

**CLONING, CHARACTERIZATION AND
DIRECTED EVOLUTION OF A XYLOSIDASE FROM
*Aspergillus niger***

Bibi Khadija Khan

Submitted in complete fulfilment for the Degree of Master of Science (Applied Science) in
the Department of Biotechnology and Food Technology, Faculty of Applied Sciences,
Durban University of Technology, Durban, South Africa

FINAL COPY APPROVED FOR SUBMISSION

**PROF KUGEN PERMAUL
SUPERVISOR**

DATE:

**PROF SUREN SINGH
CO-SUPERVISOR**

DATE:

PREFACE

The work described in this thesis was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, under the supervision of Prof Kugen Permaul and Prof Suren Singh.

Bibi Khadija Khan (Candidate)

Date

As the candidates supervisors we agree to the submission of this thesis.

Prof Kugen Permaul (Supervisor)

Date

Prof Suren Singh (Co-supervisor)

Date

DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Applied Sciences, to the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa. It has not been submitted before for any degree or dissertation to any other institute

Bibi Khadija Khan
2016

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ACKNOWLEDGEMENTS

All thanks is due to the Almighty Allah (SWT) for giving me the patience, humility, strength and courage to persevere.

I would like to express my deepest appreciation to Prof Kugen Permaul who expertly guided me through my undergraduate studies, and for his criticism, patience, and advice throughout this project.

To friends that became family, Evashni, Kerisha, Vashni, Sanjana, Malvin, Faez, Ashira and Meng - thank you for making the darkest days brighter.

The staff at the Department of Biotechnology and Food Technology for their invaluable assistance and advice throughout my studies.

The Stellenbosch team for providing the *S. cerevisiae* strain and plasmid used in this study

Technology Innovations Agency for their award of the scholarship that funded my postgraduate studies.

I am indebted to my parents, brothers and husband for their love and encouragement, financial support, and relentless cheering, this would not have been possible without you.

“it always seems impossible until it’s done”

– Nelson Mandela

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ABSTRACT

β -xylosidases catalyse the hydrolyses of xylooligosaccharides into the monosaccharide sugar, xylose. In this study we report the production of xylose under different conditions in *Pichia pastoris* and *Saccharomyces. cerevisiae*, and its conversion to bioethanol using *Pichia stipitis*. The aim of this study was to change the characteristics of the *A. niger* 90196 β -xylosidase through random mutagenesis and increase expression under the control of different promoter systems in yeasts *P. pastoris* and *S. cerevisiae*. The recombinant library created through random mutagenesis was screened for changes in activity and subsequently pH and temperature stability. One variant showed an increase in enzyme expression, thermostability, and a change in amino acid sequence at residue 226. The enzyme was then cloned, expressed and characterized in *P. pastoris* GS115 and *S. cerevisiae*.

β -xylosidase was constitutively expressed in *P. pastoris* using the GAP promoter and the inducible AOX promoter. In *S. cerevisiae* the enzyme was expressed using the constitutive PGK promoter and inducible ADH2 promoter systems. Enzyme functionality with the different expression systems was compared in both hosts. The GAP system was identified as the highest-producing system in *P. pastoris*, yielding 70 U/ml after 72 hours, followed by the PGK system in *S. cerevisiae*, with 8 U/ml. A 12% SDS-PAGE gel revealed a major protein band with an estimated molecular mass of 120 kDA, and the zymogram analysis revealed that this band is a fluorescent band under UV illumination, indicating enzyme activity. Stability characteristics was determined by expressing the enzyme at different pH and temperatures. Under the control of the GAP promoter in *P. pastoris*, enzyme activity peaked at pH4 while retaining 80% activity between pH 3 – 5. Highest activity of 70 U/ml xylosidase was recorded at 60°C.

Due to the high enzyme production in *P. pastoris*, the co-expression of this enzyme with a fungal xylanase was evaluated. The xylanase gene from *Thermomyces lanuginosus* was cloned with the GAP promoter system and expressed together with the β -xylosidase recombinant in *P. pastoris*. Enzyme activities of the co-expressed recombinant revealed a decrease in enzyme activity levels. The co-expressed xylanase production decreased by 26% from 136 U/ml to 100 U/ml while the xylosidase expression decreased 86% from 70 U/ml to 10 U/ml. The xylose

produced from the hydrolysis of birchwood xylan was quantified by HPLC. The monosaccharide sugar was used in a separate saccharification and fermentation strategy by *P. stipitis* to produce bioethanol, quantified by gas chromatography. Bioethanol production peaked at 72 h producing 0.7% bioethanol from 10 g/l xylose. In conclusion a β -xylosidase from *Aspergillus niger* was successfully expressed in *P. pastoris* and was found to express large quantities of xylosidase, that has not been achieved in any prior research to date. The enzyme was also successfully co-expressed with a *Thermomyces* xylanase and is now capable of bioethanol production through xylan hydrolysis. This highlights potential use in industrial applications in an effort to reduce the world dependence on petroleum and fossil fuels. However the technical challenges associated with commercialization of bioethanol production are still significant.

CHAPTER 1 : INTRODUCTION AND LITERATURE REVIEW

With the world's human population growing daily, life has completely transformed the globe. In order to support this growth increase and maintain the biosphere, there is a need for agriculture and industry to function in a sustainable manner, and this will require developments and improvements in biotechnology. Global warming and dwindling fossil fuel reserves have become a great concern (Wakelin, 2009) for the environment. Fossil carbon represents an important raw material for energy generation, but its oxidation product, carbon dioxide, is an important greenhouse gas. Attempts at reducing fossil carbon consumption, either by improving energy efficiency or by substituting alternative resources will result in lowering carbon dioxide production and reducing global warming. These problems can also be overcome by promoting cleaner manufacturing, the use of biological methods, cheap bioremediation processes when the environment has become polluted, thereby promoting a better quality of life (Wackett and Bruce, 2000). Biotechnology can thus offer greatly reduced dependence on non-renewable fuels and other resources by way of enzymes (Gavrilescu and Chisti, 2005).

Enzymes are already being used in several applications in the food, feed, agriculture, paper, leather, textile and pharmaceutical industries. To curb production costs, there is a growing trend to embrace enzyme technology to reduce environmental pollution. The pulp and paper industry has benefited greatly from the progress achieved in enzyme technology. A suitable example is "industrial sustainability" where xylanases play a major role as an environmentally safe alternative to toxic chlorine bleaching employed in the pulp and paper industry (Gavrilescu and Chisti, 2005).

Enzymes are indispensable and found abundantly in nature, but in some instances have to be tailored to perform desired functions, thereby enabling new technologies such as genetic engineering to produce pharmaceuticals, disease-resistant plants, cloned animals and industrial products. Directed evolution has been used for over 20 years as an alternative to rational design and through on-going research can now improve enzyme activity, alter

enzyme selectivity and evolve function *de novo* (Tao and Cornish, 2002). The possibility to alter enzyme characteristics by directed evolution and gene shuffling, and efficient methods to screen for new enzymes in the environment, makes it cost effective to use enzymes that are specifically suited to their application and process conditions (Beilen and Li, 2002).

The aim of this research was to change the characteristics of xylosidase through random mutagenesis by creating a mutant library to screen for constructs with altered characteristics such as improved xylosidase activity and thermostability. The second part of this research aimed to investigate production levels by expressing the enzyme using different promoters in different host systems. The effect of constitutive and inducible systems were tested in *Saccharomyces cerevisiae* and *Pichia pastoris*, and the fermentable sugar produced was used in the conversion to bioethanol.

1.1 INDUSTRIAL ENZYMES

Enzyme functionality has been used for decades in many biological processes. Their existence was associated with the history of prehistoric Greece where enzymes from microorganisms were used in baking, brewing, alcohol production, cheese manufacturing etc. (Haki and Rakshit, 2003). Enzymes carry out a myriad of biochemical reactions under ambient conditions, which make their use eco-friendly and at times are the best alternative to polluting chemical technologies. Enzymatic treatment can provide the same level of output that is achieved through conventional methods that use harsher chemicals (Dhiman *et al.*, 2008).

The application of enzymes in the commercial sector is widening and it is therefore important to understand their nature and properties for efficient and effective usage (Dhiman *et al.*, 2008). Enzymes are gradually entering the chemical industry as catalysts for numerous reactions. As established by Novozymes, the enzyme market is roughly divided into three major sections: technical enzymes, detergent enzymes and food enzymes.

Technical enzyme sales include enzymes in the leather, textile and paper and pulp industries. Food enzymes are the second largest segment and includes enzymes used in the dairy, wine,

brewing, and baking industries. Enzymes used in household care encompasses the majority, with 36% of the market (Fig. 1.2) with enzyme sales increasing from 8% in 2009 to about 12% in 2013 (Fig. 1.3).

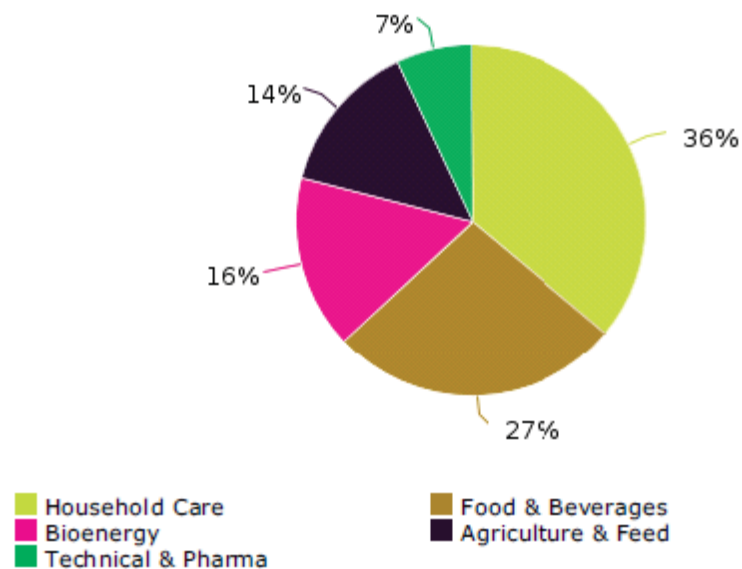


Figure 1.1: Worldwide Novozyme enzyme sales for 2013 indicating the percentage spent on enzymes related products in various segments (Novozyme).

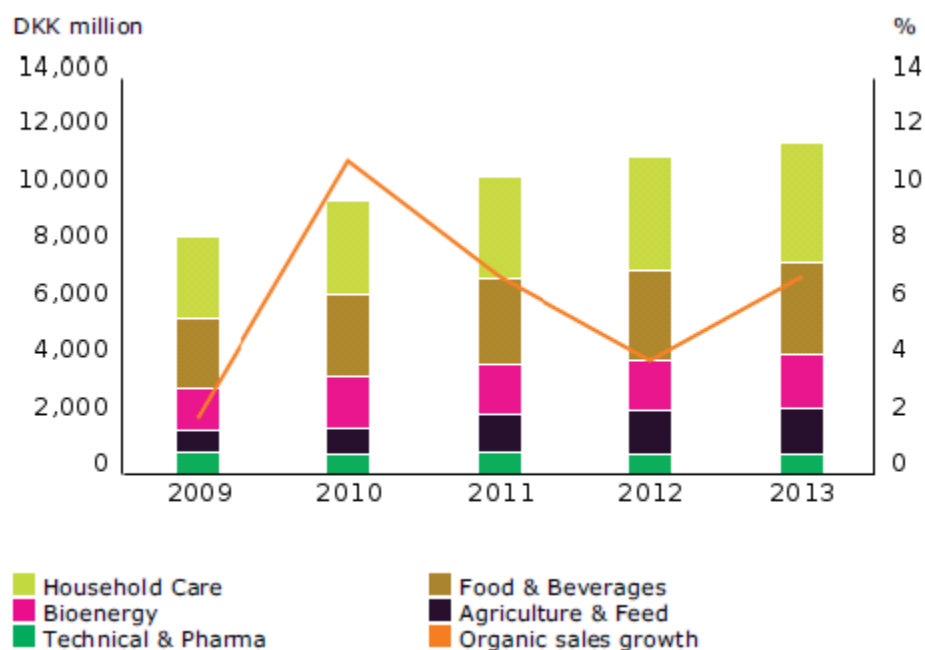


Figure 1.2: Worldwide Novozyme related sales in various application sector from 2009 to 2013. 1 DKK is equivalent to R2.01 (Novozyme).

1.2 HEMICELLULOSE-DEGRADING ENZYMES

Hemicelluloses are located between the lignin and cellulose fibres and consists of a number of natural polysaccharide monomers such as xylose, mannose, glucose, galactose and arabinose. In nature, hemicellulases produced by a wide variety of microorganisms including bacteria and fungi are either cell-associated or secreted into the surroundings (Knob *et al.*, 2010). Degradation of hemicellulose requires the synergistic action of multiple enzymes (Gray *et al.*, 2006; Hibbert *et al.*, 2005). These enzymes include endo-xylanase, β -xylosidase, α -glucuronidase, α -arabinofuranosidase and acetylxylan esterase (Juturu and Wu, 2012)

1.2.1 Xylanase

Plant cell walls contain xylan, the second most abundant polymer after cellulose. This complex structure requires the combination of xylanases and xylan de-branching enzymes for the efficient utilization of these complex materials (Ho, 2014; Juturu and Wu, 2012; Patel and Savanth, 2015). The complex structure of xylan requires different enzymes for complete hydrolysis. From an industrial point of view, filamentous fungi are the main and most effective producers of xylanase due to the extracellular release (Goswami and Pathak, 2013; Ho, 2014). Xylanases have been primarily classified as GH 10 and 11 glycoside hydrolases based on their amino acid sequence similarities. Glycoside hydrolase family 10 is composed of endo-1,4- β -xylanases and endo-1,3- β -xylanases (EC 3.2.1.32) and GH11 is composed only of xylanases (EC 3.2.1.8), leading to their consideration as “true xylanases,” as they are exclusively active on D-xylose-containing substrates (Motta *et al.*, 2013)

There is an increasing demand for cost-effective microbial xylanolytic enzymes which benefits the industrial segment and are produced commercially (Patel and Savanth, 2015). They are used for production of bulk chemicals, enzymatic treatment of animal feeds to release pentose sugars, as food additives in baking industry and improving the general economics of processing lignocellulosic materials for the generation of liquid fuels and chemicals (Kulkarni *et al.*, 1999). In the baking industry, xylanases have been used for decades in improving dough

stability, flexibility, increasing bread volume and improving crumb structure (Dutron *et al.*, 2012) and can also increase wheat flour separation into wheat and gluten. In the feed industry, xylanase is used to improve the assimilation of ruminant feed (Paës *et al.*, 2012). In the paper and pulp industry, there is a great potential for application in the pre-bleaching of kraft pulp which leads to significantly lower chemical consumption, with almost no loss in pulp yield or quality. The removal of ink from paper is a major problem in the recycling process and in recent years the use of microbial enzymes such as xylanases, cellulases and lipases have been used on an industrial scale for the deinking process (Chutani and Sharma, 2015). Many studies considered xylan as one of the new substrates in the production of biofuels, pharmaceuticals and solvents and considerable attention has been focused on the use of microorganisms in industrial fermentation of xylan.

1.2.2 Xylosidase

β -Xylosidases are grouped into eight families (3, 30, 39, 43, 52, 54, 116, and 120) of glycoside hydrolases (Huy *et al.*, 2013; Lagaert *et al.*, 2014). GH 3 is one of the largest families according to the CAZy database. Xylosidases are enzymes in the xylanolytic system that hydrolyzes complex alpha or beta linkages and heterogeneous hemicellulose (Kousar *et al.*, 2013). β -xylosidase hydrolyses xylobiose and xylooligosaccharides to fermentable xylose. The xylobiose and xylooligosaccharides are produced through the action of β -xylanases from the non-reducing end (Díaz-Malvárez *et al.*, 2013; La Grange *et al.*, 2001)

β -xylosidases have been produced from plant, animal and microbial sources, however the level of production and biochemical characteristics of a particular enzyme varies between species and strains of the same species. In the fungal kingdom, a majority of both xylanase and β -D-xylosidase enzyme producing organisms belong to the genus *Aspergillus*, many of which have been well characterized. La Grange *et al.* (2001) isolate, cloned and characterised the β -xylosidase from *A. niger* 90196. The enzyme activity was found to be as low 3.5 nkat.ml, but remained active at temperatures as high as 60°C. These filamentous fungi have mostly been used for high productivity and stability of their enzymes in industrial application and are main producers of xylosidases due to extracellular release of xylanases, higher yield in comparison to bacteria and yeast and production of numerous auxiliary enzymes that are

necessary for debranching of the substituted xylanase (Kousar *et al.*, 2013; Patel and Savanth, 2015).

Many bacterial and fungal β -xylosidases have been purified and characterized. Functional expression of β -xylosidases is more challenging which harshly affects their functional expression in heterologous hosts including yeasts (Juturu and Wu, 2012). In a study by Choengpanya *et al.* (2015), the coding sequence of a glycoside hydrolase family 3 β -xylosidase from *Aspergillus niger* ASKU28 (AnBX) was cloned and expressed in *Pichia pastoris* as an N-terminal fusion protein with the α -mating factor signal sequence (α -MF) and a poly-histidine tag. The enzyme displayed an optimal activity at 70° C and at pH 4.0–4.5 and results suggested that AnBX may be useful for degradation of lignocellulosic biomass in bioethanol production.

1.3 APPLICATIONS OF HEMICELLULASES

The use of enzymes as biocatalysts in many industries has increased and evolved with time (Cobb *et al.*, 2013). Due to enzyme specificity, unwanted reactions can be minimized by determining the correct enzyme for production of certain products (Hasan *et al.*, 2006). Since enzymes are biodegradable, their contribution to the biochemical oxygen demand in environmental waste streams is reduced. Compared to enzymes derived from plants and animals, microbial enzymes tend to be of major importance due to the variety of catalytic activity, high yields, ease of manipulation, regular supply due to lack of seasonal fluctuations and rapid growth of microorganisms. The food, feed, fuel, pharmaceutical, detergent and paper industries now rely on the use of enzymes as replacements for harsh chemicals and the rapid demand for these enzymes has become the driving force in research based on their potential future applications.

1.3.1 Pulp and Paper

The current bleaching process in the pulp and paper industry requires large quantities of chlorine and chlorine-based chemicals. The by-products produced are toxic and mutagenic. An alternative to the chemical bleaching process is the use of enzymes such as cellulase-free xylanase that have application in the removal of hemicellulose from kraft-pulp to give

brightness to the paper. This process is less toxic than conventional chemical treatment (Khristova *et al.*, 2006; Techapun *et al.*, 2003). The pulp and paper industry has benefited greatly from the progress achieved in enzyme technology and provides a suitable example for “industrial sustainability”. However for enzymatic pulp bleaching cost to be competitive, enzymes should be produced at less than US\$2.25 per gram and the finishing cost of enzyme treatment should not surpass \$4.50 per tonne of pulp (Techapun *et al.*, 2003).

1.3.2 Bioethanol

The energy demand worldwide is increasing dramatically due to the growth in population. The use of non-renewable sources to meet this demand has over time led to an increase release in greenhouse gases (Gupta and Verma, 2015). This has directed the focus towards the use of renewable sources such as ethanol derived from corn or sugarcane. But, with the demand for bioethanol increasing rapidly, there are concerns that these crops will become limiting factors in the near future due to their primary role as food and feed (Gray *et al.*, 2006; Gupta and Verma, 2015)

Biodegradation of lignocellulose can be one of nature's most important biological processes. Saccharification of lignocelluloses to sugars can be used for the production of organic solvents. Proper pre-treatment methods can increase concentrations of fermentable sugars after enzymatic saccharification, thereby improving the efficiency of the whole process and can are therefore seen as an attractive feedstock to alleviate the problem of utilising food sources for ethanol production (Okeke and Obi, 1995).

Production in the USA is estimated to increase in the next 10 years with 1.9535×10^7 gallons in 2009 to 3.6×10^7 gallons in 2020 (Rizzatti *et al.*, 2004). Generally, bioethanol is used to generate both power and heat and in recent years as liquid biofuel for motor vehicles. This has been gradually increasing around the world and is still in the early stages of development South Africa (Amigun *et al.*, 2011)

While biofuel production on the African continent is minimal, biofuels in South Africa have the potential to diversify the energy supply and reduce the dependence on imported fuels

(Pradhan and Mbohwa, 2014). However, the South African government is challenged with issues related to food security, commodity prices, economic and social concerns, and effects of land use changes on the environment. One of the routes the country has taken to research alternative sources of fuel is the proposed bioethanol project in Cradock, South Africa. While it may have not reached the use of agro residues, there is an opportunity for South African grain farmers to cultivate available lands for bioethanol crops (Nasterlack *et al.*, 2014).

Across the globe, the DuPont cellulosic ethanol facility opened in Nevada on October 30, 2015 and is said to be the largest cellulosic ethanol plant in the world. The facility is anticipated to produce about 30 million gallons of cellulosic ethanol per year from corn stover. DuPont together with National renewable energy laboratory (NREL) focused on two key areas to improve biomass pre-treatment and ethanol production technologies. The first was the optimization and scale-up of *Zymomonas mobilis*, a bacterium that enhances the fermentation of biomass sugars for production of biofuels. The second area focused on developing a mild ammonia pre-treatment process suitable for corn stover residues. The completion of this biorefinery is a key milestone in the emerging production of cellulosic ethanol in the United States and sets the pace for future projects around the globe (www.dupont.com).

1.4 HETEROLOGOUS PROTEIN EXPRESSION

Advances in genetic engineering have made possible the production of pharmaceutical proteins and commercial enzymes of industrial interest, in the form of recombinant proteins. The significant need of pharmaceuticals and enzymes can be met by heterologous production of these recombinant proteins. Heterologous production of proteins and enzymes involves two major steps: (a) Introduction of foreign DNA into the host cells (b) optimization of expression of foreign DNA for protein synthesis in the chosen expression system (Ceponoyte *et al.*, 2008; Dominguez *et al.*, 1998; Rai and Padh, 2001)

There are two general systems for protein production: prokaryotic and eukaryotic systems. Both systems have their advantages and disadvantages. *E. coli* is the most widely used host and its dominance in this field is a reflection of the depth of information about its genetic and

biochemical systems that has accumulated over many decades of research. Bacterial systems remain the most attractive as hosts due to their: (a) low cost; (b) high productivities; and (c) ease of use (Terpe, 2006).

1.5 PROKARYOTIC EXPRESSION SYSTEMS

1.5.1 *Escherichia coli*

E. coli is the preferred host for recombinant protein expression due to: (a) their ease of genetic manipulation; (b) inexpensive culturing; (c) well established isotope labelling protocols and; (d) fast expression, typically the production of protein in a single day. The ease of use of *E. coli* for heterologous protein production is best highlighted by the wide selection of commercial products available for the *E. coli* expression system (Peti and Page, 2007), mainly due to the understanding that has developed over the years such as complete genomic sequence which aids in gene cloning and cultivation. However, the *E. coli* system has some disadvantages when compared to eukaryotic systems, which need to be overcome for efficient production of proteins. These include the inability to perform post-translational modification, production of unglycosylated proteins and endotoxin production (Demain and Vaishnav, 2009; Rai and Padh, 2001). Proposed solutions are summarized in Table 1.1.

Table 1.1: Additional problems associated with heterologous protein production in *E. coli* and possible solutions (Terpe, 2006)

POSSIBLE PROBLEM	SYMPTOMS	SOLUTION
Toxic protein	Cell death or no colonies	Tightly controlled promoter system
Misfolded protein	No activity	Minimize reduction in cytoplasm, Accumulation in the periplasm, Attenuate expression, Change affinity tag
Reduction of disulphide bonds	Insoluble disulphide protein (inclusion bodies)	Minimize reduction in cytoplasm

1.5.2 *Bacillus* Expression Systems

Bacillus seems to be the most popular expression system after *E. coli*. These gram positive cultures are preferred for heterologous production of protease and amylase. Some of the advantages of *Bacillus* expression systems are their ease of manipulation, extracellular protein production and superior growth characteristics (Demain and Vaishnav, 2009). However, a problem that was noticed is the production of many proteases that can sometimes destroy the recombinant proteins being expressed. Among the *Bacillus* species, heterologous gene expression in *B. megaterium* seems to be an interesting alternative system. Zheng *et al.* (2012) expressed a xylanase gene (*xynG1-1*) from *Paenibacillus campinasensis* G1-1 in *Bacillus megaterium* MS941. 304.26 IU/ml xylanase activity was achieved after induction with 0.5% xylose. The recombinant xylanase revealed optimal activity at 60 °C and pH 7.0. Other *Bacillus* hosts include *B. subtilis* and *B. brevis*. Some of the recombinant proteins successfully expressed are cellulase with *B. brevis*, lipase A with *B. subtilis* and toxin A with *B. megaterium* (Demain and Vaishnav, 2009)

1.6 YEAST EXPRESSION SYSTEMS

Yeasts are an attractive group of lower eukaryotic microorganisms used in numerous industrial processes including brewing, baking, and the production of biochemical compounds. These single-celled eukaryotic organisms have developed as host organisms for the production of foreign proteins not produced well in *E. coli*. The two most utilized yeasts are *Saccharomyces cerevisiae* and the methylotrophic yeast *Pichia pastoris*. Some advantages that make yeast a favourable choice include high yield, robustness, cost effectiveness, and the ability to glycosylate proteins. (Demain and Vaishnav, 2009; Dominguez *et al.*, 1998).

1.6.1 *Saccharomyces cerevisiae*

Saccharomyces as a host system offers ease with which it can be genetically manipulated, and has extensive amount of information accumulated about its molecular biology and physiology. Despite this, there are some limitations associated with the *S. cerevisiae* expression system (Cereghino and Cregg, 2006):

(a) product yields are usually low;

- (b) proteins are rather cell bound; and not secreted into the culture medium
- (c) the inability to ferment xylose.

Saccharomyces cerevisiae is widely utilized in basic research as a model eukaryotic organism and in biotechnology as a host for heterologous protein production. This organism has many advantages as a cloning host since it has a history of use in industrial fermentations, extracellular heterologous protein secretion when proper signal sequences are attached and carries out glycosylation of proteins. Some of the products expressed in *S. cerevisiae* are insulin, hepatitis B surface antigen, urate oxidase and glucagons (Ceponoyte *et al.*, 2008; Demain and Vaishnav, 2009; Quintero *et al.*, 2007).

