xylans are found mainly in the secondary cell walls of plants, where they act as one of the major components of woody tissue (Thomson 1993). The degradation of hemicellulose is performed by a complex set of enzymes, such as xylanases, and could contribute to wine aroma by increasing the amount of monoterpenyldiglycoside precursors in the must. These compounds can be released from the walls of grape cells by the action of these enzymes. Since cellulose and hemicellulose represent the primary structural polysaccharides of the plant cell wall, a portion of them might be released into the wine after acid hydrolysis. This could lead to filtration and clarification problems (Zoecklein *et al.* 1995). The presence of cellulase and hemicellulase in the wine can solve this problem. Lichenase activity can also play an important role in the filterability of a wine, since a major portion of the grape and *Botrytis* glucans contain 1,3-1,4 linkages. Cellulase activity can play a role in extracting more colour and flavour from the skins of the grapes. It is also possible that this activity can change the tannin composition of the wine or even shorten the time needed for fermentation on the skins. Enzyme preparations containing cellulases and hemicellulases, in addition to pectinase activities, are known as macerating enzymes. These preparations are used to improve juice yields by degrading the structural polysaccharides that interfere with juice extraction, clarification and filtration.

When comparing the isolates from the different areas, the *C. stellata* and *K. apiculata* strains isolated from the Constantia area were shown to possess a much more complex enzyme profile. It is well established that wine fermentations, whether they develop naturally or after inoculation with *S. cerevisiae*, are characterised by the significant growth of indigenous species of non-*Saccharomyces* yeast. It is also believed and has been proven that these yeasts must effect the chemical composition and final sensory quality of the wine. Our study revealed the potential of indigenous wine yeasts to produce a wide range of extracellular enzymes. These findings indicate the need to determine the impact that such activities may have on the sensory properties of wine. In ongoing research, we are using some of these strains in co-cultures with *Saccharomyces* strains to see what effect these strains have on wine aroma. From the preliminary results, it seems that the strains with the most enzymatic activities are the ones that have the greatest effect on wine aroma. Therefore, future research should focus on the activity of these enzymes in wine fermentations and a better knowledge of the physiological and metabolical features of non-*Saccharomyces* yeast is required.

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3.5 ACKNOWLEDGEMENTS

The authors thank the South African wine industry (Winetech), the National Research Foundation (NRF) and THRIP for funding.

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CHAPTER 4

RESEARCH RESULTS

The heterologous expression of polysaccharase genes with oenological relevance in *Saccharomyces cerevisiae*

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RESEARCH RESULTS

The heterologous expression of polysaccharase genes with oenological relevance in *Saccharomyces cerevisiae*

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Abstract: The aim of this study was to construct a wine yeast, *S. cerevisiae*, that will secrete polysaccharases, such as pectinases, xylanases and glucanases. This paper will also look at the effects of these enzymes at the vinification of different varieties of wine grapes.

Methods & Results: The *S. cerevisiae* wine yeast strain, VIN13, was transformed with the plasmid pEXS, containing an endoglucanase gene and an endoxylanase gene. Another VIN13 was transformed with the plasmid pPPK, containing the polygalacturonase and pectate lyase genes. Pinot noir, Cinsaut and Muscat d'Alexandria wines were made with these two transformed yeasts and tests were conducted on these wines. Expression of glucanase and xylanase caused a decrease in turbidity of the wine, especially in Pinot noir, making it easier to filter, and also caused a distinct increase in colour intensity. The pectinases caused a decrease in phenolics in Pinot noir, whereas the glucanase and xylanase caused an increase in phenolics due to the degradation of the grape skins. In the Muscat d'Alexandria and Cinsaut, the differences between the control and transformants were less intense, showing that these enzymes have a different effect on different cultivars. In all three cultivars, the enzymes did not seem to cause significant alterations in the chemical composition of the wines.

Conclusions: This study clearly showed the potential of a polysacharase-secreting wine yeast to produce polysaccharases directly into the wine, thus solving many of the problems experienced during the winemaking process without the addition of industrial enzymes.

Significance and Impact of the Study: A polysaccharase-secreting wine yeast can have an economical impact on the winemaking process, as well as an influence on the quality of wine.

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4.1 INTRODUCTION

Originally, all wines were made by taking advantage of the natural microflora for spontaneous fermentation. Various yeasts found on the surface of grape skins and the indigenous microbiota associated with winery surfaces participate in these natural wine fermentations. While the controlled growth of these indigenous species may, in some circumstances, enhance the chemical complexity and sensory quality of wines, there are other circumstances in which their contribution may result in spoilage (Fleet 1992). Today, most winemakers use pure *Saccharomyces cerevisiae* cultures to produce wine of a more reproducible quality. This microbiological simplification of the wine fermentation process has opened the way for the use of genetic engineering techniques in wine yeast.

Enzymes play a definitive role in the production of wine, which could be viewed as the product of enzymatic transformation of the grape juice (for a review see Van Rensburg and Pretorius 2000). The enzyme activities originate from the grape itself, and also from yeast and other microorganisms. The winemaker also needs to reinforce and extend the action of these endogenous enzymes through the use of exogenous, industrial enzyme preparations (Fleet 1993). Polysaccharides can influence the clarification and stabilisation of must and wine. Polysaccharides, which are found in wines at levels between 300 and 1000 mg/l, originate in the grape itself, from the fungi on the grape and from the microorganisms present during winemaking (Whitaker 1990). The main polysaccharides responsible for turbidity, viscosity and filter stoppages are pectins, glucans (a component of cellulose) and, to a lesser extent, hemicelluloses (mainly xylans). Industrial enzyme preparations that degrade these polysaccharides play an important role in improving juice yields, increasing the release of colour and flavour components, improving clarification and filtration of wine, etc (Colagrande *et al.* 1994). Most of the commercial pectinase and glucanase preparations are derived from *Aspergillus* and *Trichoderma* respectively (Canal-Llauberes 1993). Since the addition of these commercial enzyme preparations can be quite expensive, some researchers have looked at the native pectinases and glucanases of *S. cerevisiae* (Pretorius 1997). Since these endogenous polysaccharase activities of *S. cerevisiae* are not sufficient, a programme is in place at our Institute to introduce a wide variety of heterologous polysaccharase genes into *S. cerevisiae*.

