Transcriptional regulation of the endo-polygalacturonase-encoding gene in *Saccharomyces cerevisiae*

by

Campbell Trout Louw

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Promoter: Prof P van Rensburg
Co-promoter: Dr B Divol

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Declaration

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

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Summary

Wine fermentation with a yeast strain able to degrade grape cell polysaccharides can result in improved processability and an increase in wine quality by improving extraction of essential compounds from the grapes during the maceration stage. Pectin is the only important cell wall polysaccharide that can be degraded by wild-type *Saccharomyces cerevisiae* strains. Pectin is degraded by a polygalacturonase (PG) encoded by the *PGU1* gene (ORF YJR153W). Only certain *S. cerevisiae* strains can degrade pectin and PG activity is thus strain specific. The lack of activity in certain strains has been attributed to a number of factors: (1) the complete absence of the *PGU1* gene, (2) the *PGU1* gene is present but the allele is dysfunctional and (3) the *PGU1* gene is present but not transcribed. The lack in transcription has been shown to be due to the gene having a dysfunctional promoter or to regulatory differences between strains. Results published in the literature are contradictory. The primary aim of this investigation was to clarify the regulation of PG activity in *S. cerevisiae* and to determine why there are differences in PG activity between different strains. Regulation of PG activity between several wine and laboratory strains with varying PG activities was compared by looking at the sequence of the *PGU1* gene and its promoter as well as transcription levels of this gene and its main transcription factors, *TEC1* and *STE12*. In order to identify regulatory factors influencing PG activity, the *S. cerevisiae* genome was screened for activators and inhibitors of PG activity. Fourteen inhibitors and two activators of PG activity were identified during this screen. Real-time PCR analysis showed that the PG activity is regulated by transcription of the *PGU1* gene. A linear relationship was demonstrated between *PGU1* and its two transcription factors *TEC1* and *STE12*. Some of the genes identified as inhibitors of *PGU1* transcription are involved in gene silencing by Telomere Position Effect (TPE) indicating that *PGU1* is possibly silenced due to its subtelomeric location within 25 kb from the right telomere of chromosome X. Moving the *PGU1* gene with its native regulatory machinery to a different position away from its telomere resulted in an increase in *PGU1* transcription and PG activity, demonstrating the epigenetic influence on *PGU1* regulation. Results from this study suggested that the strain related difference in *PGU1* expression occurs at an epigenetic level, with steric hindrance preventing RNA polymerase access to the *PGU1* promoter and thus inhibiting transcription of this gene in some strains.

Understanding regulation of PG activity can potentially lead to the development of more effective strategies to improve PG degradation by *S. cerevisiae.* The genetic model describing regulation of *PGU1* transcription was extended by this study and a novel mechanism of regulation of PG activity was identified.

The secondary aim of this study written as an addendum to this thesis, focussed on degradation of another grape cell wall polysaccharide xylan by recombinant strains of *S. cerevisiae.* These strains were enabled to degrade this polysaccharide through heterologous expression of novel xylanase encoding genes from various origins. Xylanase activity of the recombinant strains generated was compared. Overexpressing the complete gene *xynA* of *Ruminococcus flavefaciens,* the functional domain *xynAa* or the functional domain *xynAc* within optimal conditions for these enzymes all conferred very low xylanase activity to *S. cerevisiae*, with *xynAc* resulting in the highest xylanase activity. Since overexpression of the *R. flavefaciens xynA* gene yielded very low activity under optimal conditions activity in wine making conditions would be negligible. The genes *XYN2* and *XYN4* from *Trichoderma reesei* and *Aspergillus niger* respectively yielded higher levels of activity. According to these results, only the expression of *XYN2* and *XYN4* could have a potential effect on wine

An effective strategy for improving pectin degradation can in future potentially be combined with heterologous expression of a xylanase encoding gene in *S. cerevisiae* in order to engineer a wine yeast strain with improved polysaccharase abilities.

Opsomming

Gisting van druiwe met polisakkaried-afbrekende gisrasse kan lei tot ‘n verbetering in wyn prosessering en tot die produksie van hoër kwaliteit wyne deur die ekstraksie van belangrike wynkomponente uit druifselle te verbeter. Pektien is die hoof komponent van die druifselwand wat deur wilde tipe *Saccharomyces cerevisiae* giste afgebreek kan word en word afgebreek deur ‘n poligalaktoronase (PG) wat deur die *PGU1* (YJR153W) geen gekodeer word. Slegs spesifieke gisrasse kan pektien afbreek en die ensiem aktiwiteit is dus ras-spesifiek. Die gebrek aan PG aktiwiteit in sekere rasse is al omskryf as gevolg van die afwesigheid van die geen, die teenwoordigheid van ‘n nie-funksionele alleel of dat die geen wat teenwoordig is nie uitgedruk word nie. Transkripsie is al bewys om nie plaas te vind nie a.g.v. die teenwoordigheid van ‘n nie-funksionele promotor of a.g.v. ‘n verskil in regulering van transkripsie tussen rasse. Sommige studies wat PG regulering ondersoek het, het teenstrydige resultate verkry. Die hoofdoel van hierdie studie was om PG regulering te ondersoek en te bepaal waarom daar verskille in PG aktiwiteit tussen verskillende gisrasse voorkom. Regulering van PG aktiwiteit is ondersoek tussen wyn en laboratorium gisrasse met wisselende vlakke van PG aktiwiteit deur die DNS volgorde van die *PGU1* geen en sy promotor, so wel as die DNS volgorde van die geen se hoof transkripsie faktore *TEC1* en *STE12* te bepaal. Om reguleerders van PG aktiwiteit te identifiseer is die genoom van die gis *S. cerevisiae* ondersoek om faktore te identifiseer wat PG aktiwiteit aktiveer of inhibeer. “Real-time PCR” het bewys dat PG aktiwiteit gereguleer word deur transkripsie van die *PGU1* geen en dat daar ‘n lineêre verhouding tussen die transkripsie van die *PGU1* geen en sy twee hoof transkripsie faktore *TEC1* en *STE12* bestaan. Sommige van die gene wat geïdentifiseer is as inhibeerders van PG aktiwiteit is voorheen bewys om betrokke te wees by die inhibering van transkripsie deur middel van die telomeer posisie effek, dit dui daarop dat transkripsie van die *PGU1* geen moontlik geïnhibeer word as gevolg van die geen se subtelomeriese posisie binne 25 kb vanaf die regter telomeer van chromosoom X. Die *PGU1* geen is met sy natuurlike regulerings elemente na ‘n ander posisie in die genoom, weg van sy naaste telomeer geskuif, die verandering in posisie van die geen het gelei tot ‘n toename in PG aktiwiteit en transkripsie van die *PGU1* geen en het dus bewys regulering word beïnvloed deur ‘n epigenetiese effek. Die resultate van hierdie studie het daarop gedui dat die verskil in transkripsie van die *PGU1* geen plaasvind op ‘n epigenetiese vlak waartydens die chromatien struktuur

toegang van die RNA polimerase tot die *PGU1* geen voorkom en dus word transkripsie van die geen sodoende in sommige rasse voorkom.

Die tweede doelwit van hierdie studie het gefokus op die afbraak van ‘n ander komponent van die druif selwand, xilaan, deur *S. cerevisiae.* Hierdie navorsing vorm ‘n addendum aan die tesis en Xylanase aktiwiteit van verskeie rekombinante rasse is in hierdie studie vergelyk. Baie lae xylanase aktiwiteit is verleen aan rekombinante giste wat die volledige *xynA* geen gekloneer van die bakteriee *Ruminococcus flavefaciens,* asook twee aktiewe domeins van die geen, domein *xynAa* en domein *xynAc* uitdruk. Van die voorafgenoemde giste het die uitdrukking van die domein *xynAc* die rekombinante gis ras met die hoogste aktiwiteit tot gevolg gehad. Ooruitdrukking van die gene *XYN2* en *XYN4* wat gekloneer is van die fungi *Trichoderma reesei* en *Aspergillus niger* onderskeidelik, het beide gisrasse wat oor hoë vlakke van xylanase aktiwiteit beskik tot gevolg gehad. Hierdie resultate dui dus daarop dat van die gene ondersoek in die studie, slegs *XYN2* en *XYN4* potensiaal het om xylanase aktiwiteit van wyngiste te verbeter.

Deur die regulering van PG aktiwiteit te bestudeer kan meer effektiewe strategieë potensieel ontwikkel word om PG aktiwiteit in *S. cerevisiae* te verbeter. Hierdie studie het die genetiese model wat PG regulering omskryf uitgebrei deur ‘n nuwe meganisme van regulering van toepassing op *PGU1* te identifiseer.

As ons die regulering van die *PGU1* goed verstaan kan dit in die toekoms gekombineer word met ‘n effektiewe strategie om ‘n gis aan te pas om xylaan af te breek, om sodoende ‘n wyngis geneties te verbeter om beide xylaan en pektien te kan afbreek.

This dissertation is dedicated to my mother Maryna

Biographical sketch

Campbell Louw was born on September 20, 1977 in Cape Town, South Africa. He attended the Primary School Swartland in Malmesbury and matriculated at High School Swartland in 1995. In 1997 he enrolled for a BSc-degree at the University of Port Elizabeth and obtained the degree in 1999, majoring in Microbiology and Biochemistry. In 2000 he obtained an HonsBSc-degree in Microbiology from the University of Port Elizabeth. In 2004 he obtained an MSc-degree in Wine Biotechnology at the University of Stellenbosch. In 2005 he enrolled for a PhD-degree in Wine Biotechnology at the University of Stellenbosch.

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Preface

This dissertation is presented as a compilation of seven chapters. Each chapter is introduced separately and is written according to the style of the journal FEMS Yeast research in which chapter 3 was published, chapter four was submitted for publication in the same journal.

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CHAPTER 6. GENERAL DISCUSSION AND CONCLUSION

ADDENDUM: COMPARING XYLANASE ACTIVITY OF RECOMBINANT *SACCHAROMYCES CEREVISIAE* STRAINS WITH XYLANASE ACTIVITY THROUGH HETEROLOGOUS EXPRESSION OF DIFFERENT XYLANASE ENCODING GENES OF VARIOUS ORIGINS (RESEARCH CHAPTER)

Chapter 1

Introduction and project aims

1.1 Introduction

Throughout the winemaking process various biochemical reactions are catalysed by enzymes originating from the grapes and associated microorganisms. During alcoholic fermentation, yeasts do not only convert sugars to ethanol and carbon dioxide; they also produce a range of minor but oenological relevant volatile metabolites that contribute to the aroma profile of the wine. Many grape-derived compounds are released or modified by the action of flavour-active yeast and bacteria. Winemakers could thus adjust the flavours and aromas of their wines through appropriate yeast strain selection, allowing them to tailor their preferred style of wine.

The cell walls of grape berries, especially from the epidermal cells of the skin, form a barrier to the diffusion of components such as aroma compounds and polyphenols which are important for the quality of wines (Barnavon *et al.*, 2001). The polysaccharides pectin, hemicellulose and cellulose, constitute the largest portion of cell walls in fruit cells (Kilcast, 2004). Enzyme preparations consisting of pectinases, cellulases and hemicellulases are known as macerating enzymes in the beverage industry. These preparations are used to improve wine quality by increasing the juice yield and improve wine processing by improving clarification and reducing filtration time during winemaking by degrading polysaccharides that interfere with juice extraction (Haight & Gump, 1994). Breaking open the grape berry cells by degrading the structural polysaccharides in the grape cell walls can also result in an improvement in wine quality through the release of intracellular colour and aroma compounds, improving colour intensity, stability and aroma profile (Louw *et al.,* 2006). The addition of commercial enzyme preparations is expensive and may contain impurities or side activities which can adversely affect the quality of the wine (Van Rensburg *et al*., 2007).

A wine yeast strain with polysaccharide degrading ability would thus result in an improvement in wine quality and processing without the addition of these commercial preparations. *Saccharomyces cerevisiae* strains that can degrade multiple polysaccharides through heterologous expression of a variety of genes have been created and fermentation with these strains indeed resulted in an improvement in wine quality and processing (Louw *et al.,* 2006, Van Rensburg *et al.*, 2007). Pectin is the only important cell wall polysacchararide that can be degraded by specific wild type *S. cerevisiae* strains. Polygalacturonase (PG) activity is the main pectolytic activity in *S. cerevisiae* and has been reported in several strains (Blanco *et al.*, 1994). A PG-encoding gene, *PGU1* (ORF YJR153W), was cloned and sequenced from *S. cerevisiae* (Blanco *et al.*, 1994, Hirose, 1998). Recombinant *S. cerevisiae* strains with pectolytic

abilities have been constructed through heterologous expression of pectinase encoding genes, cloned from pectin degrading organisms (Van Rensburg *et al.,* 2007). PG activity has also been increased by overexpressing the native *PGU1* gene in *S. cerevisiae* (Blanco *et al.*, 1998; Hirose *et al.*, 1998; Jia & Wheals, 2000) and by chemical mutation of strains (Radoi *et al.*, 2005a).

Some of these recombinant and mutant strains with improved PG activity are wine yeast strains and were created with the aims of improving wine quality and processing (Vilanova *et al.,* 2000; Radoi *et al.*, 2005a). Overexpressing the native *PGU1* gene under control of a native *S. cerevisiae* promoter is a self cloning strategy as no "foreign" genes are introduced into the recombinant strain. Self-cloning indeed describes genetic modification by gene transfer within the same species (Heller, 2006). In Japan and in Europe more flexible legislation is applicable to organisms developed by self-cloning strategies compared to other genetically modified organisms (Querol & Fleet, 2006). Since these countries are very important wine markets, strains complying with this legislation should be constructed. Fermentation with these strains had positive effects on wine quality and processing, juice extraction (Fernández-González *et al.*, 2004, Radoi *et al.*, 2005a), colour extraction, filtration (Radoi *et al.*, 2005a, Vilanova *et al.*, 2000) and alterations in the chemical profiles (Van Wyk & Divol*,* 2010) of wines fermented with these strains compared to wines fermented with the wild type strains.

Only certain strains of *S. cerevisiae* possess natural PG activity. The inability of certain strains to degrade pectin can in some cases be attributed to the PG-encoding *PGU1* gene being absent from these strains (Fernández-González *et al.*, 2004, Divol & Van Rensburg, 2007). Nevertheless, some of the strains in which the *PGU1* gene is present are also devoid of pectolytic activity; this lack in activity is attributed to mutations in the *PGU1* gene (Gognies *et al.*, 1999) and transcription of *PGU1* (Jia & Wheals, 2000). Inhibition of transcription has been shown to be due to differences in the promoter region of *PGU1* or due to differences in the genetic background of the strain. Multiple studies showed that overexpressing the *PGU1* gene in strains without PG activity enabled these strains to degrade pectin (Blanco *et al.*, 1998; Hirose *et al.*, 1998; Jia & Wheals, 2000).

PG activity has also been shown to be regulated by various environmental factors in *S. cerevisiae.* The promoter for the *PGU1* gene contains three binding sites for the *TEC1* transcription factor. *TEC1* transcription is activated by the Kss1p Mitogen activated protein kinase (MAPK) (Madhani *et al.*, 1999). This regulatory pathway also regulates transcription of genes responsible for filamentous growth. This co-regulation

of genes responsible for filamentous growth and polysaccharide degradation during starvation conditions correlates with the proposal that filamentous growth is coordinated foraging behaviour in the wild (Gimeno *et al.*, 1992; Madhani *et al.*, 1999).

Pectolytic activity has also been found to be regulated by carbon source (Blanco *et al*., 1997a). Radoi *et al.* (2005b) found pectolytic activity to be induced by galactose in certain wine yeast strains. A Gal4p binding site was identified 600 and 632 bp upstream of the *PGU1* coding region by Radoi *et al.* (2005b). Dissolved oxygen present in the medium has also been shown to influence PG activity. The effect of the amount of dissolved oxygen on PG activity varies from strain to strain in *S. cerevisiae*. Certain strains have PG activity when grown without aeration, while other strains lose PG activity with high aeration (Blanco *et al.*, 1999). The *PGU1* gene is subtelomeric since it is located within 25 kb of the right telomere of chromosome X, indicating this gene could possibly be regulated by epigenetic mechanisms. Epigenetics is the study of heritable changes in gene expression that are not mediated at the DNA sequence level. Molecular mechanisms that mediate epigenetic regulation include DNA methylation and chromatin/histone modifications (Cheung & Lau, 2005). In *S. cerevisiae* genes placed near telomeres are transcriptionally repressed. This phenomenon is referred to as Telomere Position Effect (TPE) (Mondoux & Zakian, 2007). Gene silencing by TPE in yeast has mainly been demonstrated by inserting reporter genes in close proximity of a telomere (Gottschling *et al.*, 1990). It has been shown that the *FLO* genes, responsible for filamentous growth, are under genetic and epigenetic control (Halme *et al.*, 2004). Four of the five *FLO* genes regulating cell surface variation are located in subtelomeric positions, 10 to 40 kb from telomeres and have been shown to be under epigenetic control (Halme *et al.,* 2004). Both pectin degradation and filamentous growth phenotypes have been implicated in *S. cerevisiae* functioning as a plant pathogen (Gognies & Belarbi, 2002; Juana, 2001). Based on the position of the *PGU1* it is possible that these two phenotypes are not only co-regulated by the same regulatory pathway, but also co-regulated on an epigenetic level. Figure 1.1 illustrates regulation of *PGU1* transcription according to literature. Transcription has been shown to be regulated by the mating MAPK and the filamentous and invasive growth MAPK.

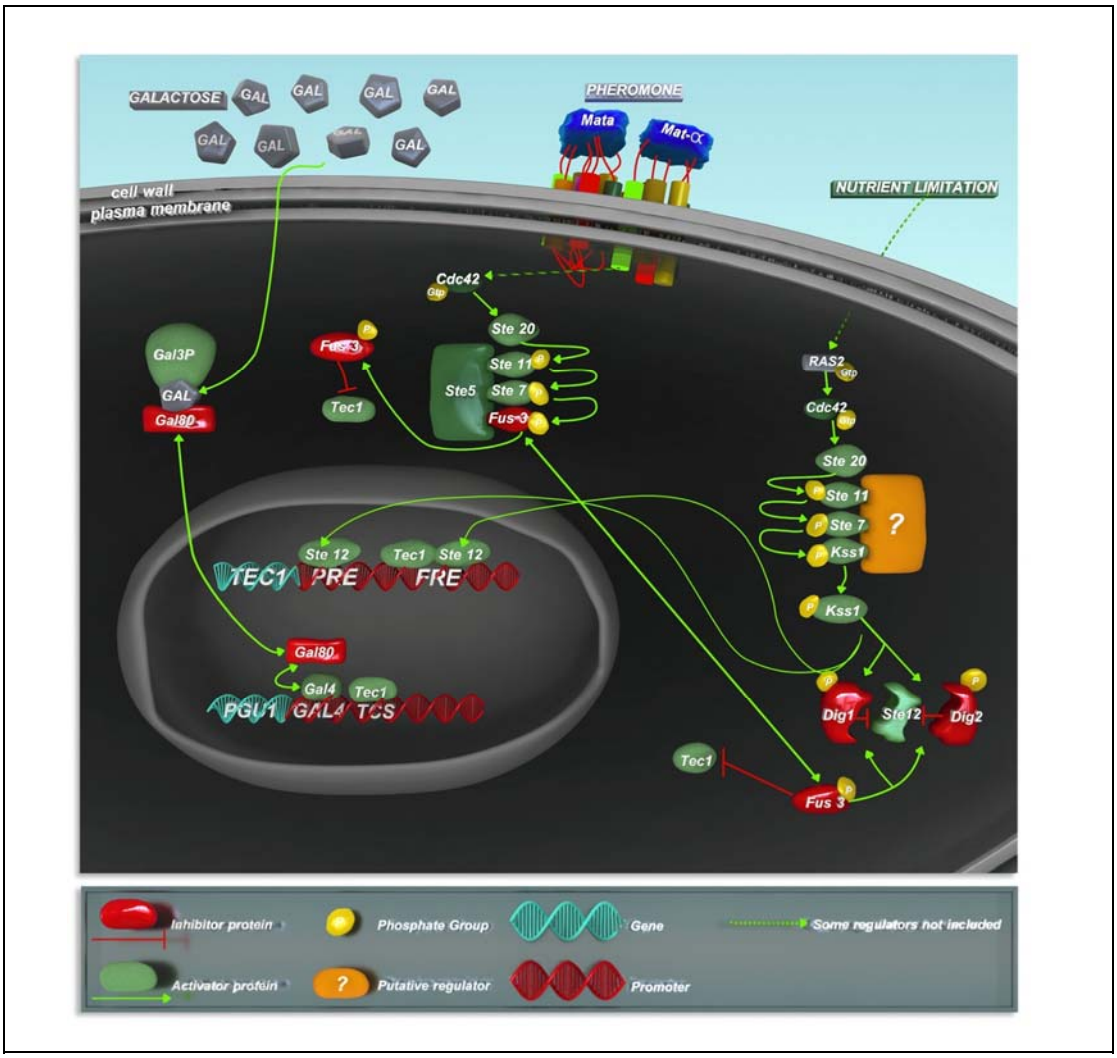

Figure 1.1 Regulatory pathways influencing transcription of the *PGU1* gene in *S. cerevisiae* drawn from literature up to date. Activation and signal transduction by phosphorylation of various kinases through the mating and invasive growth MAPK pathways is demonstrated as described by Madhani (2006). Activation of genes containing a Gal4 binding domain is demonstrated according to the description from Peng & Hopper (2002).

Understanding the regulation of *PGU1* transcription is important. This knowledge can be used to develop more effective strategies to improve PG degradation by *S. cerevisiae.* Since *PGU1* has been implicated in *S. cerevisiae* acting as phytopathogen towards *Vitis vinifera,* studying this gene can also have significant ecological importance, shedding light on how certain yeast strains survive in the vineyard.

Wines fermented with recombinant wine yeast strains that possess different combinations of glucanase, pectinase and xylanase activity resulted in improvements in wine quality and processing compared to wines fermented with the wild type strains (Louw *et al.,* 2006; Van Rensburg *et al.,* 2007). Since *S. cerevisiae* does not possess any xylanase encoding genes, the only way to construct xylan degrading strains, is through heterologous expression of xylanase encoding genes, cloned from organisms that can degrade xylan. In an addendum to this thesis xylan degradation by several

recombinant *S. cerevisiae* strains are investigated. In previous studies xylan degrading wine yeasts were created through heterologous expression of xylanase encoding genes, these genes include *XYN A, B* and *C* cloned from *A. niger* and *XYN2* cloned from *T. reesei* (Ganga *et al.*, 1999; la Grange *et al.*, 1996; Luttig *et al.*, 1997). Fermentation with recombinant strains overexpressing *XYN2*, and *XYNC* cloned both made a significant impact on wine quality by influencing colour intensity, aroma profile and wine processing by improving the percentage of free flow wine released (Louw *et al.*, 2006).

Very few xylanase encoding genes with bacterial origin have been expressed in yeast. Rumen bacteria play an extensive role in the degradation of plant cell wall polysaccharides in the rumen of herbivorous mammals, making these bacteria a good source to mine for novel polysaccharase encoding genes. Investigating xylanase activity of novel xylanase encoding genes expressed in *S. cerevisiae* can potentially result in production of recombinant wine yeast strains with improved xylanolytic abilities.

Ruminococcus flavefaciens is one of three species of anaerobic, cellulolytic bacteria that play a major role in the degradation plant cell wall polysaccharides in the rumen. This bacterium has been found to produce at least six different xylanases, XynA is one of these xylanases. It is a bifunctional enzyme with two different catalytic domains capable of degrading xylan (Flint *et al.*, 1994). In this addendum chapter we made an attempt to construct yeast strains more efficient in xylan degradation. Different truncations of the *XynA* gene in *S. cerevisiae* and comparedSince this enzyme has two catalytic domains that differ in substrate specificity, degradation products released and mode of action, this gene is a good target to overexpress in *S. cerevisiae* in order to.

1.2 Project aims

The main objective of this study was to investigate the regulation of PG activity in *S. cerevisiae* by using genetic and phenotype analysis to identify regulatory mechanisms that control *PGU1* transcription and PG activity. The secondary objective was to construct a xylanase secreting *S. cerevisiae* strain through heterologous expression of the *XynA* gene previously cloned from the rumen bacteria *R. flavefaciens,* in order to identify novel xylanase encoding genes to improve xylanase activity of wine yeast strains through heterologous expression.

More specifically, the aims included:

(i) investigating the relationship between PG activity and the transcription level of *PGU1* and its main transcription factors *TEC1* and *STE12* in the various *S. cerevisiae* strains

(ii) screening the *S. cerevisiae* EUROSCARF deletion library for activators and inhibitors of PG activity.

(iii) determining if *PGU1* is silenced in strains lacking PG activity due to its subtelomeric position.

(iv) constructing-xylanase degrading strains by overexpressing truncations of the *R. flavefaciens XynA* gene.

(v) comparing xylanase activity of strains overexpressing various truncations of *XynA* with strains overexpressing xylanase genes from fungal origin.

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Chapter 2

Literature review

Pectolytic activity in *Saccharomyces cerevisiae*

2.1 Introduction

Polysaccharides in wine originate from the grape berries and microorganisms that occur in the must. Degradation of grape polysaccharides plays an important role in wine processing and wine quality. The most widely available enzymes used in the wine making process are pectinases, hemicellulases, glucanases and glycosidases. Pectolytic enzymes have been used in grape processing to improve juice yield and aid in pressing and clarification since the 1950's (Cruess *et al.*, 1955) and is now commonly used to improve wine quality and flavour. Acidic pectinases used in the fruit juice and wine industry are mainly produced by *Aspergillus* species (Aguilar, 1987). Yeast is an alternative source to filamentous fungi for the production of commercial enzymes. *S. cerevisiae* secretes a polygalacturonase (PG) encoded by the *PGU1* gene, present in a single copy per haploid genome on chromosome X (Blanco *et al.*, 1998). PG activity in *S. cerevisiae* is highly strain dependent and the absence in activity in certain strains has been attributed to either the absence of the *PGU1* gene, mutations in the structural gene or a lack in transcription of *PGU1.*

This review gives a brief overview of different polysaccharides involved in the wine making process. Since this study mainly focused on pectin, degradation of this polysaccharide constituted the bulk of the literature review. The role of xylan degradation is briefly metioned and investigating degradation of this polysaccharide by *S. cerevisiae* is investigated in a addendum to the thesis. The role and regulation of the *PGU1* gene in *S. cerevisiae* is reviewed. Differences in PG activity between strains is discussed, focusing on the *PGU1* structural gene, its promoter sequence and the regulatory pathways and environmental factors influencing *PGU1* transcription. A brief overview is also given of recombinant *S. cerevisiae* strains with improved PG activity constructed for integrated bioprocessing during wine making.

2.2 Polysaccharides in wine

Polysaccharides are found in wines at levels ranging from 0.3 to 1 g l^{-1} and display great structural diversity (Pretorius, 2000). These polysaccharides originate from the cell walls of grape berries and from microorgansims associated with the winemaking process. Polysaccharides are released from the grape berry by various vinification treatments or from yeast cell walls during autolysis following alcoholic fermentation (Boulet *et al.*, 2007). Polysaccharides originating from microorganisms can also play an important role in wine spoilage, two of the main spoilage organisms secreting polysaccharides into wine being the fungus *Botrytis cinerea* and the bacterium *Pediococcus damnosus. P.*

damnosus is a spoilage bacterium that cause ropy wines by secreting β-D-glucan, a glucose homopolymer consisting out of trisaccharide repeating units. These polysaccharides cause wines to have an abnormal viscosity that is very detrimental to wine quality (Canal-Llaubères, 1993). *Botrytis cinerea* is a necrotrophic fungus and during *Botrytis* infection a high molecular weight glucan consisting of a β-D-1,3-linked backbone with very short β-D-1,6-linked side chains is secreted into wine, resulting in problems with the filtration and fining of wine (Villettaz *et al.*, 1984).

The grape berry is the main source of wine polysaccharides and the tissue can be divided into the skin (exocarp) and the pulp (endocarp). The skin consists of the epidermis, hypodermis and cuticle. Growing cells that make up the grape berry tissues are surrounded by and thus isolated from each other by a polysaccharide rich primary cell wall. The cell walls are mainly responsible for the integrity and texture of tissues and therefore play an important role in fruit processing (Barnavon *et al.*, 2001). The primary cell wall is also responsible for determining and maintaining cell shape, to resist internal turgor pressure, to control rate and direction of growth, carbohydrate storage, protection against pathogens and dehydration, it is a source of biologically active signaling molecules and important in cell-cell interactions. The concentration of grape polysaccharides in wine depends on the vinification method used and on the stage of berry maturity and treatments of harvested grapes (Boulet *et al.*, 2007).

The main grape polysaccharides degraded in order to improve wine quality are present in the skin and flesh cell walls of the berry and are degraded through enzyme degradation during maceration (Louw *et al.,* 2006). In grape cells the primary cell wall is mostly comprised of cellulose fibers called micro fibrils, in a matrix of pectin, hemi cellulose and proteins (Figure 2.1a). The cellulose fibrils are connected to pectin by xyloglucan, and the pectin is connected by arabinogalactan to serine residues in the cell wall. The secondary cell wall is much thicker then the primary cell wall and is largely made up of cellulose micro fibrils (40-80%) organized in parallel bundles, the secondary cell wall also contain hemicellulose (20-40%), pectin and some lignin (5-10%) (Pinelo *et al.*, 2006). The middle lamella binds neighbouring cells and consists mainly of pectin in the form of calcium pectate and magnesium pectate (Jayani, *et al.,* 2005). Hydrolysis of pectin results in the dissolving of the middle lamella leaving the individual cells floating free (Gilbert & Cruiess 1929).

Degradation of polysaccharides results in an increase in free flow juice by breaking down the cell walls that act as a barrier between the juice containing vacuole and the outside of the cell (Lourens & Pellerin, 2004). Wine quality is improved by

polysaccharide degradation, mainly due to an increase in release of cell wall bound and intracellular components from the grape cell. More intense colour is due to an increase in anthocyanins released and th aroma profile of the wine is extended due to an increase in release of monoterpenes into the wine. Colour stability is improved by more tannins being released, these tannins can polymerize with anthocyanins, leading to increased colour stability (Somers & Wescome, 1982). Treating the must with pectinase has also been shown to have health benefits. Reduction of the particle size of grape pomace upon pectinase treatment can lead to an increase in recovery of phenols. Previous studies have shown that wine phenols significantly retard human low-density lipoprotein oxidation in vitro, functioning as an antioxidant that may reduce atherogenesis and prevent coronary heart disease (Meyer *et al.*, 1998).

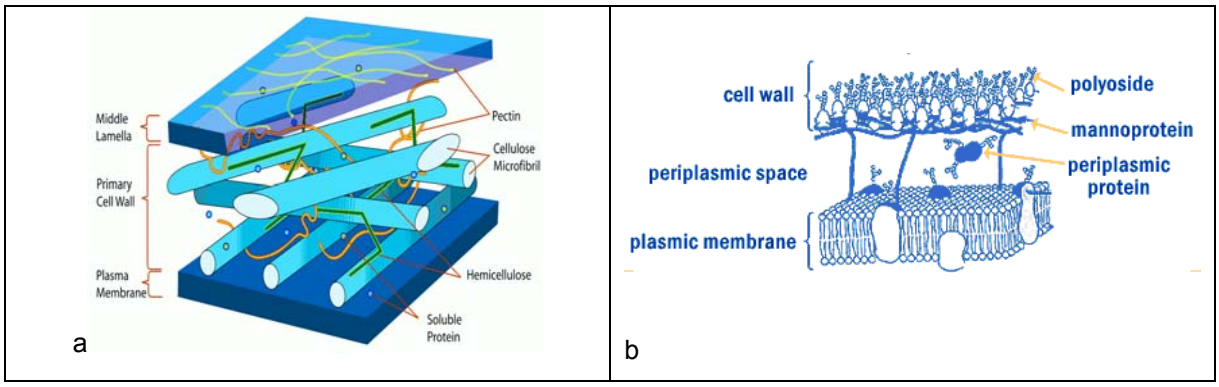

Figure 2.1 The main polysaccharides that play a role in wine production and wine quality are: (a) pectin. cellulose and hemicellulose from the grape berry cell walls (www.wpclipart.com/plants/diagrams/Plant_cell_wall_diagram.png) and (b) mannoproteins that originate from the yeast cell walls (www.eurasyp.org/public.levure.ecorce.screen)

Beta-glucanes and mannoproteins make up the polysaccharide group that originates from yeast. Mannoproteins from yeast cell walls form another large family of polysaccharides (Figure2.1b) and occur at about 150 mg/l in wines (Ribéreau-Gayon, *et al.*, 2000). Yeast polysaccharides are mainly released into dry white wines when aged on the lees. Polysaccharide complexes are mainly released into red wine after fermentation, during high temperature maceration. This only takes place for a short period, because most of the yeast lees are separated from the wine during racking. Two types of exocellular proteins are released by the yeast during fermentation and ageing on the lees, mannoproteins and glucomannoproteins. Mannoproteins form 80% of the exocellular polysaccharides and consists of 90% mannose and 10% proteins. Glucomannoproteins make up the other 20% of exocellular polysaccharides and contain 25% glucose, 25% mannose and 50% protein (Ribéreau-Gayon, *et al.,* 2000). Yeast cell wall mannoproteins play a very important role in the overall vinification process,

because of its important role in inhibition of tartrate salt crystallization (Jackson, 2000), reduction of protein haze (Dupin *et al.*, 2000), stimulation of malolactic fermentation (Guilloux-Benatier *et al.*, 1995), interaction with flor wines (Dos Santos *et al.*, 2000) and autolysis in sparkling wines (Klis *et al.*, 2002).