Xiong *et al.* (2014), engineered *S. cerevisiae* to express xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*) genes from *P. stipitis* and was able to produce ethanol from xylose (Fig. 1.3). While *S. cerevisiae* is extensively used for the conversion of glucose to ethanol, a major disadvantage is its inability to naturally utilize xylose. Some of the limitation to this species has now been relieved by the genetic engineering of *S. cerevisiae* and the development of expression systems in other yeast species (Dominguez *et al.*, 1998; La Grange *et al.*, 2001).

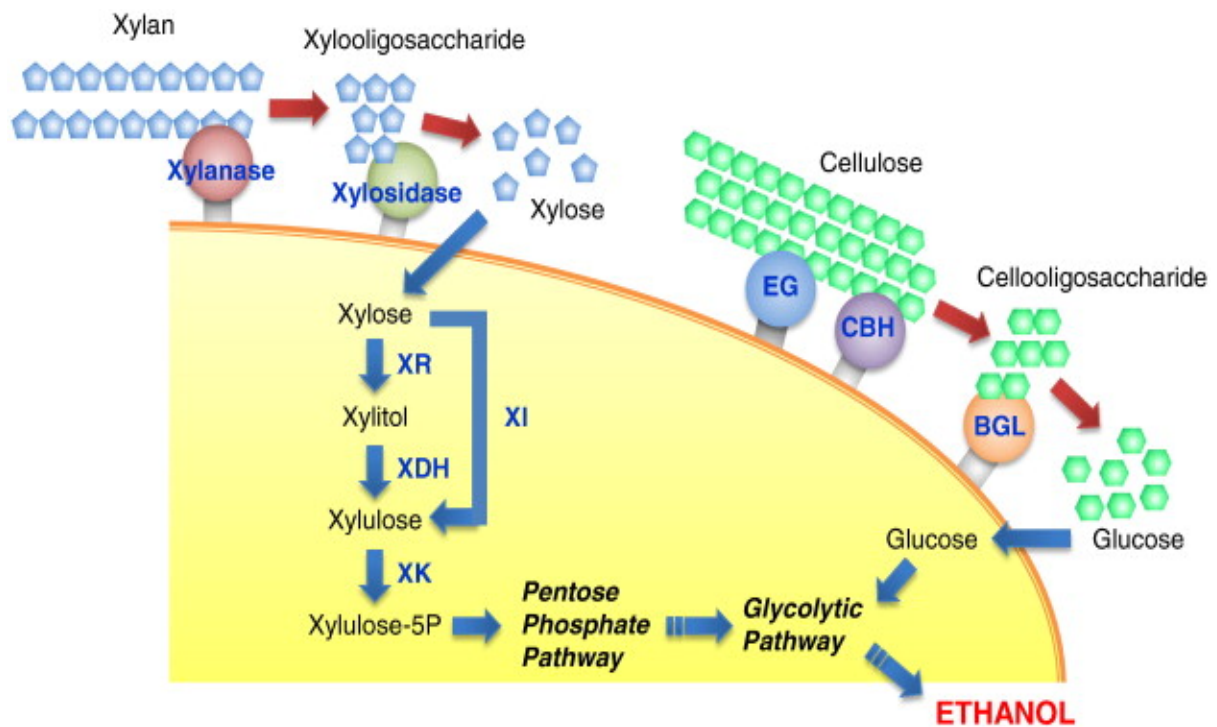


Figure 1.3: Conversion of xylan and cellulose to ethanol by *S. cerevisiae* (Hasunuma and Kondo, 2012)

1.6.2 *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast capable of growing in methanol as its sole carbon source. This organism is suited for the production of foreign proteins for three important reasons:

- (a) ease of manipulation;
- (b) the ability to perform complex post-translational modifications; and
- (c) high expression levels (intracellular and extracellular).

Pichia pastoris was initially developed by Phillips Petroleum Company (Cereghino and Cregg, 1999) for the production of single cell proteins and is now the most widely used non-conventional yeast in which the most proteins have been expressed. *S. cerevisiae* has usually been the yeast of choice, but an increasing number of alternative non-*Saccharomyces* yeasts has now become available for modern molecular techniques (Cereghino *et al.*, 2002; Cos *et al.*, 2006; Dominguez *et al.*, 1998). *P. pastoris* has been used extensively over the years to express recombinant proteins and is a useful system for minute quantities of a protein which can be scaled up to fermentation to meet larger demands.

There are two alcohol oxidase genes in *P. pastoris* that code for the alcohol oxidase enzyme. These are responsible for more than 90% of the enzyme in the cell (Cos *et al.*, 2006; Dominguez *et al.*, 1998). The use of inducible and constitutive promoters and the combined usage of these promoters enhanced the expression of heterologous protein in this organism (Fichser *et al.*, 1999; He *et al.*, 2008; Li *et al.*, 2007), and has been characterized and introduced into a series of commercially available *P. pastoris* expression vectors (Li *et al.*, 2007; Romonas, 1995).

1.6.3 *Pichia pastoris* as a Suitable Host for Protein Expression

P. pastoris was not always considered to be genetically amenable as *S. cerevisiae*, but genetic advances over the years has paved the way for *P. pastoris* (Spohner *et al.*, 2015). *P. pastoris* is a single-celled and is easy to manipulate and culture. Over the last decade, research has focused towards genetics and use in the pharmaceuticals industry (Cregg *et al.*, 2000; Demain and Vaishnav, 2009).

The yeast *Pichia pastoris* has two major advantages over *Saccharomyces cerevisiae*. Firstly, the presence of the methanol-inducible alcohol oxidase 1 gene (AOX1). This is a tightly regulated gene and is repressed in the absence of methanol, and when used to drive heterologous protein production, avoids toxic effects of heterologous protein expression until expression of the product is induced by methanol. Secondly, *Pichia* can be grown to higher densities than *S. cerevisiae* (Cereghino and Cregg, 2006).

Elgharbi *et al.* (2015) cloned and expressed cDNA of the β -1,4-endoxylanase of *Aspergillus niger* US368 in *Pichia pastoris* under control of the constitutive GAP promoter. The maximum activity obtained was 41 U/ml, 3-fold higher than that obtained with the native species. Enzyme assays revealed optimum activity at pH 4.0 and 50°C. The gene encoding xylanase activity produced by *T. lanuginosus* 195 was expressed in *P. pastoris* by Gaffney *et al.* (2009), following 120 h induction with 0.5% methanol a maximum of 26.8 U/ml was obtained. A thermotolerant β -xylosidase from *Aspergillus* sp. BCC125 was expressed in *P. pastoris* KM71 by Wongwisansri *et al.* (2013) and demonstrated synergism of xylanolytic activities of *P. pastoris* expressed xylanase and xylosidase towards birchwood xylan. As more

is learned about this organism, its use as an expression system will become progressively more efficient and user-friendly (Cereghino *et al.*, 2002; Demain and Vaishnav, 2009).

1.7 *Pichia stipitis* as a Suitable Host for Bioethanol Production

Scheffersomyces (Pichia) stipitis is an ascomycetous yeast, extensively investigated for the fermentation of xylose to ethanol (Kurtzman *et al.*, 2011). The use of *P. stipitis* has increased over the years and unlike *S. cerevisiae*, it has the capability to convert pentoses such as xylose to ethanol. This inability by *S. cerevisiae* to ferment xylose is due to the lack of a pathway from xylose to xylulose (Amore *et al.*, 1991). The conversion of xylose to xylulose in yeast is performed by a two-step reduction and oxidation mediated by xylose reductase (XYL1) and xylitol dehydrogenase (XYL2) (Jeffries, 2006). The *P. stipitis* genome has provided important information to better understand its biology, metabolism and regulation of numerous genes for lignocellulose bioconversion (Jeffries *et al.*, 2007). Increasing the *P. stipitis* fermentation rate could greatly improve its usefulness in commercial processes and aid researchers to improve xylose metabolism in *S. cerevisiae*.

The fermentation of hemicellulosic sugars is crucial for the conversion of lignocellulose to ethanol (Van Vleet and Jeffries, 2009). Wongwisansri *et al.* (2013) demonstrated the simultaneous saccharification and fermentation of *P. stipitis* by pre-incubating xylanase and xylosidase with the xylan substrate followed by fermentation with *P. stipitis* inoculum to determine ethanol production by HPLC. This conversion to ethanol is more difficult with cellulose and hemicellulose as they require several steps for complete hydrolysis.

In yeast and fungi, a two step oxidation-reduction process is employed for the conversion of xylose to ethanol. The xylulose produced from this process is metabolized by the pentose phosphate pathway (PPP). The glyceraldehyde-3-phosphate produced is subsequently converted to ethanol by fermentative reactions of the Embden-Meyerhoff-Parnes pathway (Jeffries, 2006; Kuhad *et al.*, 2011).

Various studies summarised by Kuhad *et al.* (2011) highlights the use of *P. stipitis* to ferment xylose hydrolysates from different lignocellulosic material. Useful information into how the

organism ferments xylose under micro-aerobic conditions is provided by the genome sequence of *P. stipitis* sequenced by Jeffries *et al.* (2007). The challenge therefore lies in creating a cost effective process to obtain high yields of fermentable sugars.

1.8 DIRECTED EVOLUTION

Life on earth is ubiquitous; organisms have evolved to handle the wide ranges of temperature, pressure, pH and water activity by mutative adaptation of proteins to their changing environmental conditions (Jaenicke and Závodszky, 1990). Arnold (1996) proposed mimicking the key processes of Darwinian evolution in a test tube, where the functions of enzymes can be explored free from the constraints of functionality within a living system. The specificity of enzymes in biological systems that allow multiple reactions to proceed simultaneously inside a living cell, along with their capability of carrying out chemical reactions, whilst producing no waste products is of great interest to scientists and chemists around the world (Arnold, 1996).

Directed evolution has over the years emerged as a powerful tool for improving the characteristics of enzymes. By pairing various protocols for generating large variant libraries of genes, together with high-throughput screens that select for specific properties of an enzyme, such as thermostability and substrate specificity, it is now possible to optimize biocatalysts for specific applications (Turner, 2003).

The performance of naturally-occurring enzymes can be improved through molecular evolution to optimize desired characteristics of enzymes. Enzymes are often used in many industrial applications that require harsh conditions, such as high temperature and pH. Molecular evolution has led to modified enzymes that can withstand harsh conditions that are not always met and therefore be applied in industry (Cherry and Fidantsef, 2003).

The evolutionary mechanisms at work in nature ensure adaptability to ever-changing environments and does not work toward any particular direction. The underlying processes occur rather spontaneously during reproduction and survival (Arnold, 1998). Laboratory evolution on the other hand has a defined method – mutagenesis. Evolution comprises

repeated cycles of random gene mutagenesis in a Darwinistic sense for the purpose of improving the catalytic profile of an enzyme (Reetz *et al.*, 2007). In some ways, the basic algorithm of directed evolution, mimics that of natural evolution. The two key steps are generating molecular diversity and identifying the improved variants. Two widely-used approaches to generating diversity are *in vitro* recombination and random point mutagenesis. An error-prone version of the polymerase chain reaction (PCR) (Fig. 1.4), where under the correct conditions the polymerase will mis-incorporate bases pairs at a controllable rate during gene amplification is mostly convenient for the latter (Otten and Quax, 2005).

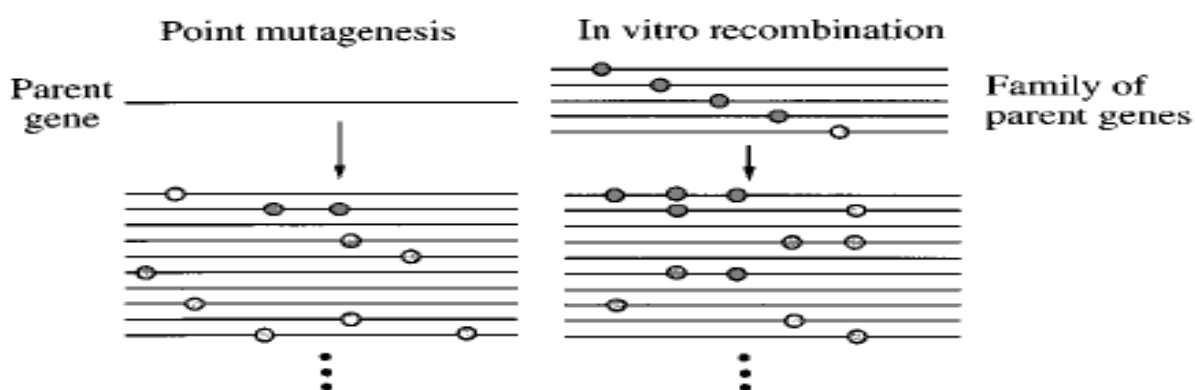


Figure 1.4 Error prone PCR generates a library of genes containing point mutations, arising from a single parent sequence. Recombination generates gene libraries with different combinations of the mutations from a pool of parent sequences (Arnold, 1998).

Antarctic ice fields and volcanic pools are home to organisms capable of surviving these harsh conditions, and enzymes from these organisms can therefore be applied to industrial processes similar to conditions of their natural environments. Sequence comparison indicate that these extremophilic enzymes are similar to their mesophilic counterparts sharing a common inherited trait and have accumulated mutations that allow them to adapt over millions of years (Arnold *et al.*, 2001). Directed evolution has made it reasonably feasible to tailor-make enzymes for a range of applications and in addition to build enzymes with new features and functions (Arnold, 1996). This option has become a developed tool in molecular biology due to various methods that are involved in generating mutant libraries (Otten and

Quax, 2005). Screening and selection can be used to target the enzyme towards the desired direction. To date, this technique is broadly used in two major applications: (a) industrial biocatalysis, where enzymes are engineered to produce suitable biocatalysts for use in an industrial environment; and (b) research, to quickly engineer new enzymes for every possible catalytic step, thereby creating a universal biotechnological toolbox (Otten and Quax, 2005). An example of direction evolution is well demonstrated in the research carried out by Stephens et al (2007). The *xynA* gene from *Thermomyces lanuginosus* was modified by ep-PCR and DNA shuffling (Stephens *et al.*, 2014) to obtain a single robust enzyme with enhanced activity and stability.

The numerous ideas of how to engineer enhanced enzymes stem from studying their creation in nature; such as their evolutionary patterns. From this it was learnt that they are constantly changing molecules that can adapt to new environments and they can take on new tasks by the processes of random mutation, recombination, and natural selection (Arnold, 1996). Limitations of enzymes include high temperature, unnatural substrates, poor stability, and expensive growth requirements. The goal is to consequently generate desired changes in enzyme performance, to enhance production and eliminate the above mentioned problems (Farinas *et al.*, 2001). The direction of evolution can be changed by controlling mutagenesis, the rate and types of changes made and the accompanying selection pressures. As a result, the screening process may take as long as 2 or 3 years (Arnold, 1996).

It is a known fact that enzymes have evolved over the years and have accustomed themselves to their specific surroundings. Because enzymes in nature is so diverse from that of an industrial environment, certain characteristics need to be satisfied to create an ideal industrial enzyme catalyst. Directed evolution is one of the methods used, where the sequence of the gene is changed in order to change the normal functions, leading to various changes in enzymatic properties. Understanding this will be key to the future implementation of industrial processes and ultimately, a combination of approaches will enable better implementation of effective biocatalytic processes (Farinas *et al.*, 2001; Hibbert *et al.*, 2005; Hibbert and Dalby, 2005).

Recombinant DNA technology provides a very powerful method for combining diverse genetic capabilities. It allows the genetic engineering of organisms with specific catalytic capabilities. The success of enzymes at industrial level depends on their robustness and application at high temperatures as well as specificity, stability and activity at process conditions, directed evolution has therefore become an essential tool in development of such enzymes (Zhao *et al.*, 2002). However as mentioned, an understanding of the trade-off between directed evolution and process techniques will allow for improvement on the industrial front. Despite the significant advances to date on many industrially relevant enzymes, the need to improve directed evolution strategies and develop generic screening or selection tools which make the process of identifying novel enzyme activities more feasible, still exists (Hibbert and Dalby, 2005).

This research aimed to improve the characteristics of the *A. niger* β -xylosidase. Directed evolution by ep-PCR created a mutant library that was screened for a change in enzyme function. These changes were characterised to determine their effects on the enzyme. Part of the characterisation was to determine pH and thermostability by enzyme assays, followed by DNA sequencing confirmation. β -xylosidase was also expressed under the control of different promoter systems and different hosts. The constitutive GAP-promoter and inducible AOX promoters were used to measure xylosidase enzyme activity in *P. pastoris*, while the constitutive PGK promoter and inducible ADH2 promoters were used to measure xylosidase activity in *S. cerevisiae*. The high xylosidase production in *P. pastoris* led to the co-expression of the *A. niger* β -xylosidase with the *T. lanuginosus* xylanase. The co-expressed enzymes were used to completely digest xylan. The xylose produced was used in a fermentation with *P. stipitis* to produce bioethanol.

2.1 INTRODUCTION

β -xylosidases are hydrolytic enzymes that play an important role in xylan degradation by catalysing the hydrolysis of xylobiose and xylooligosaccharides to release xylose (Dobberstein and Emeis, 1991; Matsuo *et al.*, 1998). Due to its complexity, a variety of enzymes are required for complete degradation of xylan. Xylanases are glycosidases which catalyze the endohydrolysis of 1,4- β -D-xyloside linkages in xylan. These enzymes act on main-chain polysaccharides, while accessory enzymes act on side-chains of these structures, releasing xylose monomers from the non-reducing end (Tony *et al.*, 2005). This enzymatic relationship exists in bacteria and fungi, with filamentous fungi being of particular importance as they are known to secrete xylosidase into the medium (Knob *et al.*, 2010)

Eukaryotic systems provide an attractive expression platform over prokaryotes. The two most utilized yeasts are *Saccharomyces cerevisiae* and the methylotrophic yeast *Pichia pastoris*. Both have become important tools for the expression of foreign proteins and the ability to perform post-translational modifications, such as glycosylation. The methylotrophic yeast *P. pastoris* has, over the years, been developed into a successful system for the production of heterologous proteins and has been used extensively to express recombinant proteins with a high degree of success due to ease of genetic manipulation and simple fermentation design (Cereghino and Cregg, 2006; Li *et al.*, 2007).

P. pastoris can be used to over-express proteins and contains low levels of secreted non-recombinant proteins, which makes purification easier (Tsai and Huang, 2008). The *P. pastoris* system provides competitive and reproducible processes for producing relevant compounds on an industrial and laboratory scale. The GAP promoter system provides strong constitutive expression, thereby omitting the need for methanol induction. However, in some cases, this may not be a good choice for the production of proteins that may be toxic to the yeast (Li *et al.*, 2007).

P. pastoris strains vary with their degree of methanol utilization. Three commercial strains that have different methanol utilization properties exist: Mut⁺ (methanol utilization phenotype), Mut⁻ (methanol utilization minus phenotype) and Mut^s (methanol utilization slow phenotype). The *AOX1* promoter, induced by methanol, has been used to express many foreign proteins. In some cases, the use of this promoter system may not be appropriate, for example, in the food industry (Scientific, 2014)

The *xlnD* gene from *Aspergillus niger* was originally placed under the control of the strong yeast promoter system (ADH2). This work was carried out by La Grange *et al.* (2001), with a total expression of 5.3 nkat/ml. ADH2 is one of the promoters regulated by glucose repression (Romanos *et al.*, 2004) Glucose repression, regulates the expression of genes by the availability of glucose which is utilized by yeast via the glycolytic pathway (Donoviel *et al.*, 1995). Glycolytic genes from yeast are of great importance due to their ability to produce high levels of proteins (Hitzeman *et al.*, 1982). These include 3-phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAP). The xylosidase gene used in this study was originally isolated from *Aspergillus niger* 90196 by La Grange *et al.* (2001) and expressed in *S. cerevisiae*. The aim of this chapter were to improve express and characterize this xylosidase gene from *A. niger*, under the control of different promoters in *S. cerevisiae* and *P. pastoris*, to increase expression levels, as well as co-expression of this gene with an evolved *Thermomyces lanuginosus* xylanase (pBGP1-S340) (Stephens *et al.*, 2007) to assess xylan degradation.

2.2 MATERIALS AND METHODS

2.2.1. Media and Culture Conditions

E. coli XL1 Blue (Stratagene) was cultivated on Luria-Bertani medium at 37°C, supplemented with ampicillin (100 µg/ml) for plasmid selection. *S. cerevisiae* Y294 obtained from W.H. van Zyl, Stellenbosch University, was cultured on synthetic complete (SC) medium (20 g/l dextrose, 6.7 g/l yeast nitrogen base without amino acids [Difco], 2 g/l drop-out amino acid without uracil [US Biological]). *P. stipitis* obtained from the Department of Microbiology (University of Kwa-Zulu Natal) and *P. pastoris* GS115 (Invitrogen) was cultivated on YPD medium (10 g/l yeast extract, 20 g/l peptone, 10-20 g/l dextrose). All yeast cultures were incubated at 30°C for 3 days. Solid media contained 15% agar. For long-term storage, cultures were stored in 30% glycerol at -80°C.

2.2.2. Plasmids

The plasmids used in this study are listed in Table 2.1. Plasmid pBGP1 (Fig. 2.2) and pHIL-S1 (Fig. 2.3) were used for expression in *P. pastoris* and plasmid pJC1 (Fig. 2.5) and pDLG55 (Fig. 2.4) were used for expression in *S. cerevisiae*. The pTZ57R/T (Fig. 2.1) cloning vector was used to create the pTZ57R/T-*xlnD* construct for use in further restriction digestions.

Table 2.1: Plasmids used in this study

Name	Characteristics	Source/Reference
pDLG55	Amp ^R , <i>URA3</i> , <i>ADH2</i> promoter and termination cassette	La Grange <i>et al.</i> (2001)
pJC1	Amp ^R , <i>URA3</i> , <i>PGK</i> promoter and termination cassette	W. H. Van Zyl
pBGP1	Amp ^R , Zeocin ^R , <i>GAP</i> promoter and termination cassette	Lee et al., 2005
pHIL-S1	Amp ^R , <i>HIS</i> , <i>AOX1</i> promoter and termination cassette	Invitrogen
pTZ57/R	Amp ^R T7/T9 promoter and termination cassette	Thermo Fisher Scientific
pBGP1-S340	Amp ^R , Zeocin ^R , <i>GAP</i> promoter and termination cassette, xylanase gene (S340)	DUT, Enzyme Technology Group

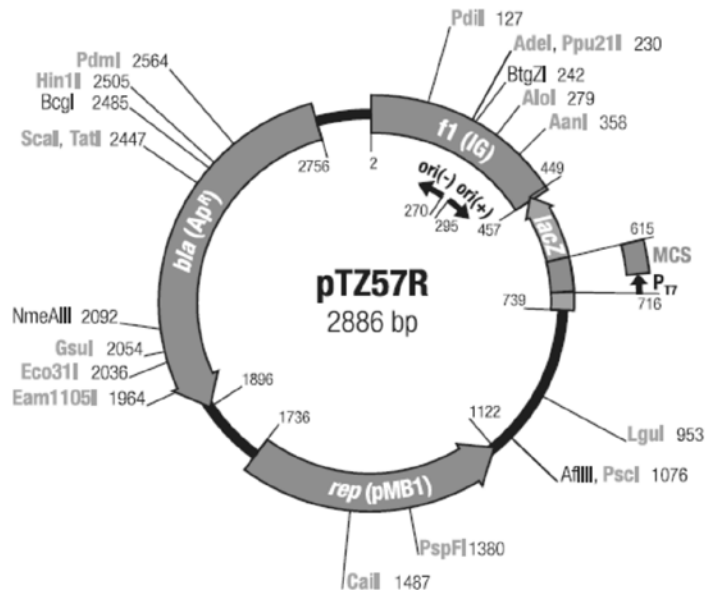


Figure 2.1: Map of the pTZ57R cloning vector (Thermo Fisher Scientific).

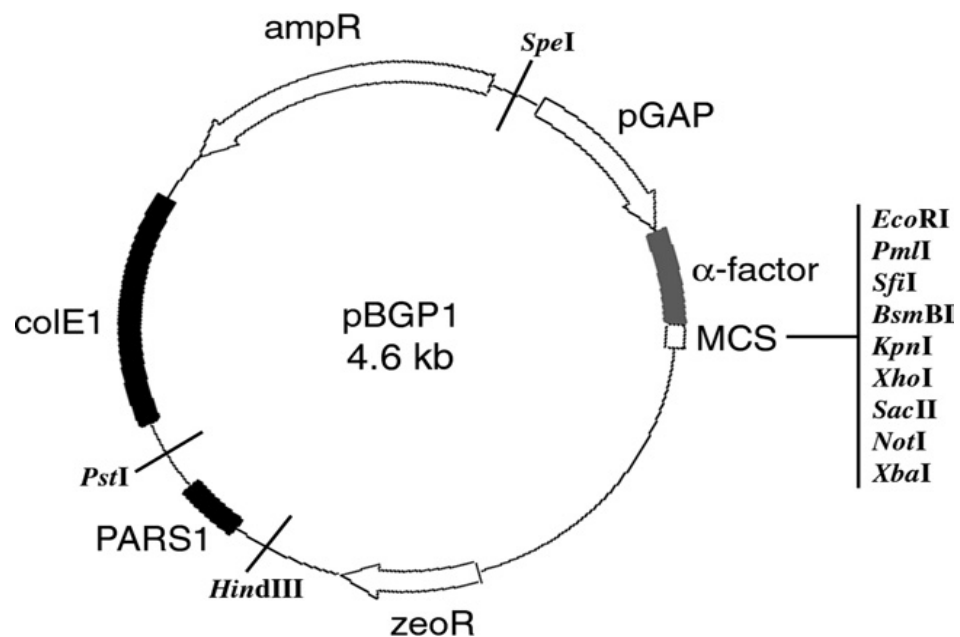


Figure 2.2: Map of the pBGP1 vector showing the location of the ampicillin resistance (Amp^R) gene, zeocin resistance (Zeo^R) gene, GAP promoter (pGAP), secretion signal (α -factor), multiple cloning site (MCS), *E. coli* replicon (colE1) and *Pichia* autonomous replication sequence (PARS1)(Lee *et al.*, 2005).