In this paper, we describe the construction of two recombinant wine yeast strains. In the one strain, we integrated the endo-β-1,4-glucanase gene (*end1*) from *Butyrivibrio fibrisolvens*, together with the endo-β-1,4-xylanase gene (*xyn4*) from *Aspergillus niger*. The second wine yeast strain was integrated with the pectate lyase-encoding gene (*pelE*) from *Erwinia chrysanthemi* and with the polygalacturonase-encoding gene (*peh1*) from *E. carotovora* subsp. *carotovora*. All the genes were fused with the *ADH1* promoter, the *Mf*α*1* secretion sequence and the *TRP5* terminator, except for *XYN4*, which contains the *ADH1* terminator and its

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native signal sequence. A further aim was to determine the extent of influence of these recombinant strains on the wines made.

4.2 MATERIALS AND METHODS

4.2.1 MICROBIAL STRAINS, PLASMIDS AND GROWTH CONDITIONS

The bacterial and yeast strains used in this study are listed, with their sources and relevant genotypes, in Table 1.

Bacterial transformants of *Escherichia coli* were grown in Luria Bertani broth (LB) (Sambrook *et al.* 1989) supplemented with ampicillin at a concentration of 100 μg/ml. *Saccharomyces cerevisiae*, transformed with plasmid pEXS containing the *SMR1* (sulphometronmethyl resistance) marker gene, was grown in liquid YPD (1% yeast extract, 2% peptone and 2% glucose) and on YPD agar. Transformants containing the (sulphometronmethyl resistance) marker gene were selected on YPD agar containing sulphometronmethyl with concentrations ranging between 50 and 100 μg/ml. Transformants containing the *KanMX* (geneticin resistance) marker gene were selected on YPD agar containing geneticin with concentrations ranging between 10 and 100 μg/ml.

Standard methods for the manipulation and subcloning of DNA fragments, plasmid DNA isolations, transformation of *E. coli* DH5α (Sambrook *et al.* 1989) and yeast cell electroporation were used. DNA amplification by the polymerase chain reaction (PCR) technique and Southern blot hybridisation were performed as described by Laing and Pretorius (1992) and Sambrook *et al.* (1989).

Table 1 Yeast strains, bacterial strains and plasmids used in this study.

Strains	Relevant genotype	Source or reference
Yeast strains:		
Saccharomyces cerevisiae (VIN13)	Diploid industrial wine yeast	Anchor Yeast (SA)
Escherichia coli strains :		
E.coli DH5α	*sup*E44 Δ*lac*U169 (Φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Sambrook *et al.* (1989)

4.2.2 CONSTRUCTION OF RECOMBINANT *E. COLI*-YEAST SHUTTLE PLASMIDS

The sulphometronmethyl gene (*SMR1*) was cut from plasmid pDLG31 with *Bam*HI and ligated into the *Bam*HI site of the yeast integrating plasmid YIp5.

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The 3,2 kb sulphometronmethyl gene (*SMR1*) was then cut from plasmid YIp5(SMR1) with *Eco*RV and ligated into the *Sma*I site of plasmid pEX, resulting in plasmid pEXS (Fig. 1).

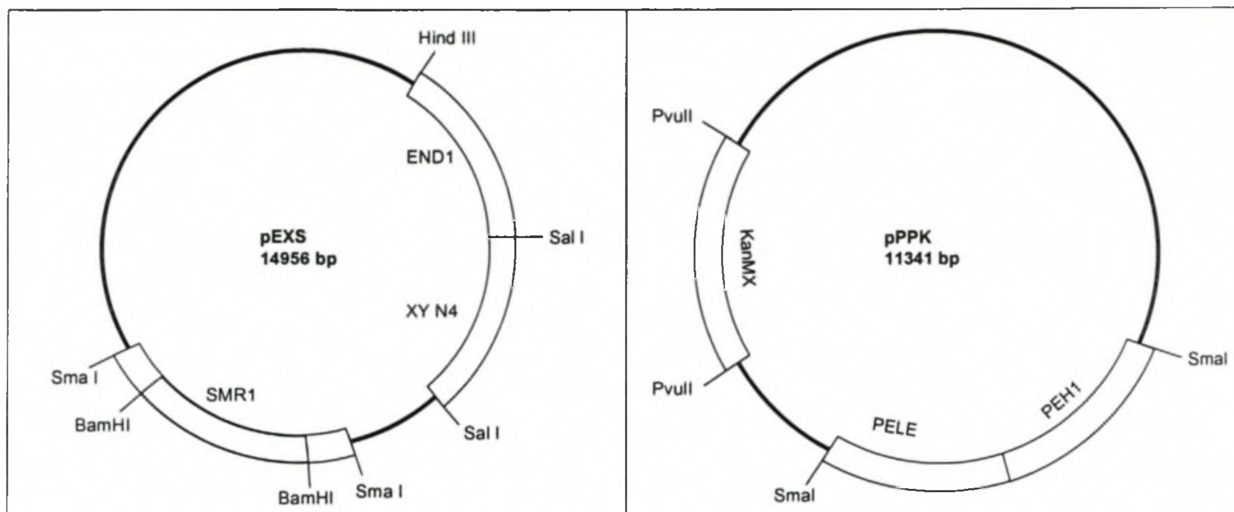

Fig. 1 Plasmid pEXS. This plasmid is the yeast integrating plasmid YIp5 with a 4.7 kb DNA fragment containing the *SMR1* gene, a 2.6 kb DNA fragment containing the $ADH1_P$ $MF\alpha1_S$ *end1* $TRP5_T$ cassette and a 2.6 kb fragment containing the $ADH2_P$ *xynC* $ADH2_T$ cassette.