Mannoproteins also have various positive effects on the sensorial properties of red wines, including colour stabilization, reduction of astringency (Escot *et al.*, 2001), increased body and mouthfeel (Vidal *et al.*, 2004) and interaction with aromatic compounds (Chalier *et al.*, 2007).

2.3 Pectin structure

Pectin is a structural heteropolysaccharide that occurs mainly in the middle lamellae and primary cell walls of higher plants (de Vries & Visser, 2001). Pectin is a generic name for a mixture of pectic substances with different compositions, but with pectinic acid as the major component (Kashyap *et al.*, 2001). The molecules are made up of alternating smooth and hairy regions. In the smooth regions the backbone of homogalacturonan is made up of D-galacturonic acid residues linked by α-1,4-glycosydic bonds, the galacturonic acid residues can be acetylated at O-2 or O-3 or methylated at O-6. The hairy regions consist of Rhamnogalacturonan or xylogalacturononan. There are two kinds of rhamnogalacturonan, I and II. In Rhamongalacturonan I about every twenty fifth galacturonic acid residue in the homogalacturonan backbone is replaced by (1,2)-linked L—rhamnose. From rhamnose, sidechains of various neutral sugars branch of to form hairy regions. Different ramifications are found, composed of L—rhamnose, arabinose, galactose or xylose. Some regions consist of alternating smooth galacturonic acid and hairy rhamnose regions with a lower density of rhamnose smooth regions. Rhamnogalacturonan II is another kind of pectin that is more highly branched. The American Chemical Society classified pectic substances into four main types: protopectin, pectic acid, pectinic acid and pectin (polymethyl galacturonate) (Jayani *et al.*, 2005). Protopectin is the water insoluble pectic substance present in intact tissue that yields pectin or pectic acids upon hydrolysis. Pectic acid is a soluble polymer made up of galacturonans that contain insignificant amounts of methoxyl groups, normal or acid salts of pectic acid is known as pectates. Pectinic acids is the polygalacturonan chains that contain less then 75% methylated galacturonate, the normal or acid salts of pectinic acids is known as pectinates. Pectin (polymethyl galacturonate) is the polymeric material in which 75% or more of the carboxyl groups of the galacturonate units are esterified with methanol,

pectin confers rigidity on the cell wall when it is bound to cellulose in the cell wall (Jayani *et al.*, 2005). Grape pectins are 70-80% esterified with methanol (Ribéreau-Gayon, *et al.,* 2000). Figure 2.2 shows the chemical structure of different pectic substances.

2.4 Enzymatic pectin degradation

The enzymes that hydrolyze pectic substances are known as pectic enzymes, pectinases, or pectinolytic enzymes. The pectinolytic enzymes can be devided into three groups: protopectinases, esterases and depolymerases. The enzymes in these groups can then be subdivided according to action mechanism, pattern of action, primary substrate and products released (Table. 2.1).

Table 2.1 Classification of pectinolytic enzymes (Jayani *et al.*, 2005)

Enzyme	E.C. no.	Action mechanism	Action pattern	Primary substrate	Product
Esterase					
Pectin methyl esterase	3.1.1.11	Hydrolysis	Random	Pectin	Pectic acid+methanol
Depolymering enzymes					
a. Hydrolases					
1. Protopectinases		Hydrolysis	Random	Protopectin	Pectin
2.Endopolygalacturonase	3.2.1.15	Hydrolysis	Random	Pectic acid	Oligogalacturonates
3. Exopolygalacturonase	3.2.1.67	Hydrolysis	Terminal	Pectic acid	Monogalacturonates
4.Exogalacturonan-di galacturonohydrolase	3.2.1.82	Hydrolysis	Penultimate bonds	Pectic acid	Digalacturonates
5. Oligogalacturonate hydrolase		Hydrolysis	Terminal	Trigalacturonate	Monogalacturonates
6. 4:5∆ Unsaturated oligogalacturonate hydrolases		Hydrolysis	Terminal	∆4:5 $(Galacturonate)_n$	Unsaturated monogalacturonates & saturated (*n*-1)
7. Endopoly methyl-galacturonases		Hydrolysis	Random	Highly estirified pectin	Oligomethyl galacturonates
8. Endopoly methyl-galacturonases		Hydrolysis	Terminal	Highly estirified pectin	Oligogalacturonates
b. Lyases					
1.Endopolygalacturonase lyase	4.2.2.2	Trans-elimination	Random	Pectic acid	Unsaturated oligogalacturonates
2.Exopolygalacturonase lyase	4.2.2.9	Trans-elimination	Penultimate bonds	Pectic acid	Unsaturated digalacturonates
3.Oligo-D-galactusiduronate lyase	4.2.2.6	Trans-elimination	Terminal	Unsaturated digalacturonates	Unsaturated monogalacturonates
4. Endopolymethyl -D-galactusiduronate lyase	4.2.2.10	Trans-elimination	Random	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methyloligogalacturonates
5.Exopolymethyl-D-galactusiduronate lyase		Trans-elimination	Terminal	Unsaturated poly-(methyl-D-digalacturonates)	Unsaturated methylmonogalacturonates

Protopectinases degrade the insoluble protopectin with highly polymerized soluble pectin as end product (Jayani *et al.*, 2005). Pectin esterases, such as pecin methyl esterase (PME) catalyze the de-esterification of pectin by the removal of methoxy groups from high methoxy pectin to give low methoxy pectin and methanol. Depolymerases catalyze the hydrolytic cleavage of the α-(1 → 4)-glycosidic bonds in

the D-galacturonic acid moieties of the pectic substances (Jayani *et al.*, 2005). Depolymerases can cleave pectin by one of two different mechanisms, hydrolysis or trans-elimination reactions. During hydrolysis depolymerases catalyze the cleavage reaction by introducing water across the oxygen bridge. In trans-elimination lysis, the glycosidic bond is broken by a trans-elimination reaction without any participation of water molecule. Polygalacturonase (PG) and polymethylgalacturonase (PMG) break down pectate and pectin, respectively by the mechanism of hydrolysis. Pectin lyases (PL), polygalacturonate lyase (PGL), and polymethylgalacturonate lyase (PMGL) breakdown pectate and pectin by β elimination respectively. Polygalacturonase is the most extensively studied among pectinolytic enzymes. It has been shown that fermenting grape must with *S. cerevisiae* with PG activity greatly facilitates clarification and can reduce filtration time with 50% during winemaking (Fernandez-Gonzalez *et al.*, 2004). It can also assist in the extraction of colour during red wine fermentation and have a positive effect during ageing of wine. PG activity is desired since it confers these advantages, without liberating toxic methanol (as is done by PME) or lead to colour loss (which can result from β-glycosidase being present), both these unwanted enzyme activities is often present in commercial pectinase preparations (Radoi *et al.*, 2005a). Depending upon the pattern of action, *i.e*. random or terminal, these enzymes are termed as Endo or Exo enzymes, respectively.

2.5 Pectin degrading organisms

Pectin degrading enzymes are naturally produced by a wide variety of organisms including plants, filamentous fungi, bacteria, insects, nematodes, protozoa and some yeast. Pectinases occur naturally in all fruit, including grapes and are importantly in the ripening process. Grape pectinases are however inactive under the pH and SO_2 conditions associated with winemaking, while Fungal pectinases are resistant to these winemaking conditions (Lourens & Pellerin, 2004). Pectinase production has been thoroughly studied in bacteria and fungi because they play an important role in plant pathology (da Silva *et al.*, 2005). Pectic enzymes are produced by a wide variety of bacteria, with the most important being species of *Bacillus, Clostridium, Erwinia, Pseudomonas* and *Xanthomonas* (Rombouts, 1980). Most well known pectic enzymes are produced by the soft rot-causing *Erwinia* species *E. carotovora, E. chrysanthemi* and *E. arideaia,* these bacteria produce large amounts of endo-pectate lyase and endo-polygalacturonase. Filamentous fungi have been used for more than 50 years for the production of commercial enzymes and usually produce a variety of enzymes

simultaneously. Acidic pectinases used in the fruit juice and wine industry are produced by the *Aspergillus* species, mainly Aspergillus *niger,* but also by *A. oryzyzae* and *A. wentii* (Aguilar, 1987). *A niger* produces various pectinases, including PME, PG and PL. Commercial pectinases produced by *A.niger* are often low in PG and high in PL and PME activity. Due to high PME content treatment with a pectinase preparation from *A. niger* can result in production of toxic methanol.

In early studies done by (Gilbert & Cruess, 1929) they found that yeast cultures tested, including *S. cerevisiae,* were unable to degrade pectin. In a later study however (Luh & Phaff, 1954) found 6 cultures of yeast (*Saccharomyces fragilis, Saccharomyces thermantitnum, Torulopsis kefyr, Candida pseudotropicalis,* and *Candida pseudolis* var. *lactosa)* able to degrade pectin by clarifying an opaque pectin solution. Figure. 2.2 demonstrates the different kinds of pectinases and the yeast species that secrete these enzymes. The enzyme responsible for pectin degradation was classified as a yeast polygalacturonase. This enzyme was found to differ from polygalacturonase from mold origin in that the pectic substrate was only partially hydrolyzed (Demain & Phaff, 1954). Pectolytic enzymes from yeasts are mainly endo-PG, that hydrolyze the α-1,4 glycosidic linkages in a random way, the end-product is always oligosaccharides with a varying number of galacturonic acid residues and no free galacturonic acid residues, which is the endproduct of exo-PG. Pectolytic enzymes with different functions have been reported in a few cases (Blanco *et al.*, 1999). This was confirmed in a study done by Evânia Geralda *et al.* (2005), three hundred yeasts isolated from tropical fruits were screened for secretion of pectinases. Twenty-one of the 300 isolates produced polygalacturonase and among them seven isolates could secrete pectin lyase. None of the isolates was able to secrete pectin methylesterase. The enzyme secreted was an endo-polygalacturonase in all but one strain, the polygalacturonase secreted by *Stephanoascus smithiae* was suggested to function by an exo-splitting mechanism (Evânia Geralda *et al.*, 2005). A *S. Bayanus* strain, SCPP, showing PG, PE and PL activity has been reported. Production of pectin methyl esterase activity has been detected in *Rhodoturula* sp. associated with the softening of olives (Vaughn *et al.*, 1969). Yeast pectinases are mainly exocellular glycoproteins with varying molecular mass, isozymes have been shown in *S. fragilis* (Lim, 1980). Yeast pectic enzymes have an acidic optimal pH ranging between 3.5 and 5.5 and an optimal temperature between 25 and 55°C depending on species (Blanco *et al.*, 1999). These enzymes preferentially degrade pectate (pectic acid salts) to pectin, and the activity of the enzyme increase with a decrease in methylation. The production of pectinase in yeast has been found to

be constitutive and that it is repressed by glucose and aeration. Two different functions has been attributed to pectinolytic activity in yeast, organisms that can use galacturonic acid as carbon source will secrete pectinase to breakdown the cell walls in order to use breakdown products of pectin to sustain growth. Since many types of yeast can't use pectin or its hydrolysis products as carbon source, pectinase can have an ecological function, when the plant tissue is degraded; sugar is released allowing yeast to colonise the plant (Blanco *et al.*, 1999).

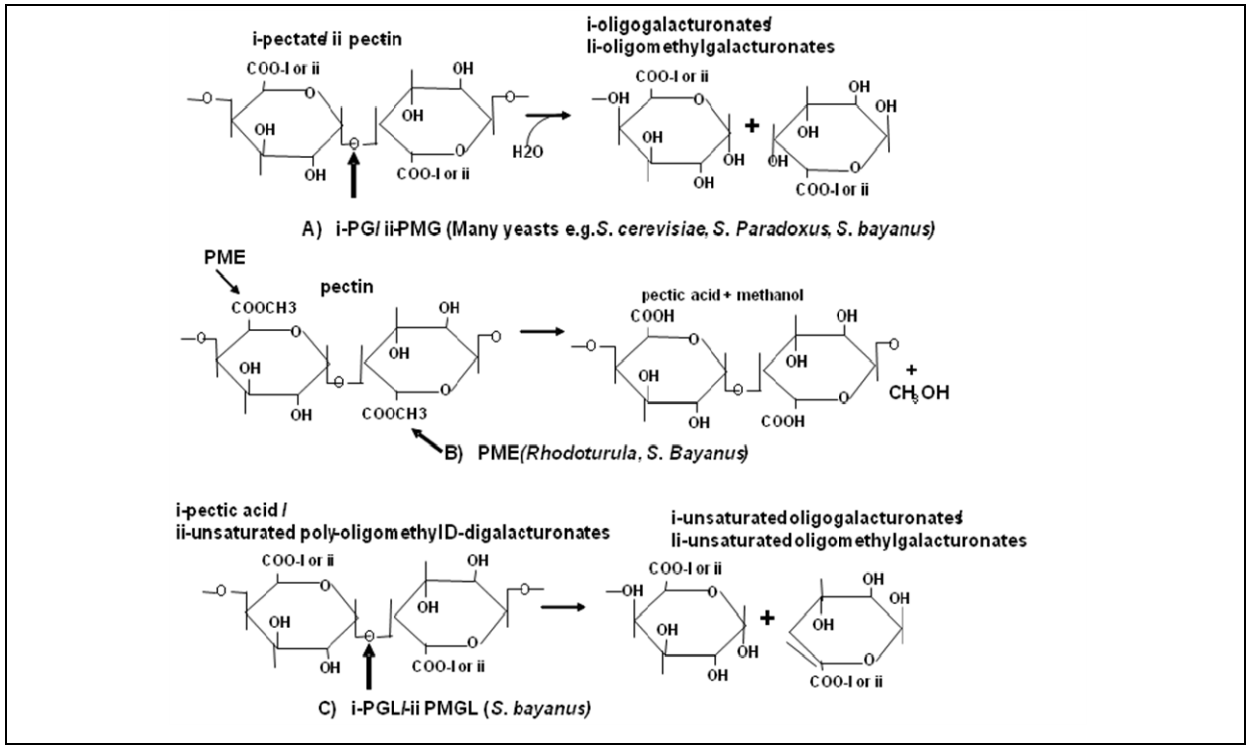

Figure 2.2 Structure of pectic substances, action of different pectolytic enzymes and some of the yeast species that posses these abilities. Depolymerases can cleave pectin by one of two different mechanisms, hydrolysis during which water is introduced across the oxygen bridge (A) or the bond can be broken without the participation of water by trans-elimination reactions (C). During hydrolysis (A), polygalacturonase (PG) will act on pectate as substrate (i-H) to release oligogalacturonates as degradation products and Polygalacturonate Lyase wil act on (i) pectic acid to release unsaturated oligogalacturonates. During transelimination (C) Polygalacturonate Lyase Pectin Lyase (PGL) and polymethylgalacturonate lyase (PMGL) breakdown pectate and pectin by β elimination respectively. This figure was drawn based on structures enzyme actions drawn and described by Jayani *et al.* (2005).

2.6 Applications of microbial pectinases

The main industrial application of pectinases is in fruit juice extraction and clarification. Pectinase treatment reduces viscosity and turbidity of fruit juice. During wine making grape juice is clarified by pectinase treatment before fermentation. Pectin molecules carry a negative charge and form a protective layer around positively charged grape molecules after crushing. Pectinase will break the protective pectin layer down exposing the underlying positive charged molecule. The positively charged particles will be attracted to the negative layers of other molecules, forming bigger aggregates that will settle out (Lourens & Pellerin, 2004) (Figure 2.3).

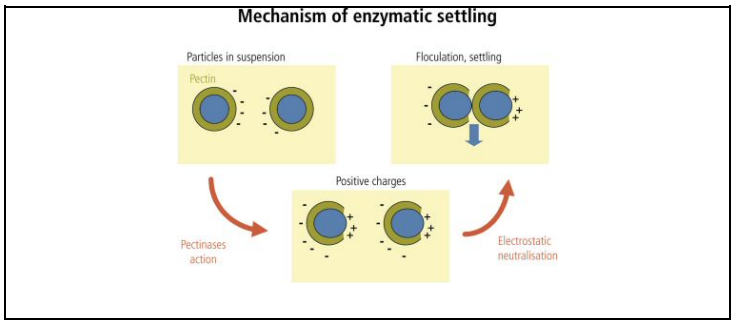

Figure 2.3 Pectinase treatment results in degradation of the negative charged pectin layer, exposing the haze forming grape particle core that carries a positive charge. Consequently these particles aggregate and settle out (Lourens& Pellerin, 2004).

Pectinase treatment can decrease filtration time with up to 50% and treating pulp has showed to increase fruit juice volume from banana, grapes and apples (Jayani *et al.*, 2005). It has been shown that fermenting must with a recombinant strain secreting polygalacturonase and pectin lyase results in greater quantities of higher alcohols, short to medium chain ethyl esters, aldehydes and terpenes than fermentation with the wild type strains (Van Rensburg *et al.,* 2007). Terpenes are important components in establishing varietal aroma. The release of anthocyanins and tannins from the vacuole during enzyme action is demonstrated in figure 2.4, these components have an impact on red wine colour.

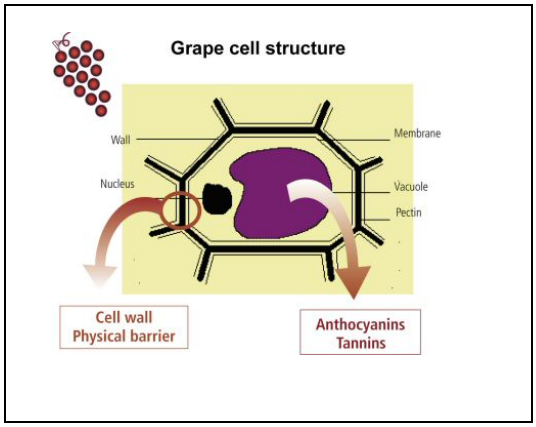

Figure 2.4 Pectinase treatment results in degradation of the grape cell, cell walls. Breaking open the cells increases the volume of juice extracted from the cells and improves wine quality by improving the release of flavour, aroma and colour compounds from the grape skins (Lourens & Pellerin, 2004).

Yeast is an alternative source to filamentous fungi for the production of commercial enzymes. Yeast has the advantages that it is unicellular, growth is simple, the growth medium does not require an inducer and that gene manipulation and cloning is less complicated in yeast, this could allow for the commercial production of most enzymes in yeast. The fact that yeast does not possess a variety of enzymes in its pectolytic arsenal confers advantages and disadvantages to these organisms for industrial production of pectinases. Applications of yeast pectolytic enzymes are limited since hydrolysis of some substrates require the concerted action of several enzymes and

yeasts often only posses some pectolytic activities (Blanco *et al.*, 1999). This limited pectinase range also offers certain advantages. Since pectinase produced by most yeast do not include PME, resulting in methanol production, these enzymes would be well suited for wine clarification (da Silva *et al.*, 2005). Pectolytic enzymes from yeast could be used for industrial purposes as enzyme mixtures, offering the advantage that such mixtures could be prepared with different proportions of each enzyme suited for the enzyme mixtures specific need.

2.7 Pectinase production by *Saccharomyces cerevisiae*

Pectinolytic activity was described in *S. cerevisiae* 20 years ago (McKay, 1990). The enzyme responsible for pectin degradation is an extracellular endopolygalacturonase (PG) with an optimal temperature and pH for enzyme activity of 45°C and 5.5, respectively (Blanco *et al.*, 1994). A strain used for making champagne has been isolated that posses PG, PME and PL activity (Gainvors *et al.*, 1994). This strain has however since been re-classified as *Saccharomyces bayanus* SCPP (Naumov *et al.*, 2001). Thus PG activity remains the only type of pectolytic enzyme identified in *S. cerevisiae.*

Blanco *et al*. (1997b) compared PG degradation between the industrial strain *S.cerevisiae* 1389 and the lab strain IM1-8b. They determined with classical genetic techniques that one gene is responsible for PG production in the lab strain and 2 genes in the industrial strain. By using cation-exchange chromatography they isolated the PG secreted by the IM1-8b lab strain, two different polygalacturonases were secreted and isolated from the 1389 industrial strain. The three enzymes isolated had different K_m values, molecular masses, and optimal pH's for activity (Blanco *et al.*, 1997b). In a subsequent study the enzymes secreted by these two strains were compared, both enzymes were found to be endo-polygalacturonases, but with different biochemical properties. The PG isolated from the industrial strain was more efficient in degrading a pectin solution than the enzyme isolated from the laboratory strain (Blanco *et al.*, 1997b).

Only one PG encoding gene has however been cloned and sequenced in *S. cerevisiae.* The PG encoding gene, *PGU1* (ORF YJR153w) was cloned and sequenced from *S.cerevisiae* at about the same time by two groups (Blanco *et al.*, 1998; Hirose, 1998).

2.8 The PG encoding gene, *PGU1,* in *S. cerevisiae*

The sequence of the PG encoding gene *PGU1,* also known as *PGL1* or *PSM1,* was obtained by sequencing the strain ATCC S288C, a strain without pectolytic activity, this sequence is available on the *Saccharomyces* Genome Database. The gene is present in a single copy per haploid genome on chromosome X. The PG enzyme was purified, characterised and named pglP. PglP was shown to be N-glygosylated, it has a M_r of 42 kDa, is active from pH 3 to 5.5 with an optimum temperature at 25°C and hydrolyses polygalacturonic acid as an endo-PG (Gainvors *et al.*, 1994). The predicted protein comprises 361 amino acids, with a signal peptide between residues 1 and 18. The putative active site has a conserved histidine in position 222. This PG shows 54% homology with fungal ones and 24% with plant and bacterial PG's (Blanco *et al.*, 1998).

The *PGU1* gene and the processes of depolymerisation of pectin have been related to plant pathogenesis (Gognies *et al.*, 1999). During plant attack the pathogen must breach the polysaccharide rich cell wall and invade its plant host. Yeast can penetrate the cell wall by secreting PG, macerating plant host tissue. Tissue is invaded by yeast undergoing a morphological change to a pseudohyphal growth form. A study carried out by Gognies & Belarbi (2002) on *Vitis vinefera* plantlets as model, showed pectinolytic activity in *S. cerevisiae* is required for pseudohyphae development and invasive growth. A strain that did not possess the PG encoding *PGU1* gene could not penetrate *Vitis vinefera* or form pseudohyphae, when *PGU1* was however overexpressed in this strain it obtained both these characteristics (Gognies & Belarbi, 2002). In a subsequent study it was however shown that the presence of PG activity enhances plant penetration by macerating plant tissue, but is not required for yeast to switch to the filamentous growth form, and that yeast can switch to the filamentous growth form in the absence of the *PGU1* gene (Gognies *et al.*, 2006). Pectolytic activity would enable the natural yeast flora of a plant to reach metabolites, such as sugars and amino acids needed for plant growth, this will facilitate yeast invasion of fruits and thus confers an ecological role to *PGU1* in *S. cerevisiae*.

2.9 Regulation of PG activity in *S.cerevisiae*

PG activity in *S. cerevisiae* is highly strain dependent and the absence in activity in certain strains has been attributed to the absence of the *PGU1* gene, mutations in the structural gene and a lack in transcription of *PGU1*.

2.9.1 Presence of the *PGU1* gene

To determine if the absence of PG activity could be attributed to the PG encoding *PGU1* gene being absent in these strains, 61 strains of which 60 originate from a wine environment, were screened for the presence of the *PGU1* gene and tested for PG activity (Fernandez-Gonzalez *et al.*, 2004). Nine strains lacked the *PGU1* gene and were devoid of PG activity, but 17 of the 52 strains, that did possess the *PGU1* gene, were not able to degrade pectin. This result indicated that strains that possess the gene can lack activity due to structural mutations, lack of expression or post-transcriptional regulation. Sequence mutations seems like an unlikely level for regulation to occur at since the *PGU1* gene sequence was found to be very well conserved over millions of years (Veiga-Crespo *et al.*, 2004).

Divol & Van Rensburg (2007) investigated the *PGU1* locus to try to establish why the gene was missing in some strains. It was found that in strains without the *PGU1* gene a part of the chromosome that overlaps with *PGU1,* from 40 bp upstream of the *PGU1* locus, until 1404 bp upstream of YJR154W (the locus upstream of *PGU1*) was replaced with a region of DNA that had 98% identity with transposon Ty1 in the cosmid 8142A. All chromosomal rearrangements in *S. cerevisiae* are said to be as a result of Ty elements in transpositions, duplications, deletions and inversions (Mieczkowski *et al.*, 2006). According to Divol & Van Rensburg (2007) the translocation of a short fragment of a truncated POL (polyprotein) like region attached to an LTR (long terminal repeat) has never been reported in the literature. They hypothesised that this translocation could have been caused by the insertion of a Ty mobile element just upstream of the *PGU1* gene start codon, followed later by the disruption of a large part of this mobile element (Divol & Van Rensburg, 2007). They noted that the Ty1 transposon is activated by the same transcription factors that activate *PGU1* transcription and speculated that this could somehow be involved in the transposition of *PGU1.* Since all the strains lacking *PGU1* seems to have undergone the same transposition phenomenon and are European wine isolates they attempted to prove that strains in which the gene is absent originated from a common phylogenetic origin. Fingerprinting only allowed grouping of a few of these strains in a common group (Divol & Van Rensburg, 2007). Van Wyk (2009) restored the *PGU1* gene in its original locus in some of the strains in which the gene was absent. Since only some of these strains recovered PG activity upon reconstitution of the *PGU1* gene it indicated that these strains would have lacked PG activity even if the gene was present and contributed the lack in PG activity in strains in strains that did

not recover PG activity to the presence of a trans-acting factor inhibiting *PGU1* transcription.

2.9.2 Functionality of the *PGU1* gene

The majority of studies found the *PGU1* gene to be functional in strains irrespective of PG activity. Hirose *et al.* (1998) compared the *PGU1* gene sequence between that of a strain without PG activity (DKD-5DH) and that of a mutant strain with PG activity (SSM52). They found the *PGU1* gene sequences to be identical. Two other studies, using different strains also found that overexpressing the *PGU1* gene from strains without PG activity in the same strain conferred PG activity to these strains, thereby showing that the lack of activity in the wild type strains was not due to a dysfunctional allele of *PGU1* being present in these strains (Blanco *et al.*, 1998; Jia & Wheals, 2000). It was however found by Gognies *et al.* (1999) that certain strains were unable to degrade pectin because of a dysfunctional allele of *PGU1* being present in these strains. These authors found that when they overexpressed the *PGU1* gene, cloned from a strain without PG activity, *S. cerevisiae* X2180, in another strain lacking PG activity, FL100, the strain remained without PG activity. When they however cloned a *PGU1* allele from a yeast with PG activity, *S. bayanus* SCPP, and overexpressed it in the strain without PG activity, FL100, this strain gained the ability to degrade pectin. The difference between the Pgu1p from the strain with and without pectolytic activity was only 3 amino acids. None of the changes were situated in potential glycosylation sites or in highly conserved sequences. Since the *PGU1* gene cloned from the strain with and without PG activity was cloned into the same expression vector and transformed into the same strain, the difference in activity was attributed to the allele cloned from the strain without PG activity, X2180, being dysfunctional and thus possibly the reason why this strains lacked PG activity. This result however contradicted what was found in several other studies, the sequence cloned from the X2180 strain was identical to the S288C sequence from the *Saccharomyces* Genome Database (Gognies *et al.*, 1999). An identical allele was found to be functional in other studies (Blanco *et al.*, 1998; Hirose, 1998).

Van Wyk (2009) cloned the *PGU1* gene from *Saccharomyces paradoxus* RO88, she found this strain to contain significant PG activity. Overexpressing the *S. paradoxus* gene in *S. cerevisiae* was found to result in higher PG activity then when the *S. cerevisiae* gene was overexpressed in the same strain. Upon sequencing several single nucleotide differences were found between the *S. cerevisiae* and *S. paradoxus*

sequences. These differences resulted in differences in amino acid sequence, one of these sites were located in a putative secretion signal, while none of the changes occured in the active sight (Van Wyk, 2007).

2.9.3 Expression of the *PGU1* gene.

Multiple studies showed that overexpressing the *PGU1* gene in strains without PG activity enabled these strains to degrade pectin, indicating that the *PGU1* gene is functional and attributed the lack in PG activity to an absence of transcription of the *PGU1* gene (Blanco *et al.*, 1998; Hirose *et al.*, 1998; Jia & Wheals, 2000). Jia & Wheals (2000) compared *PGU1* transcription by Northern blot analysis and found the gene only to be transcribed in strains with PG activity, The different *S. cerevisiae* strains they compared were variety uvarum, variety chevalieri and FY1679. Two of these strains have now been re-classified and these three strains are now classified as different species: *Saccharomyces uvarum, S. cerevisiae chevalieri* and *S. cerevisiae* strain FY1679. Since the *PGU1* gene is present in these *Saccharomyces* yeasts, with and without PG activity, but is only transcribed in strains with activity it indicates that regulation is at transcriptional level in these yeasts (Jia & Wheals, 2000).

2.9.3.1 Differences in *PGU1* promoter sequence

Small differences in the *PGU1* promoter region were detected between laboratory and industrial *S. cerevisiae* strains (Radoi *et al.*, 2005b). These authors suggested that the difference in gene expression patterns between laboratory and industrial strains might be due to these differences in the promoter region.

To determine if differences in *PGU1* regulation were due to differences in the *PGU1* promoter region, Hirose *et al.* (1999) sequenced and compared the *PGU1* promoter region between a strain without PG activity (DKD-5DH) and a mutant strain with activity, SSM52, the two promoter regions were found to be identical.

Gognies *et al.* (2001) cloned the promoter region of *PGU1* from a strain without PG activity, X2180-1B, and a *S. bayanus* strain with PG activity, SCPP. Activation of different promoters was compared by making *LacZ* fusions and by then measuring the resulting β-galactosidase activity (Gognies *et al.*, 2001). The result was the opposite of what was expected, both promoters were active, with the regulatory region cloned from the yeast without PG activity resulting in higher β-galactosidase activity than that of the promoter cloned from the strain that did possess activity.

The *PGU1* promoter region was sequenced in *Saccharomyces* yeasts with varying *PGU1* transcription, a number of Single Nucleotide Polymorphisms (SNPs) were detected in these promoter regions (Jia & Wheals, 2000). It has not been determined if the variation in transcription is due to these sequence differences or because of differences in the genetic background of strains such as different levels of transcription factors, epigenetic regulation or post transcriptional regulation.

2.9.3.2 Factors regulating PG activity

Functionality of a cell is tightly coupled to the external environment. Gene expression plays a central role in the adaptation to changing conditions. Transcription in eukaryotic cells is controlled by transcription factors that bind to specific regulatory sequences and modulate the activity of RNA polymerase to transcribe the downstream gene. Transcription factors thus control the expression of specific genes by binding to promoter regions on the DNA. Regulation of transcription allows cells to integrate and respond to different intracellular and extra cellular signals (Hermsen *et al.*, 2006). Signal transduction pathways are the molecular mechanisms responsible for detecting and transmitting changes in the surrounding environment to the nucleus where appropriate responses are generated (Navarro-Garcia *et al.*, 2001). The transcriptome in *S. cerevisiae* is dramatically modified by changes in nutrient availability, growth conditions, temperature, and a variety of other environmental conditions (Levy *et al.*, 2007).

PG activity is regulated by carbon source (Blanco *et al.*, 1994; Gognies *et al.*, 1999) disolved oxygen in the medium (McKay, 1990) and nutrients that also induce filamentous and invasive growth (Blanco *et al.*, 1994).

2.9.3.2.1 Influence of carbon source

The influence of carbon source on PG activity in several wine and laboratory yeast strains was compared. None of the strains showed any PG activity when grown on glucose, while several wine strains had PG activity when cultured on galactose, suggesting inhibition of PG activity by glucose and/or activation by galactose (Radoi, *et al.,* 2005b). It was shown in this study that there are small differences in the promoter region between wine and laboratory strains and the authors speculated that these differences in promoter region may be responsible for the different pattern in gene expression between wine and laboratory strains when grown on galactose (Radoi *et al.*, 2005b). The effect of carbon source on *PGU1* transcription was also evaluated in

another study by fusing the *LacZ* reporter gene with the *PGU1* promoter region. An increase in glucose concentration resulted in a concomitant decrease in β-galactosidase activity, indicating that glucose inhibited *PGU1* expression (Gognies *et al.*, 1999).

In some other organisms the presence of pectins induces secretion of pectin degrading enzymes (Ortega, 1994). Pectin resulted in activation of *PGU1* transcription in *S. cerevisiae*. Since pectin is to big too passively penetrate the cell and activate a regulatory pathway by triggering transcription, activation of transcription by the smaller pectin breakdown products were investigated. Galactose resulted in a slight increase in transcription while di-galacturonic acid, the main breakdown product, resulted in a significant activation of the promoter. Activation of transcription by galactose and di-galacturonic acid was more significant when lactose, a non-fermentable carbon source, was used to sustain growth (Gognies *et al.*, 1999).

GAL4 binding sites were identified *in silico* 610 and 632 bp upstream of the *PGU1* gene (Radoi *et al.*, 2005b). The *PGU1* promoter could thus activate transcription in the presence of galactose due to an increase in Gal4p. The transcription factors Gal4p, Gal80p, and Gal3p regulates transcription of the galactose structural genes; *GAL1*, *GAL10*, *GAL7* and *GAL2*. In particular, Gal4p is the key transcriptional activator required for transcription of all *GAL* genes. In the absence of galactose the transcriptional activation function of Gal4p is blocked by its association with Gal80p (Bhat & Hopper, 1992). The Gal80 repressor protein binds to a 28 amino acid core activation region at the very C-terminus of Gal4p, blocking interactions of the activation domain with the transcriptional machinery (Wu *et al.*, 1996). In the presence of galactose, Gal3p sequesters the transcriptional repressor Gal80p in the cytoplasm, thereby relieving inhibition of Gal4p and resulting in *GAL* gene expression (Peng & Hopper, 2002). If the Gal4p binding sites identified *in silico* by Radoi *et al.* (2005) is indeed binding sites for the Gal4p it would explain how galactose activates transcription of *PGU1* (Fig 2.5).