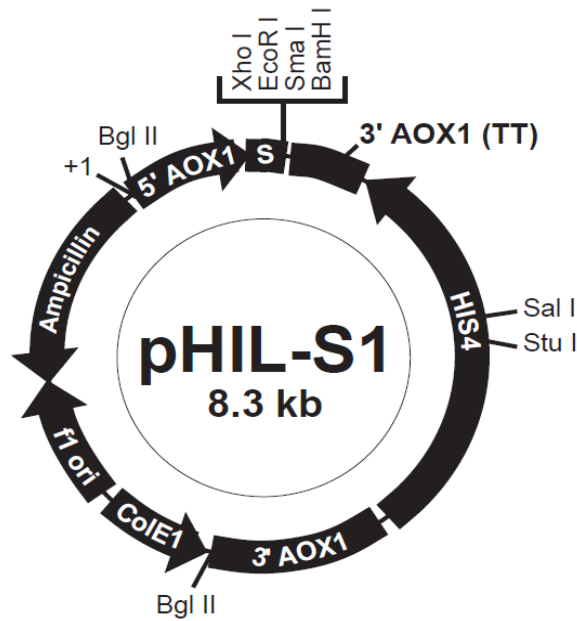


Figure 2.3: Map of the pHIL-S1 vector showing the location of the ampicillin resistance (Amp^R) gene, histidine selectable marker (HIS4), AOX1 promoter, secretion signal (S), multiple cloning site (MCS), and *E. coli* replicon (colE1) (Life Technologies).

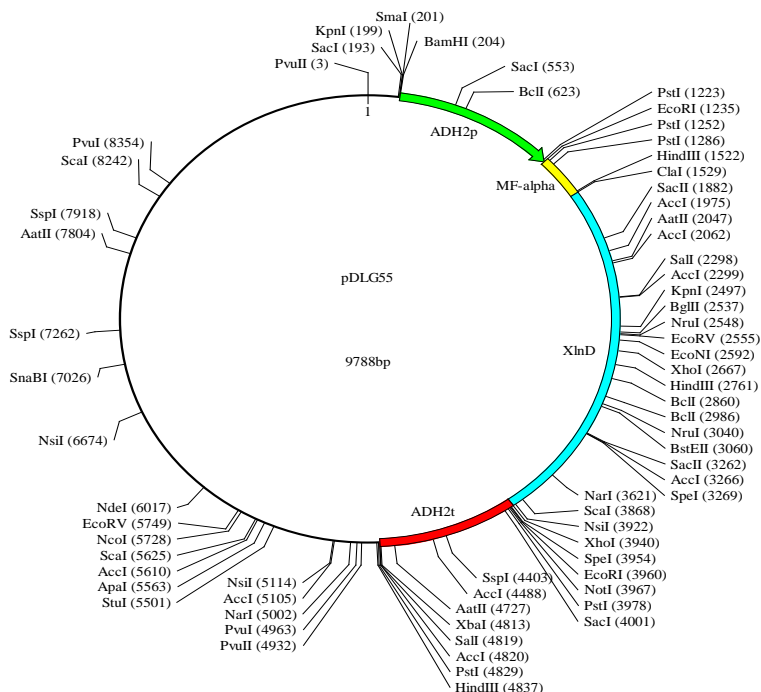


Figure 2.4: Map of pDLG55 vector showing the location of the ampicillin resistance (Amp^R) gene, uracil selectable marker (URA3), ADH2 promoter and terminator cassette (ADH2_p, ADH2_t), and origin of replication (2μ) (La Grange *et al.*, 2001).

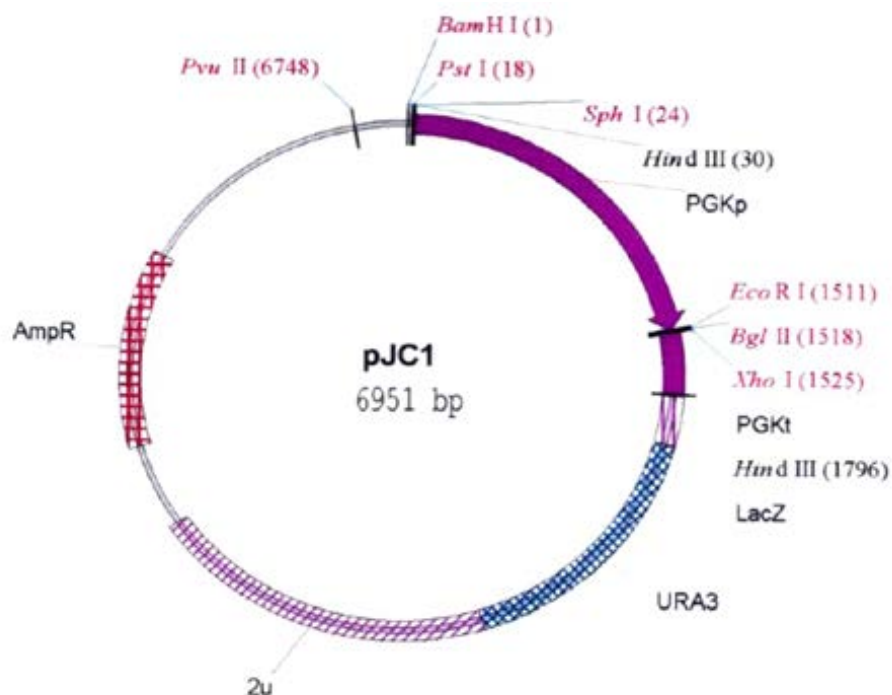


Figure 2.5: Map of pJC1 vector, containing and origin of replication (2 μ), Amp^R gene, uracil selectable marker (URA3) and *PGK1* promoter and termination cassette.

2.2.3. Plasmid DNA Isolation

The alkaline lysis method of Birnboim and Doly (1979), was used to isolate plasmid DNA. *E. coli* clones were inoculated in 5 ml LB medium containing 100 μ g/ml ampicillin and grown for 12 - 16 h at 37°C. Cells were harvested by centrifugation at 4000 $\times g$ for 5 min. The pellets were re-suspended in 100 μ l of re-suspension solution (25 mM Tris-HCl; 50 mM glucose; 10 mM EDTA, pH 8) and transferred to 1.5 ml microcentrifuge tubes. A 200 μ l volume of freshly-prepared lysis solution (0.2 N NaOH; 1% SDS) was added to the tube to disrupt the cell membranes due to the anionic detergent, SDS. NaOH was responsible for the denaturation of DNA. Plasmid DNA was re-annealed and chromosomal DNA and cellular proteins were precipitated by addition of 150 μ l of cold neutralization solution (3 M sodium acetate, pH 4.8). The tubes were then placed on ice for 5 minutes and centrifuged for 15 minutes at 14 000 $\times g$ to precipitate cell debris. After centrifugation, DNA in the supernatant was precipitated by the addition of 100% ethanol and incubated at -70°C for 15 minutes. Precipitated DNA was then recovered by centrifugation for 15 minutes. The DNA pellet was then washed with 1 ml of 70% ethanol. The pellet was air-dried and dissolved in 20 μ l TE buffer (pH 8) and stored at

-20°C. For specialized applications like ep-PCR and DNA sequencing, where DNA of a higher purity was necessary, the GeneJet Plasmid Mini Kit (Thermo Scientific) was used, according to the manufacturer's instructions.

2.2.4 DNA Quantification

DNA concentration was assessed spectrophotometrically at 260 nm and calculated on the principle that an absorbance of 1 at 260 nm corresponds to 50 ng DNA/μl. Purity of the DNA sample was predicted using the absorbance ratio measured at 260 nm and 280 nm. A ratio greater than 1.7 was considered ideal purity for procedures like DNA sequencing

2.2.5 Agarose Gel Electrophoresis

DNA molecules were separated on 0.8% agarose gels, based on their size. The three forms of plasmid DNA molecules typically viewed on an agarose gel after plasmid DNA isolation are: linear, circular and covalently closed circular. Covalently closed circular bands migrate the furthest due to their compact size, followed by linear and then circular DNA, for the plasmids used in this study. The desired amount of agarose was weighed and dissolved in 1× TAE buffer, which was diluted from a 50× TAE stock solution (242 g Tris base; 57.1 ml glacial acetic acid; 100 ml 0.5 M EDTA, pH 8). The solution was microwaved until the agarose dissolved, poured into a casting tray and allowed to set. The DNA sample was mixed with 6× gel loading dye (Thermo Fisher Scientific) and loaded into the wells of the gel and electrophoresis was conducted at 110 V for approximately 1 h. Ethidium bromide (0.25 μg/ml) was used to stain the gel for 15 minutes. The gel was later de-stained in distilled water for a further 5 minutes, if necessary. The interaction of double stranded DNA with ethidium bromide resulted in a strong, UV-excitable orange fluorescence, which indicated the location of the DNA bands on the agarose gel. Gels were viewed using the Gel Doc XR gel documentation system (Bio-Rad Laboratories), using Quantity One imaging software.

2.2.6 Polymerase Chain Reaction (PCR)

Two sets of PCR primers were designed for amplification of the *xlnD* gene; Set A – ECORF and XYNR was used to amplify *xlnD* from pDLG55 (Fig. 2.4), Set B – XXF and XXR was used to

amplify the *xlnD* gene including the GAP promoter and terminator. Primer sequences (Table 2.2) were synthesized by Inqaba Biotech (Set A) and Whitehead Scientific (Set B),.

Table 2.2: Primers used in this study

	Forward	Reverse
Set A	5'GCTT ATCGAT ACACCAGCT ATGTCGATTAC3'	5'CTCGAGACATCGTATCACTCT AGACAC 3'
Set B	5'GAGGAG GGATCC AGATCTT TTTTGTAGA3'	5'GTGGGG GGATCC GCACAAACG AAGGTC3'

Restriction endonuclease recognition sites are indicated in bold (ATCGAT – ClaI, TCTAGA – XbaI, GGATCC – BamHI)

The first PCR reaction was carried out on pDLG55 containing the *xlnD* gene. The *xlnD* gene was amplified together with the promoter and terminator using primer Set A, to yield an approximately 2700 bp fragment. The reaction was carried out using 10 ng of pDLG55 plasmid DNA, 0.5 µM of each primer, 1 U KAPA *Taq* DNA Polymerase, 2× KAPA *Taq* Buffer, 0.2 mM of each dNTP and 1.5 mM MgCl₂ in a total volume of 50 µl. The following cycling conditions were used: denaturation at 95°C for 30 seconds, followed by primer annealing at 55°C for 30 seconds and primer extension at 72°C for one minute.

The second amplification reaction was carried out using primer set B to amplify the S340 xylanase gene using the same conditions mentioned above. The PCR reactions were carried out for 30 cycles using the GeneAmp PCR System 9700 (Applied Biosystems). The resulting PCR products were then analysed on agarose gels to determine if the genes were successfully amplified. The PCR products were purified using the QIAquick Gel Extraction Purification Kit (Qiagen). 5 µl of the purified product was then analysed on an agarose gel to verify successful purification and the DNA was quantified spectrophotometrically at 260 nm.

2.2.7 Restriction Analysis

Restriction endonucleases were used for different applications in this study. Restriction digestion of both the vector and the insert DNA with suitable restriction enzymes was used to create compatible sticky ends for ligation with DNA ligase. Recombinant plasmids were then restricted with the same enzymes to determine if they contained the insert after

transformation into *E. coli*. Standard protocols were followed for restriction analysis. Plasmid DNA was incubated with suitable restriction enzymes (Fermentas) at 37°C for a specified time. Restricted DNA was then analysed on 0.8% agarose gels.

Firstly, pDLG55-*xlnD* was restricted with *EcoRI* to release a sticky-ended fragment of approximately 2700 bp fragment, compatible with the similarly-digested pJC1 vector to enable ligation between vector and insert. Secondly, the *xlnD* fragment was amplified from pDLG55-*xlnD* and cloned using the InsTAclone PCR Cloning Kit (Thermo Fisher Scientific). Successful clones were confirmed by digestion with *EcoRI* and *XbaI* to yield the approximately 2700 bp fragment. This *xlnD* insert was cloned into pBGP1 digested with the same restriction endonucleases.

Thirdly, pTZ57R/T-*xlnD* plasmid was digested with restriction enzymes *EcoRI* and *BamHI* for cloning into similarly-digested vector pHIL-S1. *P. pastoris* is capable of integrating multiple copies of transforming DNA via recombination into the genome at sites of sequence homology. Transformation of *P. pastoris* GS115 with *StuI*-linearized constructs favours recombination at the *HIS4* locus (Invitrogen, 2014). Successful clones were identified using restriction endonucleases *EcoRI* and *BamHI*. The positive clone was linearized with restriction enzyme *StuI* to favour isolation of His⁺, Mut⁺ recombinants of *P. pastoris* GS115. Restriction reactions were purified using either the DNA Clean and Concentrate Kit (Zymo Research) or the QIAquick Gel Extraction Purification Kit (Qiagen), according to the manufacturer's instructions, and small aliquots were examined on agarose gels to confirm successful purification.

Clone pBGP1-S340, created in a previous experiment, was used to amplify the xylanase gene together with the GAP promoter and terminator using primer set B. The amplified region was cloned using the InsTAclone PCR Cloning Kit and restricted using restriction enzyme *BamHI* to release a 1986 bp fragment. This fragment was cloned into similarly digested pBGP1-*xlnD* to create pBGP1-*xlnD*-GAPS340, a recombinant plasmid containing both xylanase and xylosidase genes. This construct was designated as pBGP1-XX1.

2.2.8 Dephosphorylation

To prevent plasmid recircularization prior to ligation, linearized plasmid was dephosphorylated with calf intestinal alkaline phosphatase (CIAP, Inqaba Biotech). This was carried out by adding 1 µg of restricted plasmid DNA, 2 µl of 10× CIAP buffer (Thermo Scientific), followed by 1 U of CIAP enzyme to a sterile eppendorf tube. The reaction mixture was incubated at 37°C for 30 minutes, followed by heating to 85°C for 15 minutes to stop the reaction. The mixture was then purified using the QIAquick Gel Extraction Purification Kit (Qiagen), according to the manufacturer's instructions.

2.2.9 Ligation

The ligation reactions were carried out at a molar vector: insert ratio of 1:3. The purified restricted PCR inserts and purified restricted pJC1, pBGP1 and pHIL-S1 vectors were ligated using the Rapid DNA Ligation kit (Thermo Scientific), according to the manufacturer's instructions. The ligation was incubated at 22°C for 5 minutes. One µl of the ligation reaction was used to transform 50 µl of electrocompetent *E. coli* XL1 Blue cells.

2.2.10 Preparation of Electrocompetent Cells

Prior to transformation, electrocompetent bacterial cells were prepared by growing a single colony of *E. coli* XL1 Blue in 5 ml LB broth overnight at 37°C in a shaking incubator. One ml of this culture was used to inoculate 29 ml of fresh LB broth and further shaken at 37°C, until an optical density of 0.500 at 590 nm was reached. Cells were centrifuged at 4000× *g* for 10 minutes and the supernatant was discarded. Throughout the subsequent procedures, the cells were kept chilled by placing on ice. Cells were re-suspended in 10 ml of ice-cold 10% glycerol. The cell suspension was once again centrifuged at the same speed for a further 10 min, the supernatant was discarded, and the cells were re-suspended in 5 ml ice-cold 10% glycerol. This step was repeated once more using 2 ml ice-cold 10% glycerol. The final step involved re-suspension of the competent cells in 200 µl of ice-cold 10% glycerol and dispensing 50 µl aliquots into sterile eppendorf tubes. These cells were stored at -80°C, and used within 3 months, as prolonged storage decreases the transformation efficiency (Ausubel, 2002).

2.2.11 Transformation and Screening in *E. coli*

After ligation, 1 µl of the reaction mixtures were used to transform 50 µl electrocompetent *E. coli* XL1 Blue cells. The electrocompetent cells and DNA were incubated on ice and then transferred to a chilled 0.2 cm GenePulser electroporation cuvette (Bio-Rad). The cells were then electroporated using the GenePulser Xcell (Bio-Rad) under the following conditions: 2.5 kV, 200 Ω and 25 µF. Time constants of approximately 5 msec were an indication of successful pulse deliveries. One ml of LB broth was immediately added to the cuvette, the contents were transferred to an eppendorf tube and incubated at 37°C for 1 h. During this incubation period, the transformed cells were allowed time to recover from the treatment, amplify the plasmid and express antibiotic resistance and other genes present on the plasmid. 100 µl of the transformation mixture was plated on LB agar plates (containing 100 µg/ml ampicillin). Colonies that grew on the plates were deemed resistant to the antibiotic and could contain the recombinant plasmid

2.2.12 Preparation of *S. cerevisiae* and *P. pastoris* Electrocompetent Cells

Yeast host cells were made competent by treating with DTT and stabilizing in sorbitol. Both *S. cerevisiae* and *P. pastoris* were grown for 3 days at 30°C on YPD plates. A single colony was used to inoculate 200 ml of YPD broth and incubated at 30°C overnight until an OD₆₀₀ of approximately 1.4 was reached. Cells were then centrifuged at 4000x *g* for 10 min. For the preparation of *S. cerevisiae* competent cells, 40 ml YPD, 1 ml DTT (1 M) and 8 ml HEPES buffer (1 M, pH 8), was used to re-suspend the pelleted cells and incubated at 30°C for 15 min. The cells were then washed three times by suspension and centrifugation at 4000x *g* with 200 ml sterile water, 100 ml and then 8 ml aliquots of 1 M sorbitol. The cells were finally re-suspended in 400 µl 1 M sorbitol and dispensed in aliquots of 80 µl for use on the same day. *P. pastoris* cells were harvested at 4000x *g* for 10 min, followed by re-suspension and centrifugation in 250 ml ice-cold distilled water, 125 ml and 20 ml ice cold sorbitol (1 M). Cells were finally re-suspended in 500 µl 1 M sorbitol and aliquots of 80 µl each were dispensed and used on the same day.

2.2.13 Transformation and Screening in *P. pastoris* and *S. cerevisiae*

Electroporation of *P. pastoris* and *S. cerevisiae* was carried out using the GenePulser Xcell (Bio-Rad) under the following conditions: 1.5 kV, 200 Ω and 25 μ F. Eighty microlitres of competent yeast cells were mixed with 10 μ l of plasmid DNA in a 1.5 ml sterile tube. Cells were then transferred to a cold 0.2 cm electroporation cuvette (Bio-Rad) and transformed. One ml of ice cold sorbitol (1 M) was added immediately after electroporation and the cells were incubated on ice for 15 min. One ml of YPD was then added followed by incubation for 1 h at 30°C. One hundred microlitre aliquots was plated onto YPD plates containing 100 μ g/ml zeocin for *P. pastoris* containing the pBG1 vector and histidine minus plates for *P. pastoris* containing the pHIL-S1 vector. YPD plates deficient in uracil were used for screening of *S. cerevisiae*. All plates were incubated at 30°C for 3-4 days.

2.2.14 Plate Screening for β -xylosidase activity

β -xylosidase screening was carried out on synthetic complete (SC) medium for constructs pJC1-*xlnD*, pHIL-S1-*xlnD* and pDLG55 (control) and YPD medium supplemented with zeocin (100 μ g/ml) for construct pBGP1-*xlnD*. 4-Methylumbelliferyl- β -D-xyloside (3 mg/ml) (MUX, Sigma) was spread onto the surface of the agar plates. Yeasts were point-inoculated on the agar surface and the plates incubated at 30°C for 24 h. The hydrolysis of MUX by the action of β -xylosidase activity results in the release of 4-methylumbelliferone (MU), which can be visualised under UV illumination as fluorescent halos surrounding the colonies (Manzanares *et al.*, 1999).

2.2.15 Xylosidase Expression and Extraction

Selected yeast colonies showing xylosidase activity were plated on YPD media and a single colony was used to inoculate 20 ml of YPD broth containing 100 μ g/ml zeocin for recombinant pBGP1-*xlnD*. A 1% pre-inoculum was used to inoculate 200 ml volume of media for xylosidase production. For the induced expression of xylosidase in pHIL-S1, buffered methanol complex media (BMMY) was used with 1% methanol induction. Samples were taken every 6 hours and expression was terminated after 60 h for constitutive production, and 96 h for induction. *S. cerevisiae* samples were tested every 4 hours and terminated after 48 hours of production.

Optical density of samples was measured at 600 nm for growth analysis, and these samples were then centrifuged at 10 000x *g*. The supernatants obtained from *P. pastoris* samples were stored at 4°C for subsequent enzyme analysis. To determine overall enzyme production, enzyme assays were carried out on the intracellular, extracellular and membrane-bound components obtained from *S. cerevisiae*. Intracellular enzyme extraction was carried out using YeastBuster Protein Extraction Reagent (Novagen). Cells were harvested by centrifugation at 4000x *g* for 10 min at 4°C. The cell pellet was re-suspended with 5 ml of YeastBuster reagent and incubated for 20 min in a shaking incubator at room temperature. The cells were then centrifuged at 10 000x *g* for 30 min at 4°C. The supernatant was stored at 4°C for enzyme analysis. For membrane-bound activity, cells were centrifuged at 4000x *g* for 10 min, and resuspended in equal volume citrate buffer. Culture supernatant was used for extracellular enzyme expression.

2.2.16 Co-expression of β -xylanase and β -xylosidase

Colonies showing both β -xylanase and β -xylosidase activity were inoculated into 20 ml volumes of YPD media containing 100 μ g/ml zeocin for 16 hours. A 1% pre-inoculum was used to inoculate a 200 ml volume of media, supplemented with zeocin for xylanase and xylosidase production. Samples were taken every 6 hours and expression was terminated after 60 hours. Optical density of samples was measured at 600 nm for growth analysis, and then centrifuged at 10 000x *g*. The supernatant obtained from *P. pastoris* samples were stored at 4°C for subsequent enzyme analysis.

2.2.16.1 β -xylosidase Enzyme Activity Assays

β -xylosidase activity was determined in triplicate in 50 mM sodium citrate buffer at pH 5, and at a temperature of 50°C for 5 min. The culture supernatant was used as a source of β -xylosidase from *P. pastoris* GS115 and intact yeast cells from *S. cerevisiae* Y294. β -xylosidase activity was measured using 0.1% chromophoric substrate *p*-nitrophenyl- β -D-xylopyranoside (PNPX, Sigma). Two hundred and fifty microlitres of the enzyme solution was added to 250 μ l of substrate, mixed, and incubated for 5 min at 50°C. Five hundred μ l of sodium carbonate (1 M) was added to stop the reaction. The release of *p*-nitrophenol was measured

spectrophotometrically at 405 nm against a reagent blank (buffer and substrate PNPX) and corrected for background colour against an enzyme blank (buffer and enzyme). A *p*-nitrophenol standard curve was used to calculate enzyme activity. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μ mol of *p*-nitrophenol per minute at 50°C (Saha, 2003).

2.2.16.2 β -1,4-Xylanase Enzyme Activity Assays

β -1,4-xylanase activity was assayed according to Bailey *et al.* (1992). One hundred μ l of enzyme extract was incubated with 900 μ l of a 1% birchwood xylan substrate (Sigma) in citrate buffer (0.05 M, pH 5.0) at 50°C for 5 min. The reaction was terminated by the addition of 1.5 ml of the DNS reagent (10 g/l 3,5-dinitrosalicylic acid, 16 g/l NaOH, 300 g/l sodium potassium tartrate). The solution was then boiled for 5 min and allowed to cool in water. This absorbance was measured against an enzyme blank and expressed as IU using a xylose standard curve. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μ mol of xylose per minute at 50°C.

2.2.17 Temperature, pH Optima and Stability of Enzymes

β -xylosidase activity was assayed by incubating equal volume of substrate and enzyme extract in citrate buffer (50 mM, pH 5) at 50°C for 5 min. The reactions were terminated by the addition of 500 μ l sodium carbonate (1 M). pH and temperature optima of the enzymes produced in *P. pastoris* and *S. cerevisiae* were determined by incubation at temperatures ranging from 40°C – 70°C and pH 3.0 – 7.0. (pH 3.0 – 5.0 using citrate buffers, pH 6.0 – 7.0 using phosphate buffers at a 0.1 M concentration). Samples were removed at 15 min intervals, stored on ice and assayed for residual xylosidase activity. pH stability was determined by incubating the enzyme at optimum temperature at pH 3, 4, 5, 6 and 7, with sampling every 15 min.

2.2.18 SDS–PAGE and Zymogram Analysis

Protein samples were analysed by SDS-PAGE and zymogram using a 12% resolving gel and a 5% stacking gel. Samples were prepared by adding equal volumes of loading buffer (50 mM

Tris-HCL, pH 6.8, 100 mM dithiothreitol, 0.1% bromophenol blue and 10% glycerol) and supernatant, followed by boiling for 10 min. Samples were then loaded onto the gel and electrophoresed for approximately 30 minutes at 50 mA in electrophoresis buffer (25 mM Tris, 250 mM glycine and 0.1% SDS). The gel was then stained for 30 min in staining solution containing 0.25% Coomassie Brilliant Blue in 90 ml methanol: water (1:1 v/v) and 10 ml glacial acetic acid, then de-stained overnight in a de-staining solution containing 90 ml methanol: water (1:1 v/v) and 10 ml glacial acetic acid (Laemmli, 1970). A similar procedure was followed for the zymogram analysis, 0.1% MUX was used as the substrate and added to the gel before allowing it to set. These gels were not stained and the proteins in the gel were re-natured in a solution of 1% Triton X-100 and 50 mM citrate buffer pH 5 for 30 min, followed by washing with citrate buffer pH 5. The gel was then incubated at 50°C for 10 minutes and viewed using a UV transilluminator and the gel image was captured using the Quantity One Imaging Software. A pre-stained protein ladder (Thermo Scientific) was used to determine the molecular weights of the proteins.