Fig. 2 Plasmid pPPK. This is also the yeast integrating plasmid YIp5. This plasmid, however, was transformed with a 1.5 kb fragment containing the *KanMX* gene. It was also transformed with a 4.3 kb fragment containing the $ADH1_P$ $MF\alpha1_S$ *pelE* $TRP5_T$, $ADH1_P$ $MF\alpha1_S$ *peh1* $TRP5_T$ cassette.

For the construction of plasmid pPPK, the yeast integrating plasmid YIp5 was used as base plasmid. Plasmid pUG6 was digested with *Pvu*II and *Eco*RV to obtain the 1.5 kb *KanMX* gene. This gene then was integrated into the *Pvu*II site of YIp5, generating plasmid pK. The 4.3 kb DNA fragment containing the pectate lyase gene (*pelE*) and polygalacturonase gene (*peh1*) was cut from plasmid pAR5 with *Not*I and ligated into the *Sma*I site of plasmid pK, resulting in plasmid pPPK (Fig. 2). The plasmids used and created in this study are listed in Table 2.

4.2.3 PLATE ASSAYS

Pectinase activity was determined as follows: *S. cerevisiae* transformants were grown in 10 ml of YPD for 24 hours. 10 μl of the pre-culture was then plated 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 overnight at 25°C. They were then stained with 0.02% ruthenium red for 60 min at 37°C, whereafter the ruthenium red was washed off with water. Transformants with pectinase activity showed a clear zone around the colony.

Glucanase activity was tested by spotting the transformants onto 0.1% barley β-glucan (Sigma) or 0.4% lichenan (Sigma) plates. The plates were incubated for three to five days, whereafter they were stained with 0.03% Congo red (Teather and Wood

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1982). After destaining with water, positive transformants showed a clear zone around the colonies.

Table 2 Plasmids used and created in this study

Plasmid	Relevant genotype	Source or reference
Ylp5	Ap^R Tc^R URA3	Struhl *et al.* (1979)
pUG6	Ap^R $KanMX^R$	Güldener *et al.* (1996)
pDLG31	Ap^R $SMR1^R$ $PGK1_P$ *LKA1* $PGK1_T$	Gundlapalli Moses *et al.* (2002)
pAR5	Ap^R Gt^R *LEU2* $ADH1_P$ $MF\alpha1_S$ *end1* $TRP5_T$ $ADH1_P$ $MF\alpha1_S$ *pelE* $TRP5_T$ $ADH1_P$ $MF\alpha1_S$ *peh1* $TRP5_T$	Van Rensburg *et al.* (1994)
pEX	Ap^R Tc^R *URA3* $ADH1_P$ $MF\alpha1_S$ *end1* $TRP5_T$ $ADH2_P$ *xynC* $ADH2_T$	Petersen *et al.* (1998)
Ylp5(SMR1)	Ap^R Tc^R *URA3* $SMR1^R$	This work
pK	Ap^R Tc^R *URA3 KanMX*	This work
pEXS	Ap^R Tc^R *URA3* $SMR1^R$ $ADH1_P$ $MF\alpha1_S$ *end1* $TRP5_T$ $ADH2_P$ *xynC* $ADH2_T$	This work
pPPK	Ap^R Tc^R *URA3 KanMX* $ADH1_P$ $MF\alpha1_S$ *pelE* $TRP5_T$ $ADH1_P$ $MF\alpha1_S$ *peh1* $TRP5_T$	This work

4.2.4 MICROVINIFICATION EXPERIMENTS

Fermentation assays were carried out in duplicate using 6.5 L of must for each sample. The varieties used were Pinot noir, Cinsaut and Muscat d'Alexandria. A total of 20 kg of grapes of each variety were destemmed and crushed, and 40 ppm of SO_2 added. Yeast were then inoculated to a final concentration of 1 to 2 x 10^6 cells/ml for each sample. The must of the red varieties was fermented at 25°C and that of the white variety at 15°C. Samples (20 ml) were collected at four stages of fermentation from the Cinsaut and Pinot noir for colour and phenolics testing: at the beginning of fermentation, at the end of fermentation, after pressing and after filtration. This was done by measuring the density of the samples at different wavelengths (280, 420 and 520 nm) (Zoecklein *et al.* 1990). The Muscat d' Alexandria was only tested after filtration for total hydroxycinnamates and total phenolics. Total volumes of free flow

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wine and pressed wine were also determined in the Cinsaut and Pinot noir. This was done by physically measuring the volumes.

4.2.5 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 using standard methods. The values obtained were confirmed by using a WineScan FT120 Type 77310, FOSS Electric A/S, Hillerød, Denmark. The results are shown in Table 3.

4.2.6 GAS-LIQUID CHROMATOGRAPHY

Apparatus: HP 6890 series gas chromatograph, fitted with a FID (flame ionisation detector), fitted with a split-splitless injector, and automatic sampler 7683 were used. Column: Supelco SPB5, 60 m x 0.32 mm i.d., 0.25 μm film thickness. Chromatographic conditions: carrier gas, He; head pressure, 140 kPa; flow, 12.5 ml/min; purge flow, 7.0 ml/min; injector and detector temperature, 250°C; initial column temperature, 50°C, held for 2 min and then raised to 150°C at 10°C/min, then to 160°C at 5°C/min and then to 220°C at 10°C/min and held for 10 min; make-up gas, N_2 at 30 ml/min; detector FID, H_2 at 40 ml/min; air, 450 ml/min; injected volume, 2 μl.