In a study done by Blanco *et al.* (1997a) contradictory results were found, this group found that *S. cerevisiae* had PG activity in glucose, fructose and sucrose but found that PG activity was inhibited by galactose.

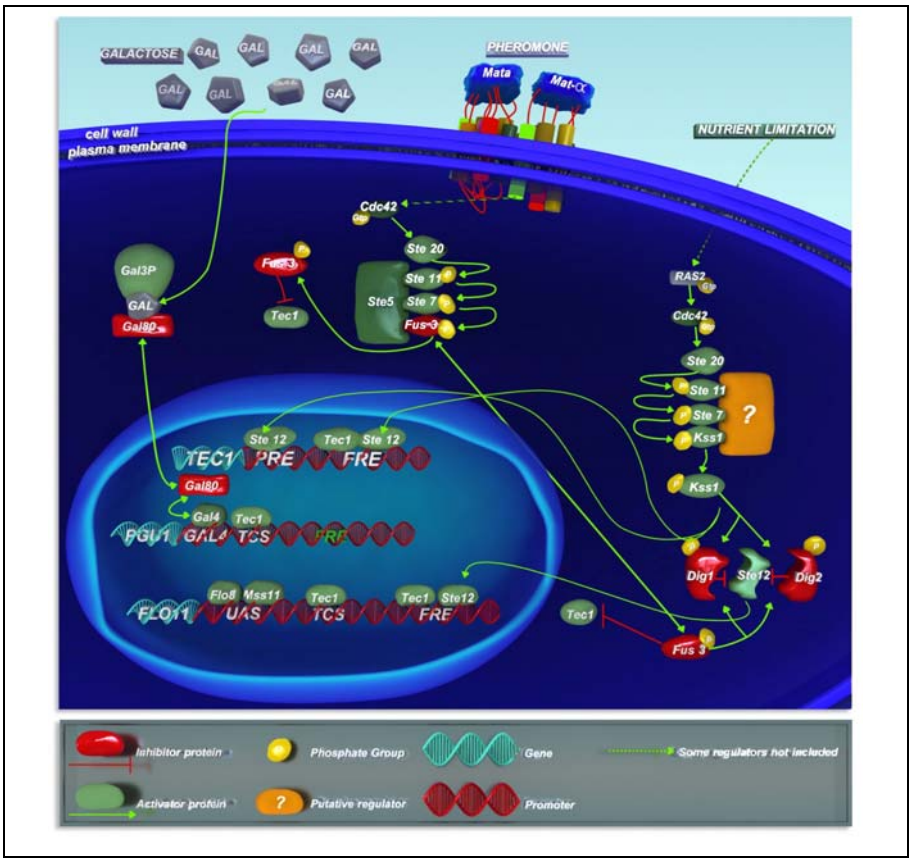

Figure 2.5 Regulatory pathways influencing transcription of the *PGU1* gene in *S. cerevisiae.* Activation and signal transduction by phosphorylation of various kinases through the mating and invasive growth MAPK pathways. Activation of Dig1p and Dig2p through phosphorylation of the MAPK's Kss1p and Fus3p, results in Ste12p to be released from inhibition by the Dig proteins. Ste12p binds to PRE elements in the promoters of *FLO11* and *TEC1* activating transcription of these genes. Ste12p together with Tec1p binds to FRE elements in the promoters of the *FLO11* and *TEC1* and Tec1p binds to TCS elements in the promoter of *PGU1.* Activation and signal transduction by phosphorylation of various kinases through the mating and invasive growth MAPK pathways is demonstrated as described by Madhani (2006). Galactose has been speculated to activate *PGU1* transcription by Gal4p binding to a putative Gal4p binding site located in the *PGU1* promoter. In the presence of galactose activation would then function as follows: Gal3p sequesters the transcriptional repressor Gal80p in the cytoplasm, thereby relieving inhibition of Gal4p and resulting in *PGU1* expression.

2.9.3.2.2 Influence of disolved oxygen

PG activity in yeast is also regulated by the amount of disolved oxygen present in the medium. In the yeast *Saccharomyces fragilis,* PG activity is completely inhibited when the Disolved Oxygen Tension (DOT), an indication of how much oxygen is available to the yeast, saturates less than 60% of the air. Decreasing this percentage by replacing O_2 with CO_2 or N_2 resulted in complete recovery of PG activity (Wimborne & Rickard, 1978). The effect of the amount of disolved oxygen on PG activity varies from strain to strain in *S. cerevisiae*. The strains IM1-8b and 1389 had PG activity when they were grown without aeration, while both these strains lost PG activity with high aeration (Blanco *et al.*, 1999). On the other hand oxygen has been shown not to affect PG activity in the strains NCY 373 and NCY 365 (McKay, 1990).

2.9.3.2.3 Influence of the Kss1 MAPK signalling pathway

The promoter region of the *PGU1* gene contains binding sites for the Tec1 transcription factor that is also required for filamentous and invasive growth (Kohler *et al.*, 2002). *TEC1* is activated by the Kss1 Mitogen Activated Protein Kinase (MAPK) signalling pathway (Figure 2.5). *PGU1* was identified as a target of Kss1 MAPK signaling pathway by comparing transcription profiles of null mutants in single components of the pathway (Madhani *et al.*, 1999). The MAPK's signaling pathways are found in plants, animals and fungi and allow yeast cells to rapidly adapt to changes in its environment. A MAPK pathway generally consists of three protein kinases that act in series. When the cascade is activated, the MAP kinase kinase kinase (MAPKKK or MEKK) phosphorylates the MAP kinase kinase (MAPKK or MEPK). This kinase in turn phosphorylates the MAPK. MAPK's pathways can be switched on by extra or intracellular signals. Upon activation, the MAPK or MEK is thought to move from the cytosol to the nucleus and phosphorylate target proteins such as transcription factors. Since MAPK pathways often regulate transcription factors, these signaling pathways regulate transcription of specific genes in response to signals received by the cell (Gustin, 1998). *S. cerevisiae* possesses five independent MAPK pathways individually regulating pheromone response, filamentous and invasive growth, growth in high osmilarity, cell integrity and spore wall assembly (Gustin *et al.*, 1998). Filamentous and invasive growth usually occurs in diploid yeast cells, but haploid cells can also be induced to invade a solid medium (Gimeno *et al.*, 1992) A dimorphic switch occurs and the yeast differentiates to form pseudohyphae that grows as filaments of extended and connected cells that form rough edged colonies invading solid media. In this growth form the bud site of cells change from bi-polar to uni-polar and the cells elongate (Gimeno *et al.*, 1992). This growth form change to filamentous growth is triggered by nitrogen limitation in diploid cells (Gimeno *et al.*, 1992), while induction in haploid cells happens during glucose limitation (Cullen & Sprague, 2000). Two related phenotypes; filamentous growth and PG activity have both been shown to be activated in low glucose concentrations, since these phenotypes are both activated by the kss1 MAPK (Madhani *et al.*, 1999); it indicates that glucose concentration influences *PGU1* transcription via this regulatory pathway. The mating and filamentous growth MAPK mediates signal transduction from two small GTP binding proteins, Ras2p and Cdc42p to the nucleus. Cdc42p acts downstream of Ras2p and is required for the function of the Ste20p, activating the filamentation-invasion pathway (Mösch *et al.*, 1996). The

filamentous and invasive growth MAPK have several components upstream of the MAPK in common with the mating MAPK. Both signaling pathways use a core module comprising the upstream protein kinase Ste20, the MAPKKK Ste11 and the MAPKK Ste7. In the mating pathway Ste7 phosphorylates the MAPK Fus3p, while Kss1p is the MAPK in the filamentous growth pathway (Madhani, 2006). The nucleoproteins Dig1p and Dig2p repress the mating and filamentous growth responses of *S. cerevisiae* by directly inhibiting Ste12. Activation of Fus3 or Kss1 by phosphorylation may cause release of Ste12 from these inhibitor proteins thereby activating Ste12-dependent transcription (Tedford *et al.*, 1997).

The *PGU1* gene was induced in a *DIG1*Δ*DIG2*Δ mutant (Breitkreutz *et al.*, 2003). Fus3, activates mating but counteracts filamentous growth, while the other MAPK, Kss 1, preferentially activates filamentous growth. Both kinases activate the transcription factor, Ste 12, which can stimulate gene expression specific to each of the pathways, the filamentous growth pathway also activates the transcription factor Tec1 (Tedford *et al.*, 1997). Several mechanisms are present to match input signal to a output response. When pheromone binds to its receptor (Ste2/Ste3) it induces dissociation of the heterotrimeric G protein into Gpa1p (Gα) and the Ste4p/Ste18p (G_{β}/G_{γ}) complex, this complex then activates the Ste5 scaffold protein (Gustin *et al.*, 1998; Madhani *et al.*, 1999). The Ste5 scaffold protein tethers the mating kinase proteins Ste11, Ste7 and Fus3 together so that one kinase can phosphorylate the next. This increases the specificity of the cascade, inhibiting inappropriate interactions with other related cascades (Madhani, 2006). A similar scafold protein that has not yet been identified might be present in the filamentous growth pathway (Madhani, 2006). It seems that 'leaking' might occur between these two pathways if Ste7 phophorylates the wrong MAPK, matching activation of transcription factors with the wrong input signal. To prevent such cross talk occuring between the mating and filamentous growth pathways, active Fus 3 phosphorylates and induces Tec1 ubiquitination and degradation through the SCF^{Cdc4} ubiquiton ligase (Chou *et al.*, 2004). Activating the mating pathway will thus result in the destruction of tec1p, the transcription factor required for activation of filamentous and invasive growth genes. Deletion of *FUS3* results in a significant increase in PG activity (Madhani *et al.*, 1999). Micro array analysis confirmed this result and showed a significant increase in *PGU1* transcription in a *Fus3*Δ mutant (Roberts *et al.*, 2000). The increase in Tec1p upon *Fus3* deletion can also be the result of inappropriate substitution of Kss1p for Fus3p. In the absence of Fus3p, Kss1p is wrongly activated instead of Fus3p; it has been shown that pheromone treatment

resulted in a ten fold increase in Tec1p activation in a *fus3* mutant compared to the wild type strain. Once recruited in this manner, Kss1p thus activates two pathways, the mating response MAPK and the invasive growth MAPK (Madhani *et al.*, 1997). Both these mechanisms are responsible for the increase in *PGU1* transcription in a *fus3*Δ mutant. From the study done by Madhani *et al.,* (1999) it could be seen that a *fus3*Δ *kss1*Δ mutant shows increased PG activity, the increase in transcription could not be due to inappropriate substitution of kss1p for fus3p, since *KSS1* was also deleted and thus indicates that this increase *PGU1* transcription is due to more Tec1p being present, since it is not being tagged for destruction by fus3p. Since the *fus3* mutant showed much higher PG activity than the *fus3*Δ *kss1*Δ mutant, it indicates that the increase in PG activity in this situation was due to inappropriate substitution of kss1p for fus3p.

Micro array analysis showed an increase in *PGU1* transcription during pheromone treatment, suggesting that an overlap can occur between these two signaling pathways (Roberts *et al.*, 2000). Genes regulated by the filamentous growth MAPK contain two types of *cis*-acting regulatory sequences in their promoters, a binding site for the transcription factor Ste12p, termed a Pheromone Response Element (PRE) and a Tec1-binding-site (TCS) (Gustin *et al*., 1998). When PRE and TCS elements are in close proximity to one another, they form a Filamentation Response Element (FRE) together. Tec1 and Ste12 usually activate transcription by binding on this FRE in a synergistic fashion. It has however been shown that haploid invasive and diploid pseudohyphal growth are not only regulated by this cooperative binding to FRE elements. Tec1p can activate gene expression in the absence of Ste12p through a mechanism known as TCS control. *TEC1* expression can in turn be activated by only Ste12p via clustered PREs located in the *TEC1* promoter (Kohler *et al.*, 2002).

The *PGU1* promoter region has been found to contain three TCS elements, but no neighboring PREs. A typical FRE element was thus not found (Kohler *et al.*, 2002). *PGU1* expression was found to be independent of Ste12p activation and was only indirectly up regulated via an increase in Tec1p. Microarray analysis showed that deleting the transcription factor *TEC1* or the upstream Mitogen Activated Protein Kinase Kinase (MAPKK) *STE7,* caused a decrease in *PGU1* expression and overexpressing *TEC1* resulted in a significant increase in *PGU1* transcription (Madhani *et al.*, 1999). PG activity was lost in mutants of the invasive growth MAPK genes *ste7*Δ, *ste11*Δ, *ste20*Δ and that of the transcription factors activated by this pathway *ste12*Δ and *tec1*Δ (Madhani *et al.*, 1999). The main strategies used to maintain pathway specificity between these two pathways are thus the presence of a scaffolding protein (Ste5p), a

pathway specific transcription factor (Tec1p) and degradation of this transcription factor upon activation of the neighboring pathway (Fus3p degradation) (Figure 2.5).

The main targets of the kss1 MAPK have been shown to be *CLN1* encoding a morphogenic regulator essential for pseudohyphal growth, *FLO11* encoding a glycoprotein that regulates cell-cell and cell-surface adhesion and *PGU1* encoding a plant cell wall degrading polygalacturonase (Madhani *et al.*, 1999) Since PG activity and invasive growth have both been shown to play a role in plant pathogenesis (Gognies & Belarbi, 2002), co-regulation of the genes controlling these phenotypes makes sense.

Activation of *PGU1* transcription by various transcription factors is illustrated in Figure 2.5.

2.9.3.3 Could *PGU1* transcription be regulated on a epigenetic level?

Epigenetics is the study of heritable changes in gene expression that are not mediated at the DNA sequence level. Due to its postion in the genome it is possible that *PGU1* is regulated by an epigenetic mechanism. Molecular mechanisms that mediate epigenetic regulation include DNA methylation and chromatin/histone modifications (Cheung & Lau, 2005). DNA is packaged into chromatin through association with histone proteins. The basic repeating unit of chromatin is the nucleosome (Figure 2.6). It consists of 146 base pairs of DNA wrapped around an octameric histone core containing two copies each of the histones H2A, H2B, H3 and H4 (Kent *et al.*, 2001). The DNA that has wrapped around the nucleosome is kept in place by associating with histone H1. The H1 protein binds to the "linker DNA" (approximately 80 nucleotides in length) region between the histone beads, helping stabilize the zig-zagged 30 nm chromatin fiber (Berezney & Jeon, 1995).

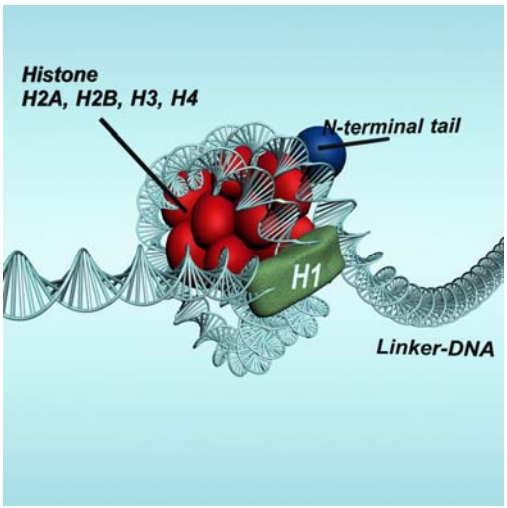

Figure 2.6 A nucleosome made up of a 146 base pairs of DNA wrapped around an octameric histone core containing two copies each of histones H2A, H2B, H3, and H4. The DNA is kept in place by associating with the histone H1. The H1 protein binds to the "linker DNA" (approximately 80 nucleotides in length) region between the histone beads. This nucleosome was drawn according to a description by Kent *et al.,* 2001).

S. cerevisiae possess a single H1 linker histone encoding gene *HHO1* (Veron *et al.*, 2006). DNA is further packaged by higher order looping and folding of the chromatin fiber (Cheung & Lau, 2005).

Diversity in the histone/nucleosome structures is generated by a variety of posttranslational modifications, such as acetylation, phosphorylation, methylation, and ubiquitination. The organisation of chromatin physically restricts access of nuclear proteins, such as transcription factors and RNA polymerase, to the underlying DNA and posttranslational modifications of histone proteins can alter chromatin conformation playing direct regulatory roles in gene expression (Cheung & Lau, 2005). In higher eukaryotes genomic DNA is organised into heterochromatin and euchromatin. Heterochromatin is condensed through the cell cycle and generally not transcribed, while euchromatin is more relaxed and gene transcription is active (Iida & Araki, 2004). Subtelomeric DNA from *S. cerevisiae* has been compared to heterochromatin of higher organisms, also containing repetitive sequences and few transcribed genes (Barton & Kaback, 2006). Packaging of genes into a condensed heterochromatin-like structure at the telomeric tract and subtelomeric region can result in silencing of genes located in these areas irrespective of their promoters (Martin *et al.*, 2004)

The *PGU1* gene is subtelomeric since it is located within 25 kb of the right telomere of chromosome X (Galibert, *et al.,* 1996) (Figure 2.7).

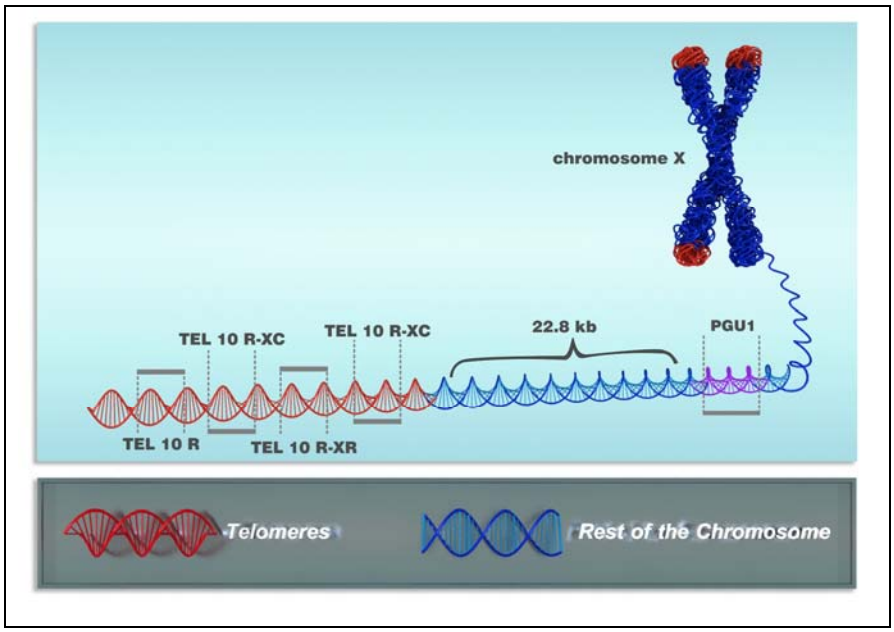

Figure 2.7 The *PGU1* gene is located 22.8 kb from the right telomere of chromosome X. The position of the *PGU1* gene was located according to the Saccharomyces Genome Database.

In *S. cerevisiae,* the telomeres consist of tandem repeats about 350-bp in length, of the sequence $C_{1\text{-}3}A$. Together with proteins that bind to this region known as telomere silencing factors, the telomeres form a non-nuleosomal DNA-protein complex called the telosome (Wright & Shay, 1992). Rap1p, the sequence specific duplex DNA binding protein is the major protein in the telosome. Rap1p binds to the DNA repeat sequences and interacts with other proteins including Rif1, Rif2, Sir2, Sir3 and Sir4. These proteins interact with each other and subtelomeric nucleosomes via the N-termini of histones H3 and H4, thus packaging the telomeric region into a complex heterochromatin-like structure (Vega-Palas *et al.*, 2000). Genes are silenced by the Sir proteins (Silence Information Regulator proteins) binding to other silencing factors up to 8kb from the telomeres (Wyrick *et al.*, 1999).

In *S. cerevisiae* genes placed near telomeres are transcriptionally repressed, this is called the Telomere Position Effect (TPE) (Mondoux & Zakian, 2007). Gene silencing by TPE in yeast has mainly been demonstrated by inserting reporter genes in close proximity of a telomere (Gottschling *et al.*, 1990). The only naturally occurring yeast genes that TPE has been demonstrated for are, *HMR* the silenced copy of the mating type locus on chromosome III (Thompson *et al.*, 1994), the TY5-1 retrotransposon, YFR057W an uncharacterized ORF located next to the telomere R-VI (Vega-Palas *et al.*, 2000) and *IMD* a pseudogene encoding an IMP dehydrogenase ortholog, located next to the telomere R-I (Barton & Kaback, 2006). Different methods were used to demonstrate that these genes are silenced by TPE. *HMR* was found to be derepressed

when the H3 histone was mutated and the gene was moved far away from the telomere. The H3 mutation did not lead to a significant increase in transcription of *HMR* when the gene was integrated adjacent to a telomere (Thompson *et al.*, 1994). TY5-1 and YFR057W expression were derepressed by mutating genes that alleviate TPE, *SIR2, SIR3* and *SIR4* (Vega-Palas *et al*., 2000). *IMD1* expression was derepressed by removing the telomere adjacent to this gene from chromosome I; the expression of this gene also increased in a *sir3* Δ mutant (Barton & Kaback, 2006).

Unlike the telomeric tract, sub-telomeric DNA is organised into nucleosomes (Tham & Zakian, 2002). Histones can contribute to Sir independent silencing of subtelomeric genes upstream of the Sir binding region (Wyrick *et al.*, 1999). According to the histone code hypothesis combinations of covalent histone modifications lead to varied transcriptional outputs (Dion *et al.*, 2005). DNA regulatory sequences thus only provide a partial explanation of gene regulation. Deciphering the complexity of histone modifications is thus very important in understanding transcriptional regulation. Post-translational modification of histone has been proposed to demarcate transcribed and silent regions (Martin *et al.*, 2004). In an experiment where individual lysines of the H3 histone were substituted with the uncharged residue glutamine to mimick acetylation, the unacetylated N-terminus of H3 repressed transcription, while acetylation relieved repression. The positive charge of the N terminal tails, due to the lysine side chains, was neutralised by acetylation. This neutralisation was proposed to weaken the interaction with the negatively charged DNA, increasing exposure of DNA to transcription factors and altering nucleosome confomation (Mizzen & Aliss, 1998).

The anti-promiscuity model that explains how post translational modification of histones regulates transcription of different chromosomal regions was proposed by Van Leeuwen & Gottschling (2002) (Figure 2.8). This model is based on the following observations: binding of the Sir proteins is not restricted to the chromosome ends (Georgel *et al.*, 2001). Over expression of *SIR3* results in the silenced region to spread further from the telomere, inward along the chromatin fiber (Hecht *et al.*, 1996). *In vitro*, Sir3p binds stronger to the unacetylated forms of histones, and in histone H3 and H4 every acetylable lysine residue in the histone tails is unacetylated in silent chromatin (Suka *et al.*, 2001). The model proposes that post-translational modification of histones marks active chromatin and limits silencing to discrete domains by preventing the promiscuous binding of Sir proteins elsewhere along the genome. Methylation of histone H3-K79 has been shown to be important in marking active chromatin and limits silencing to discrete domains by preventing the promiscuous binding of Sir proteins

elsewhere along the genome (Van Leeuwen & Gottschling, 2002). *DOT1*, which has been shown to be important in silencing, encodes an histone methyltransferase responsible for modifying Lys79 on histone H3 (H3-K79). Over expression of Dot1p leads to a virtually 100% tri-methylation of Lys79, resulting in a increase in expression at many loci, since Sir proteins are largely excluded from binding to chromatin due to histone modifications; they can only bind and silence near the strongest silencers *i.e. HM* loci. A *dot1* Δ mutant does not show inhibition of transcription of all genes, since the limited supply of Sir proteins would be diluted among all possible binding sites if H3 is hypomethylated throughout the genome (Van Leeuwen & Gottschling, 2002). It has been proposed that the anti-promiscuity hypothesis is not necessarily restricted to H3-K79 methylation and that it may be extended to other histone residues and modifications as well (Van Leeuwen & Gottschling, 2002). The *SET1* gene is solely responsible for H3-K4 methylation in *S. cerevisiae* (Nagy *et al.*, 2002), since there is a strong correlation between methylated Lys4 in open coding regions and transcriptional activity. It was proposed that H3-K4 methylation may also prevent promiscuous binding of silencing proteins, separating transcribed from silent regions (Van Leeuwen & Gottschling, 2002). Results indicated that lysine-4, -9, -18, 23-, and -27 in H3 are involved in subtelomeric gene repression (Martin *et al.*, 2004). These residues were hypo-metylated or –actetylated in subtelomeric regions while being hyper-methylated or –actetylated in the coding regions of active genes. These results led to a model similar to the anti-promiscuity model to be proposed by these authors (Martin *et al.*, 2004).

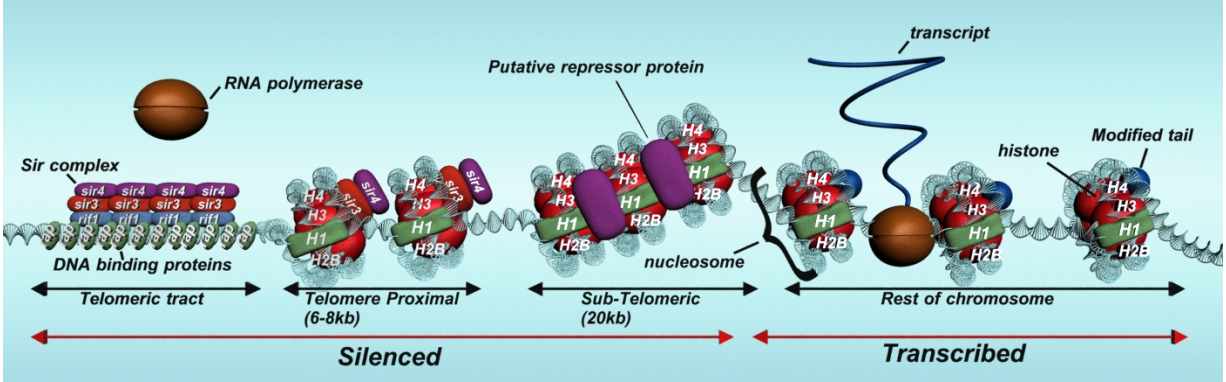

Figure 2.8 The chromosome can be divided into silent and transcribed regions. The telomeric tract and telomere proximal region is silenced due to steric hindrence of RNA polymerase by SIR proteins binding to the DNA or histone tails. According to the anti-promiscuity model (Van Leeuwen & Gottschling, 2002, Martin *et al.*, 2004) subtelomeric DNA is silenced by binding of repressor proteins. The rest of the chromosome is transcribed due to post translational modification of histone tails inhibiting binding of repressor proteins.

2.10 Recombinant *S. cerevisiae* strains with improved pectolytic activity

Genetic modification has not been a widely used technique in the development of industrial yeast strains outside the pharmaceutical industry and many countries have banned the use of GM strains for wine production. Two recombinant wine yeast strains have been given Generally Regarded As Safe' (GRAS) status from the Food and Drug Administration (FDA) in the United States of America and are commercialized. The first *S. cerevisiae* strain developed to be given this status is the "malolactic strain" ML01 (GRN 000120), containing the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *Oenococcus oeni* malolactic gene (*mleA*) under control of the *S. cerevisiae PGK1* promoter and terminator sequences integrated into the *URA3* locus of an industrial wine yeast (Husnik *et al.*, 2006). The wine yeast ECMo01 commercialised by First Venture Technologies Corporation, was constructed by a self-cloning strategy and also received GRAS status (GRN 000175). The introduced sequence consists of the *DUR1, 2* gene under the control of *PGK1* promoter and terminator sequences for high expression. Ethyl carbamate (urethane), a suspected human carcinogen, can be formed in wine by a chemical reaction that occurs between urea and ethanol. The increase in production of the *DUR1, 2* encoded urea amidolyase reduces the production of ethyl carbamate indirectly by lowering the levels of urea substrates available to react with ethanol in the wine (Coulon *et al.*, 2006).

Degradation of the structural polysaccharides by carbohydrases can result in an improvement in juice yield, clarification and filterability during winemaking. The commercial enzyme preparations used during maceration are typically blends of pectinases, cellulases, hemicellulases and other enzymes with carbohydrase activities (Haight & Gump, 1994). Since the endogenous polysaccharase activity of *S. cerevisiae* is very limited, the heterologous expression of specific polysaccharase genes in wine yeast can improve certain aspects of the winemaking process. Therefore, it might be possible to produce higher quality wines without the addition of expensive commercial enzyme preparations. Since only the desired enzymes are secreted by the recombinant strain, there will be no undesired side activities detrimental to wine quality (Louw *et al.*, 2006). A *S. cerevisiae* strain that can degrade multiple polysaccharides through heterologous expression of a variety of genes has been created. The pectate lyase gene (*PEL5)* cloned from *Erwinia chryasanthemi* and polygalacturonase gene (*PEH1*) cloned from *Butyrivibrio fibriosolvens* were overexpressed in *S. cerevisiae* in conjuction with an endo-∆-1,4-D-glucanase (*END1*), a cellobiohydrolase (*CBH1*), a exo-∆-1,3-D-glucanase (*EXG1*), a ∆-glucosidase (*BGL1*) and a endo-∆-1,4-D-xylanase (*XYN4*)

(Peterson *et al.*, 1998). The resulting strain could use all these carbon sources to sustain growth. Such recombinant strains can be used in Consolidated Bioprocessing (CBP) degrading and fermenting substrates in an integrated process (Pretorius *et al.*, 2003). Cellulose and hemicellulose degrading wine yeast strains have been created through heterologous expression of fungal genes, making wine with these strains by using different grape cultivars, showed several improvements concerning wine quality and processing (Louw *et al.*, 2006, Van Rensburg *et al.*, 2007). Wines produced by a strain overexpressing the *Trichoderma reesei, XYN2,* xylanase encoding gene in tandem with the *Butyvibrio fibriosolvens, end1,* endoglucanase encoding gene resulted in the most significant alteration in chemical profile, most intense stable colour and some of the wines made by using this strains were preferred by a tasting panel, compared to wines fermented with the wild type strains (Louw *et al.*, 2006).

Several pectolytic strains have been created by over expressing pectinase encoding genes cloned from different organisms in *S. cerevisiae* (Table 2.2).

Van Wyk (2009) constructed recombinant wine yeast strains with pectolytic ability by re-integrated the *PGU1* gene in its original locus in some of the yeast strains lacking the *PGU1* gene. No significant differences were found between wines fermented with the recombinant and wild type strains, there was however no skin contact during fermentation, thus no time was allowed for the PG to act during fermentation. Wine yeast strains were also made by overexpressing *PGU1* cloned from *S. cerevisiae* and *Saccharomyces paradoxus* RO88. Fermentation with these recombinant strains resulted in small differences in chemical profile compared to fermentation with the wild type strain, but no significant differences were found in the wine fermented with these two strains (Van Wyk, 2009).

Table 2.2 Recombinant wine yeast strains with pectolytic ability used for wine making

Engineering strategy used	Enzyme	Wine yeast strain engineered	Result of fermentation compared to wild type strain	Reference
Heterologous expression *PELE* (*E.chryasanthemi)* and *PEH1* (*E.carotovora)*	Pectate lyase Polygalacturonase	VIN13	Improved juice extraction, chemical profile of the wine altered	(Van Rensburg, *et al.,* 2007)
Heterologous expression *PELE* (*E.chryasanthemi)* and *PEH1* (*E.carotovora)*	Pectate lyase Polygalacturonase	VIN13	chemical profile of the wine altered	(Louw, *et al.,* 2006)
Heterologous expression *PELA (Fusarium solani*)	Pectate lyase	T_{73}	No effect	(Gonzalez-Candelas *et al*., 1995)
Heterologous expression native *PGU1*	Polygalacturonase	M-20	10 fold reduction in filtration	(Vilanova *et al*., 2000)
Heterologous expression native *PGU1*	Polygalacturonase	UCLMS-1M	Increase in juice extraction	(Fernandez-Gonzalez *et al*., 2004)
Chemical mutation	Polygalacturonase	Uvaferm, KW4	improved filtration, increase in wine colour	(Radoi *et al*., 2005a)
Re-introduce *PGU1* in strains lacking the gene	Polygalacturonase	UCLMS-1, -3 and -4	chemical profile of the wine altered	(Van Wyk, 2009)
Heterologous expression of native *PGU1* and *S. paradoxus PGU1*	Polygalacturonase	VIN13	chemical profile of the wine altered	(Van Wyk, 2009)

A pectin degrading strain was constructed by over expressing the pectate lyase - encoding gene (*PELE*) cloned from *Erwinia chryasanthemi* together with a polygalacturonase (*PEHL*) cloned from *Erwinia carotovora* on a yeast centromeric plasmid (Laing & Pretorius, 1993). A recombinant wine yeast strain with pectolytic abilities was constructed by integrating the two aforementioned genes into the genome of the wine yeast strain VIN13 (Van Rensburg *et al.*, 2007). Fermenting with this pectolytic yeast strain resulted in an increase in juice extraction in all three cultivars fermented compared to the wild type strain. Fermentation of red grape cultivars had a negative impact by increasing turbidity compared to fermentation with the wild type strain. Fermentation with the recombinant strain also resulted in alterations in the chemical profile of the must, the compounds altered are implicated in increasing fruity aromas, but no formal sensory analysis was conducted (Van Rensburg *et al.*, 2007). Gonzalez-Candelas *et al.* (1995) constructed a pectolytic wine yeast strain by overexpressing the *Fusarium solani* pectate lyase gene (*PELA*). In small scale microvinifation experiments wine produced by this recombinant strain was indistinguishable from wine produced by the wild type strain (Gonzalez-Candelas *et al.*, 1995).

