Analysis of endo-polygalacturonase activity in a recombinant yeast containing a reconstituted *PGU1* gene

by

Herine van Wyk

Thesis presented in partial fulfilment of the requirements for the degree of
Master of Science

at
Stellenbosch University
Institute for Wine Biotechnology, Faculty of AgriSciences

Supervisor: Dr Benoit Divol

March 2009

Declaration

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

Date: 05/12/2008

Copyright © 2009 Stellenbosch University
All rights reserved

Summary

The *PGU1* gene encodes an endo-polygalacturonase, an enzyme that degrades pectin. Although the presence and function of this gene is well characterized in *Saccharomyces cerevisiae*, its regulation is very complex and not yet fully understood. Yeast producing a highly active polygalacturonase (PG) during alcoholic fermentation could potentially improve filtration and turbidity and also enhance extraction of certain aroma compounds. This could replace the addition of expensive commercial enzyme preparations that often contain unwanted enzymes.

The first objective of this study was to evaluate *PGU1* expression in recombinant strains of *S. cerevisiae* that originally lacked the *PGU1* gene. A functional *PGU1* gene and its promoter were successfully re-introduced into their native position in the genomes of five wine strains. Three of these strains recovered PG activity while two did not transcribe the gene and subsequently lacked activity. The three strains that recovered activity were used in microvinification experiments to determine the effect of PG-producing yeast on the aroma profile of the wine. No significant differences were observed in the volatile compounds production between the recombinants and their respective wild types, but some tendencies arose, especially for the monoterpene geraniol.

The second objective of this study was to analyze the *PGU1* gene and promoter from *Saccharomyces paradoxus* RO88 (a strain that exhibits high PG activity) and to compare it to those of *S. cerevisiae* S288C in order to identify differences that could potentially be responsible for the difference in their PG activities. Comparison of the gene sequences revealed several amino acid differences, one of which was in the peptide secretion signal. Analyses of the promoters also indicated some potentially important differences. Furthermore, *S. cerevisiae* strain VIN13, RO88 as well as two interspecies hybrids (all displaying varying PG activities) were compared under winemaking conditions. Clear differences were observed for the production of certain compounds. RO88 and the hybrids produced higher concentrations of certain volatile compounds, although they were not strong fermenters. Two recombinants, each containing a *PGU1*-overexpressing plasmid (one with the *PGU1* gene from *S. paradoxus* and the other from *S. cerevisiae*), were also used in vinification to determine the effects of the different *PGU1* gene on the aroma profile of the wine. Unfortunately, the plasmids were unstable and lost during the fermentation. Nevertheless, some tendencies were observed that indicated possible higher production of certain compounds by the recombinants compared to their wild types.

This study identified that regulation of the *PGU1* gene differs between strains with different genetic backgrounds. Certain differences were observed in the *PGU1* gene and promoter

sequences between *S. cerevisiae* and *S. paradoxus* that could potentially be the reason for the difference in their PG activities. From an oenological point of view, the presence of *PGU1* in the genome of a fermenting strain tends to increase the aromatic potential of wine. These results provide a good platform for further studies on the *PGU1* gene.

Opsomming

Die *PGU1* geen kodeer vir ‘n endo-polygalakturonase, ‘n ensiem wat pectien afbreek. Alhoewel die teenwoordigheid van hierdie geen goed gekaraktiriseer is in *Saccharomyces cerevisiae*, is die regulering daarvan uiters kompleks en gedeelteliks onbekend. ‘n Gis wat ‘n hoogs aktiewe polygalakturonase (PG) tydens alkoholiese fermentasie produseer, kan potensieël die filtrasie en turbiditeit van die wyn verbeter asook die ekstraksie van sekere aromatiese verbindings verhoog. Dit kan moontlik ook die byvoeging van duur kommersiële ensiem-voorbereidings, wat gewoonlik ander ongewenste ensieme bevat, vervang.

Die eerste objektief van hierdie studie was om die uitdrukking van *PGU1* in rekombinante giste van *S. cerevisiae*, wat nie oorspronklik die geen bevat het nie, te evalueer. ‘n Funksionele *PGU1* geen en promotor is suksesvol in hul oorspronklike lokus geintegreer in die genome van vyf wyngisrasse. PG aktiwiteit is herstel in drie van hierdie gisrasse terwyl die ander twee nie die geen uitgedruk het nie en gevolglik geen aktiwiteit getoon het nie. Die gisrasse waarin aktiwiteit herstel is, is gebruik in klein-skaalse fermenteringseksperimente om die effek van ‘n PG-produserende gis op die aromatiese profiel van die wyn te evalueer. Geen beduidende verskille is opgemerk in die produksie van vlugtige aromakomponente tussen die rekombinante en hul respektiewe wilde tipe rasse nie, maar sekere neigings is wel opgemerk.

Die tweede objektief was om die *PGU1* geen en promotor van *S. paradoxus* RO88 (‘n gisras wat hoë PG aktiwiteit toon) te analiseer en te vergelyk met díe van *S. cerevisiae* S288C met die doel om verskille te identifiseer wat moontlik die oorsaak is vir die verskil in PG aktiwiteite tussen die twee rasse. Vergelyking van die twee geenvolgordes het verskeie verskille getoon, waarvan een in die sekresiesein van die geen was. ‘n Paar verksille is ook gevind tussen die promoters wat moontlik belangrik kan wees. Verder is *S. cerevisiae* VIN13, RO88 en twee interspesiehibriede (waarvan die PG aktiwiteite almal verskil) vergelyk onder wynmaak-toestande. Duidelike verskille is opgemerk vir die produksie van sekere verbindings. RO88 en die hibriede het hoër konsentrasies van sommige verbindings produseer, alhoewel hulle nie sterk fermenteerders is nie. Twee rekombinante, wat elk ‘n *PGU1*-ooruitdrukkingsplasmied bevat (een met die *PGU1* geen van *S. paradoxus* en die ander die *S. cerevisiae* geen), is ook gebruik in kleinskaalse fermentasies om die effek van die verskillende *PGU1* gene op die aromaprofiel van wyn te evalueer. Ongelukkig was die plasmiede onstabiel en is verlore tydens die fermentasie. Nietemin is neigings wel waargeneem vir hoër produksie van sekere verbindings deur die rekombinante in vergelyking met hul wilde tipes.

Hierdie studie het geidentifiseer dat die *PGU1* geen verskillend gereguleer word in gisrasse met verskillende genetiese agtergronde. Sommige verskille is opgemerk tussen die *PGU1* gene en

promoters van *S. cerevsiae* en *S. Paradoxus* wat potensieël die oorsaak kan wees vir hul verskillende PG aktiwiteite. Vanuit 'n wynkundige oogpunt blyk dit of die teenwoordigheid van die *PGU1* geen in 'n fermenterende gis die aromatise profiel van die wyn verbeter. Hierdie resultate dien as 'n goeie basis vir toekomstige studies van die *PGU1* geen.

This thesis is dedicated to my parents

Hierdie tesis is opgedra aan my ouers

Biographical sketch

Herine van Wyk was born in Bellville, South Africa on the 18th of January 1984. She attended Eversdal Primary School and matriculated from Stellenberg High School in 2002.

Herine enrolled at Stellenbosch University in 2003 and obtained a BSc degree in Molecular and Cellular Biology in 2005. In 2006, she obtained the degree HonsBSc (Wine Biotechnology) at the Institute for Wine Biotechnology, Stellenbosch University where she also enrolled for an MSc degree in 2007.

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following persons and institutions:

- **VINCENT VAN EMMENIS**, for his love, support, patience and encouragement.
- **MY PARENTS**, for their love and the opportunities they presented me.
- **DR BENOIT DIVOL**, for acting as my supervisor, guidance, time and valuable inputs throughout my studies.
- **STAFF AND STUDENTS** at the Institute for Wine Biotechnology, for their assistance and inputs in various fields.
- **MY FAMILY** and **FRIENDS**, for believing in me.
- The **NATIONAL RESEARCH FOUNDATION**, **WINETECH**, **THRIP** and the **INSTITUTE FOR WINE BIOTECHNOLOGY** for financial support.
- **THE ALMIGHTY**, for making all this possible.

Preface

This thesis is presented as a compilation of 6 chapters. Each chapter is introduced separately and is written according to the style of the journal FEMS Yeast Research to which Chapter 4 will be submitted for publication.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**
Endo-polygalacturonases produced by *Saccharomyces* spp.: production, function and potential biotechnological applications in wine

Chapter 3 **Research results**
Overexpression of *PGU1* and *TEC1* in various *Saccharomyces cerevisiae* strains and its effect on polygalacturonase activity

Chapter 4 **Research results**
Recovery of endo-polygalacturonase activity in wine yeast and its effect on wine aroma

Chapter 5 **Research Results**
Comparison of endo-polygalacturonase activity between *Saccharomyces cerevisiae* and *Saccaromyces paradoxus*

Chapter 6 **General discussion and conclusions**

Contents

Chapter 1. General introduction and project aims **1**

1.1 General introduction **1**
1.2 Project aims **2**
1.3 References **2**

Chapter 2. Literature review: Endo-polygalacturonases produced by *Saccharomyces* Spp.: production, function and potential biotechnological applications in wine **4**

2.1 Introduction **4**
2.2 Wine yeast and alcoholic fermentation **4**
2.3 Pectic substances **6**
2.3.1 Structure 6
2.3.2 Presence in grapes throughout ripening 6
2.3.3 Pre-fermentative conditions 7
2.4 Enzymes used in winemaking **7**
2.4.1 Importance of enzymes in winemaking 7
2.4.2 Pectinases 8
2.4.2.1 Diversity 8
2.4.2.2 Polygalacturonases 8
2.4.2.3 Production of pectinases by *Saccharomyces cerevisiae* 10
2.4.2.3.1 General considerations 10
2.4.2.3.2 Endo-polygalacturonase 11
2.5 The *PGU1* gene and its protein **11**
2.5.1 Gene and protein sequences 11
2.5.2 Presence in *S. cerevisiae* 12
2.6 *PGU1* transcription and regulation **13**
2.6.1 General regulation 13
2.6.2 *PGU1* promoter 15
2.7 Improvement of wine yeasts **16**
2.7.1 Targets for improvement 16
2.7.2 Ways of improving yeast 17
2.7.3 Transformation of industrial yeast strains 18
2.7.4 Removable selection markers used in industrial yeast transformation 19
2.8 Recombinant *PGU1* strains (previous studies on yeast) **20**
2.9 GMOs and the consumer **21**
2.10 General conclusions **22**
2.11 References **23**

Chapter 3. Research results: Overexpression of *PGU1* in various *Saccharomyces cerevisiae* strains and its effect on polygalacturonase activity **27**

3.1 Introduction **27**
3.2 Materials and methods **28**
3.2.1 Strains, media and culture conditions 28
3.2.2 Isolation of plasmid and genomic DNA 29
3.2.3 PCR amplification 29
3.2.4 Construction of the plasmids 29
3.2.5 Bacterial transformation 31
3.2.6 Yeast transformation 31
3.2.7 Polygalacturonase plate assay 32
3.3 Results and discussion **32**
3.3.1 Plasmid construction 32
3.3.2 Yeast transformation 33
3.3.3 Determination of polygalacturonase activity 33
3.4 Conclusions **35**
3.5 References **36**

Chapter 4. Research results: Recovery of endo-polygalacturonase activity in wine yeast and its effect on wine aroma **38**

4.1 Introduction **38**
4.2 Materials and methods **40**
4.2.1 Strains, media and culture condition 40
4.2.2 Isolation of genomic DNA 40
4.2.3 PCR amplification 41
4.2.4 Construction of the plasmids 42
4.2.5 Bacterial transformation 43
4.2.6 Sequencing 43
4.2.7 Yeast transformation 44
4.2.8 Identification of positive transformants 44
4.2.9 Polygalacturonase plate assay 45
4.2.10 RNA isolation and reverse transcription 45
4.2.11 Microvinifications 46
4.2.12 Extraction of wine components 47
4.3 Results and discussion **48**
4.3.1 Construction of plasmids 48
4.3.2 Yeast transformation 50
4.3.3 Evaluation of transcription and enzyme activity 52
4.3.4 Microvinifications 54
4.3.5 Analyses of aroma compounds in wine 56
4.4 Conclusions **58**
4.5 Acknowledgements **60**
4.6 References **60**

Chapter 5. Research results: Comparison of endo-polygalacturonase activity between *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* 62

5.1 Introduction **62**
5.2 Materials and methods **63**
5.2.1 Strains, media and culture conditions 63
5.2.2 Isolation of plasmids and genomic DNA 64
5.2.3 PCR amplification 64
5.2.4 Sequencing 65
5.2.5 Construction of the plasmids 65
5.2.6 *E. coli* transformation 66
5.2.7 Yeast transformation 66
5.2.8 Identification of transformants 66
5.2.9 Polygalacturonase plate assay 66
5.2.10 Liquid polygalacturonase assay 67
5.2.11 Microvinifications 67
5.2.12 Extraction of wine components 68
5.3 Results and discussion **69**
5.3.1 PCR amplification 69
5.3.2 Sequences of the *S. paradoxus PGU1* gene and promoter 69
5.3.3 Obtaining recombinant yeasts 72
5.3.4 Polygalacturonase assays 72
5.3.5 Microvinifications 74
5.3.6 Analyses of aroma compounds from wine 76
5.4 Conclusions **81**
5.5 References **82**

Chapter 6. General discussion and conclusions 84

6.1 Discussion, conclusions and future prospective **84**
6.2 References **88**

Chapter 1

Introduction and project aims

1. General introduction and project aims

1.1 General Introduction

Pectin is the name given to a group of diverse and complex heteropolysaccharides. It basically consists of a D-galacturonic acid backbone to which a variety of complex side chains are attached. Pectinases are enzymes that degrade pectin and are divided into classes according to their mode of action. Endo-polygalacturonases specifically cleave the links between non-methylated D-galacturonic acid residues by acting randomly within the chain (Kester *et al*., 1996). Polysaccharides (such as pectin, glucan and xylan) are found in wine at concentrations between 300 – 1000 mg L^{-1} and are often responsible for turbidity, viscosity and filter stoppages (Van Rensburg & Pretorius, 2000). Natural pectinases in wine (derived from grapes and micro-organisms) have low activity under winemaking conditions. For this reason, commercial enzyme preparations are often added. These enzymes are produced commercially by fungi and often contain other unwanted enzymes (Whitaker, 1984; Blanco *et al*., 1997; Whightman *et al*., 1997). The production of pure petinases by yeast would therefore be advantageous.

The *PGU1* (*PGL1*) gene encodes an endo-polygalacturonase in *Saccharomyces cerevisiae*. It was first believed that *S. cerevisiae* does not produce pectinases (Luh & Phaff, 1951). However, in 1956, polygalacturonase (PG) activity was detected in certain strains (Bell & Etchells, 1956). Since then, the presence the *PGU1* gene and PG activity has been characterized in various strains. Most strains of *S. cerevisiae* possess the *PGU1* gene, except a few that were identified to lack it (Fernández-González *et al*., 2004; Radoi *et al*., 2005; Divol & van Rensburg, 2007). The latter authors also indicated that the *PGU1* gene was replaced by a partial mobile element in these strains. Despite the presence of the *PGU1* gene, PG activity still varies between strains. Some strains possess the gene, yet lack the ability to degrade pectin (Fernández-González *et al*., 2004). Certain strains that do not degrade pectin, are able to when *PGU1* is expressed on a plasmid (Blanco *et al*., 1998) suggesting a non-functional gene or promoter in their genomes. However, no (or very little) difference was observed in the gene and promoter sequences between strains exhibiting PG activity and those that do not (Hirose *et al*., 1998; Hirose *et al*., 1999). The latter authors proposed regulation of the *PGU1* gene through a trans-acting factor inhibiting *PGU1* transcription. Regulation of *PGU1* is very complex. It is regulated through a mitogen-activated protein kinase (MAPK) pathway (Madhani *et al*., 1999) and linked to filamentation and invasive growth (Gognies *et al*., 2001). The Tec1 protein was identified as the main transcription factor of *PGU1* (Madhani *et al*., 1999). The *PGU1* promoter indeed contains several binding sites for the Tec1p (Köhler *et al*., 2002).

The effects of yeast-derived polygalacturonases on wine have been studied. Blanco *et al.* (1997) used strains exhibiting PG activity to perform alcoholic fermentation and found a decreased filtration time in some cases. A recombinant *S. cerevisiae* strain, expressing *PGU1* on a multi-copy plasmid under control of the *PGK1* promoter, was found to reduce the filtration time and increase the concentration of a few volatile compounds in the wine (Vilanova *et al*., 2000). Another group integrated the *PGU1* gene under control of the *PGK1* promoter into the genome of a yeast that lacks PG activity. On the contrary, these authors observed no decrease in filtration time or increase in aroma compounds (Fernández-González *et al*., 2005). *S. paradoxus* strain RO88 has also been evaluated under winemaking conditions and found to possess good oenological properties. The strain also influenced the production of certain aroma compounds and contributed to the final quality of the wine (Madjak *et al*., 2002). Additionally, RO88 exhibits high PG activity (Mocke, 2005).

1.2 Project aims

The reason for the deletion of the *PGU1* gene in certain strains remains unknown. It could be a way of silencing the gene as part of directed evolution, or it could simply be a random phenomenon. The fundamental aim of this study was to re-introduce the *PGU1* gene into its native position in strains lacking the gene in order to determine if this will allow these strains to recover PG activity. From an oenological point of view, the study also aimed at determining the effects of a good oenological strain with PG activity on the aroma compounds in wine. A yeast that secretes an active endo-polygalacturonase under winemaking conditions could alter the final aroma profile of the wine (Pretorius, 2000) and potentially eliminate the need to add expensive commercial enzyme preparation.

S. paradoxus was found to perform well during alcoholic fermentation and alter the aroma profile of the wine (Madjak *et al*., 2002). A further objective of this study was to analyze the *PGU1* gene and promoter from *S. paradoxus* RO88 and compare it to those of *S. cerevisiae* in order to determine the possible reason for the difference observed in their PG activities. RO88 was also evaluated under winemaking conditions to confirm previous results and to compare it to *S. cerevisiae* as well as two interspecies hybrids (Mocke, 2005).

1.3 References

Blanco P, Sieiro C, Díaz, A Reboredo NM & Villa TG (1997) Grape juice Biodegradation by polygalacturonases from *Saccharomyces cerevisiae*. *Int Biodeterior Biodegradation* **40:** 115-118.

Blanco P, Sieiro C, Reboredo NM & Villa TG (1998) Cloning, molecular characterization, and expression of an endo-polygalacturonase-encoding gene from *Saccharomyces cerevisiae* IM1-8b. *FEMS Microbiol Lett* **164:** 249-255.

Bell TA & Etchells JL (1956) Pectin hydrolysis by certain salt-tolerant yeasts. *Appl Microbiol* **4:** 196-201.

Divol B & van Rensburg P (2007) *PGU1* natural deletion is responsible for the absence of endo-polygalacturonase activity in some wine strains of *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7:** 1328-1339.

Fernández-González M, Úbeda JF, Vasudevan TG, Cordero Otero RR & Briones AI (2004) Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains. *FEMS Microbiol Lett* **237:** 261-266.

Fernández-González M, Úbeda JF, Cordero-Otero RR, Thanvanthri V & Briones AI (2005) Engineering of an oenological *Saccharomyces cerevisiae* strain with pectinolytic activity and its effect on wine. *Int J Food Microbiol* **102:** 173-183.

Gognies S, Simon G & Belarbi A (2001) Regulation of the expression endopolygalacturonase gene *PGU1* in *Saccharomyces*. *Yeast* **18:** 423-432.

Hirose N, Fujii M, Kishida M, Kawasaki H & Sakai T (1999) Isolation and genetic determination of a *Saccharomyces cerevisiae* mutant that produces an endo-polygalacturonase. *J Biosci Bioeng* **87:** 594-597.

Hirose N, Kishida M, Kawasaki, H & Sakai T (1998) Molecular cloning and expression of a polygalacturonase gene in *Saccharomyces cerevisiae*. *J Ferment Bioeng* **86:** 332-334.

Kester HCM, Kusters-Van Someren MA, Müller Y & Visser J (1996) Primary structure and characterization of an exopolygalacturonase from *Aspergillus tubingensis*. *Eur J Biochem* **240:** 738-746.

Köhler T, Wesche S, Taheri N, Braus GH & Mösch H (2002) Dual role of the *Saccharomyces cerevisiae* TEA/ATTS family transcription factor Tec1p in regulation of gene expression and cellular development. *Eukaryot cell* **1:** 673-686.

Luh BS & Phaff HJ (1951) Studies on polygalacturonase of certain yeasts. *Arch Biochem Biophys* **33:** 213-227.

Madhani HD, Galitski T, Lander ES & Fink GR (1999) Effectors of a developmental mitogen-activated protein kinase cascade revealed by expression signatures of signaling mutants. *Proc Natl Acad Sci USA* **96:** 12530-12535.

Majdak A, Herjavec S, Orlić S, Redžepović S & Miroševic N (2002) Comparison of wine aroma compounds produced by *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* strains. *Food Technol Biotechnol* **40:** 103-109.

Mocke BA (2005) The breeding and characterisation of starter culture yeast strains for red wine making. MSc Thesis, Stellenbosch University, Stellenbosch.

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16:** 675-729.

Radoi F, Kishida M & Kawasaki H (2005) Endo-polygalacturonase in *Saccharomyces* wine yeasts: effect of carbon source on enzyme production. *FEMS Yeast Res* **5:** 663-668.

Van Rensburg P & Pretorius IS (2000) Enzymes in winemaking: Harnessing natural catalysts for efficient biotransformations – a review. *S Afr J Enol Vitic* **21:** 52-73.

Vilanova M, Blanco P, Cortés S, Castro M, Villa TG & Sieiro C (2000) Use of a *PGU1* recombinant *Saccharomyces cerevisiae* strain in oenological fermentations. *J Appl Microbiol* **89:** 876-883.

Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. *Enzyme Microb Technol* **6:** 341-349.

Wightman JD, Price SF, Watson BT & Wrolstad RE (1997) Some effects of processing enzymes on anthocyanins and phenolics in Pinot Noir and Cabernet Sauvignon wines. *Am J Enol Vitic* **48:** 39-49.

Chapter 2

Literature review

Endo-polygalacturonases produced by *Saccharomyces* spp.: production, function and potential biotechnological applications in wine

2. Literature review

2.1 Introduction

The polysaccharide content in wines can result in certain processing problems such as filter stoppages and viscosity. Since pectinases (enzymes that degrade pectic substances) have relatively low activity under winemaking conditions, commercial enzymes are usually added by the winemaker. These fungi-derived enzymes are often not pure and may have certain side effects in the wine. A solution to this would be the production of pure enzymes by the yeast strain conducting alcoholic fermentation. This property can be introduced into wine yeast through genetic engineering. Due to strict legislation and consumer distrust of Genetically Modified Organisms (GMOs), yeast strains have generally been improved through classical breeding techniques. These techniques all have their limits that can be overcome by using well-established biotechnological methods to improve wine yeast. All the above-mentioned aspects will be discussed in this review.

2.2 Wine yeasts and alcoholic fermentation

Wine is the alcoholic beverage obtained by fermenting grape juice (also referred to as must). The basic steps of winemaking are presented in Fig. 1.

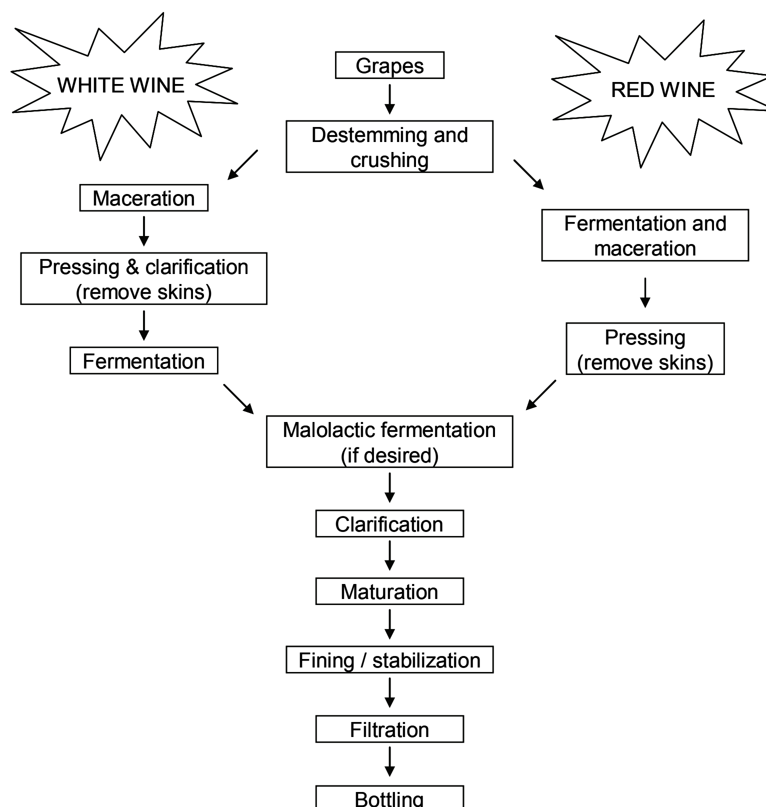

Fig. 1. The main steps of winemaking (adapted slightly from Pretorius, 2000).

In most wineries, the fermentation nowadays is conducted by a specific yeast strain selected by the winemaker for its fermentation ability and specific contribution to the aroma profile of the wine. These wine strains are more resistant than other yeast to the unfavourable conditions of winemaking such as low temperature, low pH, high initial sugar concentration, high alcohol at the end of fermentation as well as the presence of various inhibitors such as SO_2. A yeast strain also contributes to the wine aroma profile and its overall quality by producing more compounds that contribute positively to the wine (such as higher alcohols and esters) and less unwanted compounds (such as H_2S). These compounds are produced through metabolism of sugar, amino acids and sulphur by the yeast (Pretorius, 2000) as indicated in Fig. 2. For this reason, it is common practice nowadays to inoculate the wine must with a specific, pure yeast culture to ensure its dominance during alcoholic fermentation and the production of a desired, "reproducible" wine. Because the yeast plays such an essential role in winemaking, the development of new yeast strains is an important research field to ensure the production of top-quality and competitive wines in agreement with the consumers' taste.

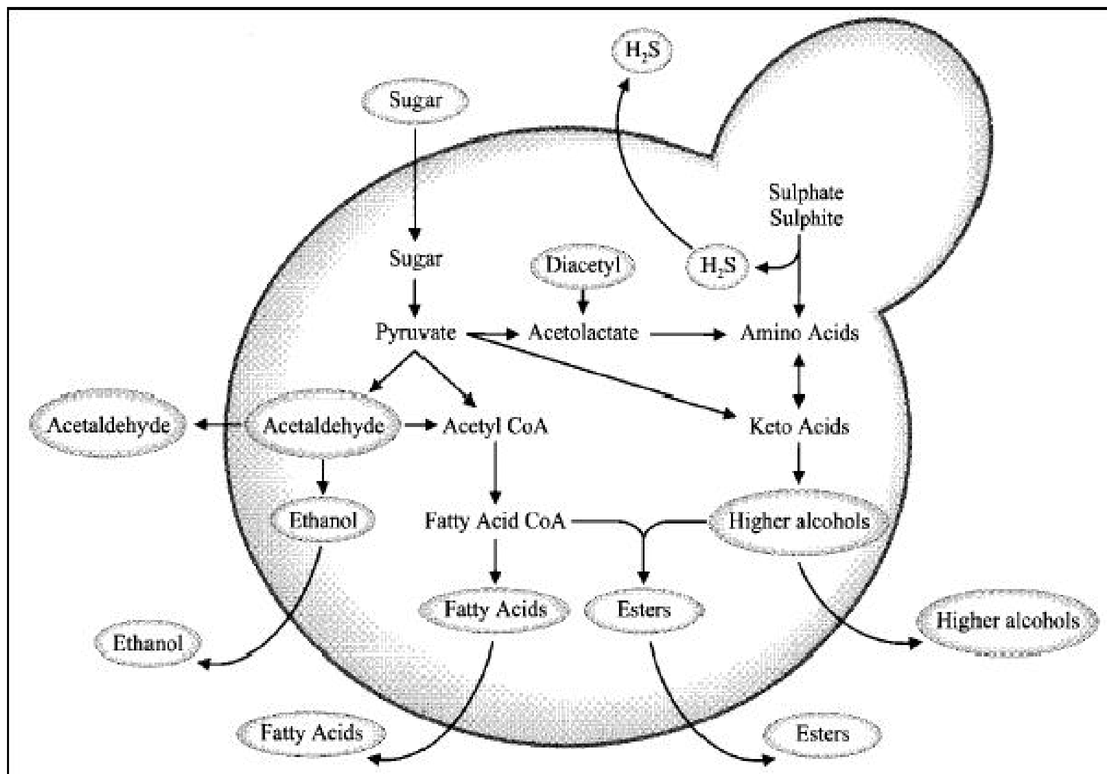

Fig. 2. Schematic representation of aroma compounds produced by yeast during fermentation (Pretorius, 2000).

2.3 Pectic substances

2.3.1 Structure

Pectin refers to a group of heteropolysaccharides that are quite diverse and very complex. It is present in the cell walls of plants, including the primary cell wall of grapes. Structurally, it can be divided into two regions (De Vries & Visser, 2001): The first is called the “smooth” region. It consists of a backbone of D-galacturonic acid residues joined by α-1,4-linkages and is also known as homogalacturonan or polygalacturonic acid. The second region, the “hairy” region, consists of two polysaccharides. The first is called rhamnogalacturonan I. The homogalacturonan skeleton is interrupted by L-rhamnose residues which usually have chains of neutral sugars, such as arabinose and glucose, attached to it. The D-galacturonic acid residues can also be methylated or acetylated. The second polysaccharide in this region was confusingly named rhamnogalacturonan II because its backbone does not contain L-rhamnose residues as the name suggests. It consists of a homogalacturonan backbone with complex and structurally diverse side chains attached to the D-galacturonic acid residues. Although this is the commonly accepted structure of pectin, an alternative was proposed by Vincken *et al*. (2003). They suggested that homogalacturonan was not part of the pectin backbone, but rather a side chain of rhamnogalacturonan I.

The D-galacturonic acid residues in homogalacturonan may also be methyl-esterified or acetylated. The degree of this esterification and acetylation has a large impact on the functional properties of the pectin molecule (Willats *et al*., 2006). The fine structures of pectin vary considerably, even within a cell wall. In rhamnogalacturonan I, the length of the side chains as well as the sugar composition vary tremendously. In contrast, rhamnogalacturonan II seems to have a relatively conserved structure (Willats *et al*., 2006). Pectin is, indeed, a very complex polysaccharide.

2.3.2 Presence in grapes throughout ripening

Initially, at the beginning of grape development, the cell wall consists primarily of cellulose. At *véraison*, a considerable amount of pectin is synthesized. These pectin molecules are usually highly methylated. During maturation of the berry, the insoluble pectins are solubilized through various enzymatic reactions. This leads to an increase in the soluble pectin content. The methyl groups are removed by pectin methyl esterases, leaving the pectin molecule available for degradation by other enzymes such as polygalacturonases and pectin-lyases (Ribéreau-Gayon *et al*., 2006a). As the berry ripens, some of the pectin chains are hydrolyzed and the pectin content decreases towards the end of ripening. In comparison to other fruits, ripe grapes have

relatively low concentrations of pectic substances. In some cases however, the grapes lack endogenous pectinase activity and the concentration of soluble pectic substances increases throughout ripening (Ribéreau-Gayon *et al*., 2006b).

2.3.3 Pre-fermentative conditions

Pectic substances are mostly in the cell walls of the skins and thus relatively small amounts of soluble pectins are found in the must. Harvest treatments can extract some insoluble pectins which are then usually degraded by grape pectinases. Additionally, during solid maceration, enzymes act on various macromolecules of the grapes. The enzymes are however not always highly active due to the duration of pre-fermentative treatments and sub-optimal conditions such as the pH or SO_2 concentrations (Ribéreau-Gayon *et al*., 2006a). For this reason, additional enzymes are added by the winemaker.

2.4 Enzymes used in winemaking

2.4.1 Importance of enzymes in winemaking

Polysaccharides (pectin, glucan and xylan) can be found in wine at concentrations between 300 – 1000 mg L^{-1}. They are often responsible for turbidity, viscosity and filter stoppages (Van Rensburg & Pretorius, 2000). Pectinases from grapes and wine-related micro-organisms have low activity under winemaking conditions. For this reason, it is common practice nowadays to add commercial enzyme preparations during the winemaking process. Normally, these enzymes are added after pressing to improve clarification. Alternatively, they can be added before pressing to improve juice and colour yield and possibly also enhance the extraction of aroma compounds or precursors. The addition of enzymes during winemaking is an old practice. Cruess *et al*. (1951) studied the effect of pectic enzyme treatments from 1936 – 1950. They observed several improvements in the wine. These include a higher juice yield, an effect on the colour of white juice/wine, faster maturation of wines, more compact lees, shortened filtration times, lower foam formation and less bentonite needed for clarification.

The enzymes approved for commercial use are produced by fungi (most by *Aspergillus niger).* Three of the major enzymes that are available (hemicellulases, glucanases and glycosidases) are usually sold as blends with pectinases. The type of enzymes used depends on the type of wine that is made (Lourens & Pellerin, 2004). For white wine, settling enzymes are added after crushing. These are pectinases that break the large pectin molecules into smaller components and help particles to sediment. They act mainly on the insoluble pectins (mostly homogalacturonan) in the pulp. White wines undergoing short skin contact before alcoholic

fermentation contain more complex pectin molecules that are extracted from the skins. The enzyme preparations used in this case are more concentrated and have more side activities, to degrade the complex side chains, than the "normal", commonly used settling enzymes. Red skin contact enzymes differ from white skin contact enzymes in that they have a higher concentration of hemicellulases for improved maceration. In the case of application in red wine, it is also important that these preparations have low or no anthocyanase activity which may cause colour instability in the wine.

Commercial preparations often contain other enzymes such as arabinofuranosidase that causes turbidity (Whitaker 1984), pectin methyl esterase (PME) that produces the toxic alcohol methanol (Blanco *et al*., 1997a), and ß-glycosidase that cleaves the sugar from anthocyanins, leading to colour loss in red wine (Wightman *et al*., 1997). The production of pure, yeast-derived pectinases will be advantageous to the winemaking process. Yeasts usually do not produce PME and can therefore be used without the production of methanol (Fernández-González *et al*., 2004).

2.4.2 Pectinases

2.4.2.1 Diversity

Pectinases, also referred to as pectic, pectolytic or pectinolytic enzymes, play an important role in the juice and food (Kashyap *et al.*, 2001) as well as the paper and pulp (Viikari *et al*., 2001) industries and have been reported to account for 25% of the world's food enzyme sales (Jayani *et al*., 2005). They are also the main enzymes used in winemaking.