2.2.19 Xylan Degradation and Quantification by HPLC

β -xylanase and β -xylosidase-producing as well as co-producing cultures were grown in YPD for 96 hours. Xylan hydrolysis was first determined by *P. pastoris* GS115 (*xlnD*) and *T. lanuginosus* DSM5826 (*xynA*) cultures in 1 ml volumes at 50°C. A 1 % birchwood xylan mixture dissolved in citrate buffer (0.05 M, pH 5.0) was thermally equilibrated before adding 100 μ l of a 144 h-old culture of *T. lanuginosus* (*xynA*) and 100 μ l of a 72 h-old culture of *P. pastoris* GS115 (*xlnD*). The individual activities of the enzyme mixture on xylan used in the above reaction was 100 U/ml xylanase and 20 U/ml xylosidase. The reaction was stopped at different time intervals and 100 μ l aliquots were filtered for HPLC analysis. Secondly, xylan hydrolysis was analysed by *P. pastoris* GS115 co-expressing β -xylanase (*xynA*) and β -xylosidase (*xlnD*). A 1 M birchwood xylan mixture dissolved in citrate buffer (0.05 M, pH 5.0) was thermally equilibrated at 50°C. 100 μ l of a 72 h culture was added to 900 μ l xylan substrate and incubated at 50°C. The individual activity of the enzyme mixture was 30 U/ml xylanase and 8 U/ml xylosidase. 100 μ l aliquots were taken at different time intervals and filtered. The amount of xylose produced was quantified by HPLC (Hitachi, Merck) with the Aminex HPX-87H (organic acid column, BioRad) at 40°C using de-ionised water (Millipore,

Merck) as an eluent (0.6 ml/min) with an RI detector. Data was analysed using the Clarity software. Xylobiose and xylotriose (Megazyme) was used together with monosaccharide xylose (Sigma) as HPLC standards.

2.2.20 Separate Hydrolysis and Fermentation by *P. stipitis*

Enzymes were produced by growing *T. lanuginosus* (xylanase) and *P. pastoris* recombinant (xylosidase and co-expressed xylanase and xylosidase). The culture supernatants and enzymes were used to measure xylanase and xylosidase activity. Enzymatic hydrolysis was firstly carried out by incubating 1% birchwood xylan with single enzymes and co-expressed enzymes at 30°C for 48 h. Samples were aliquoted from at regular intervals to estimate the sugar released (2.2.19). Supernatant was collected after 48 h and inoculated with a 10% v/v culture suspension of *P. stipitis* to initiate fermentation of the degraded products. The fermentation was incubated at 30°C for 72 h. Fermentation products were measured by HPLC at 24 h intervals.

2.3 RESULTS

2.3.1 PCR Amplification of *xlnD*

The *xlnD* gene was originally cloned into plasmid pDGL55, under the control of the *ADH2* promoter and expressed in *S. cerevisiae* Y294. pDGL55 containing the *xlnD* gene was isolated as described in section 2.2.3 and subjected to PCR. A single discrete band was observed on agarose gels (Fig. 2.6). In lane 2, the amplification product of the approximately 2700 bp fragment was seen. The high intensity of the band indicated that the PCR reaction was successful and contained sufficient PCR product for further procedures.

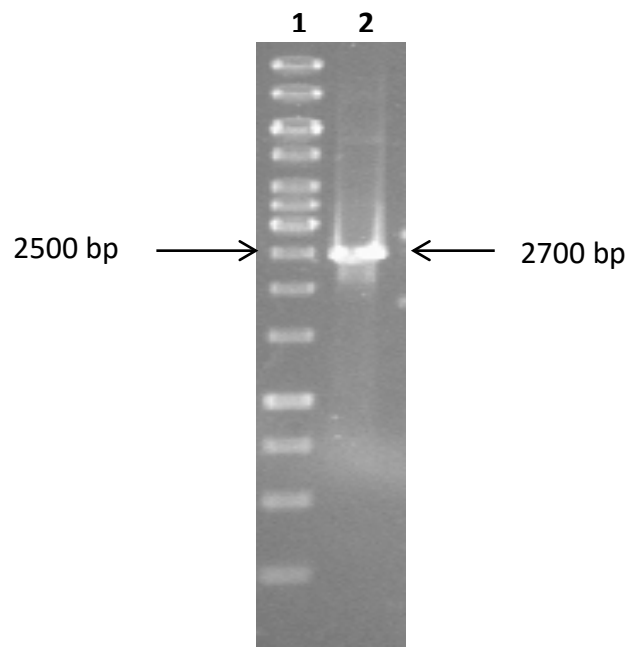


Figure 2.6: PCR amplification of the *xlnD* gene. Lane 1: 1 kb DNA ladder. Lane 2: The 2700 bp *xlnD* product.

2.3.2 Construction of pBGP1-*xlnD* and pHILS1-*xlnD*

The purified PCR product obtained in section 2.3.1 was initially ligated to cloning vector pTZ57/R, and this construct was used for subsequent cloning procedures. pBGP1, pHIL-S1 and pTZ57/R-*xlnD* were isolated as described in section 2.2.3. The 5600 bp pTZ57/R-*xlnD* (Fig. 2.7, lane 3), was restricted using restriction enzymes *EcoRI* and *XbaI* for ligation to similarly-digested vector pBGP1. Since the size of plasmid pTZ57/R (2900 bp) and insert *xlnD* (2700 bp) were similar, a triple-digest was performed (including restriction enzyme *ScaI*) to further cleave the plasmid and allow better visualization of the *xlnD* band and to aid in band excision from the gel. This restriction digestion produced the 2700 bp *xlnD* fragment (Fig. 2.7, lane 4.) and 2 smaller vector bands. Lanes 5 and 6 contained linearized pBGP1 and pHIL-S1 corresponding to sizes 4600 bp and 8300 bp, respectively.

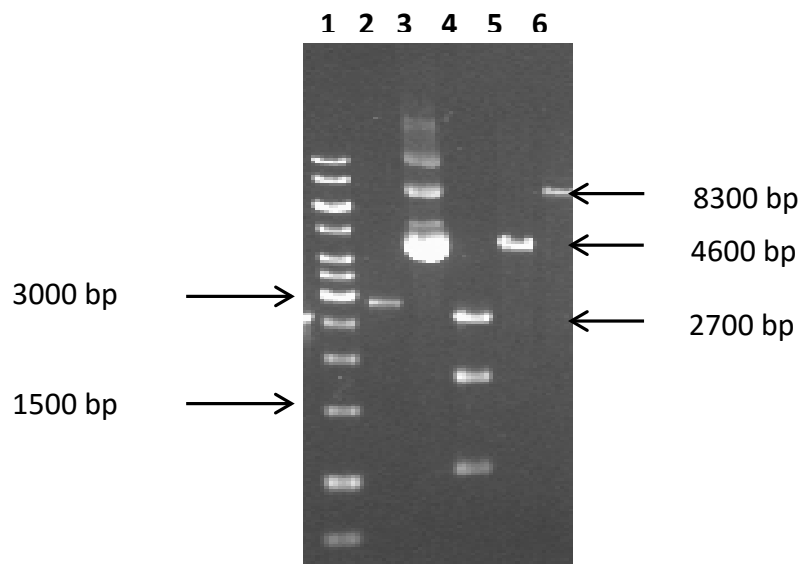


Figure 2.7: Agarose gel electrophoresis of ligation reactants pBGP1-*xlnD* and pHILS1-*xlnD* cassettes. Lane 1: DNA molecular weight marker. Lane 2: Linearized pTZ57/R cloning vector. Lane 3: pTZ57/R-*xlnD* construct. Lane 4: triple digest of pTZ57/R-*xlnD* to release the *xlnD* fragment. Lane 5: Double digest of plasmid pBGP1 using enzymes *EcoRI* and *XbaI*. Lane 6: double digest of plasmid pHILS1 using enzymes *EcoRI* and *BamHI*.

2.3.3 Construction of pJC1-*xlnD*

Plasmid pDLG55 and pJC1 were isolated as described in Section 2.2.3. The *xlnD* gene was isolated from plasmid pDLG55 by digestion using *EcoRI* (Fig. 2.8, lane 2), to yield the 2700 bp fragment. The larger band corresponds to the pDLG55 (7000 bp). Plasmid pJC1 was linearized using the same restriction enzyme *EcoRI*, and visualized as a 6900 bp vector band (Fig. 2.8, lane 3).

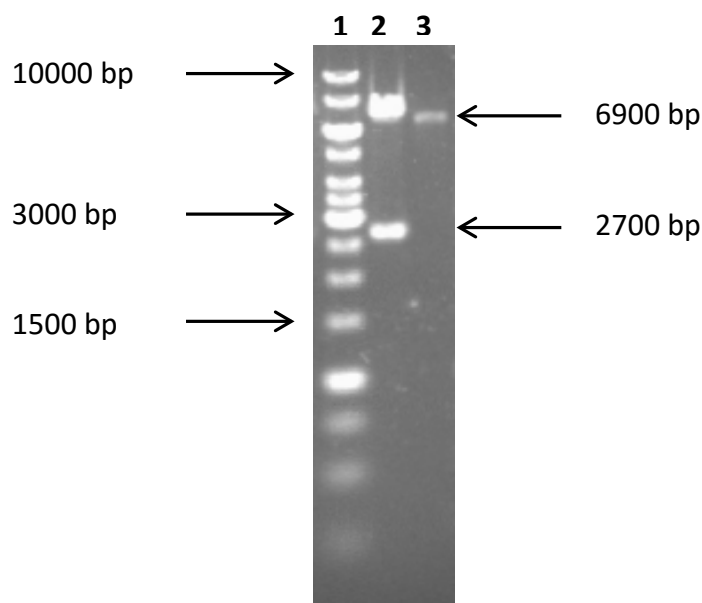


Figure 2.8: Reactants used to construct pJC1-*xlnD*. Lane 1: DNA molecular weight marker. Lane 2: pDLG55 restricted with *EcoRI*. Lane 3: pJC1 linearized with *EcoRI*.

2.3.4 Restriction Analysis of Selected Clones

Restriction analysis was performed on recombinant plasmids isolated from *E. coli* after the *xlnD* fragment was cloned into pBGP1, pHIL-S1, pJC1 and pTZ57/R (2.3.1). Vector pTZ57/R and the *xlnD* fragment were approximately the same size, therefore 3 restriction enzymes, (*EcoRI*, *ScaI* and *XbaI*) were used for the analysis of pTZ57/R-*xlnD* clones (Fig. 2.9, lane 4).

Colonies obtained from ligation reactions with vectors pBGP1 and pHIL-S1 were inoculated in media and their plasmids isolated and analysed based on their molecular weights. Successful pBGP1-*xlnD* transformants were restricted using enzymes *EcoRI* and *XbaI*. This restriction yielded two distinct bands (Fig. 2.9, lane 7). The larger band corresponds to the 4600 bp

vector, and the smaller band corresponds to the 2700 bp *xlnD* gene cassette. This also corresponds to the *xlnD* fragment observed in lane 4. This gene was successfully cloned into the pHIL-S1 vector (Fig. 2.9, Lane 10). This was confirmed using restriction enzymes *EcoRI* and *BamHI* to yield an 8300 bp vector band, and a 2700 bp insert band. An increase in size seen in lanes 3, 6 and 9 is due to the difference in size of the vectors used.

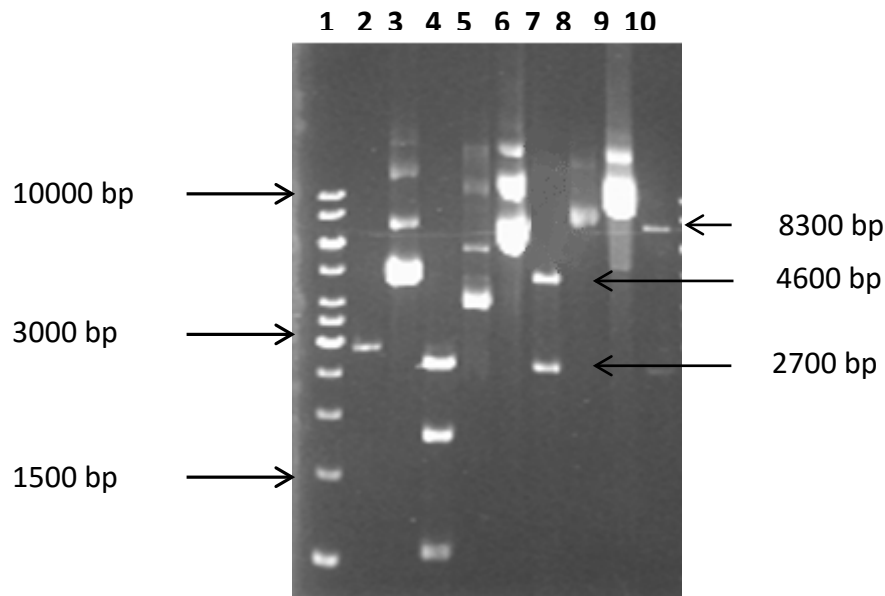


Figure 2.9: Restriction analysis of plasmids isolated from *E. coli* XL1 Blue transformants. Lane 1: DNA molecular weight marker. Lane 2: Linearized plasmid pTZ57/R. Lane 3: pTZ57/R-*xlnD*. Lane 4: pTZ57/R-*xlnD* restricted with *EcoRI*, *XbaI* and *Scal*. Lane 5: Plasmid pBGP1. Lane 6: pBGP1-*xlnD*. Lane 7: Clone pBGP1-*xlnD* restricted with enzymes *EcoRI* and *XbaI*. Lane 8: Plasmid pHILS1. Lane 9: pHILS1-*xlnD*. Lane 10: clone pHILS1-*xlnD* digested with enzymes *EcoRI* and *BamHI*.

Plasmids were also isolated from pJC1-*xlnD*. These plasmids extracted from the clones were restricted with *EcoRI* to confirm successful ligation (Fig. 2.10). The larger band observed in lane 7 corresponds to the 6.9 kb vector band (Fig. 2.10, lane 5), whilst the smaller band in lane 7 corresponds to the 2700 bp *xlnD* gene. This 2700 bp fragment corresponded to the band in lane 3. The larger bands corresponding to the vector (pDLG55), and the smaller band corresponding to the *xlnD* fragment.

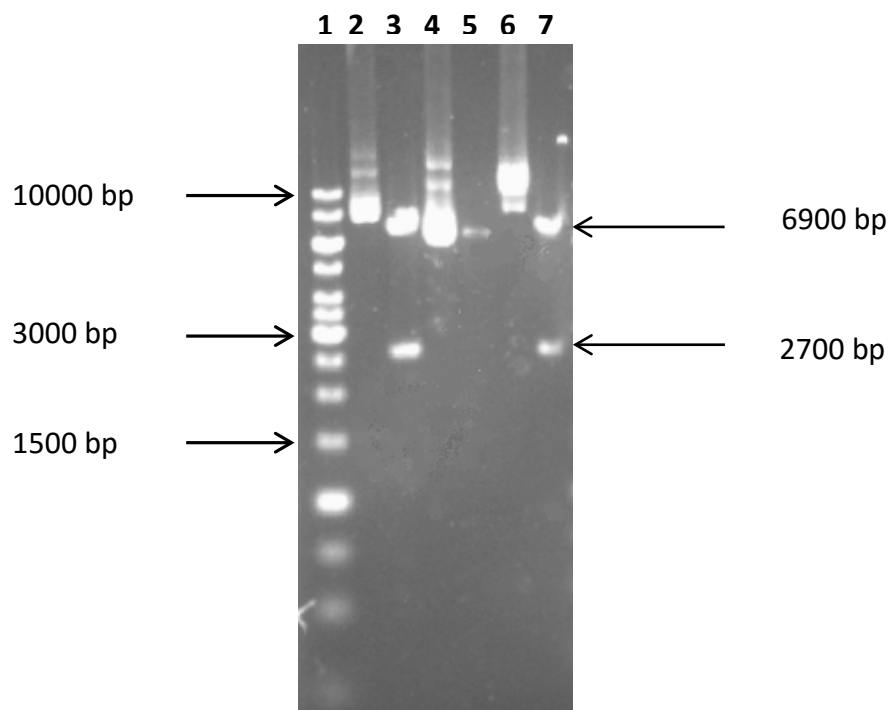


Figure 2.10: Restriction analysis of *E. coli* pJC1-*xlnD* clones. Lane 1: DNA molecular weight marker. Lane 2: plasmid pDLG55. Lane 3: Plasmid pDLG55 digested with enzyme *EcoRI*. Lane 4: Plasmid pJC1. Lane 5: Plasmid pJC1 linearized with enzyme *EcoRI*. Lane 6: Uncleaved positive clone (pJC1-*xlnD*). Lane 7: pJC1-*xlnD* digested with enzyme *EcoRI*.

2.3.5 Homologous Recombination

To isolate His⁺ Mut⁺ transformants, construct pHLS1-*xlnD* was linearized with *Stu*I. A small portion was analysed by agarose gel electrophoresis to confirm complete digestion. This was transformed into electrocompetent *P. pastoris* cells as described in section 2.2.13. Total DNA was isolated from fluorescent colonies, followed by PCR using xylosidase specific primers (Set A). Plasmid pDLG55 containing the 2700 bp *xlnD* gene served as a positive control for the PCR reaction (Fig. 2.11, lane 8). Genomic DNA was isolated from *P. pastoris* transformed with pHLS1 and subjected to PCR (Fig. 2.11, lane 6). This served as a negative control due to the absence of the *xlnD* gene. The *xlnD* fragment obtained after PCR of genomic DNA (Fig. 2.11, lane 7) corresponded to the same molecular weight as the positive control (Fig. 2.11, lane 8). This confirmed the presence of *xlnD* in the genome of *P. pastoris*.

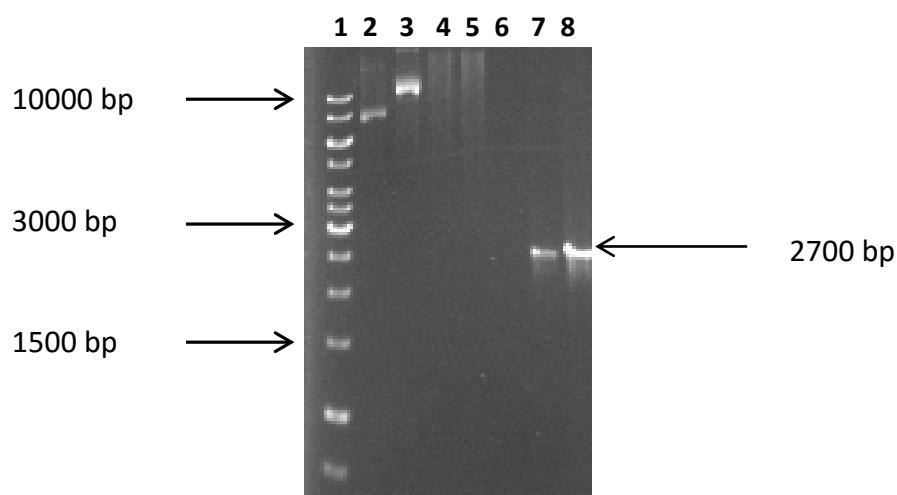


Figure 2.11:

Confirmation of *P. pastoris* pHLS1-*xlnD* transformants. Lane 1: DNA molecular weight marker. Lane 2: plasmid pHLS1. Lane 3: Unrestricted pHLS1-*xlnD* plasmid. Lane 4: gDNA isolated from *P. pastoris* pHLS1. Lane 5: gDNA isolated from pHLS1-*xlnD* positive clone. Lane 6: PCR amplification of *P. pastoris* pHLS1 gDNA, using *xlnD* specific primers. Lane 7: PCR amplification of gDNA from *P. pastoris* pHLS1-*xlnD*, using *xlnD* specific primers. Lane 8: PCR amplification of plasmid pDLG55, positive control.

2.3.6 PCR Amplification of the *xynA* Construct

The *T. lanuginosus* xylanase was modified by random mutagenesis by Stephens *et al.* (2007). This modified xylanase was cloned into the yeast shuttle vector pBGP1 and designated pBGP1-S340. This recombinant was isolated as described in section 2.2.3 and subjected to PCR using primers Set B (Table 2.1). Primer design allowed the amplification of the pBGP1 GAP promoter and terminator region. An aliquot of the PCR product was analysed by electrophoresis to determine if the construct was successfully amplified. A single discrete band was observed on an agarose gel (Fig. 2.12, lane 2), as a 1986 bp fragment. The intensity of the band from the PCR reaction was sufficient for purification of PCR product for subsequent cloning procedures.

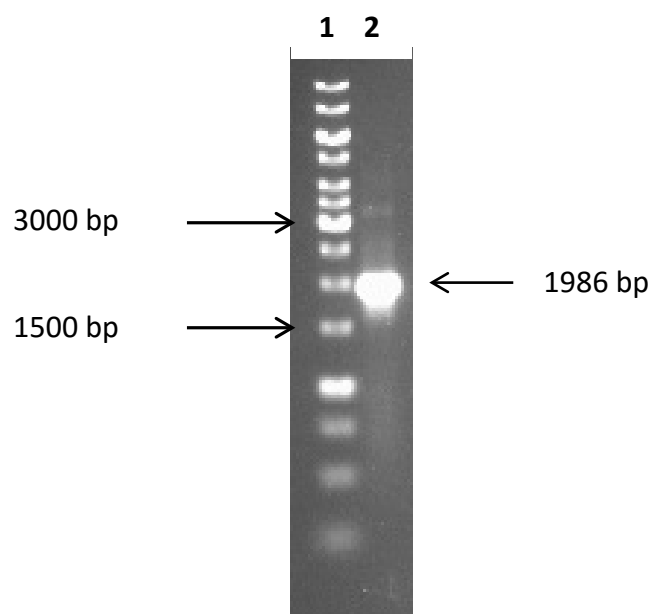


Figure 2.12: PCR analysis of pBGP-*xlnD* construct containing the GAP-S340 fragment. Lane 1: 1 kb DNA ladder used to estimate the molecular weight of the bands produced. Lane 2: 1986 bp GAP-S340 amplification product.

2.3.7 Amplification of pTZ57/R-GAP-S340 Cassette

The purified PCR product obtained in section 2.3.6 was initially ligated to cloning vector pTZ57/R, and this construct was used as template DNA for subsequent cloning procedures. To confirm successful insertion of the S340 gene and GAP promoter and terminator, plasmid isolated from presumptive positive colonies were restricted with *Bam*HI (Fig. 2.13, lane 4).

These restriction sites were designed on primer set B (Table 2.1). This digestion released a 2900 bp vector band, and a smaller band containing the S340 gene and GAP promoter and terminator region (1986 bp). This clone was designated pTZ57/R-S340.

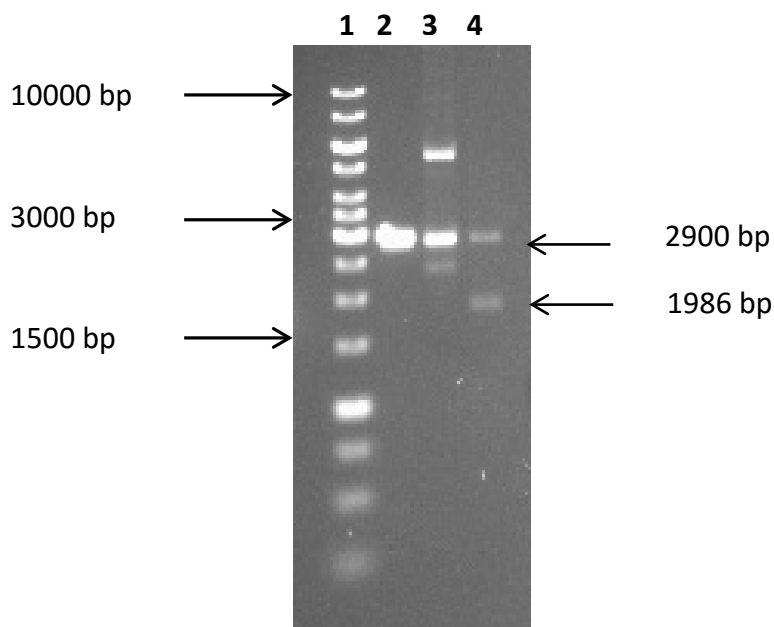


Figure 2.13: Confirmation of the construction of pTZ57/R-GAP-S340. Lane 1: DNA molecular weight marker. Lane 2: Linearized pTZ57/R cloning plasmid. Lane 3: Uncleaved pTZ57/R-S340 clone used as template. Lane 4: pTZ57/R-GAP-S340 restricted to release the GAP-S340 fragment.

2.3.8 pBGP1-*xlnD*-S340

Plasmid DNA pBGP1-*xlnD* and pTZ57/R-S340 (Fig. 2.14, lanes 2 and 3 respectively), were isolated as described in section 2.2.3 and digested using *Bam*HI to produce cohesive ends for successful cloning to pBGP1-*xlnD*. pTZ57/R-S340 yielded two bands, with one band corresponding to the 1900 bp GAP-S340 fragment (Fig. 2.14, Lane 4). Colonies obtained from ligation reactions with pBGP1-*xlnD* were analysed by gel electrophoresis. Plasmid was isolated from possible clones and restricted with *Eco*RV, to confirm successful insertion of the GAP-S340 fragment to pBGP1-*xlnD* recombinant. The *Eco*RV recognition site is located on vector pBGP1 and is also found within the *xlnD* gene. Restriction with this enzyme yielded a 5900 bp fragment and a smaller 3300 bp fragment (Lane 7). The pBGP1-*xlnD*-S340 clone was analysed by PCR to further confirm the presence of the xylanase gene (S340), which was

observed in lane 9 as a 786 bp fragment. Lane 10 served as a positive control for amplification of the xylanase gene from pBGP1-S340.