Since *S. cerevisiae* contains a functional PG encoding gene, the pectin degrading ability of this yeast can be improved without expressing genes cloned from other organisms.

Self-cloning describes genetic modification by gene transfer within the same species (Heller, 2006), such as changing gene expression by substituting a genes native promoter with a promoter cloned from the same organism resulting in a different expression profile, thus no DNA cloned from another species is introduced. Increasing PG activity by improving *PGU1* transcription without introducing DNA cloned from another species is thus genetic modification by a self cloning strategy. In Japan and in Europe more flexible legislation is applicable to organisms developed by self-cloning strategies (Querol & Fleet, 2006). Overexpressing the *PGU1* gene in a variety of *S. cerevisiae* laboratory strains devoid in PG activity, conferred PG activity to these strains (Blanco *et al.*, 1998; Hirose, *et al.,* 1998; Jia & Wheals, 2000). The gene was overexpressed in all these strains by using *S. cerevisiae* constitutive glycolytic pathway promoters and auxotrophic markers, no foreign DNA was thus introduced. A *S. cerevisiae* wine yeast strain with PG activity (M-20) was created by over expressing *PGU1* under control of the constitutive *PGK1* promoter on a multi-copy plasmid (Vilanova *et al.*, 2000). Since selective pressure was not maintained during fermentation, some of the cells lost the plasmid, it was shown the plasmid was maintained in 70% of the cells. Fermenting with this recombinant strain resulted in a 10 fold reduction in filtration time compared to fermenting with the wild type. Terpenes with a positive contribution to wine aroma were also increased. Fermenting with the wild type strains, with addition of commercial macerating enzyme preparations had a similar impact on the wine made, but also showed increased levels of specific terpenes, esters and methanol that confers negative characters to the wine (Vilanova *et al.*, 2000). Fernandez-Gonzalez *et al.* (2004) also constructed a wine yeast strain (UCLMS-1M) by over expressing the *PGU1* gene under control of the *PGK1* promoter, they however integrated the cassette into a mutated allele of the yeast acetolactate synthase gene (*ilv2*) that provides resistance to the herbicide sulphometuron as dominant selectable marker. No bacterial DNA was present in the integrated structure, resulting in a strain conforming to GMO regulation in the USA and Europe (Fernandez-Gonzalez *et al.*, 2004). The patent for this strain was filed in Spain on 01/07/2003 (publication nr WO/2004/00519). Fermentation with this strain did not affect the taste or flavour of the wine, but did result in an increase in juice extraction from the pulp. The less profound effect on processing and quality during wine making with this strain (UCLMS-1M)

compared to the M-20 strain (Vilanova *et al.*, 2000) might be due to less enzyme being secreted into the must by UCLMS-1M baring a single integrant, compared to the multiple copy plasmid present in M-20. Mutant wine yeast strains were produced by chemical mutation with 5% ethyl-methanesulfonate and were selected for their ability to degrade pectin (Radoi *et al.*, 2005a). Fermentation with these strains resulted in improved filtration and increase in wine colour compared to wines fermented with the wild type strain. Since *PGU1* is the only PG encoding gene identified in *S. cerevisiae* the increase in PG activity can be assumed to be due to an increase in *PGU1* transcription. This shotgun approach can increase PG activity in many different ways, *e.g.* mutating the structural gene, the promoter sequence or affecting regulatory pathways regulating activation of *PGU1* transcription factors. Regulatory pathways that can impact PG activity can also activate genes that may be detrimental to wine quality.

The advantage of genetic modification strategies is that only specific pathways are altered, even so central metabolism can still be rerouted to produce complex side effects.

Systems biology aproaches can be used to construct engineered and mutated strains with improved properties for wine fermentation (Borneman, *et al.,* 2007).

Systems biology is a whole genome aproach integrating technology, biology, and computation to study an organism by analysing all its components and their interactions in order to understand the functioning of the system as a whole. *In silico* analysis of the transcriptome of mutant wine yeast strains, isolated and engineered, with improved PG activity can be used to identify all biochemical pathways altered by the mutation. The influence of the newly constructed strains on wine quality can thus be predicted decreasing the amount of strains that have to be screened for the desired phenotypes. Comparitive genomic studies between the recombinant and wild type strains can also be important on a fundamental level, identifying new pathways involved in PG regulation.

2.11 Conclusion

Wine quality and processing can be improved by degrading grape derived polysaccharides during vinification. This is accomplished through the addition of commercially produced enzymes in the normal wine making process. The grape polysaccharide, pectin, plays an important role during vinification. Pectin can be degraded by some strains of *S. cerevisiae* by secreting a polygalacturonse during

fermentation. This phenotype is strain specific because the PG encoding gene, *PGU1,* is absent or present but not transcribed in some strains. Expression of *PGU1* can be influenced by environmental factors and by factors that influence the mating and invasive growth phenotypes. Differences in transcription of *PGU1* between strains are usually due to differences in the genetic background of strains affecting regulation and not due to variation in the sequence of the regulatory region. Recombinant *S. cerevisiae* strains with pectolytic abilities have been constructed by heterologous expression of pectinase encoding genes, cloned from pectin degrading organisms and by increasing secretion of the native polygalacturonase. Native PG activity has been improved by increasing *PGU1* secretion through self-cloning strategies and by chemical mutation.

Understanding the processes involved in regulation of *PGU1* transcription is important, since the knowledge can be used to develop more effective strategies to improve PG degradation by *S. cerevisiae.* Since *PGU1* has been implicated in *S. cerevisiae.* acting as phytopathogen towards *Vitis vinifera,* studying this gene can also have significant ecological importance, shedding light on how certain yeast strains survive in the vineyard.

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Chapter 3

Research results

Regulation of endo-polygalacturonase activity in *Saccharomyces cerevisiae*

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Regulation of endo-polygalacturonase activity in *Saccharomyces cerevisiae*.

Campbell Louw[1], Philip R. Young[1], Pierre Van Rensburg[1, 2] & Benoit Divol[1]

[1] Institute for Wine Biotechnology, Stellenbosch University, Matieland, South Africa;
[2] Distell Corporation, Stellenbosch, South Africa

3.1 Abstract

Pectolytic activity in *Saccharomyces cerevisiae* is due to the secretion of a endo-polygalacturonase encoded by the *PGU1* gene. The ability to degrade polygalacturonic acid has been shown to vary between different strains. In this study we tried to elucidate how pectolytic activity is regulated in *S. cerevisiae* and to determine if the means of regulation differ between strains. *S. cerevisiae* strains from different genetic backgrounds, with varying ability to degrade pectin were compared. Activity was found not to be regulated by sequence differences in the *PGU1* gene, but by the transcription level of the gene. Expression of *PGU1* was found to be determined by the transcription level of its two transcription factors *TEC1* and *STE12*. The activation of *PGU1* transcription by galactose was found to be strain specific, independent of the strain being an industrial or domesticated strain. The EUROSCARF yeast deletion library was screened for genes encoding inhibitors and activators of PG activity. Fourteen strains were identified in which deletion of a specific gene resulted in a recovery of PG activity, these genes were identified as encoding inhibitors of PG activity, and two activators were identified.

3.2 Introduction

Pectin is a structural heteropolysaccharide that occurs mainly in the middle lamellae and primary cell walls of higher plants. It is composed of a backbone of D-galacturonic acid residues linked by α-1,4-glycosydic bonds (Hoondal *et al.*, 2002). Pectinases are enzymes that are able to degrade pectic substances. Endopolygalacturonase specifically degrades the pectic substrate by splitting α -1, 4-glycosydic bonds between two non-methylated galacturonic acid residues. *Saccharomyces cerevisiae* was initially thought to be devoid in pectolytic activity (Luh & Phaff, 1951), but certain strains have been shown to be able to degrade pectin (Luh & Phaff, 1954). Polygalacturonase (PG) activity is the main pectolytic activity in *S. cerevisiae* and has been reported in several strains (Blanco *et al.*, 1994; Blanco *et al.*, 1998; Radoi *et al.*, 2005). A PG-encoding

gene, *PGU1* (ORF YJR153W), was cloned and sequenced from *S. cerevisiae* by several groups (Blanco *et al.*, 1994; Blanco *et al.*, 1997; Hirose, 1998). The inability of certain strains to degrade pectin can in some cases be attributed to the PG-encoding *PGU1* gene being absent in these strains (Fernandez-Gonzalez *et al.*, 2004). Some of the strains in which the *PGU1* gene is present are also devoid in pectolytic activity; this absence in activity is attributed to mutations in the *PGU1* gene (Gognies *et al.*, 1999), inhibition of transcription of *PGU1* (Jia & Wheals, 2000) or to post-transcriptional regulation (Hirose *et al.*, 1999).

Gognies *et al*. (1999) found that the inability of certain strains to degrade pectin was due to a dysfunctional allele of *PGU1* being present in these strains. The dysfunctional allele identified in this study was cloned from the X2180-1B strain and was found to be 100% identical to the sequence in the *Saccharomyces* Genome Database (SGD) originating from the S288C strain, a strain also devoid in PG activity. The gene cloned from *S. bayanus* SCPP a with strong PG activity, was found to be functional. The difference in Pgu1p between S288C and SCPP was only three amino acids. One is a conservative mutation and the other two could have some functional significance, although one of the changes was in potential glycosylation sites or in highly conserved sequences (Gognies *et al*. 1999). Jia & Wheals (2000) however showed that the nucleotide sequence of *PGU1* is identical between strains regardless of their PG activity. They also confirmed that the inability to degrade polygalacturonic acid was not due to structural mutations by over-expressing the *PGU1* gene cloned from a strain without PG activity in the same strain, resulting in a strain able to hydrolyze polygalacturonates. The latter authors showed that *PGU1* was not expressed in a strain without PG activity and was expressed in a strain with activity. As the *PGU1* gene is present in all strains, but is only transcribed in strains with activity, it suggested that regulation is at transcriptional level (Jia & Wheals, 2000).

It has been shown that *PGU1* transcription is regulated by the Kss1p Mitogen activated protein kinase (MAPK) that regulates filamentous growth (Madhani *et al.*, 1999). This co-regulation of genes responsible for filamentous growth and polysaccharide degradation during starvation conditions correlates with the proposal that filamentous growth is a coordinated foraging behaviour in the wild (Gimeno *et al.*, 1992; Madhani *et al.*, 1999). Genes regulated by the filamentous growth MAPK contain two types of cis-acting regulatory sequences in their promoters, a binding site for the transcription factor Ste12p, termed a Pheromone Response Element (PRE) and a Tec1-binding-site (TCS) (Gustin *et al*., 1998). PRE and TCS in close proximity to one another,

together form a Filamentation Response Element (FRE). Tec1 and Ste12 usually activate transcription by acting on this FRE in a synergistic fashion. Tec1p can activate gene expression in the absence of Ste12p through a mechanism known as TCS control. *TEC1* expression can in turn be activated by only Ste12p via clustered PREs located in the *TEC1* promoter (Kohler *et al.*, 2002). The *PGU1* promoter region has been found to contain three TCS elements, but no neighbouring PREs, a typical FRE element was thus not found (Kohler *et al.*, 2002). *PGU1* expression was found to be independent of Ste12p activation and was only indirectly up-regulated via up regulation of *TEC1.* Microarray analysis showed that deleting the transcription factor *TEC1* or the upstream Mitogen Activated Protein Kinase Kinase (MAPKK) *STE7* caused a decrease in *PGU1* expression and overexpressing *TEC1* resulted in a significant increase in *PGU1* transcription (Madhani *et al.*, 1999). Strains in which any one of the invasive growth MAPK genes, *STE7, STE11* and *STE20* or the transcription factors activated by this pathway *STE12* and *TEC1* has been deleted, lost all pectolytic activity (Madhani *et al.*, 1999).

Radoi *et al*. (2005) showed that PG activity is repressed by glucose and activated by galactose and polygalacturonate in wine yeast strains. This induction in PG activity by galactose, was however not found in domesticated strains. The absence of PG induction by galactose in laboratory strains was speculated to be due to the presence of an unidentified gene silencer, that is absent in wine yeast strains.

Although the various authors did not use comparable strains, the results published are clearly contradictory. They could nevertheless indicate that regulation of pectolytic activity is complex and takes place on multiple levels in different strains, varying between functionality and presence of the *PGU1* gene, differences in the *PGU1* promoter region or differences in the genetic background of the strain, leading to silencing of *PGU1*.

The aim of this study was to unravel the regulation of polygalacturonase activity in different strains of *S. cerevisiae.* Five domesticated and two industrial strains were investigated. Strains were compared by sequencing the *PGU1* gene and its promoter and by investigating the transcription of *PGU1* and its two transcription factors *TEC1* and *STE12* using quantitative real time PCR (qRT-PCR). The EUROSCARF yeast deletion mutants library was screened in order to identify genes or regulatory pathways inhibiting pectolytic activity, based upon the fact that the wild-type reference strain BY4742 has no natural PG activity. The influence of carbon source on PG activity was

also investigated in these seven strains by looking at PG activity and transcription of *PGU1* in strains grown on galactose versus glucose.

3.3 Materials and Methods

3.3.1 Strains, plasmids and culture conditions

Bacteria and yeast strains used in this study are summarised in Table 3.1**.**

Plasmids were constructed and amplified in *Escherichia coli* DH5α, grown in Luria Bertani (LB) medium (Biolab diagnostics, Wadenville, South Africa). The medium was supplemented with 100 mg L^{-1} ampicillin for the selection of resistant bacteria when appropriate. *S. cerevisiae* wild type strains were grown in Yeast Peptone Dextrose (YPD) Broth (Biolab diagnostics) at 30°C on a rotary shaker at 150 r.p.m. For the selection of yeast transformants, Synthetic Complete (SC) medium containing 6.7 g L^{-1} yeast nitrogen base (Difco Scientific group, Waterfall Park, South Africa) and 20 g L^{-1} glucose supplemented with the appropriate amino acids to apply auxotrophic pressure.

Table 3.1 Microbial strains used in this study.

Strains	Genotype/description	Reference
S. cerevisiae strains		
BY4742 (S288C Background)	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*dls1*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; dls1Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*pgu1*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*; *pgu1Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*isw2*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; isw22Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*fus3*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3; fus3Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*set2*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; set2Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*qcr8*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; qcr8Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*vps25*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; vps25Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*ctk1*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; ctk1Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*vps32*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; vps32Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*vps33*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; vps33Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*hts3*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; hts33Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*esc1*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; esc13Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*dig1*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; dig1Δ0*	(Brachmann *et al.*, 1998)
BY4742 Δ*sir3*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; sir3Δ0*	(Brachmann *et al.*, 1998)

Table 3.1 (cont.)

Strains	Genotype/description	Reference
BY4742 Δ*dhh1*	*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; dhh1Δ1*	(Brachmann *et al.*, 1998)
W303	*MAT*a *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(Veal *et al.*, 2003)
CENPK42	*MAT*a *ura3-52, trp1-289, leu2-3_112, his3 Δ1, MAL2-8C, SUC2*	(Van Dijken *et al.*, 2000)
TCY1	*MATa; lys2Δ0; ura3Δ*	(Cunningham *et al.*, 1994)
VIN 13	Commercial wine yeast strain	Anchor Yeast, Cape Town, South Africa
L2323 Lalvin Rhône 2323	Commercial wine yeast strain	Lallemand, South Africa
∑1278b	*MATα; ura3-52; trp1Δ::hisG; leu2Δ::hisG; his3Δ::hisG;*	(Van Dyk *et al.*, 2005)
Escherichia coli strain		
DH5α	[F-φ80lacZΔM15Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk−, mk+) phoA supE44 thi-1 gyrA96 relA1 λ]	GIBCO-Invitrogen Life technologies, Mowbray, South Africa)

S. cerevisiae strains from the EUROSCARF deletion library were grown in YPD supplemented with 200 mg L^{-1} geneticin. Solid media contained 20 g L^{-1} agar. Bacteria and yeasts were cultured at 37 and 30°C, respectively.

All strains listed in Table 3.1 and BY4742 overexpressing *PGU1* or *TEC1,* respectively were cultured for RNA isolation. For each strain, a single colony was inoculated into 5 mL of Synthetic Complete (SC) media and incubated in a roller drum overnight at 30°C. The cells were pelleted by centrifugation at 5000g for 5 min. The pellet was washed in water and used to inoculate 10 mL of SC medium to an OD_{600nm} of 0.1**.** The following strains were inoculated into SC medium containing either 2% (w/v) glucose or 2% (w/v) galactose: L2323, TCY1, CENPK42 and BY4742. Cells were harvested during the logarithmic growth phase at OD_{600nm} of 1.0. The pellet was washed in water and flash frozen in liquid nitrogen.

3.3.2 DNA preparation and analysis

Chromosomal DNA from *S. cerevisiae* strains was isolated from overnight cultures grown in YPD at 30°C (Ausubel *et al.*, 1995).

All genes studied were amplified by using the polymerase chain reaction (PCR). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). All genes were amplified from genomic DNA, using an Applied Biosystems 2720 thermal cycler. Takara ExTaq enzyme and Takara buffer with $MgCl_2$ were used (Separations, Randburg, South Africa). The reaction mixture contained 250 µM of each nucleotide (dNTP), 200 ng DNA, 0.25 µM of each primer, and 0.2 mM $MgCl_2$.

DNA fragments to be sequenced (Table 3.2) were first cloned into a pGEM®-T Easy vector (pGEM®-T Easy Vector Systems, Promega, Whitehead Scientific, Cape Town, South Africa) according to the manufacturer's instructions. Plasmid DNA was isolated from positive transformants of *E. coli* DH5α. Both strands were sequenced in an ABI 3130XL Genetic Analyzer at the Central Analytical Facility (Stellenbosch University) using the universal M13 forward and reverse primers. PCR programs are listed in Table 3.3.

Table 3.2 DNA amplified

Gene/ DNA	Strain	Goal of PCR	Primers used for cloning	PCR program	Reference
PGU1	BY4742, ∑1278b, CENPK42, TCY1, FY23, L2323, VIN13,W303	cloning	5'PPGU1-BglII pP2 CTCGAGTTAACAGCTTGCACCAGA TCCAGATG 3'PPGU1-XhoI pP2 AGATCTATGATTTCTGCTAATTCATTACT TATTTCC	1	This study
TEC1	BY4742	cloning	Tecp2fw: 5'- GATCCTCGAGTTAATAAAAGTTCCCATG CGATTG Tecp1revv: 5'- AGTGAATTCATGAGTCTTAAAGAAGACG ACTTTGG	2	This study
1375 bp upstream of *PGU1*	BY 4742, L2323 CENPK42, TCY1	cloning	5'-PGU1PROM: 5'- TACGTCTAGAGGACAAGTCGACTTGTCC TGCCT 3'-PGU1PROM: 5'- GGATCCGGTCATTGCGTTTGTCCATCAA TGTGGGTAGA	3	Goetze and Van Rensburg (unpublished study)
ACT1	BY4742, ∑1278b, CENPK42, TCY1, FY23, L2323, VIN13,W303	qRT-PCR	Act fw 5'-TACCGGCCAAATCGATTCTC Act rev 3' CACTGGTATTGTTTTGGATT	4	(Divol *et al.*, 2006)
PGU1	BY4742, ∑1278b, CENPK42, TCY1, FY23, L2323, VIN13,W303	qRT-PCR	Pgu1fw2 5'- GTGCTTCGGGACATACCATT Pgu1rev2 3'- CGTCAACGCCAACTTTACAA	4	(Divol & Van Rensburg, 2007)
TEC1	BY4742, ∑1278b, CENPK42, TCY1, FY23, L2323, VIN13,W303	qRT-PCR	Tec1fw TCAAGCACAAAACCAACGAG Tec1rev ATGATAGGGTCAGCGAGTCC	4	This study

Table 3.3 PCR programs used

Program nr	Initial denaturation		Iteration			Final elongation	
	Temperature	Time	Cycle	Temperature	Time	Temperature	Time
1	94°C	10 min	30	95°C	30 s	72°C	7 min
				51°C	40 s		
				72°C	60 s		
2	94°C	10 min	30	95°C	30 s	72°C	7 min
				53°C	40 s		
				72°C	90 s		
3	94°C	10 min	30	95°C	30 s	72°C	7 min
				57°C	40 s		
				72°C	90 s		
4 [a]	95°C	10min	40	95°C	15 s		
				60°C	1 min		

[a] This reaction had an activation stage of 50°C for 2 min. Preceeding the initial denaturation and was followed by dissociation curve analysis: 95°C for 15 s, 60°C for 1 min and 95°C for 15 s**.**

3.3.3 Constructing overexpression vectors

Plasmids were constructed in order to overexpress the *TEC1* and *PGU1* genes in *S. cerevisiae* (Table 3.4). Correct cloning was verified by restriction analysis and sequencing.

Table 3.4 Plasmids used in this study

Plasmid	Description	Reference
pCEL13	Ap^R *URA3* $PGK1_P$ [a]-$PGK1_T$ [b]	(Gundllapalli *et al.*, 2006)
pHVXII	Ap^R *LEU2* $PGK1_P$ -$PGK1_T$	(Volschenk *et al.*, 1997)
pCP2	Ap^R *URA3 MFα* $PGK1_P$-*PGU1* -$PGK1_T$ (*PGU1* gene cloned from BY4742)	This study
pCP9	Ap^R *URA3* $PGK1_P$-*PGU1* $PGK1_T$ (*PGU1* gene cloned from VIN13)	This study
pTEC	Ap^R *LEU2* $PGK1_P$-*TEC1* -$PGK1_T$	This study

[a] $PGK1_P$ Phosphoglycerate kinase promoter
[b] $PGK1_T$ Phosphoglycerate kinase terminator

All genes were first cloned into the pGEM®-T Easy vector, sequenced and subcloned into the appropriate expression vector. The *PGU1* gene was excised from the pGEM®-T Easy plasmids by restriction with *Bgl*II and *Xho*I (Roche Diagnostics, Randburg, South Africa) and ligated into the corresponding sites of the pCEL13 expression vector. *TEC1* was excised from the pGEM® T-easy vector by with *Eco*RI and *Xho*I (Roche Diagnostics) and ligated into the corresponding sites of pHVXII. Restriction endonuclease-digested DNA was eluted from agarose gels by using the Zymoclean™ gel recovery kit (Zymo research, Orange, CA, USA) according to the manufacturer's instructions. Standard methods were used for the restriction and ligation of DNA, plasmid transformation into *E. coli*, and agarose-gel electrophoresis (Maniatis *et al.*, 1989).

All constructs were amplified in *E. coli* DH5α and isolated with the Qiaprep® Spin Mini-prep Kit (Qiagen) for yeast transformation.

3.3.4 Yeast transformation

S. cerevisiae, strain BY4742, was transformed with pCP2, pCP9 and pTEC. Deletion mutants, in which the deleted gene is normally activated by Tec1p, were transformed with pTEC. All transformations were carried out using the lithium-acetate method described by (Gietz & Schiestl, 1991). The plasmids were maintained as autonomously replicating plasmids in the yeast cells by maintaining auxotrophic selective pressure. Transformation was verified by colony PCR analysis (results not shown).

3.3.5 Screening for Polygalacturonase activity

Strains were screened for PG activity with a modified plate assay described by Masoud & Jespersen (2006). Five µL of an overnight culture containing 10^4 cells were spotted on PG plates (1.25% polygalacturonic acid (Sigma), 0.67% yeast nitrogen base (YNB, Difco™), 1% glucose (Merck), 2% agar (difco), 0.68% Potasium phosphate pH 4.0) and incubated for three days at 30°C. In order to determine the effect of galactose on PG activity, cells were cultured in Yeast Nitrogen Broth (YNB, Difco™), with 1% (w/v) galactose replacing glucose as carbon source. Plates were supplemented with the necessary amino acids to maintain auxotrophic pressure in transformed strains. Degradation halos were visualised by staining plates with 6M HCl after washing the colonies of with distilled water. Three independent replicate experiments were performed for each strain. This assay was semi-quantitative and only used to compare PG activity between strains. Surface area of degradation halos was measured and more than a 25% change in surface area was seen as a significant change in activity.

3.3.6 Quantitative real-time PCR (qRT-PCR)

3.3.6.1 RNA isolation

qRT-PCR was carried out to check the expression level of *PGU1* as well as *TEC1* and *STE12,* transcription factors that bind in the *PGU1* promoter. RNA was isolated in late exponential phase. Results are given as relative quantification, relating the PCR signal of target transcript to the transcription level of the same gene in the BY4742 strain. *ACT1* was used to normalize data obtained using qRT-PCR.

Total RNA was isolated for qRT-PCR from all the strains mentioned in Table 3.1 and BY4742 overexpressing *PGU1* and *TEC1,* respectively. RNA was isolated from the

yeast cells using acid phenol as described for the hot phenol RNA isolation protocol (Ausubel *et al.*, 1995). The quality of RNA was evaluated by gel electrophoresis and by measuring absorbance at 260 nm and 280 nm. Absorbance at 260 nm was used to determine the concentration.

3.3.6.2 Reverse transcription

RNA samples were treated with Dnase I (Roche) to remove any residual DNA contamination, following the manufacturer's instructions. The absence of DNA was confirmed by end point PCR. RNA was converted into cDNA by using the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. 1 µg of total RNA was used as template.

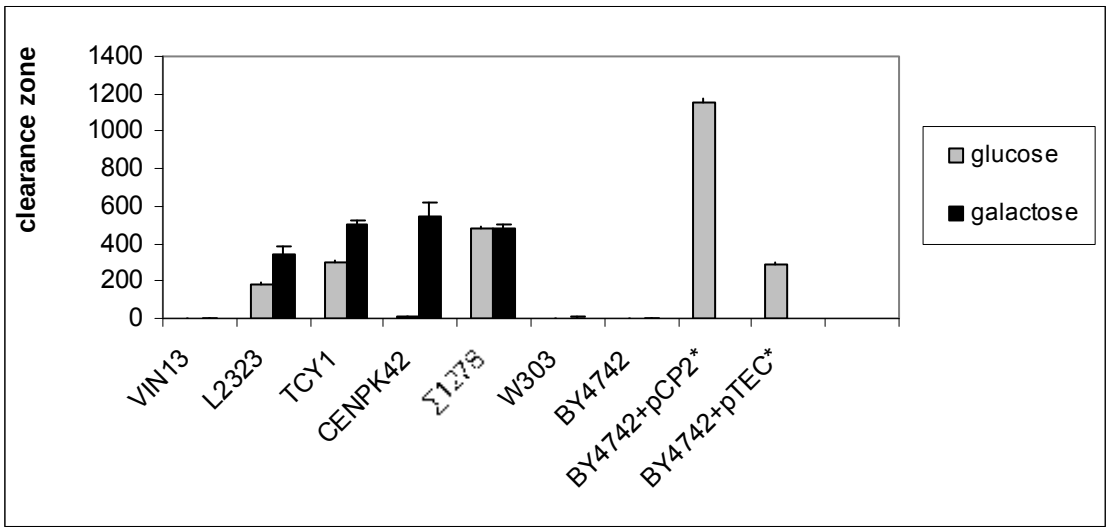
3.3.6.3 qRT-PCR

qRT-PCR were performed on cDNA samples originating from two independent replicate experiments. The experiments were carried out using SYBR-Green dye in a 7500 Real-Time PCR System (Applied Biosystems). Reactions contained Power KAPATaq Ready Mix (KAPA Biosystems, Cape Town, South Africa), forward and reverse primers (0.1 µM each) and a cDNA template (20 ng). Primers in Table 3.2 and PCR reaction conditions in Table 3.3 were used. For each PCR product, melting curves were determined according to the ABI guidelines, ensuring specific amplification of the target gene. Quantitative values were obtained as the threshold PCR cycle number (Ct) when the increase in the fluorescent signal of the PCR product showed exponential amplification. Transcription of each gene was normalized to that of *ACT1* in the same sample. The cycle threshold (Ct) value for each reaction was determined using the Sequence Detection System, 7500 Real-Time PCR System software package (Applied Biosystems). Ct values were used to calculate the expression of *PGU1* and *TEC1* relative to that of the same gene in the BY4742 strain. Fold change was calculated via the $2^{-\Delta\Delta Ct}$ method for each sample in triplicate, in which 1 indicates no change in abundance (Livak & Schmittgen, 2001).

3.4 Results

3.4.1 Polygalacturonase activity of different *Saccharomyces cerevisiae* strains

Polygalacturonase activity was compared between strains grown on glucose or galactose by PG plate assays (Figure 3.1).



* PG activity of these strains was only measured on glucose.

Figure 3.1 Semi quantitative plate assay comparing PG activity between different *S. cerevisiae* strains. Activity was compared between wild type strains using either glucose or galactose as carbon source, as well as the BY4742 strain overexpressing *TEC1* (BY4742+pTEC) and *PGU1* (BY4742+pCP2). Activity was measured as surface area of clearance zone in mm^2 on PG plates after addition of HCl

The only strains that had significant activity when cultured on glucose were the domesticated strains Σ 1278b and TCY1 and the wine yeast strain L2323. When cultured on galactose the strains TCY1, CENPK42 and L2323 showed a significant increase in PG activity compared to when cultured on glucose as carbon source. The strains that showed an increase in PG activity when cultured on galactose, TCY1 and CENPK42, had an significantly slower growth rate when cultured on galactose than any of the other domesticated strains (data not shown). Overexpressing *TEC1* conferred pectolytic ability to the BY4742 strain. Overexpressing *PGU1* resulted in a BY4742 strain with stronger PG activity than any of the wild type strains studied (Figure 3.1).

The presence of the *PGU1* gene was confirmed in all the strains listed in Table 3.2 (data not shown). Analysis was performed by PCR, yielding a product of 1086 bp with the primers designed to amplify the entire *PGU1* gene.

3.4.2 Sequence analysis of *PGU1*

The nucleotide sequence of *PGU1* for the following laboratory strains: BY4742, ∑1278b, TCY1, CENPK42, W303 and wine yeast strains VIN13 and L2323 was determined. The coding region was confirmed to be 1086 bp long in each strain. The nucleotide sequence was found to be 100% to that of strain S288C for all strains except for the wine yeast strain, VIN13 (Figure 3.2).

a (ATG)1….853 877 900

PGU1(BY 4742) GTTATCGAAGGCGATTATTTGAATAGTAAGACTACTGGAACTGCTACA

PGU1(VIN13) GTTATCGAAGGCGATTATTTGAATGGTAAGACTACTGGAACTGCTACA

GTTATCGAAGGCGATTATTTGAAT GTAAGACTACTGGAACTGCTACA

b

Pgp(BY4742) VIEGDYLNSKTTGTATG

Pgp(VIN13) VIEGDYLNGKTTGTATG

Consensus VIEGDYLN KTTGTATG

Figure 3.2 a) Partial nucleotide sequence of the polygalacturonase gene, *PGU1,* 853-900 bp downstream of the ATG start codon from strains BY4742 and VIN13, showing a single nucleotide polymorphism, 877 bp downstream of the ATG start codon. b) Deduced amino acid sequence of this region.

The VIN13 *PGU1* had one single nucleotide polymorphism when compared to that of BY4742; Adenine substituted Guanine 877 bp downstream of the ATG start codon (Figure 3.2a). This nucleotide change resulted in an amino acid change: Serine $_{293}$ substituted Glycine $_{293}$ (Figure 3.2b). Only the region of the gene in which the polymorphism occurred is shown. To determine if the lack in PG activity in the VIN13 strain was due to the amino acid change shown in Figure 3.2, rendering the *PGU1* allele of VIN13 defective; the PG activity of polygalacturonase encoded by *PGU1* from VIN13 and BY4742 was compared. This was done by overexpressing each gene in BY4742. Plasmids were constructed to overexpress the *PGU1* alleles cloned from BY4742 (pCP2) and VIN13 (pCP9). Activity resulting from overexpressing *PGU1* cloned from BY4742 and VIN13 were found to be identical (Figure 3.3).