Pectinases can be divided into two main classes: esterases and depolymerases (Table 1). Esterases release the methoxyl residues from the galacturonic acid units (Blanco *et al*., 2002) while the depolymerases act on the main chain. Depolimerases are sub-divided into hydrolases and lyases according to their mechanism of degradation. Hydrolases act by hydrolysis, which is the cleavage of bonds with the introduction of a water molecule. Lyases degrade the chain through trans-elimination lysis by breaking glycosidic bonds without the participation of water molecules (Codner, 2001). Depending on the pattern of action, enzymes are termed endo or exo. Endo-acting enzymes act randomly within the chain while exo-acting enzymes reduce the chain by acting on the terminal residues (Jayani *et al.,* 2005).

2.4.2.2 Polygalacturonases

Polygalacturonases fall under the class of depolymerases. These enzymes hydrolyse the α-1,4-bonds between two non-methylated galacturonic acid residues of the pectin backbone (Fogarty & Ward, 1974). Furthermore, polygalacturonases are grouped according to the pattern by which

Table 1. Summary of the classification of pectinolytic enzymes (Jayani *et al.,* 2005).

Enzyme	Action mechanism	Action pattern	Primary substrate	Product
Esterase				
1. Pectin methyl esterase	Hydrolysis	Random	Pectin	Pectic acid + methanol
Depolymerizing enzymes				
a. Hydrolases				
1. Protopectinases	Hydrolysis	Random	Protopectin	Pectin
2. Endopolygalacturonase	Hydrolysis	Random	Pectic acid	Oligogalacturonates
3. Exopolygalacturonase	Hydrolysis	Terminal	Pectic acid	Monogalacturonates
4. Exopolygalacturonan-digalacturono hydrolase	Hydrolysis	Penultimate bonds	Pectic acid	Digalacturonates
5. Oligogalacturonate hydrolase	Hydrolysis	Terminal	Trigalacturonate	Monogalacturonates
6. Δ4:5 Unsaturated oligogalacturonate hydrolases	Hydrolysis	Terminal	Δ4:5(Galacturonate)$_n$	Unsaturated monogalacturonates & saturated (*n*-1)
7. Endopolymethyl-galacturonases	Hydrolysis	Random	Highly esterified pectin	Oligomethylgalacturonates
8. Endopolymethyl-galacturonases	Hydrolysis	Ternimal	Highly esterified pectin	Oligogalacturonates
b. Lyases				
1. Endopolygalacturonase lyase	Trans-elimination	Random	Pectic acid	Unsaturated oligogalacturonates
2. Exopolygalacturonase lyase	Trans-elimination	Penultimate bond	Pectic acid	Unsaturated digalacturonates
3. Oligo-D-galactosiduronate lyase	Trans-elimination	Terminal	Unsaturated digalacturonates	Unsaturated monogalacturonates
4. Endopolymethyl-D-galactosiduronate lyase	Trans-elimination	Random	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methyloligogalacturonates
5. Exopolymethyl-D-galactosiduronate lyase	Trans-elimination	Terminal	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methylmonogalacturonates

they break down the pectin chain. Exo-polygalacturonase act by releasing galacturonic residues from the non-reducing end of the chain (Kester *et al*., 1996), producing monomeric galacturonic acids. This results in a relatively slow reduction in the chain length. Endo-polygalacturonases attack the chain randomly and can break any non-methylated bonds within the backbone (Kester & Visser, 1990), producing a range of different sized oligomers. This results in a much faster degradation of the polysaccharide and subsequent reduction in viscosity.

2.4.2.3 Production of pectinases by *Saccharomyces cerevisiae*

2.4.2.3.1 General considerations

Many organisms have been identified to produce pectinases. At first, it was thought that *S. cerevisiae* does not produce any pectinolytic enzymes (Luh & Phaff, 1951). This was later proven wrong by the discovery that some strains indeed show pectinolytic activity (Bell & Etchells, 1956). Now the production of pectinases in *S. cerevisiae* is characterised in various strains through studies by various authors. McKay *et al*. (1990) published the first report of polygalacturonic acid degradation by two *S. cerevisiae* strains. An endo-polygalacturonase was characterized by Blanco *et al*., (1994) and comparison between two strains revealed a difference in their ability to degrade polygalacturonic acid. Gainvors *et al*. (1994) identified several pectinase activities in the strain SCPP which was isolated from Champagne wines during alcoholic fermentation. This strain was however later identified to be a *S. bayanus* strain and the enzyme activities observed should be noted with some caution. Nevertheless, it resembles *S. cerevisiae* closely as all the different physiological and molecular tests performed on this strain by Gainvors *et al*. (1994) identified it as *S. cerevisiae*.

After further investigation and characterization it was found that the production of pectinases in yeasts was not induced by pectin, polygalacturonic acid or galacturonic acid, but rather by galactose (Blanco *et al*., 1994). It was also found to be repressed by glucose and complete inhibition was observed with a glucose concentration of 2% (Blanco *et al*., 1994). The amount of dissolved oxygen in the growth medium also appears to have an influence on the production of these enzymes in certain strains of *S. cerevisiae*. For some strains the production is inhibited by moderate or higher aeration (Blanco *et al*., 1999) while in other strains it has no effect on enzyme production (McKay *et al*., 1990). This could make the anaerobic winemaking conditions favourable to the production of pectinases, depending on the strain used during fermentation. The polygalacturonase activity of *S. cerevisiae* strains are also inhibited by ethanol (Fernández-González *et al*., 2004).

2.4.2.3.2 Endo-polygalacturonase

The endo-polygalacturonase produced by *S. cerevisiae* is an extracellular enzyme (Gainvors *et al*., 1994). An endo-polygalacturonase was isolated from *S. cerevisiae* and characterized by Gainvors *et al*. (2000). According to these authors, this enzyme has an apparent molecular mass of 42 kDa and shows activity within the pH range of 3 to 5 with optimal activity at pH 4. A functional enzyme at such a low pH is particularly useful in the beverage (including wine) industry because of the acidity of fruit juice. They also found that the optimum temperature for its activity was 25°C, which was similar to that observed for endo-polygalacturonases from *Aspergillus japonicus* (Ishii & Yokotsuka, 1972) and *Lactobacillus plantarum* (Sakellaris *et al*., 1989). Furthermore, Gainvors *et al*. (2000) found the enzyme to be a glycoprotein with an *N*-linked carbohydrate moiety. They observed that the deglycosylated protein still possessed endo-polygalacturonase activity. Although the exact function of this moiety is unknown, it is thought to be necessary for the secretion of the enzyme as was observed for *Fusarium oxysporum* (Di Pietro & Roncero, 1996). Several *S. cerevisiae* strains have been identified that produce an endo-polygalacturonase (Blanco *et al*., 1994; Blanco *et al*., 1999; Gainvors *et al*., 1994) which might be of particular interest to the winemaking industry.

2.5 The *PGU1* gene and its protein

2.5.1 Gene and protein sequences

The *PGU1* gene (also known as *PGL1*) encodes for an endo-polygalacturonase in *S. cerevisiae*. The coding region of the gene is 1086 bp long and the whole protein consists of a total of 361 amino acids (Blanco *et al*., 1998; Gognies *et al*., 1999). The first 18 amino acids represent a peptide secretion signal that directs the protein to be transported out of the cell (Blanco *et al*., 1998). The latter authors also compared the amino acid sequence of *S. cerevisiae* Pgu1p to that of other organisms. They found a 24% similarity to polygalacturonases from plants and bacteria and 54% similarity to that of fungi. Similar results were obtained by Gognies *et al*. (1999). The putative active site in *S. cerevisiae* is a histidine residue at position 222 and two possible N-glycosylation sites are found at residues 318 and 330 (Blanco *et al*., 1998). Through site-directed mutagenesis, certain amino acid residues were identified as important for polygalacturonase activity (Blanco *et al*., 2002). These are highly conserved amino acid residues at positions 179, 200, 201 and 222. The results corresponded to certain important amino acids identified in the filamentous fungus *Aspergillus niger* (Van Santen, 1999; Armand, 2000).

2.5.2 Presence in *S. cerevisiae*

The *PGU1* gene is present in most strains of *S. cerevisiae*. A single copy of the gene is present per haploid genome and it is situated on chromosome X (Blanco *et al*., 1998). The gene has been isolated from several strains by different research groups. It was found that most strains possess the gene, although not all are able to degrade polygalacturonic acid. Fernández-González *et al*. (2004) screened 61 strains and found 52 to have the *PGU1* gene in their genomes. Of these strains, only 36 showed polygalacturonase (PG) activity. Some strains that do not degrade polygalacturonic acid naturally were found to do so when *PGU1* was expressed on a plasmid under control of the *PGK1* or *GAL1* promoter (Blanco *et al*., 1998). These authors suggested that these strains possess a non-functional *PGU1* gene or promoter in their genomes, preventing them from producing a functional enzyme. However, genes cloned from strains that produce endo-polygalacturonase (PG+) and those that do not (PG-) were found to have no (Hirose *et al*., 1998) or very small (Gognies *et al*., 1999) differences in their sequences. They concluded that the gene sequence itself is not responsible for the lack of endo-polygalacturonase activity in certain strains, but rather the regulation of the gene. Jia & Wheals (2000) also tested several strains from *S. cerevisiae* and *Kluyveromyces marxianus* and found that all strains that expressed the *PGU1* gene also secreted a functional enzyme. Despite several studies on the *PGU1* gene, no explanation has yet been given to explain the different phenotypes observed between strains that possess the *PGU1* gene.

Although the *PGU1* gene is present in most strains of *S. cerevisiae*, a few wine strains have been identified that lack the gene. These strains include UCLMS-1, -3, -4, -31, -58, -59, -63, -67, -72 (Fernández-González *et al*., 2004), OC2, KW1 and L2226 (Radoi *et al*., 2005), Collection Cépage Cabernet and Collection Cépage Merlot (Divol & Van Rensburg, 2007). The latter authors also indicated that the *PGU1* gene was replaced by a partial Ty mobile element in these strains (except OC2, KW1 and UCLMS-31 which were not tested). They hypothesise that this happened by insertion of the Ty element followed by partial deletion thereof (Fig. 3). The part of the locus that was replaced stretches from about 40 bp upstream of the *PGU1* (YJR153W) ORF up to about 1403 bp upstream the YJR154W ORF. The 334 bp at the 5' end of the insertion matched a δ element and the 3' end matched a POL-like region from the Ty element. A similar phenomenon was observed in *Saccharomyces bayanus* var. *uvarum* (SCPP) by Gognies *et al*. (2001). An insertion of 337 bp was identified 40 bp upstream the ATG of the *PGU1* ORF, which was also identified as a δ element from a Ty2 transposon. In this strain, the *PGU1* gene is still present though. The *PGU1* gene sequences from *S. cerevisiae* and *S. bayanus* were found to be very similar. This could indicate either a common ancestor for these two species or the transfer of genetic material between them. Pulsed-field gel electrophoresis (PFGE) was performed on several *S. cerevisiae* strains (Divol & Van Rensburg, 2007). The fingerprints were

used to obtain a phylogenetic tree which indicated a close relationship between UCLMS-1, -3, -4, L2226, CC Cabernet and CC Merlot.

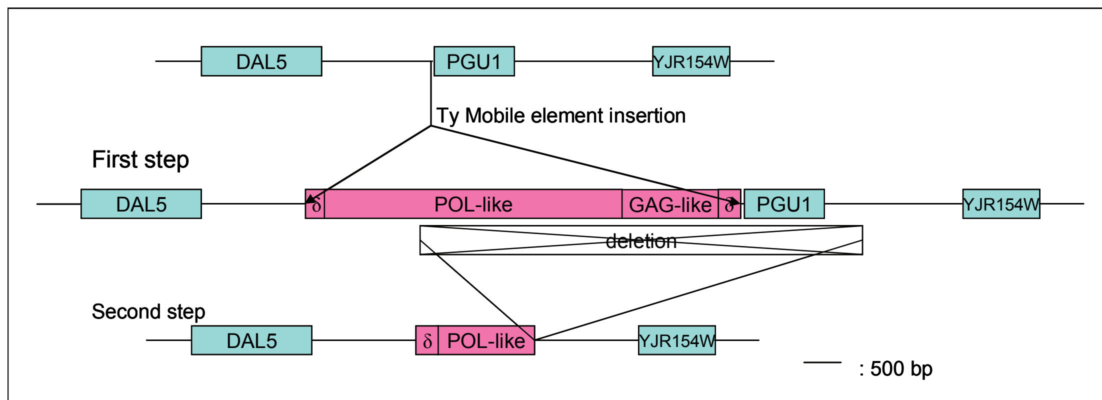

Fig. 3. Proposed insertion and partial deletion of the Ty mobile element in the *PGU1* region of certain strains of *S. cerevisiae* (Divol & Van Rensburg, 2007).

2.6 *PGU1* transcription and regulation

2.6.1 General regulation

The Pgu1p is believed to function in invasive growth by degrading the plant cell wall (Madhani *et al*., 1999). Regulation of the *PGU1* gene is very complex. It is regulated through the Mitogen-activated Protein Kinase (MAPK) pathway that controls filamentous growth (Madhani *et al*., 1999; Gognies *et al*., 2001). The latter authors observed that when cells were found in conditions of starvation, *S. cerevisiae* switched to filamentation or invasive growth, which is induced by the filamentation MAPK pathway. Simultaneously, the expression of *PGU1* also increased. The filamentation pathway is illustrated in Fig. 4.

Under “normal” conditions, when nutrients are not limited, the filamentation MAPK pathway is not activated. The unphosphorylated Kss1p binds to Ste12p and, together with Dig1p and Dig2p, inactivates Ste12p (Bardwell *et al*., 1998). When nutrients (especially nitrogen) are limited and cells are found in a state of starvation, this pathway is activated. Through a series of phosphorylation steps, the phosphorylated Kss1p phosphorylates Dig1p and Dig2p, preventing them from binding to Ste12p (Gustin *et al*., 1998). Ste12p can then activate the transcription of certain genes, including the *TEC1* gene which in turn activates transcription of the *PGU1* gene (Madhani *et al*., 1999).

The *PGU1* gene seems to be constitutively expressed as no pectin, polygalacturonic acid or galactutonic acid is required for polygalacturonase synthesis (Blanco *et al*., 1999). Certain sugars however do seem to increase or decrease polygalacturonase activity, although some contradictions are found in the literature regarding this. PG activity was found to be repressed

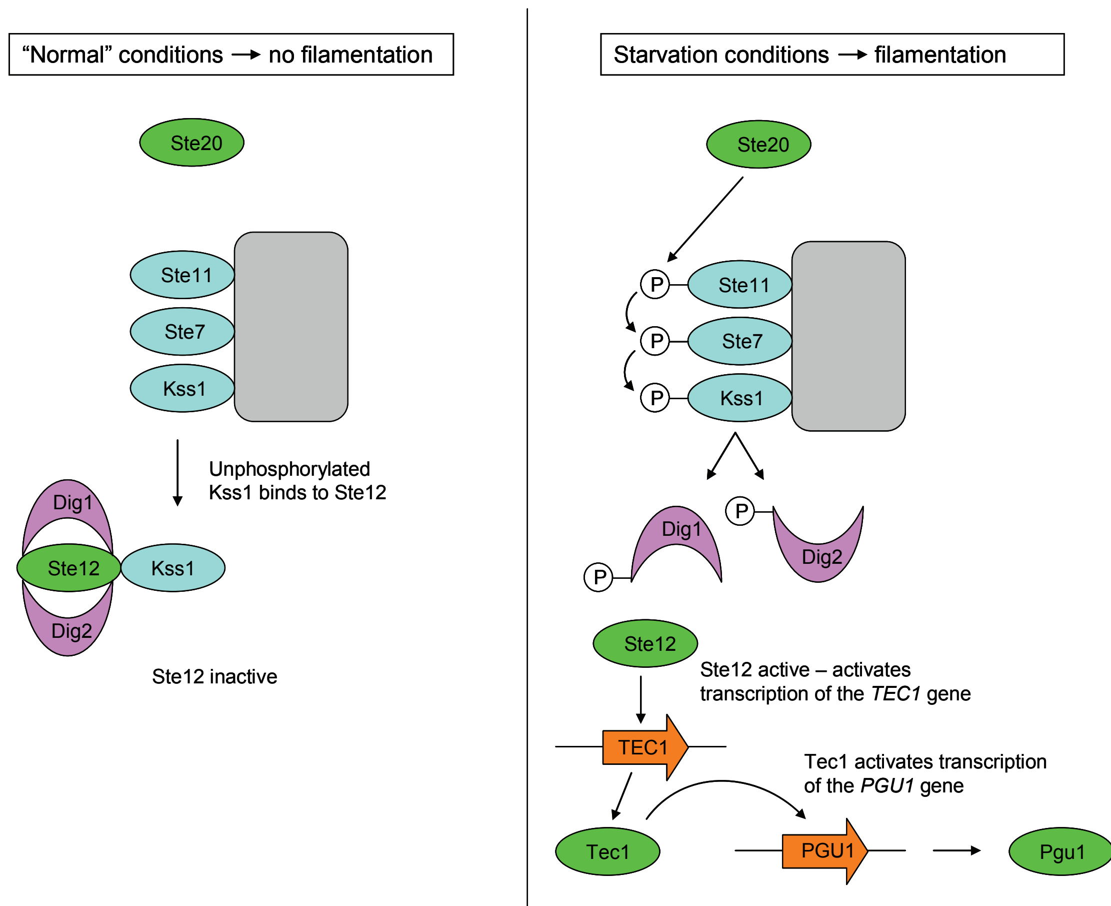

Fig. 4. Illustration of the filamentation MAPK pathway under "normal" (non-starvation) and starvation conditions.

by glucose (Blanco *et al*., 1994; Gognies *et al*., 2001; Radoi *et al*., 2005) and induced by galactose (Radoi *et al*., 2005). Other authors also observed a higher PG activity by cells grown in the presence of galactose compared to glucose (Blanco *et al*., 1994; Oliveira *et al*., 2006). This higher activity observed in the galactose medium could be due to the lack of repression by glucose rather than induction by galactose. The same regulation by glucose and galactose was observed for *Neurospora crassa* (Crotti *et al*., 1998), *Botrytis cinerea* (Wubben *et al*., 2000), *Candida albicans* and *Kluyveromyces marxianus* (Blanco *et al*., 1999). The glucose/galactose regulation of *PGU1* is thought to be similar to that found for *GAL1*, *GAL7* and *GAL10* (genes involved in galactose metabolism) in *S. cerevisiae* laboratory strains (Radoi *et al*., 2005). They also proposed similar regulation in wine strains because of a putative cis-element bound to Gal4p (the regulator of galactose induction) 610 and 632 bp upstream the ORF of *PGU1*.

Furthermore, Blanco *et al*. (1994) found that the lower the substrate (polygalacturonic acid) concentration, the higher the PG activity and that the highest activity was revealed in the presence of galacturonic acid. Strangely, Radoi *et al*. (2005) found polygalacturonase production to be induced by polygalacturonic acid. When pectin is used as substrate, the actual final product after degradation by endo-polygalacturonase is not galacturonic acid, but rather

oligogalacturonides such as tri- or digalacturonic acid (Gognies *et al*., 2001). These authors found that the addition of digalacturonic acid induced the *PGU1* promoter. The amount of dissolved oxygen in the medium also has an effect on the production of endo-polygalacturonase in certain strains of *S. cerevisiae*. For some strains, production is lowered or inhibited, depending on the intensity of aeration (Blanco *et al*., 1994; Blanco *et al*., 1997b), while shaking had no effect on enzyme production in other strains (McKay, 1990). Other possible inhibitors for enzyme activity include ethanol (Fernández-González *et al*., 2004) and SO_2 (Takayanagi *et al*., 2001).

2.6.2 *PGU1* promoter

Most authors ascribe the lack of PG activity in certain strains to mutations in the promoter sequence (Hirose *et al*., 1998) or lack of transcription of the *PGU1* gene (Blanco *et al*., 1998; Hirose *et al*., 1998; Jia & Wheals, 2000). Studies on the *PGU1* promoter have shown small differences between promoter areas of different strains (Hirose *et al*., 1999; Radoi *et al*., 2005). The first mentioned authors concluded that regulation of the *PGU1* gene was dependent on a trans-acting factor that represses the expression of the *PGU1* gene. They further suggested that this repression might be defective in strains with natural PG activity. Some differences were observed between the promoter regions of laboratory and wine strains (Radoi *et al*., 2005). The latter authors indicated that these differences may determine whether polygalacturonase production is induced by galactose and polygalacturonic acid or not. The wine strains identified not to possess the *PGU1* gene, were found to still have the promoter at the expected position, except for the last 40 bp or so that were replaced along with the gene (Divol & Van Rensburg, 2007). The filamentation MAPK pathway is complex and identified to regulate the expression of *PGU1*. Madhani *et al*. (1999) identified the regulatory effect of *TEC1* on *PGU1*. Tec1p acts as a transcription factor for *PGU1*. The *PGU1* promoter was found to have binding sites for Tec1p (Köhler *et al*., 2002).

It is still not clear why some strains have PG activity and some not and if it even is due to a single reason. There are different potential reasons why a strain might lack activity and it could be strain specific. It could be that some strains possess a non-functional *PGU1* gene as suggested by some authors. The majority of studies performed on *PGU1* rather suggest the promoter to be responsible for the lack of activity in some strains. Another possibility is the regulation by a trans-acting factor on the promoter as proposed by Hirose *et al*. (1999). An interesting observation was made by Gognies *et al*. (1999). They identified an out-of-phase ATG at position -13/-11 to the initiation codon of the *PGU1* gene in *S. cerevisiae* strain S288C. This could prevent translation of the *PGU1* gene in this strain as well as several laboratory strains derived from S288C.

2.7 Improvement of wine yeasts

2.7.1 Targets for improvement

Winemaking has come a long way from the traditional methods used many years ago and has to be adaptable to the demands of the producer- and consumer-driven market. Winemakers must be able to produce competitive wines in an ever-improving industry. They have to ensure reproducible, good quality wines with minimum input. Consumers have become much more health-conscious and, as the market expands, are looking for healthy, good quality wines at affordable prices. In order to produce such a product, continuous improvement is necessary. One way is to improve the yeast performing the fermentation. As the yeast contributes quite significantly to the final quality of the wine, for example by producing or alternating molecules such as glycerol, acetate, succinate, pyruvate and several esters as well as the release of cell components like proteins and polysaccharides (Cebollero *et al*., 2007), there are many factors that can potentially be enhanced. The majority of these targets are listed in Table 2.

Table 2. Some important targets for yeast improvement (Pretorius, 2000; Pretorius & Bauer, 2002)

A. Improved quality control and strain handling	**D. Improvement of wine flavour and other sensory qualities**
1. Strain maintenance (genetic stability)	1. Enhanced liberation of grape terpenoids
2. Molecular marking	2. Enhanced production of desirable volatile esters
B. Improvement of fermentation performance	3. Optimized fusel oil production
1. Improved viability and vitality of active dried wine yeast starter cultures	4. Enhanced glycerol production
2. Efficient sugar utilization	5. Bio-adjustment of wine acidity
3. Improved nitrogen assimilation	6. Elimination of phenolic off-flavour
4. Improved ethanol tolerance	7. Reduced sulphite and sulphide production
5. Increased tolerance to antimicrobial compounds	**E. Improvement of wine wholesomeness**
6. Reduced foam formation	1. Increased resveratrol production
C. Improvement of processing efficiency	2. Reduced formation of ethyl carbamate
1. Improved protein and polysaccharide clarification	3. Reduced formation of biogenic amines
2. Controlled cell sedimentation and flocculation	4. Decreased levels of alcohol
3. Controlled cell flotation and flor formation (Sherry production)	

There are many aspects in yeast that can be enhanced in order to improve the final quality of the wine. Depending on the wine to be made, a yeast with specific characteristics is chosen to

perform alcoholic fermentation. Ideally, these traits can be combined in a single strain for optimum performance. This particular study focused on the improvement of yeasts to enhance the sensory quality of the wine, specifically the extraction and alteration of aroma compounds such as higher alcohols, esters and monoterpenes.

2.7.2 Ways of improving yeast

Traditionally, improved yeast strains for winemaking were obtained by isolating new yeast from grapes and wine and screening them for the desired characteristic. This was the origin of the majority of the wine yeast strains commercially available today (Cebollero *et al.*, 2007). Because of the limited selection criteria, the limits of this strategy have been reached and alternative methods have been developed to obtain new, improved yeast strains. Yeast have generally been improved by classic genetic techniques, such as through sexual breeding, parasexual hybridization or random mutagenesis (Pretorius, 2000; Cebollero *et al.*, 2007). Sexual breeding is rarely used to improve micro-organisms (including wine yeast) as they either lack a sexual cycle or it is difficult to manipulate, expensive and time-consuming. There are four techniques to hybridize yeast. The first is through mating of haploids of opposite mating types (a or α) to yield a heterozygous diploid. Secondly, wine yeast strains that do not express a mating type can be forced to mate with a haploid strain of specific mating type. This is known as rare-mating. A third technique is cytoduction. This is when cytoplasmic genetic elements are introduced into the wine strain without the transfer of nuclear DNA. The fourth hybridization technique is spheroplast fusion where the cell walls of yeasts are removed and the spheroplasts (protoplasts) are fused. Random mutagenesis is where yeasts are treated with chemical or physical agents to increase the frequency of mutations in a yeast population. Cells can then be selected for a specific characteristic. All of these techniques can be used to improve or combine traits in the yeast. Importantly, yeast improved by these methods are not considered genetically modified organisms (GMOs) and are therefore easily accepted by the consumers (Pretorius & Bauer, 2002). The downside of these methods is that it is difficult to add or remove a characteristic without changing the yeast’s performance and other characteristics (Dequin, 2001).

Another way of improving yeast strains is by genetic engineering using biotechnological techniques. This is achieved through cloning and transformation. This is beneficial because the modifications can be well controlled in order to produce a targeted change. Specific characteristics can be altered without influencing others. Desired characteristics can be introduced, existing ones changed or unwanted traits removed. For this however, extensive knowledge is needed about the genes and mechanisms involved.

2.7.3 Transformation of industrial yeast strains

There are important genomic and physiological differences between domesticated (laboratory) and industrial yeast strains. Most laboratory strains are either haploid or diploid, while wine strains can be diploid or aneuploid and occasionally polyploid (Bakalinsky & Snow, 1990). Industrial wine yeasts are usually prototrophic and thus the auxotrophic markers used for transformation of laboratory strains can not be used for selection. Selection of transformants has to be on dominant markers. To overcome this, a second gene that confers resistance to some chemical (often an antibiotic) is usually transformed along with the gene of interest. Several dominant selectable markers have been developed for transformation of wine yeast. Some of these are listed in Table 3.

Table 3. Genes used as selectable markers for transformation of wine yeast and the chemicals selected on.

Gene used as selectable marker	Chemical that resistance is acquired against	Reference
kan^{r}	Kanamycin or G418	Jimenez & Davies (1980); Wach *et al*. (1994)
CYH2 (mutated)	Cycloheximide	Pérez-González *et al*. (1993)
SMR1-410	Sulfometuron	Petering *et al*. (1991)
ARD4-OFP	*p*-fluorophenylalanine (PFP)	Cebollero & González (2004)
bsd	Blasticidin	Fukuda & Kizaki (1999)
SFA1	Formaldehyde	Van den Berg & Steensma (1997)
dehH1	Fluoroacetate	
hph	Hygromycin B	Goldstein & McCusker (1999)
nat1	Nourseothricin	
pat	Bialaphos	

Puig *et al*. (1998) proposed the construction of auxotrophic wine yeast strains through interruption of both (all) copies of the *URA3* gene. After transformation, selection can be done using *URA3* as would be done for laboratory strains. The only problem is that this would have to be done for every strain to be transformed which will be very time-consuming. Another example is the use of the K1 killer toxin gene as a dominant selectable marker (Boone *et al*., 1990). Transcription of this gene produces the killer toxin and also conveys resistance to the yeast against the toxin. In this case, the yeast K1 killer toxin gene is integrated into the genome of a wine yeast along with the gene of interest. Transformants are then selected on resistance to the killer toxin. This has additional benefits: firstly, it improves the yeast's anticontaminant properties against K1 killer toxin sensitive species and secondly, it kills cells that lose the K1 killer toxin gene allowing, in theory, only the transformant to survive during the fermentation.

There are different methods available for transformation of yeast. The most commonly used methods found in the literature are through electroporation or heat shock after making the cells competent with lithium acetate. Both methods have been used successfully for the transformation of laboratory and industrial yeast strains with either a plasmid or an integration cassette. Sometimes, a method using lithium chloride is also used. One group, Pérez-González *et al*. (1993) used three different methods to transform wine strains: electroporation, polyethylene glycol-mediated transformation of protoplasts and a lithium acetate method. The latter was the only method that provided them with positive results. It could be that certain transformation methods are more effective for certain yeast strains or integration rather than plasmid transformation. This however has not been established.

2.7.4 Removable selection markers used in industrial yeast transformation

In order for any modified industrial yeast to be accepted by the public, it will have to be free from any foreign DNA. If the recombinant yeast contains any *E. coli* antibiotic resistance markers or yeast drug-resistant markers, it would have to be removed before the yeast would be accepted and approved for commercial use. Examples of selection systems used for yeast transformation are the *ura3*/5-FOA, Flp/FRT and Cre-loxP systems (Akada, 2002). The first is a method to select for ura3$^-$ auxotrophic mutants. The enzyme encoded by the *URA3* gene converts 5-fluoro-orotic acid (5-FOA) into a toxic metabolite. Thus, URA$^+$ wild types are unable to grow in plates while ura3$^-$ cells can. The latter two systems are based on recombinase-induced recombination at a specific site, which eliminates the unwanted sequence (usually a drug resistance gene). Both these systems require multiple transformations. The Flp/FRT system is based on the recombination between Flp recombinase Recognition Target (FRT) sites (Storici *et al*., 1999) while Cre-recombinase induces recombination between two loxP sites (Sauer, 1987). Another novel counter-selection system was developed (Kawahata *et al*., 1999; Olesen *et al*., 2000). These markers each consisted of a growth inhibitory gene under control of an inducible promoter. If the promoter is repressed, the cells grow normally. When the promoter is induced, the inhibitory gene is overexpressed and the cells that contain the marker cannot grow while those that lost the marker can.

The Cre-loxP system has been used in obtaining GM plants (Hare & Chua, 2002). It was also used in this study to obtain recombinant wine yeast. G418 is an antibiotic with a wide range inhibitory activity. The *kan*^{r} gene from *E. coli* transposon Tn903 confers resistance to G418 (Jimenez and Davies, 1980). Wach *et al*. (1994) constructed a kanMX cassette by fusing the *kan*^{r} gene to the *TEF* promoter and terminator sequences from *Ashbya gossypii*. The Cre-loxP system of bacteriophage P1 shows efficient recombination between two loxP sites flanking a marker gene, resulting in the excision of that gene (Sauer, 1987). A loxP-kanMX-loxP cassette

was constructed by combining the kanMX cassette with the Cre-loxP system (Güldener *et al*., 1996). The loxP-kanMX-loxP cassette can be integrated into the desired locus, conferring resistance to G418, and used as a selectable marker. Upon expression of Cre-recombinase (by transformation with a *cre* expression plasmid), recombination takes place between the two loxP sites, excising the kanMX module. A single loxP sequence is left in the genome.

2.8 Recombinant *PGU1* strains (previous studies on wine yeast)

The *PGU1* gene from *S. cerevisiae* strain IM1-8b (a strain that exhibits PG activity) was cloned under control of the constitutive *PGK1* promoter on a multicopy plasmid (Blanco *et al*., 1998). Vilanova *et al*. (2000) transformed *S. cerevisiae* strain M-20 (a wine yeast that exhibits no PG activity) with this plasmid in order to overexpress *PGU1* in this strain. They used the recombinant strain in microvinification experiments alongside the wild type strain as well as the wild type with added commercial enzyme. They found a 10-fold reduction in filtration time with the recombinant compared to the wild type. This was comparable to the results observed when commercial enzyme was added. Sensory analysis of the wine showed an increase in the terpenes nerol and geraniol which contribute to the typical aroma of the cultivar. Higher alcohols and esters did not show any difference. Wine made with addition of commercial enzymes showed an increase in certain terpenes, higher alcohols and esters. It did however also increase the amount of certain unwanted compounds that have a negative effect on the wine.

Another group (Fernández-González *et al*., 2005) also expressed the *PGU1* gene under control of the *PGK1* promoter and used the plasmid to transform the wine yeast UCLMS-1 which does not possess the *PGU1* gene (and subsequently lacks PG activity). The *PGU1* gene used in this experiment was amplified from *S. cerevisiae* strain UCLMS-39, a wine yeast that exhibits PG activity. In this study, the cassette was integrated into the genome of the yeast. The integration was stable and the strain recovered polygalacturonase activity. Microvinification with the recombinant and subsequent analysis of the wine did not show any difference in filtration time, viscosity or major volatile components. This was contradictory to what Vilanova *et al*. (2000) observed. A possible explanation could be that the expression of the *PGU1* gene was not high enough when a single copy was present in the genome of the yeast to have a significant effect on the aroma profile of the wine. On the contrary, a multicopy plasmid would ensure high enough expression of *PGU1* to enhance the extraction of certain aroma compounds during vinification.

2.9 GMOs and the consumer

S. cerevisiae is an important commercial organism with GRAS (Generally Regarded As Safe) status, especially in the baking, brewing and winemaking industries. In the wine industry specifically, some wineries are known for their traditional image. Most new wine strains are obtained through isolation and screening of strains either naturally found in wine or obtained through directed evolution in cultures kept under selective pressure. Alternatively, new strains are obtained through various breeding techniques as mentioned previously. These can be time-consuming processes for adding or removing a characteristic without changing the fermenting ability of the yeast. Biotechnology offers a much faster and target-specific alternative. Unfortunately, it is difficult to get genetically modified (GM) products on the market due to international, national and local regulations, consumer distrust and activist opposition. It is therefore crucial for the public to be informed about the potential benefits of recombinant DNA technology and reassured about a safe, good quality product that is clearly beneficial and not in any way harmful to their health or the environment. No scientifically reputable test has shown any GM food on the shelf to be toxic thus far (Pretorius, 2000).