Method: 10 ml of wine were introduced into the extraction tube. 200 μl of Freon 113 (1,1,2-Trichloro-1,2,2-trifluoroethane, obtained from Aldrich) was added as extracting agent, as well as 2 μl of a solution of 2,6-dimethylheptenol (400 mg/l in ethanol as internal standard). 1,2 g of NaCl was also added. The tubes were capped and shaken for 30 min in an automatic shaker at maximum speed. The tubes were centrifuged (5 min at 3000 rpm) and the organic phase was recovered with a pasteur pipette, transferred over 50 mg of Na_2SO4 into a HP 2 ml vial with a 200 μl glass insert, and analysed under the chromatographic conditions as described above. After the chromatographic analysis, the relative areas or heights of the calibrated peaks were interpolated from calibration graphs created with synthetic wine solutions (ethanol 12% for white wine, 16% for red wine v/v; tartaric acid 6 g/l; pH 3.2) with an alcohol content similar to that of the analysed wine.

4.3 RESULTS

4.3.1 GENETIC TRANSFORMATION OF THE INDUSTRIAL WINE YEAST STRAIN VIN13

The plasmid pEXS digested by *Nco*I, containing the *ADH1*$_P$-*MF*α*1*$_S$-*end1-TRP5*$_T$ and *ADH1*$_P$-*xynC-ADH2*$_T$ cassettes and the *SMR1* marker gene, as well as plasmid pPPK cut by *Stu*I, harbouring the *ADH1*$_P$-*MF*α*1*$_S$-*peh1-TRP5*$_T$ and *ADH1*$_P$-*MF*α*1*$_S$-*pelE-TRP5*$_T$ cassettes and the *KanMX* marker gene, were transformed separately by elctroporation, and integrated into the *URA3* locus of *S. cerevisiae* VIN13 strain.

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Expression of the various gene cassettes were confirmed by screening on selective agar plates. Positive transformants were also confirmed by PCR (data not shown).

4.3.2 MICROVINIFICATION

Microvinification experiments were carried out in duplicate with the transformants. The *S. cerevisiae* strain, VIN13, was used as control. Spectrophotometric tests were done during the fermentation to determine colour densities (Fig. 3) and the amount of phenolics (Fig. 4). These experiments were done on both red varieties, Pinot noir and Cinsaut, to determine the different effects on the different varieties.

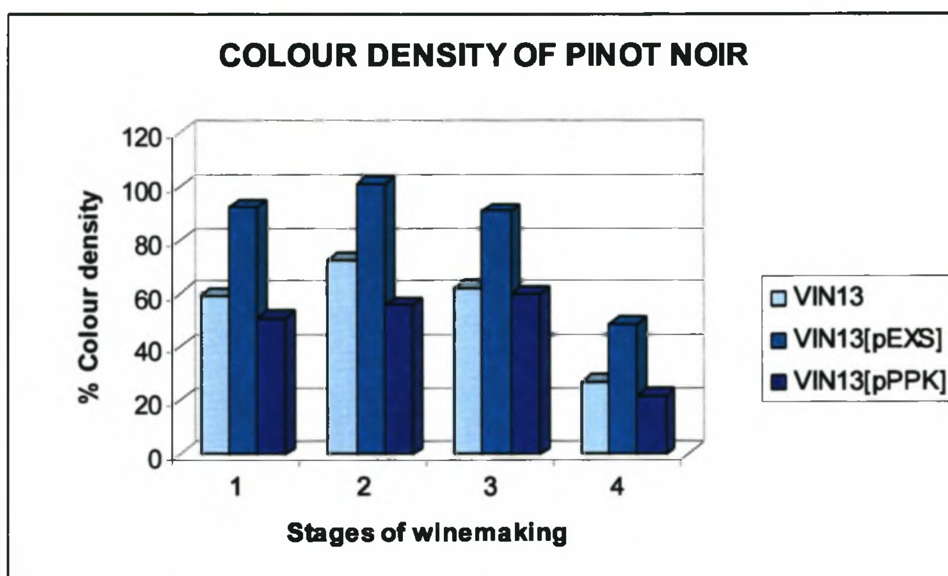

Fig. 3 The colour density of Pinot noir at different stages of fermentation. 1 – beginning of fermentation, 2 – end of fermentation, 3 – before pressing, 4 – after pressing.

In the case of Pinot noir, it is known that the extraction of sufficient colour from the skin tends to be a problem. The transformant VIN13[pEXS] caused an increase in colour density. Speculatively, this is due to the better degradation of the grape skin, leading to a higher concentration of colour pigments in the wine. The transformant VIN13[pPPK] also was expected to give more colour, but less colour compared to the control was obtained. This could be caused by the fact that some pectinases are capable of reducing red wine colour through pigment modification and subsequent degradation (Wightman *et al.* 1997). No real difference in colour density was found with the Cinsaut. Enough colour pigments are extracted from Cinsaut grapes under normal conditions, which means that it might not be possible to extract more pigments from the skins, but it produces a lot of juice, making the juice lighter in colour.

Due to the increased degradation of the grape skin by the endoglucanase and endoxylanase produced by the VIN13[pEXS] transformant, a higher concentration of phenolics is released into the wine made from Pinot noir (Fig. 4).

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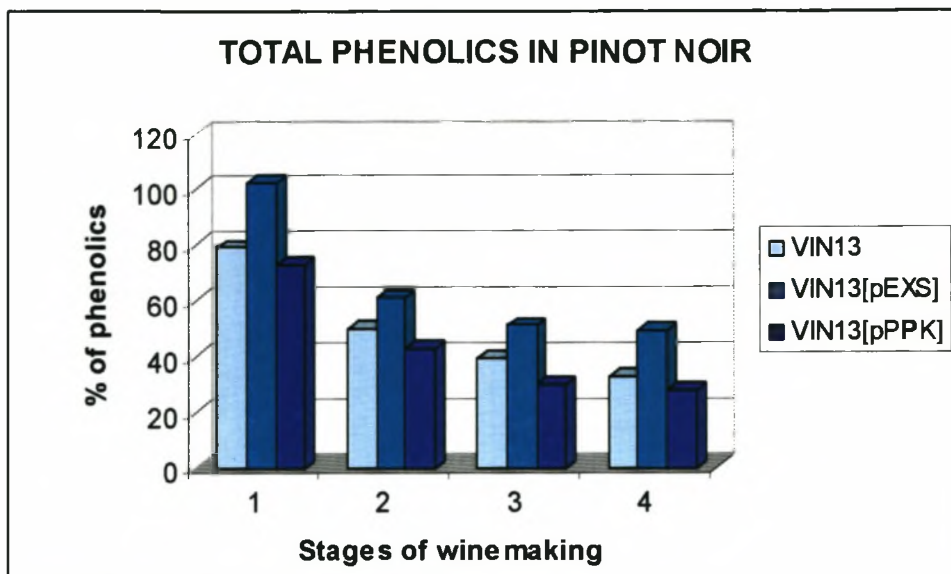

Fig. 4 The total phenolics in Pinot noir during the winemaking process. 1 – beginning of fermentation, 2 – end of fermentation, 3 – before pressing, 4 – after pressing.