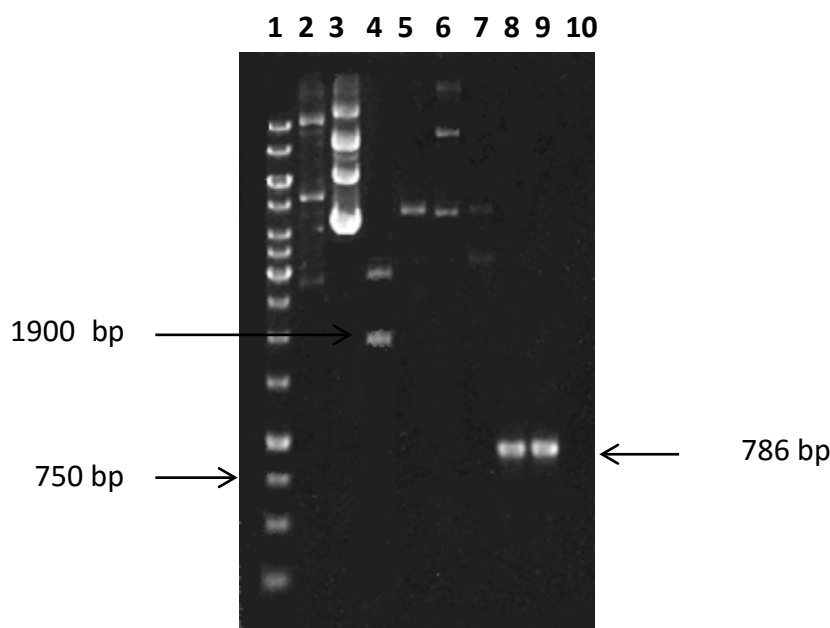


Figure 2.14: Cloning reactants used for the construction of pBGP1-*xlnD*-S340. Lane 1: DNA molecular weight marker. Lane 2: Uncleaved plasmid pTZ57/R-GAP-S340. Lane 3: Uncleaved plasmid pBGP1-*xlnD*. Lane 4: pTZ57/R-S340 restricted with *Bam*HI. Lane 5: pBGP1-*xlnD* linearized with *Eco*RV. Lane 6: Presumptive positive clone (pBGP1-*xlnD*-GAP-S340). Lane 7: Clone pBGP1- *xlnD*-S340 restricted with enzyme *Eco*RV. Lane 8: Positive PCR confirmation of xylanase gene (S340) using pBGP1-*xlnD*-S340 cassette as template DNA. Lane 9: Positive control, amplification of xylanase gene S340 using pBGP1-S340 as template. Lane 10: Negative control, PCR amplification from pBGP1-*xlnD* construct.

2.3.9 Screening for Xylosidase Production

The xylosidase gene was cloned into three vectors viz., pBGP1, pHLS1 and pJC1 followed by transformation into their respective hosts. Media deficient in uracil was used for selection in *S. cerevisiae*. For selection in *P. pastoris*, medium deficient in histidine was used for the selection of clones that contained the pHLS1-*xlnD* construct after transformation and YPD supplemented with zeocin was used to identify clones containing the plasmid pBGP1. Selection plates were supplemented with 0.1% MUX as described in section 2.2.14. Colonies positive for xylosidase production fluoresced in the presence of MUX (Fig. 2.15), this was due to the hydrolysis of 4-methylumbelliferyl- β -D-xyloside by xylosidase which resulted in the release of 4-methylumbelliferone. The fluorescence appeared to be related to the size of the

colony, larger colonies fluoresced more than smaller colonies. The fluorescence visualized by construct pBGP1-*xlnD* in *P. pastoris* was much brighter than that obtained in *S. cerevisiae* constructs.

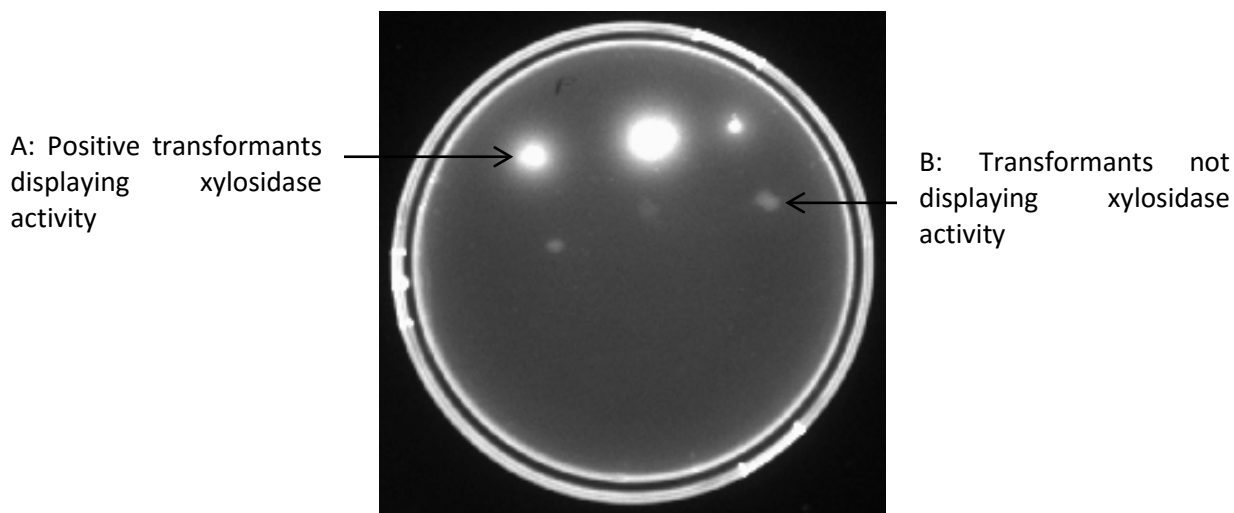


Figure 2.15: MUX screening plate used to determine β -xylosidase activity of positive and negative *P. pastoris* pBGP1-*xlnD* transformants, A: transformants displaying xylosidase activity, indicated by fluorescence. B: non xylosidase producer, showing no fluorescence.

Identification of positive clones was based on their ability to co-express both xylanase and xylosidase. YPD media supplemented with zeocin containing RBB-xylan for xylanase production, and MUX for xylosidase production aided in the screening for positive clones. Successful transformants displaying xylosidase activity were visualised under UV illumination as fluorescence surrounding the colony (Fig. 2.16). These transformants were simultaneously cultured on RBB xylan plates. Positive xylanase transformants were identified by the fluorescence around the colony, which meant that the colony was producing a xylanase that hydrolysed the RBB-xylan complex. RBB-xylan is a blue coloured complex, and degradation leads to the formation of a colourless product.

The fluorescence produced by *P. pastoris* were sufficient to confirm xylosidase expression. The control (pBGP1-*xlnD*) and clone pBGP1-XX1 both produced xylosidase whilst the negative control (pBGP1-S340), as expected, did not produce xylosidase (Fig. 2.16). The intensity of the fluorescence is almost identical, indicating that xylosidase production by pBGP1-XX1 could be equivalent to the control (pBGP1-*xlnD*).

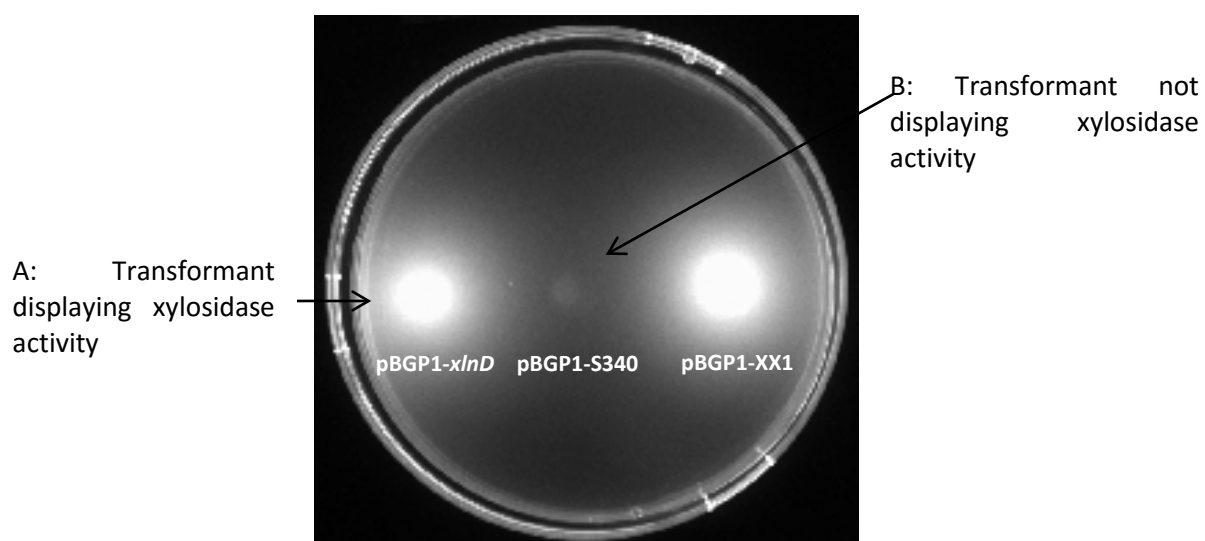


Figure 2.16: Confirmation showing positive xylosidase expression in yeast transformants spotted on YPD zeocin plate supplemented with MUX. A: *P. pastoris* recombinant transformed with pBGP1-*xlnD* expressing xylosidase, indicated by fluorescent halo. B: *P. pastoris* recombinant transformed with pBGP1-S340.

The fluorescence produced by *P. pastoris* transformants confirmed xylanase production (Fig. 2.17), the control (pBGP1-S340) and pBGP1-XX1 both produced xylanase, whilst the negative control (pBGP1-*xlnD*) did not. Even though pBGP-S340 and pBGP-XX1 both produced zones of hydrolysis, pBGP1-S340 produced a noticeably bigger zone when compared to construct pBGP1-XX1. This was a possible indication of the difference in xylanase activity produced by both constructs.

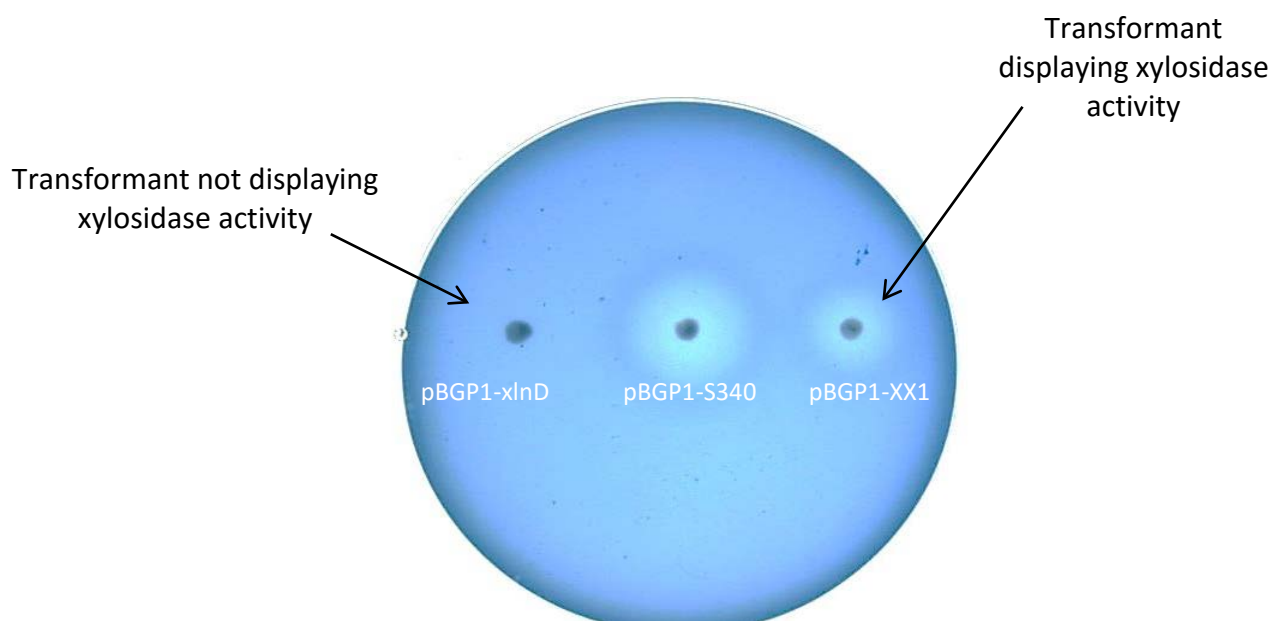


Figure 2.17: Positive and negative xylanase expression in *P. pastoris* transformants spotted on a YPD plate supplemented with zeocin and RBB xylan plate.

2.3.10 SDS-PAGE and Zymogram Analysis in *P. pastoris* and *S. cerevisiae*

In order to compare protein production, aliquots obtained from shake flask experiments with *S. cerevisiae* and *P. pastoris*, were analysed using SDS-PAGE. The *P. pastoris* recombinant showed a single band of approximately 120 kDa (Fig. 2.18, Lane 4). The clone co-expressing xylanase and xylosidase displayed 2 protein bands, one corresponding to the 120 kDa xylosidase (Fig. 2.18, Lane 3) and the other corresponding to a 24 kDa band corresponding to the xylanase reported by Mchunu *et al.* (2009). Protein activity was confirmed by zymogram analysis of the bands seen on SDS-PAGE gels. Samples were run concurrently, and a single band was obtained in *P. pastoris* as an active xylosidase (Fig. 2.18, lane 6 and 7) fluorescing in the presence of MUX.

pDLG55-xlnD and pJC1-xlnD expressed in *S. cerevisiae* were highly contaminated with intracellular proteins, as expected. The cell sample was lysed to release cell bound proteins and numerous bands were observed after SDS-PAGE analysis. The zymogram and SDS-PAGE

samples analysed showed a single active xylosidase band approximately 120 kDa (Fig. 2.19, lane 6 and 7), and no active bands were seen in the other clones.

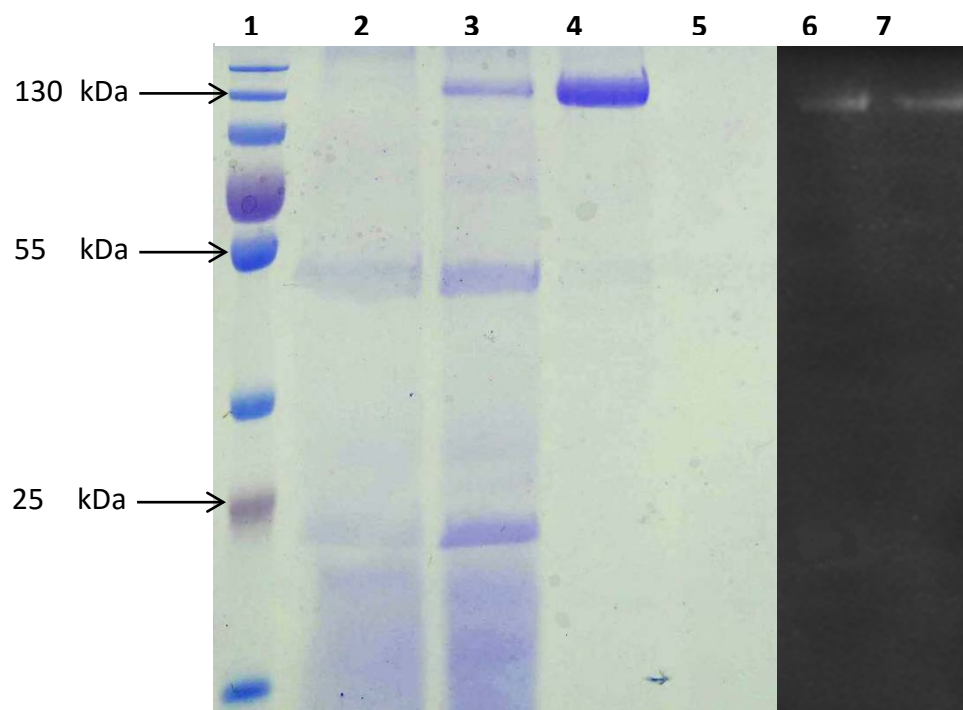


Figure 2.18: SDS-PAGE and zymogram of *P. pastoris* clones. Lane 1: Protein marker, Lane 2: pBGP1-S340 control Lane 3: pBGP1-xlnD-S340 clone, Lane 4: pBGP1-xlnD clone, Lane 5: *P. pastoris* GS115 transformed with pBGP1, Lane 6: Zymogram analysis confirming xylosidase activity of pBGP1-xlnD-S340, Lane 7: Zymogram analysis confirming xylosidase activity from pBGP1-xlnD.

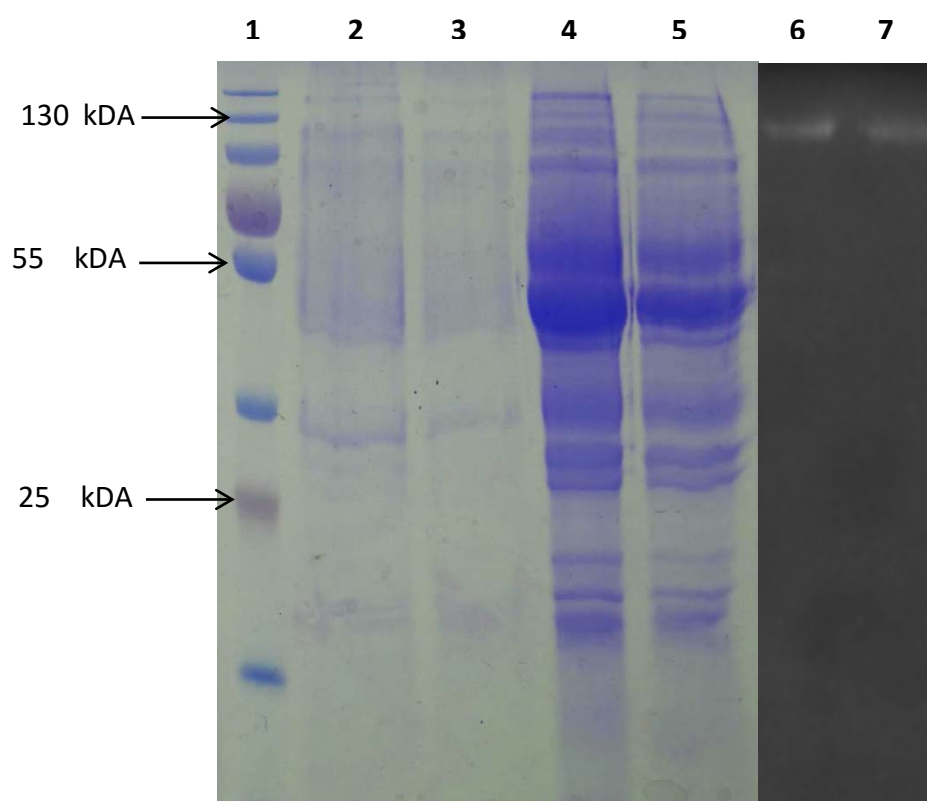


Figure 2.19: SDS-PAGE and zymogram of *S. cerevisiae* clones. Lane 1: Protein marker, Lane 2: *S. cerevisiae* Y294 transformed with pDLG55, Lane 3: *S. cerevisiae* Y294 transformed with pJC1, Lane 4: pDLG55-xlnD clone, Lane 5: pJC1-xlnD clone, Lane 6: Zymogram analysis confirming xylosidase production from pDLG55-xlnD clone. Lane 7: Zymogram analysis confirming xylosidase activity from pJC1-xlnD clone.

2.3.11 Effect of Glucose Concentration on Xylosidase Activity

The ADH2 promoter, known to be one of the most powerful *S. cerevisiae* promoters is highly repressed in the presence of glucose (Hitzeman *et al.*, 1982). Therefore, different concentrations of glucose was tested with construct pDLG55-*xlnD* in *S. cerevisiae*. Three different concentrations were used, 1%, 1.5%, and 2%. The highest xylosidase activity was obtained using 1% glucose. 1% and 1.5% glucose showed comparable results; reaching levels of up to 3.5 nkat/ml. However the use of 2% glucose highlighted the repression of high levels on xylosidase activity, reaching a maximum of 2 nkat/ml after 48 h (Fig. 2.20).

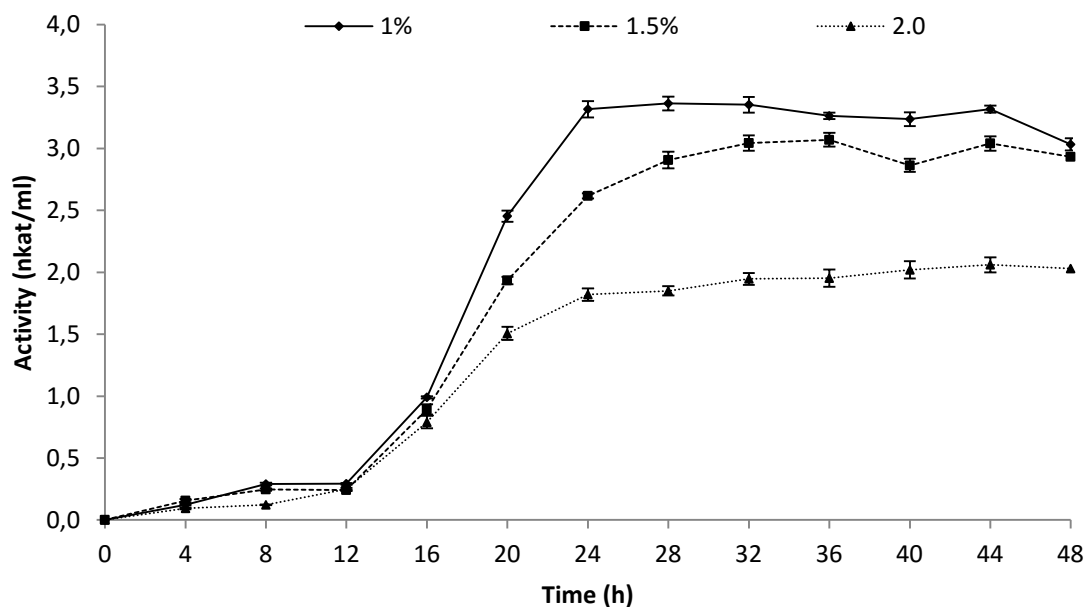


Figure 2.20: The effect of varying glucose concentration on xylosidase activity in *S. cerevisiae* ADH2 promoter using pDLG55-*xlnD*

2.3.12 Expression of xylosidase by *P. pastoris*

2.3.12.1 Constitutive and Induced Expression

P. pastoris shake flask cultivations were carried out in triplicate for the pBGP1-xlnD construct. The selective pressure was maintained by the addition of the antibiotic zeocin. Shake flask cultivations were carried out over 72 hours with highest aeration. Enzyme production occurred between 18 h and 72 h with an increase in optical density. Xylosidase production in *P. pastoris* produced the highest activity of 70 U/ml after 72 hours was recorded. This was at a final OD₆₀₀ of 30 (Fig. 2.21). pBGP1 maintains the glycolytic GAP promoter system, constitutively producing high expression levels on glucose, glycerol and methanol media (Cereghino and Cregg, 1999). Xylosidase production was extracellular, and almost no activity was detected from the extraction of intracellular contents.

The AOX1 promoter regulating the production of alcohol oxidase is used to drive heterologous protein expression in *Pichia*. In contrast, this promoter is highly repressed by glucose and other carbon sources. Shake flask cultivations with the pHIL-S1 construct were grown in BMMY medium for 96 hours and were sampled every 6 hours (Fig 2.22). Methanol was added to a final concentration of 0.5% every 24 hours. A close correlation between optical density and activity was noticed, enzyme activity increased with optical density and was noticeable from 18 h. Growth and enzyme production plateaued around 48 h, producing around 0.5 U/ml of xylosidase.

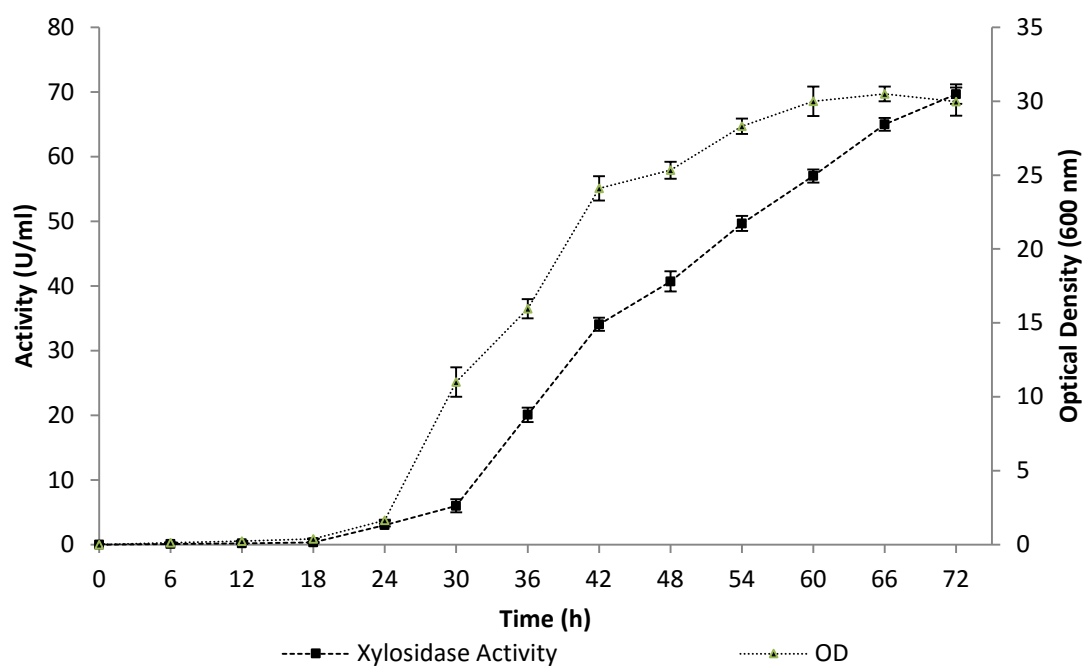


Figure 2.21: Constitutive xylosidase production in *P. pastoris* with pBGP1-xInD, in YPD broth supplemented with zeocin. Xylosidase production was maintained over 72 h at 30°C shaking at 200 rpm.