The use of genetically modified organisms (GMOs) for food applications is strictly regulated in most countries. Except for the general regulations, GMO wine also has to be accepted by the International Organisation of Vine and Wine (OIV) (Cebollero *et al*., 2007). GMO technologies therefore have to be carried out in such a way that it abides by the regulations in order to promote the acceptance of the product, not only by the authorities, but also by the consumer. In order to achieve this, there are certain important aspects to focus on. Firstly, the amount of foreign (non-yeast) DNA should be reduced to a minimum, if not avoided at all, and the GMO should not contain any antibiotic resistance markers for selection. This eliminates the possibility of the transfer of drug resistance markers from a GMO to other organisms. Researchers should also create genetically stable transformants by integrating the desired DNA into the genome of the yeast, rather than to use a self-replicating plasmid (Akada, 2002; Cebollero *et al*., 2007). This will avoid the need of maintaining selective pressure. Plasmid transformations can be unstable and plasmids lost when the yeast is not under selective pressure (Cebollero *et al*., 2007). The first step towards acceptance of GMOs in food is the exemption of “self-cloned” organisms from GMO legislation. Self-cloning is the cloning of a gene or genes within the organism itself. The modified organism thus contains no foreign DNA and is therefore not considered a GMO (Akada, 2002). This eliminates the concern about antibiotic resistance markers and allergen or toxic product formation by the organism. It is interesting to note that protoplast fusion, a hybridization technique sometimes used to develop new strains, is considered as “genetic engineering” under the GMO regulations in the European Union (Cebollero *et al*., 2007).

Two recombinant *S. cerevisiae* strains received approval for commercial use from the British Government (Dequin, 2001). The first is a baker's yeast in which the uptake and digestion of maltose was improved to increase the rate of CO_2 release (CO_2 allows the dough to rise). This was achieved by cloning more effective promoter before the maltose permease and maltase genes (Aldhous, 1990). The second is a brewer's yeast expressing the *STA2* gene to produce exocellular glucoamylase (Hammond, 1995). There is however a big difference between approval and the actual use of the organism commercially as consumers remain skeptic even after official government approval. A third strain, a recombinant sake yeast strain, was approved by the Japanese Government as a "self-cloned" organism in 2001 (Akada, 2002). Consequently, the strain did not have to be treated as a GMO. It also received GRAS status from the Food and Drug Administration (FDA) in 2006. The first recombinant wine yeast strain was approved for winemaking in three countries: USA, the Republic of Moldova (Cebollero, 2007) and Canada. This is a *S. cerevisiae* strain that can perform malolactic fermentation. The strain produces the malate permease from *Schizosaccharomyces pombe* and the malolactic enzyme from *Oenococcus oeni* (Husnik *et al*., 2006). It is commercialized by Lesaffre as ML01. A second recombinant wine strain has also received GRAS status. The strain was named $S22^{EC-}$ and reduces ethyl carbamate formation in wine via efficient urea degradation (Coulon *et al*., 2006).

Biotechnology research in the food industry is rapidly expanding and becoming ever more important for the producer to keep up with the consumer demand. Although consumers are still very skeptic about GM products and GMOs, the approval of some GM yeast strains indicate a movement in direction of acceptance. The difficult part is still convincing the general public about the safety of the approved products and their benefit to society. A possible way to convince the consumer might be through a systems biology approach to better understand the cell as a whole and control whether the gene that was introduced has an overall effect on the biological functioning of the cell. Strict regulation in this field is a necessity to ensure that all GM products are safe and have a clear benefit to both the producer and the consumer. It is therefore the responsibility of the researchers to develop products that abide by the legislation and also to be honest and open to the public about the GM organism or product regarding its construction, function, safety and benefits. If the product is beneficial and the consumer convinced, the applications of biotechnology are endless!

2.10 General conclusions

Endo-polygalacturonases are enzymes that degrade pectin, a component of plant cell walls that can be detrimental to the winemaking process. These enzymes are produced by various micro-

organisms, including *Saccharomyces* species. The enzyme activities observed in these species vary greatly between strains. The *PGU1* gene, which encodes an endo-polygalacturonase in *S. cerevisiae*, is regulated through the filamentous MAPK pathway, indicating the role of the enzyme in filamentation and invasive growth of the pathogen yeast on its plant host. During winemaking, polygalacturonases can assist in reducing filtration time and viscosity and potentially enhance extraction of certain aroma compounds and precursors. Yeast with strong oenological properties that can produce active endo-polygalacturonases during alcoholic fermentation could be beneficial for the winemaking industry by eliminating the need to add expensive commercial enzyme preparations that are often contaminated with other unwanted enzymes. Such a yeast can be obtained through biotechnological techniques. By introducing a yeast *PGU1* gene into a wine strain, this self-cloned recombinant can be exempted from the strict GMO legislation and potentially used in commercial wineries.

2.11 References

Akada R (2002) Genetically modified industrial yeast ready for application. *J Biosci Bioeng* **94:** 536-544.

Aldhous P (1990) Modified yeast fine for food. *Nature* **344:** 186.

Armand S, Wagemaker MJM, Sánchez-Torres P, Kester HCM, Van Santen Y, Dijkstra BW, Visser, J & Benen JAE (2000) The active site topology of *Aspergillus niger* endopolygalacturonase II as studied by site-directed mutagenesis. *J Biol Chem* **275:** 691-696.

Bakalinsky A & Snow R (1990) The chromosomal constitution of wine strains of *Saccharomyces cerevisiae*. *Yeast* **6:** 367-382.

Bardwell L, Cook JG, Zhu-Shimoni JX, Voora D & Thorner J (1998) Differential regulation of transcription: repression by unactivated mitogen-activated protein kinase Kss1 requires Dig1 and Dig2 proteins. *Proc Natl Acad Sci USA* **95:** 15400-15405.

Bell TA & Etchells JL (1956) Pectin hydrolysis by certain salt-tolerant yeasts. *Appl Microbiol* **4:** 196-201.

Blanco P, Sieiro C, Diaz A & Villa TG (1994) Production and partial characterization of an endopolygalacturonase from *Saccharomyces cerevisiae*. *Can J Microbiol* **40:** 974-977.

Blanco P, Sieiro C, Díaz, A Reboredo NM & Villa TG (1997) Grape juice Biodegradation by polygalacturonases from *Saccharomyces cerevisiae*. *Int Biodeterior Biodegradation* **40:** 115-118.

Blanco P, Sieiro C Diaz A & Villa TG (1997) Differences between pectic enzymes produced by laboratory and wild-type strains of *Saccharomyces cerevisiae*. *World J Microbiol Biotechnol* **13:** 711-712.

Blanco P, Sieiro C, Reboredo NM & Villa TG (1998) Cloning, molecular characterization, and expression of an endo-polygalacturonase-encoding gene from *Saccharomyces cerevisiae* IM1-8b. *FEMS Microbiol Lett* **164:** 249-255.

Blanco P, Sieiro C & Villa TG (1999) Production of pectic enzymes in yeasts. *FEMS Mircobiol Lett* **175:** 1-9.

Blanco P, Thow G, Simpson CG, Villa TG & Williamson B (2002) Mutagenesis of key amino acids alters activity of a *Saccharomyces cerevisiae* endo-polygalacturonase expressed in *Pichia pastoris*. *FEMS Microbiol Lett* **210:** 187-191.

Boone C, Sdicu AM, Wagner J, Degré R, Sanchez C & Bussey H (1990) Integration of the yeast K1 killer toxin gene into the genome of marked wine yeasts and its effect on vinification. *Am J Enol Vitic* **41:** 37-42.

Cebollero E & Ganzalez R (2004) Comparison of two alternative dominant selectable markers for wine yeast transformation. *Appl Environ Microbiol* **70:** 7018-7023.

Cebollero E, Gonzalez-Ramos D, Tabera L & Gonzalez R (2007) Transgenic wine yeast technology comes of age: is it time for transgenic wine? *Biotechnol Lett* **29:** 191-200.

Codner RC (2001) Pectinolytic and cellulolytic enzymes in the microbial modification of plant tissues. *J Appl Bacteriol* **84:** 147-160.

Coulon J, Husnik JI, Inglis DL, van der Merwe GK, Lonvaud A, Erasmus DJ & van Vuuren HJJ (2006) Metabolic engineering of *Saccharomyces cerevisiae* to minimize the production of ethyl carbamate in wine. *Am J Enol Vitic* **57:** 113-124.

Crotti LB, Terenzi HF, Jorge JA & Lourdes MTM (1998) Regulation of pectic enzymes from the *exo-1* mutant strain of *Neurospora crassa*: effects of glucose, galactose and galacturonic acid. *J Basic Microbiol* **38:** 181-188.

Cruess WV, O 'Neal R, Chong G & Uchimoto D (1951) The effect of pectic enzymes in wine making. *Am J Enol Vitic* **2:** 59-75.

Dequin S (2001) The potential of genetic engineering for improving brewing, wine-making and baking yeasts. *Appl Microbiol Biotechnol* **56:** 577-588.

De Vries RP & Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol Mol Biol Rev* **65:** 497-522.

Di Pietro A & Roncero MIG (1996) Endopolygalacturonase from *Fusarium oxysporum* f. sp. *Lycopersici*: Purification, characterization and production during infection of tomato plant. *Phytopathology* **86:** 1324-1330.

Divol B & van Rensburg P (2007) *PGU1* natural deletion is responsible for the absence of endo-polygalacturonase activity in some wine strains of *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7:** 1328-1339.

Fernández-González M, Úbeda JF, Vasudevan TG, Cordero Otero RR & Briones AI (2004) Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains. *FEMS Microbiol Lett* **237:** 261-266.

Fernández-González M, Úbeda JF, Cordero-Otero RR, Thanvanthri V & Briones AI (2005) Engineering of an oenological *Saccharomyces cerevisiae* strain with pectinolytic activity and its effect on wine. *Int J Food Microbiol* **102:** 173-183.

Fogarty WM & Ward OP (1974) Pectinases and pectic polysaccharides. *Prog Ind Microbiol* **13:** 59-119.

Fukuda K & Kizaki Y (1999) A new transformation system of *Saccharomyces cerevisiae* with blasticidin S deaminase gene. *Biotechnol Lett* **21:** 969-971.

Gainvors A, Frézier V, Lamaresquier H, Lequart C, Aigle M & Belarbi A (1994) Detection of polygalacturonase, pectin-lyase and pectin-esterase activities in a *Saccharomyces cerevisiae* strain. *Yeast* **10:** 1311-1319.

Gainvors A, Nedjaoum N, Gognies S, Muzart M, Nedjma M & Belarbi A (2000) Purification and characterization of acidic endo-polygalacturonase encoded by the *PGL1-1* gene form *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **183:** 131-135.

Gognies S, Gainvors A, Aigle M & Belarbi A (1999) Cloning, sequence analysis and overexpression of a *Saccharomyces cerevisiae* endopolygalacturonase-encoding gene (*PGL1*). *Yeast* **15:** 11-22.

Gognies S, Simon G & Belarbi A (2001) Regulation of the expression endopolygalacturonase gene *PGU1* in *Saccharomyces*. *Yeast* **18:** 423-432.

Goldstein AL & McCusker JH (1999) Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* **15:** 1541-1553.

Güldener U, Heck S, Fiedler T, Beinhauer J & Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24:** 2519-2524.

Gustin MC, Albertyn J, Alexander M & Davenport K (1998) MAP Kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **62:** 1264-1300.

Hammond JRM (1995) Genetically-modified brewing yeasts for the 21st century. Progress to date. *Yeast* **11:** 1613-1627.

Hare PD & Chua N (2002) Excision of selectable marker genes from transgenic plants. *Nat Biotechnol* **20:** 575-580.

Hirose N, Fujii M, Kishida M, Kawasaki H & Sakai T (1999) Isolation and genetic determination of a *Saccharomyces cerevisiae* mutant that produces an endo-polygalacturonase. *J Biosci Bioeng* **87:** 594-597.

Hirose N, Kishida M, Kawasaki, H & Sakai T (1998) Molecular cloning and expression of a polygalacturonase gene in *Saccharomyces cerevisiae*. *J Ferment Bioeng* **86:** 332-334.

Husnik JI, Volschenk H, Bauer J, Colavizza D, Luo Z & van Vuuren HJJ (2006) Metabolic engineering of malolactic wine yeast. *Metab Eng* **8:** 315-323.

Ishii S & Yokotsuka (1972) Purification and properties of endo-polygalacturonase from *Aspergillus japonicus*. *Agric Biol Chem* **36:** 1885-1893.

Jayani RS, Saxena S & Gupta R (2005) Microbial pectinolytic enzymes: A review. *Process Biochem* **40:** 2931-2944.

Jia J & Wheals A (2000) Endopolygalacturonase genes and enzymes from *Saccharomyces cerevisiae* and *Kluveromyces marxianus*. *Curr Genet* **38:** 264-270.

Jimenez A & Davies J (1980) Expression of a transposable antibiotic resistance element in *Saccharomyces. Nature* **287:** 869-871.

Kashyap DR, Vohra PK, Chopra S & Tewari R (2001) Applications of pectinases in commercial sector: a review. *Bioresour Technol* **77:** 215-227.

Kawahata M, Amari S, Nishizawa Y & Akada R (1999) A positive selection for plasmid loss in *Saccharomyces cerevisiae* using galactose-inducible growth inhibitory sequences. *Yeast* **15:** 1-10.

Kester HCM and Visser J (1990) Purification and characterization of polygalacturonases produced by the hyphal fungus *Aspergillus niger*. *Biotech Appl Biochem* **12:** 150-160.

Kester HCM, Kusters-Van Someren MA, Müller Y & Visser J (1996) Primary structure and characterization of an exopolygalacturonase from *Aspergillus tubingensis*. *Eur J Biochem* **240:** 738-746.

Köhler T, Wesche S, Taheri N, Braus GH & Mösch H (2002) Dual role of the *Saccharomyces cerevisiae* TEA/ATTS family transcription factor Tec1p in regulation of gene expression and cellular development. *Eukaryot cell* **1:** 673-686.

Lourens K & Pellerin P (2004) Enzymes in winemaking. *Wineland* Nov 2004: 94-97.

Luh BS & Phaff HJ (1951) Studies on polygalacturonase of certain yeasts. *Arch Biochem Biophys* **33:** 213-227.

Madhani HD, Galitski T, Lander ES & Fink GR (1999) Effectors of a developmental mitogen-activated protein kinase cascade revealed by expression signatures of signaling mutants. *Proc Natl Acad Sci USA* **96:** 12530-12535.

McKay A (1990) Degradation of polygalacturonic acid by *Saccharomyces cerevisiae. Lett Appl Microbiol* **11:** 41-44.

Olesen K, Johannesen PF, Hoffmann L, Serensen SB, Gjermansen C & Hansen J (2000) The pYC plasmids, a series of cassette-based yeast plasmid vectors providing means of counter-selection. *Yeast* **16:** 1035-1043.

Oliveira KF, Malavolta L, Souza CS, Vicente EJ & Laluce C (2006) Pectinolytic activity secreted by yeasts isolated from fermented citrus molasses. *J Appl Microbiol* **100:** 633-640.

Pérez-González JA, González R, Querol A, Sendra J & Ramón D (1993) Construction of a recombinant wine yeast strain expressing ß-(1,4)-endoglucanase and its use in microvinification processes. *Appl Environ Microbiol* **59:** 2801-2806.

Petering JE, Henschke PA & Langridge P (1991) The *Escherichia coli* β-glucuronidase gene as a marker for *Saccharomyces* yeast strain identification. *Am J Enol Vitic* **42:** 6-12.

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16:** 675-729.

Pretorius IS & Bauer FF (2002) Meeting the consumer challenge through genetically customized wine-yeast strains. *Trends Biotechnol* **20:** 426-432.

Puig S, Ramon D & Perez-Ortin JE (1998) Optimized method to obtain stable food-safe recombinant wine yeast strains. *J Agric Food Chem* **46:** 1689-1693.

Radoi F, Kishida M & Kawasaki H (2005) Endo-polygalacturonase in *Saccharomyces* wine yeasts: effect of carbon source on enzyme production. *FEMS Yeast Res* **5:** 663-668.

Ribéreau-Gayon P, Dubourdieu D, Donèche B & Lonvaud A (2006a) *Handbook of enology, Vol 1. The microbiology of wine vinifications.* John Wiley & Sons, Ltd., Chichester, UK.

Ribéreau-Gayon P, Glories Y, Maujean A & Dubourdieu D (2006b) *Handbook of enology, Vol 2. The chemistry of wine stabilization and treatments.* John Wiley & Sons, Ltd., Chichester, UK.

Sakellaris G, Nikolaropoulos S & Evangelopoulos AE (1989) Purification and characterization of an extracellular polygalacturonase from *Lactobacillus plantarm* strain BA11. *J Appl Bacteriol* **67:** 77-85.

Sauer B (1987) Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* **7:** 2087-2096.

Storici F, Coglievina M & Bruschi CV (1999) A 2-μm DNA-based marker recycling system for multiple gene disruption in the yeast *Saccharomyces cerevisiae*. *Yeast* **15:** 271-283.

Takayanagi T, Uchibori T & Yokotsuka K (2001) Characteristics of yeast polygalacturonases induced during fermentation on grape skins. *Am J Enol Vitic* **52:** 41-44.

Van den Berg MA & Steensma HY (1997) Expression cassettes for formaldehyde and fluoroacetate resistance, two dominant markers in *Saccharomyces cerevisiae*. *Yeast* **13:** 551-559.

Van Rensburg P & Pretorius IS (2000) Enzymes in winemaking: Harnessing natural catalysts for efficient biotransformations – a review. *S Afr J Enol Vitic* **21:** 52-73.

Van Santen Y, Benen JAE, Schröter K, Kalk KH, Armand S, Visser J & Dijkstra BW (1999) 1.68-Å crystal structure of endopolygalacturonase II from *Aspergillus niger* and identification of active site residues by site-directed mutagenesis. *J Biol Chem* **274:** 30474-30480.

Viikari L, Tenkanen M & Suurnakki A (2001) Biotechnology in the pulp and paper industry. *Biotechnology, Vol. 10* (Rehm HJ, ed), pp. 523-546. Wiley-VCH, Chichester, UK.

Vilanova M, Blanco P, Cortés S, Castro M, Villa TG & Sieiro C (2000) Use of a *PGU1* recombinant *Saccharomyces cerevisiae* strain in oenological fermentations. *J Appl Microbiol* **89:** 876-883.

Vincken JP, Schols HA, Oomen RJFJ, McCann MC, Ulvskov P, Voragen AGJ & Visser RGF (2003) If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant physiol* **132:** 1781-1789.

Wach A, Brachat A, Pöhlmann R & Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10:** 1793-1808.

Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. *Enzyme Microb Technol* **6:** 341-349.

Wightman JD, Price SF, Watson BT & Wrolstad RE (1997) Some effects of processing enzymes on anthocyanins and phenolics in Pinot Noir and Cabernet Sauvignon wines. *Am J Enol Vitic* **48:** 39-49.

Willats WGT, Knox JP & Mikkelsen JD (2006) Pectin: new insights to an old polymer are starting to gel. *Trends Food Sci Tech* **17:** 97-104.

Wubben JP, ten Have A, van Kan JAL & Visser J (2000) Regulation of endo-polygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. *Curr Genet* **37:** 152-157.

Chapter 3

Research results

Overexpression of *PGU1* and *TEC1* in various *Saccharomyces cerevisiae* strains and its effect on polygalacturonase activity

3. Research results

Overexpression of *PGU1* and *TEC1* in various *Saccharomyces cerevisiae* strains and its effect on polygalacturonase activity

Herine van Wyk and Benoit Divol

Institute for Wine Biotechnology, Stellenbosch University, Matieland, South Africa

Abstract

The *PGU1* gene encodes an endo-polygalacturonase, an enzyme that cleaves poly-galacturonic acid. Most strains of *Saccharomyces cerevisiae* possess the gene, yet their polygalacturonase (PG) activity varies greatly. Due to the complex regulation of the gene, it is not clear why some strains do not exhibit PG activity when they have the gene in their genomes. Tec1p was found to be the main transcription factor for *PGU1*. This study evaluated the effect of *PGU1* or *TEC1* overexpression on the PG activities of several strains. As expected, all the strains showed an increase in activity when *PGU1* was overexpressed. The same tendency was observed for all the strains that have the *PGU1* gene in their genomes when *TEC1* was overexpressed. The only exception was the strain Σ1278b which did not show an increase with *TEC1* overexpression. This study demonstrated that PG activity can be enhanced or recovered by modifying the transcriptional regulation of the *PGU1* gene.

3.1 Introduction

Pectin refers to a group of diverse and complex heteropolysaccharides. These polysaccharides basically consist of a D-galacturonic acid backbone to which side chains can be attached. Structurally, it can be divided into two regions (De Vries and Visser, 2001): a “smooth” region that consists exclusively of D-galacturonic acid residues linked by α-1,4-bonds and a “hairy” region where diverse side chains can be attached to the backbone and where some D-galacturonic acid residues may be methylated. Pectinases is the general term used for enzymes that degrade pectin. Endo-polygalacturonases are pectinases that specifically cleave the links between non-methylated D-galacturonic acid residues by attacking the chain randomly (Kester *et al*., 1996).

The *PGU1* gene (also known as *PGL1*) encodes for an endo-polygalacturonase in *Saccharomyces cerevisiae*. It is situated on chromosome X and a single copy is present per haploid genome (Blanco *et al*., 1998). Most strains of *S. cerevisiae* possess this gene, although not all are able to degrade polygalacturonic acid (Fernández-González *et al*., 2004). Some strains that do not degrade polygalacturonic acid naturally, can recover this enzyme activity when the *PGU1* gene is expressed under plasmid promoter control (Blanco *et al*., 1998),

suggesting a non-functional *PGU1* gene or promoter in their genomes. However, through sequencing and Southern blot analyses, it was found that the gene sequence is not responsible for the lack of activity in certain strains (Hirose *et al*., 1998). *In silico* analyses of the *PGU1* promoter regions of strains able to degrade polygalacturonic acid and of those that are not, revealed very few differences between promoters (Hirose *et al*., 1999). These authors concluded that *PGU1* regulation was dependent on a trans-acting factor that represses the expression of the *PGU1* gene. They suggested that this repression may be defective in strains that produce an endo-polygalacturonase.

Although it is present in most strains of *S. cerevisiae*, a few strains lacking the *PGU1* gene have been identified. These include UCLMS-1, -3, -4, -31, -58, -59, -63, -67, -72 (Fernández-González *et al*., 2004), OC2, KW1, L2226 (Radoi *et al*., 2005), Collection Cépage Cabernet and Collection Cépage Merlot (Divol & van Rensburg, 2007). The latter authors also indicated that the *PGU1* gene was replaced by a partial Ty mobile element in all these strains (except OC2, KW1 and UCLMS-31 which were not tested).

Regulation of the *PGU1* gene is very complex and not yet fully understood. The gene was found to be regulated through a mitogen-activated protein kinase (MAPK) pathway (Madhani *et al*., 1999) and regulation is linked to filamentation and invasive growth (Gognies *et al*., 2001). The Tec1 protein was identified as the main transcription factor of *PGU1* (Madhani *et al*., 1999). The *PGU1* promoter indeed contains several binding sites for the Tec1p (Köhler *et al*., 2002).

In this study, *PGU1* and *TEC1* were overexpressed independently in various strains of *S. cerevisiae* in order to determine the effect of each on the polygalacturonase (PG) activity of each specific strain. Some of these strains naturally have polygalacturonase activity, while others do not and some even lack the *PGU1* gene completely. The aim was to determine if PG activity would be enhanced by overexpressing either or both of the genes and if strains lacking activity would also gain activity to ultimately determine why certain strains are not capable of degrading polygalacturonic acid.

3.2 Materials and methods

3.2.1 Strains, media and culture conditions

The bacteria used for transformation was *Escherichia coli* DH5α (F-φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-). All *E. coli* cultures were grown at 37 °C in liquid Luria Bertani (LB) Broth (Biolab Diagnostics, Wadeville, South Africa) or on LB plates, both containing 100 μg mL^{-1} Ampicillin (Ampicillin sodium salt, Sigma, Missouri, USA) for selection. Various *Saccharomyces cerevisiae* strains were

transformed with the final constructs as presented in Table 1: Collection Cépage Cabernet (CC Cabernet), Collection Cépage Merlot (CC Merlot), L2226, L2323, VIN13, BY4742 and Σ1278b. All yeast cultures were grown in liquid Yeast Peptone Dextrose (YPD) medium and on YPD plates containing 200 µg mL^{-1} Geneticin (G418 disulphate salt, Sigma) and incubated at 30°C.

Table 1. *S. cerevisiae* strains used for transformation. PG+ strains exhibit polygalacturonase activity while PG- strains do not.

Strain	Relevant characteristics	Reference
Collection Cépage Cabernet*	Wine yeast; PG-	CIVB, Bordeaux, France
Collection Cépage Merlot*	Wine yeast; PG-	CIVB, Bordeaux, France
L2226*	Wine yeast, PG-	Lallemand
L2323	Wine yeast; PG+	Lallemand
VIN13	Wine yeast; PG-	Anchor Yeast (South Africa)
BY4742	Laboratory strain; PG-	Brachmann *et al*. (1998)
Σ1278b	Laboratory strain, PG+	This laboratory

**PGU1* gene replaced by a transposon

3.2.2 Isolation of plasmid and genomic DNA

Plasmid DNA was isolated from overnight *E. coli* cultures using the QIAprep® Spin Miniprep Kit (Qiagen, Southern Cross Biotechnology, Cape Town, South Africa) as instructed by the supplier. For the isolation of genomic DNA from yeast cultures, the alternative protocol for rapid isolation of yeast chromosomal DNA according to the method described by Cryer *et al*. (1975) and Davis *et al*. (1980) was used.

3.2.3 PCR amplification

The *PGU1* gene was amplified from *S. cerevisiae* L2323 using the primers 5'PPGL1-BGLIIpP2 and 3'PPGL1-BGLIIpP2 (Table 2). The reaction took place in a total volume of 50 µL, using 1U Extaq Polymerase from Takara (SEPARATIONS, Randburg, South Africa). The PCR program started with a denaturation step at 95°C for 5 minutes. A denaturation step at 94°C for 30 s, annealing temperature of 55°C for 30 s and an elongation step at 72°C for 1 minute were repeated for 30 cycles. The program ended with a final elongation step at 72°C for 10 minutes.

3.2.4 Construction of the plasmids

For overexpression, the *PGU1* and *TEC1* genes were cloned into pCEL13, a plasmid containing the *PGK1* promoter and terminator (Gundllapalli *et al*., 2006). The *PGU1* gene was amplified from *S. cerevisiae* L2323 by PCR and cloned into pGEM®-T Easy from Promega (Whitehead Scientific, Cape Town, South Africa) as instructed by the supplier.

Table 2. A list of all primers used in this study for PCR amplification

Primer name	Sequence 5' – 3'	Reason for use	Reference
5'PPGL1-BGLII pP2	AGATCTATGATTTCTGCTAATTCATTACTTATTTCC	Amplification of the *PGU1* gene from *S. cerevisiae* L2323	This study
3'PPGL1-BGLIIpP2	CTCGAGTTAACAGCTTGCACCAGATCCAGATG (*Xho*I)		This study
Pgu1fw2	GTGCTTCGGGACATACCATT	Amplification of the *PGU1* gene for verification of the final construct (pP3-loxP)	Divol & Van Rensburg (2007)
Pgu1rev2	CGTCAACGCCAACTTTACAA		Divol & Van Rensburg (2007)
Tec1fw	TCAAGCACAAAACCAACGAG	Amplification of the *TEC1* gene for verification of the final construct (pT1-loxP)	This study
Tec1rev	ATGATAGGGTCAGCGAGTCC		This study
loxPfw	ATCGAGGCCTGGAATTCGATTGATCGCATGC (*Stu*I)	Amplification of the loxP cassette for verification of the final constructs (pP3-loxP and pT1-loxP)	This study
loxPrev	ATCGAGGCCTCGAATTCACTAGTGATAGATCGGATCC (*Stu*I)		This study
5'-KPNPGK-631	GGGGTACCCTTTATTTTGGCTTCACCC	Screening for positive transformants together with Pgu1rev2	Volschenk *et al.* (2004)

It was then released by enzymatic digestion with *Bgl*II and *Xho*I (Roche, Penzberg, Germany). The pCEL13 vector was also digested with these enzymes and the gene sub-cloned into pCEL13 using T4 DNA ligase from Fermentas (Inqaba Biotechnical Industries (PTY) Ltd., Pretoria, South Africa) as indicated by the supplier. The *TEC1* gene was released from a pHVX2 plasmid, previously constructed in our laboratory, by enzymatic digestion with *EcoR*I and *Xho*I (Roche). The pCEL13 vector was then also digested with these enzymes and *TEC1* sub-cloned into pCEL13 using the same concentrations in the ligation mixture as with *PGU1*. A loxP cassette, containing the kan^r gene, was also cloned into these plasmids. This was done by excising the loxP cassette from a pGEM®-T vector (Malherbe DF, unpublished) by enzymatic digestion with *Apa*I and *Nde*I (Roche). The plasmids pP3 (pCEL13-PGU1) and pT1 (pCEL13-TEC1) were also digested with these enzymes. The loxP cassette was then sub-cloned into each of these plasmids with T4 DNA ligase (Fermentas), as indicated by the supplier. The reaction took place at 4°C overnight. The final constructs were named pP3-loxP and pT1-loxP respectively.

The constructs were verified by PCR and enzymatic digestion. The PCR programs are summarised in Table 3. Two reactions were carried out for each construct, one to amplify the specific gene and one to amplify the loxP cassette. Colonies that showed amplicons for both the gene and loxP cassette were then digested with *Nco*I and *Sca*I (Roche) for final verification.

Table 3. PCR programs used to verify the final constructs, pP3-loxP and pT1-loxP, by amplification of the *PGU1* gene, *TEC1* gene and loxP cassette respectively

Template	Primers used	Target	Temp	Time	Number of Cycles
pP3-loxP	Pgu1fw2 & Pgu1rev2	*PGU1* gene	95°C	5 min	1
			94°C	30 s	30
			55°C	30 s	
			72°C	30 s	
			72°C	10 min	1
pT1-loxP	Tec1FW & Tec1REV	*TEC1* gene	95°C	5 min	1
			94°C	30 s	30
			56°C	30 s	
			72°C	40 s	
			72°C	10 min	1
pP3-loxP & pT1-loxP	loxPfw & loxPrev	loxP cassette	95°C	5 min	1
			94°C	30 s	30
			64°C	30 s	
			72°C	1 min 30 s	
			72°C	10 min	1

3.2.5 Bacterial transformation

Blank competent *E. coli* DH5α cells were transformed with the constructs following the transformation protocol from the Promega Technical Manual (TM042).

3.2.6 Yeast transformation

The two plasmids (pP3-loxP and pT1-loxP) were used to transform the seven strains mentioned in Table 1. All the strains were transformed with both plasmids individually using a LiAc method as described by Agatep *et al*. (1998). The transformants were incubated on YPD plates containing 200 μg mL^{-1} G418 for three days.

Positive transformants were identified by colony PCR. Five colonies for each strain were picked from the YPD plates containing 200 μg mL^{-1} G418 for selection and each resuspended in 50 μL sterile water. 1 μL of this was used as template for the PCR reaction. The primers used were 5'-KPNPGK-631 and Pgu1rev2 (refer to Table 1). The reaction took place in a total volume of 50 μL, using Supertherm Polymerase (Southern Cross Biotechnology). The PCR program started with a denaturation step at 95°C for 10 minutes. This was followed by 35 cycles of

denaturing at 94°C for 30 s, annealing at 58°C for 30 s and elongating at 72°C for 1 minute. It was ended with a final elongation step at 72°C for 10 minutes.

3.2.7 Polygalacturonase plate assay

Recombinants were tested for their PG activity by a plate assay that indicates polygalacturonic acid breakdown. The method was adapted from McKay (1988). Plates containing 1.25 % polygalacturonic acid (Sigma), 0.68% potassium phosphate, pH 3.5, 0.67% Bacto™ Yeast Nitrogen Base without amino acids (Difco, BD, Johannesburg, South Africa), 1% glucose and 2% agar were prepared. The plates also contained 20 µg mL^{-1} L-histidine, 60 µg mL^{-1} L-leucine, 30 µg mL^{-1} L-lysine and 20 µg mL^{-1} Uracil to allow the growth of BY4742, a strain auxotrophic for these amino acids. Overnight cultures of the recombinants were grown in 5 mL YPD at 30°C. The next morning, the cultures were diluted to 1×10^6 cells mL^{-1}. From these dilutions, 5 µL were spotted on a plated and incubated at 30°C for three days. The cells were washed off with water and the plate covered with 6 M HCl. Clear zones appeared around the spots which indicated PG activity in the specific strain. The assay was performed in triplicate.

3.3 Results and discussion

3.3.1 Plasmid construction

Two plasmids were constructed as mentioned in the materials and methods section. The first plasmid is pP3-loxP. This plasmid contains the *PGU1* gene amplified from L2323 and cloned into pCEL13. Sequencing of this gene confirmed that it matches perfectly that of the S288C strain (Saccharomyces Genome Database; Data not shown). The plasmid also includes a loxP cassette with the *kan*^{r} gene for resistance to G418. The second plasmid is pT1-loxP. It contains the *TEC1* gene amplified from BY4742 (which is the same as that of S288C) and with the loxP cassette cloned in pCEL13. The plasmids are presented in Fig. 1.

The plasmids were verified firstly by PCR amplification of the specific gene (*PGU1* or *TEC1*) and the loxP cassette as mentioned in the previous section (see Table 2) and secondly by enzymatic digestion. Correct amplicons as well as the correct digestion fragments were obtained for both plasmids (data not shown).

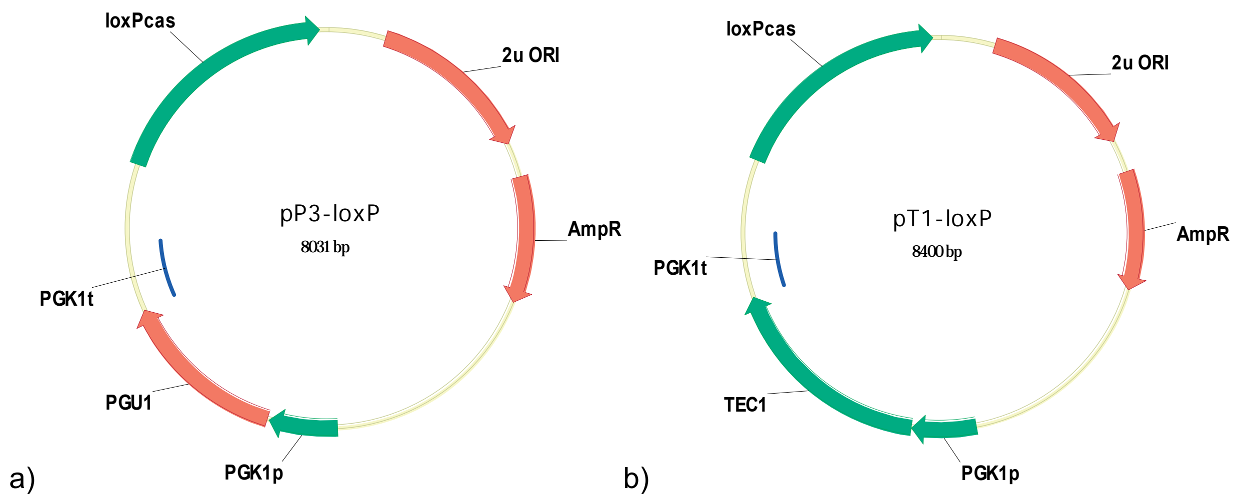

Fig. 1. The final constructs, pP3-loxP (a) and pT1-loxP (b), that were used to transform the different yeast strains. The genes are under the control of the *PGK1* promoter allowing for constitutive expression.