In Cinsaut, the same transformant seemed to cause no difference in the amount of phenolics. This may be that the phenolics are bound differently in Cinsaut than in Pinot noir. In both varieties, a decrease in phenolics was observed towards the end of fermentation. This can be caused by the binding of the phenols.

Regarding the correlation between the amount of free flow wine and pressed wine, the two cultivars showed some similarities (Fig. 5). In both varieties, the two transformants (VIN13[pEXS] and VIN13[pPPK]) gave more free flow wine than the control (VIN13) due to the degradation of the grape skins. The transformant VIN13[pPPK] gave better results by degrading the pectins in the cell walls.

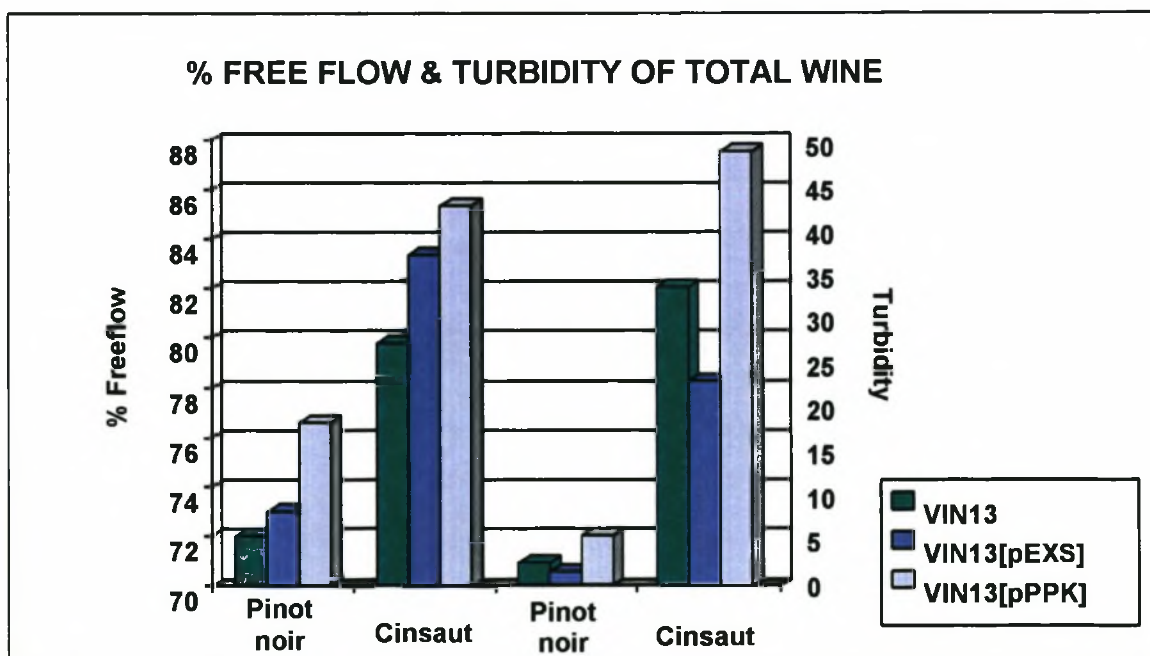

Fig. 5 The percentage of free flow wine versus the pressed wine, and the differences in turbidity between Pinot noir and Cinsaut.

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The degradation of the pectins is carried out by the polygalacturonase and pectate lyase secreted by the transformed wine yeast. In the experiment, the transformants showed more activity in Cinsaut than in Pinot noir. This indicates that enzymes secreted by the wine yeasts do not have the same effects on all varieties.

The general norm with regard to the turbidity was that the wines fermented with the transformant VIN13[pEXS] showed less turbidity, whereas the wines fermented with the transformant VIN13[pPPK] showed higher turbidity than the control (Fig. 5).

Experiments were also done with both transformants on a white variety, Muscat d'Alexandria. This variety is very fleecy, making it very difficult to press the juice from the skins. The continuous pressing of the grapes results in the extraction of a lot of phenolics and other components, giving a bitter taste to the juice. The results (Fig. 6A+B) show clearly that fermentation with VIN13[pEXS] results in a wine with less phenolics and hydroxycinnamates than the control. Fermentation with VIN13[pPPK] showed no real difference in comparison with the control.

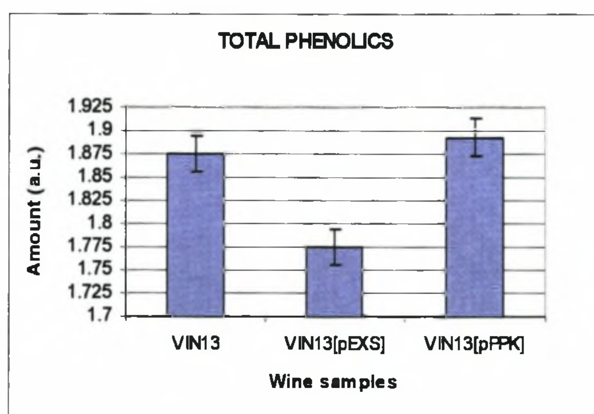

Fig. 6A The total phenolics in the wines made with the three different wine yeasts.