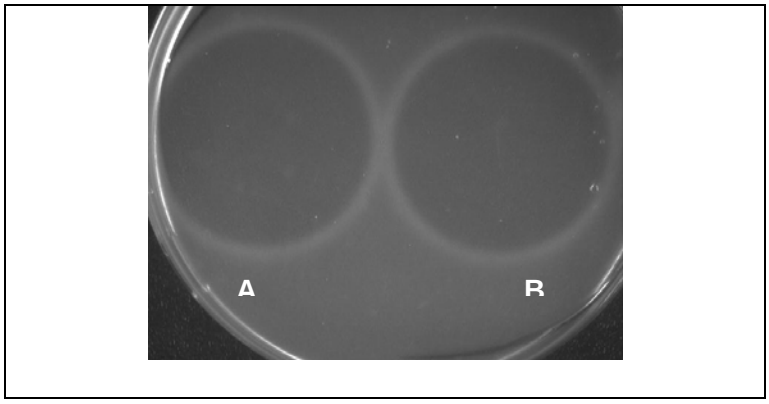
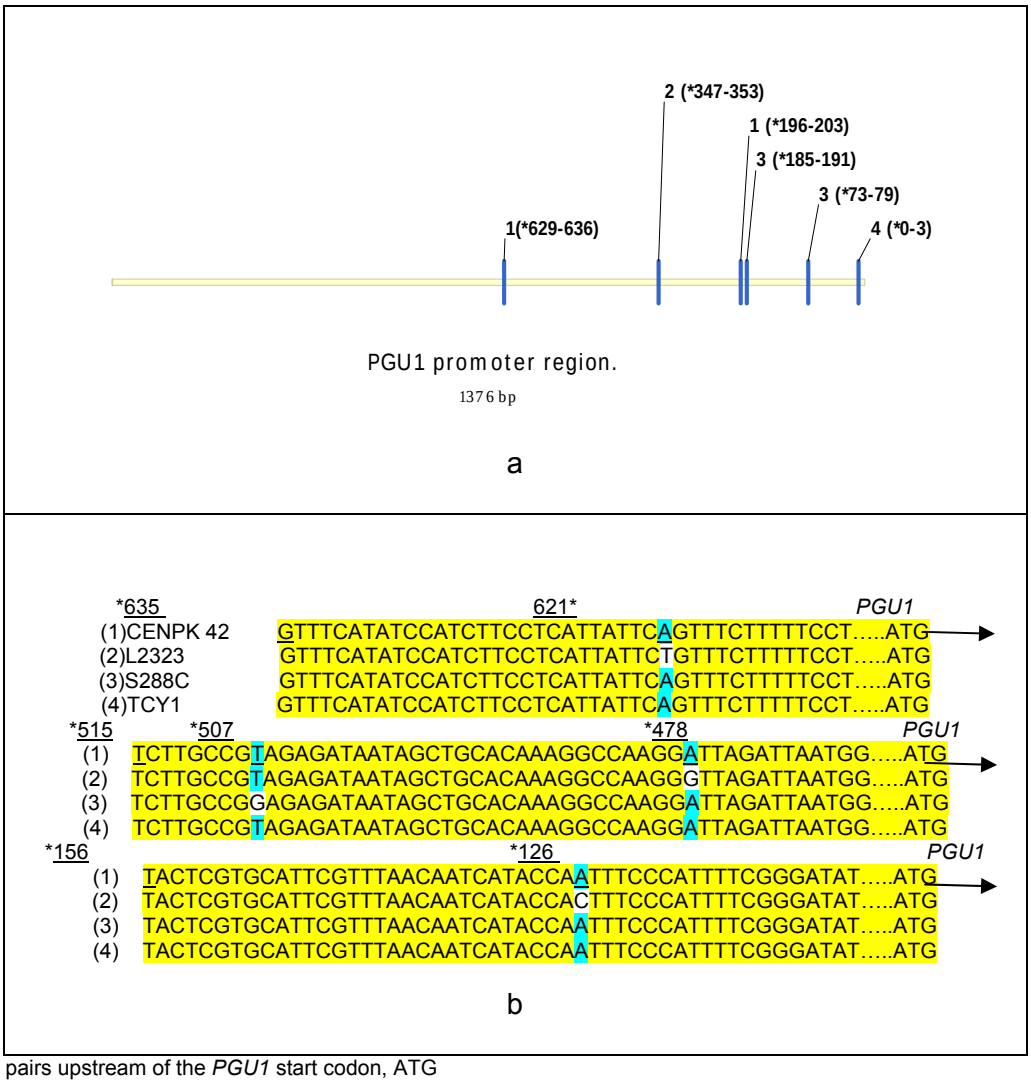


Figure 3.3 PG plate assay comparing PG activity between BY4742 transformed with **a)** pCP2 and **b)** pCP9.

To determine if upregulation of *PGU1* transcription by galactose in certain strains was due to differences in the promoter region of *PGU1* in these strains, 1375 bp

upstream of the *PGU1* start codon were sequenced from strains that showed an increase in PG activity when cultured on galactose. CENPK42, TCY1 and L2323 showed such an increase and were compared with strains that did not show an increase, BY4742 and S288C. The sequence of this region was identical for the CENPK42, TCY1 and BY4742 strains; the wine yeast strain L2323 had three Single Nucleotide Polymorphisms (SNPs) when compared to these two strains (Figure 3.4b). None of these SNPs were in putative transcription factor binding sites. The promoter region of eight different yeast strains was sequenced (data not shown). All promoters shared a common polymorphism when compared with the S288C strain. 507 bp upstream of the ATG; Thiamine was substituted with Guanine (Figure 3.4b). Except for this one nucleotide the CENPK42 and TCY1 promoter regions were identical to that of BY4742. The Transcription Element Search System (TESS) was used to predict transcription factor binding sites in the *PGU1* regulatory region (http://www.cbil.upenn.edu/cgi-bin/tess/tess). A putative Gal4p cis-binding site, two PRE and three TCS elements were identified (Figure 3.4a). Two of the TCS elements were in the reverse orientation compared to the other transcription factor binding sites.



* base pairs upstream of the *PGU1* start codon, ATG

Figure 3.4 a) The regulatory region of *PGU1* in the S288C strain, showing the following putative binding sites: PRE (1) - AGTTTCA, TCS (2) – CATTCT, the reverse complement of TCS (3)- AGAATG, and the *PGU1* start codon (4) –ATG.
b) Alignment of regions in the promoter area of the *PGU1* gene in the strains CENPK42, TCY1, S288C and L2323 strains that showed variation between these strains.

3.4.3 Transcription of *PGU1* and transcription factors *TEC1* and *STE12*

Wine yeast strains had higher transcription of *PGU1* than laboratory strains with the same PG activity. Transcription of *PGU1* increased alongside PG activity for all strains studied, except W303. This strain had high *PGU1* transcription (Figure 3.5) and almost no PG activity (Figure 3.2).

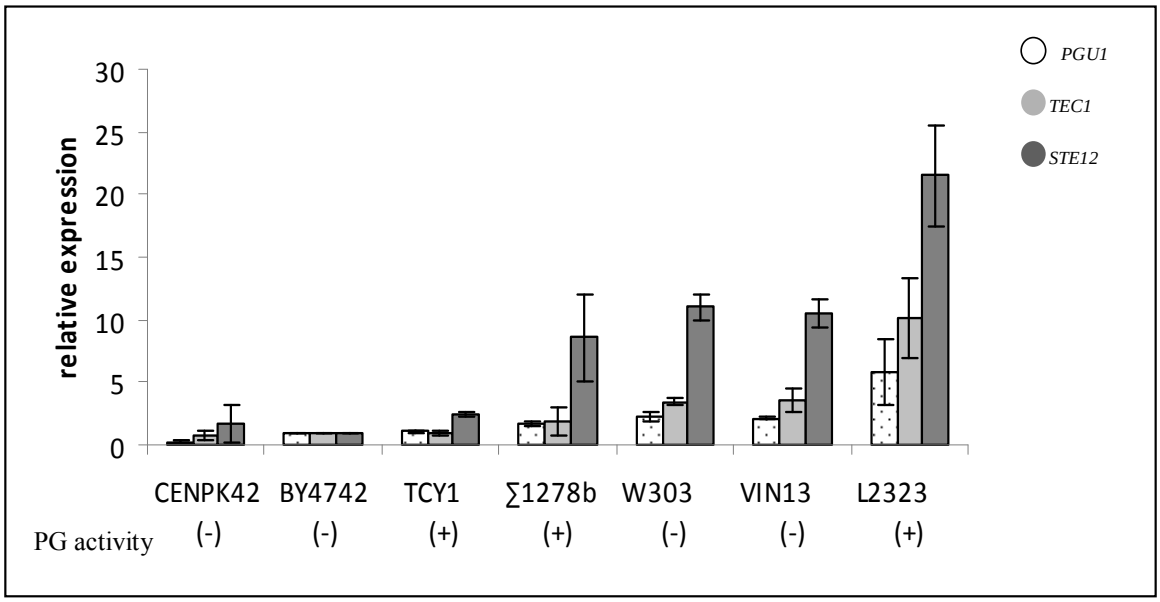


Figure 3.5: Quantitative PCR comparing the transcription level of the *PGU1, TEC1* and *STE12* genes between different strains. Results are given as relative quantification, relating the PCR signal of target transcript to the transcription level of the same gene in the BY4742 strain. PG activity positive (+) or negative (-) is indicated for each strain.

There was a linear relationship between transcription of *PGU1* and that of its transcription factors *TEC1* and *STE12* between strains (Figure 3.5). Transcription of *PGU1* was compared between BY4742 and the same strain overexpressing *PGU1* from pCP2 and *TEC1* from the pTEC plasmid respectively, both genes were overexpressed by fusion with the *PGK1* promoter (Figure 3.6).

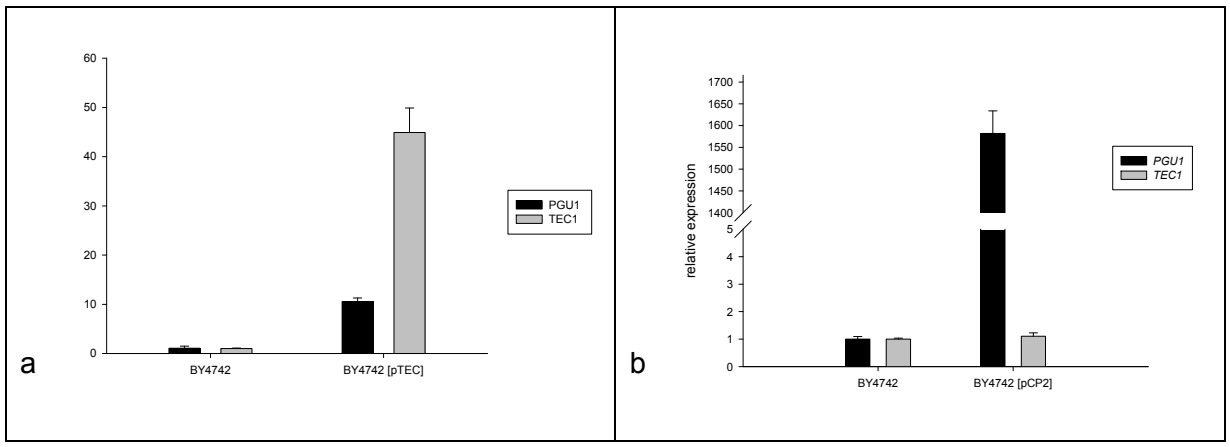


Figure 3.6 Quantitative PCR focusing on the *PGU1* and *TEC1* genes in: a) BY4742 wild type strain and in same the strain overexpressing *TEC1* and b) BY4742 wild type strain and in the same strain overexpressing *PGU1.* Results are given as relative quantification, relating the PCR signal of target transcript to the transcription level of the same gene in the BY4742 strain. (This figure was not included in the manuscript published in FEMS Yeast Research)

Overexpressing *PGU1* conferred PG activity to *S. cerevisiae,* confirming the result obtained by Jia & Wheals (2000). Overexpressing *TEC1* resulted in a sufficient

increase in *PGU1* transcription to confer PG activity to BY4742 (Figure 3.1) confirming the result obtained by Madhani *et al.* (1999).

3.4.4 Influence of carbon source on *PGU1* transcription

TCY1, CENPK42 and L2323 were the only strains that showed an increase in PG activity when grown on galactose compared to glucose (Figure 3.1). *PGU1* transcription was compared between strains grown on glucose and galactose. Figure 3.7 shows the increase in *PGU1* transcription when grown on galactose compared to glucose.

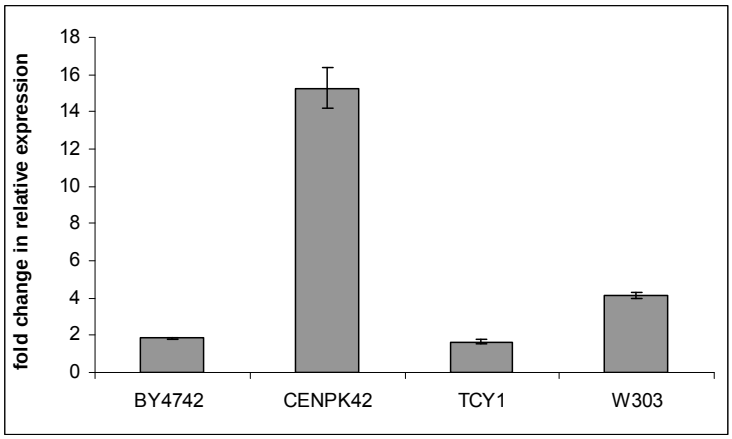


Figure 3.7 The increase in transcription of the *PGU1* gene when strains were cultured on galactose compared to glucose. Increase was calculated as fold change in transcription of the *PGU1* gene between cells grown on glucose and galactose. Fold changes were calculated as transcription when grown on galactose/Transcription when grown on glucose. All transcription levels were relative to BY4742 grown on glucose.

The CENPK42 and TCY1 strains showed a significant increase in PG activity when cultured on galactose compared to glucose (Figure 3.1). Replacing glucose with galactose resulted in the following increases in *PGU1* transcription: 15 fold for CENPK42, 2-fold for TCY1 and 4-fold for W303 (Figure 3.7). Transcription of *PGU1* in the strain BY4742 also increased 2-fold even though this strain showed no increase in activity.

3.4.5 Screening for activators and repressors of PG activity

The EUROSCARF deletion mutant library was screened for strains in which the deletion of a single gene resulted in an increase in pectolytic activity. Strains were screened for PG activity by semi-quantitative plate assays as described. Many genes were found responsible for the recovery of a weak PG activity (data not shown). The mutants in which the genes in Table 3.5 were deleted showed the recovery of the strongest PG activity.

Table 3.5 Gene Ontology of genes identified as encoding inhibitors of PG activity

Standard name	Systemic name	PG activity in BY4742 deletion mutant	Molecular function	Biological process
FUS3	YBL016W	strong	MAP kinase activity	Cell cycle arrest, invasive growth, negative regulation MAPKKK cascade
DIG1	YPL0149C	strong	Transcription binding factor, DNA binding	Invasive growth during glucose limitation
CTK1	YKL139W	strong	Cyclin dependant protein kinase activity	mRNA processing
VPS32	YLR025W	strong	Protein binding	Vacuole transport, Protein catabolism
SIR3	YLR442C	weak	Chromatin binding, DNA binding, nucleosomal histone binding, structural constuent of chromatin	heterochromatin formation, Chromatin silencing at the mating type loci and telomeres, loss of chromatin silencing during ageing
DLS1	YJL065C	weak	Unknown	Chromatin silencing at the telomeres
HST3	YOR025W	weak	DNA binding, histone deacetylase activity	Chromatin silencing at the telomeres, histone deacetylation, fatty acid metabolism
ESC1	YMR219W	weak	Unknown	Chromatin silencing at the telomeres
ISW2	YOR304W	weak	ATPase activity	Chromatin remodelling, Chromatin silencing at telomeres
QCR8	YJL166W	weak	Cytochrome c reductase activity	Aerobic respiration, Ubiquinol to cytochrome c
SET2	YJL168C	weak	Histone methyltransferase	Histone methylation, Transcription regulation, RNA elongation
VPS25	YJR102C	weak	Protein binding	Negative regulation of transcription by glucose, protein targeting to vacuaole, ubiquitin dependent protein catabolism
VPS33	YLR396C	weak	ATP binding, contributes to phosphoinositide binding	Golgi to endosome transport, late endosome to vacuole transport, piecemeal microautophagy of nucleus, vacuole fusion, non-autophagic
Dubious ORF [a]	YJL064W	weak	Unknown	Unknown

[a] overlaps *DLS1*

All accessions from Saccharomyces Genome Database (SGD)

It has been shown that over-expressing *TEC1* in the strain BY4742, enables this strain to degrade polygalacturonic acid (Madhani, *et al.,* 1999). Enhancers of *PGU1* transcription activated by *TEC1* were identified by looking for strains in which a single gene deleted resulted in a strain with lower PG activity then the wild type BY4742 strain overexpressing *TEC1*. All genes activated by *TEC1* were identified by the YEASTRACT

website (www.yeastract.com). BY4742 strains in which these genes were individually deleted were obtained from the EUROSCARF yeast deletion library and were transformed with the pTEC plasmid (Table 3.4). Enhancers were defined as genes whose deletion resulted in more than 25% difference in PG activity compared with that of the wild type strain and with a significance threshold below 0.05 according to the Student's test. *DHH1* (YDL160C) and *PUF4* (YGL014W) were identified as enhancers of PG activity by this screen (Figure 3.8).

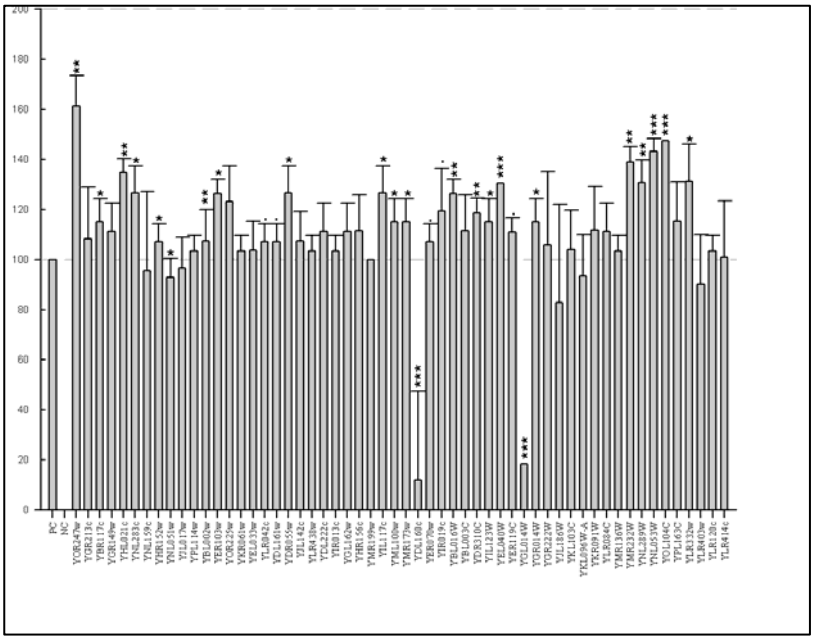
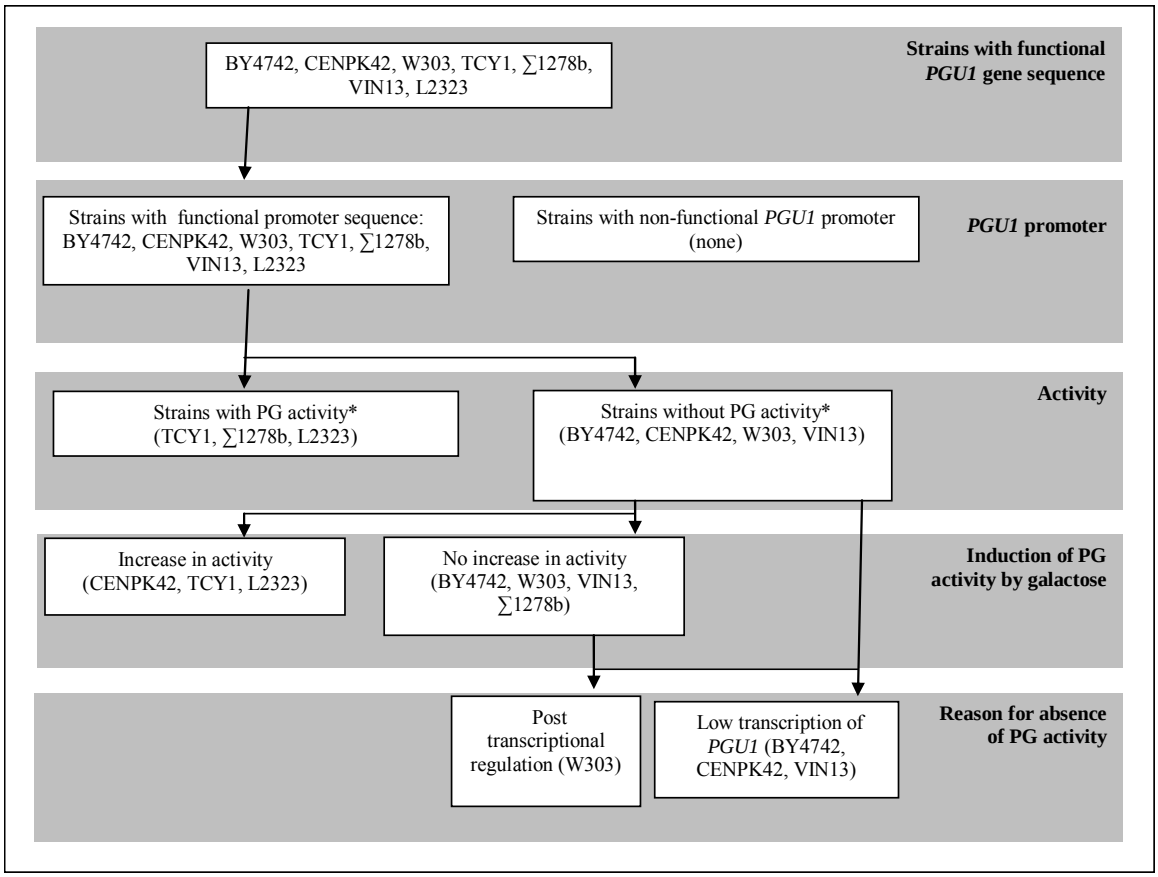


Figure 3.8 Comparison between the surface area (mm^2) of the polygalacturonate-degradation zones of BY4742 deletion mutants overexpressing *TEC1* and the wild-type strain overexpressing this gene. Activity is expressed as the percentage activity of the deletion mutant when compared to the wild type strain. *TEC1* was over-expressed in all strains compared. The names of the ORFs indicate the corresponding deletion mutants. Differences significance: p<0.1; *:p<0.05;**p<0.01; ***: p<0.001 (Student's test, BY4742 wild-type was used as the reference).

3.5 Discussion

Based on contradictory results found in literature we tried to elucidate how pectolytic activity is regulated in *S. cerevisiae* and to determine whether this regulation differs between strains. Regulation of PG activity in different strains of *S. cerevisiae* investigated in this study is summarized in Figure 3.9.



*When grown in glucose.

Figure 3.9 Flow diagram illustrating how PG activity is regulated in different strains of *S. cerevisiae*.

The *PGU1* sequence of the S288C strain published in the *Saccharomyces* Genome Database (www.yeastgenome.org) was identical to the *PGU1* alleles found in six of the seven strains investigated in this study. Gognies *et al*. (1999) described this allele as dysfunctional. Contradicting this, the results of our study proved that this allele was functional by overexpression. The wine yeast strain VIN13 had one single nucleotide polymorphism when compared with this sequence. This strain lacks PG activity. In order to determine if the lack in activity in this strain is due to this mutation, the *PGU1* gene was cloned from both the VIN13 and BY4742 strains. Both these alleles of the *PGU1* gene were over-expressed on multicopy plasmids under control of the *PGK1* promoter in the BY4742 strain, a strain that cannot degrade polygalacturonic acid. Both these strains gained the same PG activity (Figure 3.3) showing the polygalacturonase encoded by *PGU1* in VIN13 was just as efficient in degrading polygalacturonic acid as the gene possessed by other strains and was thus not the reason why this strain lacked PG activity. Since a mutation in the *PGU1* gene was not the reason for variation in PG activity between any of the laboratory or wine yeast

strains studied, it is reasonable to assume variation in *PGU1* gene sequence is not a common mechanism responsible for variation in PG activity.

In previous studies, the expression of the *PGU1* gene was compared between strains by cloning the *PGU1* promoter regions of different strains, making *LacZ* fusions and by then measuring the resulting β-galactosidase activity (Gognies *et al.*, 2001). The plasmids harbouring these *PGU1* promoter-*LacZ* fusions were not always transformed into the strain from which the promoter was cloned. Transcription of the *PGU1* gene is not only dependent on the promoter sequence but can be influenced by the genetic background of the strain (Hirose *et al.*, 1999). Fusing promoters cloned from various strains with a reporter gene will therefore not give an accurate reflection of the transcription level of the gene in each strain. Corresponding with the study done by Hirose *et al.* (1999), we found strains with identical *PGU1* sequence, but varying polygalacturonase activity to have identical promoter sequences. The strains TCY1, CENPK42 and BY4742 have varying PG activity (Figure 3.1), but identical *PGU1* promoter sequences (Figure 3.4b). We quantified and compared transcription of *PGU1* between different strains by qRT-PCR (Figure 3.5). Since *PGU1* transcription varies between strains with identical *PGU1* promoter sequences, this confirms that variation in *PGU1* transcription is due to differences in the genetic backgrounds of these strains and not due to differences in the *PGU1* promoter sequence. PG activity is regulated by *PGU1* transcription: higher *PGU1* transcription resulted in higher PG activity, in all but the W303 strain. Since PG activity increases with an increase in *PGU1* transcription it indicates that the level of PG activity is regulated by *PGU1* transcription. This was confirmed by increasing *PGU1* expression by different levels. It has been shown that overexpressing *TEC1* confers PG activity to a strain that does not usually have this ability (Madhani, *et al.,* 1999). In our study higher transcription of *PGU1* was obtained when the gene was directly overexpressed compared to when *TEC1* was overexpressed, resulting in higher activity (Fig 3.6).

In the W303 strain, the lack of PG activity was not due to an absence of *PGU1* transcription. It can be seen from Figure 3.5 that higher *PGU1* expression levels are required by wine yeast strains to have PG activity than domesticated strains. Heterologous overexpression of *PGU1* seems to confer PG activity to all strains investigated (Van Wyk, 2009). Since PG activity was absent in the W303 strain, even though this strain had higher expression than strains with activity, expression could be below the threshold required to confer PG activity to this strain due to differences in post-transcriptional regulation of *PGU1.*

The filamentous growth MAPK pathway has been shown to control *PGU1* expression. The *PGU1* promoter region has been found to contain three TCS elements, but no neighboring PRE; a typical FRE element was thus not found (Madhani *et al.*, 1999). However, by *in silico* analysis of the promoter sequence, we found two PRE elements of which one was in close proximity of a TCS element potentially forming a FRE (Figure 3.4a). These PRE elements were in the reverse orientation when compared with other transcription factors binding sites. Since filamentous growth MAPK genes are required for pectolytic activity, the transcription level of these genes could be responsible for the difference in pectolytic activity between strains. The transcription level of the *TEC1* and *STE12* transcription factors were compared between strains. There was a direct correlation between the transcription level of *TEC1* and *STE12* and that of *PGU1* (Figure 3.5). Strains with higher PG activity had higher *PGU1* transcription. Transcription level of *PGU1* between strains varied alongside the transcription level of the transcription factors *TEC1* and *STE12,* showing these transcription factors determined the level of PG activity in different strains.

In agreement with the study conducted by Radoi *et al.* (2005) we also found pectolytic activity to be induced by galactose in certain strains. However in contrast with the findings by the latter authors, we found the potential induction by carbon source to be independent on whether the strain is a domesticated or industrial yeast strain. In this study, we showed that this increase in enzymatic activity was due to an increase in *PGU1* transcription (Figure 3.7). The domesticated CENPK42 and TCY1 strains showed a significant increase in PG activity when glucose was replaced by galactose as carbon source (Figure 3.1). Galactose did not induce pectolytic activity in all wine yeast strains, as seen for the wine yeast strain VIN13 (Figure 3.1). CENPK42 showed the largest increase in PG activity in the presence of galactose. This strain also showed the most significant increase in *PGU1* transcription in the presence of galactose. There was however no difference in the promoter sequence between strains, whether pectolytic activity could be induced by galactose or not (Figure 3.4b). Galactose only activated PG activity in CENPK42, TCY1 and the wine yeast strain L2323. The strain W303 showed a significant increase in *PGU1* transcription when grown on galactose compared to glucose (Figure 3.7), but did not show any increase in activity. This again confirms that *PGU1* activity is regulated at post-transcriptional level in this strain.

A Gal4p binding site was identified 600 and 632 bp upstream of the *PGU1* coding region by Radoi, *et al.* (2005). The sequence for this Gal4p binding site was however not shown by the authors. The 17-mer Gal4p binding site:

CGGAAGACTCTCCTCCG, identified by Giniger & Ptashne (1988) was not found to be located upstream of the *PGU1* coding region in any of the strains investigated in this study. The region in which the putative gal4p binding site was located according to Radoi, *et al.* (2005) was identical in the promoter region of all domesticated strains in this study; one SNP was found in the promoter region of the wine yeast strain L2323 in our study and it was not at this location (Figure 3.4b).

Since the promoter regions of most of the yeast strains investigated were identical, differences in activation of transcription by galactose might be due to strain related differences in regulation of Gal4p activation. Further investigation is necessary to unravel the potential role of Gal4p.

In order to identify *S. cerevisiae* genes encoding inhibitors of pectolytic activity the EUROSCARF deletion library was screened for mutants in which the deletion of a single gene resulted in an increase in pectolytic activity. The deleted gene responsible for the recovery of PG activity was identified as an inhibitor of PG activity. Fourteen main inhibitors of PG activity were identified by this screen. Two of the PG inhibitors, *DIG1* and *FUS3* are components of the mating and filamentous growth MAPKs. *FUS3* deletion has previously been shown to result in an increase in PG activity (Madhani *et al.*, 1999). Fus3p induces ubiquitination of Tec1p (Chou *et al.*, 2004). *FUS3* deletion has been shown to result in hyperactivation of Ste12p and Tec1p (Madhani *et al.*, 1999). This deletion thus resulted in an increase in *PGU1* transcription and PG activity. Dig1p also inhibits Ste12p (Roberts *et al.*, 2000). Lower Ste12p can subsequently result in a decrease in *PGU1* transcription.

The other genes identified by screening the deletion library did not have such an obvious relationship with *PGU1* transcription and a global effect would be expected if any of these genes are mutated. An alternative explanation might be foreseen. In a genome-wide screening for genes affecting telomere length, 150 genes were previously identified (Askree *et al.*, 2004). The separate deletion of three genes, *CTK1, VPS25* and *VPS32,* resulted in a shortening of telomeres. These genes were also identified as resulting in an increase in PG activity when we screened the deletion library. *PGU1* is located at position 722806..723891. It is subtelomeric since its location is within 25 kb of the right telomere of chromosome X (Barton *et al.*, 2003; Galibert *et al.*, 1996). Elongated telomeres can result in repression of transcription of subtelomeric genes (Park & Lustig, 2000). Between different strains, the mean length of telomeres can vary as much as two-fold (Constable *et al.*, 1990). Since telomere length affects transcription level of subtelomeric genes, the variation in telomere length can be responsible for

variation in *PGU1* expression between strains. We identified 5 genes (*DLS1, ISW2, SIR3, HST3* and *ESC1)* involved in gene silencing by telomere position effect (tpe), as inhibitors of PG activity in the BY4742 strain, indicating this is a possible method of gene silencing in *S. cerevisiae.* YJL064W codes for an unknown open reading frame (ORF) and was also identified as an inhibitor of PG activity in the deletion library screen. Since YJL064W overlaps with *DLS1,* its deletion results in a partial deletion of *DLS1*. It is most likely that the increase in PG activity observed after deleting YJL064W was due to the deletion of *DLS1.*

Invasive growth has been shown to be induced in a BY4742 ∆ *isw2* mutant (Trachtulcova, *et al.,* 2004), in this study we showed this mutation also induced PG activity. *ISW2* codes for a chromatin remodelling complex known to be involved in silencing of subtelomeric genes (Iida & Araki, 2004). Compacting DNA into heterochromatin limits the access of proteins that drive transcription on the DNA template. Since genes responsible for PG activity and invasive growth is located at subtelomeric positions, the deletion of this gene could result in activation of both these phenotypes due to silent chromatin structure not being established at these subtelomeric areas. Moreover, *SET2* was also identified as an inhibitor of PG activity. Set2 is a histone methyl transferase (HMT) and is involved the establishment of silent chromatin structure through post translational modification of histones (Strahl *et al.*, 2002).

Several of the genes identified as inhibitors of PG activity (i.e. *SET2, DLS1, ISW2, SIR3, HST3* and *EST1)* are involved in epigenetic regulation of transcription. Some of these genes have been shown to inhibit transcription through the Telomere Position Effect (TPE) or to be involved in determining telomere length. *PGU1* borders the telomere periphery, the inhibitors of PG activity identified by this study indicates that TPE and variation in telomere length between strains could have an influence on strain specific PG activity, further investigation is needed.

Enhancers of *PGU1* transcription activated by *TEC1* were identified by looking for deletion mutants with lower PG activity than the wild type strain when *TEC1* was overexpresssed in these strains. Mutants, in which the genes that are activated by *TEC1* were separately deleted, were screened for PG activity. Enhancers were identified as genes that resulted in more than 25% decrease in activity when compared to the control (Figure 3.8). Two genes were identified by this screening: *DHH1* and *PUF4*. *DHH1* is important in mRNA export and translation (Coller *et al.*, 2001). It has been shown that Dhh1p controls yeast filamentation by regulating *STE12* translation

(Ka *et al.*, 2008). A Δ*dhh1* mutant caused a decrease in Ste12p level, while *STE12* transcription level remained the same. The decrease in Ste12p resulted in severe mating defects (Ka *et al.*, 2008). This decrease in Ste12p upon deletion of *DHH1* explains the decrease in PG activity in this strain. This further confirms that both transcription factors of *PGU1* (i.e. *TEC1* and *STE12*) are required for PG activity. Since a Δ*dhh1* mutation results in loss of both invasive growth (Minhan Ka, 2008) and PG activity via a decrease in the amount of Ste12p available, it again shows that these two phenotypes are co-regulated. *PUF4* is a member of the PUF protein family; these proteins possess Pumillio homology domains that confer RNA binding activity. Puf4p preferentially binds mRNAs encoding nucleolar ribosomal RNA processing factors (Gerber *et al.*, 2004). It was recently noticed that a Σ1278 strain loses the filamentous and invasive growth phenotypes upon deletion of *PUF4* (Jin *et al.*, 2008). The loss in PG activity upon deletion of *PUF4* again highlights the co-regulation of filamentous growth and PG degradation.

3.6 Conclusion

It has been proposed that polysaccharide degradation and invasive growth are coordinated foraging behavior by *S. cerevisiae.* In this study we showed that expression of *PGU1* in different strains is directly related to expression of the transcription factors *STE12* and *TEC1* in each strain. These transcription factors regulate transcription of both *PGU1* and *FLO11*, co-regulating these phenotypes at a genetic level. It has been shown that epigenetic mechanisms control expression of virulence genes in a variety of pathogens. The *FLO* gene family responsible for flocculation and pseudohyphal growth has been shown to be under epigenetic regulation.