3.3.2 Yeast transformation

All seven strains were transformed successfully with pP3-loxP and pT1-loxP respectively. The transformation efficiency was high for all the strains and a single transformation attempt was sufficient to ensure positive recombinants for all the strains. This proved the LiAc method effective for plasmid transformations in both wine and laboratory strains of *S. cerevisiae*. Positive recombinants were identified by colony PCR as described in the previous section. In order not to amplify the native *PGU1* gene in certain strains, a forward primer in the *PGK1* promoter was used (5'-KPNPGK-631) along with a reverse primer in the *PGU1* gene (Pgu1rev2). Almost all the colonies that were screened tested positive for the plasmid.

3.3.3 Determination of polygalacturonase activity

In this experiment, the plate assay was considered semi-quantitative. All recombinants were spotted on plates containing polygalacturonic acid to test their polygalacturonase activity (ability to break down polygalacturonase). The size of the clear zone corresponds to the activity of the strains: the larger the zone, the higher the activity. On each plate, the respective wild type strains were also spotted in order to determine the increase in the size of the clear zone in the recombinants. On all plates, L2323 wild type was spotted as a positive control and BY4742 as a negative control. The diameters of the zones that appeared on the plates were measured to obtain a numerical value for each strain's activity. The results are presented in Table 4.

Overexpression of the *PGU1* gene led to a general increase in PG activity in the yeast, even in the strains that have no natural activity such as CC Cabernet, CC Merlot, L2226 (which do not possess the *PGU1* gene), VIN13 and BY4742 (that do possess the gene in their genomes, but

Table 4. Diameters of clear zones formed on polygalacturonic acid plates by the different recombinants and their wild types. The values in the "Before" column indicate the size of the zone of the wild type strain before it was transformed with a plasmid. The column "After" indicates the diameters after transformation. The last column indicates the difference between the before and after measurements, thus showing the increase (if any) in the zone size after transformation.

Strain	Plasmid	Before (zones)	After (zones)	Difference
CC Cabernet	**pP3-loxP**	0 mm	43 mm	43 mm
	pT1-loxP	0 mm	0 mm	-
CC Merlot	**pP3-loxP**	0 mm	42 mm	42 mm
	pT1-loxP	0 mm	0 mm	-
L2226	**pP3-loxP**	0 mm	41 mm	41 mm
	pT1-loxP	0 mm	0 mm	-
L2323	**pP3-loxP**	15 mm	41 mm	26 mm
	pT1-loxP	13 mm	25 mm	12 mm
VIN13	**pP3-loxP**	0 mm	39 mm	39 mm
	pT1-loxP	0 mm	23 mm	23 mm
Σ1278b	**pP3-loxP**	14 mm	40 mm	26 mm
	pT1-loxP	12 mm	12 mm	0 mm
BY4742	**pP3-loxP**	0 mm	36 mm	36 mm
	pT1-loxP	0 mm	18 mm	18 mm

exhibit no PG activity). This indicated that the lack of activity in these latter two strains is not due to post-transcriptional regulation, as a functional enzyme is secreted when *PGU1* is under plasmid promoter control. This is in accordance with the results obtained by Blanco *et al*. (1998). It rather suggests a non-functional *PGU1* promoter or gene for strains lacking PG activity. This was the case even for the strains that naturally lost the *PGU1* gene: the experiment clearly showed that if the gene is re-introduced by genetic engineering in these strains, PG activity should be recovered. However, the *PGU1* genes and promoters of the various strains were not analysed in this study. For this reason, it is also possible that the *PGU1* genes of VIN13 and BY4742 may be kept inactive by a transacting factor as proposed by Hirose *et al*. (1999).

Tec1p is a transcription factor for the *PGU1* gene. It is thus expected that overexpressing this gene would result in an increase of *PGU1* transcription and therefore allow the yeast to have higher PG activity. Overexpression of *TEC1* did indeed increase PG activity in L2323 and also allowed VIN13 and BY4742 to exhibit activity. In these latter strains, the native Tec1p is unable to activate *PGU1* transcription, while the plasmid–derived protein can. This is probably due to an inhibitory factor acting on the native *TEC1* promoter, inhibiting *TEC1* transcription in these strains. The plasmid-derived *TEC1* gene is under control of the *PGK1* promoter which is not inhibited. Subsequently, this gene is transcribed and its protein able to activate transcription of *PGU1* in the genome of the yeast. The overexpression of *TEC1* in CC Cabernet, CC Merlot and L2226 did not lead to an increase in PG activity. This was also expected as these strains do not

possess the *PGU1* gene in their genomes. A surprising result was the fact that Σ1278b did not show an increase in activity when *TEC1* was overexpressed. This strain naturally exhibits PG activity. This can be attributed to a functional *PGU1* gene and promoter. The difference could be that this strain exhibits post-transcriptional regulation on *TEC1*, thereby preventing a functional protein from binding to the *PGU1* promoter. The regulation has to be on *TEC1* (or another factor that acts on the Tec1 protein) as the strain did show an increase in activity when *PGU1* was overexpressed.

3.4 Conclusions

As a first attempt to verify the contradictory results and hypotheses found in the literature, the effect of overexpressing either *PGU1* or *TEC1* on polygalacturonase activity was tested in several strains of *S. cerevisiae*. These strains included laboratory as well as wine strains. Some of these strains have natural PG activity while some do not. Of those that show no activity, three strains do not possess the *PGU1* gene, while the other two do. Two plasmids were constructed. Both constructs had pCEL13 as a backbone. The first contained the *PGU1* gene under control of the constitutive *PGK1* promoter while the second had the *TEC1* gene.

The recombinant strains and their respective wild types were subjected to a polygalacturonase plate assay to test the effect of the plasmids (and thus overexpression of the particular genes) on the PG activity of the yeasts. It was found that overexpression of the *PGU1* gene leads to an increase in PG activity in all the strains, including those that naturally lack *PGU1*. This study therefore opened the way to the re-introduction of the *PGU1* gene in these strains. *TEC1*, a transcription factor for *PGU1*, did not have any effect on the three strains that do not possess the *PGU1* gene, as expected. It increased the PG activity of all the other strains, but not as much as the overexpression of the *PGU1* gene did. The only exception was Σ1278b which did not show any increase in activity when *TEC1* was overexpressed.

This study showed the effect of two genes on the PG activity of several *S. cerevisiae* strains. The results were mostly as expected. Through sequencing analyses, this study found that the lack of PG activity in BY4742 is not due to a non-functional *PGU1* gene. The *PGU1* gene sequence for BY4742 was identical to that of L2323, a strain that exhibits PG activity. BY4742 (as well as all the other strains tested) also does not seem to exhibit any post-transcriptional regulation on the Pgu1p as was indicated by an increase in PG activity through overexpression of the *PGU1* gene. This confirmed the results obtained by Hirose *et al.* (1998) and Hirose *et al.* (1999) that proposed the *PGU1* gene to be regulated by a trans-acting factor inhibiting *PGU1* transcription. Σ1278b was however not affected by the overexpression of *TEC1*. This is

probably due to some post-transcriptional regulation on the Tec1 protein in this strain. This needs to be confirmed and investigated further.

3.5 References

Agatep R, Kirkpatrick RD, Parchaliuk DC, Woods RA & Gietz RD (1998) Transformation of *Saccharomyces cerevisiae* by the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol. *Technical Tips Online* (http://tto.trends.com).

Blanco P, Sieiro C, Reboredo NM & Villa TG (1998) Cloning, molecular characterization, and expression of an endo-polygalacturonase-encoding gene from *Saccharomyces cerevisiae* IM1-8b. *FEMS Microbiol Lett* **164:** 249-255.

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P & Boeke JD (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14:** 115-132.

Cryer DR, Eccleshal R & Marmur J (1975) Isolation of yeast DNA. *Methods Cell Biol* **12:** 39-44.

Davis RW, Thomas M, Cameron J, St John TP, Scherer S & Padgett RA (1980) Rapid DNA isolations for enzymatic and hybridization analysis. *Methods Enzymol* **65:** 404-411.

De Vries RP & Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol Mol Biol Rev* **65:** 497-522.

Divol B & van Rensburg P (2007) *PGU1* natural deletion is responsible for the absence of endo-polygalacturonase activity in some wine strains of *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7:** 1328-1339.

Fernández-González M, Úbeda JF, Vasudevan TG, Cordero Otero RR & Briones AI (2004) Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains. *FEMS Microbiol Lett* **237:** 261-266.

Gognies S, Simon G & Belarbi A (2001) Regulation of the expression endopolygalacturonase gene *PGU1* in *Saccharomyces*. *Yeast* **18:** 423-432.

Güldener U, Heck S, Fiedler T, Beinhauer J & Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24:** 2519-2524.

Gundllapalli SB, Otero RRC & Pretorius IS (2006) Development of a screening method for the identification of a novel *Saccharomyces cerevisiae* mutant over-expressing *Trichoderma reesei* cellobiohydrolase II. *Ann Microbiol* **56:** 143-150.

Hirose N, Kishida M, Kawasaki, H & Sakai T (1998) Molecular cloning and expression of a polygalacturonase gene in *Saccharomyces cerevisiae*. *J Ferment Bioeng* **86:** 332-334.

Hirose N, Fujii M, Kishida M, Kawasaki H & Sakai T (1999) Isolation and genetic determination of a *Saccharomyces cerevisiae* mutant that produces an endo-polygalacturonase. *J Biosci Bioeng* **87:** 594-597.

Jimenez A & Davies J (1980) Expression of a transposable antibiotic resistance element in *Saccharomyces. Nature* **287:** 869-871.

Kester HCM, Kusters-Van Someren MA, Müller Y & Visser J (1996) Primary structure and characterization of an exopolygalacturonase from *Aspergillus tubingensis*. *Eur J Biochem* **240:** 738-746.

Köhler T, Wesche S, Taheri N, Braus GH & Mösch H (2002) Dual role of the *Saccharomyces cerevisiae* TEA/ATTS family transcription factor Tec1p in regulation of gene expression and cellular development. *Eukaryot cell* **1:** 673-686.

Madhani HD, Galitski T, Lander ES & Fink GR (1999) Effectors of a developmental mitogen-activated protein kinase cascade revealed by expression signatures of signaling mutants. *Proc Natl Acad Sci USA* **96:** 12530-12535.

McKay AM (1988) A plate assay method for the detection of fungal polygalacturonase secretion. *FEMS Lett* **56:** 355-358.

Radoi F, Kishida M & Kawasaki H (2005) Endo-polygalacturonase in *Saccharomyces* wine yeasts: effect of carbon source on enzyme production. *FEMS Yeast Res* **5:** 663-668.

Sauer B (1987) Functional expression of the cre-lox site-specific recombination system in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* **7:** 2087-2096.

Volschenk H, Viljoen-Bloom M, van Staden J, Husnik J& van Vuuren HJJ (2004) Genetic engineering of an industrial strain of *Saccaromyces cerevisiae* for L-malic acid degradation via an efficient malo-ethanolic pathway. *S Afr J Enol Vitic* **25:** 63-73.

Wach A, Brachat A, Pöhlmann R & Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10:** 1793-1808.

Chapter 4

Research results

Recovery of endo-polygalacturonase activity in wine yeast and its effect on wine aroma

This manuscript will be submitted for publication in
FEMS Yeast Research

4. Research results

Recovery of endo-polygalacturonase activity in wine yeast and its effect on wine aroma

Herine van Wyk and Benoit Divol
Institute for Wine Biotechnology, Stellenbosch University, Matieland, South Africa

Abstract

The *PGU1* gene, which encodes an endo-polygalacturonase, is present in most strains of *Saccharomyces cerevisiae*. In some strains that lack activity, the *PGU1* gene was replaced by a partial transposon. In an attempt to restore enzyme activity, the *PGU1* gene was successfully re-introduced into its native position in five of these strains. PG activity was recovered in strains UCLMS-1, -3 and -4, while Collection Cépage Cabernet and L2226 did not show any transcription of the gene after integration. Small scale fermentations, under conditions mimicking those of winemaking, were carried out with the recombinant strains to determine the effect of an endo-polygalacturonase-producing wine strain on the wine aroma. Some tendencies were observed for the production of certain compounds to be higher for the recombinant strains than their wild types. However, none of the differences observed were significant and we propose the experiment be repeated under more optimal conditions for the enzyme activity.

4.1 Introduction

Polysaccharides, such as pectin, glucan and xylan, can be found between 300 and 1000 mg L^{-1} in wine and are often responsible for turbidity, viscosity and filter stoppages (Van Rensburg & Pretorius, 2000). Pectinases derived from the grapes and wine-related micro-organisms generally have a low activity under winemaking conditions. As a result, commercial enzyme preparations are often added before pressing to improve juice and colour yield and possibly enhance extraction of aroma compounds or precursors. Alternatively, the enzyme preparations are added after pressing to improve clarification.

The *Saccharomyces cerevisiae PGU1* gene (also known as *PGL1*) is situated on chromosome X and only a single copy is present per haploid genome. This gene encodes for an endo-polygalacturonase – a pectinolytic enzyme that cleaves the bonds between non-methylated galacturonic acid units in pectin.

At first, it was thought that *S. cerevisiae* does not produce the pectinolytic enzymes needed for pectin degradation (Luh & Phaff, 1951). In 1956 however, polygalacturonase (PG) activity was detected in certain strains (Bell & Etchells, 1956). Since then, the presence of this gene in *S. cerevisiae* has been characterised in various strains. Although most strains of *S. cerevisiae* possess the *PGU1* gene, their activity varies. Some strains do have the gene in their genomes, yet lack the ability to degrade pectic substances (Fernández-González *et al*., 2004) and some were even identified to express the gene but still lack activity (Gognies *et al*., 1999). It was observed that some strains that cannot degrade pectin are able to do so when *PGU1* was introduced on a plasmid (Blanco *et al*., 1998). The gene and promoter sequences of these strains showed no or very little differences when compared to that of strains able to degrade polygalacturonic acid naturally (Hirose *et al*., 1998; Hirose *et al*., 1999). The latter authors proposed the *PGU1* gene to be regulated by a trans-acting factor that inhibits *PGU1* transcription.

Some effects of yeast-derived polygalacturonases on wine have been studied. Blanco *et al*. (1997) used *S. cerevisiae* strains with PG activity in vinification and found in some cases a decrease in filtration time. Another group (Vilanova *et al*., 2000) constructed a strain that overexpressed *PGU1* (by using a plasmid that contain the *PGU1* gene under expression of the constitutive *PGK1* promoter) and also found a shorter filtration time as well as an increase in a few volatile compounds.

A few strains actually lack the *PGU1* gene, resulting in the absence of PG activity in these particular strains. Fernández-González *et al.* (2004) studied 61 strains, and found that nine of them do not have the gene. They identified certain strains that lack the gene, namely UCLMS-1, -3, -4, -31, -58, -59, -63, -67 and -72. The strains OC2, KW1 and L2226 were also found not to possess the *PGU1* gene (Radoi *et al*., 2005). Divol & van Rensburg (2007) showed that in these strains the *PGU1* gene was replaced by a partial mobile Ty-element while the promoter is still at its expected location. They tested and verified the absence of the gene in the above-mentioned strains and also identified Collection Cépage Cabernet (CC Cabernet) and Collection Cépage Merlot (CC Merlot) to exhibit the same phenomenon.

The reason for the natural deletion of the *PGU1* gene in certain strains is still unknown. These strains could have lost the gene by directed evolution, where the gene was silenced because the yeast no longer had a need for it. Another possibility is that the replacement by a mobile element was simply a random phenomenon. The fundamental aim of this study was to re-introduce the *PGU1* gene into its native position in some of these strains that lost the gene and to test if it allowed the strains to recover PG activity. This would then indicate if the lack of activity in these strains is purely because they do not possess the gene, or whether there is

additionally some transcriptional or post-transcriptional regulation that prevents the yeast from secreting a functional enzyme.

From an oenological point of view, the study also aimed to show the effects of a good oenological strain with PG activity on the aroma compounds of wine. A yeast that secretes an active endo-polygalacturonase under winemaking conditions could facilitate the extraction of aroma compounds or precursors and alter the final aroma profile of the wine (Pretorius, 2000). The *PGU1* gene was re-introduced into wine strains with strong oenological properties, but lacking the *PGU1* gene, to obtain good oenological strains with PG activity. This would potentially eliminate the need to add expensive commercial enzyme preparations.

4.2 Materials and methods

4.2.1 Strains, media and culture conditions

The bacterium used for transformations was *Escherichia coli* DH5α (F-φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-). All transformed *E. coli* were plated out onto Luria-Bertani (LB) agar (Biolab Diagnostics, Wadeville, South Africa) plates containing 100 µg ml^{-1} Ampicillin (Ampicillin sodium salt, Sigma, Missouri, USA) for selection. Selected colonies were then grown in LB medium, also containing 100 µg ml^{-1} Ampicillin, at 37°C. For yeast transformations, several industrial *S. cerevisiae* strains were used: CC Cabernet, CC Merlot, L2226, UCLMS-1, -3, -4, -57, -58, -59, -63 and -72. The *PGU1* gene was amplified from *S. cerevisiae* L2323. This wine strain exhibits a relatively high PG activity (not shown). Yeast cultures were grown on Yeast Peptone Dextrose (YPD) agar plates and YPD medium (Biolab Diagnostics) containing various concentrations of G418 (G418 disulphate salt, Sigma) for selection. The concentrations ranged from 180 – 250 µg mL^{-1}. Yeast cultures were incubated at 30°C.

4.2.2 Isolation of genomic DNA

Genomic DNA was isolated from yeast using the alternative protocol for rapid isolation of yeast chromosomal DNA according to the method described by Cryer *et al*. (1975) and Davis *et al*. (1980).

DNA was also isolated from wine isolates for direct PCR analyses by using the Whatman® Indicating TFA® Classic Card (Sigma-Aldrich, Johannesburg, South Africa). The protocol was followed as instructed by the supplier, except that the washing steps with FTA Purification Reagent and 1X TE were not repeated. The 1.2-mm punch was used.

4.2.3 PCR amplification

All primers used for PCR amplification are listed in Table 1. The *PGU1* gene (as well as a small part of the *PGU1* promoter and the complete terminator) was amplified form *S. cerevisiae* L2323 using the primers intPGfw and intPGrev. Genomic DNA was used as template. The PCR reaction was performed in a total volume of 50 µL, using 1 U Extaq DNA Polymerase from Takara (SEPARATIONS, Randburg, South Africa).

Table 1. List of primers used for PCR amplification in this project. The restriction sites incorporated in some primers are underlined and the corresponding restriction enzyme indicated in brackets.

Primer name	Sequence 5' – 3'	Purpose in this study	Reference
intPGfw	GCTGTAACGGAAACAGAG	Amplification of the *PGU1* region from *S. cerevisiae* L2323	Divol & Van Rensburg (2007)
intPGrev	GTCTATCTGAATCGCTTATG		Divol & Van Rensburg (2007)
loxPfw	ATCGAGGCCTGGAATTCGATTGATCGCATGC (*Stu*I)	Amplification of the loxP cassette from pGEM®-T Easy plasmid	This study
loxPrev	ATCGAGGCCTCGAATTCACTAGTGATAGATCGGATCC (*Stu*I)		This study
intPGUcasFW	ATGCCCCGGGCTAGATGCATGATACTCGTGCATTCGTTTAACAATCATACCAATTTCCCATTTTCGGGATATTAAACATGAACATACTTTTTTACTGTGAGAATGTGGTTTCACAATTATTCCATACAGGTATAAAAACGCACAGAACTT (*Sma*I)	Amplification of the whole *PGU1* cassette for integration with addition of homologous sequences for recombination.	This study
intPGUcasREV	GCATCCCGGGGCCTTAAAGATATTTTGGCTTTTTTCTCACTGGCTCGATGGGTAAACAATGGTTCAAAAAAACAGGGCTATTATTTGAGTCAGCGAAGTTTCACAATTGTGTCATATTGGTAGATGCATTTAAACATAAAATGGAGAAGG (*Sma*I)		This study
PGregionFW2	CAGATACACTTTATGAAATAG	Amplification of the *PGU1* region from the recombinant BY4742 to obtain the modified integration cassette.	Divol & Van Rensburg (2007)
PGregionREV	GAATCTGTGTTCATCGTATG		Divol & Van Rensburg (2007)
5'PPGL1-HINIII	AAGCTTATGATTTCTGCTAATTCATTACTTATTTCCAC (*Hind*III)	Amplification of the *PGU1* gene for screening *E. coli* transformants.	This study
3'PPGL1-BGLII	AGATCTTTAACAGCTTGCACCAGATCCAGATG (*Bgl*II)		This study
Dal5promfw	CGAAAGATAAAGAGTGCTGGCTCA	Used for screening for integration of PGU1-loxP cassette in yeast	This study
Pgu1rev2	CGTCAACGCCAACTTTACAA		Divol & Van Rensburg (2007)
S. cerevisiae actin frw	TAAAGCCGGTTTTGCCGGTGAC	Amplification of the *ACT1* gene	This study
ACTRev	CGATAGATGGACCACTTTCGTCG		This study
Delta 12	TCAACAATGGAATCCCAAC	Fingerprinting recombinant and wild type yeasts	Legras & Karst (2003)
Delta 21	CATCTTAACACCGTATATGA		Legras & Karst (2003)

The program started with an initial denaturation step for 5 min at 95°C. A denaturation step at 94°C for 30 s, annealing step at 54°C for 30 s and an elongation step at 72°C for 2 min 30 s was repeated for 35 cycles. A final elongation step took place at 72°C for 10 min.

The loxP-KanMX-loxP cassette was also amplified from a pGEM®-T Easy plasmid previously constructed (Malherbe DF, unpublished). For this reaction, the primers loxPfw and loxPrev were used, which have built-in *Stu*I restriction sites. All concentrations in the reaction were the same as for amplification of the *PGU1* gene. The same program was used, but the annealing temperature was changed to 60°C and the elongation time shortened to 1 min 40 s. Primers were designed to amplify the whole integration cassette (PGU1-loxP) from the constructed plasmid. These primers (intPGUcasFW and intPGUcasREV) have 120 bp flanking regions that are specific to areas just outside the PG region in the strains to be transformed. They also contain *Sma*I restriction sites at each end. The primers were designed for binding at specific areas on the plasmid and consequently had very low Tm values (41°C for the area binding to the plasmid). To optimize the PCR reaction for amplification of the integration cassette, a temperature gradient PCR was performed with annealing temperatures ranging from 41°C to 55°C. The program started with an initial denaturation step at 95°C for 5 min. Then a denaturation step at 94°C for 30 s, annealing step between 41 – 55°C (each column in the PCR block had a different temperature) for 30 s and elongation step at 72°C for 3 min was repeated for 5 cycles. Another 30 cycles were then repeated with the same denaturation and elongation steps, but with an annealing step at 60°C (This higher annealing temperature is the Tm of the whole primer, including the flanking regions).

4.2.4 Construction of the plasmids

A pGEM®-T Easy vector from Promega (Whitehead Scientific, Stikland, South Africa) containing the Ty region cloned from *S. cerevisiae* L2226 was used as backbone to construct the plasmid. It was constructed by amplifying the whole Ty region from L2226 using the primers intPGfw and intPGrev and then cloning it into a pGEM®-T Easy vector according to the method described by the provider. This plasmid was digested with *Bln*I and *Cla*I (both from Roche, Penzberg, Germany) to remove the largest, middle part of the Ty region. The *S. cerevisiae* L2323 *PGU1* gene was amplified by PCR as described and the amplicon digested with the same enzymes. All digested DNA were separated by electrophoresis on 1% agarose gels. The correct fragments were excised and purified using the QIAquick® Gel Extraction Kit (Qiagen, Southern Cross Biotechnology, Cape Town, South Africa). The digested amplicon was cloned into the vector using T4 DNA ligase from Promega (Whitehead Scientific) according to the supplier. The reaction took place at 4°C overnight. The loxP cassette was also cloned into this plasmid. The plasmid was digested with *Stu*I (Roche) and treated with Antarctic Phosphatase (New England Biolabs, Laboratory Specalist Services, Clareinch, South Africa) as indicated by the supplier to remove the 5' phosphate groups, thus preventing the vector from self-ligating. The loxP cassette amplicon was also digested with *Stu*I. It was cloned into the plasmid with T4 DNA ligase. The final construct was named pGPL and is presented in Fig. 1a.

The plasmid was used as template for PCR amplification of a PGU1-loxP integration cassette using the primers intPGUcasFW and intPGUcasREV (Table 1). The PCR product was separated by electrophoresis on a 1% agarose gel and excised using the QIAquick® Gel Extraction Kit (Qiagen). The integration cassette was blunted and cloned into pJET1 using the GeneJET™ PCR Cloning Kit (Fermentas) as indicated by the supplier. The construct was verified by digestion with two enzymes in individual reactions: *EcoR*I and *Mun*I (both Roche). It was named pJPL (Fig. 1b).

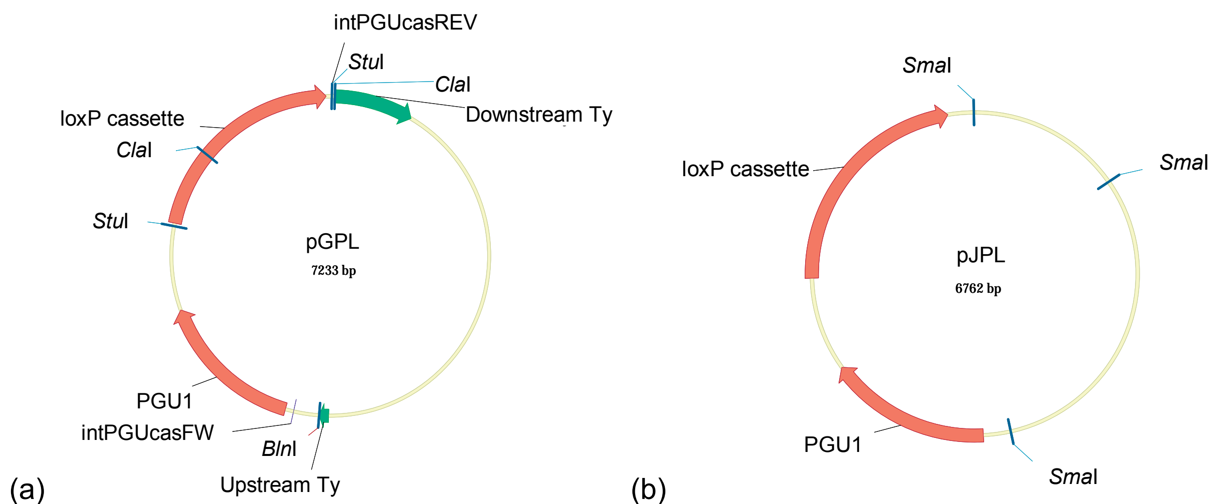

Fig. 1. The two plasmids constructed in this study. pGPL (a) has a pGEM-T Easy backbone and contains the *PGU1* gene and the loxP cassette. pJPL (b) is a pJET1 based plasmid containing the PGU1-loxP cassette that was amplified from pGPL.

4.2.5 Bacterial transformation

Chemically competent *E. coli* DH5α cells were transformed with the constructs following the transformation protocol from the Promega Technical Manual (TM042).

4.2.6 Sequencing

After completion of the pJPL plasmid, it was sent to the Central Analytical Facility at Stellenbosch University, South Africa for sequencing. This was to determine if the sequence of the integration cassette was correct before integration into the yeast strains. Several primers were supplied for the sequencing reaction. They are summarized in Table 2.

Table 2. Primers used for sequencing the integration cassette after it was cloned into pJET1.

Primer name	Sequence 5' – 3'	Description	Reference
pJET1FW	GCCTGAACACCATATCCA TCC	Forward primer that binds to pJET1, 27-47 bp upstream of the insert (integration cassette)	GeneJET™ PCR Cloning Kit (Fermentas)
Pgu1fw2	GTGCTTCGGGACATACCA TT	Forward primer that binds at position 296-315 of the *PGU1* gene	Divol & Van Rensburg (2007)
pJET1REV	GCAGCTGAGAATATTGTA GGAGATC	Reverse primer that binds to pJET1, 10-34 bp downstream of the insert (integration cassette)	GeneJET™ PCR Cloning Kit (Fermentas)
Kan-R (AS)	CTGCAGCGAGGAGCCG	Reverse primer that binds at position 200-215 of the *kan*^{r} gene in the loxP cassette	This study
Pgu1rev2	CGTCAACGCCAACTTTAC AA	Reverse primer that binds at position 377-396 of the *PGU1* gene	Divol & Van Rensburg (2007)

4.2.7 Yeast transformation

The plasmid pGPL was linearised by digestion with *Bsa*I (Roche) and used to transform the yeast strains using a LiAc method (Agatep *et al*., 1998). The pJPL plasmid was digested with *Sma*I (Roche) which released the insert (integration cassette). The yeast strains were transformed with the integration cassette using the same LiAc method as mentioned previously, with the following changes: firstly, no pre-culture was grown overnight and re-inoculated in a larger volume the next morning. Instead, a single yeast colony was inoculated in 200 mL of YPD and incubated overnight. This was directly used the next morning. Secondly, three times the amount of cells were used than indicated in the protocol.
The cassette was also integrated into BY4742, using the LiAc method. The DNA from this strain was used as template to amplify a second integration cassette, using the primers PGregionFW2 and PGregionREV (Table 1). This second cassette was used to transform the wine strains using an electroporation method by Volschenk *et al*. (2004). For all yeast transformations 1-4 µg of DNA was used per strain.

4.2.8 Identification of positive transformants

All transformants were identified by PCR. For transformed *E. coli*, a simple colony PCR was performed. Several colonies were picked from the plates and each resuspended in 50 µL sterile water. 1 µL was used as template for the PCR reaction. The primers 5'PPGL1-HINIII and 3'PPGL1-BGLII were used to amplify the *PGU1* gene using GoTaq polymerase (Promega). The reactions took place in a total volume of 50 µL. The annealing temperature was 55°C and the elongation time 1 min. The rest of the reaction was the same as for previous PCR amplifications. For colonies that tested positive, the rest of the colony suspension was inoculated in 5 mL LB broth and grown overnight.

To verify integration of the PGU1-loxP cassette in yeast, a PCR reaction was done using a forward primer in the genome of the yeast upstream of the integration position (Dal5promFW) and a reverse primer in the *PGU1* gene (pgu1rev2). Genomic DNA was isolated from several yeast colonies and used as template. The volumes of the reaction were the same as for screening *E. coli* transformants, except that 0.3 mM dNTPs were used. The annealing temperature was 56°C and the elongation time 4 min 30 s.

A fingerprint PCR was also carried out on all recombinants to confirm that the recombinant and the wild type are indeed the same strain. The program was modified slightly from that described by Legras & Karst (2003): an annealing temperature of 55°C and an elongation time of 30 s were used. The reactions were performed in a total volume of 50 µL using 1.25 U ExTaq Polymerase (Takara). The primers used were Delta 12 and Delta 21 (Table 1).

4.2.9 Polygalacturonase plate assay

This assay was adapted slightly from the protocol described by McKay (1988). Plates containing 1.25% polygalacturonic acid (Sigma), 0.68% potassium phosphate, pH 3.5, 0.67% Bacto^{TM} Yeast Nitrogen Base without amino acids (Difco, BD, Johannesburg, South Africa), 1% glucose, and 2% agar were prepared. The following amino acids were then added: 20 µg mL^{-1} L-histidine, 60 µg mL^{-1} L-leucine, 30 µg mL^{-1} L-lysine and 20 µg mL^{-1} Uracil (the amino acids were added to allow growth of the laboratory strain BY4742 on the plates). The recombinant yeasts were grown in 5 mL YPD overnight at 30°C. The next morning the OD_{600nm} of the cultures were measured and appropriately diluted in order to obtain 2 x 10^6 cells ml^{-1}. 5 µL of each dilution were spotted on a polygalacturonic acid plate. On every plate, *S. cerevisiae* L2323 was spotted as a positive control and BY4742 was spotted as negative control. The plates were incubated at 30°C for 3 days. It was then removed from the incubator and the cells washed off with water. The plates were covered with 6M HCl and left at room temperature until a clear zone appeared around the spots. All plate assays were carried out in triplicate.

4.2.10 RNA isolation and reverse transcription

RNA was isolated from all recombinant strains and their wild types. The method was adapted from a protocol for small scale RNA isolation from tobacco (Joubert, 2004). Yeast cultures were grown overnight in 5 mL YPD. The next morning 1-2 mL of the culture was centrifuged and the supernatant removed. The pellet was resuspended in 1 mL extraction buffer (0.1 M Tris/HCl pH 8.0, 1.5% SDS, 300 mM LiCl, 10 mM Na_2EDTA, 1% (w/v) Na-deoxycholate, 5 mM Thiourea, 1% (w/v) Na-Metabisulphite). The extraction buffer was mixed with phenol (Tris-buffered, pH 8.0) in a 7:3 ratio before being added to the yeast cells. The resuspended cells were transferred to a

screw cap tube containing 200 µL glass beads. The samples were vortexed for 5 minutes at room temperature. 200 µL chloroform was added and vortexed vigorously for 2 minutes. All the samples were then centrifuged at 12 000 *g* for 10 minutes and the aqueous phase transferred to a fresh tube. An equal volume of isopropanol was added and the samples were incubated at room temperature for 10 minutes. They were centrifuged at 12 000 *g* for 10 minutes and the supernatant discarded. The resultant RNA pellet was washed with 1 mL 70% ethanol, mixed well by inversion and centrifuged again. The supernatant was discarded. The pellet was dried at 55°C for about 5 minutes and the RNA pellet resuspended in 50 µL DEPC-treated water. To each sample, 1 µL Ribolock™ RNase Inhibitor (Fermentas) was added and the samples were stored at -20°C.

Each sample was treated with 10 units DNaseI recombinant, RNase-free (Roche) for 1 hour in a 37°C waterbath. A PCR reaction was performed to determine if there was any DNA left in the RNA samples. For this reaction, the primers *S. cerevisiae* actin frw and ACTRev (refer to Table 1) were used that amplify the *ACT1* gene. The PCR reaction was the same as that used for screening *E. coli* transformants. The only difference was the annealing temperature, which was changed to 57°C. The RNA was then reverse transcribed using the ImProm-II ™ Reverse Transcription System (Promega) as described by the supplier. For each reaction, 1 µg of RNA was used as template along with the Oligo$(dT)_{15}$ primers of the kit. The synthesized cDNA was used as template for two individual PCR reactions. The first reaction was to amplify the *PGU1* gene in the same way as for screening *E. coli* transformants. In the second reaction, the *ACT1* gene was amplified as described above. ExTaq Polymerase (Takara) was used for both reactions.