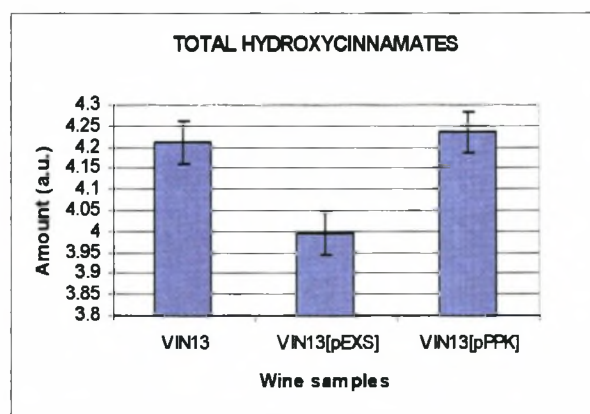

Fig. 6B The total hydroxycinnamates in the wines made with the control and the two transformed wine yeasts

In the case of Muscat d'Alexandria, the VIN13[pEXS] transformant will definitely be a better choice if the results obtained are taken into consideration.

Finally, the GC results showed no significant differences in the wine composition profiles of the two transformants and the control (Tables 4, 5 and 6). Regarding the ethanol concentration, it is seen that the pEXS transformant in both Cinsaut and Pinot noir produced higher ethanol concentrations than the control (Table 3). In both Pinot noir and Muscat d'Alexandria, transformant pPPK showed a lower alcohol concentration than pEXS and the control (Table 3).

VIN13[pPPK] seemed to produce more higher alcohols than the control in Pinot noir and Cinsaut (Tables 4 and 5). In Muscat d'Alexandria, VIN13[pPPK] also produced more higher alcohols and more short to medium chain ethyl esters, aldehydes and terpenes. Both transformants produced less long chain ethylesters than the control (Table 6).

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Table 3 Analysis of wines.

Sample ID	Ethanol (% v/v)	R. sugar (g/i)	pH	VA (g/l)	TA (g/l)	Malic acid (g/l)	Lactic acid (g/l)
Cinsaut Vin13 (1)	11.62	0.57	3.08	0.23	6.43	2.85	0.14
Cinsaut Vin 13 (2)	11.5	0.49	3.04	0.19	6.61	3.27	0.08
Cinsaut pPPK(1)	12.23	0.38	3.18	0.14	5.63	2.09	0.23
Cinsaut pPPK(2)	11.51	0.58	3.14	0.2	5.61	1.94	0.25
Cinsaut pEX5 (1)	12.43	0.58	3.15	0.16	6.22	2.81	0.3
Cinsaut pEX5 (2)	12.3	0.61	3.18	0.2	6.09	2.78	0.23
Pinot noir Vin13 (1)	11.11	0.46	2.95	0.19	7.11	4.35	0.12
Pinot noir Vin 13 (2)	10.85	0.46	2.95	0.23	7.04	3.97	0.09
Pinot noir pPPK(1)	10.16	0.42	2.92	0.19	7.33	4.81	0.06
Sample ID	Ethanol (% v/v)	R. sugar (g/i)	pH	VA (g/l)	TA (g/l)	Malic acid (g/l)	Lactic acid (g/l)
Pinot noir pPPK(2)	10.29	0.32	3	0.22	6.9	4.21	0.09
Pinot noir pEX5 (1)	11.77	0.72	2.94	0.22	7.02	3.3	0.14
Pinot noir pEX5 (2)	11.55	0.52	2.96	0.17	6.91	3.41	0.14
Muscat d'Alexandria Vin13 (1)	12.15	3.04	3.22	0.09	4.82	2.32	0.13
Muscat d'Alexandria Vin 13 (2)	12.2	0.62	3.17	0.04	4.99	2.37	0.27
Muscat d'Alexandria pPPK(1)	12.04	0.36	3.11	0.04	5.11	2.14	0.25
Muscat d'Alexandria pPPK(2)	12.1	0.36	3.14	0.07	5.11	2.17	0.27
Muscat d'Alexandria pEX5 (1)	12.16	0.54	3.14	0.04	5.07	2.31	0.25
Muscat d'Alexandria pEX5(2)	12.17	0.51	3.16	0.05	5.02	2.33	0.26

Table 4 The volatile components as found by microextraction of Cinsaut wines. All values are in mg/l.

Volatile components - Cinsaut	Control VIN13	VIN13[pEXS]	VIN13[pPPK]
Butanol-3-methyl	92.25	102.39	112.40
Butanol-2-methyl	29.03	29.73	36.77
Acetic acid-2-phenyl ester	0.48	0.42	0.63

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Table 5 The volatile components as found by microextraction of Pinot Noir wines. All values are in mg/l.

Volatile components - Pinot Noir	Control VIN13	VIN13[pEXS]	VIN13[pPPK]
3-Methyl-1-butanol	82.13	107.04	116.35
2-Methyl-1-butanol	36.54	36.55	37.78
Butanoic acid-3-methylethyl ester	0.04	0.05	-
Acetic acid-2-phenyl ester	0.03	0.07	0.09
Hexanol	1.55	1.65	1.75
Decanoic acid ethyl ester	0.04	0.09	0.08

Table 6 The volatile components as found by microextraction of Muscat d'Alecandria wines. All values are in mg/l.

Volatile components - Muscat d'Alexandria	Control VIN13	VIN13[pEXS]	VIN13[pPPK]
Butanol-3-methyl	81.20	73.57	122.82
Butanol-2-methyl	28.93	25.61	34.74
Acetic acid isobutyl ester	1.37	1.58	1.83
Hexanoic acid ethyl ester	1.20	1.31	1.28
Furfural	0.02	0.25	0.03
Linalool	0.42	0.44	0.47
Nonaldehyde	0.04	0.04	0.05
Octanoic acid ethyl ester	1.04	0.96	1.00
Terpineol	0.14	0.14	0.16
Citronellol	0.02	0.02	0.02
Decanoic acid ethyl ester	0.30	0.29	0.26

4.4 DISCUSSION

Until recently, studies directed at wine improvement concentrated on the selection of new grape varieties and viticultural practices or on fermentation and winemaking practices. Little attention has been paid to the genetic improvement of the organisms that play a vital role in the whole winemaking process, namely the wine yeasts.