Screening the *S. cerevisiae* genome for activators and inhibitors of PG activity identified genes indicating that an epigenetic effect might be involved in regulating *PGU1* transcription. Results obtained in this study indicate that invasive growth and pectin degradation are co-regulated by genetic and potentially epigenetic mechanisms.

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3.8 References

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Chapter 4

Research results

Epigenetic regulation of *PGU1* transcription in *Saccharomyces cerevisiae*

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Epigenetic regulation of *PGU1* transcription in *Saccharomyces cerevisiae*.

Campbell Louw[1], Philip R. Young[1], Pierre Van Rensburg[1, 2] & Benoit Divol[1]

[1] *Institute for Wine Biotechnology, Stellenbosch University, Matieland, South Africa;*
[2] *Distell Corporation, Stellenbosch, South Africa*

4.1 Abstract

The *PGU1* gene of *Saccharomyces cerevisiae* has been shown to encode a polygalacturonase (PG). PG activity in *S. cerevisiae* is strain specific. There are no significant differences in the *PGU1* promoter regions of strains with and without PG activity. The *PGU1* gene is subtelomeric since it is located within 25 kb of the right telomere of chromosome X. Expression of genes located in subtelomeric regions in the yeast *S. cerevisiae* are inhibited compared to the rest of the genome. In this study we show that the deletion of genes involved in telomere silencing enhances PG activity. *PGU1* transcription and PG activity is increased when *PGU1* is moved to a different location in the genome, away from the telomere located close to this gene and that the depletion of the histone H4 leads to an increase in *PGU1* transcription. We concluded that *PGU1* is silenced in strains without PG activity due to an epigenetic effect.

Results of this study suggest that *PGU1* is silenced by being folded into a heterochromatin-like structure at its subtelomeric position on chromosome X. Formation of this silent structure is dependent on the Isw2p chromatin remodeling complex, its histone fold motif containing subunit Dls1p and the N-terminal tail of the H4 histone.

4.2 Introduction

Pectin is a generic name for a mixture of pectic substances with different compositions, but with a polygalacturonic acid backbone. It is a structural heteropolysaccharide that occurs mainly in the middle lamellae and primary cell walls of higher plants. Pectin is degraded by a group of enzymes collectively called pectinases. These enzymes are classified according to their substrate, mode of action, action pattern and cleavage products (Jayani *et al.*, 2005).

Pectolytic activity enables yeast to reach plant metabolites and thus facilitate yeast invasion of fruits (Gognies *et al.*, 1999). Polygalacturonic acid is degraded by an endo-

polygalacturonase (PG) encoded by the *PGU1* gene in *Saccharomyces cerevisiae* (Blanco *et al.*, 1994). The *PGU1* gene is absent in some *S. cerevisiae* strains and consequently these strains do not possess PG activity (Fernández-González *et al.*, 2004). Some of the strains that possess a functional *PGU1* gene lack activity because the gene is not expressed (Jia & Wheals, 2000). The frequency at which a gene is transcribed is determined by its promoter sequence and by the presence of transcription factors that bind to this promoter in order to activate transcription. Transcription factors that activate expression of the *PGU1* gene are regulated by the mating and filamentous growth Mitogen Activated Protein Kinase pathway (MAPK) (Madhani *et al.*, 1999). Gene expression is however not only affected by the promoter sequence of a gene, but also by its position in the genome (Flagfeldt *et al.*, 2009).

PGU1 is located at position 722806..723891, within 25 kb of the right telomere of chromosome X and is therefore subtelomeric (Louw *et al.,* 2009).

Transcription of genes inserted near telomeres is frequently repressed by an epigenetic effect known as Telomere Position Effect (TPE) (Vega-Palas *et al.*, 2000). Packaging of genes into a condensed heterochromatin-like structure at the telomeric tract and subtelomeric region can result in silencing of genes irrespective of their promoters (Martin *et al.*, 2004). In *S. cerevisiae*, the telomeres consist of tandem ($C_{1\text{-}3}A$) repeats approximately 350-bp in length. Together with the telomere silencing factors, the telomeres form a non-nucleosomal DNA-protein complex called the telosome (Wright & Shay, 1992). Rap1p, the sequence specific duplex DNA binding protein is the major protein in the telosome. Rap1p binds to the DNA repeat sequences and interacts with other proteins including Rif1p, Rif2p, Sir2p, Sir3p and Sir4p. These proteins interact with each other and subtelomeric nucleosomes via N-termini of histones H3 and H4, thus packaging the telomeric region into a complex heterochromatin-like structure (Vega-Palas *et al.*, 2000). Genes are silenced by the Sir proteins (Silence Information Regulator proteins) binding up to 8kb from the telomeres (Wyrick *et al.*, 1999). Unlike the telomeric tract, sub-telomeric DNA is organised into nucleosomes (Tham & Zakian, 2002). Histones can contribute to Sirp independent silencing of subtelomeric genes upstream of the Sirp binding region (Wyrick *et al.*, 1999). According to the histone code hypothesis, combinations of covalent histone modifications lead to varied transcriptional outputs (Dion *et al.*, 2005). DNA regulatory sequences thus only provide a partial explanation of gene regulation. Deciphering the complexity of histone modifications is thus essential in understanding transcriptional regulation in the subtelomeric region.

A model that explains how post-translational modification of histones regulate transcription of different chromosomal regions has been proposed (Van Leeuwen & Gottschling, 2002; Martin *et al.*, 2004). The model suggests that a repressor protein can only bind to the N-terminal tail of histones with hypoacylated or hypomethylated lysine residues, since the presence of these functional groups would inhibit binding of the protein. The repressor protein would thus be restricted to regions of the chromosome in which histones are hypomethylated and hypoacetylated such as subtelomeric regions (Martin *et al.*, 2004). Gene silencing by TPE in yeast has mainly been demonstrated by inserting reporter genes in close proximity of a telomere (Gottschling *et al.*, 1990). The only naturally occurring yeast genes that TPE has been demonstrated for are: (1) *HMR* the silenced copy of the mating type locus on chromosome III (Thompson *et al.*, 1994); (2) the TY5-1 retrotransposon, (3) YFR057W an uncharacterized ORF located adjacent to the telomere on R-VI (Vega-Palas *et al.*, 2000); and (4) *IMD1* a pseudogene encoding an IMP dehydrogenase ortholog located next to the right telomere of chromosome I (Barton & Kaback, 2006). Different methods were used to demonstrate that these genes are silenced by TPE. *HMR* was found to be derepressed when the H3 histone was mutated and the gene was moved away from the telomere. The H3 mutation did not lead to a significant increase in transcription of *HMR* when the gene was integrated adjacent to a telomere (Thompson *et al.*, 1994). TY5-1 and YFR057W expression were derepressed by mutating genes that alleviate TPE (i.e. *SIR2, SIR3* and *SIR4)* (Vega-Palas *et al*., 2000). *IMD1* expression was derepressed by removing the telomere adjacent to this gene from chromosome I; the expression of this gene also increased in a *sir3Δ* mutant (Barton & Kaback, 2006).

Both pectin degradation and filamentous growth phenotypes have been implicated in *S. cerevisiae* functioning as a plant pathogen (Gognies & Belarbi, 2002; Juana, 2001). These two phenotypes are co-regulated by the same regulatory pathway (Madhani *et al.*, 1999). It has been shown that the *FLO* genes, responsible for filamentous growth, are under genetic and epigenetic control (Halme *et al.*, 2004). Four of the five *FLO* genes regulating cell surface variation are located in subtelomeric positions, 10 to 40 kb from telomeres and have been shown to be under epigenetic control (Halme *et al.,* 2004). *PGU1* is also sub-telomeric. Deletion of five genes *DLS1, ISW2, SIR3, HTS3* and *ESC1* involved in gene silencing by TPE in *S. cerevisiae* results in an increase in PG activity, indicating that *PGU1* transcription might be inhibited by telomeric silencing in the *S. cerevisiae* BY4742 strain (Louw *et al.,* 2009).

In this study we investigate if *PGU1* transcription is also under epigenetic control by moving the *PGU1* gene with its native regulatory sequences to a different position in the genome, away from its telomere and comparing *PGU1* transcription and PG activity to that of the wild type strain. To determine if the chromatin remodeling complex *DLS1* only inhibits PG activity in the BY4742 strain, the gene is knocked out in other strains normally devoid of PG activity and the strains are screened for recovery in PG activity. In order to identify histone modifications that affect *PGU1* transcription, microarray data of studies in which different histones were mutated are analysed.

4.3 Materials and Methods

4.3.1 Strains, plasmids and culture conditions

Bacteria and yeast strains used in this study are summarized in Table 4.1**.**

Table 4.1 Microbial strains used in this study

Strains	Genotype/description	Reference
S. cerevisiae strains		
BY4742 (S288C Background)	*MATα; his3-1; leu2Δ0; lys2Δ0; ura3Δ0*	(Brachmann *et al.*, 1998)
BY4742 *hst3*Δ0	*MATα; his3-1; leu2Δ0; lys2Δ0; ura3Δ0; hst3::kanMX4*	(Brachmann *et al.*, 1998)
BY4742 *isw2*Δ0	*MATα; his3-1; leu2Δ0; lys2Δ0; ura3Δ0; isw2::kanMX4*	(Brachmann *et al.*, 1998)
BY4742 *sir3*Δ0	*MATα; his3-1; leu2Δ0; lys2Δ0; ura3Δ0; sir3::kanMX4*	(Brachmann *et al.*, 1998)
BY4742 *dls1*::*KanMX4*	*MATα; his3-1; leu2Δ0; lys2Δ0; ura3Δ0;* yjl064w*::kanMX4*	(Brachmann *et al.*, 1998)
BY4742 *pgu1*Δ0	*MATα; his3-1; leu2Δ0; lys2Δ0; ura3Δ0; pgu1::kanMX4*	(Brachmann *et al.*, 1998)
FY23	*MAT***a** *ura3-52 trp1-63 leu2-1 GAL2*	(Winston *et al.*, 1995)
FY23 *dls1*::*KanMX4*	*MATa ura3-52 trp1-63 leu2-1 GAL2;* yjl064w*::kanMX4*	This study
FY23 *pgu1*Δ0	*MAT***a** *ura3-52 trp1-63 leu2-1 GAL2 pgu1::kanMX4*	This study
FY23-1	*MAT***a** *ura3-52 trp1-63 leu2-1 GAL2 pgu1::kanMX4; URA3::PGU1*	This study
W303-1A	*MAT*a *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	(Veal *et al.*, 2003)
W303-1A *dls1*::*KanMX4*	*MAT*a *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15,* yjl064w*::kanMX4*	This study
∑1278b	*MATα; ura3-52; trp1::hisG; leu2::hisG; his3::hisG;*	(Van Dyk *et al.*, 2005)
∑1278b *pgu1*Δ0	*MATα; ura3-52; trp1::hisG; leu2::hisG; his3::hisG; pgu1::kanMX4*	This study
∑1278b-1	*MATα; ura3-52; trp1::hisG; leu2::hisG; his3::hisG; pgu1::kanMX4, URA3::PGU1*	This study
Escherichia coli strain		
DH5α	*[F-φ80lacZΔM15Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk−, mk+) phoA supE44 thi-1 gyrA96 relA1 λ]*	GIBCO-Invitrogen Life technologies, Mowbray, South Africa)

Plasmids were constructed and amplified in *Escherichia coli* DH5α grown in Luria Bertani (LB) medium (Biolab diagnostics, Wadenville, South Africa). The medium was supplemented with 100 mg L^{-1} ampicillin for the selection of resistant bacteria when appropriate.

S. cerevisiae wild type strains were grown in Yeast Peptone Dextrose (YPD) Broth (Biolab diagnostics) at 30°C on a rotary shaker at 150 rpm. Yeast transformants were grown in Synthetic Complete (SC) medium containing 6.7 g L^{-1} Yeast Nitrogen Base (YNB) (Difco Scientific group, Waterfall Park, South Africa) and 20 g L^{-1} glucose supplemented with the appropriate amino acids to apply auxotrophic pressure. Geneticin resistant transformants were selected on appropriate media supplemented with 200 mg L^{-1} geneticin (Sigma-Aldrich, St. Louis). Solid media contained 20 g L^{-1} agar. Bacteria and yeasts were cultured at 37 and 30°C, respectively.

4.3.2 Yeast strain construction

The wild type yeast strains ∑1278b, W303-1A and FY23 (S288C genetic background) were used for the construction of recombinant strains. Deletion mutants were generated by transforming the wild type strains with PCR-generated knock-out cassettes (Table 4.2).

Table 4.2 List of primers used for PCR amplification in this study.

Primer name	Sequence 5'-3'	Template	PCR purpose and locus amplified an	Reference:
5'YJL064w knock out	TCAGTAACGTCTTCGTCGT CGTCTTCGT	BY4742 ∆yjl064w	Constructing knockout cassette ∆yjl064w::*kanMX4*	This study
3'YJL064w knockout	TTGAAAGAACAGCGCTAAC AATGTGC	BY4742 ∆yjl064w	Constructing knockout cassette ∆yjl064w::*kanMX4*	This study
PGL1PROM	AAGCTTGGACAAGTCGACT TGTCCTGC	BY4742 ∆*pgu1*	Constructing knockout cassette ∆*pgu1*::*kanMX4*	(Divol & Van Rensburg, 2007)
PGU-nat-term-rev	CGAACTATGGCGAAGGTTG ATGAGA	BY4742 ∆*pgu1*	Constructing knockout cassette ∆*pgu1*::*kanMX4*	This study
5'PGU1+1600BP FLANKS	GGGTTCCCTGAAGAAACAG AGAAT	BY4742	Amplifying native *PGU1* expression cassette	This study
3'PGU1+1600bp FLANKS	CAATCTTG CTCTTTTCCAACG	BY4742	Amplifying native *PGU1* expression cassette	This study
5'PPGL1- BGLII pP2	AGATCTATGATTTCTGCTAA T TCATTACTTATTTCC	BY4742	Preparing DIG labelled dUTP probe for *PGU1*	(Louw *et al.,* 2009)
3'PPGL1- BGLII pP2	CTCGAGTTAACAGCTTGCA CCAGATCCAGATG	BY4742	Preparing DIG labelled dUTP probe for *PGU1*	(Louw *et al.,* 2009)
Act fw	TACCGGCCAAATCGATTCT C	BY4742, ∑1278b, FY23, FY23-1, ∑1278b-1	qRT *ACT1*	(Divol *et al.;* 2006)
Act rev	CACTGGTATTGTTTTGGATT	BY4742, ∑1278b, FY23, FY23-1, ∑1278b-1	qRT *ACT1*	(Divol *et al.,* 2006)
Pgu1fw2	GTGCTTCGGGACATACCAT T	BY4742, ∑1278b, FY23, FY23-1, ∑1278b-1	qRT *PGU1*	(Divol & Van Rensburg 2007)
Pgu1rev2	CGTCAACGCCAACTTTACA A	BY4742, ∑1278b, FY23, FY23-1, ∑1278b-1	qRT *PGU1*	(Divol & Van Rensburg 2007)

Each gene disruption was replaced with a *KanMX4* module (Wach *et al.*, 1994). Deleting YJL064W resulted in a partial deletion of *DLS1,* generating the *dls1*::*KanMX4* genotype. To move the *PGU1* gene to another locus the *pgu1*Δ strains were transformed with the YIplac211-PGU1 plasmid linearized with the restriction enzyme *ApaI* (Roche Diagnostics) generating FY23 *pgu1*Δ0*, ura3::PGU1* (FY23-1) and ∑1278b *pgu1*Δ0*, ura3::PGU1 (*∑1278b-1). All strains were transformed by electroporation (Volschenk *et al.*, 2004). Deletion of genes with the *KanMX4* knockout cassettes was verified by PCR analysis (results not shown). Integration of *PGU1* into the *URA3* locus was verified by Southern blot analysis. Genomic DNA was isolated from the *S. cerevisiae* strains analysed (Ausubel *et al.*, 1995). DNA was digested with *PstI* and *ClaI* (Roche Diagnostics) separated in a 1% (w/v) agarose gel and blotted to a Nylon membrane, positively charged™ (Roche Diagnostics). λ DNA digested with *BstEII* restriction enzyme (Roche Diagnostics) was loaded as molecular weight marker. Southern hybridizations were carried out as described in the digoxygenin (DIG) Application Manual (Roch Molecular Biochemicals). A DIG -labelled probe was prepared for the detection of *PGU1* by PCR labelling with the PCR DIG probe synthesis kit (Roche diagnostics). λ DNA was labelled with the DIG DNA labelling kit (Roche Applied Science) to probe the molecular weight marker.

4.3.3 Constructing knockout and integration cassettes

The knock-out cassettes for YJL064W and *PGU1* were obtained through PCR amplification of the corresponding disrupted genes of the mutants from the BY4742 (Brachmann *et al.*, 1998) mutant collection supplied by EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis, http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html).

The *PGU1* expression cassette was amplified by using polymerase chain reaction (PCR). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). All genes were amplified from genomic DNA, using an Applied Biosystems 2720 thermal cycler. Takara ExTaq enzyme and Takara buffer with MgCl2 were used (Separations, Randburg, South Africa). The reaction mixture contained 250 µM of each nucleotide (dNTP), 200 ng DNA, 0.25 µM of each primer, and 0.2 mM MgCl2. Primers used are listed in Table 4.2.

The *PGU1* expression cassette was digested with *XhoI* and *HindIII* (Roche Diagnostics, Randburg, South Africa) and ligated into the corresponding sites of the

Yeast Integrating Plasmid YIplac-211 (Gietz & Sugino, 1988), to generate the plasmid YIplac211-PGU1. Correct cloning was verified by restriction analysis and sequencing (Central Analytical Facility, Stellenbosch University).

4.3.4 Screening for Polygalacturonase activity

Strains were screened for PG activity with a modified plate assay described by Masoud & Jespersen (2006). All yeast strains listed in Table 4.1 were screened for PG activity. PG activity could be seen as halos surrounding colonies where polygalacturonic acid was degraded. Five µL of an overnight culture containing 10^4 cells were spotted on PG plates (1.25% polygalacturonic acid (Sigma), 0.67% YNB, 1% glucose (w/v) (Merck), 2% agar (w/v) (Difco), 0.68% Potasium phosphate pH 4.0) and incubated for three days at 30°C. Degradation halos were visualized by washing colonies off with distilled water and staining plates with 6M HCl.

4.3.5 Quantitative real-time PCR (qRT-PCR)

4.3.5.1 RNA isolation

Total RNA was isolated from *S. cerevisiae* strains FY23 and FY23-1 as described in the hot phenol RNA isolation protocol (Ausubel *et al.*, 1995). For each strain, a single colony was inoculated into 5 mL SC media and incubated in a roller drum overnight at 30°C. The cells were pelleted by centrifugation at 5000xg for 5 min. The pellet was washed in water and used to inoculate 10 mL of SC**.** Cells were harvested during the logarithmic growth phase at OD_{600nm} of 1.0.

The quality and concentration of RNA was evaluated by gel electrophoresis and by measuring absorbance, absorbance was determined at 260 nm for quantification and the 260 nm /280 nm ratio to determine purity.

4.3.5.2 cDNA synthesis

RNA samples were treated with DNase I (Roche Diagnostics) to remove any residual DNA contamination, following the manufacturer's instructions. The absence of DNA was confirmed by end-point PCR. One µg of each RNA sample was converted into cDNA by using the ImProm-II Reverse Transcription System (Promega, Madison, USA) following the manufacturer’s instructions. Total RNA was used as template.

4.3.5.3 qRT-PCR

qRT-PCR was performed on cDNA samples originating from two independent replicate experiments. The experiments were carried out using SYBR-Green dye in a 7500 Real-Time PCR System (Applied Biosystems, Johannesburg, South Africa). Reactions contained Power KAPATaq Ready Mix (KAPA Biosystems, Cape Town, South Africa), forward and reverse primers (0.1 µM each; Table 4.2) and 20 ng cDNA template. Primers described in Table 4.2 were used. For each PCR product, melting curves were determined according to the ABI guidelines, ensuring specific amplification of the target gene. Quantitative values were obtained as the threshold PCR cycle number (Ct) when the increase in the fluorescent signal of the PCR product showed exponential amplification. Transcription of each gene was normalized to that of *ACT1* in the same sample. The cycle threshold (Ct) value for each reaction was determined using the Sequence Detection System, 7500 Real-Time PCR System software package (Applied Biosystems). Ct values were used to calculate the expression of *PGU1* in the recombinant strains relative to that of the same gene in the wild type strain. Fold change was calculated via the $2^{-\Delta\Delta Ct}$ method for each sample in triplicate, in which 1 indicates no change in abundance (Livak & Schmittgen, 2001).

4.4 Results

4.4.1 PG Activity in *S. cerevisiae* deletion mutants

Derepression of PG activity in the following BY4742 mutants listed in Table 4.1 *dls1*::*KanMX4, isw2Δ, sir3Δ, hst3Δ* and *esc1Δ* were compared by plate assays. Disruption of *DLS1* and *ISW2* resulted in the most significant increase in PG activity, the *hst3Δ* mutant had medium PG activity and the *sir3Δ* and *esc1Δ* mutants showed a slight recovery in PG activity (results not shown). To determine if *DLS1* acts as repressor of PG activity in other strains lacking PG activity, W303-1A *dls1*::*KanMX4* and FY23 *dls1*::*KanMX4* mutants were generated. PG activity of these two mutant strains and the BY4742 *Δdls1*::*KanMX4* strain obtained from the EUROSCARF deletion mutant library were compared with the corresponding wild type strains (Figure 4.1).

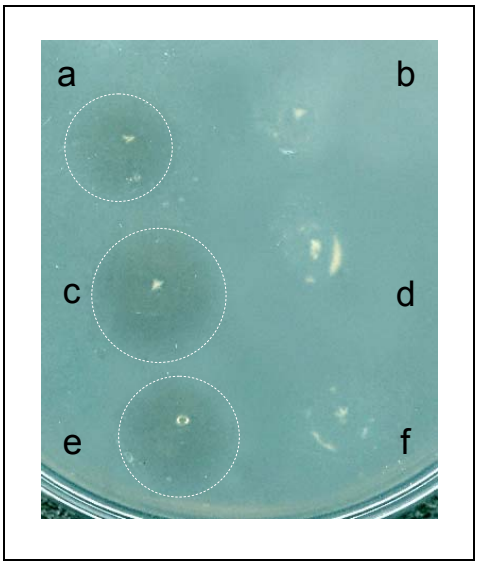

Figure 4.1. PG plate assay comparing PG activity between a) BY4742 *dls1*::*KanMX4,* b) BY4742, c) FY23 *dls1*::*KanMX4*, d) FY23*, e*) W303-1A *dls1*::*KanMX4* and f) W303-1A. Deletion of the *DLS1* gene resulted in the recovery of PG activity in all three strains.

It can be seen from Figure 4.1 that the BY4742, FY23 and W303-1A wild type strains had no PG activity. Deletion of the *DLS1* gene however conferred PG activity upon all three strains.

4.4.2 Moving the *PGU1* gene to a different locus

To confirm that transcription of the *PGU1* gene was inhibited by an epigenetic effect in some *S. cerevisiae* strains, the gene with its native promoter and terminator was moved to a different (non-telomeric) locus. *PGU1* was deleted in locus YJR153w with a *KanMX4* containing cassette in strains FY23 (isogenic to BY4742) and ∑1278b, deletion was confirmed by PCR (results not shown). YIplac211-PGU1 was subsequently integrated into *URA3* generating FY23, *pgu1::kanMX4; URA3::PGU1* (FY23-1) and ∑1278b, *pgu1::kanMX4; URA3::PGU1* (∑1278b-1). The presence of a single copy of *PGU1,* moved from the YJR153W locus on chromosome X to the YEL021W locus on chromosome V, was confirmed by Southern blot analysis (Figure 4.2).

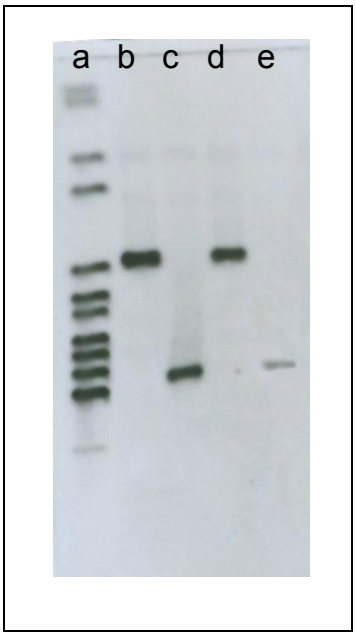

Figure 4.2. Southern blot analysis of genomic DNA isolated from the FY23 and ∑1278b wild type strains and FY23-1and ∑1278b-1 recombinant strains, probed for the *PGU1* gene, showing a single copy of *PGU1* present in all strains but located in different loci for wild type and recombinant strains. Lane a) Marker lane (λ DNA digested with *BstEII*) Lane b) FY23, Lane c) FY23-1, Lane d) ∑1278b, Lane e) ∑1278b-1.

4.4.3 PG activity in strains in which the *PGU1* gene has been moved to a different locus

PG activity of the wild type strains was compared with the mutant strains in which *PGU1* was moved to a different locus (Figure 4.3).

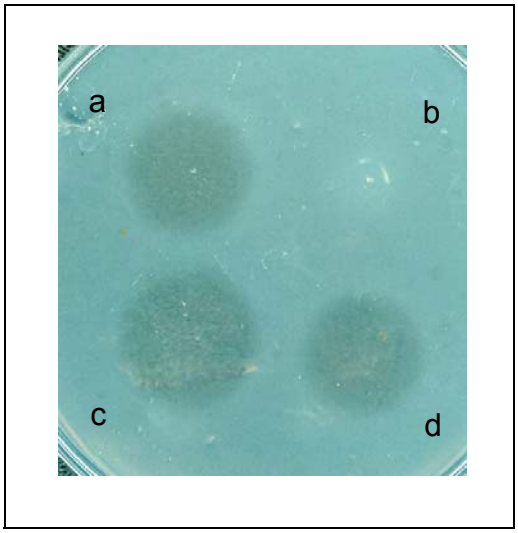

Figure 4.3 PG plate assay comparing PG activity between a) FY23-1, b) FY23, c) ∑1278b-1 and d) ∑1278b. Moving the *PGU1* gene to a different position in the genome, away from its telomere, resulted in a recovery in PG activity in the FY23-1 strain while the ∑1278b-1 maintained its activity.

From Figure 4.3 it can be seen that FY23, a *S. cerevisiae* strain without PG activity was able to degrade polygalacturonic acid when *PGU1* was moved to a non-telomeric locus generating FY23-1. When ∑1278b, a *S. cerevisiae* strain with PG activity was engineered in the same manner, the resulting recombinant strain ∑1278b-1 showed PG activity similar to that of the wild type strain.

4.4.4 Transcription of *PGU1* in FY23-1

qRT-PCR was carried out to confirm that the recovery in PG activity in the FY23-1 strain was due to an increase in transcription when the gene was moved to a different locus. Transcription of *PGU1* increased 2.34 fold (+-0.14) in the FY23-1 strain compared to the FY23 wild type strain.

4.5 Discussion

PG activity has been shown to be regulated by transcription of the *PGU1* gene. The gene has however been shown to have an identical promoter region in strains with and without PG activity (Jia & Wheals, 2000). DNA regulatory sequences only provide a partial explanation of gene regulation (Martin *et al.*, 2004). Screening the *S. cerevisiae* genome for activators and inhibitors of PG activity revealed that 5 genes implicated in telomeric silencing (i.e. *DLS1, ISW2, SIR3, HST3* and *ESC1*) are involved in regulating *PGU1* transcription (Louw *et al*. , 2009).Since *DLS1* deletion resulted in the most significant recovery in PG activity in BY4742 among the mutants identified by the deletion library screen (results not shown) we focused on this chromatin remodeller as an inhibitor of PG activity in other strains that lack PG activity. In this study we proved that *PGU1* transcription is indeed influenced by an epigenetic effect. Moving the *PGU1* cassette from its sub telomeric position at locus YJR153W on chromosome X to YEL021W (*URA3*) on chromosome V enabled FY23, a *S. cerevisiae* strain without PG activity to degrade polygalacturonic acid. Real-time PCR analysis confirmed that this recovery in activity was due to a doubling in *PGU1* transcription. This result was in agreement with that of Flagveldt *et al.* (2009) that found an increase in transcription when moving a marker gene from a subtelomeric position to the *URA3* locus.

The position of *PGU1* in the genome could possibly explain epigenetic silencing in some strains. Post-translational modification of histones lead to the division of chromosomes into silenced and transcribed regions (Shahbazian & Grunstein, 2007). *PGU1* is located at a subtelomeric position ~25 kb from the right telomere of

chromosome X. It has been shown by Wyrick *et al.* (1999) that genes located in this region can be silenced due to post-translational modification of histones

PGU1 is located beyond the 3-4 kb extent from the telomere within which genes can be silenced by Sir protein binding. A slight increase in PG activity was however found upon deletion of *SIR3* by Louw *et al.* (2009) and was confirmed in this study.

The *sir3* deletion can result in an increase in PG activity by an indirect effect. Microarray analysis showed that a deletion of *SIR3* results in 65.9-fold decrease in *FUS3* transcription (Wyrick *et al.*, 1999). *FUS3* is the MAPK (Mitogen Activated Protein Kinase) in the mating MAPK pathway and has been shown to act as an inhibitor of *PGU1* (Madhani *et al.*, 1999). *FUS3* is not involved with epigenetic regulation but the decrease in *FUS3* expression could potentially lead to an increase in *PGU1* transcription. Fus3p induces ubiquitination of Tec1p, the main transcription factor regulating *PGU1* transcription, through the SCFCdc4 ubiquitin ligase during mating (Chou *et al.*, 2004). A decrease in Fus3p will thus result in more Tec1p being available in the cell, resulting in higher expression of *PGU1.*

To determine if *DLS1* only inhibits PG activity in the BY4742 strain, this gene was also mutated in two other strains without PG activity FY23 and W303-1A. From Figure 4.1 it can be seen that BY4742 *dls1*::*KanMX4,* FY23 *dls1*::*KanMX4* and W303-1 *dls1*::*KanMX4* recovered PG activity, while the corresponding wild type strains could not degrade polygalacturonic acid. Dls1p plays a critical role in Isw2p dependent repression of a wide variety of genes *in vivo* (McConnell *et al.*, 2004). *DLS1* has been shown to be required for Isw2p dependent chromatin remodeling of genes at the telomeric regions (Iida & Araki, 2004). Chromatin remodelers can restructure, mobilize or eject nucleosomes, allowing exposure of DNA in chromatin (Cairns, 2007). *ISW2* is an ortholog of the human CHRAC (Chromatin Accessibility Complex) and functions by sliding mononucleosomes from the end to the centre of DNA (Dang *et al.*, 2007). Chromatin analysis revealed that Dls1p is required in formation of repressive chromatin structure subsequent to crosslinking of Isw2p with chromatin (McConnell *et al.*, 2004). In this study it has been shown that *PGU1* transcription is inhibited due to its position in the genome of strains lacking PG activity. Since *DLS1* and *ISW2* are required for formation of repressive chromatin structure and deletion of either one of these genes results in derepression of PG activity, it indicates *PGU1* transcription is inhibited due to ISW2p dependent formation of repressive chromatin structure at the native *PGU1* locus YJR153w in strains possessing the *PGU1* gene but lacking PG activity. Deletion of the *ISW2* gene has been shown to result in Flo11p independent invasive growth

(Trachtulcova *et al.*, 2004). The phenotypes invasive growth and PG degradation are co-regulated by the invasive growth *KSS1* MAPK pathway activating the transcription factors Tec1p and Ste12p. It seems that a mutation affecting both phenotypes would do so by affecting this regulatory pathway. This is however not the case since invasive growth increases in ∆*kss1*∆*isw2* and ∆*tec1*∆*isw2* mutants (Roberts *et al.*, 2000). It has been shown that the Isw2-Itc1 complex could play a role in reorganizing genomic expression during metabolic adaptation to starvation conditions (Kent *et al.*, 2001). Pectolytic activity and invasive growth are coordinated foraging behaviors during starvation conditions by the yeast (Madhani, *et al.,* 1999). Regulation of transcription of the genes responsible for these phenotypes by the same chromatin remodeling complex shows that these genes are not only regulated by the same transcription factors, but are also regulated on a epigenetic level. TPE is the transcriptional repression of genes located near telomeres (Mondoux & Zakian, 2007). Telomeres facilitate silencing of nearby promoters through propagation of the Sir silencing complex (Perrod & Gasser, 2003). Histones have however been shown to make Sir independent contributions to telomeric silencing (Wyrick *et al.*, 1999). Histone N-terminal domains play important roles in regulating chromatin structure and gene transcription (Parra *et al.*, 2006). Genomic chromatin structure is organized into condensed, silent heterochromatin and more relaxed active euchromatin regions. The nucleosome is the repeating unit in chromatin and consists of two copies of each of the four histone proteins, forming the histone octamer, with 147 base pairs of DNA wrapped in nearly two turns of a superhelix around the core octamer (Shahbazian & Grunstein, 2007). Post-translational modification of histones has been proposed to demarcate regions of heterochromatin and euchromatin in *S. cerevisiae* (Martin *et al*., 2004).Previously published microarray data sets were investigated in order to determine the influence of different histones on chromatin structure that influence *PGU1* transcription (Parra & Wyrick, 2007; Parra *et al.*, 2006; Martin *et al.*, 2004; Wyrick *et al.*, 1999). Table 4.3 shows the effect that mutation of different histones had on *PGU1* transcription.

Table 4.3 Fold change in *PGU1* transcription upon mutation of different histones.