4.2.11 Microvinifications

Three recombinant yeast strains (UCLMS-1, -3 and -4) as well as their respective wild types were used to ferment grape must from two different cultivars, Chenin Blanc, and Shiraz. The grapes were pressed and 30 ppm SO_2 added to the juice. To each cultivar, 60 g hL^{-1} Nutrivin (Anchor, Eppindust, South Africa) was added and 1.5 L aliquots were fermented in 2-L glass bottles at 15°C. All fermentations were performed in triplicate. Table 3 summarises the sugar and titratable acid (TA) measurements performed on the must before inoculation.

Table 3. Sugar concentration and titratable acid in the different cultivar musts before inoculation.

Cultivar	Sugar concentration (°B)	TA (g L^{-1})
Chenin blanc	20.5	5.82
Shiraz	27.15	4.93

Yeast strains used were grown in 500 mL YPD at 30°C with shaking for 2 – 3 days. The must was inoculated directly with the pre-cultures at 1 x 10^6 cells mL^{-1}. The progress of the fermentations was monitored by measuring the weight loss, due to CO_2 release, on a daily basis. When there was no change in the weight for 3 consecutive days, the fermentation was considered as finished and a sample was taken. The sample was used to make a dilution series up to a 1/100000 dilution. 100 µL of the 1/10000 and 1/100000 dilutions were plated out onto two plates each: a YPD plate and a YPD plate containing 200 µg mL^{-1} G418 (Sigma). The plates were incubated for about three days. Cell counts were performed and a comparison was made between growth on plates with and without G418. The colonies were also screened by PCR (as described previously) to verify that alcoholic fermentation was indeed performed by the strain inoculated in the must.

4.2.12 Extraction of wine components

After filtration and bottling, certain aroma compounds were extracted from the wines.

Extraction of the volatile compounds from wine:
Five mL of wine with an internal standard, 4-Methyl-2-Pentanol, (100 µL of 0.5 mg L^{-1} solution in soaking solution) were extracted with 1 mL of diethyl ether by placing the ether/wine mixture in an ultrasonic bath for 5 minutes. The wine/ether mixture was then centrifuged at 4000 rpm for 3 minutes. The ether layer was removed and dried on $NaSO_4$. This extract was then injected into the GC-FID.

Extraction of the monoterpenes from wine:
The C-18 (Varian, HF Bond ElutLRC-C18 OH, 500 mg) cartridges were conditioned firstly with 4 mL of dichloromethane, then with 4 mL of methanol and finally with 4 mL of wine simulant (distilled water, 12% ethanol, 2.5 g L^{-1} tartaric acid, pH 3.5 adjusted with NaOH). Care was taken to ensure that the flow rate was not too high and that the cartridges never ran dry. Then 50 mL of wine with 50 µL (of a 2.5 µg L^{-1} solution in ethanol) internal standard was passed through the cartridges without them running dry. The cartridges were washed with 4 mL distilled water and left to dry under vacuum for 15 minutes. The vacuum chamber was cleaned firstly with distilled water and secondly with ethanol and dried with a paper towel. Glass tubes into which the samples were eluted were placed in the tube rack in the chamber. The monoterpenes were eluted with 2 mL of dichloromethane. The extract was dried with $NaSO_4$. The dichloromethane was removed from the $NaSO_4$ and placed into a GC-vial ensuring there was no salt in the vial.

4.3 Results and discussion

4.3.1 Construction of plasmids

The pGPL (pGEM-T-PGU1-loxP) and pJPL (pJET1-PGU1-loxP) constructs were completed as described in the previous section. The construction of pGPL is summarised in Fig. 2a and 2b. The backbone of this construct was prepared by cloning a Ty region into a pGEM®-T Easy vector as indicated on the right in Fig. 2a. The largest, middle part of the Ty region was then removed through enzymatic digestion and replaced by the *PGU1* gene amplified from *S. cerevisiae* L2323. The parts of the Ty region that remained on the plasmid were the areas used for homologous recombination. The shorter of the two parts is only 71 bp long while the longer part is 583 bp. The left side in Fig. 2a shows the amplification of the *PGU1* gene from *S. cerevisiae* L2323. A PCR amplicon of 2620 bp was obtained, using the primers intPGfw and intPGrev. The amplicon was digested with *Bln*I and *Cla*I (Roche) to obtain ends compatible to the vector. This digested amplicon consisted of the *PGU1* gene, 247 bp upstream (which is the end of the *PGU1* promoter) and 633 bp downstream (including the *PGU1* terminator of unknown size) of the gene. The fragment was cloned into the vector to yield the construct indicated at the bottom in figure 2a.

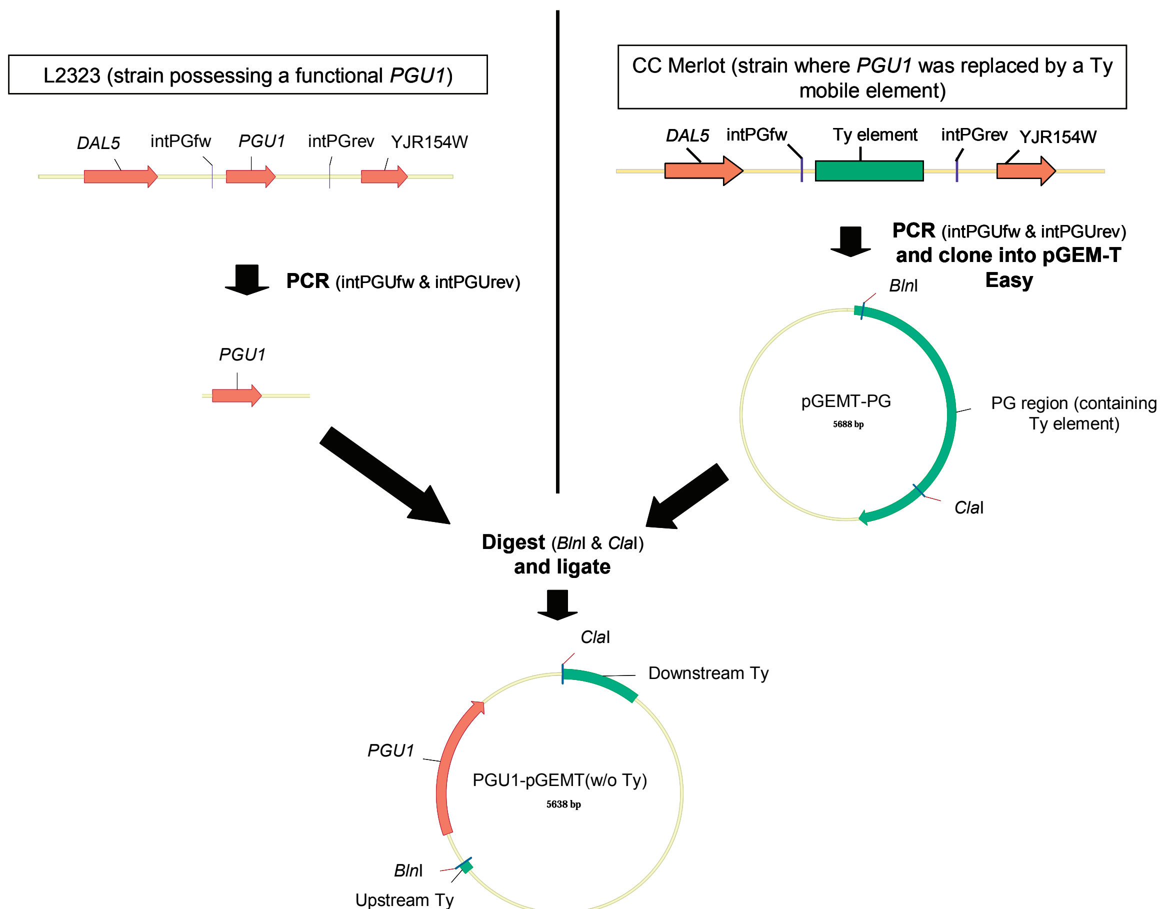

Fig. 2a. Construction of the pGPL plasmid: cloning the *S. cerevisiae* L2323 *PGU1* gene into the pGEM-T Easy based vector.

A loxP-KanMX-loxP cassette (Güldener *et* al., 1996) was also introduced into this construct. This cassette contains the *kan*^{r} gene that confers resistance to the antibiotic G418 (Jimenez & Davies, 1980) flanked by direct repeats on both sides. The addition of this cassette allows for selection of positive transformants on YPD plates containing G418. This was necessary as the strains transformed were all prototrophic wine strains and thus the auxotrophic markers used for transformation of laboratory strains could not be used for selection. The loxP cassette was amplified by PCR form a plasmid constructed previously as shown on the left of Fig. 2b. The primers used (loxPfw and loxPrev) were designed with *Stu*I restriction sites at the ends. The plasmid constructed in Fig. 2a (PGU1-pGEMT(w/o Ty)) as well as the loxP amplicon were digested with *Stu*I and the loxP cassette cloned into the vector. The final construct was verified firstly by PCR amplification of the *PGU1* gene and secondly by enzymatic digestion, using several different enzymes (not shown).

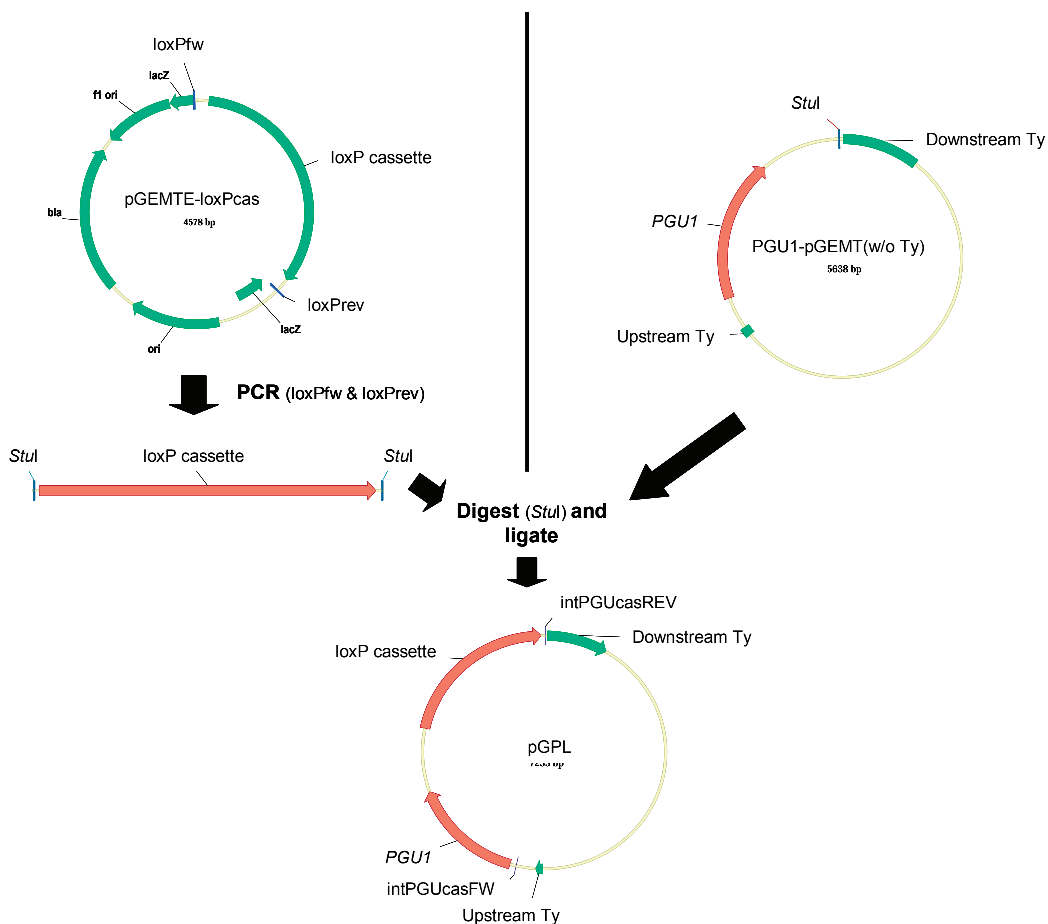

Fig. 2b. Construction of the pGPL plasmid: cloning the loxP cassette into the vector.

Fig. 2c summarises the construction of the pJPL plasmid. An integration cassette was amplified by means of a gradient PCR, blunted and cloned into a pJET1 vector as described in the previous section. The pJET1 vector from the kit is linear with blunt ends and is directly available for cloning. The construct was verified by enzymatic digestion with various enzymes as well as sequencing of the insert with a range of primers (data not shown).

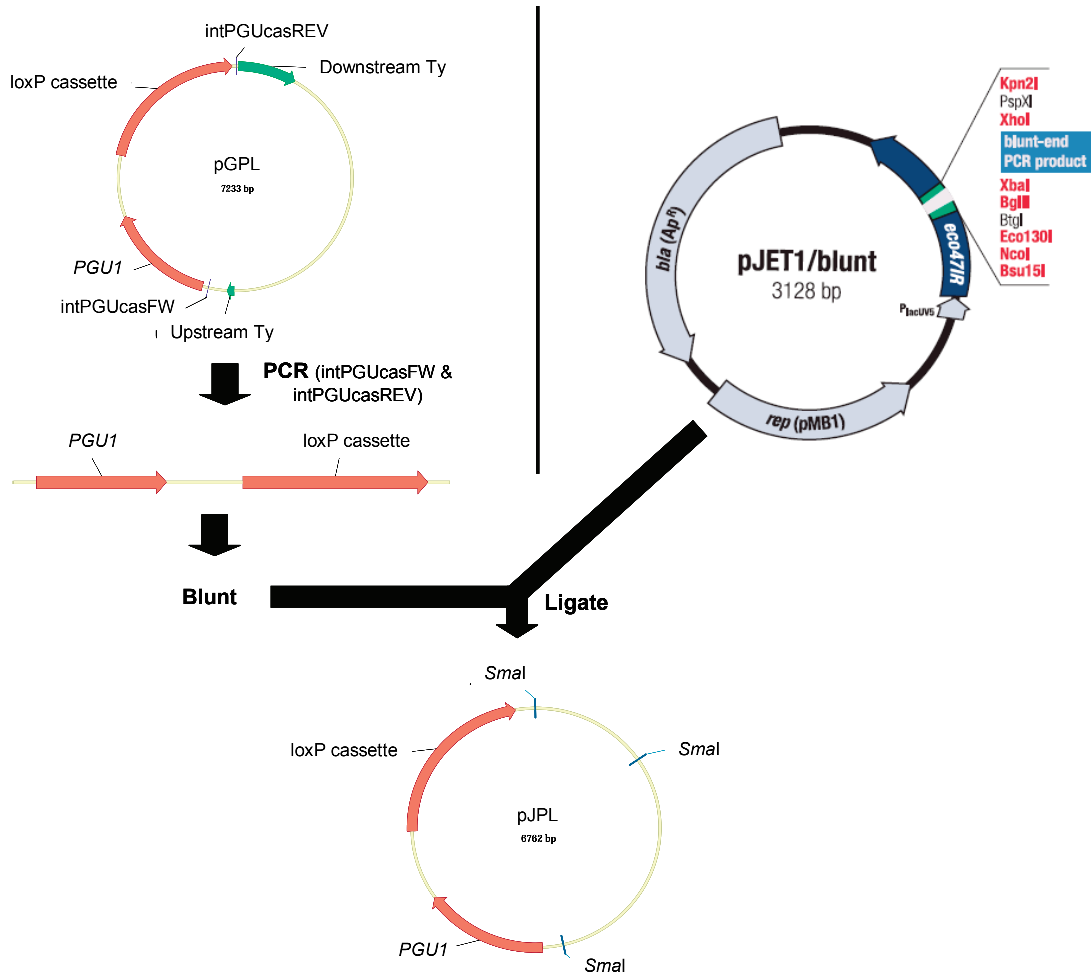

Fig. 2c. Construction of the pJPL plasmid. The integration cassette (containing the *PGU1* gene and the loxP cassette) was amplified by PCR, blunted and cloned into the pJET1 vector.

4.3.2 Yeast transformation

The pGPL plasmid was linearised with *Bsa*I (Roche) and used to transform the yeast strains using a LiAc method. This did not deliver any desired results. We speculate that either the one homologous sequence (71 bp) was not long enough and/or that the target was not specific enough because the Ty element is found throughout the genome. After several unsuccessful attempts, it was decided to change the strategy.

The integration cassette was amplified with the specially designed primers to have larger homologous sequences that were also more specific to the site we wanted to integrate, not targeting the Ty element as previously. The cassette was cloned into pJET1 as described previously, yielding the pJPL plasmid. A purified integration cassette, obtained by digesting pJPL with *Sma*I, was used to transform the yeast strains using the same LiAc protocol as for the first attempt with the linearised pGPL plasmid. After many attempts, the cassette was successfully integrated into one strain, CC Cabernet. The transformation efficiency was still very

low and only a single recombinant was obtained for this strain. Even after optimisation of the transformation protocol, no other positive transformants were obtained.

Finally, a third strategy was tested. The cassette was integrated into the laboratory strain BY4742. A single attempt produced several recombinants. This confirmed that it is easier to integrate into domesticated (laboratory) strains than into wine strains of *S. cerevisiae*, although no viable explanation was found thus far. It could be due to the fact that wine strains are genetically more complex than domesticated strains because of their ploidy. Another reason could be that their cell walls are complex, making them harder to penetrate.

Genomic DNA from this recombinant BY4742 was used as template to amplify the whole *PGU1* region. This second cassette was easily amplified and a single amplicon was obtained. The cassette included the complete *PGU1* promoter, gene and terminator, the loxP cassette as well as additional sequences both up- and downstream of the *PGU1* region. The primers amplified the whole region from the 3'-end of *DAL5* (ORF upstream of *PGU1*) to the 5'-end of *YJR154W* (ORF downstream of *PGU1*). This second cassette had larger regions for homologous recombination than the first cassette. In addition to a modified cassette, electroporation was used for the transformation.

The new cassette was successfully integrated into the genomes of 4 more yeast strains: L2226, UCLMS-1, -3 and -4. Positive transformants were identified by PCR amplification, using one primer that binds in the native genome of the yeast and one that binds in the cassette that was integrated into the genome. The forward primer, Dal5promfw, binds to the promoter area of the *DAL5* gene (the ORF upstream to *PGU1*), which is native to all the yeast strains. The reverse primer, Pgu1rev2, binds 377 bp into the *PGU1* gene, which is only present in the recombinants, as the *PGU1* gene is absent in the wild type yeasts. Fig. 3a shows the PCR reaction that was performed on all the recombinant strains and their wild types. As expected, all the recombinant strains produced an amplicon, while none of the wild types did. Divol & van Rensburg (2007) carried out PFGE on several wine yeast strains. The phylogenetic tree obtained from the patterns showed a close relationship between UCLMS-1, -3, -4, L2226, CC Cabernet and CC Merlot. It was interesting to see that, with the exception of CC Merlot, these were the only strains we managed to integrate into. This could support the argument that the genetic background of a strain has an influence on the difficulty of integrating into its genome. Fig. 3b shows the fingerprint PCR of all the wild type and recombinant yeasts. This indicated that the recombinant yeast is indeed the same strain as its original wild type.

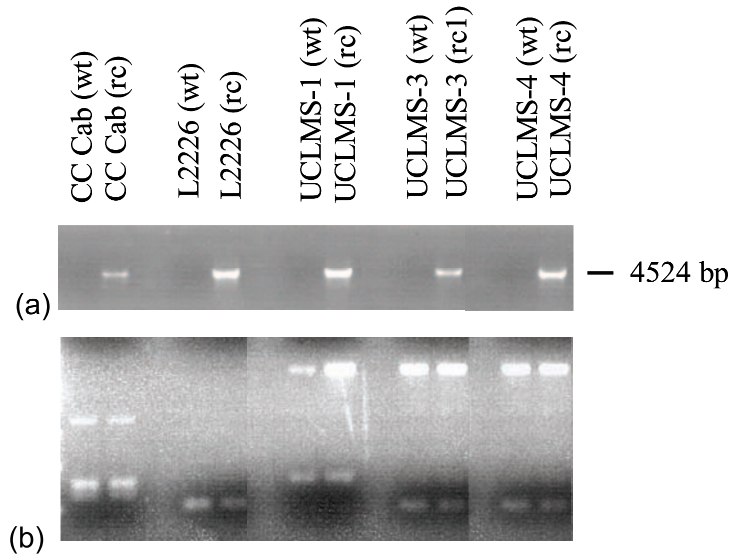

Fig. 3. All the wild type strains and their recombinants. (a) Integration of the cassette as identified by PCR. (b) Fingerprint PCR of all the strains.

4.3.3 Evaluation of transcription and enzyme activity

RNA was isolated from all recombinant yeasts and their wild types. Reverse transcription was performed and the cDNA used as template for a PCR reaction. The *ACT1* gene was amplified as housekeeping gene and was present in all the cDNA samples. The amplification also showed that the same amount of *ACT1* was present in all the samples, indicating that the same concentration of RNA was used for reverse transcription in all the samples. Because of this, the amounts of *PGU1* transcript could be compared between the different recombinants. Fig. 4 shows the results after amplification from the cDNA.

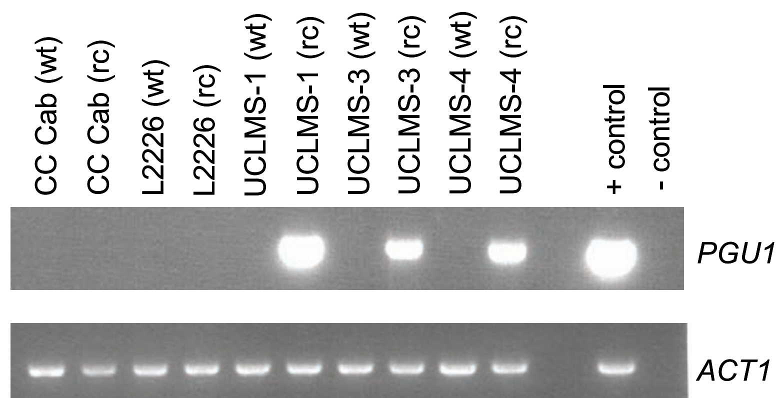

Fig. 4. RT-PCR results. The top indicates amplification of the *PGU1* gene and the bottom the *ACT1,* used as housekeeping gene. The wild type (wt) was always followed by its recombinant (rc).

None of the wild type yeasts showed an amplicon, which is as expected because they do not possess the *PGU1* gene. CC Cabernet (rc) and L2226 (rc) exhibited no amplicon either. The re-introduced *PGU1* gene was clearly not transcribed in these strains.

These latter strains did however recover PG activity when *PGU1* was expressed on a plasmid under control of the *PGK1* promoter (See Chapter 3). The same was observed by Blanco *et al*. (1998) and they proposed a non-functional *PGU1* gene or promoter. This was however not the case in this study as a functional gene and promoter were integrated into the genomes of these strains. The results rather suggest the regulation by a trans-acting factor that acts on *PGU1* as proposed by Hirose *et al*. (1999). This would also explain expression of *PGU1* in these strains when the gene was under control of the constitutive *PGK1* promoter and not its native promoter. UCLMS-1 (rc) showed high transcription of the *PGU1* gene, indicated by a bright PCR band. UCLMS-3 (rc) and UCLMS-4 (rc) also showed transcription, although slightly less than UCLMS-1 (rc). These three strains do not exhibit the same regulation on *PGU1* as CC Cabernet and

L2226 and the lack of PG activity in these strains is probably only due to the loss of the *PGU1* gene through the transposon insertion.

A plate assay was carried out to determine whether polygalacturonase activity was recovered in the recombinant yeasts. It was found that only the UCLMS-1, -3 and -4 recombinants showed a clear zone on the plates, which indicates PG activity in these strains (Fig. 5).

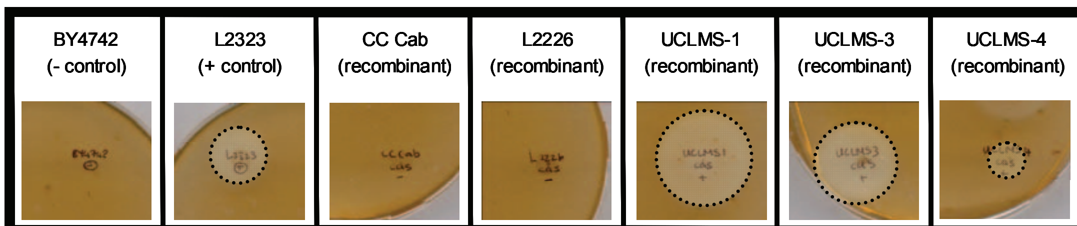

Fig. 5. The plate assay to test for polygalacturonase activity in the recombinant yeast strains. BY4742 has no activity and was used as negative control while L2323 was used as positive control because of its natural strong activity.

The assay is not quantitative, but provides an approximation of the activity: the larger the zone on the plate, the higher the activity of the corresponding strain. UCLMS-1 (recombinant) had the highest activity, followed by UCLMS-3 (recombinant) and finally UCLMS-4 (recombinant) indicating very low activity. This low activity exhibited by UCLMS-4 (recombinant) is contrasting to the high transcription of *PGU1* obseved for this strain. This suggests that UCLMS-4 could exhibit post-transcriptional regulation on the Pgu1 protein which results in very low PG activity. The CC Cabernet (CC Cab) and L2226 recombinants indicated no activity on the plates. This correlated well to the RT-PCR results, which indicated no transcription of the *PGU1* gene in these strains.

In addition, CC Cabernet (which did not recover PG activity after the integration) was transformed with a plasmid overexpressing the *TEC1* gene. *TEC1* is the main transcription factor for *PGU1* and overexpression of this gene is known to result in a higher transcription of *PGU1* (Madhani *et al*., 1999). In this case, the overexpression of *TEC1* did not lead to recovery of PG activity for the CC Cabernet recombinant (results not shown). This further suggests the presence of a trans-acting factor that inhibits *PGU1* transcription, even when the main transcription factor was present in abundance. This proposed inhibitory factor could possibly prevent transcription factors from binding to the promoter. Further studies should be conducted to evaluate this.

4.3.4 Microvinifications

Fermentations were carried out with the UCLMS-1, -3 and -4 recombinants and their respective wild types. For each strain, three bottles of each cultivar were inoculated from the same yeast culture. The bottles were all weighed on a daily basis in order to follow the fermentations. The accumulated weight loss was determined for each fermentation and the average of the triplicates are presented in Fig. 6. All recombinant strains fermented at approximately the same rate as their wild types, except for UCLMS1(rc) and UCLMS4(rc) that fermented slightly slower than their respective wild types in the Shiraz must. It was noted that UCLMS-3 fermented on average about 5 days longer than UCLMS-1 and -4.

To ensure that the strain used to inoculate the fermentation was indeed the strain still present at the end of fermentation, a sample of the wine was plated onto normal YPD as well as YPD containing 200 μg mL^{-1} G418. It was found that there was no growth on the G418 plates for fermentations conducted with the wild type strains. For the recombinant strains, equal amounts of cells were found on the YPD and YPD G418 plates. This showed that the recombinant strains did indeed complete the fermentation and that the integration was stable. For each strain, 10 colonies were picked randomly from the plate and a fingerprint PCR was done to compare the pattern to the strain used for inoculation. As an example, the fingerprints for the UCLMS-1 wild type and recombinant strains isolated from the Shiraz wine are presented in Fig. 7a and 7b respectively. All the colonies isolated from the wine had the same pattern as the original colonies that were used for integration and fermentation. For the positive control, genomic DNA was isolated from the strain that was used to inoculate the specific fermentation and used as template in the PCR reaction.

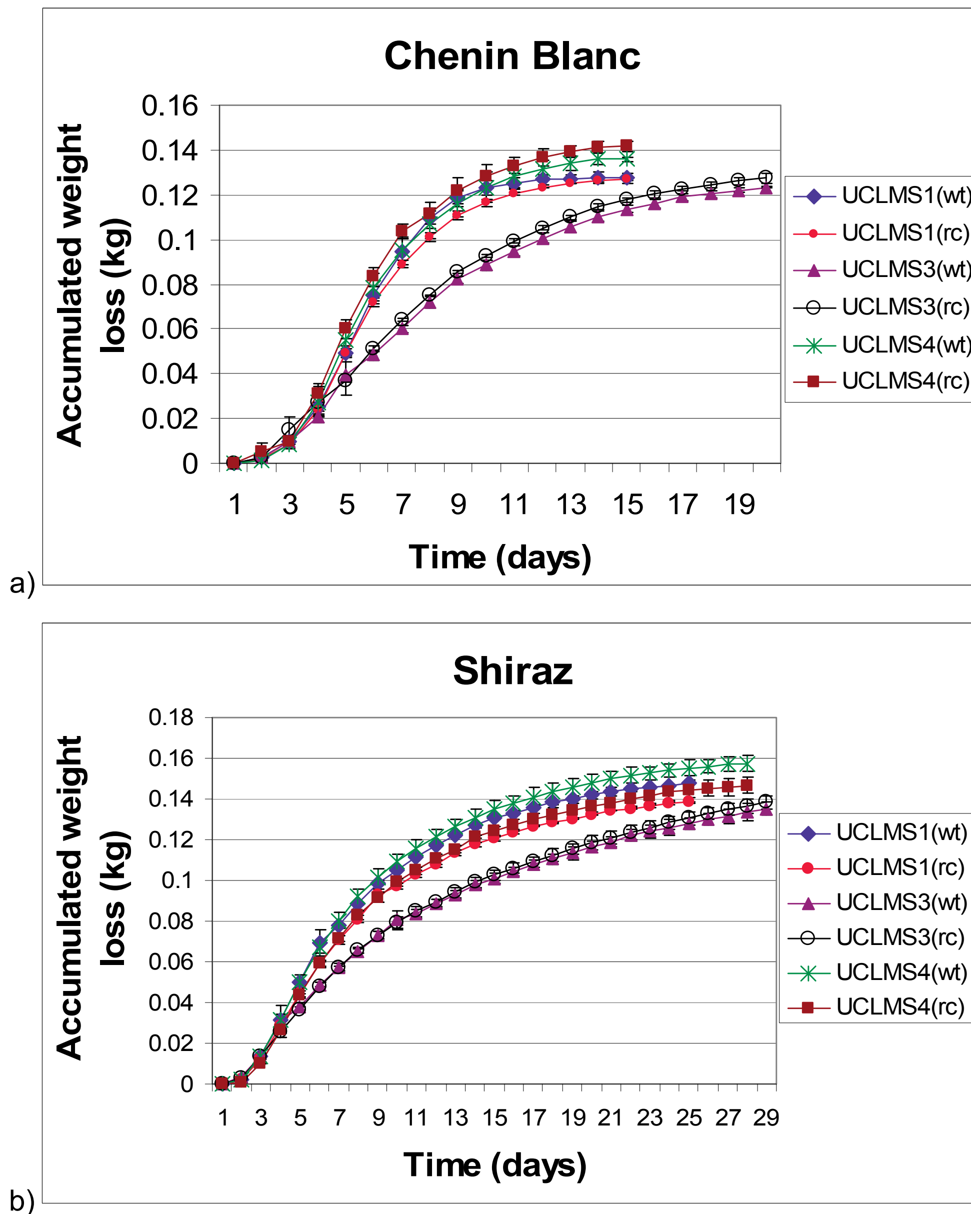

Fig. 6. Accumulated weight loss during the fermentations in Chenin Blanc (a) and Shiraz (b) musts. The values presented are the average of the triplicates. wt = wild type; rc = recombinant.

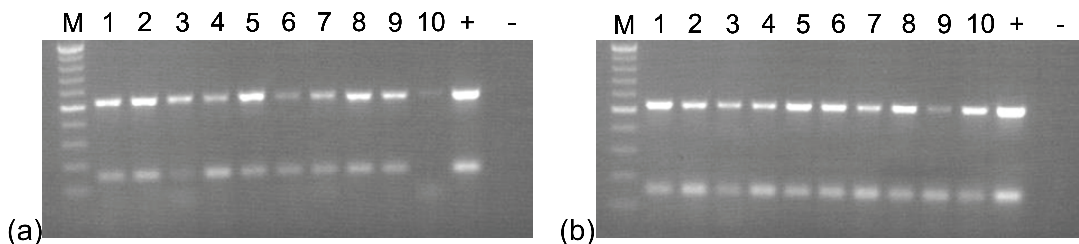

Fig. 7. Fingerprints of colonies isolated from UCLMS-1 wild type (a) and recombinant (b) fermentations in Shiraz must. M = DNA ladder (GeneRuler^{TM} 100bp DNA Ladder, Fermentas); 1-10 = isolates; + = positive control (UCLMS-1 genomic DNA); - = PCR negative control.

4.3.5 Analyses of aroma compounds in wine

After the fermentation was completed, a 50-mL sample of each wine was tested for the major biochemical parameters using a Winescan instrument. The parameters included pH, volatile acidity, total acid, malic acid, glucose, fructose, ethanol and glycerol. No significant differences were seen between wines made by the wild types and their recombinant yeasts (data not shown). The integration cassette therefore had no effect on the normal fermentation ability of the strains as none of the major parameters were changed. No metabolic pathways were changed which could have led to an increase or decrease of certain compounds that are always produced during fermentation.

Some volatile compounds and monoterpenes were also extracted from the wines in order to compare the aroma profiles of the recombinant yeasts to the wild types. The results are summarized in Fig. 8 and 9. The values presented are the average for the three triplicates of the specific strain.