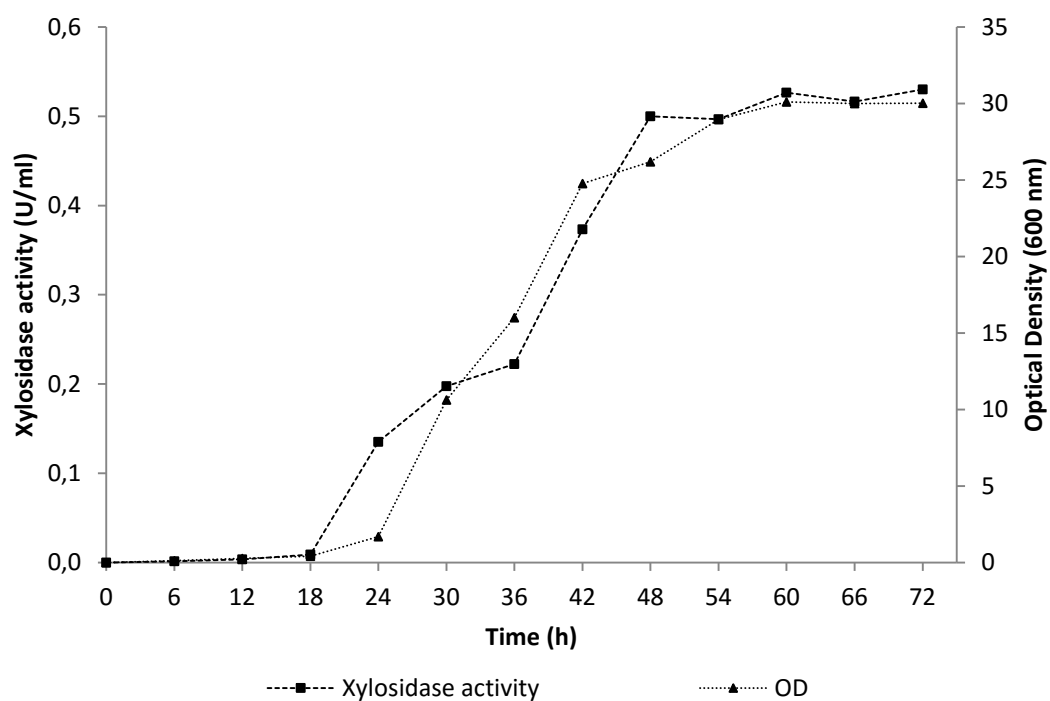


Figure 2.22: Induced xylosidase production in *P. pastoris* grown in BMMY broth. Methanol induction was constant at a final concentration of 0.5%. Xylosidase production was maintained over 96 h at 30°C shaking at 200 rpm.

2.3.13 Expression of Xylosidase by *S. cerevisiae*

2.3.9.1 Constitutive and Induced Production

Shake flask cultivations were carried out in triplicate to compare the two promoter systems, ADH2 and PGK, with constructs pDLG55-xlnD and pJC1-xlnD respectively. For the ADH2 promoter dependent on glucose concentration, a 1% concentration was maintained. Enzyme activity and optical density were similar for the first 12 h, after which the optical density increased but activity did not. Clone pDLG55 served as a control in this study and produced 5.0 μ kat/ml of cell bound enzyme whilst reaching a maximum OD₆₀₀ of 8.0 after 48 h (Fig. 2.23). It is likely that if the fermentation was carried out for a longer period, the activity would have increased.

The glycolytic PGK promoter system is constitutive and therefore able to express protein without induction. The pJC1-xlnD clone was grown in triplicate (2.2.18) and xylosidase activity measured every 4 hours. The increase in optical density and the increase in activity were proportional for the first 12 h. After 12h, enzyme activity increased at a faster rate than optical density. Even though the rate of increase of enzyme activity was much higher than optical density, the optical density continues to increase while enzyme activity reaches maximum and then declines. Maximum production was 8 U/ml after 48 hours, after reaching an OD₆₀₀ of 8.0 (Fig. 2.24).

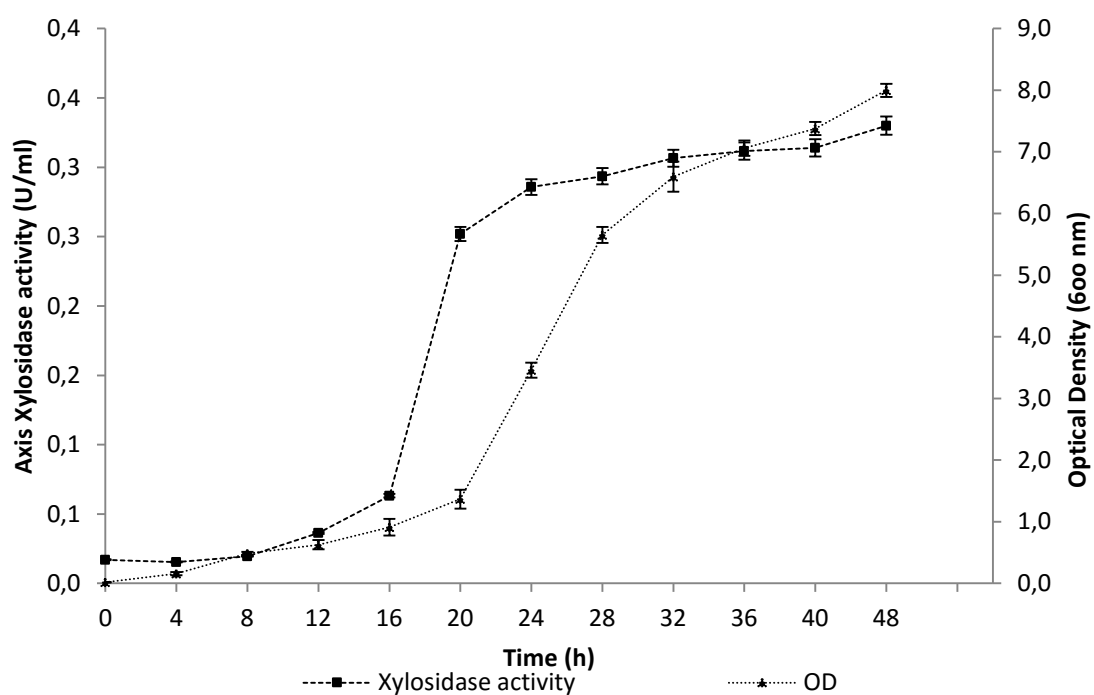


Figure 2.23: Induced xylosidase production in *S. cerevisiae* using pDLG55, grown in YPD medium. Xylosidase production was carried out over 48 h at 30°C shaking at 200 rpm.

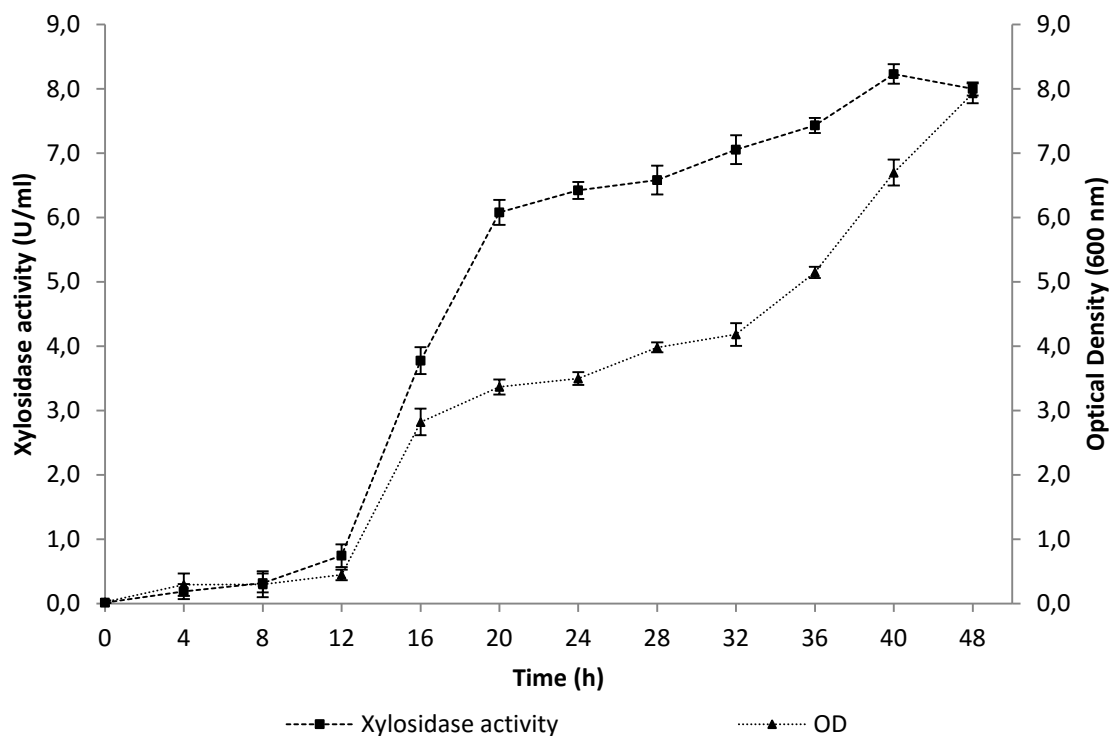


Figure 2.24: Constitutive xylosidase production in *S. cerevisiae* using pJC1-xlnD, grown in YPD medium. Xylosidase production was determined over 48 h at 30°C shaking at 200 rpm.

2.3.14 Determination of pH and Temperature Optima

Enzyme characterization was performed with *P. pastoris* and *S. cerevisiae* clones. The pH and temperature optimum of xylosidase was tested between pH 3 – 8 and 40°C – 70°C.

2.3.14.1 pH and Temperature Optima of Xylosidase from *P. pastoris*

Xylosidase activity peaked at pH 4 (Fig. 2.25). 80% activity was retained between pH 3 - 5. Enzyme activity characteristics was studied at the optimum pH between 30°C to 80°C. The highest xylosidase activity was obtained at 60°C (Fig. 2.26). 77% activity was maintained at 50°C and 58% activity at 40°C. At 70°C, enzyme activity decreased and enzyme activity completely terminated at 80°C. Enzyme production was the highest at this temperature, producing 70 U/ml xylosidase. Characterization of xylosidase was carried out at standard assay conditions (pH 5, and 50°C).

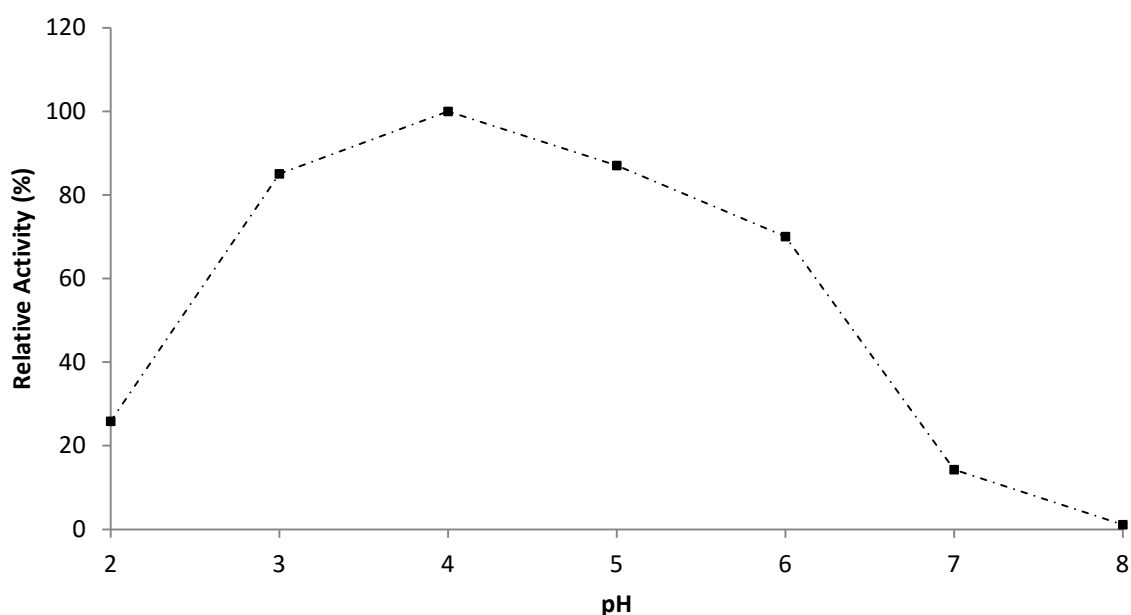


Figure 2.25: Xylosidase activity from pBGP1-xInD, expressed in *P. pastoris*. Activity was measured from pH2 to pH8 at 50°C for 5 min. The remaining activity was expressed as percentage of original activity.

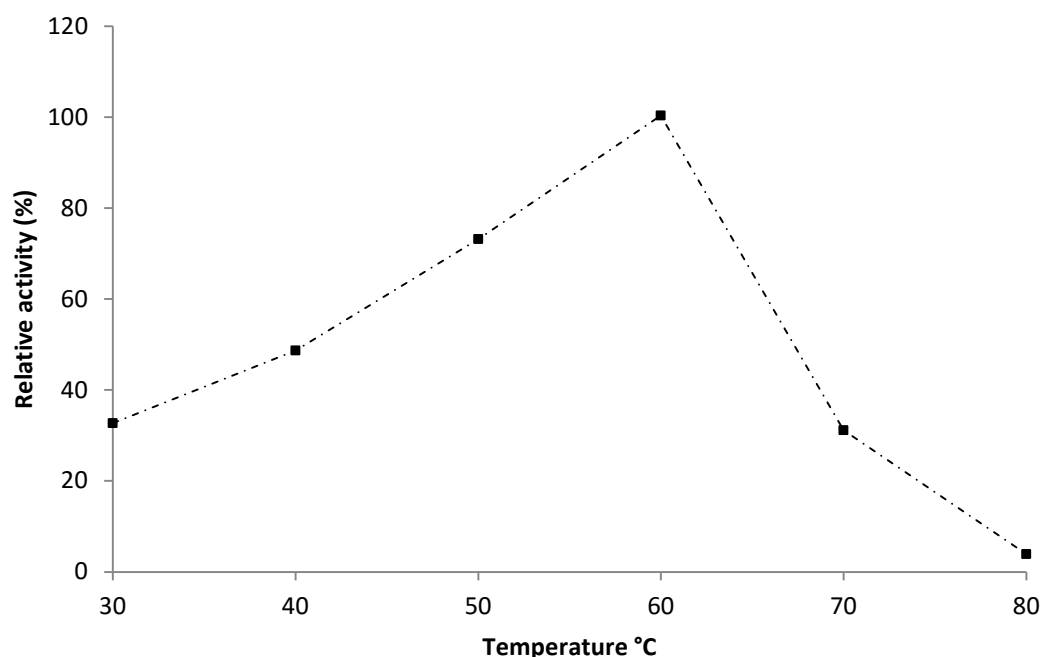


Figure 2.26: The effect of temperature on xylosidase activity. Xylosidase activity was measured from pBGP1-xlnD, expressed in *P. pastoris*. The temperature ranged from 30°C – 70°C for 5 min, pH 4. The remaining activity was expressed as percentage of original activity.

2.3.10.2 pH and Temperature Stability of Xylosidase from *S. cerevisiae*

Enzyme stability assays were carried out for *S. cerevisiae* expressing the pJC1-xlnD construct and *P. pastoris* expressing the pBGP-xlnD construct, to determine differences between the two strains. Thermostability of the enzyme at pH 5 over a range of different temperatures revealed retention of almost 92% of activity at 40°C after 2 h (Fig. 2.27). At 50°C, the enzyme retained 84% activity after 2 h. 60% of activity was retained at optimum temperature after 30 minutes, followed by a steady decline to 38% after 2 h. At 70°C the enzyme lost all activity after 30 min. Due to the sudden drop between 60°C and 70°C, stability of the enzyme was also analysed at 65°C. These results were intermediate for those at 60°C and 70°C, indicating that the rapid activity loss at 70°C was valid. Alkaline stability of xylosidase was tested at standard assay temperature over the pH range 3 – 7 (Fig. 2.28). The enzyme was most stable at pH 5, retaining 95% activity after 2 hours. At pH 4, the enzyme retained 90% activity.

However, towards the more acidic pH, the stability decreased. At pH 6, 39% retention of activity was observed after 2 h.

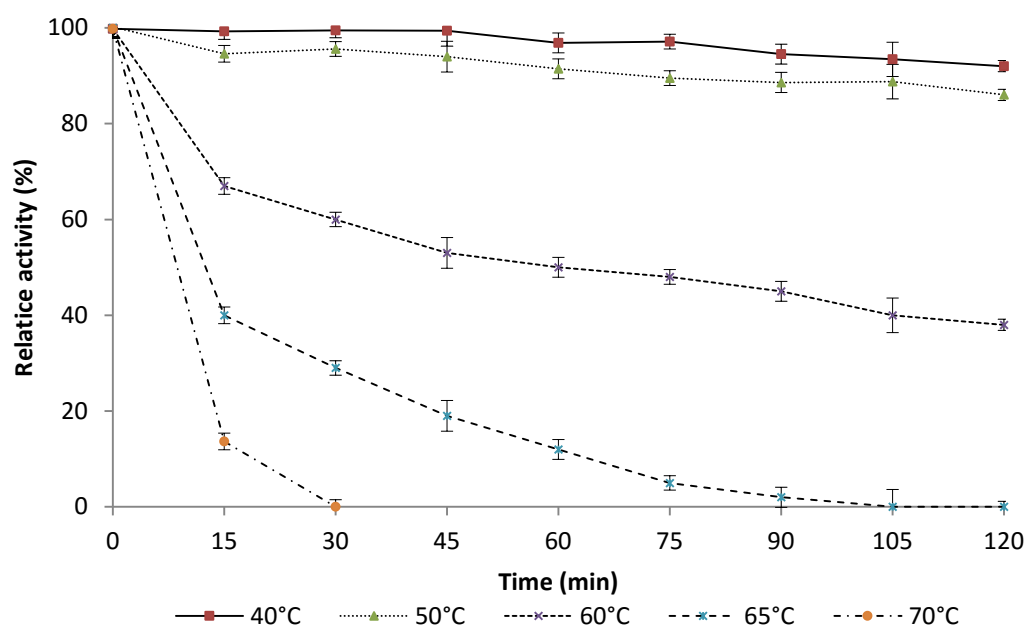


Figure 2.27: Thermo-stability of xylosidase expression in *S. cerevisiae* at pH 5. The remaining activity was expressed as percentage of original activity.

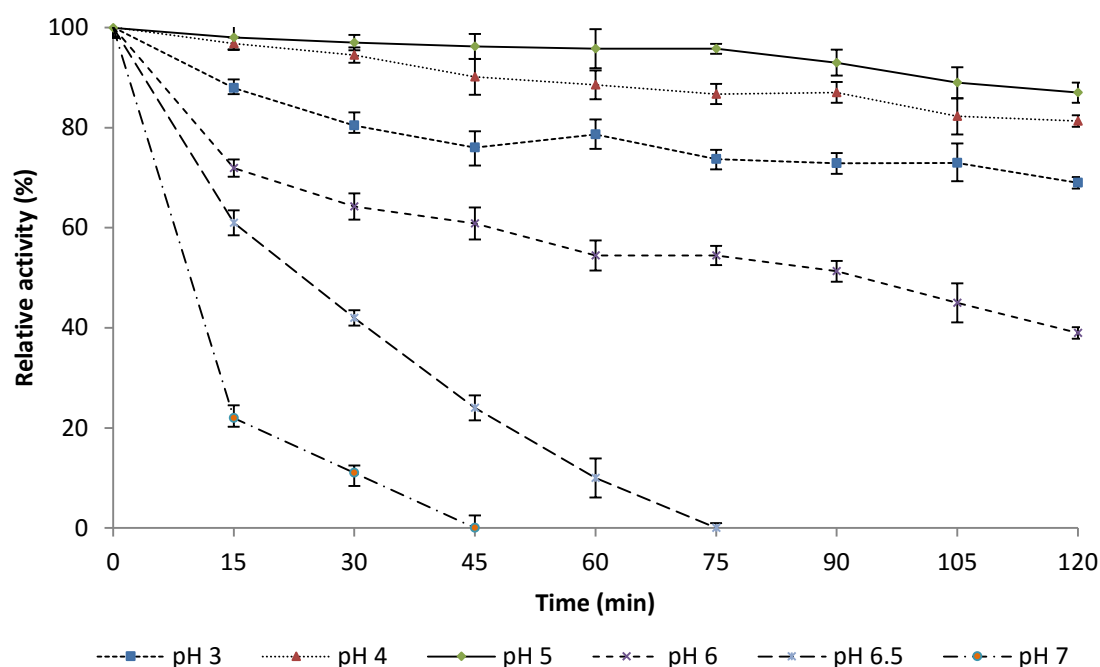


Figure 2.28: Alkaline stability of xylosidase expressed in *S. cerevisiae* at 50°C for 2 h. The remaining activity was expressed as percentage of original activity.

2.3.10.3 pH and Temperature Stability of Xylosidase from *P. pastoris*

Thermostability of xylosidase produced by *P. pastoris* was tested at pH 5 over a range of different temperatures. The enzyme retained 91% activity at 40°C and 89% activity at 50°C after 2 h. At optimum temperature, the enzyme maintained 55% of its total activity (Fig. 2.29), this was higher than the activity retained in *S. cerevisiae*. However at 70°C, the enzyme lost more than 50% of its activity after min but retained this activity for 45 minutes. Alkaline stability of xylosidase was tested at standard assay temperature over the pH range 3 – 7. The enzyme was most stable at pH 5, and retained 95% activity over 2 hours, similar to results obtained for *S. cerevisiae*. At pH 4, xylosidase retained 90% activity (Fig. 2.30). However, as seen with *S. cerevisiae* as well, at the more acidic pH's the enzyme lost activity. At pH 7, the activity dropped to 50% in 15 minutes and almost 30% in 30 minutes. Enzyme stability in both hosts showed similar results and maintained the same trend at acidic and neutral conditions. Nonetheless, the enzyme expressed in *P. pastoris* proved to be more resilient to pH and temperature changes than the enzyme expressed in *S. cerevisiae*.

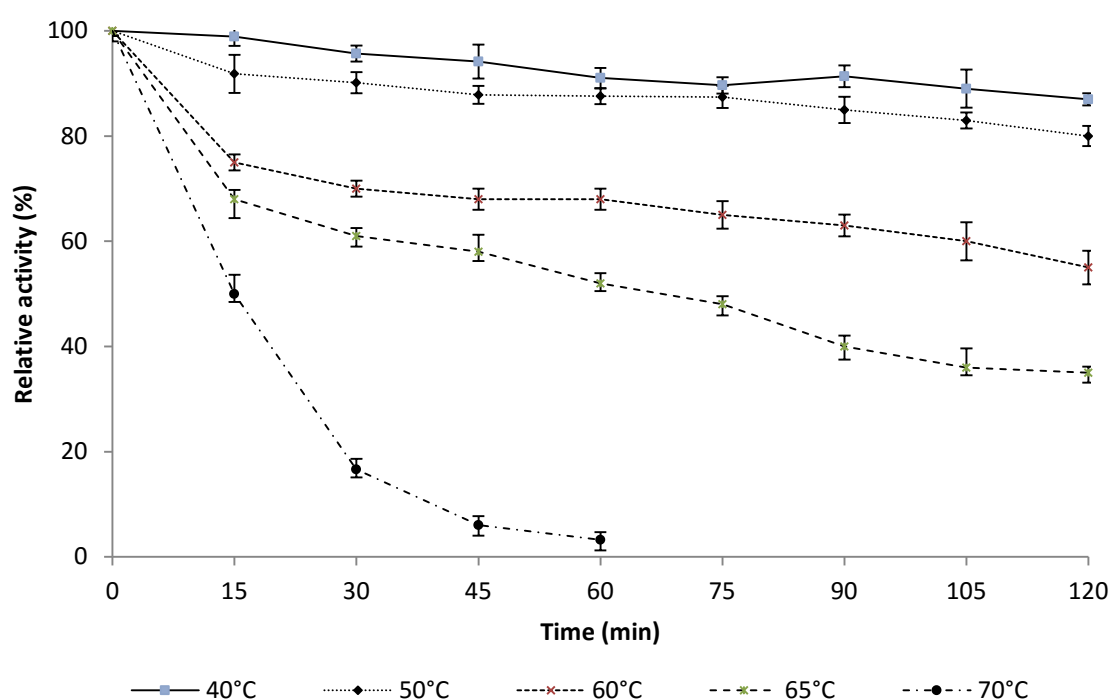


Figure 2.29: Thermostability of xylosidase expression in *P. pastoris* at pH 5. The remaining activity was expressed as percentage of original activity.

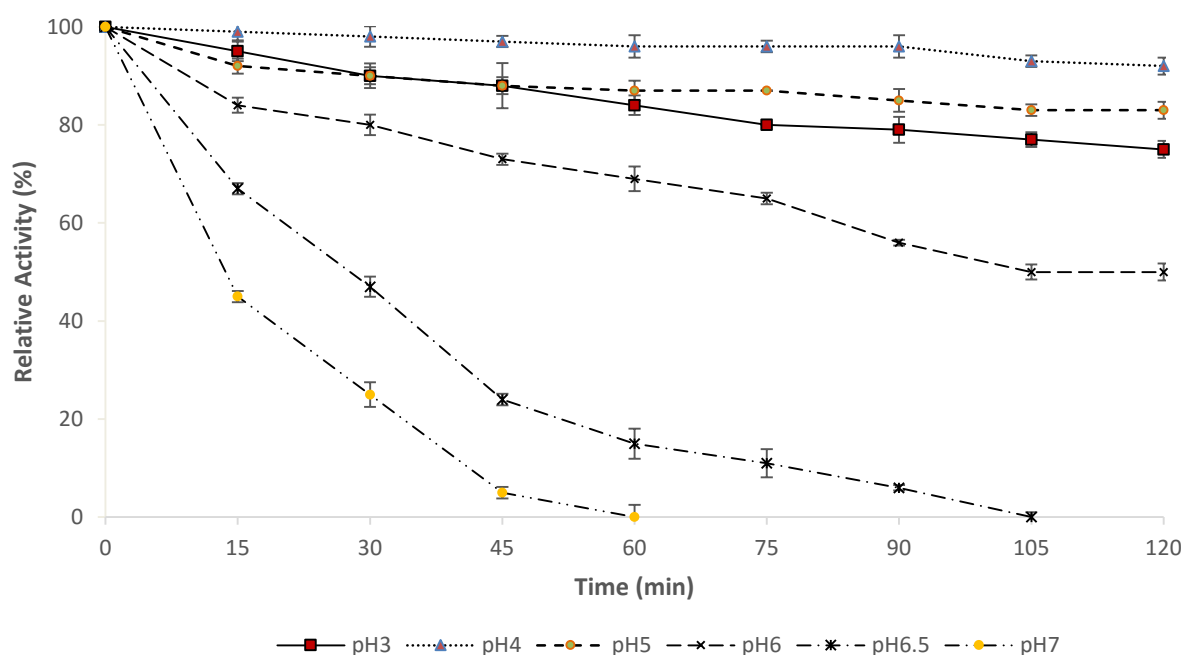


Figure 2.30: Alkaline stability of xylosidase expressed in *P. pastoris* at 50°C for 2 h. The remaining activity was expressed as percentage of original activity.