Mutation	Resulting fold change increase in *PGU1* transcription	Reference
H2A N-terminal tail	none	(Parra & Wyrick, 2007) http://wyrick.sbs.wsu.edu/histoneH2A/
H2B N-terminal tail	none	(Parra *et al.*, 2006) vhttp://wyrick.sbs.wsu.edu/histoneH2B/
H3 N-terminal tail (lysine-9, 14, 18,23 to glysine)	2.13	(Martin *et al.*, 2004) http://wyrick.sbs.wsu.edu/histoneH3/
H4 N-terminal tail (lysine-5, 8, 12, 16 to glysine)	5.18	(Martin *et al.*, 2004) http://wyrick.sbs.wsu.edu/histoneH3/
H4 depletion	19.38	(Wyrick *et al.*, 1999) http://jura.wi.mit.edu/cgi-bin/young_public/lists.cgi

From Table it 4.3 can be seen that the N-terminal domains of H3 and H4 play a role in repression of *PGU1* expression. Mutating different residues in the N-terminal tails of H2A and H2B had no effect on *PGU1* transcription. Changing lysines 9, 14, 18 and 23 to glysine in the N-terminal tails of H3 resulted in a 2.13 fold increase in *PGU1* transcription compared to the wild type strain. Changing lysines 5, 8, 12 and 16 to glysine in the N-terminal tails of H4 resulted in a 5.18 fold increase in *PGU1* transcription compared to the wild type strain and depletion of this histone resulted in the largest increase in *PGU1* transcription (Table 4.3). *PGU1* expression has been shown to increase 20-fold upon depletion of the histone H4. Since *PGU1* expression seems to be silenced due to the proximity of the gene to the right telomere of chromosome X, it would be expected that genes located between *PGU1* and the telomere would also be silenced. Table 4.4 shows the increase in transcription of genes located in the subtelomeric region of X-R, upon depletion of H4.

Table 4.4 Increase in transcription of genes located between *PGU1* and the telomere X-R upon depletion of the histone H4 according to Wyrick *et al.* (1999)*. Genes are ordered as they occur on the chromosome, proceeding away from the telomere.

Gene	Distance located from the right telomere of chromosome X (kb)*	Increase in transcription (fold change)*
YJR162C	0.49	9.1
COS5	1.75	1.41
YJR160C	5.93	2.7
SOR1	9.7	3.7
HXT16	13	13.3
YJR157W	15.23	14.3
THI11	16.15	5.8
AAD10	18.34	3.2
YJR154W	19.96	1.5
PGU1	22.88	19.38

* http://jura.wi.mit.edu/cgi-bin/young_public/telgenesdisp.cgi?s=2

It can be seen from Table 4.4 that transcription of the cluster of genes located between *PGU1* and the telomere were derepressed upon depletion of H4 (Wyrick *et al.*, 1999). Analyzing genome wide gene expression, Martin *et al*. (2004) found transcriptional repression to be dependend on the histone H3 N-terminal domain, but not the histone H4 N-terminal domain. In contrast with this analysis of the complete genome, analyzing microarray results by focusing on the X-R sub telomeric region, we found transcriptional repression of *PGU1* to be dependent on H4 N-terminal domain and transcription of all sub-telomeric genes on chromosome X to increase upon depletion of H4.

Our results showed that *PGU1* was silenced due to its subtelomeric position on chromosome X. The Isw2p chromatin remodeling complex and its histone fold motif containing subunit Dls1p is important in establishing this repressed state. Transcriptional repression of *PGU1* is dependent on the H4 N-terminal domain as microarray analysis indicates that H4 is involved in inhibition of transcription of all the genes in the subtelomeric area of chromosome X.

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Chapter 5

RESEARCH NOTE

Phenotypic expression of PCR generated random mutations generated in *PGU1* overexpressed in *Saccharomyces cerevisiae*

Phenotypic expression of PCR generated random mutations generated in *PGU1* overexpressed in *Saccharomyces cerevisiae*.

Campbell Louw[1], Pierre Van Rensburg[1, 2] & Benoit Divol[1]

[1] Institute for Wine Biotechnology, Stellenbosch University, Matieland, South Africa;
[2] Distell Corporation, Stellenbosch, South Africa

5.1 Introduction

The sequence of the Polygalacturonase (PG) encoding gene *PGU1,* also known as *PGL1* or *PSM,* was obtained by sequencing the strain ATCC S288C, a strain without pectolytic activity and is available on the *Saccharomyces* Genome Database. The gene is present in a single copy per haploid genome on chromosome X. The PG enzyme was purified, characterized and named Pgu1p. Pgu1p was shown to be N-glycosylated. It has a M_r of 42 kDa, is active in a pH range comprised between 3 and 5.5 with an optimum temperature at 25°C and hydrolyzes polygalacturonic acid as an endo-PG (Gainvors *et al.*, 1994). The predicted protein comprises 361 amino acids, with a signal peptide between residues 1 and 18. A conserved histidine in position 222 is the putative active residue in the active site. This PG shows 54% homology with fungal equivalent homologues and 24% with plant and bacterial PG's (Blanco *et al.*, 1998).

The majority of studies found a functional copy of the *PGU1* gene to be present in strains irrespective of PG activity (Hirose *et al* 1998, Blanco *et al.*, 1998; Jia & Wheals, 2000). It was however found by Gognies *et al.* (1999) that certain strains were unable to degrade pectin because of a dysfunctional allele of *PGU1* being present in these strains. The difference between the Pgu1p from the strain with and without pectolytic activity was only 3 amino acids. None of the changes were situated in potential glycosylation sites or in highly conserved sequences.

In order to identify important regions for PG activity in Pgu1p, site-directed mutagenesis was used to mutate nucleotides in the *PGU1* gene coding for amino acid residues shown to have an important role in PG activity in filamentous fungi (Blanco 2002). Mutated alleles of the *PGU1* gene were overexpressed in *Pichia pastoris*.

The level of PG activity achieved when overexpressing *PGU1* in *P. pastoris* was significantly higher compared to activity when the same gene was overexpressed in *S. cerevisiae*. Expressing the mutated genes showed that the following amino acids are important for activity of Pgu1p: aspartic acid residues at positions 179, 200, and 201,

and histidine at position 222. Mutation of the two potential glycosylation sites in *PGU1* showed that the two residues individually (N318D, N330D) did not affect secreted enzyme activity, but the double mutant caused a 50% reduction in enzyme activity when compared to the wild-type. In the case of this double mutant it is possible that changing two amino acids in close proximity had a more deleterious effect on enzyme structure, compromising catalytic activity and possibly protein stability (Blanco, *et al* 2002).

Thermal stable, *Taq* DNA polymerase, from *Thermus aquaticus* is the most common polymerase used to carry out the Polymerase Chain Reaction (PCR). This polymerase lacks 3'-5' proofreading exonuclease activity and therefore this enzyme has an error rate varying between 10^{-4} and 10^{-5} errors per nucleotide synthesised.

Generating Single Nucleotide Polymorphisms (SNPs) in a gene is a technique that can be used to identify coding regions in the gene that is important for enzyme structure, function and secretion. The analysis of how structure influences the function of a protein benefits from the availability of a spectrum of point mutations in the encoding gene. The randomness of nucleotide substitutions by thermostable polymerases during PCR makes it a good candidate for the generation of such mutations (Kok *et al.*, 1999). The investigation of how random mutations may change the properties of a protein benefits from a biological system in which their individual phenotypes may be both selected and screened *in vivo*.

In order to verify correct cloning of the *PGU1* gene, multiple clones were sequenced. Single Nucleotide Polymorphisms (SNPs) were detected in some of these clones. These mutations in the *PGU1* gene were attributed to the gene being cloned with *Taq* DNA polymerase lacking 3'-5' proofreading exonuclease activity. In order to determine if any of these mutations were located in regions important to the function and secretion of the Pgu1p clones bearing such mutations were overexpressed in a *S. cerevisiae* strain naturally lacking PG activity and was compared to that of a strain overexpressing the wild type *PGU1* gene.

5.2 Materials and methods

5.2.1 Strains and culture conditions

Plasmids were constructed and amplified in *Escherichia coli* DH5α (*End A1 hsd r17 (r^- m_k^+) sup E44 thi-1λ^-recA1 gyrA relA* Δ *(lacZYA- arg F) U169 (ψ80lacZΔM15)* (GIBCO-Invitrogen Life technologies, Mowbray, South Africa), grown in Luria Bertani (LB) medium (Biolab diagnostics, Wadenville, South Africa). The medium was supplemented with 100 mg L^{-1} ampicillin for the selection of resistant bacteria when appropriate. *S.*

cerevisiae BY4742 strains (Brachmann *et al.*, 1998) were grown in Yeast Peptone Dextrose (YPD) Broth (Biolab diagnostics) at 30°C on a rotary shaker at 150 r.p.m. For the selection of yeast transformants, Synthetic Complete (SC) medium containing 6.7 g L^{-1} yeast nitrogen base (Difco Scientific group, Waterfall Park, South Africa) and 20 g L^{-1} glucose supplemented with the appropriate amino acids to apply auxotrophic pressure.

5.2.2 DNA preparation and analysis

Chromosomal DNA from *S. cerevisiae* strains was isolated from overnight cultures grown in YPD at 30°C (Ausubel *et al.*, 1995).

Multiple copies of *PGU1* were individually amplified by using the polymerase chain reaction (PCR). Primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Each copy was amplified from genomic DNA, using an Applied Biosystems 2720 thermal cycler. Takara ExTaq enzyme and Takara buffer with $MgCl_2$ were used (Separations, Randburg, South Africa). The reaction mixture contained 250 µM of each nucleotide (dNTP), 200 ng DNA, 0.25 µM of each primer, and 0.2 mM $MgCl_2$.

DNA fragments to be sequenced were first cloned into a pGEM®-T Easy vector (pGEM®-T Easy Vector Systems, Promega, Whitehead Scientific, Cape Town, South Africa) according to the manufacturer's instructions. Plasmid DNA was isolated from positive transformants of *E. coli* DH5α. Both strands were sequenced in an ABI 3130XL Genetic Analyzer at the Central Analytical Facility (Stellenbosch University) using the universal M13 forward and reverse primers.

5.2.3 Constructing overexpression vectors

Plasmids were constructed in order to overexpress the mutated and wild type alleles of *PGU1* in *S. cerevisiae*. Correct cloning was verified by restriction analysis and sequencing.

The mutated *PGU1* alleles to be overexpressed were subcloned from the pGEM®-T Easy vector into the pCEL13 expression vector (Gundllapalli *et al.*, 2006). The pCEL13 expression vector carries a Ampicillin selecTable marker for selection of positive transformants in *E. coli* and a *URA3* marker for auxotrophic selection in yeast. The *PGU1* gene was excized from the pGEM®-T Easy plasmids by restriction with *Bgl*II and *Xho*I (Roche Diagnostics, Randburg, South Africa) and ligated into the corresponding sites of the pCEL13 expression vector. Standard methods were used for the restriction and ligation of DNA, plasmid transformation into *E. coli*, and agarose-gel electrophoresis (Maniatis *et al.*, 1989).

All constructs were amplified in *E. coli* DH5α and isolated with the Qiaprep® Spin Mini-prep Kit (Qiagen) for yeast transformation.

5.2.4 Yeast transformation

S. cerevisiae, strain BY4742, was transformed with the various expression vectors constructed (Table1). All transformations were carried out using the lithium-acetate method described by (Gietz & Schiestl, 1991). The plasmids were maintained as autonomously replicating plasmids in the yeast cells by maintaining auxotrophic selective pressure. Transformation was verified by colony PCR analysis (results not shown).

5.2.5 Screening for Polygalacturonase activity

Strains were screened for PG activity with a PG plate assay modified from that described by Masoud & Jespersen (2006). Plates were supplemented with the necessary amino acids to maintain auxotrophic pressure in transformed strains. Five µL of an overnight culture containing 10^4 cells were spotted on PG plates and incubated for three days at 30°C. Degradation halos were visualized by staining plates with 6N HCl after washing the colonies of with distilled water and diameter of clearance zones were measured in millimetres. Three independent replicate experiments were performed for each strain. This assay was considered semi-quantitative and only used to compare PG activity between strains. Surface area of degradation halos was measured and more than a 25% change in surface area was regarded as a significant change in activity.

5.3 Results

5.3.1 Sequence analysis of *PGU1*

The nucleotide sequence of multiple *PGU1* clones was determined after cloning into the pGEM T vector. Single Nucleotide Polymorphisms (SNPs) were detected in five of the clones generated (Table 5.1). Plasmids were constructed for overexpression of the *PGU1* alleles (Table 5.1).

Table 5.1 Vectors constructed for overexpression of mutated alleles of the *PGU1* gene.

PGU1 allele	Nucleotides mutated	Resulting amino acid change	Overexpression plasmid generated
PGU1 (wild type)	none	none	pCP2
PGU1-1	21 Adenine to Cytosine 525 Thymine to Adenine 566 Thymine to Cytosine	Leucine to Phenylalanine Valine to Alanine No change	pPM1
PGU1-2	421 Adenine to Guanine	Asparagine to Aspartic Acid	pPM2
PGU1-3	26 Threonine to Guanine	Isoleucine to Serine	pPM3
PGU1-4	1040 Adenine to Guanine	Tyrosine to cysteine	pPM4

5.3.2 PG activity of PCR generated random mutations in *PGU1*

To determine if the nucleotides that were mutated are located in important regions for Pgu1p function, the overexpression vectors constucted were transformed into the BY4742 strain (a strain without PG activity) and activity conferred upon this strain by the various alleles were compared (Figure 5.1).

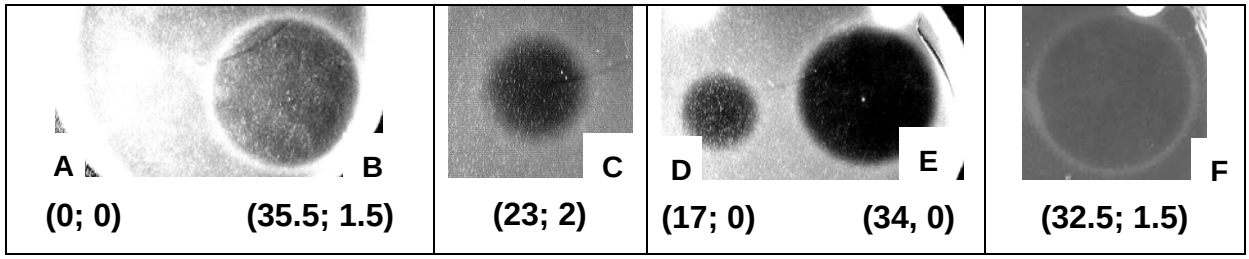

Figure 5.1 Semi-quantitative PG plate assay comparing PG activity of the *S. cerevisiae* BY4742 strain overexpressing the *PGU1* gene to the same strain overexpressing various mutated alleles in which different SNPs were generated. 3 Independent experiments were carried out for each strain, resulting in similar differences in activity between strains. Genes overexpressed: A – negative control, B - *PGU1* wild type gene, C - *PGU1*-1, D - *PGU1*-2, E - *PGU1*-3, F - *PGU1*-4. Diameter of the clearance zones in mm is indicated below each zone followed by the standard deviation between replicates.

From Figure 5.1 it can be seen that overexpressing the *PGU1* alleles carrying mutations 3 and 4 did not result in any change in PG activity when compared to the same strain overexpressing the wild type gene. Mutation 2 resulted in the most dramatic decrease in activity upon overexpression. Overexpressing allele 1 that had 3 SNPs in different regions resulted in a slight decrease in PG activity compared to overexpression of the wild type *PGU1* gene.

5.4 Discussion

From Figure 5.1 it can be seen that overexpression of two of the mutated genes conferred lower PG activity upon the BY4742 strain than when the wild type gene was overexpressed. The mutated allele *PGU1*-1 showed lower activity upon overexpression (Figure 5.1C) than the wild type gene (Figure 5.1B). Three nucleotides were mutated in *PGU1*-1, only two of these nucleotide changes resulted in amino acid changes. The Adenine that was mutated to Cytosine 21 bp downstream of the start codon resulted in leucine being replaced by a phenylalanine and the thymine that was mutated to cytosine 566 base pairs downstream of the start codon resulted in Valine being replaced by Alanine. All these amino acids are nonpolar hydrophobic amino acids and the amino acid changes should thus not have resulted in a significant change in the tertiary structure of the protein. These 3 SNPs did thus probably not lead to a significant change in tertiary structure, only resulting in a slight loss of activity. In a similar fashion the Adenine to Guanine mutation in *PGU1*-4, resulted in an amino acid with a nonpolar side chain replacing another nonpolar amino acid. Tyrosine was replaced by Cysteine; both these amino acids carry nonpolar side chains, with a neutral charge at pH 7. From Figure 5.1F it can be seen that this mutation did not lead to a loss in PG activity. The Threonine to Guanine mutation 26 bp downstream of the ATG start codon in *PGU1*-3 resulted in Isoleucine changing to Serine in Pgu1p. This mutation thus resulted in a nonpolar amino acid changing to a polar amino acid, and at a pH of 7 the side chains possessed by both these amino acids are neutral. The signal peptide of Pgu1p occurs between residues 1 and 18 of the protein (Blanco *et al.*, 1998). Since this amino acid change occured in the signal peptide of Pgu1p, this change in secretion signal could interfere with transport of the protein to the endoplasmic reticulum, interfering with processing and secretion and thus PG activity. From Figure 5.1F it can however be seen that overexpression of this mutated allele did not result in a change in activity compared to overexpression of the wild type gene. Overexpression of *PGU1*-2 resulted in a significant decrease in PG activity when compared to overexpression of the wild type gene. It indicates that this amino acid plays a significant role in enzyme function. The Adenine to Guanine mutation occured 421bp downstream of the ATG start codon. This mutation resulted in Asparagine replacing Aspartic Acid. Both these amino acids are polar, with Aspartic Acid being negatively charged at pH 7 and Asparagine being neutral. Since this amino acid change causes a change in charge and hydrophobicity, this change in primary structure of the protein could result in a change in tertiary structure affecting PG activity.

The structure of Pgu1p is not well studied. A putative active site (Blanco *et al.*, 1998) and potential glycosylation sites (Blanco, *et al.,* 2002) were identified by site directed mutagenesis. Sites to target with site directed mutagenesis were identified by comparing *PGU1* with polygalacturonase encoding genes from filamentous fungi (Blanco, *et al.,* 2002). In this study we identified a potentially important amino acid in the structure of Pgu1p by random PCR mutagenesis.

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Chapter 6

General discussion and conclusions

6.1 Concluding remarks and perspectives

The yeast *Saccharomyces cerevisiae* is one of the most useful microorganisms in industry and has been used in baking, brewing and winemaking since ancient times due to its fermentive capabilities. It has also been successfully used for many years as a model organism to unravel biological processes in higher eukaryotes. This yeast has indeed several traits making it a favourable organism to use as model organism to study eukaryotic cell biology, biochemistry and genetics (Borneman *et al.*, 2007). It is a eukaryotic organism with a short generation time, its genetics is well studied and published and cellular mechanisms are well conserved between *S. cerevisiae* and multicellular organisms and genetic manipulation of this yeast is simple. The complete yeast genome is known since 1996 and comprises ~6200 genes located on 16 chromosomes, from which more than 60% have an assigned function (Goffeau *et al.*, 1996, Mao *et al.*, 2008). Several tools such as the gene-deletion mutant collection (EUROSCARF), gene-expression libraries, DNA microarrays and protein chips are available for systematic genome-wide screenings that can be used to characterize molecular events and biological pathways and understanding the fundamental cellular functions and structures.

30% of human genes with a recognized involvement in human diseases have orthologs in yeast and studies in yeast have thus been essential for understanding fundamental cellular processes (Galao *et al.*, 2007). Since this yeast is commonly used in industry, studying this organism also has many potential economic benefits.

During the winemaking process, addition of commercial polysaccharide-degrading enzymes from fungal origin is common practice to improve wine processing and wine quality. Breaking open the grape berry cells by degrading the structural polysaccharides in the grape cell walls can result in an improvement in wine quality through the release of intracellular colour and aroma compounds, improving colour intensity, stability and aroma profile (Louw *et al.,* 2006). The addition of commercial enzyme preparations is expensive and may contain traces of other enzymes generating undesired side activities (Van Rensburg *et al.,* 2007).

A wine yeast strain with polysaccharide degrading ability will thus result in an improvement in wine quality and processing without the addition of these commercial preparations.

Pectin is a generic name for a mixture of pectic substances with different compositions, but all built on a polygalacturonic acid backbone. It is a structural heteropolysaccharide that occurs mainly in the middle lamellae and primary cell walls of

higher plants. Pectin is degraded by a group of enzymes collectively called pectinases. These enzymes are classified according to their specific substrate, mode of action, action pattern and cleavage products (Jayani *et al.*, 2005).

The role of pectolytic activity in yeast has been speculated to degrade plant cell walls to allow yeast to reach plant metabolites and thus facilitate yeast invasion of fruits (Gognies *et al.*, 1999). Polygalacturonic acid is degraded by an endo-polygalacturonase (PG) encoded by the *PGU1* gene in *Saccharomyces cerevisiae* (Blanco *et al.*, 1994). Only certain *S. cerevisiae* strains can degrade polygalacturonic acid and PG activity is thus strain specific. Various reasons for the absence of PG activity in certain strains have been described in literature. The *PGU1* gene is absent in some *S. cerevisiae* strains and consequently these strains do not possess PG activity (Fernández-González *et al.*, 2004). The absence of activity has been attributed to a dysfunctional allele of the *PGU1* gene being present (Gognies *et al*., 1999). It has also been shown that some strains lacking activity possess a functional copy of the *PGU1* gene, but the gene is not transcribed (Gognies *et al.*, 2001; Hirose *et al.*, 1999). This absence of transcription has been attributed to differences in the promoter region of the gene (Gognies *et al.*, 2001) and to differences in the genetic background of the strains (Hirose *et al.*, 1999).

In this study we tried to elucidate why there are differences in PG activity between *S. cerevisiae* strains with varying PG activities by looking at the sequence of the *PGU1* gene, transcription of this gene and its transcription factors and by screening the genome for activators and inhibitors of activity.

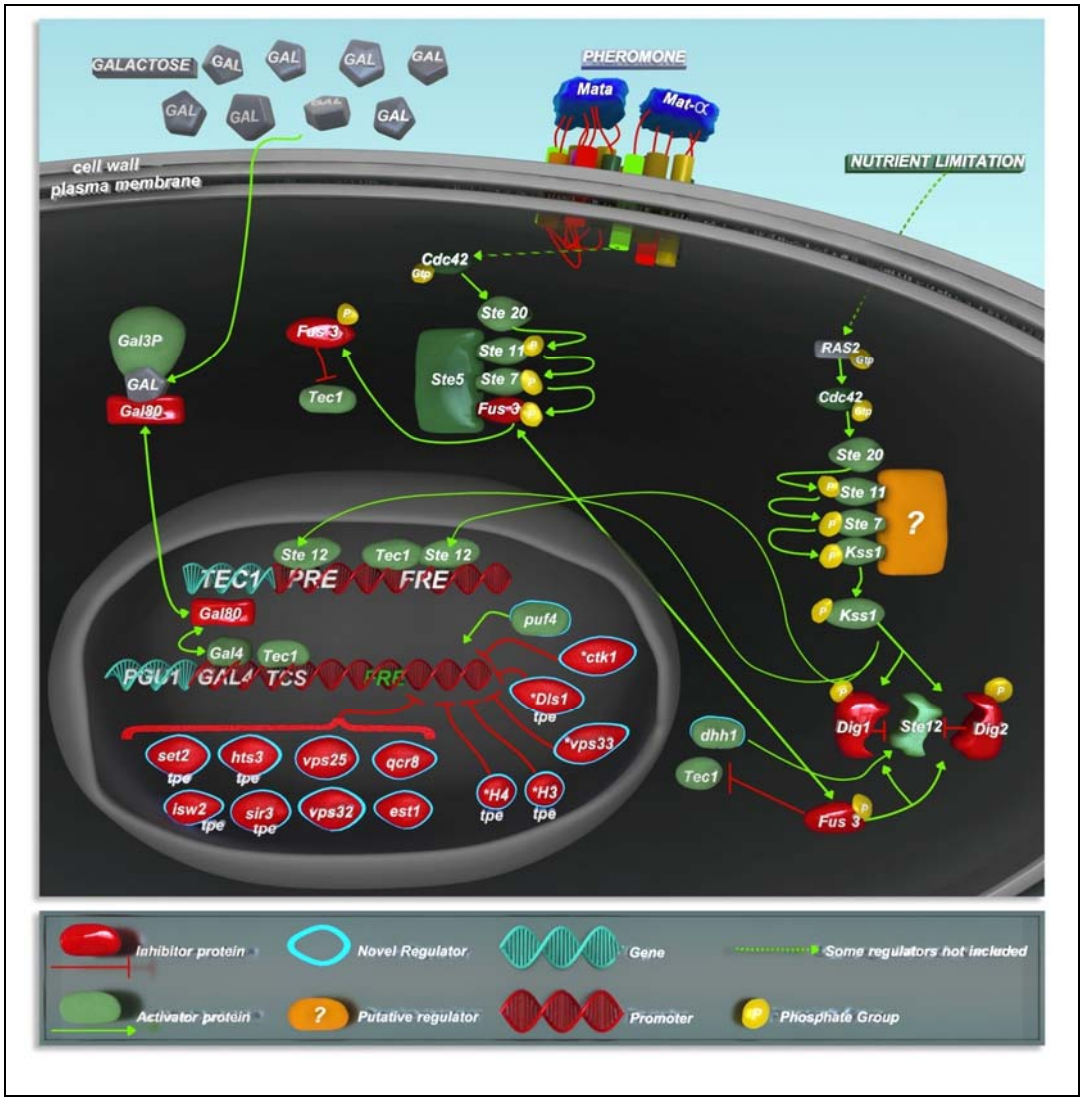
We compared various wine and laboratory yeast strains that did possess the *PGU1* gene and found variation in the *PGU1* structural gene not to be the reason for the absence of PG activity in any of the strains we investigated. Our study showed that all the strains transcribed the *PGU1* gene and confirmed that PG activity increased with an increase in *PGU1* transcription. Random mutagenesis was used to identify potential important regions in Pgu1p. We showed that there was a linear relationship between *PGU1* transcription and that of its two transcription factors *TEC1* and *STE12*. The *S. cerevisiae* genome library was screened for activators and inhibitors of PG activity. Enhancers of *PGU1* transcription activated by *TEC1* were identified by looking for deletion mutants with lower PG activity than the wild type strain when *TEC1* was overexpresssed in these strains. Two genes were identified by this screening: *DHH1* and *PUF4*. *DHH1* affects Ste12p level, while Puf4p also affects invasive and

filamentous growth, these genes probably influenced PG activity through an indirect effect on *PGU1* transcription.

Fourteen inhibitors of PG activity were identified by this screen, two of these inhibitors are components of the mating and filamentous growth MAPKs, and inhibit *PGU1* transcription by decreasing transcription of *TEC1* and *STE12*. Five genes identified as inhibitors of *PGU1* transcription (*DLS1, ISW2, SIR3, HST3* and *ESC1)* are involved in gene silencing by Telomere Position Effect (TPE). Transcription of genes inserted near telomeres is frequently repressed by an epigenetic effect known as tpe (Vega-Palas *et al.*, 2000). *PGU1* is located at position 722806..723891. It is therefore subtelomeric since its location is within 25 kb of the right telomere of chromosome X (Barton *et al.*, 2003; Galibert *et al.*, 1996). The separate deletion of three genes identified as inhibitors, *CTK1, VPS25* and *VPS32,* has previously been shown to result in the shortening of telomeres (Askree *et al.*, 2004). Shorter telomeres can potentially lead to an increase in transcription of subtelomeric genes. The fact that eight genes involved in telomeric silencing were identified as inhibitors of PG activity suggests that *PGU1* transcription is inhibited due to its subtelomeric position in the BY4742 strain. We showed *PGU1* transcription is inhibited in FY23, a strain isogenic to BY4742, due to its subtelomeric position by moving the *PGU1* gene with its native regulatory machinery to a different position away from its telomere, *PGU1* transcription and PG activity increased. PG activity also increased upon deletion of *DLS1,* a gene involved in telomeric silencing in other strains lacking PG activity, indicating the position of *PGU1* was involved in the lack of PG activity in these strains. Post-translational modification of histones lead to division of chromosomes into silenced and transcribed regions (Shahbazian & Grunstein, 2007). Genes are silenced in the region up to 25 kb from the telomeres due to post-translational modification of histones (Wyrick *et al.*, 1999). Previously published microarray data sets were investigated in order to determine the influence of different histones on chromatin structure that influence *PGU1* transcription. The amino terminal tails of histones 3 and 4 were found to be involved in silencing the *PGU1* gene.

Screening the *S. cerevisiae* genome for genes influencing PG activity thus revealed a number of novel or unexpected genes regulating this phenotype. This strategy identified a novel mechanism for regulation of PG activity in *S. cerevisiae*. Our findings corresponded with Hirose *et al.* (1999) in that transcription varies between strains and that this difference is due to differences in the genetic background of the strains and not due to differences in the promoter region of *PGU1.* The absence in activity in certain

strains have been speculated to be due to a trans-acting factor inhibiting *PGU1* transcription, this factor has been suggested to be absent in strains with PG activity (Hirose *et al.,* 1999). In this study we demonstrate that the difference in regulation of *PGU1* transcription occurs at a epigenetic level. RNA polymerase is prevented access to the *PGU1* promoter due to DNA being folded into a highly condensed structure and due to post-translational modification of histones demarcating the region in which *PGU1* is located as a silent region. Galactose induces PG activity in certain strains and this has been speculated to be due to a increase in transcription of the *PGU1* gene, as a result of Gal4p binding to an putative Gal4p binding site identified in the *PGU1* promoter. In this study we showed transcription of the *PGU1* gene increased in certain strains in the presence of galactose.



*Deletion or mutation of genes encoding these proteins was shown to result in a significant increase in *PGU1* transcription.

Figure 6.1 . Regulatory pathways influencing transcription of the *PGU1* gene and its main transcription factor *TEC1* in *S. cerevisiae.* This Figure illustrates activation and signal transduction by phosphorylation of various kinases through the mating and invasive growth MAPK pathways. Binding of transcription

factors to regulatory elements in the promoters of *PGU1* and *TEC1* is shown. The putative regulatory mechanism by which galactose activates *PGU1* transcription is also illustrated. Novel activators and inhibitors of PG activity identified by this study are presented. All these regulatory factors are proposed to regulate transcription of *PGU1.* Genes encoding inhibitors of PG activity identified by this study, that have previously been shown to be involved in gene silencing by Telomere Position Effect (TPE) are all marked tpe. A putative FRE element identified in the *PGU1* promoter region is shown.

Figure 6.1 illustrates how *PGU1* is regulated, this Figure is based on that presented in the general introduction of this dissertation. It has now been updated in view of the results reported in this study. All regulating factors identified at molecular level up to date belong to the mating and filamentous growth MAPK's, many of the novel inhibitors identified is proposed to inhibit *PGU1* transcription through telomeric silencing. The histones, H3 and H4 have also been shown to be involved in regulating transcription of *PGU1.*

Mutant wine yeast strains with pectolytic ability have been produced by chemical mutation and fermentation with these strains resulting in an improvement in wine quality and processing (Radoi *et al.*, 2005a). In order to construct pectolytic wine yeast strains this is a future option since strains produced by mutation are not classified as GMO's and strains selected with desired phenotypes can be used in the wine industry. This applied study in concert with whole genome investigation can also answer some fundamental questions concerning PG regulation. The transcriptome of the mutant and wild type strains can be compared with one another and with wild type strains with and without activity in order to identify alteration of which metabolic pathways resulted in the increase in PG activity in the mutant strains. Comparison with the wild type strains would indicate if differences in the same mechanisms were responsible for differences in activity between different wild type strains. In order to further investigate the epigenetic mechanism of *PGU1* regulation, the telomere length of the right arm of chromosome X can be compared between strains with and without PG activity by doing Southern blots. Attempts can be made to identify the putative inhibiting protein binding to the subtelomeric area according to the anti-promiscuity model suggested by Van Leeuwen & Gottschling (2002) and Martin *et al.* (2004), by doing complex immunoprecipitation (Co-IP). Antibodies can be selected to target histones identified as playing a role in inhibition of *PGU1* transcription (H3 and H4). Complexes formed with H3 and H4 can be compared between strains with and without PG activity in order to prove *PGU1* transcription is inhibited by this mechanism in certain strains. Since invasive growth and PG activity is co-regulated on more then one level, the effect of PG regulating proteins identified by this study on invasive growth should be investigated.

The influence of factors influencing invasive growth, such as nitrogen limitation, should be investigated upon PG activity. DNA footprinting analysis can be done on the *PGU1* promoter region to determine if Gal4p binds to this promoter. The galactose regulatory mechanism can be compared between different strains to elucidate if differences in this regulatory mechanism is responsible for the differential expression of *PGU1* in the presence of galactose in different *S. cerevisiae* strains.

In a addendum to this thesis, it is attempted to improve the xylanolytic ability of *S. cerevisiae* through heterologous expression of various xylanase encoding genes In order to engineer a wine yeast strain with good oenological properties that is able to degrade both pectin and xylan a strategy can be suggested. A yeast strain with desired oenological properties can be mutagenised to confer pectolytic ability to this strain. The xylanase expression cassette that yields the most optimimum xylan degradation within winemaking parameters can be cloned into the pectolytic wine yeast.

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Addendum

Comparing xylanase activity of recombinant *Saccharomyces cerevisiae* strains with xylanase activity through heterologous expression of different xylanase encoding genes of various origins

Comparing xylanase activity of recombinant *Saccharomyces cerevisiae* strains through heterologous expression of different xylanase encoding genes of various origins.