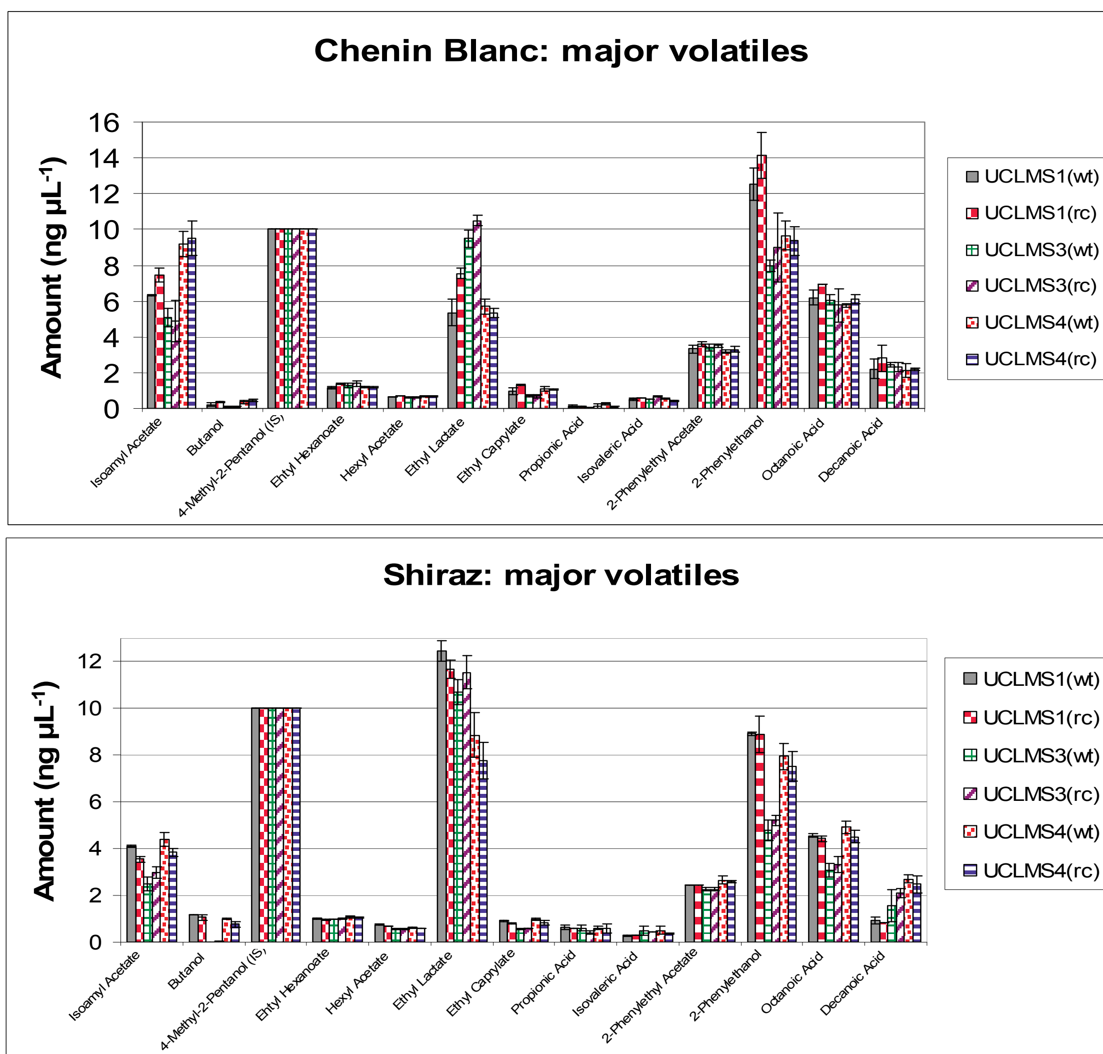

Fig. 8. The major volatile compounds extracted from the wines as analysed by GC-FID. For each strain, the wild type (wt) was followed by its recombinant (rc).

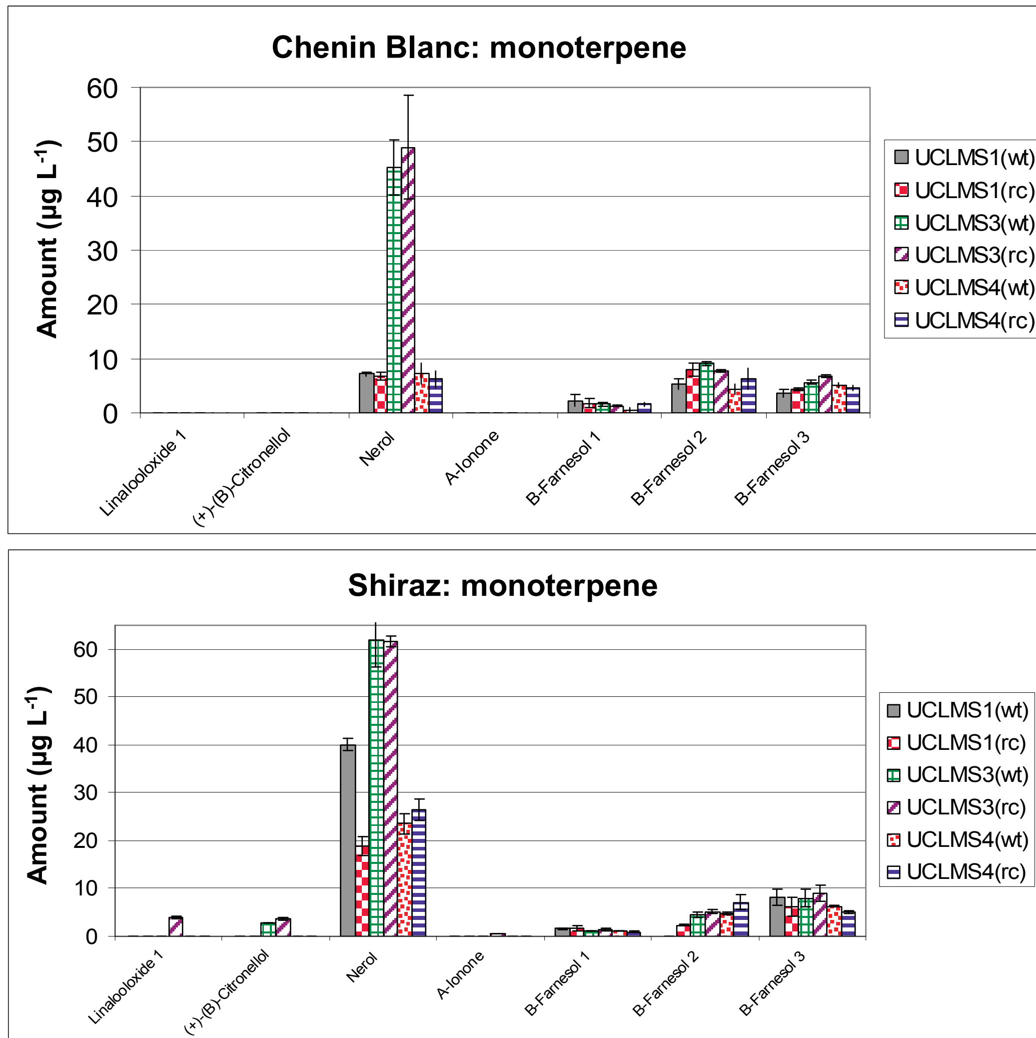

Fig. 9. Some monoterpenes extracted from the wines as analysed by GC-FID. For all strains, the wild type (wt) was followed by its recombinant (rc).

In the Chenin Blanc wines, there seemed to be a tendency towards higher production of certain compounds by the recombinants than their wild types, especially for UCLMS1(rc). It also seemed that all the recombinants tended to produce slightly more of the monoterpene geraniol in both wines (Fig. 10). Vilanova *et al*. (2000) also found a significant increase in geraniol during vinification with a *PGU1* recombinant. In their study, *PGU1* was constitutively expressed on a plasmid under the control of the *PGK1* promoter.

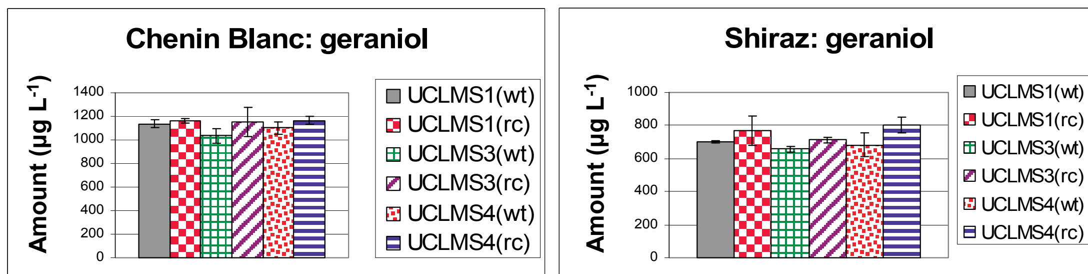

Fig. 10. Geraniol produced by the different strains in Chenin Blanc and Shiraz musts. The wild type (wt) was always followed by its recombinant (rc).

However, very few of the slight differences seen in our study were significant. Several possible explanations were foreseen: firstly, the winemaking conditions (low temperature and high sugar concentration) were probably sub-optimal conditions for the enzyme, resulting in the enzyme not being active during the fermentation. It has been established that the optimum temperature for the yeast endo-polygalacturonase is 25°C (Gainvors *et al*., 2000). It has also been demonstrated that the transcription of the *PGU1* gene is inhibited by high concentrations of glucose (Blanco *et al*., 1994). This could also explain why the Shiraz wines (which had a very high sugar concentration) did not show the same tendency towards higher production of certain compounds by the recombinants as was seen in the Chenin Blanc wines. Secondly, the gene might have been properly expressed, but the expression might not have been high enough to have any significant effect on the wine. The assays done in the laboratory may be sensitive enough to detect activity, but the activity might not be strong enough to change the wine composition. A similar study was conducted by Fernández-González *et al*. (2005) to determine the effect of a *PGU1* recombinant strain on the wine aroma. In their study, the gene was also integrated into the genome of the yeast, but under control of the *PGK1* promoter. They found no significant differences in the major volatile content between the control and recombinant wines (except for an increase in methanol), even in red wine fermented at 24-25 °C. Vilanova *et al*. (2000) found increased production of methanol, nerol and geraniol when *PGU1* was expressed on a plasmid. A single integration may not produce sufficient endo-polygalacturonase to have a noteworthy effect on the wine aroma, while a multi-copy plasmid may. Thirdly, it could also be that inefficient presursors were extracted to show any differences produced by the yeast used to conduct the fermentation due to a lack of skin contact.

In order for this to be resolved, the experiment should be repeated in more optimal conditions. Firstly, a red wine should be made by fermentation on the skins in order to ensure a high pectin content in the must. The fermentation should be performed at a higher temperature, around 25°C, which is optimal for the enzyme. Secondly, samples should be taken periodically throughout the fermentation to test for enzyme activity. This will confirm whether the enzyme is secreted or not. Unfortunately, according to our knowledge, there is no method available to test polygalacturonase activity during fermentation. Such a method should be implemented. Sensorial analyses could also be conducted through tastings to determine any differences in the wine. The GC-FID might not detect all the aroma compounds that were altered due to a limitation on the available methods for analysis.

4.4 Conclusions

The *PGU1* gene was successfully integrated into its native position in 5 wine strains that lost this gene after a transposon insertion. We realized that these strains are closely related

according to a phylogenetic tree drawn from PFGE results by Divol & van Rensburg (2007). This suggests that the genetic background of a strain has an influence on how easily DNA can be integrated into its genome. Only the strains UCLMS-1, -3 and -4 recovered activity after re-introduction of the gene as indicated by a plate assay. CC Cabernet and L2226 showed no transcription for the integrated gene and consequently no activity. The reason for this was not because of a non-functional gene or promoter as was proposed by Blanco *et al.* (1998) for certain strains that lack PG activity, nor was the result of post-transcriptional regulation as the strains did recover activity when *PGU1* was expressed under plasmid control (this study). The lack of activity is probably due to regulation by a trans-acting factor that inhibits *PGU1* transcription in certain strains as proposed by Hirose *et al*. (1999).

The UCLMS-1, -3 and -4 recombinants and their wild type strains were used to perform small scale fermentations in Chenin Blanc and Shiraz musts. The fermentations were monitored by weight loss and it was found that all the recombinants fermented at more or less the same rate as their respective wild types. UCLMS-3 also fermented on average 5 days longer than UCLMS-1 and -4. Yeast isolated from the wines at the end of fermentation were cultivated on selective plates to prove stability of the integrations. The colonies were also identified by fingerprint PCR which confirmed that it was indeed the inoculated strains that completed the alcoholic fermentations.

Several volatile compounds and monoterpenes were extracted from the bottled wines and measured by GC-FID. Some tendencies were observed for recombinants to produce higher amounts of certain compounds, especially for the UCLMS-1 recombinant. Unfortunately, very few of the differences were significant. We proposed several reasons for this. Firstly, the winemaking conditions (low temperature and high sugar concentration) could have inhibited the enzyme. Secondly, the expression of the *PGU1* gene might not have been high enough to have any effect on the aroma compounds in the wine. And thirdly, the pectin content of the must might have been too low to allow for any detectable changes to be made by the yeast during fermentation. We suggest that the experiment be repeated with certain important changes made to ensure the most optimal conditions for the enzyme.

This study provides insight into the regulation of the *PGU1* gene in certain strains of *S. cerevisiae* and would serve as a good basis for further analyses. It also confirms conclusions made by previous authors on the subject. Furthermore, it indicates the possible effects of a pectinolytic wine strain on the wine aroma and the benefit to the wine industry.

4.5 Acknowledgements

The authors wish to thank D. F. Malherbe for providing some plasmids and advice, K. Roux for assistance with the GC-FID analyses as well as the National Research Foundation, Winetech and THRIP for financial support.

4.6 References

Agatep R, Kirkpatrick RD, Parchaliuk DC, Woods RA & Gietz RD (1998) Transformation of *Saccharomyces cerevisiae* by the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol. *Technical Tips Online* (http://tto.trends.com).

Bell TA & Etchells JL (1956) Pectin Hydrolysis by certain salt-tolerant yeasts. *Appl Microbiol* **4:** 196-201.

Blanco P, Sieiro C, Díaz A & Villa TG (1994) Production and partial characterization of an endopolygalacturonase from Saccharomyces cerevisiae. *Can J Microbiol* **40:** 974-977.

Blanco P, Sieiro C, Díaz A, Reboredo NM & Villa TG (1997) Grape juice biodegradation by polygalacturonases from *Saccharomyces cerevisiae*. *Int Biodeterior & Biodegradation* **40:** 115-118.

Blanco P, Sieiro C, Reboredo NM & Villa TG (1998) Cloning, molecular characterization, and expression of an endo-polygalacturonase-encoding gene from *Saccharomyces cerevisiae* IM1-8b. *FEMS Microbiol Lett* **164:** 249-255.

Cryer DR, Eccleshal R & Marmur J (1975) Isolation of yeast DNA. *Methods Cell Biol* **12:** 39-44.

Davis RW, Thomas M, Cameron J, St John TP, Scherer S & Padgett RA (1980) Rapid DNA isolations for enzymatic and hybridization analysis. *Methods Enzymol* **65:** 404-411.

Divol B & van Rensburg P (2007) *PGU1* natural deletion is responsible for the absence of endo-polygalacturonase activity in some wine strains of *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7:** 1328-1339.

Fernández-González M, Úbeba JF, Vasudevan TG, Cordero Otero RR & Briones AI (2004) Evaluation of polygalacturonase activity in *Saccharomyces cerevisiae* wine strains. *FEMS Microbiol Lett* **237:** 261-266.

Fernández-González M, Úbeba JF, Cordero Otero RR, Thanvanthri Gururajan V & Briones AI (2005) Engineering of an oenological *Saccharomyces cerevisiae* strain with pectinolytic activity and its effect on wine. *Int J Food Microbiol* **102:** 173-183.

Gainvors A, Nedjaoum N, Gognies S, Muzart M, Nedjima M & Belarbi A (2000) Purification and characterization of acidic endo-polygalacturonase encoded by *PGL-1* gene from *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **183:** 131-135Luh BS & Phaff HJ (1951) Studies on polygalacturonase of certain yeasts. *Arch Biochem* **33:** 212-227.

Gognies S, Gainvors A, Aigle M & Belarbi A (1999) Cloning, sequence analysis and overexpression of a *Saccharomyces cerevisiae* endopolygalacturonase-encoding gene (*PGL1*). *Yeast* **15:** 11-22.

Güldener U, Heck S, Fiedler T, Beinhauer J & Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24:** 2519-2524.

Hirose N, Fujii M, Kishida M, Kawasaki H & Sakai T (1999) Isolation and genetic determination of a *Saccharomyces cerevisiae* mutant that produces an endo-polygalacturonase. *J Biosci Bioeng* **87:** 594-597.

Hirose N, Kishida M, Kawasaki, H & Sakai T (1998) Molecular cloning and expression of a polygalacturonase gene in *Saccharomyces cerevisiae*. *J Ferment Bioeng* **86:** 332-334.

Jimenez A & Davies J (1980) Expression of a transposable antibiotic resistance element in *Saccharomyces. Nature* **287:** 869-871.

Joubert DA (2004) Regulation of the *Vitis vinifera PGIP1* gene encoding a polygalacturonase-inhibiting protein. PhD thesis, University of Stellenbosch, Stellenbosch.

Legras JL & Karst F (2003) Optimisation of interdelta analyses for *Saccharomyces cerevisiae* strain characterization. *FEMS Microbiol Lett* **221:** 249-255.

Luh, BS and Phaff, HJ (1951) Studies on polygalacturonase of certain yeasts. *Arch Biochem* **33:** 213-227.

Madhani HD, Galitski T, Lander ES & Fink GR (1999) Effectors of a developmental mitogen-activated protein kinase cascade revealed by expression signatures of signaling mutants. *Proc Natl Acad Sci USA* **96:** 12530-12535.

McKay AM (1988) A plate assay method for the detection of fungal polygalacturonase secretion. *FEMS Lett* **56:** 355-358.

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16:** 675-729.

Radoi F, Kishida M & Kawasaki H (2005) Endo-polygalacturonase in *Saccharomyces* wine yeasts: effect of carbon source on enzyme production. *FEMS Yeast Res* **5:** 663-668.

Van Rensburg P & Pretorius IS (2000) Enzymes in winemaking: harnessing natural catalysts for efficient biotransformations – a review. *S Afr J Enol Vitic* **21:** 52-73.

Vilanova M, Blanco P, Cortés S, Castro M, Villa TG & Sieiro C (2000) Use of a *PGU1* recombinant *Saccharomyces cerevisiae* strain in oenological fermentations. *J Appl Microbiol* **89:** 876-883.

Volschenk H, Viljoen-Bloom M, van Staden J, Husnik J& van Vuuren HJJ (2004) Genetic engineering of an industrial strain of *Saccaromyces cerevisiae* for L-malic acid degradation via an efficient malo-ethanolic pathway. *S Afr J Enol Vitic* **25:** 63-73.

Chapter 5

Research results

Comparison of endo-polygalacturonase activity between *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*

5. Research results

Comparison of endo-polygalacturonase activity between *Saccharomyces cerevisiae* and *Saccharomyces paradoxus*

Herine van Wyk and Benoit Divol

Institute for Wine Biotechnology, Stellenbosch University, Matieland, South Africa

Abstract

The *PGU1* gene encodes for an endo-polygalacturonase, an enzyme that degrades pectin. Several yeast strains have been identified to possess this gene, yet their polygalacturonase (PG) activities vary greatly. *Saccharomyces paradoxus* RO88 exhibits a stronger PG activity than most strains of *Saccharomyces cerevisiae*. In this study, the *PGU1* genes and promoters from *S. cerevisiae* and *S. paradoxus* were analysed and compared to identify differences that could possibly be responsible for the difference in their PG activities. Expression of the genes was also evaluated under winemaking conditions and their effect on the aroma profile of the wine was determined. Clear differences were observed between strains for the production of certain aroma compounds. Furthermore, some tendencies were also noticed between recombinants containing the different *PGU1* genes.

5.1 Introduction

Pectin is the name given to a diverse group of heteropolysaccharides, some of which are very complex. Pectic substances can be divided into two structural regions (De Vries and Visser, 2001): “smooth” regions that consist of D-galacturonic acid units linked by α-1,4-bonds and “hairy” regions where the galacturonic acid residues can be methylated or have complex side chains attached to them. Pectinases are enzymes that degrade pectin and are classified by their mode of action (Jayani *et al*., 2005). One such class of enzymes is endo-polygalacturonases. They cleave the links between non-methylated galacturonic acid residues by acting randomly on the chain to produce oligomers of different lengths (Kester & Visser, 1990). This group of enzymes allow for faster degradation of polysaccharides than exo-acting pectinases which release residues from the non-reducing end of the chain (Kester *et al*., 1996).

The polysaccharide content of wine is usually between 300-1000 mg L^{-1} and is often responsible for turbidity, viscosity and filter stoppages (Van Rensburg & Pretorius, 2000). For this reason, it is common practice to add commercial enzyme preparations during winemaking. These enzyme preparations are produced by fungi (mostly *Aspergillus niger*) and often contain various unwanted enzymes (Whitaker, 1984; Blanco *et al*., 1997; Wightman *et al*., 1997). The

production of pure pectinases would be advantageous for winemaking. This can be achieved by using a yeast capable of producing a pectinase during alcoholic fermentation. Such a yeast could improve clarification, filtration and even release more flavour and colour compounds from the skins (Pretorius, 2000).

Alcoholic fermentation in grape must is usually performed by a *S. cerevisiae* strain. These strains are selected for their tolerance to the undesirable conditions of wine fermentations, as well as for their production of desirable aroma compounds during fermentation. Most of these strains however do not possess high (or any) PG activity. *Saccharomyces paradoxus* RO88 has been evaluated under winemaking conditions and found to have a good fermentation rate and produce dry wines. Additionally, this strain was found to influence the production of certain aroma compounds and contribute to the final quality of the wine (Majdak *et al*., 2002). It also exhibits high PG activity (Mocke, 2005).

The aim of this study was to evaluate the use of *S. paradoxus* in winemaking in order to enhance PG activity during alcoholic fermentation. Firstly, the *PGU1* gene and promoter from *S. paradoxus* RO88 were analysed and compared to those of *S. cerevisiae* BY4742 in order to highlight any differences between the two species. Two recombinant strains were obtained, one overexpressing the *PGU1* gene from BY4742 and the other expressing the gene from RO88. The polygalacturonase activities of BY4742, RO88 and the two recombinants were then quantified and compared. Finally, the strains were tested under winemaking conditions to determine the effect of their respective PG activities on the aroma profile of the wine. Two hybrids, PR7 and PR10, obtained by crossing *S. cerevisiae* VIN13 and *S. paradoxus* RO88 (Mocke, 2005), were also included in the vinification experiments.

5.2 Materials and methods

5.2.1 Strains, media and culture conditions

The *PGU1* genes studied were amplified from *S. cerevisiae* BY4742 and *S. paradoxus* RO88. For bacterial transformations, the *Escherichia coli* strain DH5α (F-φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-) was used. All recombinant *E. coli* cultures were grown overnight at 37°C in Luria Bertani (LB) medium and on LB plates (Biolab Diagnostics, Wadeville, South Africa), all containing 100 μg mL^{-1} Ampicillin (Ampicillin sodium salt, Sigma, Missouri, USA) for selection. The wine yeast strain VIN13 was used for transformation with the final constructs. All yeast cultures were grown in YPD medium (Biolab Diagnostics) and transformants grown on YPD plates containing 200 μg mL^{-1} G418

(G418 disulphate salt, Sigma) for selection. All cultures and plates were incubated at 30°C, unless specified otherwise. All yeast strains used in this study are presented in Table 1.

Table 1. Yeast strains used in this study

Strain	Relevant characteristics	Reference
S. cerevisiae BY4742	Laboratory strain. Genotype: *MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	Brachmann *et al*. (1998)
S. cerevisiae VIN13	Commercial diploid strain	Anchor Yeast (South Africa)
S. paradoxus RO88	Diploid strain isolated from grapes	Redžepović *et al*. (2003)
PR7	VIN13/RO88 hybrid	Mocke (2005)
PR10	VIN13/RO88 hybrid	Mocke (2005)

5.2.2 Isolation of plasmids and genomic DNA

Plasmids were isolated from *E. coli* using the QIAprep® Spin Miniprep Kit (Qiagen, Southern Cross Biotechnology, Cape Town, South Africa) as instructed by the manufacturer. Genomic DNA was isolated from yeast using the alternative protocol for rapid isolation of yeast chromosomal DNA according to the method described by Cryer *et al*. (1975) and Davis *et al*. (1980).

5.2.3 PCR amplification

The *PGU1* gene was amplified from *S. cerevisiae* BY4742 and *S. paradoxus* RO88 with the primers 5'PPGL1-HINIII and 3'PPGL1-BGLIIpP2. All primers used are listed in Table 2. The reaction was performed using 1 U ExTaq Polymerase from Takara (SEPARATIONS, Randburg, South Africa) in a total volume of 50 µL. The program started with a denaturing step of 5 min at 95°C. A cycle consisting of a denaturing step at 94°C for 30 s, an annealing step at 56°C for 30 s and an elongation step at 72°C for 1 min, was repeated 30 times. The program ended with a final elongation step at 72°C for 10 min.

Table 2. List of primers used for PCR amplification. The restriction sites incorporated in some primers are underlined and the corresponding restriction enzyme is indicated in brackets.

Primer name	Sequence 5' - 3'	Reason for use	Reference
5'PPGL1-HINIII	AAGCTTATGATTTCTGCTAATTCATTACTTATTTCCAC (*Hin*dIII)	Amplification of the *PGU1* genes from *S cerevisiae* BY4742 and *S. paradoxus* RO88	This study
3'PPGL1-BGLIIpP2	CTCGAGTTAACAGCTTGCACCAGATCCAGATG (*Xho*I)		This study
5'PGL1PROM	TACGTCTAGAGGACAAGTCGACTTGTCCTGCCT	Amplification of the *PGU1* promoters from *S. cerevisiae* BY4742 and *S. paradoxus* RO88	Goetzee & van Rensburg (unpublished)
3'PGL1PROM	GGATCCGGTCATTGCGTTTGTCCATCAATGTGGGTAGA		Goetzee & van Rensburg (unpublished)
5'-KPNPGK-631	GGGGTACCCTTTATTTTGGCTTCACCC	Screening for positive VIN13 transformants	Volschenk *et al*. (2004)
Pgu1rev2	CGTCAACGCCAACTTTACAA		Divol & van Rensburg (2007)
Delta 12	TCAACAATGGAATCCCAAC	Fingerprints of recombinant and wild type yeasts isolated from the wine	Legras & Karst (2003)
Delta 21	CATCTTAACACCGTATATGA		Legras & Karst (2003)

The *PGU1* promoter in *S. paradoxus* RO88 was also amplified from genomic DNA using the primers 5'PGL1PROM and 3'PGL1PROM. The same reaction mixture and PCR program was used as for amplification of the *PGU1* genes, except that the elongation time was changed to 1 min 30 s. The primer pairs used to amplify the genes and promoters were originally designed for amplification of the *PGU1* gene and promoter from *S. cerevisiae*. We tested these same primers for the amplification from *S. paradoxus*.

5.2.4 Sequencing

The *PGU1* gene and promoter of *S. paradoxus* RO88 were amplified as described, separated by electrophoresis on a 1% agarose gel, excised and purified using the QIAquick® Gel Extraction Kit (Qiagen). Each was then cloned into a pGEM®-T Easy vector from Promega (Whitehead Scientific, Stikland, South Africa) according to the manufacturer's protocol. The desired concentration of each plasmid was sent to the Central Analytical Facility at Stellenbosch University, South Africa for sequencing. The primers that were used are listed in Table 3.

Table 3. Primers used for sequencing of the *S. cerevisiae PGU1* gene and *S. paradoxus PGU1* gene and promoter

Primer name	Sequence 5' – 3'	Reason for use	Reference
T7	TAATACGACTCACTATAGGG	Initial sequencing of *S. paradoxus PGU1* gene and promoter respectively.	Universal primer
SP6	TATTTAGGTGACACTATAG		Universal primer
SPMidPGU1fw	CTCAACGGTAAGACCACTGGAAC	Second internal sequencing of the *S. paradoxus PGU1* gene	This study
SPMidPGU1rev	AGTACCACTGCTTAACCCACTTAGATC		This study
SPprom2fw	GTTACTGCTAGCACGGCCATGCAATTGG	Second internal sequencing of the *S. paradoxus PGU1* promoter	This study
SPprom2rev	CTTGGCCTTTGTGCAGCTATTATCTCTACGGC		This study

5.2.5 Construction of the plasmids

The *PGU1* gene from *S. cerevisiae* BY4742 (which is identical to that of S288C) was also purified and cloned into pGEM®-T Easy as described above for *S. paradoxus* RO88. The *PGU1* genes form BY4742 and RO88 were released from the pGEM®-T Easy plasmids by digestion with *Not*I and *Spe*I (both Roche, Penzberg, Germany). The genes were separated on a 1% agarose gel by electrophoresis. The genes were excised and purified using the QIAquick® Gel Extraction Kit (Qiagen). A plasmid, pDMPL, containing the *PGK1* promoter and terminator, as well as a loxP cassette with the *kan*^{r} gene for resistance to the antibiotic G418, was previously constructed (Malherbe, DF, unpublished). This plasmid was also digested with *Not*I and *Spe*I to

produce ends compatible with the genes. The genes were then cloned into the pDMPL using T4 DNA ligase (Promega) as instructed by the provider. The plasmids were named pDMP-SC (pDMPL containing the *PGU1* gene from *S. cerevisiae*) and pDMP-SP (pDMPL containing the *PGU1* gene from *S. paradoxus*).

5.2.6 *E. coli* transformation

Blank competent *E. coli* DH5α cells were transformed with the constructs following the transformation protocol from the Promega Technical Manual (TM042).

5.2.7 Yeast transformation

The two constructs (pDMP-SC and pDMP-SP) were used to transform VIN13 in individual reactions by electroporation as described by Volschenk *et al*. (2004).

5.2.8 Identification of transformants

All positive transformants were identified by PCR. Colonies were picked from the G418 plates and each resuspended in 50 µL sterile water. This was used as template in the PCR reaction. Each reaction was performed in a total volume of 50 µL using 1 U GoTaq Polymerase (Promega). The primers used were 5'-KPNPGK-631 and Pgu1rev2 (Table 2). The program was the same as for amplification of the *PGU1* genes, except that the initial denaturation step was changed to 10 min, an annealing temperature of 58°C and an elongation time of 1 min were used.

Transformed yeast cells were plated out onto YPD plates containing 200 µg mL^{-1} G418 for selection. Several colonies were picked from the plates and grown overnight in YPD medium. DNA was extracted and used as template for the PCR reaction. The reaction mixture and program were the same as for screening the *E. coli* colonies with the initial denaturation step shortened to 5 min.

5.2.9 Polygalacturonase plate assay

This assay was modified slightly from the one described by McKay (1988). Plates containing 1.25% polygalacturonic acid (Sigma), 0.68% potassium phosphate, pH 3.5, 0.67% Bacto^{TM} Yeast Nitrogen Base without amino acids (Difco, BD, Johannesburg, South Africa), 1% glucose and 2% agar were prepared. The plates were supplemented with 20 µg mL^{-1} L-histidine, 60 µg mL^{-1} L-leucine, 30 µg mL^{-1} L-lysine and 20 µg mL^{-1} Uracil. Yeast cultures were grown overnight

in 5 mL YPD. The next morning the OD_{600nm} of the cultures were determined and each diluted to 2×10^6 cells mL^{-1} with sterile water. From each dilution, 5 µL were spotted on a polygalacturonic acid plate and incubated at 30°C. After three days, the colonies were washed off with water and the plates covered with 6 M HCl until clear zones appeared. On all the plates, L2323 was used as a positive control and BY4742 as a negative control. Assays were done in triplicate.

5.2.10 Liquid polygalacturonase assay

A quantitative liquid assay was conducted to determine the polygalacturonase activity of the different yeasts. The assay was adjusted from a method by Lever (1972). A standard curve was plotted by measuring the OD_{410nm} of different galacturonic acid concentrations in PABA (5% 4-Hydroxy-benzhydrazide in 0.5 M HCl, mixed 1:4 with 0.5 N NaOH). The different concentrations were prepared as indicated in Table 4. The standard curve was plotted and the equation of the graph was used to extrapolate the galacturonic acid concentration from the OD_{410nm} readings of the enzyme reactions. The reaction mixture consisted of the following: 30 µL 0.5 M acetate buffer (acetic acid, diluted in water and adjusted with NaOH to pH 5), 20 µL supernatant of yeast culture (grown 3 days without shaking), 100 µL 1% polygalacturonic acid and 150 µL water. The mixture was incubated at 37°C. At time points 0, 2, 4 and 8 hours, 25 µL were withdrawn and added to 1.5 mL PABA. The tube was flicked to mix the contents and heated at 100°C for 10 min. It was allowed to cool to room temperature and the OD_{410nm} measured. A no-substrate control was also included.

Table 4. Volumes used to obtain different galacturonic acid concentrations for setting up a standard curve for the liquid polygalacturonase assay.

10 mM galacturonic acid	Water	PABA
0 µL	50 µL	1.5 mL
5 µL	45 µL	1.5 mL
10 µL	40 µL	1.5 mL
15 µL	35 µL	1.5 mL

2.5.11 Microvinification

The following strains were used in small scale fermentations: VIN13, RO88, VIN13[pDMP-SC], VIN13[pDMP-SP], PR7 and PR10. The latter two strains are hybrids between VIN13 and RO88 (Mocke, 2005). The fermentations were performed in two different cultivars: Chenin Blanc and Shiraz. The grapes were pressed and 30 ppm SO_2 was added to the juice. Prior to inoculation, the sugar concentration and titratable acidity of the juices were measured. This is presented in Table 5. Each cultivar was supplemented with 60 g hL^{-1} Nutrivin (Anchor, Eppindust, South Africa) and 1.5 L aliquots were fermented in 2-L glass bottles at 15°C.

Table 5. Sugar concentration and titrable acidity (TA) in the different cultivar musts before inoculation.

Cultivar	Sugar concentration (°B)	TA (g L^{-1})
Chenin blanc	20.5	5.82
Shiraz	27.15	4.93

The strains were grown in 500 mL YPD at 30°C with continuous shaking for 2-3 days. These cultures were used to inoculate the must at 1 x 10^6 cells mL^{-1} by adding them directly to each bottle. The fermentations were monitored by weight loss and were considered finished after three days without a change in weight. Once finished, a sample was taken from each bottle. The samples were diluted to 1/10 000 from which 100 µL were plated out onto two plates: a YPD plate and a YPD plate containing 200 µg mL^{-1} G418. The plates were incubated at 30°C for about three days. The colonies on the plates were counted for each strain. The growths on YPD and YPD-G418 were compared. Colonies were picked from the plates and verified by a fingerprint PCR. DNA was isolated from the colonies using the Whatman® Indicating FTA® Classic Card (Sigma-Aldrich, Johannesburg, South Africa). The protocol was followed as instructed by the supplier, except that the washing steps with FTA Purification Reagent and 1X TE were not repeated and the 1.2-mm punch was used. The PCR reactions were performed in a total volume of 50 µL using 1.25 U ExTaq Polymerase (Takara). The primers used were Delta 12 and Delta 21 (Table 1). The PCR program described by Legras & Karst (2003) was used with following two changes: an annealing temperature of 55°C and an elongation time of 30 s.

5.2.12 Extraction of wine components

After filtration and bottling, the major volatile compounds and monoterpenes were extracted from the wines.

Extraction of the volatile compounds from wine:
Five mL of wine with an internal standard, 4-Methyl-2-Pentanol, (100 µL of 0.5 mg L^{-1} solution in soaking solution) was extracted with 1 mL of diethyl ether by placing the ether/wine mixture in an ultrasonic bath for 5 minutes. The wine/ether mixture was then centrifuged at 4000 rpm for 3 minutes. The ether layer was removed and dried on $NaSO_4$. This extract was then injected into the GC-FID.