2.3.15 Analysis and Quantification of Enzymatic Hydrolysis Products by HPLC

Xylanolytic enzymes are known to work synergistically to hydrolyse xylan to xylose. Xylan degradation was assessed firstly using the xylosidase produced by *P. pastoris* and a fungal xylanase *xynA* from *T. lanuginosus* produced in the laboratory. Secondly, their analyses was done by using co-expressed enzymes present in the *P. pastoris* supernatant.

Degradation products released by enzymatic hydrolysis of birchwood xylan were analysed by HPLC. 1% xylotriose, xylobiose and xylose standards served as controls, and diluted aliquots were used to construct standard curves. The retention time for xylotriose, xylobiose and xylose were 10.1 min, 11.4 min, and 14.2 min, respectively (Fig. 2.31).

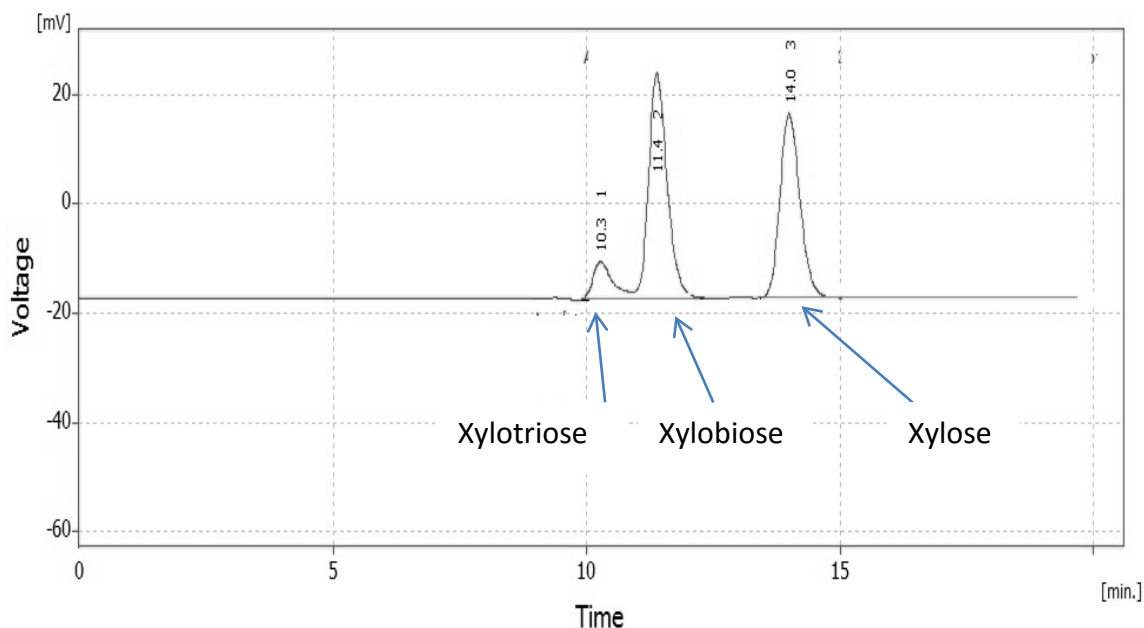


Figure 2.31: Chromatogram of 1% xylooligosaccharide standards.

Xylan degradation was analysed from the supernatants of strains producing single and co-produced xylanase and xylosidase enzymes. Xylan degradation from strains producing enzymes xylanase and xylosidase separately produced 7.2 g/l xylose from a 1% birchwood xylan after 48 hours (Fig. 2.32). A steady release of xylose was observed from 1-6 h and 12-36 h, and a more rapid release was observed for 6-12 h (Fig. 2.32). Hydrolysis of xylan by *P. pastoris* co-expressing enzymes produced 0.7 g/l xylose at 50°C after 30 minutes and 1.8 g/l xylose after 48 h from 1% birchwood xylan (Fig. 2.33). The co-expressed enzymes had an activity of 100 U/ml xylanase and 10 U/ml xylosidase as compared to the individual producing enzymes, with activities of 70 U/ml xylosidase and 136 U/ml xylanase.

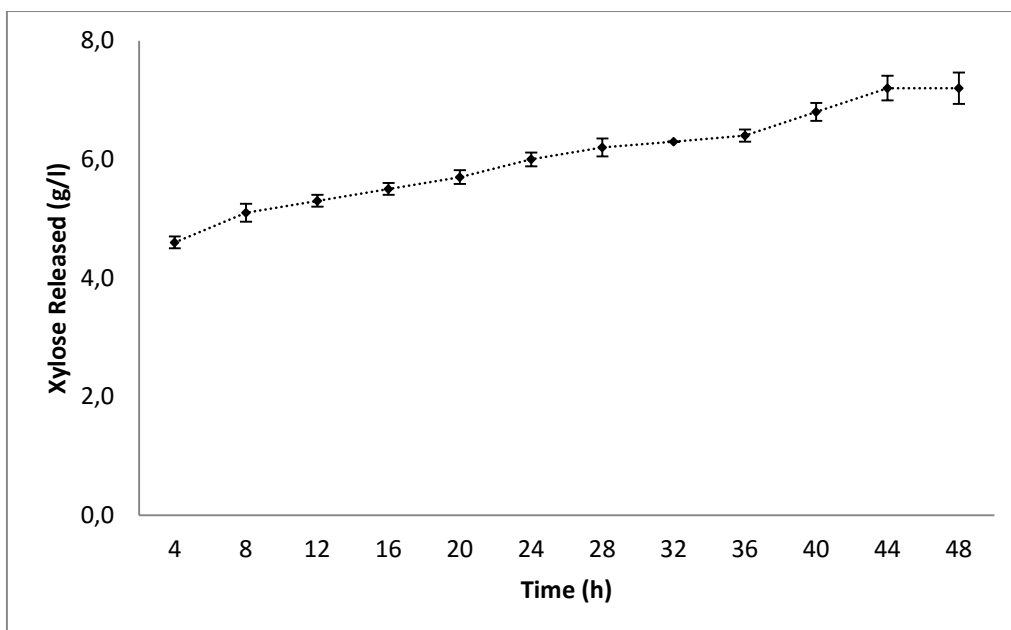


Figure 2.32: Xylan hydrolysis from 1% birchwood xylan with 100 U xylanase and 20 U xylosidase.

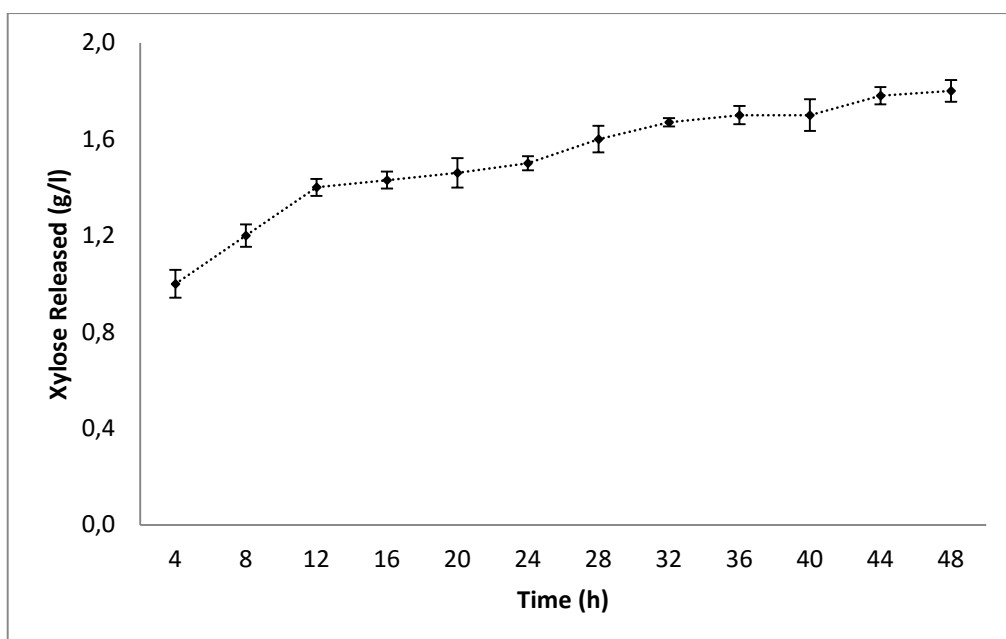


Figure 2.33: Xylan hydrolysis of 1% birchwood xylan using supernatant co-expressing xylanase and xylosidase. Supernatant contained 100 U xylanase and 10 U xylosidase.

2.3.16 Bioethanol Production by *P. stipitis*

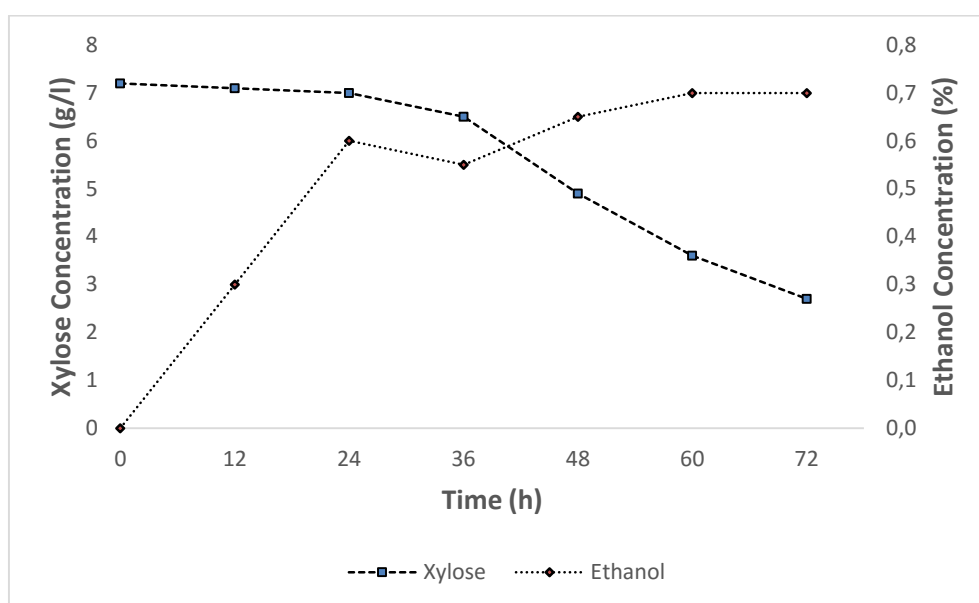


Figure 2.34: Conversion of xylose to bioethanol by *P. stipitis*.

Supernatant containing co-expressed xylanase and xylosidase was used to degrade 1% birchwood xylan at 50°C. Degradation of xylan was the highest in the first 30 minutes of incubation, after which the substrate was hydrolysed at a much slower rate. A maximum xylose production of 1.8 g/l was produced after 48 h (Fig. 2.33). The xylose contained in the supernatant was used as a carbon source for further studies and the supernatant was inoculated with *P. stipitis* to promote bioethanol production. The xylose contained in the medium was slowly converted to bioethanol (Fig. 2.34). Initial xylose concentration was 7 g/l. This decreased to 2.7 g/l after 72 h. The xylose produced was utilised by *P. stipitis* and converted to bioethanol. This conversion rate was the greatest in the first 24 h, producing 0.6% ethanol. As the xylose concentration decreased, bioethanol production increased. Bioethanol production peaked at 72 h producing 0.7% bioethanol, and continued to increase, however this data is not shown.

2.4 DISCUSSION

1,4 β -D-xylosidase is one of the enzymes from the microbial xylanolytic system essential for complete degradation of xylan (Jordan *et al.*, 2007). Conversion of xylan to monosaccharides can be accomplished by many hydrolytic enzymes-most commonly hemicellulases, a widespread group of glycoside hydrolases (Bohra, 2011). Xylanases attack the polysaccharide backbone by random hydrolysis and 1,4 β -D-xylosidase hydrolyses xylooligosaccharides to D-xylose.

The *xlnD* gene was originally isolated by La Grange *et al.* (2001) and cloned into plasmid pDGL55, under the control of the ADH2 promoter system and expressed in *S. cerevisiae* Y294. This construct reached maximum expression levels of 5.3 nkat/ml xylosidase. This expression was neither extracellular nor intracellular, but membrane-bound. Consequently, whole cells of *S. cerevisiae* were used in the enzyme assays. This construct was used as template DNA and restricted using *EcoRI* to release a 2700 bp fragment, containing the MF- α secretion signal (Fig. 2.8, Lane 2). This fragment was cloned into vector pJC1 (Fig. 2.8, Lane 3).

The *xlnD* gene was initially amplified by PCR and cloned into vector pTZ57/R (Fig. 2.9, Lane 3) and used as template DNA. pBGP1 was restricted using enzymes *EcoRI* and *XbaI* and pHIL-SI was restricted using enzymes *EcoRI* and *BamHI* (Fig. 2.9, Lane 7 and 10), respectively. Presumptive recombinants were analysed using the same restriction enzymes. Positive recombinant clone pBGP1-xlnD produced a maximum of 70 U/ml (Fig.2.21), while pHILSI-xlnD produced 0.5 U/ml after 72 h (Fig. 2.22). pBGP1-xlnD was expressed using the strong constitute promoter (GAP), while pHILSI-xlnD uses chromosomal expression, which can account for the activity obtained.

Although ligations can be done successfully, a screening procedure is crucial for the selection of positive clones, or the exercise can be cumbersome, and not succeed. A two-step screening procedure was used in this study. The first screen was using the antibiotic zeocin to screen for all transformants in *P. pastoris*. The second step entailed using 4-methylumbelliferyl- β -D-xyloside (MUX) to screen for xylosidase-producing clones. Xylosidase production was visualized under UV illumination as fluorescence due to hydrolysis of MUX to release 4-

methylumbelliferone (Fig. 2.15). The use of a substrate that xylosidase specifically degraded greatly simplified the screening procedure. The subsequent clones, pBGP1-*xlnD*, pHLS1-*xlnD* and pJC1-*xlnD* were transformed into their respective hosts and screened on selective plates containing MUX.

For *P. pastoris*, YPD-MUX plates were used containing the antibiotic zeocin. Zeocin was used to screen for clones that had taken up the plasmid. This marker could not be used solely for screening, as this marker could not discriminate between colonies that had the plasmid only, or colonies that had the plasmid with the xylosidase gene. Therefore, clones that contained an active xylosidase were identified with fluorescence, whilst colonies that had taken up the plasmid only did not fluoresce.

The same two-step screening was used for *S. cerevisiae*. This host strain was a URA auxotroph. Instead of the zeocin screen, these clones were screened on uracil-deficient media. The vector contained the complimentary *URA* gene, this was the primary screen for all positive transformants followed by the secondary screening using MUX to differentiate between colonies that had taken up the plasmid that contained the xylosidase gene, opposed to colonies that had taken up plasmid only. Comparison between these yeast clones revealed that clone pBGP1-*xlnD* expressed in *P. pastoris*, produced the most intense fluorescence, followed by pJC1-*xlnD* expressed in *S. cerevisiae*, and lastly pHLS1-*xlnD* expressed in *P. pastoris* (Fig. 2.15). This was later shown to correlate with expression levels of the enzyme (Fig. 2.21 – Fig. 2.24). This suggested that the MUX plate assay proved to be semi-quantitative in this study.

Enzyme expression experiments were carried out and showed a large difference in enzyme activities. *S. cerevisiae* clone pJC1-*xlnD* was compared to the control, pDLG55-*xlnD*, using glucose as a carbon source. The control produced 0.2 U/ml of cell-bound protein, whilst pJC1- produced 8 U/ml after 48 hours, approximately 40 times more than the control. This could be because the pDLG55 contains the ADH2 promoter. Since it is known that this promoter is repressed in the presence of glucose, the effect of different glucose concentration was tested on xylosidase activity (Fig. 2.20). After testing a range of concentrations (1%, 1.5% and 2%),

the highest xylosidase activity was obtained using 1% glucose, while 2% showed a higher level of repression on xylosidase activity, producing only 0.11 U/ml. pJC1-xlnD uses the *PGK1* promoter. This is a strong constitutive system that can express recombinant proteins at 4-10% of the total soluble proteins depending on culture conditions (Hitzeman *et al.*, 1982). These findings corresponds with work by Mchunu *et al.* (2009) expressing the xylanase gene NC38, using both promoter systems. The pDGL1 clone produced no xylanase in media containing glucose as a carbon source, even though restriction digestion proved the presence of a xylanase gene. But when glucose was substituted with galactose, xylanase activity was observed on the enzyme screening plate. In this study, the *S. cerevisiae* clone containing the pJC1 vector produced 13.4 U/ml of xylanase.

Shake flask cultivations were carried out in triplicate to compare expression levels of pBGP1-xlnD and pHLS1-xlnD expressed in *P. pastoris*. Clone pBGP1-xlnD was grown in YPD media supplemented with zeocin to maintain the plasmid and produced 70 U/ml of activity after 72 hours. This was more than 330-fold higher than the pDLG55 clone expressed in *Saccharomyces*. This can be attributed to the highly constitutive *GAP* promoter system. However, clone pHLS1-xlnD expressed only 0.5 U/ml after 96 h of induction with 0.5% methanol. This plasmid uses the *AOX1* promoter and therefore requires induction. The total activity obtained in *P. pastoris* reached levels of 70 U/ml and was much higher than in *S. cerevisiae*, where a maximum of 8 U/ml was obtained. This may be primarily due to the extracellular production of proteins in *P. pastoris*, whilst in *S. cerevisiae* the enzyme is cell-bound.

The pBGP1-xlnD clone expressed in *P. pastoris* was found to release the most xylosidase into the culture medium, with little activity found when intracellular fractions were tested. This was also validated by SDS-PAGE and zymogram analysis. The SDS-PAGE analysis displayed a band corresponding to 120 kDa. This showed a single protein band that fluoresced on the zymogram. This also served as confirmation of xylosidase activity. SDS-PAGE analysis of enzyme extracted from *S. cerevisiae* also indicated the presence of other intracellular proteins. This was expected since these cells were lysed to extract xylosidase, thereby making identification of the protein band more difficult.

The assumed molecular mass of the *A. niger* 90196 xylosidase was found to be 87.1 kDa (La Grange *et al.*, 2001). In comparison, β -xylosidases have been identified from other *Aspergillus* species. Knob and Carmona (2012), stated that most fungal xylosidases display a molecular mass above 100 kDa. Eneyskaya *et al.* (2003) reported a 250 kDa protein consisting of two identical subunits (125 kDa) from culture filtrate, whilst *A. niger*, showed a molecular mass of 253 kDa according to the gel filtration data and 122 kDa according to the data from SDS-PAGE electrophoresis (Rodionova *et al.*, 1983). Xylosidase enzyme from the yeast-like fungus *Aureobasidium pullulans* with a molecular mass of 224 kDa separates in to two subunits of equal molecular mass (Dobberstein and Emeis, 1991), whilst characterized a thermostable bacterial β -xylosidase from *Thermomonospora fusca*. This protein was an estimated 168 kDa using gel filtration chromatography. However, comprised of 3 subunits when analysed on a denaturing gel.

According to La Grange *et al.* (2001), the xylosidase used in this study had a theoretical molecular mass of 86 kDa. This was not confirmed on a denaturing gel. Results indicated by Eneyskaya *et al.* (2003), Rodionova *et al.* (1983) and Bachmann and McCarthy (1989), confirm variation between gel filtration data and data obtained from SDS-PAGE gels indicating that gel filtration results are more accurate. However both methods produce molecular weights that are higher than the theoretical molecular weight, calculated from the nucleotide sequence. This is due to the glycosylation and hypoglycosylation of proteins during post translational modification. Unlike bacterial expression systems, *P. pastoris* has the ability to perform post-translational modifications, e.g. correct folding, *O*- and *N*- linked glycosylation and processing of signal sequences (Macauley-Patrick *et al.*, 2005). The molecular mass of the recombinant enzyme on SDS gels, was estimated between 120 and 130 kDa (Fig. 2.18) and was much higher than the predicted molecular weight of 87.1 kDa (La Grange *et al.*, 2001). When analysed on NetOGly 4.0, 12 potential O-glycosylation sites were predicted for β -xylosidase. Also, SDS is known to be inaccurate for size estimates of glycosylated proteins, due to the non-uniform binding of SDS to amino acids.

Throughout the characterization procedures, plasmid pDLG55 containing the xylosidase gene expressed in *S. cerevisiae* was used as a control for comparison between hosts and promoter

systems. The pBGP1-xlnD clone produced a maximum of 70 U/ml in *P. pastoris* and the pHIL-S1-xlnD clone produced 0.5 U/ml after methanol induction. The alkaline and temperature stabilities were determined from pH 3 - 7 and 30°C - 70°C. The *S. cerevisiae* pJC1-xlnD clone produced a maximum of 8 U/ml of membrane bound protein. The pH optima (Fig. 2.25) and temperature optima (Fig. 2.26) was observed for both expression hosts. The optimum pH was found to be pH 4 and optimum temperature was 60°C. These results corresponded to the results obtained by La Grange *et al.* (2001) using pDLG55 expressed in *S. cerevisiae*, where enzyme activity peaked between pH 3-5 with highest activity obtained at pH 3.2 at a temperature of 60°C. The optima results obtained seem to be a characteristic of the enzyme and not the expression host.

Herrmann *et al.* (1997) purified a xylosidase from *Trichoderma reesei* and found its optimal pH to be 4 and temperature to be 60°C. *Aureobasidium pullulans* xylosidase activity was optimum at pH 4.5, however showed a higher temperature optima of 80°C as noted by Dobberstein and Emeis (1991). Basaran and Ozcan (2008) noted the xylosidase activity of *Pichia stipitis* mutant NP54376 to be optimal between pH 4.8 and 5.0, with optimum activity at 45°C and a maximum activity of 0.018 U/ml. The β -xylosidase isolated from *A. niger* 15 by Rodionova *et al.* (1983), exhibited a temperature optima of 70°C and the pH optimum was 3.8 - 4.0. Even though these results were obtained from natural xylosidase producers, none produced enzyme levels close to the 70 U/ml produced in this study.

Thermostability characteristics were carried out at optimum pH 4, at different temperatures for two hours. At temperatures of 30°C, 40°C and 50°C, the enzyme retained 90% activity for 2 hours in both *S. cerevisiae* and *P. pastoris*. At 60°C, the enzyme retained 60% activity in *P. pastoris* but retained only 40% in *S. cerevisiae*. The enzyme was more active at higher temperatures in *P. pastoris* (Fig. 2.29) than *S. cerevisiae* (Fig. 2.27). In addition, it lost 60% activity in 15 minutes at 65°C and lost almost all activity at 70°C. However, in *Pichia*, the enzyme retained 40% activity after 2 hours and lost 50% activity after 15 minutes at 70°C and 90% after 30 minutes. Bachmann and McCarthy (1989) characterized a bacterial xylosidase and noted that as the assay temperature was increased above 60°C, enzyme activity decreased and at 70 °C activity was approximately 50% of the maximum.

Alkaline stabilities were carried out at the optimum temperature of 60°C at different pH values. The pH stabilities also varied in both hosts. The enzyme retained 90% activity at pH 3 and 4 and 5 in *P. pastoris* for 2 hours (Fig. 2.30) but only retained these activities at pH 4 and 5 in *S. cerevisiae* (Fig. 2.28). At pH 5, the enzyme retained 76% activity. At pH 5 and 6 the enzyme still retained 50% activity after 15 minutes, compared to *S. cerevisiae* where the enzyme lost 80% activity in 15 minutes at pH 7. The *A. niger* xylosidase isolated by Rodionova *et al.* (1983) was stable at pH 3 to 8 and did not lose its activity for 1 h at temperatures up to 50°C. Some of the stability characteristics can be attributed to the enzyme folding in *P. pastoris*, which is remarkably different than in *S. cerevisiae*.

T. lanuginosus DSM 5826 is known to produce a high level of cellulase-free, thermostable xylanase. The *xynA* gene isolated from *T. lanuginosus* by was modified by directed enzyme evolution and two variants were obtained, G41 and G53 (Stephens, 2007). Thermostable variant G41 retained 75% activity at 80°C, for 90 minutes. DNA shuffling technology such as StEP was used to combine these properties into a single robust xylanase, thermo and alkali stable gene, S340 (Stephens *et al.*, 2014). The S340 gene was cloned and expressed in *P. pastoris*. Xylanase activity obtained from this clone was 136 U/ml. In order to constitutively co-express this protein together with xylosidase, primers were designed to amplify the GAP promoter and terminator region (1200 bp) together with the 786 bp S340 gene, (Fig. 12, lane 2). This fragment was initially cloned to vector pTZ57/R (Fig. 2.13, Lane 3) and then to pBGP-xlnD using restriction enzyme *Bam*HI (Fig. 2.14, Lane 6). Restriction analysis of this produced a 1900 bp fragment (Fig. 2.14. Lane 7). This recombinant clone was expressed in *P. pastoris*. Xylosidase activity was qualitatively confirmed based on their ability to hydrolyse MUX. pBGP-xlnD, pBGP-S340 and pBGP-xlnD-S340 was inoculated on selective plates containing MUX. pBGP-xlnD and pBGP-xlnD-S340 produced fluorescence, indicating xylosidase activity (Fig. 2.16). Xylanase activity similarly was determined using RBB-xylan as a substrate. Positive xylanase producers were identified by a halo surrounding the colony due to hydrolysis of the RBB-xylan complex. pBGP-xlnD, pBGP-S340 and pBGP-xlnD-S340 were inoculated on selective plates containing RBB-xylan. pBGP-S340 and pBGP-xlnD-S340 produced halos around the colony (Fig. 2.17), indicating a positive xylanase product. Positive xylanase and xylosidase activity indicated successful co-expression of both genes.