Taking genes coding for the specific enzymes from fungi has advantages, because these enzymes are normally not inhibited by the pH and temperature of must and wine or by the SO_2 added to the wine to protect it against oxidation and other microorganisms. Genes from bacterial origin usually are inhibited by the above factors, making genes from fungal origin more favourable for use in winemaking procedures.

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From the results obtained from the above experiments, it is clear enzymes directly secreted by the industrial wine yeast can play a significant role in the end product of fermentation. It also was shown that the same enzyme could have quite different effects on different grape varieties. This could be caused by the different composition of the varieties, the skins could be naturally more difficult to press or could be thicker, or some varieties could be juicier than others. There are a number of reasons why the effect of the same enzyme differs between varieties. Glucanase and xylanase clearly decreased turbidity, especially in Pinot noir, and pectinase seemed to increase turbidity in both red varieties. In Muscat d'Alexandria, there was no difference from the control. This shows that the effect differs between varieties. Pectinase, on the other hand, led to the extraction of more juice from the skins in all three varieties.

The results of the GCs show that the secretion of these four enzymes into the must during fermentation did not cause major alterations in the composition of the must and therefore did not change the chemical composition of the wines. This is very good, because the aim of this study was not to change the composition of the wines, but to obtain more juice and colour and to lessen the turbidity.

It is clear from the results obtained from the experiment that both transformed yeasts have a positive effect on different aspects of the three cultivars tested. These enzymes also gave different results with different cultivars and it therefore is important for more tests to be done on the different effects of enzymes on different cultivars. In conclusion, this study paves the way for the development of wine yeast starter cultures for the production of optimal levels of enzymes, which will improve the sensory quality of wine.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

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GENERAL DISCUSSION AND CONCLUSIONS

5.1 PERSPECTIVES

The yeast *Saccharomyces cerevisiae* is the most widely used industrial microorganism with GRAS (**G**enerally **R**egarded **A**s **S**afe) status and has been considered to be one of the most popular organisms for molecular genetic studies for many years. The reasons for this is that it is eukaryotic, giving it the advantage of having cellular processes, such as post-translational processing and secretion, and that it is single celled and therefore easy to manipulate in terms of culturing and genetics (Hadfield *et al.* 1993). *S. cerevisiae* is one of the best characterised yeasts and therefore can act as a model for other less characterized yeast systems. In the greater perspective, *S. cerevisiae* is the best characterised eukaryote and thus acts as an experimental model for eukaryotic research in general, since the basic functions of eukaryotic cells appear to be highly conserved throughout evolution. Another big advantage of *S. cerevisiae* is that it has been used in the food and alcohol industries for years, making it safe to use in experiments in which the end products have to be tested sensorialy.

One of the main disadvantages of *S. cerevisiae* in the food and alcoholic beverage industries is that it is unable to produce extracellular depolymerising enzymes that can efficiently liberate fermentable sugars from abundant polysaccharide-rich substrates to cost effectively yield heterologous proteins of biotechnological interest. Polysaccharide-degrading strains of *S. cerevisiae* would therefore hold an enormous advantage for the production of recombinant products, such as enzymes and vaccines, as well as for fuel, animal feed, food and alcoholic beverage industries.

5.2 DISCUSSION AND CONCLUSION

Chapter 1 of this thesis focuses on *S. cerevisiae* as an ideal host for genetic manipulation, as well as on the advantages of developing a polysaccharolytic strain of *S. cerevisiae*. It has been suggested that polysaccharolytic yeast strains could make a positive contribution to the quality of wine through the extraction of more colour and flavour from the grape skins. These yeast strain will also lower the total cost of the whole winemaking process by making the wines easier to clarify and filter without the addition of industrial enzyme preparations. Another focus of Chapter 1 is the enzymes produced by non-*Saccharomyces* yeasts found on grapes and in wine cellars. The reason for this was to look at the enzymes that are produced and the possible effects that they could have on wine during the initial stages of winemaking. Another reason was to look at the possibility of transforming *S. cerevisiae* with some of the genes encoding for these enzymes.

The first part of Chapter 2 focuses on the structures and hydrolysis of cellulose, as well as the use of endoglucanases in wine. Cellulose, the most abundant renewable

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polymer in nature (Béguin 1990), is a linear polymer consisting of D-anhydroglucopyranose units linked by β-1,4-glycosidic bonds (Lamed and Bayer 1998). For the degradation of crystalline cellulose, the microorganism must have a multicomponent enzyme complex (Finch and Roberts 1985) consisting of exoglucanases, endoglucanases and β-glucosidases. The endoglucanases attack the cellulose chain randomly and split β-1,4-glycosidic linkages, whereas exoglucanases (cellobiohydrolases) release cellobiose from the non-reducing end of cellulose (Bisaria and Mishra 1989). The cellobiose is then hydrolysed to glucose by β-glucosidases. Endoglucanases in wine pose a solution for the problem of different types of glucans in wine, especially the beta-glucan produced by *Botrytis cinerea*. The endoglucanases degrade these glucans to different degrees, making filtration a lot quicker and easier.