Extraction of the monoterpenes from wine:
The C-18 (Varian, HF Bond ElutLRC-C18 OH, 500 mg) cartridges were conditioned firstly with 4 mL of dichloromethane, then with 4 mL of methanol and finally with 4 mL of wine simulant (distilled water, 12% ethanol, 2.5 g L^{-1} tartaric acid, pH 3.5 adjusted with NaOH). Care was taken to ensure that the flow rate was not too high and that the cartridges never ran dry. Then, 50 mL of wine with 50 µL (of a 2.5 µg L^{-1} solution in ethanol) internal standard was passed

through the cartridges. The cartridges were washed with 4 mL distilled water and left to dry under vacuum for 15 minutes. The vacuum chamber was cleaned firstly with distilled water and secondly with ethanol and dried with a paper towel. Glass tubes into which the samples were eluted were placed in the tube rack in the chamber. The monoterpenes were eluted with 2 mL of dichloromethane. The extract was dried with $NaSO_4$. The dichloromethane was removed from the $NaSO_4$ and placed into a GC-vial ensuring there was no salt in the vial.

5.3 Results and discussion

5.3.1 PCR amplification

The *PGU1* gene and promoter were amplified, individually, from *S. paradoxus* RO88, using primers that were originally designed for *S. cerevisiae* (Fig. 1).

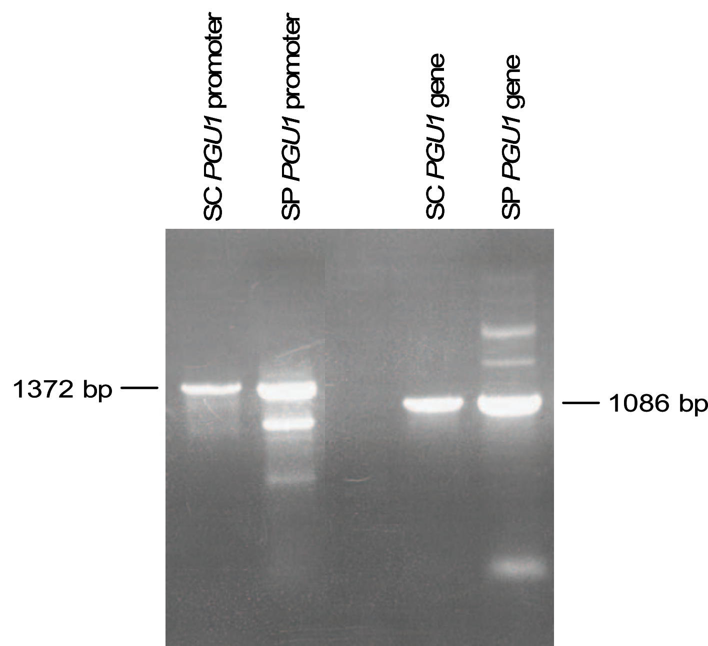

Fig. 1. Amplification of the *PGU1* promoter and gene from *S. paradoxus* (SP) with primers designed for *S. cerevisiae* (SC).

In both cases an amplicon was obtained that had the same size as the amplicon from *S. cerevisiae* despite the amplification of a few non-specific bands in the case of *S. paradoxus*. This indicated a high degree of homology for this gene between the two species.

5.3.2 Sequences of the *S. paradoxus PGU1* gene and promoter

The sequencing data for the *S. paradoxus PGU1* gene was processed and analysed to obtain a consensus sequence for the gene. To our knowledge, this sequence has not yet been

annotated. The sequence was compared to that of the *PGU1* gene of *S. cerevisiae* strain S288C (Yeast Genome Database) and some differences were observed. The sequence was then translated into an amino acid sequence and again compared to that of *S. cerevisiae* (Fig. 2). Only a few differences were revealed. Certain amino acid residues have been identified as potentially important for PG activity (Blanco *et al*., 1998). The only difference between *S. paradoxus* and *S. cerevisiae* found to be in one of these areas was residue 17. According to the authors, the first 18 amino acids constitute a peptide signal. This amino acid difference in the signal sequence could be responsible for the higher activity exhibited by RO88, via a more effective secretion of the enzyme. Alternatively, one or more of the other amino acid differences could be responsible for the difference. This should be investigated further to identify other potentially important amino acids in the *PGU1* gene.

```
PGU1(SP)    (1) MISANSLLISTLCAFAVATPLSKRDSCTLTGSSLSSLSTVKKCSSIVIKD
PGU1(BY)    (1) MISANSLLISTLCAFAIATPLSKRDSCTLTGSSLSSLSTVKKCSSIVIKD

PGU1(SP)   (51) LTVPAGQTLDLSGLSSGTTVTFEGTTTFQYKEWSGPLISISGSKISVVGA
PGU1(BY)   (51) LTVPAGQTLDLTGLSSGTTVTFEGTTTFQYKEWSGPLISISGSKISVVGA

PGU1(SP)  (101) SGHTIDGQGAKWWDGLGDNGKVKPKFVKLALTGTSKVTGLNIKNAPHQVF
PGU1(BY)  (101) SGHTIDGQGAKWWDGLGDSGKVKPKFVKLALTGTSKVTGLNIKNAPHQVF

PGU1(SP)  (151) SINKCSDLTISDITIDIRDGDSAGGHNTDGFDVGSSSNVVIQGCTVYNQD
PGU1(BY)  (151) SINKCSDLTISDITIDIRDGDSAGGHNTDGFDVGSSSNVLIQGCTVYNQD

PGU1(SP)  (201) DCIAVNSGSTIKFLNNYCYNGHGISVGSVGGRSDNTVNGFWAESNHVINS
PGU1(BY)  (201) DCIAVNSGSTIKFMNNYCYNGHGISVGSVGGRSDNTVNGFWAENNHVINS

PGU1(SP)  (251) DNGLRIKTVEGATGAVTNVNFISNKISGIKSYGIVIEGDYLNGKTTGTAT
PGU1(BY)  (251) DNGLRIKTVEGATGTVTNVNFISNKISGIKSYGIVIEGDYLNSKTTGTAT

PGU1(SP)  (301) GGVPISNLVMKDITGSVNSTAKRVKILVKNATNWQWSGVSISGGSSYSGC
PGU1(BY)  (301) GGVPISNLVMKDITGSVNSTAKRVKILVKNATNWQWSGVSITGGSSYSGC

PGU1(SP)  (351) SGIPSGSGASC
PGU1(BY)  (351) SGIPSGSGASC
```

Fig. 2. Amino acid sequences of the *PGU1* genes from *S. paradoxus* [PGU1(SP)] and *S. cerevisiae* [PGU1(BY)].

The *PGU1* promoter from *S. paradoxus* RO88 was also sequenced and compared to that of *S. cerevisiae* S288C on the Saccharomyces Genome Database. The alignment of the two promoters is presented in Fig. 3.

```
SP_PGU1prom    (1) AGAGGACAAGTCGACTTGTCCTGCCTCATACATACTTGACGATGAAGAGC
SC_PGU1prom    (1) AAAGGACAAGTCGACTTGTCCTGCCTCATACATACTTGACGATGAAGAGC

SP_PGU1prom   (51) AAGATACTGAGCACGTGAATCGAGGAACAGGTAAGGATACCTCGAGAAAT
SC_PGU1prom   (51) AAGATACTGAGCACGTGAATCGAGGAACAGGTAAGGATACCTCGAGAAAT

SP_PGU1prom  (101) GAAAAAAAAAAGTTGATCAGCCGTACTTGAAAATTAAATTTGGCGGTGAG
SC_PGU1prom  (101) GAAAAAAAAAAGTTGATCAGCCGTACTTGAAAATTAAATTTGGCGGTGAG

SP_PGU1prom  (151) AAAGACAATATCGTAATGAAAATGATTCTGACGACCCTTTGATAGTGGCA
SC_PGU1prom  (151) AAAGACAATATCGTAATGAAAATGATTCTGACGACCCTTTGATAGTGGCA

SP_PGU1prom  (201) ATGATCAAAAAGAAAAAAAAAAGATAAGACGGTAGTGTGAAGATGACATA
SC_PGU1prom  (201) ATGATCAAAAAGAAAAAAAAAAGATAAGACGGTAGTGTGAAGATGACATA

SP_PGU1prom  (251) TAGCGCTACTCTATACTCGTCCAACTTCGAAAATAATATGTGGTCGTTGG
SC_PGU1prom  (251) TAGCGCTACTCTATACTCGTCCAACTTCGAAAATAATATGTGGTCGTTGG

SP_PGU1prom  (301) TACGTTCAGATAAGAGAATACATCTCGCGCGTACGCATAATTGTGGTCTA
SC_PGU1prom  (301) TACGTTCAGATAAGAGAATACATCTCGCGCGTACGCATAATTGTGGTCTA

SP_PGU1prom  (351) AAAAACCGCTGAAATTTTCTCAATACTGAATAGAATCACGCTACTACGAC
SC_PGU1prom  (351) AAAAACCGCTGAAATTTTCTCAATACTGAATAGAATCACGCTACTACGAC

SP_PGU1prom  (401) AAGACTCGGTTACTGTGCCTAAAATAATCCTGTGATAAACGAGTTATGTT
SC_PGU1prom  (401) AAGACTCGGTTACTGTGCCTAAAATAATCCTGTGATAAACGAGTTATGTT

SP_PGU1prom  (451) AAACGCAGTACAGGGGTTAAAGGGCATTGAGTTTTTGTGAGTGGAAATGC
SC_PGU1prom  (451) AAACGCAGTACAGGGGTTAAAGGGCATTGAGTTTTTGTGAGTGGAAATGC

SP_PGU1prom  (501) CCCCGTTATAGCTTCCAGTTTAATTACAAATTATCAATTTAAGCAAATAT
SC_PGU1prom  (501) CCCCGTTATAGCTTCCAGTTTAATTACAAATTATCAATTTAAGCAAATAT

SP_PGU1prom  (551) AACTGGAGGATTGGGGAGGCGACTAAAAATGGCTACCACGCTATTAGACA
SC_PGU1prom  (551) AACTGGAGGATTGGGGAGGCGACTAAAAATGGCTACCACGCTATTAGACA

SP_PGU1prom  (601) TACAACATTGAGTATTTTATGTAATTTTGTTACTGCTAGCACGGCCATGC
SC_PGU1prom  (601) TACAACATTGAGTATTTTATGTAATTTTGTTACTGCTAGCACGGCCATGC

SP_PGU1prom  (651) AATTGGCAACTGAAAGCTATCTGACAACTTAAATGATTCTTAAAACAATG
SC_PGU1prom  (651) AATTGGCAACTGAAAGCTATCTGACAACTTAAATGATTCTTAAAACAATG
                                             1
SP_PGU1prom  (701) ACGACTATAATCTTCTCTAAGAGGTTTCATATCCATCTTCCTCATTATTC
SC_PGU1prom  (701) ACGACTATAATCTTCTCTAAGAAGTTTCATATCCATCTTCCTCATTATTC

SP_PGU1prom  (751) AGTTTCTTTTTCCTCTTGAAAGTATCGTAAAGAACAACGTCTTCACATTA
SC_PGU1prom  (751) AGTTTCTTTTTCCTCTTGAAAGTATCGTAAAGAACAACGTCTTCACATTA

SP_PGU1prom  (801) GCTATTAGAAGACCATTGAACTACCGGATATGAGTAAGAGTGATCTTGCC
SC_PGU1prom  (801) GCTATTAGAAGACCATTGAACTACCGGATATGAGTAAGAGTGATCTTGCC

SP_PGU1prom  (851) GTAGAGATAATAGCTGCACAAAGGCCAAGGATTAGATTAATGGGTGCATT
SC_PGU1prom  (851) GGAGAGATAATAGCTGCACAAAGGCCAAGGATTAGATTAATGGGTGCATT

SP_PGU1prom  (901) GTACGAAAAAAAATAGTTTACAGTCATTTATTCGCAATAAATCAATTTTT
SC_PGU1prom  (901) GTACGAAAAAAAATAGTTTACAGTCATTTATTCGCAATAAATCAATTTTT

SP_PGU1prom  (951) TTTTCAAAAAATATGTAAGTCTGATAAAAAATTCTTCACTGAAGAGAGAT
SC_PGU1prom  (951) TTTTCAAAAAATATGTAAGTCTGATAAAAAATTCTTCACTGAAGAGAGAT
                          2
SP_PGU1prom (1001) GCTTAAAGCTTAAT-CTTGAATAAAAGACTCTCTAACGCTGTGAATTCTC
SC_PGU1prom (1001) GCTTACATTCTAATTCTTGAATAAAAGACTCTCTAACGCTGTGAATTCTC

SP_PGU1prom (1050) TTTAGCTGTAACGGAAACAGAGAGTTATTCCGTAGTCACTGAATTTTTTT
SC_PGU1prom (1051) TTTAGCTGTAACGGAAACAGAGAGTTATTCCGTAGTCACTGAATTTTTTT

SP_PGU1prom (1100) TTTTTGACGCTATTATTTAAAACCTAGGATATCCGTCCCATACAAAACGG
SC_PGU1prom (1101) TTTTTGACGCTATTATTTAAAACCTAGGATATCCGTCCCATACAAAACGG
                          3         4
SP_PGU1prom (1150) CCACGAGTTTCAATCCCAGAATGTACGAGTTATAATTCTCCTAGATGCAT
SC_PGU1prom (1151) CCACGAGTTTCAATCCCAGAATGTACGAGTTATAATTCTCCTAGATGCAT

SP_PGU1prom (1200) GATGCTCGTGCATTCGTTTAACAATCATACCAATTTCCCATTTTCGGGAT
SC_PGU1prom (1201) GATACTCGTGCATTCGTTTAACAATCATACCAATTTCCCATTTTCGGGAT
                                                    5
SP_PGU1prom (1250) ATTAAACATGAACATACTTTTTTACTGTGAGAATGTGGTTTCACAATTAT
SC_PGU1prom (1251) ATTAAACATGAACATACTTTTTTACTGTGAGAATGTGGTTTCACAATTAT

SP_PGU1prom (1300) TCCATACAGGTATAAAAACGCACAGAACTTCAAACGGGAAGACTATCTAC
SC_PGU1prom (1301) TCCATACAGGTATAAAAACGCACAGAACTTCAAACGGGAAGACTATCTAC

SP_PGU1prom (1350) CCACATTGATGGACAAACGCAATGA
SC_PGU1prom (1351) CCACATTGATGGACAAACGCAATGA
```

Fig. 3. The *PGU1* promoter sequences of *S. paradoxus* RO88 (SP_PGU1prom) and *S. cerevisiae* S288C (SC_PGU1prom). Nucleotide differences are indicated in black. Boxes 1 and 3 represent Ste12p binding sites. Boxes 2, 4 and 5 represent Tec1p binding sites.

A total of 9 nucleotide differences were observed between the two promoter sequences. Two of these areas were identified as binding sites – one for Ste12p and one for Tec1p. Box number 1 in Fig. 3 represents a Ste12p binding site for S288C. There was one nucleotide difference in this sequence for RO88. Box 2 represents a Tec1p binding site for S288C. There are four nucleotide differences in the RO88 sequence and subsequently no Tec1p binding site. These

sequencing results are contradictory to what was expected. RO88 has higher PG activity than S288C, so more Tec1p and/or Ste12p binding sites were expected, as these are transcription factors for *PGU1*. This was however not the case. It could be that the promoter has binding sites for another unknown regulatory protein that increases *PGU1* transcription. Alternatively, the promoter sequence may not be the reason for RO88's higher PG activity. Transcription of *PGU1* should be evaluated in S288C and RO88 using Real-Time PCR. Additionally, transcription of *TEC1* and *STE12* should also be evaluated.

5.3.3 Obtaining recombinant yeasts

The wine strain VIN13 was transformed with the two plasmids: pDMP-SC (containing the *PGU1* gene from *S. cerevisiae* under control of the *PGK1* promoter) and pDMP-SP (containing the *PGU1* gene from *S. paradoxus* under control of the *PGK1* promoter). Both plasmids also contain the *kan*^{r} gene for selection purposes. The *kan*^{r} gene from *Escherichia coli* transposon Tn903 confers resistance to G418, an antibiotic with a large inhibitory effect (Jimenez & Davies, 1980). Wach *et al*. (1994) fused this gene to the *TEF* promoter and terminator from *Ashbya gossypii* to create the kanMX cassette. A loxP-kanMX-loxP cassette was constructed by Güldener *et al*. (1996) by combining the kanMX cassette with the cre-loxP system of bacteriophage P1. This cassette can be used for selection on G418 in recombinant yeasts. Upon expression of Cre-recombinase, recombination takes place between the two loxP sites, excising the kanMX module. The recombinant yeast is then free from any antibiotic resistance markers.

The transformants were grown on YPD plates containing G418 for selection. After 3-4 days of incubation, several colonies of each were picked from the plates and cultured in liquid YPD medium. Genomic DNA was extracted and used as template to screen for the positive transformants by PCR as described in the previous section. The primers used were 5'-KPNPGK-631 and Pgu1rev2 (refer to Table 1). 5'-KPNPGK-631 was the forward primer binding to the *PGK1* promoter and Pgu1rev2 the reverse primer binding in the *PGU1* gene. This ensured that the native *PGU1* gene of VIN13 was not amplified. Positive recombinants were identified for both plasmids. One recombinant of each was chosen for further analyses.

5.3.4 Polygalacturonase assays

The transformants were spotted on a polygalacturonic acid plate. The plasmids used for transformation had the same backbone and differed only by the sequence of the *PGU1* gene: one had the gene from *S. cerevisiae* BY4742 and the other the gene from *S. paradoxus* RO88.

As controls, the VIN13 and RO88 wild types were also spotted on the plates. Fig. 4 shows the result of the plate assay.

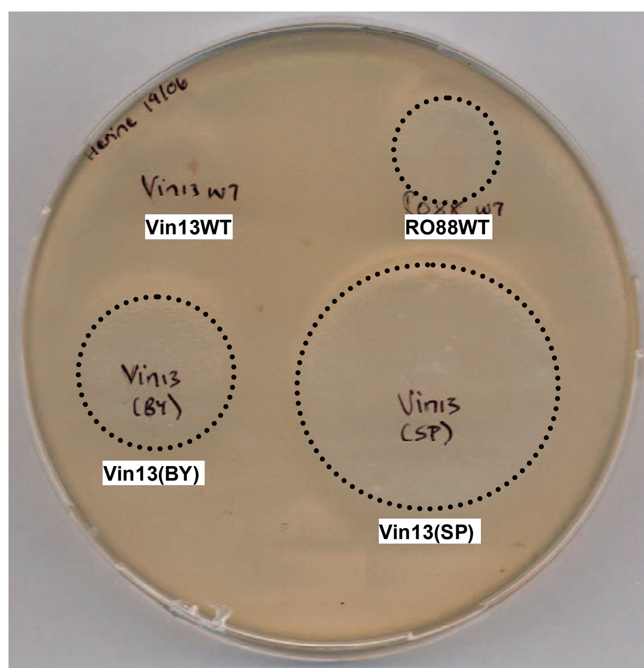

Fig. 4. Plate assay of VIN13 transformants. Vin13(BY) is the strain transformed with the *PGU1* gene from *S. cerevisiae* BY4742 and Vin13(SP) is transformed with the *PGU1* gene from *S. paradoxus* RO88. Vin13WT and RO88WT are the two wild type yeasts.

As expected, the plate assay showed that the wild type VIN13 had no PG activity and that the RO88 wild type does exhibit activity. From this assay, there is a clear increase in activity in VIN13 when the *PGU1* gene is expressed constitutively under the control of the *PGK1* promoter. The *PGU1* gene from RO88 increased the activity even more than the gene from BY4742. The only difference between the two plasmids was the sequence of the *PGU1* gene. It can therefore be concluded that the gene sequence of RO88 is responsible for the higher PG activity observed on the polygalacturonic acid plate. This assay also revealed that the *PGU1* gene of BY4742 was fully functional even though the strain had no natural PG activity.

A liquid assay was also performed in order to quantify the difference in the polygalacturonase activity between the different strains. This assay indicated the amount of galacturonic acid each strain produced from the same amount of polygalacturonic acid. The results are presented in Fig. 5.

Fig. 5 shows that Vin13(SP) had the highest activity, followed by Vin13(BY). RO88WT also showed activity while Vin13WT had none. This assay is highly sensitive. The OD_{600nm} measurements of the cultures used in the assays were probably not accurate enough to ensure the same amount of cells between biological repeats. This resulted in slightly different enzyme

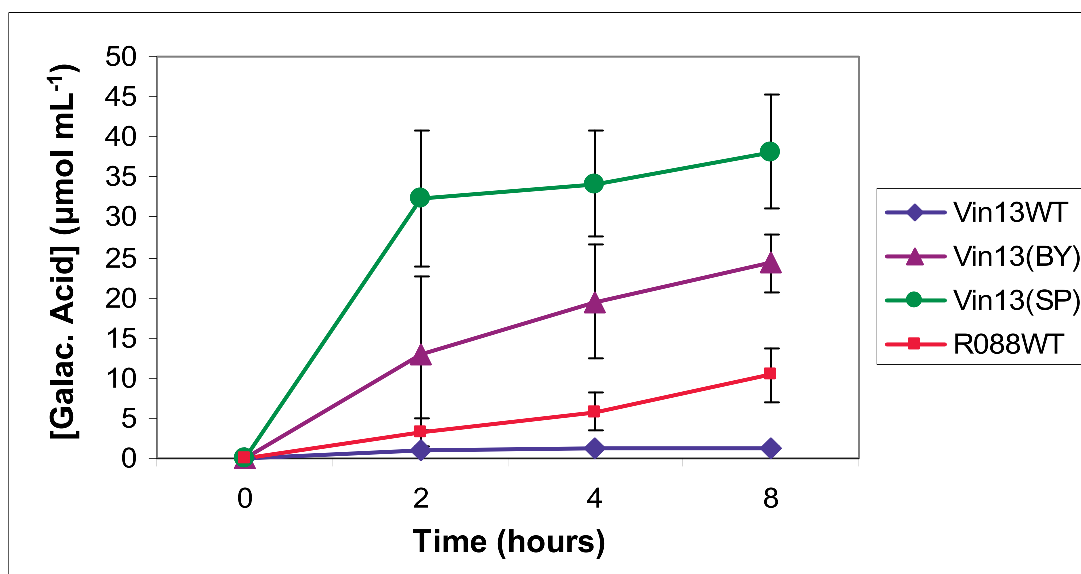

Fig. 5. Liquid polygalacturonase assay indicating the amount of galacturonic acid produced (in µmol mL^{-1}) over a certain time. The values are an average of the three biological repeats. Abbreviations as in Fig. 4.

concentrations and subsequent different concentrations of galacturonic acid. This is a potential explanation for the large standard deviations observed between the repeats. For future assays, the cultures should be plated out to ensure the same amount of cells between biological repeats. All assays should also be performed on the same day in order to minimize the influence of external factors that could affect the cell growth, enzyme production or activity measurements. Nevertheless, the differences in the activities of the different strains were significant and the trend between the strains confirmed that of the plate assay (the same trend was observed in the individual assays). A clear difference was observed between the two recombinants. Vin13(SP) exhibited a much higher activity than Vin13(BY) as indicated by the amounts of galacturonic acid produced by these strains. This was the result of the *S. paradoxus* RO88 *PGU1* gene, as explained previously. It could be that the enzyme is secreted more efficiently due to the amino acid difference in the secretion signal. Further analyses are required to confirm this. For Vin13(SP), most of the polygalacturonic acid was degraded within the first two hours, indicating a high production and secretion of the enzyme.

5.3.5 Microvinification

Wine was made from Chenin Blanc and Shiraz must using the following strains: VIN13, RO88, VIN13[pDMP-SC], VIN13[pDMP-SP], PR7 and PR10. The fermentations, performed in triplicate, were monitored by weight loss in order to compare the fermentation rates of the different strains and to detect the end of fermentation (Fig. 6).

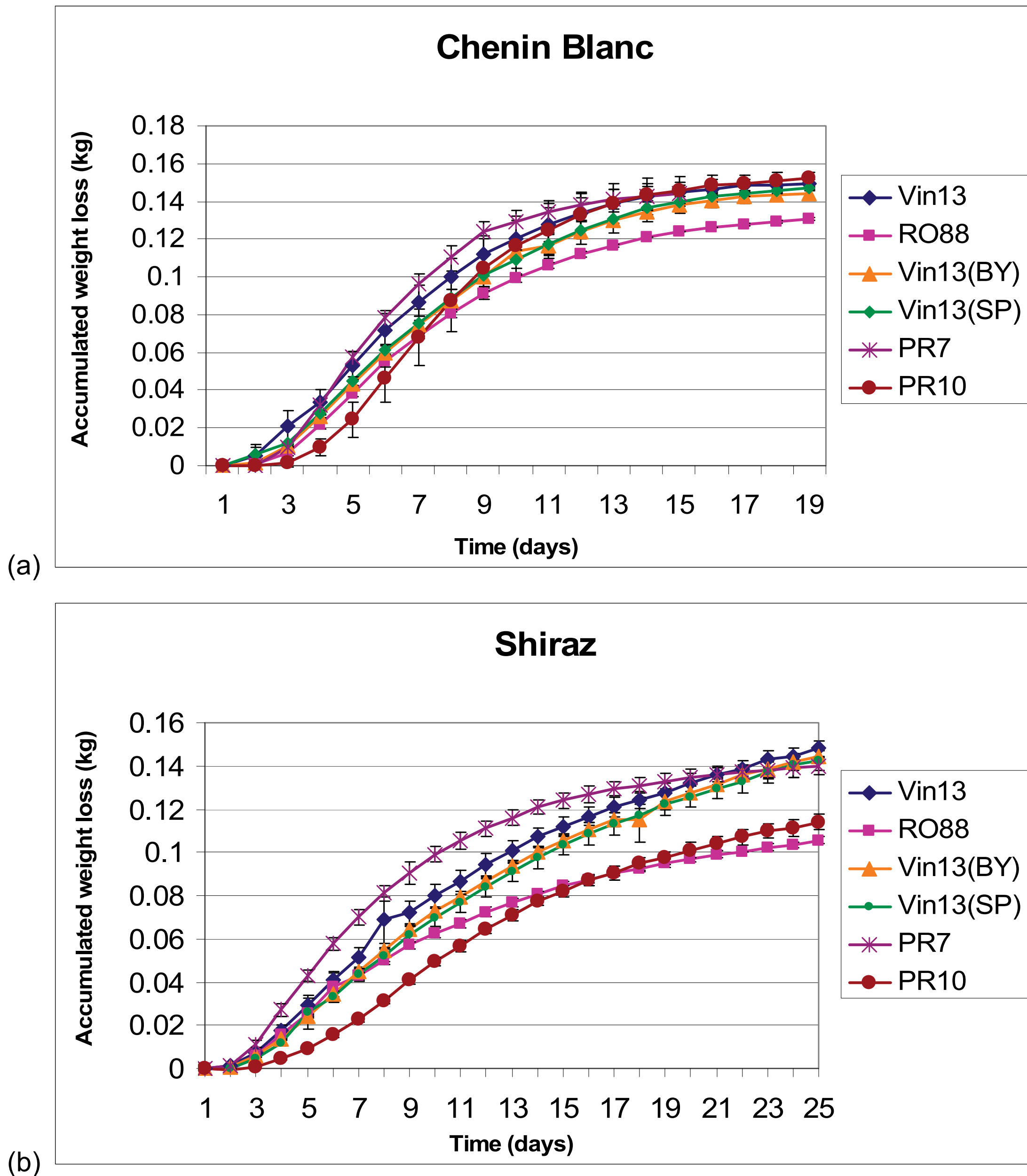

Fig. 6. Fermentation rate of the different strains in Chenin Blanc (a) and Shiraz (b) must. The fermentation rate is expressed as accumulated weight loss over time (days). Vin13(BY) is VIN13[pDMP-SC] and Vin13(SP) is VIN13[pDMP-SP].

The values that are presented are the average of the triplicates for each strain. In both cultivars, the two recombinant strains fermented at the same rate and very close to their wild type, VIN13. The presence of the plasmid did not significantly influence the performance of the recombinant strains. RO88 definitely fermented slower than VIN13. There was an even larger difference between the two strains in the Shiraz must, which had a very high sugar concentration (27.15 °B initially). RO88 seemed to be affected throughout the fermentation by this high sugar concentration. A clear difference was noticed between the two hybrids. PR10 had a slow onset of fermentation while PR7 was relatively fast. In the Chenin Blanc must, PR7 and PR10 fermented about equal amounts of sugar at the end of the fermentations. In the Shiraz must however, PR10 fermented slower than PR7 and also utilized less sugar. From this

data, PR7 seems to be the strongest fermenter of the two hybrids and would probably be the one recommended for winemaking. RO88, although it has a relatively high natural PG activity, did not perform as well as VIN13 and would therefore not be selected to perform alcoholic fermentation. It could however be used for co-inoculation with a strain like VIN13.

The wines were plated onto two types of plates at the end of fermentation: YPD plates and YPD plates containing 200 µg mL^{-1} G418. As expected, there was no growth on any of the YPD-G418 plates for VIN13, RO88 PR7 and PR10. Unfortunately, there was virtually no growth on the YPD-G418 plates for the recombinants either. It seems the plasmids were highly unstable in the recombinant yeasts and were lost during fermentation, faster than expected. Vilanova *et al*. (2000) used a recombinant strain in vinification and found that the cells gradually lost their plasmids. At the end of fermentation (after 20 days), they found 70% of the cells still retained the plasmid. To ensure stability, the construct of interest should be integrated into the genome of the strain. This can insure that up to 100% of the cells retain the construct (Fernández-González *et al*., 2005).

Colonies were picked from the plates and used for a fingerprint PCR. In this PCR reaction, the Delta 12 and Delta 21 primers were used to amplify the areas between delta elements in the yeast genome, yielding a band pattern unique to each strain (Legras & Karst, 2003). This verified that the inoculated yeast did indeed complete alcoholic fermentation.

5.3.6 Analyses of aroma compounds from wine

The major biochemical parameters of the wines were measured. These parameters included pH, volatile acidity, total acid, malic acid, glucose, fructose, ethanol and glycerol. The measurements are presented in Fig. 7.

In both cultivars, wines fermented by RO88 had a high residual fructose concentration. PR7 and PR10 also had higher residual fructose than the rest of the strains, though slightly lower than RO88. All the strains showed higher residual sugars (glucose and fructose) in Shiraz than in Chenin Blanc. This was expected as the Shiraz must had an initial sugar concentration of 27.15 °B, which is very high.

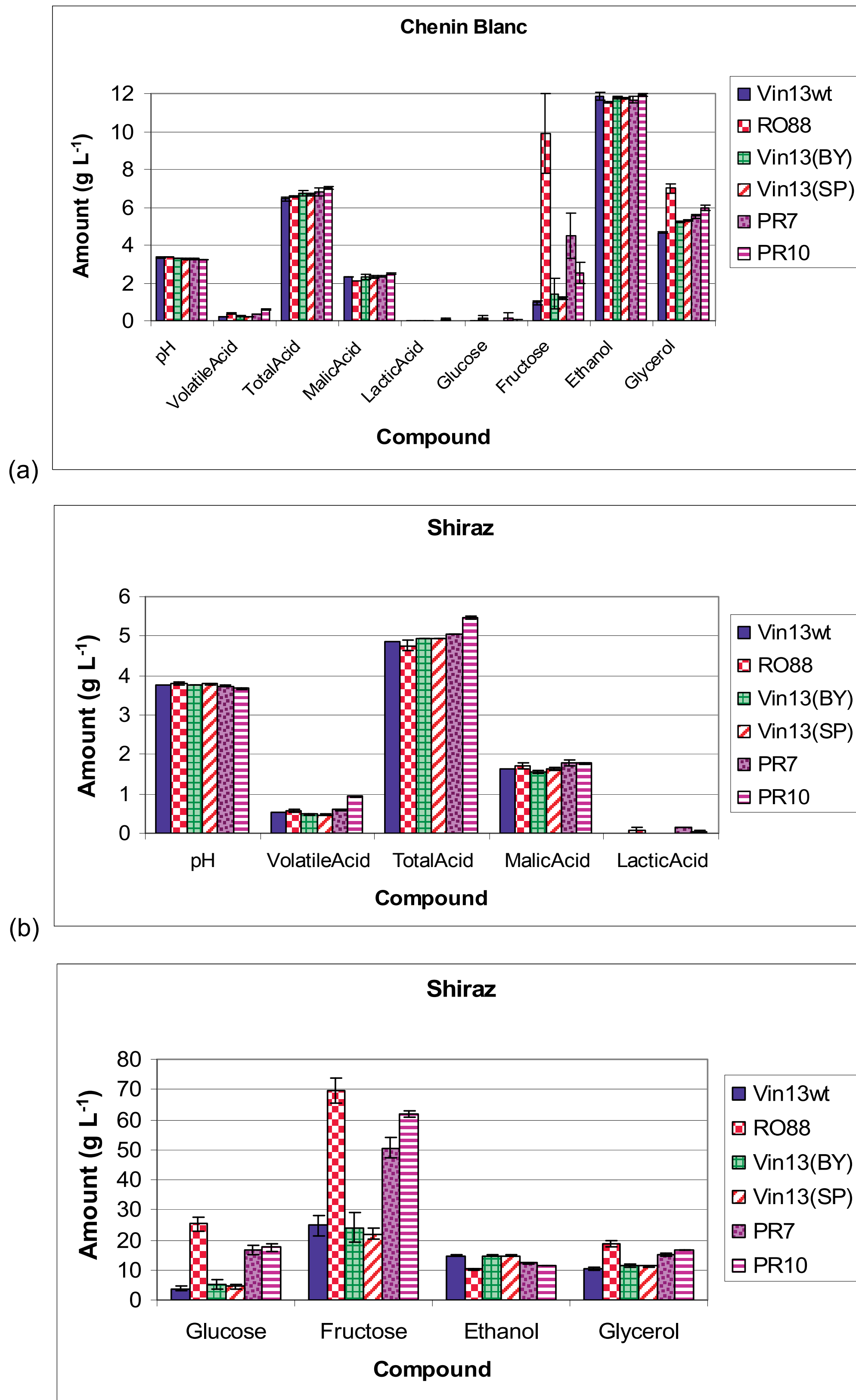

Fig. 7 The major biochemical parameters measured by the Winescan instrument for all the fermentations in Chenin Blanc (a) and Shiraz (b) must. The value for each strain is a representation of the average between the triplicates. Vin13(BY) is VIN13[pDMP-SC] and Vin13(SP) is VIN13[pDMP-SP].

None of the strains could ferment to dryness (5 g L^{-1} or less total residual sugar) in the Shiraz must. The strains were probably inhibited by the ethanol concentration as the initial sugar

concentration in this must was very high (27.15 °B). RO88 produced the lowest amount of ethanol. This suggests that VIN13 might be slightly more resistant to ethanol than RO88. Glycerol production was also slightly higher for RO88, and to minor extends for PR7 and PR10, in both cultivars, while VIN13 produced the least. It was also noted that PR10 had a slightly elevated level of volatile acidity in the Shiraz must. This only increased the total acidity of the wine by 0.5 g L^{-1} and probably has no significant effect on the wine. Although the amounts differ, the trends between VIN13, RO88, PR7 and PR10 were similar to that observed by Mocke (2005).