1 Abstract

Xylanase activity of *Saccharomyces cerevisiae* recombinant strains overexpressing different xylanase encoding genes was compared. The genes were sub-cloned and inserted into yeast expression vectors. Gene expression was controlled by the constitutive phosphoglycerate kinase 1 gene promoter ($PGK1_p$) and terminator ($PGK1_T$) sequences. The xylanase encoding genes chosen were the following: *xynA* (the complete gene) initially cloned from the rumen bacterium *Ruminococcus flavefaciens* or its two functional domains *xynAa* and *xynAc* individually, *XYN2* from *Trichoderma reesei* and *XYN4* from *Aspergillus niger.* Xylanase activity when overexpressing *XYN2* was 297.2 nkat/ml, *XYN4* 144.3 nkat/ml and *xynAc* 3.6 nkat/ml. However, no xylanase activity was detected when overexpressing *xynA* or *xynAa* with the reducing sugar assay. A weak activity was nevertheless detected using plate assay.

This study showed that heterologous expression of the *R. flavefaciens xynA* gene in *S. cerevisiae* did not result in construction of a yeast strain that was very efficient in degrading xylan. The strain constructed that was most efficient in degrading xylan was the strain overexpressing the *T. reesei XYN2* gene.

2 Introduction

Polysaccharides make up 90 – 95% of the cell walls encompassing fruit cells. These polysaccharides are mainly cellulose, hemicellulose and pectin (Kilcast, 2004). Enzyme preparations consisting of pectinases, cellulases and hemicellulases are known as macerating enzymes in the beverage industry. These preparations are used to improve juice yield, clarification and filtration during wine making by degrading polysaccharides that interfere with juice extraction (Haight & Gump, 1994). Degradation of the cell walls also result in release of more aroma compound precursors *e.g.* monoterpenes and phenolic compounds such as tannins.

Since terpenes are important constituents of the fruity aroma of grapes, hydrolysis of glycosydically bound terpenes during fermentation can enhance the aroma profile (Zoecklein *et al.*, 1997). More tannins can lead to an increase in colour intensity and

stability in ageing red wine by polymerizing with anthocyanins (Somers & Wescome, 1982).

Polysaccharide degrading wine yeast strains have been created through heterologous expression of fungal genes. Making wine with these strains by using different grape cultivars, showed several improvements concerning wine quality and processing, such as an altered chemical profile, more intense stable colour and some of the wines made by these strains were preferred by a tasting panel, compared to wines fermented with the wild type strains (Louw *et al.*, 2006). The genetically modified strains were constructed by overexpressing different combinations of pectinase, glucanase and xylanase encoding genes cloned from a variety of organisms and placing them under control of different promoters. Overexpressing combinations of glucanase and xylanase encoding genes had the biggest impact on wine making by influencing colour intensity, aroma profile and percentage free flow wine released. The xylanase encoding gene *XYN2*, cloned from the fungus *Trichoderma reesei,* and *XYN4* cloned from the fungus *Aspergillus niger,* both made an significant impact on wine quality when wine was fermented with strains overexpressing these genes (Louw *et al.*, 2006).

Xylan is a major component of hemicellulose and is comprised of a backbone of β-1, 4-linked xylopyranose residues which can be substituted with acetyl-, arabinose- and methylglucuronic acid residues. Endo-β-1,4-xylanases hydrolyze the glycosidic linkages between the xylopyranose groups in unsubstituted regions of the xylan chain, producing a mixture of xylo-oligosaccharides (Luttig *et al.*, 1997). *Saccharomyces cerevisiae* can not break down xylan, nor can it utilize the monomeric constituents of the backbone D-xylose as carbon source. The filamentous fungi *A. niger* and *T. reesei* are commonly used for commercial production of xylanases. Several xylan degrading *S. cerevisiae* strains have been created through heterologous expression of xylanase encoding genes, these genes include *XYN A, B* and *C* cloned from *A. niger* and *XYN2* cloned from *T. reesei*. (Ganga *et al.*, 1999; la Grange *et al.*, 1996; Luttig *et al.*, 1997). Several fungal xylanases have been expressed in *S. cerevisiae* but very few bacterial genes have been expressed in yeast.

Since rumen bacteria contributes extensively to the degradation of plant cell wall polysaccharides in the rumen of herbivorous mammals, these bacteria are a good source to mine for novel polysaccharase encoding genes. *Ruminococcus flavefaciens* is one of three species of anaerobic, cellulolytic bacteria that play a major role in the degradation of plant cell wall polysaccharides in the rumen. This Gram positive, anaerobic bacterium has been found to produce at least six different xylanases, ranging

in apparent molecular weight from 55 to 200 kDa (Flint *et al.*, 1994). The *xynA* gene encodes a bifunctional enzyme of 954 amino acids comprising two dissimilar xylanase domains interconnected by an aspargine and glutamine rich linker region. xynAp is thus a bifunctional enzyme with two different catalytic domains capable of degrading xylan (Flint *et al.*, 1994). The xylanases encoded by the two domains are both endo-xylanases but differ with respect to their action of xylan degradation, with domain A producing larger products than domain C. It has been speculated that the reason for the linkage of the two domains is that the larger products yielded during xylan degradation by domain A is further degraded by domain C (Flint & Zhang, 1992).

In this study, we individually overexpressed the complete *R. flavefaciens xynA* gene and its two functional domains individually as well as the *T. reesei XYN2* and *A. niger XYN4* genes in *S .cerevisiae*. The xylanase activity of these recombinant strains was compared.

3 Materials and Methods

3.1 Strains, plasmids and culture conditions

Plasmids were constructed and amplified in *Escherichia coli* DH5α *[F-φ80lacZΔM15Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk−, mk+) phoA supE44 thi-1 gyrA96 relA1 λ]* (GIBCO-Invitrogen Life technologies, Mowbray, South Africa). *E. coli* was cultured in Luria Bertani (LB) medium (Biolab diagnostics, Wadenville, South Africa), supplemented with 100 mg L^{-1} ampicillin (Ampicillin sodium salt, Sigma, Missouri, USA) for the selection of resistant bacteria. *S. cerevisiae* wild type strains were grown in Yeast Peptone Dextrose (YPD) Broth (Biolab diagnostics) at 30°C on a rotary shaker at 150 r.p.m. For the selection of yeast transformants, Synthetic Complete (SC) medium containing 6.7 g L^{-1} yeast nitrogen base without amino acids (Difco Scientific group, Waterfall Park, South Africa) and 20 g L^{-1} glucose supplemented with the appropriate amino acids to apply auxotrophic pressure. Recombinant xylanase secreting strains were constructed with the *S. cerevisiae* Σ1278b strain (Van Dyk *et al.*, 2005).

3.2 DNA manipulation and plasmid construction

All genes studied were sub-cloned by using the polymerase chain reaction (PCR). Primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA) (Table 6.1).

Table 1 Genes sub cloned

Gene cloned	Template	Organism gene originates from	Primers used	PCR program
xynA	pX4-129 (Flint *et al.*, 1991)	*R. flavefaciens*	PXYNA 5' HinIII AAGCTTGCTGATCAGCAGACC AGAGGTAACG PXYNA 3' BamHI GGATCCGCCTCACTTTGCAAGA	1
xynAa	pX4-129 (Flint *et al.*, 1991)	*R. flavefaciens*	PXYNA 5' HinIII AAGCTTGCTGATCAGCAGACC AGAGGTAACG PXYNADA 3'2 BamHI GGATCCTTAGCCACCGTTATCAGAGG AGCC	2
xynAc	pX4-129 (Flint *et al.*, 1991)	*R. flavefaciens*	PXYNA 5' 2 HinIII AAGCTTAACCAGTGGGGCGGTCAG AA PXYNA 3' BamHI GGATCCGCCTCACTTTGCAAGA	3
XYN4	pEX (Van Rensburg *et al.*, 2007)	*A. niger*	Xyn4 forward GATCAGATCTATGAAGGTCACTGCGG CTTTTG Xyn4 rev GATCCTCGAGGACTACCAAGGCACTT ATCCCTTAAG	4

Genes sub-cloned are shown in Table 6.1. DNA was amplified using an Applied Biosystems 2720 thermal cycler, Takara ExTaq enzyme and Takara buffer with MgCl2 (Separations, Randburg, South Africa). The reaction mixture contained 250 µM of each nucleotide (dNTP), 200 ng DNA, 0.25 µM of each primer, and 0.2 mM MgCl2. Table 6.2 shows PCR programs used.

Table 2 PCR programs used

Program nr	Initial denaturation		iteration			Final elongation	
	temperature	time	cycle	temperature	time	temperature	Time
1	94°C	10 min	30	95°C 57°C 72°C	30 s 40 s 180 s	72°C	7 min
2	94°C	10 min	30	95°C 55°C 72°C	30 s 40 s 60 s	72°C	7 min
3	94°C	10 min	30	95°C 52°C 72°C	30 s 40 s 60 s	72°C	7 min
4 [a]	95°C	10min	40	95°C 52°C 72°C	30 s 40 s 60 s	72°C	7 min

Plasmids were constructed in order to overexpress the various xylanase genes in *S. cerevisiae* (Table 6.3). *xynA* and its truncations were individually cloned into the multiple cloning site of pCEL15. Expression cassettes generated for expression of genes derived from *R. flavefaciens xynA* are illustrated in Figure 6.1. The *XYN4* gene was cloned into the pCEL13 vector. The *R. flavefaciens XYNA* derived genes do not code for a leader peptide that would initiate translation or mediate secretion of the xynA proteins in *S. cerevisiae* and therefore the genes were fused to the *S. cerevisiae MF*α1 secretion signal in the pCEL15 expression vector. The pCEL13 vector has no secretion signal present and the native secretion signal was maintained for *A. niger* derived *XYN4* gene ligated in to this vector.

Table 3 Plasmids used in this study

Plasmid	Strain*	Description	Reference
pCEL13		Ap^R *URA3* $PGK1_P$ [a]-$PGK1_T$ [b]	(Gundllapalli *et al.*, 2006)
pCEL15		Ap^R *URA3 MFα* $PGK1_P$-$PGK1_T$	(Gundllapalli *et al.*, 2007)
pUC13 – X4129 pEX		Ap^R *XYNA*	(Flint, *et al.,* 1991)
pDLG6	Σ1278b+ pDLG6	Ap^R *URA3* $PGK1_P$*XYN4 PGK1*	(La Grange *et al.*, 1996)
pX4	Σ1278b+ pX4	Ap^R *URA3 MFα* $PGK1_P$*xynA* $PGK1_T$	This study
pX5	Σ1278b+ pX4	Ap^R *URA3 MFα* $PGK1_P$*xynAa* $PGK1_T$	This study
pX6	Σ1278b+ pX4	Ap^R *URA3 MFα* $PGK1_P$*xynAc* $PGK1_T$	This study
pX9	Σ1278b+ pX9	Ap^R *URA3* $PGK1_P$*XYN4* $PGK1_T$	This study

- *Strain constructed when *S. cerevisiae* was transformed with the appropriate plasmid.

DNA fragments to be sequenced were first cloned into a pGEM®-T Easy vector (pGEM®-T Easy Vector Systems, Promega, Whitehead Scientific, Cape Town, South Africa) according to the manufacturer's instructions. Plasmid DNA was isolated from positive transformants of *E. coli* DH5α. Both strands were sequenced in an ABI 3130XL Genetic Analyzer at the Central Analytical Facility (Stellenbosch University) using the universal M13 forward and reverse primers. *xynA* fragments were excised from the pGEM® T-easy vector with the restriction enzymes *Bam*HI and *Hind*III and *XYN4* was excised with *Bgl*II and *Xho*I (Roche Diagnostics, Randburg, South Africa). The vector pCEL15 was linearized with *Bgl*II and *Hind*III and pCEL13 with *Bgl*II and *Xho*I (Roche Diagnostics). DNA was eluted from agarose gels by using the Zymoclean™ gel recovery kit (Zymo research, Orange, CA, USA) according to the manufacturer's instructions. Standard methods were used for the restriction and ligation of DNA, plasmid transformation into *E. coli*, and agarose-gel electrophoresis (Maniatis *et al.*, 1989). Correct cloning was verified by restriction analysis and sequencing.

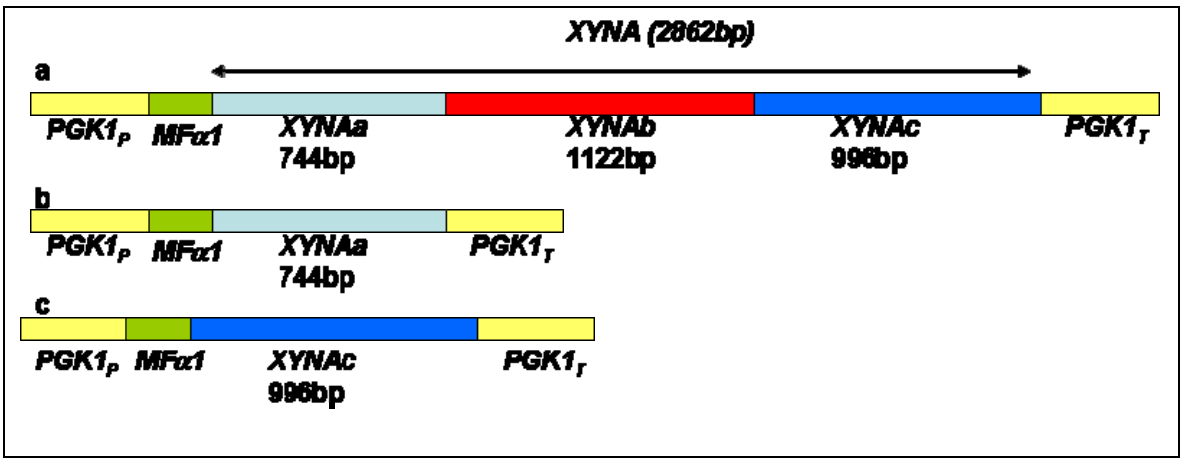

Figure 1 Expression cassettes constructed for overexpressing the *Ruminococcus flavefaciens xynA* gene and its 2 functional domains. (a) The complete gene *xynA* comprising the two functional domains *xynAa* (744bp catalytic domain), *xynAc* (996bp catalytic domain) linked by a long aspargine and glutamine rich *xynAb* linker domain, were fused with the $PGK1_P$, $PGK1_T$ and the $MF1\alpha$ secretion signal. (b) *xynAa* and (c) *xynAc* were individually cloned and expressed in the same expression cassette as the complete gene.

3.3 Yeast transformation

S. cerevisiae strain Σ1278b was transformed with plasmids constructed for overexpressing the various xylanase genes (Table 6.3). All transformations were carried out using the lithium-acetate method described by Gietz & Schiestl (1991). The plasmids were maintained as autonomously replicating plasmids in the yeast cells by maintaining auxotrophic selective pressure. Transformation was verified by colony PCR analysis: a single colony was resuspended in 100 μL dH_2O, 1 μL was used as PCR template and the primers used for cloning of the gene screened for was used.

3.4 Enzyme activity assays

Transformants were screened for xylan degrading ability on SC^{-Ura} medium containing 0.2% of 4-O-methyl-D-glucorono-D-xylan-remazol brilliant blue (RBB-xylan) (la Grange *et al.*, 1996). β-Xylanase cleaves RBB-xylan into a colourless product, leaving a clear zone around the colonies secreting xylanase.

Endo-β-1,4-xylanase activity was assayed by a method modified from that described by Baily *et al.* (1992). Samples were centrifuged at 8000 g for 5 min. after growing the cultures for 72 hours and supernatant was used as enzyme solution, 1% xylan from oatspelts (Sigma) was used as substrate. Activity was measured at 50°C and pH 5.0. The amount of reducing sugar released was determined by the dinitrosalicylic acid method describe by Miller *et al.* (1960).

4 Results

4.1 Constructing xylan degrading strains

The complete *xynA* gene and the two functional domains *xynAa* (744bp catalytic domain) and *xynAc* (996bp catalytic domain) were individually sub cloned from the plasmid pUC13 -X4129. To clone the genes *xynA* and *xynA a* (*XYNA* domain a) the region stretching from 145 bp down stream of the putative start codon, ttg, to 2940 bp and 843 bp down stream of the ttg codon was cloned for *xynA* and *xynAa* respectively. *xynA* has a putative bacterial secretion signal and start codon (Flint & Zhang, 1992). These regions were omitted during cloning.

xynAc was cloned from 1925 bp downstream of the ttg, consequently also omitting the putative secretion signal. Since the putative bacterial secretion signal was removed during cloning, truncations of the *xynA* gene, sub cloned from the plasmid pX4-129, were each fused with the *S. cerevisiae* mating factor MFα1 secretion signal to direct the recombinant xylanase proteins to the endoplasmic reticulum for secretion. This leader sequence peptide consists of a 19-amino acid (pre) sequence followed by 66-residue (pro) sequence. Three N-linked glycosylation sites and a Kex2 endopeptidase processing site are present on the (pro) sequence (Kurjan & Herskowitz, 1982). The *A. niger XYN4* gene was sub cloned from the plasmid pEX and the plasmid pDLG6 constructed by la Grange *et al.*(1996) was used for overexpressing *T. reesei XYN2*. Both these genes are from fungal origin and have previously been successfully expressed in *S. cerevisiae*. Their native secretion signals are recognized and cleaved by *S. cerevisiae* allowing for successful translocation to the endoplasmic reticulum, glycosylation and secretion of the recombinant proteins (la Grange *et al.*, 1996; Luttig *et al.*, 1997). Native xylanase secretion signals have previously been shown to be more successful in translocating xylanase enzymes out of the yeast cell, than the *S. cerevisiae* MFα1-secretion signal (Muller *et al.*, 1998). The native secretion signals were thus maintained for the fungal xylanase genes.

The PCR products were individually inserted into the pCEL15 plasmid in frame with the yeast mating factor under control of the constitutive *PGK1* promoter and terminator creating the plasmids pX4, pX5 and pX6 respectively. The *XYN4* gene was sub cloned from the plasmid pEX (Van Rensburg, *et al.,* 2007). The PCR product was inserted into the pCEL13 plasmid and placed under control of the constitutive *PGK1* promoter and terminator creating the plasmid pX9. The plasmid pDLG6 was obtained from D. La Grange (La Grange, *et al.,* 1996). These plasmids were individually transformed into the *S. cerevisiae* strain. Positive transformants were selected by auxotrophic selection on

SC^{-Ura} plates. These strains were screened for presence of the transformed gene by colony PCR (Figure 6.2).

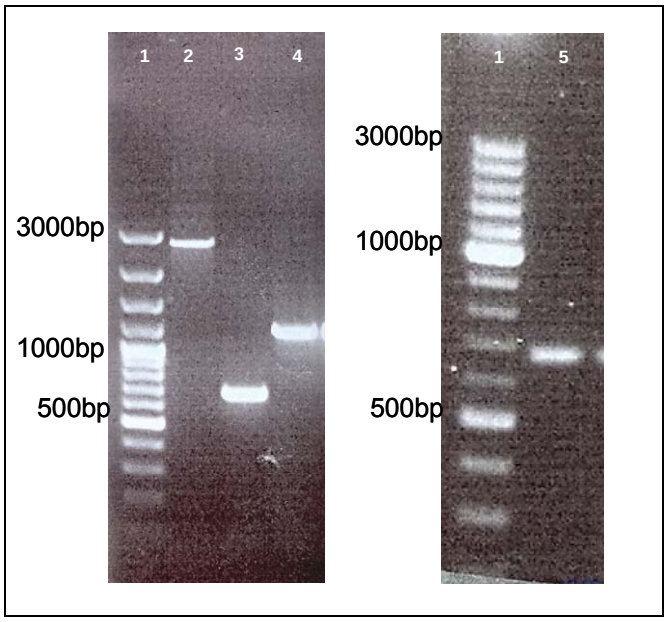

Figure 2 Colony PCR analysis showing the presence of 2. *xynA* (2862bp) in Σ1278b+ pX4, 3. *xynAa* (744bp) in Σ1278b+ pX5, 4. *xynAc* (996bp) in Σ1278b+ pX6 and 5. *XYN4* (700bp) in Σ1278b+ pX9 next to a 1) Gene Ruler Plus 100 bp DNA ladder (Fermentas Life Sciences, Glen Burnie).

4.2 Determining xylanase activity in recombinant strains.

Strains were screened for β-1, 4-xylanase activity, by spotting strains on RBB-xylan plates (Figure 6.2). It can be seen that all strains overexpressing the xylanase genes had xylanase activity, while RBB-xylan was not cleaved by the wild type Σ1278b strain. Although the plate assay is not accurately quantitative, the sizes of the halos can neverthless provide a good indication of the strength of the xylanase activity. The size of the clearance zones indicate that the most xylan was degraded by *XYN2*, followed by *XYN4*, with *xynA* and genes derived from this gene resulting in small clearance zones.

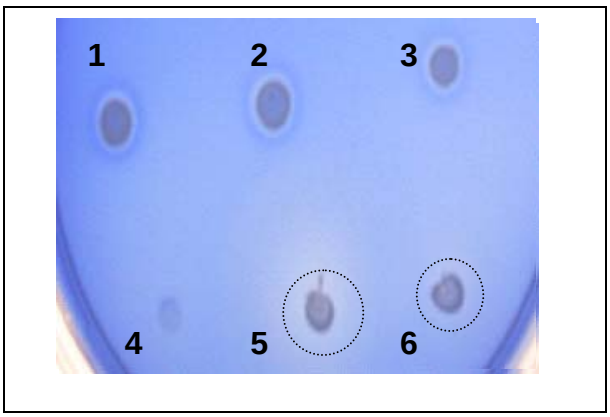

Figure 3 RBB-Xylanase plate assay showing that xylan was degraded by the strains producing active β-1, 4-xylanase through heterologous expression of 1. *xynA* (Σ1278b+ pX4), 2. *xynAa* (Σ1278b+ pX5), 3. *xynAc* (Σ1278b+ pX6), 5. *XYN2* (Σ1278b+ pDLG6) and 6. *XYN4* (Σ1278b+ pX9), compared to the wild type Σ1278b strain in 4. not resulting in xylan degradation. Xylan degradation can be seen as a colourless zone surrounding the colonies.

4.3 Enzyme activity assays

Endo-β-1,4-xylanase activity was assayed by a method modified from that described by (Baily *et al.*, 1992) using supernatant as enzyme solution (Figure 6.4) and oat spelts xylan (Sigma) as substrate. It can be seen from Figure 6.4 that the strain Σ1278b-pDLG6 (containing *XYN2* from *T. reesei*) had the highest xylanase activity (297.2 nkat/ml), followed by Σ1278b-pX9 (containing *XYN4* from *A. niger*) (144.3 nkat/ml), Σ1278b-pX6 (containing *XYNAc* from *R. flavefaciens*) transformation resulted in very low activity (3.6 nkat/ml), while the wild type Σ1278b strain and Σ1278b-pX4 and Σ1278b-pX5 (containing *xynA* and *xynAa* respectively) did not show any xylanase activity (Figure 6.4).

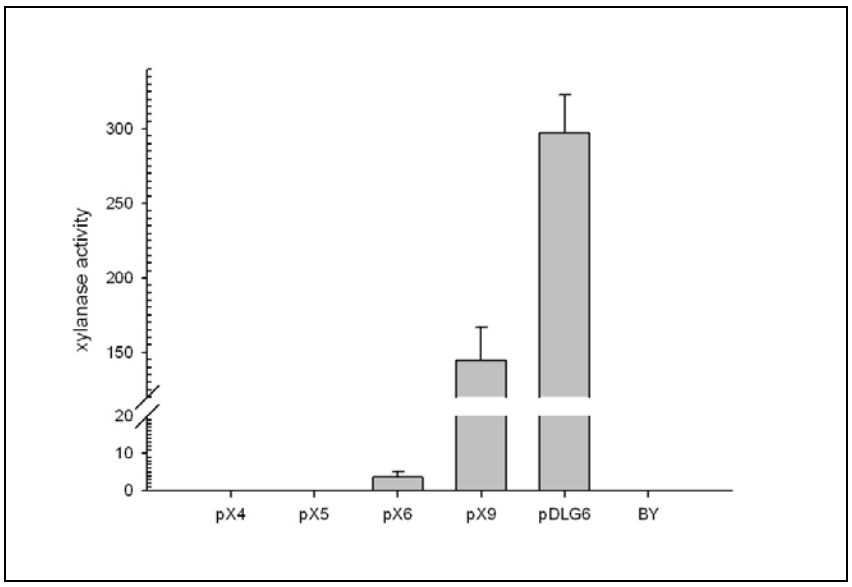

Figure 4 Xylanase activity in nkat/ml of *S. cerevisiae* recombinant stains through heterologous expression of different xylanase encoding genes. It can be seen that Σ1278b-pDLG6 overexpressing *XYN2* cloned from *T. reesei* showed the highest activity followed by Σ1278b-pX9 overexpressing *XYN4* cloned from *A. niger.* Σ1278b- pX6, overexpressing *xynA*c, a truncation encompassing the active domain C of the *xynA* gene cloned from *R. flavaciens* showed slight xylanase activity. No xylanase activity secreted could be detected from the strain overexpressing the complete *R. flavaciens xynA* gene Σ1278b-pX4 or the strain overexpressing the active domain A, Σ1278b- pX5.

5 Discussion

S. cerevisiae does not have the ability to degrade xylan. Fermenting grape must with recombinant *S. cerevisiae* wine yeast strains constructed to degrade xylan, through heterologous expression of xylanase encoding genes, have been shown to improve wine processing and wine quality compared to fermentation with the wild type strain (Louw *et al.*, 2006). With the aim of constructing yeast strains more efficient in degrading xylan, we compared xylanase activity of recombinant *S. cerevisiae* strains overexpressing the *xynA* gene previously cloned from the rumen bacteria *R. flavefaciens* and truncations of this gene, comprising its functional domains, domain A and C. Expressing *xynA* and these truncations in *E. coli* enabled this bacterium to secrete functional xylanase varying between genes (Flint & Zhang, 1992). In this study, we compared xylanase activity of strains expressing *xynA* and truncations of this gene to strains constructed, overexpressing xylanase genes previously cloned from filamentous fungi: *XYN2* from *T. reesei* and *XYN4* from *A. niger.* The ability of these strains to degrade oatspelts xylan through heterologous expression of the different xylanase encoding genes was compared. To maintain the same expression level for the xylanase encoding genes in all the recombinant strains, all these genes were place under control of the same promoter and terminator, i.e. the 3-Phosphoglycerate Kinase gene ($PGK1_P$ and $PGK1_T$). The PGK_P is a strong constitutive promoter that can direct

transcription of heterologous proteins to produce proteins at levels between 4 and 10 % of the total soluble proteins, depending on growth conditions (Hitzeman *et al.*, 1982).
The *R. flavefaciens xynA* gene and its two functional domains were sub cloned from the plasmid pX4-129 (Flint *et al.*, 1991). Different truncations of *xynA* have been previously sub cloned from this plasmid and were expressed under control of the promoter of the *LacZ* gene in *Escherichia coli* (Flint & Zhang, 1992).

From the plate assay (Figure 6.3) it can be seen that biologically active xynAp, xynAap and xynAcp were secreted by the appropriate recombinant strains resulting in slight degradation of RBB-xylan, while the strain secreting xyn4 had more activity, with the Xyn2 producing strain, secreting the most biologically active xylanase. This result corresponded with the liquid reducing sugar assay, showing the highest xylanase activity for pDLG6-*XYN2,* followed by Σ1278b-pX9, with very low xylanase activity in Σ1278b-pX6. No xylanase activity was detected for Σ1278b-pX4, Σ1278b-pX5 or the wild type Σ1278b strain. Previous studies showed that Xyn2p secreted by a recombinant *S. cerevisiae* results in about 10 times more xylan being degraded than by a strain secreting Xyn4p when both strains are grown under optimal conditions for both recombinant enzymes (Xyn2p:60°C, pH6 and Xyn4p: 60°C, pH4) (la Grange *et al.*, 1996; Luttig *et al.*, 1997). The difference in activity between the strains secreting these two enzymes in this study was only about 2 fold, which could be due to the assay conditions. The activity was assayed at 50°C, at which temperature the recombinant Xyn2p only retains about 60 % of its activity (la Grange *et al.*, 1996). Recombinant Xyn4p secreted by *S. cerevisiae* has however been shown to function with about 90 % of its maximum activity at this temperature (Luttig *et al.*, 1997). Activity of these xylanases is also influenced by pH and Xyn2p secreted by *S. cerevisiae* has been shown to retain more of its activity at pH 5, the pH used in the assay, which would increase the difference in activity between these two enzymes. Thermostability of these enzymes at 50°C has not been tested, but since Xyn2p lost about 80% of its activity within 10 minutes at 60°C, it is possible that this enzyme is more unsTable at 50°C than Xyn4p, decreasing the difference in activity between these two enzymes, to yield the two fold difference in activity detected in this study.

Several fungal xylanases have been successfully expressed in *S. cerevisiae* with expression being optimized to yield xylanase activity up to 1200 nkat/ml for expression of Xyn2p originating from *T. reesei* (La Grange *et al.*, 1996). Heterologous expression of bacterial xylanases in *S. cerevisiae* has yielded much lower activity then fungal xylanases *e.g.* heterologous expression of *xynA,* cloned from the bacterium *Bacillus*

pumilus only resulting in a maximum xylanase activity of 8.5 nkat/ml (Nuyens *et al.*, 2001). It seemed possible for the *R. flavefaciens xynA* gene to be expressed and functional enzyme to be secreted by *S. cerevisiae*, since polysaccharase encoding genes cloned from another Gram positive rumen bacteria, *Butyribrio fibriosolvens,* have previously been successfully overexpressed in *S. cerevisiae* with functional enzyme being produced (Peterson *et al.*, 1998; Van Rensburg *et al.*, 1994). The plate assay showed low amounts of active xylanase is secreted by the strains overexpressing truncations of *R. flavefaciens xynA* gene (Figure 6.3), but the DNS reducing sugar assay only detected xylanase activity from the liquid culture of Σ1278b-pX4 expressing *xynAc.* Activity was possibly to low to be detected from the Σ1278b-pX4 and Σ1278b-pX5 strains by the reducing sugar assay. Higher activity during heterologous expression of *xynAc* than during expression of the complete gene or *xynAa* could be expected, since Zhang & Flint (1992) found that overexpressing *xynAc* in *E. coli* resulted in four times higher xylanase activity than when the complete *xynA* gene was overexpressed and more than a 100 fold higher expression than during overexpression of *xynAa.* The low xylanase activity upon overexpression of the *R. flavefaciens* genes could be due to inefficient translation, post-translational processing such as glycosylation, protein folding or stability of the recombinant proteins.

Not all codons are used with the same frequency in different organisms. Since *xynA* is cloned from Gram positive bacteria, codon usage by yeast might be a problem with expression and translation of the *xynA* derived genes in *S. cerevisiae*. Heterologous expression and processing of a bacterial gene in *S. cerevisiae* has been shown to be inhibited due to the codon usage in this gene (Wiedemann & Boles, 2008). Engineering a bacterial gene to encode the same protein, but changing the codons to reflect the codon usage of *S. cerevisiae* has been shown to result in increased expression and higher levels of activity of the corresponding enzymes (Wiedemann & Boles, 2008). Significant differences were found between the different domains of the *R. flavefaciens xynA* gene.The activities associated with the two domains were shown to differ with respect to the average size of hydrolysis products formed from oat-spelt xylan, with domain C releasing relatively more xylose and domain A more xylo-oligosaccharides. The amino acid sequence of domain A of XylA closely resembled that of the *Bacillus pumilus xynA* enzyme (45% identical residues). On the other hand domain C showed significant similarity (33% to 40% identical residues) to a different group of bacterial xylanases and exoglucanases exemplified by the *Caldocellum saccharolyticum xynA* and *celB* products. Heterologous expression of *xynAa* and *xynAc* in *S. cerevisiae*

resulted in very low xylanase activity, which as was also previously shown by respective heterologous expression of *B. pumilus xynA (*4.5 nkat/ml) and *C. saccharolyticum xynA* in *S. cerevisiae* (Nuyens *et al.*, 2001, Donald *et al.*, 1994).

The efficiency of secretion of the xynA proteins might be improved by using a xylanase secretion signal instead of the MFα1-secretion signal used. When comparing the efficiency of a native xynA signal peptide from *A. pullulans* with the α-factor secretion signal from *S. cerevisiae* to direct heterologous xylanase secretion, the native secretion signal was found more capable of translocating xylanase out of the yeast cells (Li & Ljungdahl, 1996).

With the aim of constructing recombinant *S. cerevisiae* strains with improved xylan degrading abilities other fungal xylanase encoding genes could be explored.The *XYN3* gene cloned from *Aspergillus kawatchii* has been expressed and processed in *S. cerevisiae* and heterologous expression has been shown to yield very high activity (300n/kat/ml) when using the *PGK1* promoter (Crous *et al.*, 1995), This gene shows promise for expression in a wine yeast strain since the recombinant enzyme retains a lot of its activity at a low pH and this activity could be further improved by inducing transcription under control of the strong inducible *ADH2* promoter.

6 Conclusion

In this study, we investigated and compared xylanase activity of recombinant *S. cerevisiae* strains expressing various xylanase encoding genes at the same level, under the same conditions. Expressing *XYN2* originally cloned from *T. reesei* resulted in the *S. cerevisiae* recombinant strain with the highest activity, while expressing the *A. niger XYN4* gene resulted in a recombinant strain with about half the activity of a strain expressing *XYN2*.

Truncations of the *R. flavefaciens xynA* gene that confers xylanase activity to *E. coli* were overexpressed in *S. cerevisiae.* Overexpressing *S. cerevisiae* the complete gene *xynA,* the functional domain, *xynAa* and the functional domain, *xynAc* all conferred very slight xylanase activity to *S. cerevisiae*, with *xynAc* resulting in the highest xylanase activity. Since overexpression of the *R. flavefaciens xynA* gene yielded very low activity under optimal conditions activity in wine making conditions would be neglible.

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