The second part of Chapter 2 discusses the structure and hydrolysis of xylans. Xylan, which forms part of the group of polysaccharides called hemicelluloses (Puls and Schuseil 1993), is composed of β-D-1,4-linked D-xylopyranose units, forming a xylan backbone with side chains connected to it (Christov and Prior 1993). In hardwood, the side chains are 4-O-methyl-D-glucuronic acid (Eriksson *et al.* 1990; Puls and Schuseil 1993), whereas in softwood it can be 4-O-methylglucuronic, α-L-arabinofuranose or arabinose residues (Eriksson *et al.* 1990; Puls and Schuseil 1993). It has the same backbone in grasses, but it is more branched and contains large amounts of L-arabinofuranosyl units. The action of a number of hydrolytic enzymes is required for the hydrolysis of plant heteroxylans. Endo-1,4-β-xylanase attacks the backbone to produce branched xylo-oligosaccharides. Exo-xylanase and 1,4-β-xylosidase and other xylose-producing enzymes are needed for the further degradation to D-xylose (Biely 1993).

The third part of Chapter 2 discusses the structure of pectin, the bioconversion of pectin, as well as the use of pectinases in winemaking. The basic linear skeleton is composed of partially methylated α-D-1,4-linked galactopyranosiduronic acid residues (Chesson, 1980) and these chains can be interspaced by α-1,2-linked L-rhamnopyranosyl residues. The proportion and chemical structure of pectic substances may vary considerably, depending on the source, portion and age of the plant material. Pectin esterases and pectin depolymerases catalyse the degradation of pectin polysaccharides (Fogarty and Kelly 1983), while members of the saponifying and depolymerising groups of pectinases act together to complete the degradation of the pectin molecule (Rombouts and Pilnik 1980). Pectic enzymes have been used in winemaking since 1947 to increase juice yields, filtration rates and the rate of settling and for the clarification of wines. It was found that the treatment of red grapes sped up pigment extraction and led to less skin contact time, resulting in wines with good colour but fewer tannins.

The last part of Chapter 2 looks at the expression of heterologous glucanase-, xylanase- and pectinase-encoding genes in yeasts. The gene *END1*, which encodes endo-β-1,4-glucanase in *S. cerevisiae*, has been cloned and sequenced previously (Berger *et al.* 1989). The endoxyalanase gene, *XYN4*, encoding endo-β-1,4-xylanase in *S. cerevisiae*, has also been cloned and characterised previously (Luttig *et al.* 1997) as have

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the two pectinanase genes, *PELE* and *PEH1*, which encode pectate lyase and polygalacturonase in *S. cerevisiae* (Keen *et al.* 1984; Willis *et al.* 1987). *END1, PELE* and *PEH1* were put under the control of the *ADH1* promoter and *TRP5* terminator, while *XYN4* was put under the control of the *ADH1* promoter and *ADH2* terminator. The native signal sequence of *XYN4* mediated the secretion of endo-β-1,4-xylanase in *S. cerevisiae*, whereas the secretion of endo-β-1,4-glucanase, pectate lyase and polygalacturonase was directed by the signal sequence of the yeast mating pheromone α-factor. The polygalacturonase-encoding gene (*peh1*) from *E. carotovora* and the pectate lyase-encoding gene (*pelE*) from *E. chrysanthemi* were co-expressed in laboratory strains of *S. cerevisiae* (Laing and Pretorius 1993). The endo-β-1,4-glucanase-encoding genes and endo-β-1,4-xylanase-encoding genes were also co-expressed in laboratory strains of *S. cerevisiae* (Petersen *et al.* 1998).

Chapter 3 looks at the production of extracellular hydrolytic enzymes by non-*Saccharomyces* wine yeasts. A total of 237 isolates from *Kloeckera, Candida, Debaryomyces, Rhodotorula, Pichia, Zygosaccharomyces, Hanseniaspora* and *Kluyveromyces* were isolated from vineyards and wine cellars and screened for the production of extracellular pectinases, proteases, β-glucanases, lichenase, β-glucosidases, cellulases, xylanases, amylases and sulphite reductase activity. The production of all of the above enzymes, except β-glucosidase, was observed. The amount and range of these enzymes varied between the different isolates of the same species. It is a well known fact, that during the initial stages of wine fermentations, the growth of indigenous species of non-*Saccharomyces* yeasts takes place. It is thought that these yeasts have an influence on the chemical composition, as well as an effect on the sensory properties, of the wine. Further studies should be done to determine the activity of these enzymes in wine fermentations and to obtain a better knowledge of the physiological and metabolical features of non-*Saccharomyces* yeasts.

Chapter 4 describes the subcloning of the *B. fibrisolvens* endo-β-1,4-glucanase gene (*end1*), the *A. niger* endo-β-1,4-xylanase gene (*xyn4*), the *E. carotovora* polygalacturonase gene (*peh1*) and the *E. chrysanthemi* pectate lyase gene (*pelE*) into yeast integrating plasmids containing the dominant selectable Geneticin G418-resistance and Sulphumetronmethyl-resistance markers respectively. Both yeast integrating plasmids were then transformed separately in a *S. cerevisiae* industrial strain. The presence of the *PELE, PEH1, END1* and *XYN4* transcripts, together with activity on polygalacturonic acid, barley β-glucan and xylan agarose plates, indicated the extracellular production of biologically active pectate lyase, polygalacturonase, endoxylanase and endoglucanase by the *S. cerevisiae* transformants. Wine made using these two transformants, one secreting endoglucanase and endoxylanase and the other secreting polygalacturonase and pectate lyase, showed the positive effects of these enzymes.

In conclusion, these polysaccharase-encoding strains of *S. cerevisiae* showed definite effects during the winemaking process. They also showed different effects on different cultivars. This study has participated to pave the way for further projects of research on the

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effects of these polysaccharase-encoding strains on different cultivars, as well as for studies to find the reasons for these different effects. If these issues can be resolved, very few external enzymes will have to be added during the winemaking process in the future. The yeast itself can then produce the enzyme necessary for the different processes. By using different promoters and secretion signals, as well as different amounts of plasmid copies in the yeast, the secretion of these enzymes can be regulated under different circumstances. This could give to the winemaker more control over the enzymes that should be secreted from the yeast into the wine, reducing indeed the cost of vinification, as there will be fewer need for industrially produced enzymes for oenological application.

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