Certain volatile aroma compounds were extracted from the wines and analysed by GC-FID. The results are presented in Fig. 8, Fig. 9 and Fig. 10.

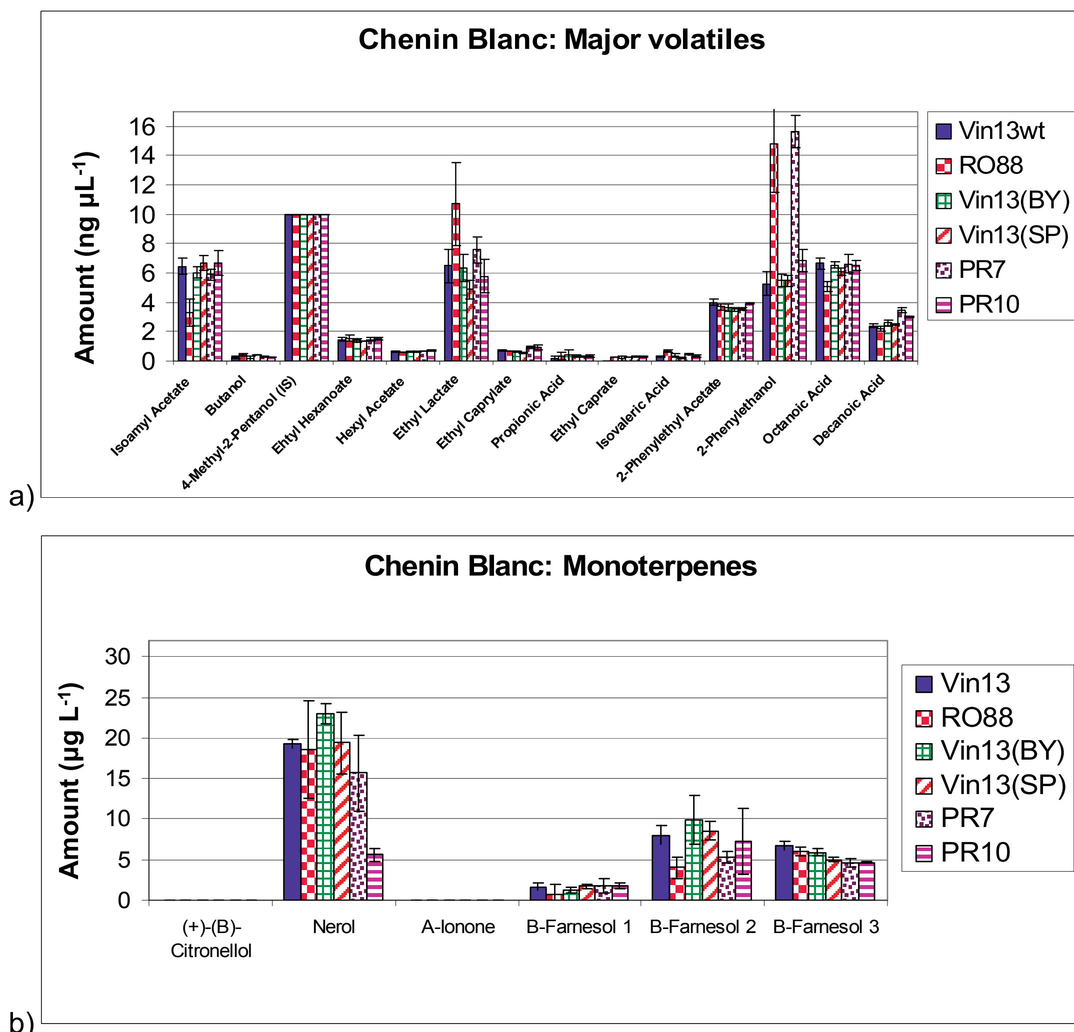

Fig. 8. Major volatiles (a) and monoterpenes (b) extracted from Chenin Blanc wines as analysed by GC-FID. The value for each strain is a representation of the average between the triplicates. Vin13(BY) is VIN13[pDMP-SC] and Vin13(SP) is VIN13[pDMP-SP].

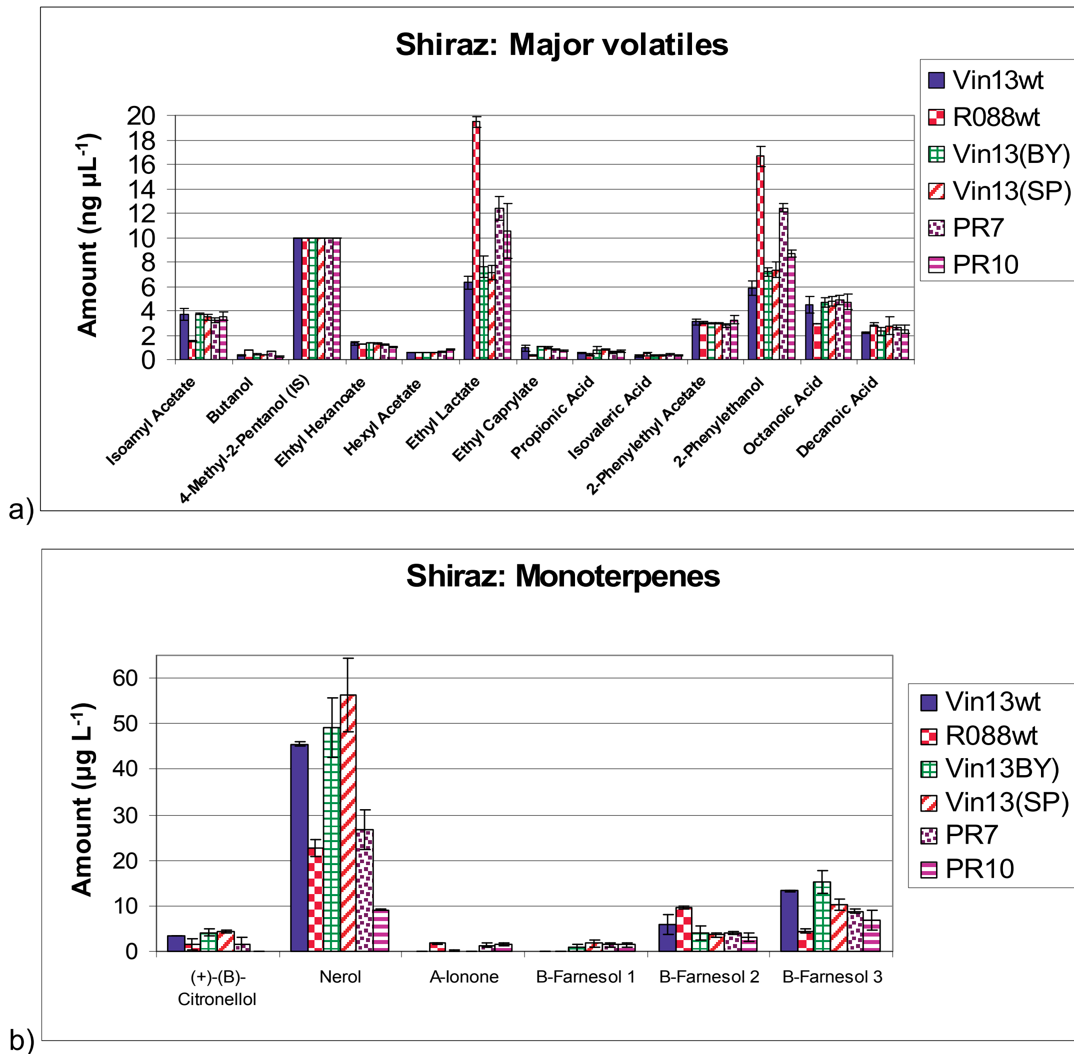

Fig. 9. Major volatiles (a) and monoterpenes (b) extracted from Shiraz wines as analysed by GC-FID. The value for each strain is a representation of the average between the triplicates. Vin13(BY) is VIN13[pDMP-SC] and Vin13(SP) is VIN13[pDMP-SP].

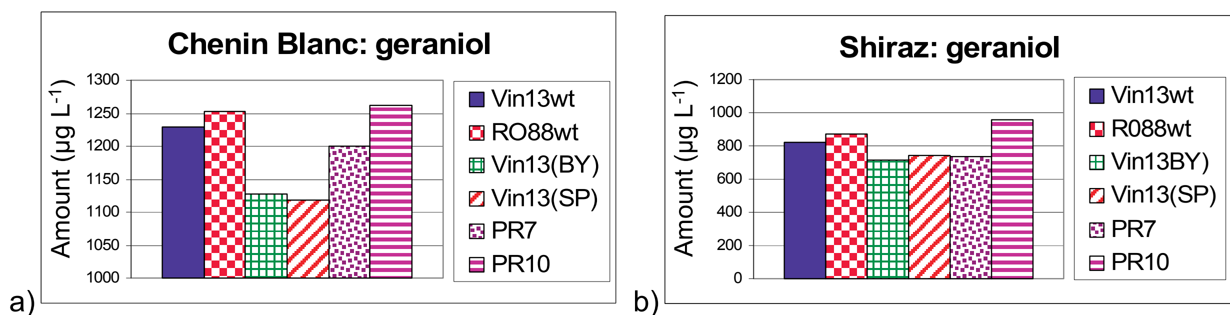

Fig. 10. Geraniol concentrations in Chenin Blanc (a) and Shiraz (b) wines. The value for each strain is a representation of the average between the triplicates. Vin13(BY) is VIN13[pDMP-SC] and Vin13(SP) is VIN13[pDMP-SP].

As explained previously, the recombinant strains lost their plasmids during fermentation. As a result, the aroma compounds for these strains did not differ significantly from that of VIN13 (their wild type strain) and unfortunately the effect of the *PGU1* gene on the wine aroma profile could not be determined by this study. However, for some compounds, some tendencies were observed. In the Chenin Blanc wines, Vin13(BY) seemed to produce higher amounts of nerol

and β-Farnesol 2. A similar trend was observed in the Shiraz wines: wines fermented by Vin13(BY) tended to have higher amounts of nerol, β-Farnesol 1 and β-Farnesol 3, while those fermented by Vin13(SP) showed increased amounts of nerol and β-Farnesol 1. Both recombinants also tended to produce slightly larger amounts of propionic acid.

RO88 produced a high amount of ethyl lactate and 2-phenylethanol in both Chenin Blanc and Shiraz. Ethyl lactate is usually formed during malolactic fermentation (Maicas *et al*., 1999) and is probably higher in wines fermented by RO88 as this strain is able to degrade malic acid (Redzepovic *et al*., 2003). It has a detection threshold of 150 mg L^{-1} and contributes to a fruity, buttery aroma (Nurgel *et al*., 2002). Production by PR7 and PR10 were also high in Shiraz (although less than RO88) and similar to VIN13 (less than RO88) in Chenin Blanc. For this compound, the results for Shiraz were similar to that found by Mocke (2005). 2-phenylethanol is responsible for a rosy aroma (López *et al*., 1999) and its threshold in wine is between 25 – 105 mg L^{-1} (Amerine & Roessler, 1976). Results for this compound differed to those observed by Mocke (2005) where PR7 showed the highest production, followed by RO88, VIN13 and finally PR10. In this study, for Chenin Blanc, PR7 and RO88 produced similar amounts. PR10 produced much less, yet slightly more than VIN13. In Shiraz, RO88 produced the highest amount, followed by PR7, PR10 and finally VIN13. Both ethyl lactate and 2-phenylethanol were found in concentrations lower than their respective thresholds in this study and therefore probably had no noticeable effect on the wine aroma.

RO88 also produced less isoamyl acetate and octanoic acid than the rest of the strains that were tested. Isoamyl acetate is responsible for a pleasant banana, pear, fruity, sweet odour and has a threshold of 0.16 mg L^{-1} (Peinado *et al*., 2004). Because of its low threshold, it could have a noticeable effect on the wine aroma when the concentration is low. The wines fermented by RO88 probably had a less sweet, fruity aroma than the other wines. PR7 and PR10 produced similar amounts to VIN13 which confirmed the results of Mocke (2005). Octanoic acid contributes a fatty, rancid odour and has a threshold of 10 mg L^{-1} (Peinado *et al*., 2004). This compound was found below its threshold value in both wines and therefore would not have a noticeable effect on the wine. These results were contradictory to those found by Mocke (2005), where RO88 produced the highest amount of this compound (PR10 was similar) and VIN13 less (PR7 was similar). In this study, RO88 produced the least amount of all the strains, while those produced by VIN13, PR7 and PR10 were similar.

The two recombinants produced much less geraniol in the Chenin Blanc wines than the rest of the strains. The difference was not as large in the Shiraz wines. PR10 also produced significantly more of this compound than PR7 in both cultivars. Furthermore, wines fermented by RO88 indicated higher geraniol levels than VIN13.

For some compounds, the hybrids clearly resemble either RO88 or VIN13. Such is the case for isoamylalcohol where both the hybrids have values close to that of VIN13, while RO88 produced much less. For other compounds, the hybrids produced amounts intermediate between their parental strains as was observed for ethyl lactate and 2-phenylethanol in Shiraz. These observations may be due to the *PGU1* gene, but it may also be the result of another gene or genes. Additional studies on the hybrids would determine if any of the above compound productions are influenced by endo-polygalacturonases. An interesting experiment would be to determine from which parent (VIN13 or RO88) each of the hybrids *PGU1* genes come from. It seems they obtained one gene from VIN13 and one from RO88 as both hybrids have good PG activity which is intermediate between that of VIN13 and RO88 (Mocke, 2005). Small scale fermentations can then be carried out again with the hybrids as well as PR7Δpgu1 and PR10Δpgu1 strains to determine the effect of the *PGU1* gene on wine aroma. Additionally, the cassette constructed in this study (or a similar cassette) can be integrated into the genome of VIN13 and used in vinification experiments.

5.4 Conclusions

The *PGU1* genes from *S. cerevisiae* BY4742 and *S. paradoxus* RO88 were amplified and cloned into a yeast expression vector. The RO88 *PGU1* gene was sequenced and compared to that of BY4742 (sequence obtained from the Saccharomyces Genome Database). A few differences were found in the amino acid sequences between the two genes. One of these differences was in the secretion signal of the gene. None of the other differences corresponded to what Blanco *et al.* (1998) identified as potentially important amino acids for PG activity.

The two genes were cloned individually into the same plasmid and used to transform the wine yeast VIN13. The recombinant strains were tested for PG activity by two distinct assays. Firstly, a semi-quantitative plate assay was performed by growing the strains on plates containing polygalacturonic acid. Clear zones formed around the colonies, which indicated PG activity and confirmed that both genes were functional. Secondly, a quantitative liquid assay was carried out to determine the amount of galacturonic acid produced by each strain from a certain amount of polygalacturonic acid. The two assays produced the same results. The yeast transformed with the gene from RO88 had the highest activity, followed by the recombinant with the BY4742 gene. It was thus concluded that the gene sequence of RO88 is responsible for its high activity, as the genes were cloned in identical plasmids. Further studies can also be conducted to determine why BY4742 has no activity, when it possesses a functional gene. It could have a non-functional promoter, and consequently the gene is not transcribed. There could also be

some sort of post-transcriptional regulation that prevents the strain from secreting a functional enzyme.

Some potentially interesting differences between the *PGU1* gene from *S. cerevisiae* and *S. paradoxus* were discovered. It also opened the door to further studies in order to determine what is responsible for these differences. The amino acids that were identified to differ between *S. cerevisiae* BY4742 and *S. paradoxus* RO88 should therefore also be studied to determine which one(s) is (are) important for a high PG activity. Analyses of the *PGU1* promoter of *S. paradoxus* RO88 revealed results contradictory to what was expected: the RO88 promoter showed less binding sites for the transcription factors Tec1p and Ste12p than *S. cerevisiae* S288C. The *PGU1* promoter of *S. paradoxus* RO88 could potentially also contribute to the high activity exhibited by the wild type yeast, by having other unknown binding sites. This can be investigated in the future.

This study identified some differences between strains with regard to their fermentation ability and production of volatile compounds during alcoholic fermentation. The strain RO88 was found to be a rather poor fermenter, as was indicated by the large amount of residual sugar at the end of fermentation. PR7 seems to be the strongest fermenter of the two hybrids, but both hybrids had higher levels of residual sugar at the end of fermentation when compared to VIN13, especially in the Shiraz wines. RO88 and the hybrids showed an increased production of certain aroma compounds and in some instances, the hybrids resembled either VIN13 or RO88. Unfortunately, no significant differences were observed in the production of volatile compounds between the recombinants and their wild type (VIN13). However, some tendencies did arise where certain compounds seem to be slightly higher in the wines fermented by the recombinants than those by VIN13. Plasmid stability should be ensured and the vinification experiment repeated to determine the true contribution of the *PGU1* gene on wine aroma.

5.5 References

Amerine MA & Roessler EB (1976) Wines: Their Sensory Evaluation. Freeman*, San Francisco*.

Blanco P, Sieiro C, Reboredo NM & Villa TG (1997) Grape juice biodegradation by polygalacturonase from *Saccharomyces cerevisiae*. *Int Bioremed* **40:** 115-118.

Blanco P, Sieiro C, Reboredo NM & Villa TG (1998) Cloning, molecular characterization, and expression of an endo-polygalacturonase-encoding gene from Saccharomyces cerevisiae IM1-8b. *FEMS Microbiol Lett* **164:** 249-255.

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P & Boeke JD (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14:** 115-132.

Cryer DR, Eccleshal R & Marmur J (1975) Isolation of yeast DNA. *Methods Cell Biol* **12:** 39-44.

Davis RW, Thomas M, Cameron J, St John TP, Scherer S & Padgett RA (1980) Rapid DNA isolations for enzymatic and hybridization analysis. *Methods Enzymol* **65:** 404-411.

De Vries RP & Visser J (2001) *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol Mol Biol Rev* **65:** 497-522.

Divol B & van Rensburg P (2007) *PGU1* natural deletion is responsible for the absence of endo-polygalacturonase activity in some wine strains of *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7:** 1328-1339.

Fernández-González M, Úbeda JF, Cordero-Otero RR, Thanvanthri Gururajan V & Briones AI (2005) Engineering of an oenological *Saccaromyces cerevisiae* strain with pectinolytic activity and its effect on wine. *Int J Food Microbiol* **102:** 173-183.

Güldener U, Heck S, Fiedler T, Beinhauer J & Hegemann JH (1996) A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res* **24:** 2519-2524.

Jayani RS, Saxena S & Gupta R (2005) Microbial pectinolytic enzymes: A review. *Process Biochem* **40:** 2931-2944.

Jimenez A & Davies J (1980) Expression of a transposable antibiotic resistance element in *Saccharomyces. Nature* **287:** 869-871.

Kester HCM, Kusters-Van Someren MA, Müller Y & Visser J (1996) Primary structure and characterization of an exopolygalacturonase from *Aspergillus tubingensis*. *Eur J Biochem* **240:** 738-746.

Kester HCM & Visser J (1990) Purification and characterization of polygalacturonases produced by the hyphal fungus *Aspergillus niger*. *Biotech Appl Biochem* **12:** 150-160.

Legras JL & Karst F (2003) Optimisation of interdelta analyses for *Saccharomyces cerevisiae* strain characterization. *FEMS Microbiol Lett* **221:** 249-255.

Lever M (1972) A new reaction for colorimetric determination of carbohydrates. *Anal Biochem* **47:** 273-279.

López R, Ferreira V, Hernández P & Cacho JF (1999) Identification of impact odorants of young red wines made with Merlot, Cabernet Sauvignon and Grenache grape varieties: a comparative study. *J Sci Food Agric* **79:** 1461– 1467.

Maicas S, Gil JS, Pardo I & Ferrer S (1999) Improvement of volatile composition of wines by controlled addition of malolactic bacteria. *Food Res Int* **32:** 491– 496.

Majdak A, Herjavec S, Orlić S, Redžepović S & Mirošević N (2002) Comparison of wine aroma compounds produced by *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* strains. *Food Technol Biotechnol* **40:** 103-109.

McKay AM (1988) A plate assay method for the detection of fungal polygalacturonase secretion. *FEMS Lett* **56:** 355-358.

Mocke BA (2005) The breeding and characterisation of starter culture yeast strains for red wine making. MSc Thesis, Stellenbosch University, Stellenbosch.

Nurgel C, Erten H, Canbaş A, Cabaroğlu T & Selli S (2002) Influence of *Saccharomyces cerevisiae* strains on fermentation and flavour compounds of white wines made from cv. Emir grown in Central Anatolia, Turkey. *J Ind Microbiol Biotechnol* **29:** 28-33.

Peinado RA, Moreno J, Bueno JE, Moreno JA & Mauricio JC (2004) Comparative study of aromatic compounds in two young white wines subjected to pre-fermentative cryomaceration. *Food Chemistry* **84:** 585-590.

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16:** 675-729.

Redžepović S, Orlić S, Majdak A, Kozina B, Volschenk H & Viljoen-Bloom M (2003) Differential malic acid degradation by selected strains of *Saccharomyces* during alcoholic fermentation. *Int J Food Microbiol* **83:** 49-61.

Van Rensburg P & Pretorius IS (2000) Enzymes in winemaking: Harnessing natural catalysts for efficient biotransformations – a review. *S Afr J Enol Vitic* **21:** 52-73.

Vilanova M, Blanco P, Cortés S, Castro M, Villa TG & Sieiro C (2000) Use of a *PGU1* recombinant *Saccharomyces cerevisiae* strain in oenological fermentations. *J Appl Microbiol* **89:** 876-883.

Volschenk H, Viljoen-Bloom M, van Staden J, Husnik J& van Vuuren HJJ (2004) Genetic engineering of an industrial strain of *Saccaromyces cerevisiae* for L-malic acid degradation via an efficient malo-ethanolic pathway. *S Afr J Enol Vitic* **25:** 63-73.

Wach A, Brachat A, Pöhlmann R & Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10:** 1793-1808.

Whitaker JR (1984) Pectic substances, pectin enzymes and haze formation in fruit juices. *Enzyme Microbl Technol* **6:** 341-349.

Wightman JD, Price SF, Watson BT & Wrolstad RE (1997) Some effects of processing enzymes on anthocyanins and phenolics in Pinot Noir and Cabernet Sauvignon wines. *Am J Enol Vitic* **48:** 39-48.

Chapter 6

General discussion and conclusions

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 Discussion, conclusions and future prospective

Commercial pectinase preparations are usually added during winemaking to improve filtration, reduce viscosity and enhance extraction of certain aroma compounds or precursors (Pretorius, 2000). These fungi-derived preparations often contain various other, unwanted enzymes that could have a negative effect on the wine. A yeast capable of producing pure pectinases during alcoholic fermentation could therefore possibly eliminate the use of commercial enzymes. Regulation of the *PGU1* gene (which encodes an endo-polygalacturonase in *Saccharomyces cerevisiae*) is very complex and not yet fully understood. This gene is present in most strains of *S. cerevisiae*, except for a few that lack it. The fundamental aim of this study was to investigate if these latter strains would recover polygalacturonase (PG) activity when the *PGU1* gene was re-introduced into its native position in their genomes in an attempt to better understand the regulation of the gene. Furthermore, *Saccharomyces paradoxus* strain RO88 exhibits a relatively high PG activity (Mocke, 2005). The *PGU1* gene and promoter of RO88 was analyzed and compared to those of *S. cerevisiae* in order to identify differences that could be responsible for the difference in their PG activities. The polygalacturonase producing strains were tested under winemaking conditions to determine their effect on the aroma profile of wine. *S. cerevisiae* and *S. paradoxus* strains were also compared.

A cassette containing a functional *PGU1* gene and promoter was successfully integrated into the *PGU1* locus of five wine yeast strains. These strains are Collection Cépage Cabernet (CC Cabernet), L2226, UCLMS-1, -3 and -4. Further integration attempts for other strains were unsuccessful. A phylogenetic tree obtained from PFGE patterns indicated a close relationship between the recombinant strains (Divol & van Rensburg, 2007). This suggests that the genetic background of a strain could influence its resistance to genomic integrations. RT-PCR indicated that the three UCLMS strains showed transcription of the re-introduced *PGU1* gene, with UCLMS-1 having the highest transcription. In two of the recombinant strains, CC Cab and L2226, the re-introduced *PGU1* gene was not transcribed. A polygalacturonase plate assay indicated that the three UCLMS strains regained PG activity while CC Cab and L2226 recombinants did not. This correlated well to the RT-PCR results. According to the plate assay, the UCLMS-4 recombinant had very low PG activity. This strain could exhibit post-transcriptional regulation on the Pgu1 protein that is not present in the other two UCLMS strains. Interestingly, CC Cabernet and L2226 did recover PG activity when *PGU1* was overexpressed on a multi-copy plasmid under control of the *PGK1* promoter. This suggests regulation of the *PGU1* gene by a trans-acting factor as proposed by Hirose *et al*. (1999). UCLMS-1, -3 and -4 do not seem to exhibit the same regulation as CC Cab and L2226 and lack of PG activity in these strains is

probably due to the loss of the *PGU1* gene. Additionally, overexpression of *TEC1* (the main transcription factor for *PGU1* (Madhani *et al*., 1999)) did not lead to the recovery of PG activity in CC Cabernet. This further supports the hypothesis of regulation through a trans-acting factor. The reason for the loss of the *PGU1* gene in certain strains remains unknown. These results suggest that the gene might have been silenced through directed evolution in strains that no longer required it. This correlates to the fact that CC Cabernet did not recover activity after integration of a functional gene and promoter. Alternatively, the replacement of the gene may also be a random phenomenon.

The UCLMS-1, -3 and -4 recombinants as well as their respective wild types were used to conduct small scale fermentations in Chenin Blanc and Shiraz must. The integration was stable throughout the fermentation as was indicated by screening on selective plates. Overall, the recombinant strains fermented at approximately the same rate as their respective wild type, except for the UCLMS-1 and -4 recombinants that fermented slightly slower than their respective wild types in the Shiraz must. It was also observed that UCLMS-3 fermented on average about five days longer than UCLMS-1 and -4. After completion of alcoholic fermentation, the major biochemical parameters of the wines were measured. No significant differences were observed between wines fermented by the wild type yeast and their respective recombinants. The integration cassette therefore had no significant influence on the normal fermentation ability of the strains. Some volatile compounds and monoterpenes were also measured at the end of fermentation. There seemed to be a tendency in the Chenin Blanc wines towards the higher production of certain compounds by the recombinants than their wild types, especially for the UCLMS-1 recombinant strain. All the recombinants also tended to produce slightly more geraniol than their respective wild types in both wines. An increase in geraniol production by *PGU1* recombinants was also observed by Vilanova *et al*. (2000). Nevertheless, very few of the slight differences observed in our study were significant. Several possible explanations were foreseen: firstly, the winemaking conditions (low temperature and high sugar concentration) were probably sub-optimal for the endo-polygalacturonase and subsequently the enzyme was not active during alcoholic fermentation. Yeast endo-polygalacturonase was found to have the highest activity at 25°C (Gainvors *et al*., 2000) and was further found to be inhibited by high concentrations of glucose (Blanco *et al*., 1994). Secondly, due to lack of skin contact, inefficient precursors were extracted to show any differences produced by the yeast that conducted the fermention. Thirdly, the *PGU1* gene might have been properly expressed under the winemaking conditions, but the amount of enzyme produced by a single copy of the gene was not high enough to have a significant effect on the aroma profile of the wine. Another group, Fernández-González *et al*. (2005), integrated the *PGU1* gene under control of the *PGK1* promoter into the genome of a wine yeast and used the stains in vinification experiments. They also did not find any significant difference in the aroma

profile of the wine compared to that produce by the wild type strain. It is possible that the expression of a single gene in the genome of the yeast is simply not high enough to alter the aroma profile of the wine, while overexpression of the gene on a multicopy plasmid will produce a high enough enzyme concentration in the wine to enhance the extraction of certain compounds as was observed by Vilanova *et al*. (2000). The experiment should be repeated in more optimal conditions for the enzyme. Additionally, a diploid recombinant with two integrated copies of the *PGU1* gene should also be used as well as a recombinant where *PGU1* is expressed on a multicopy plasmid. Together with the proper controls, this would determine the limiting factor(s) for PG activity under winemaking conditions.

Comparison of the *PGU1* gene from *S. paradoxus* to that of *S. cerevisiae* S288C on the Yeast Genome Database (SGD) revealed a few amino acid differences between the two species. Certain amino acids have been identified as potentially important for PG activity (Blanco *et al*., 1998). Only one difference observed between *S. paradoxus* and *S. cerevisiae* was found to be in one of these areas. This was residue 17, which is part of the peptide secretion signal. This amino acid could be responsible for higher PG activity in *S. paradoxus* via a more effective secretion of the enzyme. Alternatively, one or more of the other amino acid differences could result in a higher PG activity in *S. paradoxus*. A total of ten nucleotide differences were observed between the *PGU1* promoter sequences of the two species. The promoter of *S. paradoxus* (a strain which exhibits higher activity that *S. cerevisiae*) had less binding sites for the transcription factors Tec1p and Ste12p than the promoter of *S. cerevisiae*. The opposite was expected. The *PGU1* promoter of *S. paradoxus* could contain binding sites for another, unknown regulatory protein that results in subsequent higher transcription of the *PGU1* gene in this strain. *PGU1* transcription should be evaluated in the strains S288C and RO88 by means of Real-Time PCR. Simultaneously, transcription of *TEC1* and *STE12* should also be evaluated.

Alternatively, the promoter is not responsible for the higher activity exhibited by *S. paradoxus*. The *PGU1* genes from *S. paradoxus* RO88 and *S. cerevisiae* BY4742 (which has the same *PGU1* gene sequence as S288C) were cloned individually into the same plasmid and used to transform a wine yeast. The PG activity of the recombinant yeasts was measured by a semi-quantitative plate assay as well as a quantitative liquid assay. Both assays produced the same result: overexpression of the *PGU1* gene from *S. paradoxus* resulted in higher PG activity in the yeast compared to the overexpression of the *PGU1* gene from *S. cerevisiae*. This indicated that the *PGU1* gene sequence was responsible for the difference in activity between the two species as the genes were cloned in identical plasmids and expressed in the same genetic background. This also indicated that BY4742, a strain that does not exhibit PG activity, has a functional *PGU1* gene in its genome.

Certain differences in the fermentation ability of *S. cerevisiae* VIN13, *S. paradoxus* RO88, PR7 and PR10 (two interspecies hybrids between VIN13 and RO88) were identified. RO88 was found to be a poor fermenter compared to VIN13. PR7 seemed to be the strongest fermenter of the two hybrids, but RO88, PR7 and PR10 all had higher residual sugar levels than VIN13 at the end of fermentation. RO88 and the hybrids produced higher concentrations of certain aroma compounds and in some instances, the hybrids resemble either VIN13 or RO88. Unfortunately, the plasmids were highly unstable in the recombinants and were lost during the fermentation. Subsequently, no significant differences were observed between wines made by these recombinants and VIN13 (their wild type strain). A few tendencies were however observed. Construct stability should be ensured (by integration into the yeast genome for instance) and the vinification experiments repeated to determine the contribution of the *PGU1* gene to the wine aroma.

This study indicated that the *PGU1* gene is regulated differently between strains of *S. cerevisiae* with different genetic backgrounds. Additional studies should be conducted to understand the regulation of the *PGU1* gene. The experiment should be repeated for all the strains lacking the *PGU1* gene as for CC Cabernet. Transcription should be determined by Real-Time PCR and activity confirmed by a liquid polugalacturonase assay. The *PGU1* promoters of these strains should also be studied and compared to determine differences that could be responsible for transcriptional regulation in some strains. The vinification experiments should also be repeated under optimal conditions for the enzyme. Red wine should be produced to enhance pectin levels, the sugar concentration should be lower and the fermentation conducted at 25°C (the optimal temperature for the enzyme). Three different recombinants of the same strain should be compared: one containing a single, integrated copy of the *PGU1* gene under control of its native promoter, one overexpressing *PGU1* on a multicopy plasmid under control of the *PGU1* promoter and the third overexpressing *PGU1* on the same plasmid under control of the *PGK1* promoter. This would determine the inhibiting factor for high PG activity under winemaking conditions.

Several differences in the *PGU1* gene and promoter sequences between *S. paradoxus* RO88 and *S. cerevisiae* S288C were observed. These differences should be analysed by inducing single point mutations to determine which one (or more) is responsible for the difference observed in PG activity between these two species. The plasmids that were constructed containing the *PGU1* genes from the two species should be integrated into VIN13 to ensure stability. These recombinants should then be used in vinification experiments, under optimal conditions as mentioned previously, to determine the effect of PG activity on the aroma profile of the wine compared to the wild type VIN13.

These results set a platform for further studies on the *PGU1* gene, its regulation and possible applications thereof in the winemaking process to improve the overall quality of the wine.

6.2 References

Blanco P, Sieiro C, Diaz A & Villa TG (1994) Production and partial characterization of an endopolygalacturonase from *Saccharomyces cerevisiae*. *Can J Microbiol* **40:** 974-977.

Blanco P, Sieiro C, Reboredo NM & Villa TG (1998) Cloning, molecular characterization, and expression of an endo-polygalacturonase-encoding gene from *Saccharomyces cerevisiae* IM1-8b. *FEMS Microbiol Lett* **164:** 249-255.

Divol B & van Rensburg P (2007) *PGU1* natural deletion is responsible for the absence of endo-polygalacturonase activity in some wine strains of *Saccharomyces cerevisiae*. *FEMS Yeast Res* **7:** 1328-1339.

Fernández-González M, Úbeda JF, Cordero-Otero RR, Thanvanthri V & Briones AI (2005) Engineering of an oenological *Saccharomyces cerevisiae* strain with pectinolytic activity and its effect on wine. *Int J Food Microbiol* **102:** 173-183.

Gainvors A, Nedjaoum N, Gognies S, Muzart M, Nedjma M & Belarbi A (2000) Purification and characterization of acidic endo-polygalacturonase encoded by the *PGL1-1* gene form *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **183:** 131-135.

Hirose N, Fujii M, Kishida M, Kawasaki H & Sakai T (1999) Isolation and genetic determination of a *Saccharomyces cerevisiae* mutant that produces an endo-polygalacturonase. *J Biosci Bioeng* **87:** 594-597.

Madhani HD, Galitski T, Lander ES & Fink GR (1999) Effectors of a developmental mitogen-activated protein kinase cascade revealed by expression signatures of signaling mutants. *Proc Natl Acad Sci USA* **96:** 12530-12535.

Mocke BA (2005) The breeding and characterisation of starter culture yeast strains for red wine making. MSc Thesis, Stellenbosch University, Stellenbosch.

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **16:** 675-729.

Vilanova M, Blanco P, Cortés S, Castro M, Villa TG & Sieiro C (2000) Use of a *PGU1* recombinant *Saccharomyces cerevisiae* strain in oenological fermentations. *J Appl Microbiol* **89:** 876-883.