The *T. lanuginosus* xylanase expressed 136 U/ml of extracellular activity, but only produced 100 U/ml when co-expressed. The xylosidase activity also decreased to 10 U/ml compared to 70 U/ml when expressed alone. This showed a similar trend with results obtained by La Grange *et al.* (2001) using the same xylosidase. Xylanase activity decreased from 1577 nkat/ml to 860 nkat/ml when expressed unaided and co expressed with xylosidase. The xylosidase activity followed suit in *S. cerevisiae*, with a decline from 5.3 nkat/ml of activity to 3.5 nkat/ml activity when co-expressed with xylanase. This however differs from results obtained by Den Haan and Van Zyl (2003) under the control of the constitutive *P. stipitis* TKL promoter, and co-expressed with *Trichoderma reesei* (*xyn2*) and *Aspergillus kawachii* (*xynC*). Xylanase and xylosidase activities were maintained when measured independently and when co-expressed.

S. cerevisiae has been used extensively in industry for the production of ethanol, because of its ability to produce high concentration of ethanol and inherent ethanol tolerance. However the main disadvantage is that it cannot utilise or ferment D-xylose. Therefore a focus has been on adapting the xylose metabolic pathway from xylose-utilizing yeasts such as *P. stipitis*. These have been shown to rapidly ferment xylose to high ethanol yields.

This chapter focused on the comparison of xylosidase production using inducible and the constitutive systems for maximum production. The findings in this chapter highlight xylosidase production caused by expression in different hosts, and different promoter systems. When comparing expression systems, the constitutive expression system used in both hosts was found to be more suitable than the inductive system, which is largely dependent on the carbon source. *P. pastoris* rapid growth at high yield on inexpensive media, and secretion of heterologous protein into culture broth has made it an excellent host compared to *S. cerevisiae*. Industrial applications of recombinant proteins depends greatly on expression levels and ease of production as these factors determine availability and cost of the protein. The expression levels in *P. pastoris* can therefore be further increased by high cell density fermentations, which can increase production exponentially since biomass levels are directly proportional to expression levels.

3.1 INTRODUCTION

Protein engineering in biotechnology over the years has generated novel proteins with enhanced functions. However, protein engineering has proven to be, to some extent, laborious and challenging. By understanding the evolutionary structure of proteins, scientists can strategically alter proteins and enhance functionality (Socha and Tokuriki, 2013). The directed evolution of genes has become one of the most powerful tools in protein engineering, with the key motivation being the desire to turn enzymes into practical tools (Eriksen *et al.*, 2014).

Enzymes have the ability to carry out complex chemical reactions under mild conditions with little or no by-product formation. These enzymes comprise of linear chains of amino acids that fold into unique 3-dimensional structures (Fig. 3.1). The amino acid sequence determines how the enzyme functions and this sequence can be altered by protein engineering.

The use of enzymes as biocatalysts in the generation of pharmaceuticals, chemicals and biofuels has increased the need for suitably improved and evolved enzymes (Cobb *et al.*, 2013; Goldsmith and Tawfik, 2012). The goal of a directed evolution experiment is to identify clones with enhanced functionality by construction of large gene libraries. A good mutant library is arguably the most important component in a directed evolution exercise. Ideal requirements for creating a mutant gene library are highlighted in Table 3.1.

One of the tools used for generating genetic diversity, is random mutagenesis, which involves altering the DNA sequence coding for different proteins. UV irradiation, chemical mutagenesis, saturation mutagenesis and error-prone PCRs (epPCR), are often used to introduce mutations via random mutagenesis (Cadwell and Joyce, 1992). epPCR allows for quick and easy gene library creation, by altering *Taq* polymerase activity, imbalanced nucleotide concentrations, and with the use of commercial mutagenesis kits.

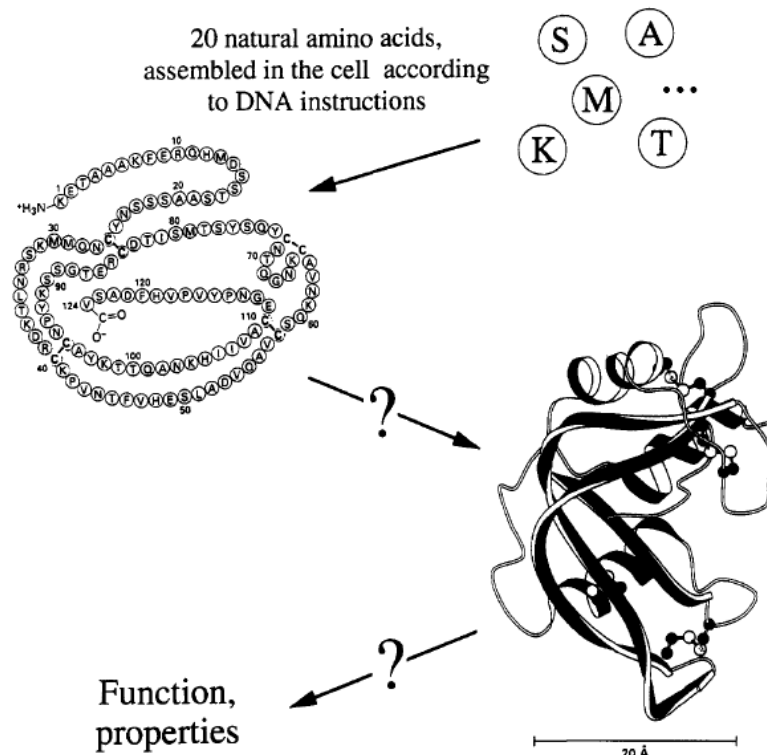


Figure 3.1: Protein engineering involves the manipulation of protein structures and functions at the level of the amino acid sequence. Significant gaps in the relationship between structure and function limit the ability to 'rationally design' new functions. (Arnold, 1996).

Table 3.1: Five requirements of an ideal gene library for directed evolution (Tee and Wong, 2013)

Requirements	Implication for genetic diversity creation methods	Implications for screening
1. Complex library to contain rare beneficial mutations	No/minimal wildtype sequence No/minimal mutational bias Consecutive nucleotide substitutions or codon-based mutagenesis	Smaller library to screen
2. Encode for mostly functional and properly folded proteins	Moderate mutation rate No/minimal prematurely truncated genes due to introduction of stop codons or frameshift mutations (<i>e.g.</i> , insertion, deletion) No/minimal structurally-disrupting mutations (<i>e.g.</i> , introducing Gly/Pro in helix)	Smaller library to screen
3. Contains mostly unique gene sequences with none or minimal genotype duplication	No/minimal mutational hotspots or preferential sites Mutations randomly distributed across the entire gene	Avoid screening identical clones (<i>i.e.</i> , more effective screening)
4. Possibility of populating certain amino acid substitutions depending on property to be evolved	Adjustable mutational spectrum	Smaller library to screen
5. Easy and cost-effective preparation	Minimal DNA manipulation No expensive kits/enzymes/chemicals Minimal number of oligonucleotides required Library can be created within a shorter time frame	Screening can commence sooner

There are limits to how much can be achieved with molecular evolution and it is vital to establish the potential and limitations of the term “directed molecular evolution”. The key to enzyme engineering is to understand their adaptive mechanisms and ability to function in different environments. Difficulties arise during cloning and screening of these libraries (Pai *et al.*, 2012), even though a myriad of selection methods have been developed, interpreting the differences in sequence, structure, function and thermodynamic properties and assigning specific sequence changes to particular enzyme behaviours becomes problematic. Despite

the intense research into the fundamentals surrounding protein function and folding, the relationship between sequence and structure, and structure and function are not fully understood (Arnold, 1996). Enzyme engineering is undergoing profound transformations and promises unprecedented application and scope of modified enzymes with desired properties. However this conventional approach requires confirmation of the mutation by sequencing, and can be tedious and expensive (Chen *et al.*, 2007).

By processes of random mutagenesis, recombination and selection enzymes are able to take on new functions and diverse capabilities to catalyse different reactions. Advancement of molecular biology has made it possible to carry out evolution of proteins with functions not found in nature. This has also made possible for enzymes to function optimally to defined conditions rather than the conditions of the host organism from which it evolved or evolution to carry out functions present in living organism. (Arnold, 1996).

In this chapter, random mutagenesis using mutagenic conditions via epPCR was used to improve the biochemical characteristics of the fungal xylosidase gene xlnD. Mutagenesis conditions were chosen based on previous work by Stephens *et al.* (2007). Clones exhibiting higher activity than the control were analysed via DNA sequencing and compared to the wild-type for changes in the nucleotide sequence.

3.2 MATERIALS AND METHODS

3.2.1. Plasmid, Media and Growth Conditions

The plasmid used in this study, pDLG55 (Fig. 3.2), containing the *A. niger xlnD* gene was obtained from W. H. van Zyl, Stellenbosch University, *E. coli* XL1 Blue cultures (Stratagene) were cultivated on Luria-Bertani medium, supplemented with ampicillin (100 µg/ml) for plasmid selection at 37°C. *S. cerevisiae* Y294 cultures were cultivated on either YPD medium (1 g/l yeast extract, 2 g/l peptone, 1 g/l or 2 g/l glucose) or selective synthetic complete (SC) medium (containing 1 g/l glucose, 2 g/l drop-out amino acid without uracil [US Biological] and 6.7 g/l yeast nitrogen base without amino acids [Difco]) containing 15% agar.

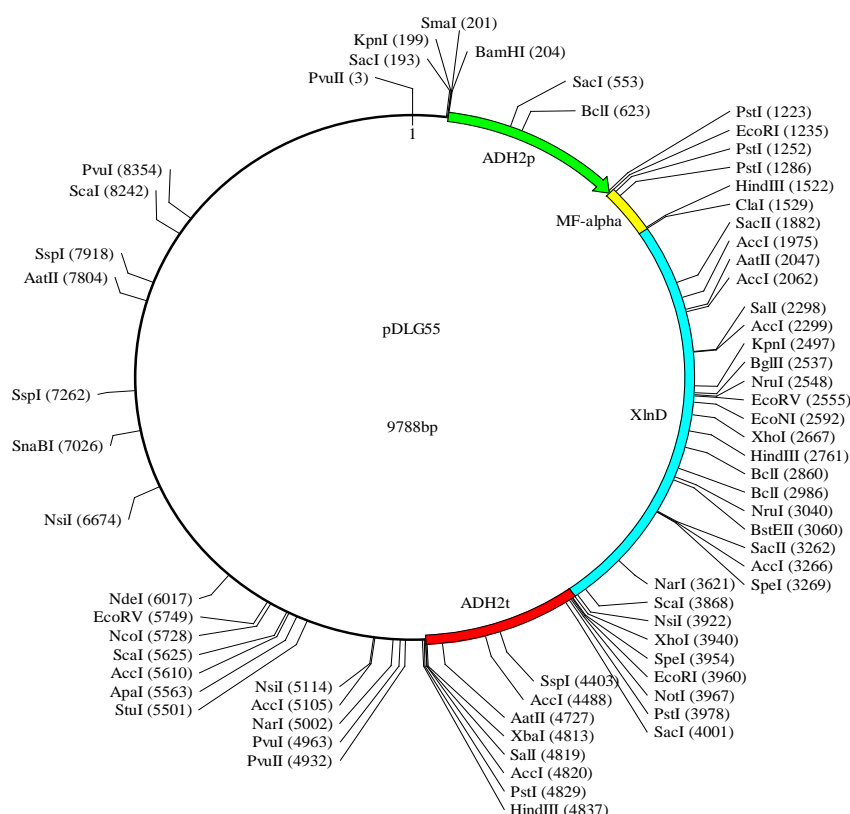


Figure 3.2: Map of pDLG55 showing the location of the ampicillin resistance (Amp^R) gene, uracil selectable marker (URA3), ADH2 promoter and terminator cassette (ADH2_p , ADH2_t), secretion signal (MF-alpha) and *xlnD* gene.

3.2.2. Plasmid DNA Isolation

A modified version of the alkaline lysis method of Birnboim and Doly (1979) was used to isolate plasmid DNA, as detailed in section 2.2.3. *E. coli* clones were inoculated in 5 ml LB medium containing 100 µg/ml ampicillin and grown for 12 - 16 h at 37°C. Cells were harvested by centrifugation at 4000× *g* for 5 min and plasmid DNA was isolated. For applications like ep-PCR and DNA sequencing, where DNA of a higher purity was necessary, the GeneJet Plasmid Mini Kit (Thermo Scientific) was used, according to the manufacturer's instructions.

3.2.3 DNA Quantification

DNA was analyzed as described in section 2.2.4, to determine concentration and assess purity.

3.2.4 Agarose Gel Electrophoresis

Cloning reactants and enzyme-digested samples were analyzed on 0.8% agarose gels, as described in section 2.2.5

3.2.5 PCR Amplification of *xlnD*

pDLG55 containing the *xlnD* fragment was amplified using primers xlnDR and xlnDF (Table 3.2), as described in section 2.2.6, to yield an approximately 2700 bp fragment. The cycling conditions were slightly different from the conditions used in 2.2.6, in that the extension time used was 2.5 minutes

Table 3.2: Primers used for amplification of *xlnD* from pDLG55

Forward Primer xlnDF:	5' GTCT ATCGAT ACACCAGCTATGTCGATTAC 3'
Reverse Primer xlnDR:	5' CGACCTGCAGG GCGCCG CGAATTCAC 3'

**Restriction endonuclease recognition sites are indicated in bold (ATCGAT – ClaI, GCGGCCGC – NotI)*

3.2.6 Error-Prone PCR

Error-prone PCR was carried out under conditions that induced an increase in the error rate. This involved performing the PCR under conditions that reduced the fidelity of nucleotide incorporation. Such conditions include the introduction of varying Mn²⁺ concentration of Mn²⁺. Mn²⁺ concentrations between 0.1 mM and 1.5 mM were tested (Table 3.3). Conditions

A to D contained increasing amounts of Mn^{2+} while the dNTP concentration remained the same. Condition E and F contained the same concentration of Mn^{2+} and varying concentration of AT and CG. Condition G contained a higher concentration of Mn^{2+} (0.5 mM).

Table 3.3: Mutagenic PCR conditions for *xlnD* amplification

Condition	Concentration (mM)		
	Mg^{2+}	Mn^{2+}	dNTPs
A	1.5	0.1	0.1
B	1.5	0.5	0.1
C	1.5	1.0	0.1
D	1.5	1.5	0.1
E	1.5	0.1	0.2 CT 0.8 AG
F	1.5	0.1	0.8 CT 0.2 AG
G	1.5	0.5	0.2 CT 0.8 AG

3.2.7 Restriction Analysis

Plasmid DNA was incubated with suitable restriction enzymes (Thermo Scientific) at 37°C as per the manufacturer's instruction. Restricted DNA was then analysed on 0.8% agarose gels. Two types of restriction analyses was carried out. Firstly, pDLG55 was linearized with *ClaI* and *NotI* to facilitate ligation with the similarly-digested PCR products after random mutagenesis. Secondly, the recombinant clones were restricted using the same restriction enzymes to confirm successful ligation.

DNA fragments were purified using either the DNA Clean and Concentrator™-100 (Zymo Research) or the QIAquick Gel Extraction Purification Kit (Qiagen), according to the

manufacturer's instructions. An aliquot of 5 µl was examined on agarose gels to visualize DNA after purification.

3.2.8 Ligation

Ligation reactions were carried out at an equimolar ratio to increase ligation efficiency as described in section 2.2.9.

3.2.9 Preparation of *E. coli* Electrocompetent Cells

Prior to transformation, bacterial cells were made electrocompetent. A single colony *E. coli* of XL1 Blue cells was grown in 5 ml LB broth shaken overnight at 37°C. Cells were made electrocompetent as described in section 2.2.10 and stored at -80°C (Ausubel, 2002).

3.2.10 Transformation and Screening in *E. coli*

After ligation, 1 µl of the reaction mixtures were used to transform 50 µl electrocompetent *E. coli* XL1 Blue cells as described in section 2.2.11. 100 µl of the transformation mixture was plated out on LB agar plates (containing 100 µg/ml ampicillin). Colonies that grew on the plates were considered resistant to the antibiotic and could contain recombinant plasmids.

3.2.11 Preparation of *S. cerevisiae* Electrocompetent cells

Yeast host cells were made competent by treating with DTT and stabilizing in sorbitol as described in section 2.2.12.

3.2.12 Transformation and Screening in *S. cerevisiae*

Electroporation of *S. cerevisiae* was carried out using the GenePulser Xcell (BioRad) as described in section 2.2.13. After transformation, cells were plated onto uracil-deficient media (SC) and incubated at 30°C for 3-4 days.

3.2.13 Plate Screening for β -xylosidase Activity

β -xylosidase screening was carried out on synthetic complete (SC) medium. MUX was spread on the surface of agar plates as described in section 2.2.14 and the fluorescence was visualised under UV illumination.

3.2.14 β -xylosidase Enzyme Activity Assays

β -xylosidase activity was determined in triplicate as described in section 2.2.16.1 at 50°C in 50 mM sodium citrate buffer at pH 5, for 5 min. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μ mol of *p*-nitrophenol per minute at 50°C.

3.2.15 DNA Sequencing

DNA was isolated (2.2.3) from clones exhibiting differences and sent to Inqaba Biotec for sequencing. DNA sequencing data was processed using the DNAMAN software package (Lynon BioSoft). The DNA sequences were then translated to protein sequences and compared to the wild-type.

3.3 RESULTS

3.3.1 PCR Amplification of *xlnD*

pDLG55 containing the *xlnD* gene was isolated as described in section 2.2.3 and amplified by PCR using primers *xlnDR* and *xlnDF*. A small aliquot of the PCR was analysed by electrophoresis to determine if the gene was successfully amplified. A single discrete band of approximately 2700 bp was observed (Fig. 3.3). The intensity of the band showed that the gene was amplified to high enough concentrations for subsequent procedures.

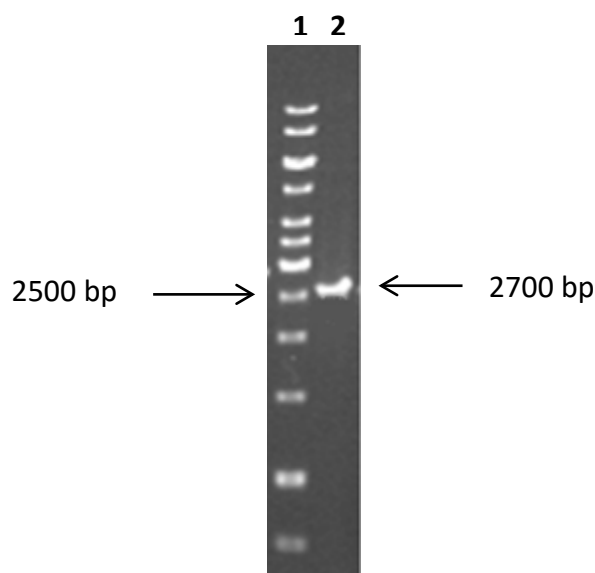


Figure 3.3: Amplification of *xlnD*. Lane 1: GeneRuler 1 kb DNA. Lane 2: *xlnD* PCR product.

3.3.2 Error-prone PCR of *xlnD*

Error-prone PCR was carried out under conditions that induced an increased error rate. The *xlnD* gene was originally isolated by La Grange *et al.* (2001) and cloned to pDLG55. The pDLG55 plasmid containing the *xlnD* gene was isolated as described in section 2.2.3 and subjected to ep-PCR using primers *xlnDR* and *xlnDF*. A small aliquot of each PCR product corresponding to the mutagenesis conditions tested were analysed by electrophoresis to determine if the gene was successfully amplified. A single discrete band of 2700 bp was obtained for all conditions (Fig. 3.4). Lanes 3, 4, 5 and 6 were mutagenic conditions using different

concentration of Mn^{2+} ranging from 0.1 mM to 1.5 mM. The control reaction in lane 2 was carried out under normal PCR conditions and does not contain Mn^{2+} . The intensity of this band, was much brighter than the other bands since no Mn^{2+} was added to the reaction, allowing *Taq* polymerase to function normally. As the Mn^{2+} concentration increased from 0.1 mM to 1.5 mM (Fig. 3.4, Lane 3-6) the intensity of the bands decreased. The varying Mn^{2+} concentration used in conditions A-D hindered the activity of *Taq* polymerase resulting in fewer amplification products.

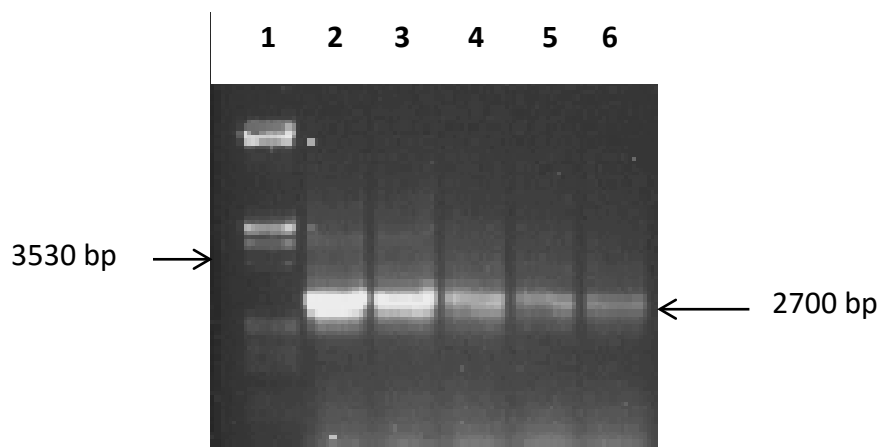


Figure 3.4: Error-prone PCR products of *xlnD* using varying concentrations of Mn^{2+} . Lane 1: λ DNA molecular weight marker restricted with *EcoRI* and *HindIII*. Lane 2: Control amplified under normal PCR conditions. Lane 3: Condition A (0.1 mM Mn^{2+}). Lane 4: Condition B (0.5 mM Mn^{2+}). Lane 5: Condition C (1.0 mM Mn^{2+}) Lane 6: Condition D (1.5 mM Mn^{2+}).

Different concentrations of dATP, dTTP, dCTP and dGTP were employed in conditions E-G. The nucleotide concentration varied in conditions E and F while the manganese concentration remained the same at 0.1 mM. This manganese concentration was chosen since it effected the fidelity of *Taq* the least (Fig. 3.4, Lane 3). Condition G differed by using the same nucleotide concentration as condition E, but increased manganese from 0.1 mM to 0.5 mM. Altering the nucleotide concentration affected the amplification of *xlnD* by *Taq* polymerase. An altered nucleotide concentration allowed for mis-incorporation at a higher frequency. The decreased nucleotide concentration resulted in unavailability of the nucleotides and therefore mis-incorporation.

These mutagenic conditions showed a difference in band intensity in comparison to the control (Fig. 3.4, lane 2). The band observed in lane 2 was amplified under normal PCR conditions and contained no Mn^{2+} . This band had the highest intensity when compared to the other bands. PCR condition E and F (Fig. 3.5, Lane 3 and 4) were similar but had varying concentrations of nucleotide. These conditions differed from conditions A-D which only varied by the Mn^{2+} concentration. PCR reaction F and G used the same nucleotide concentrations but differed by Mn^{2+} concentrations. The band intensity for the fragment obtained by reaction G (Fig. 3. Lane 5) is much lighter than the other reactions indicating that the increase in Mn^{2+} hampered the fidelity of *Taq* further resulting in decreased amplification. This was because condition G contained a higher concentration of Mn^{2+} and was further hampered altered nucleotide concentrations.

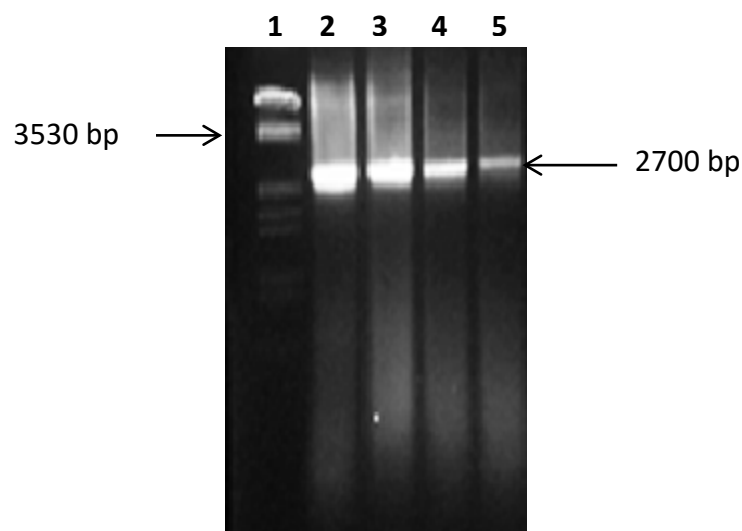


Figure 3.5: Error-prone PCR of *xlnD* using altered concentrations of nucleotides and Mn^{2+} . Lane 1: λ DNA molecular weight marker restricted with *EcoRI* and *HindIII*. Lane 2: Control amplified under normal PCR conditions. Lane 3: Condition E (1.5 Mg^{2+} , 0.1 Mn^{2+} , 0.2 mM CT, 0.8 mM AG) Lane 4: Condition F. (1.5 Mg^{2+} , 0.1 Mn^{2+} , 0.8 mM CT and 0.2 AG). Lane 5: Condition G (1.5 Mg^{2+} , 0.5 Mn^{2+} , 0.2 mM CT and 0.8 mM AG).

3.3.3 Ligation and Restriction Analysis of Clones

The purified PCR products obtained in section 3.3.2 for all mutagenic conditions were ligated to purified vector pDLG55. Two thousand seven hundred and fifty clones were isolated and analysed for the presence of a xylosidase gene. Positive transformants were identified by restriction analysis using *Clal* and *NotI* to confirm the presence of a 2700 bp fragment (Fig. 3.6). Of the 2750 clones, 2540 isolates contained the *xlnD* gene after restriction analysis. The restriction digestion produced a 7000 bp vector band and a 2700 bp band corresponding to *xlnD*.

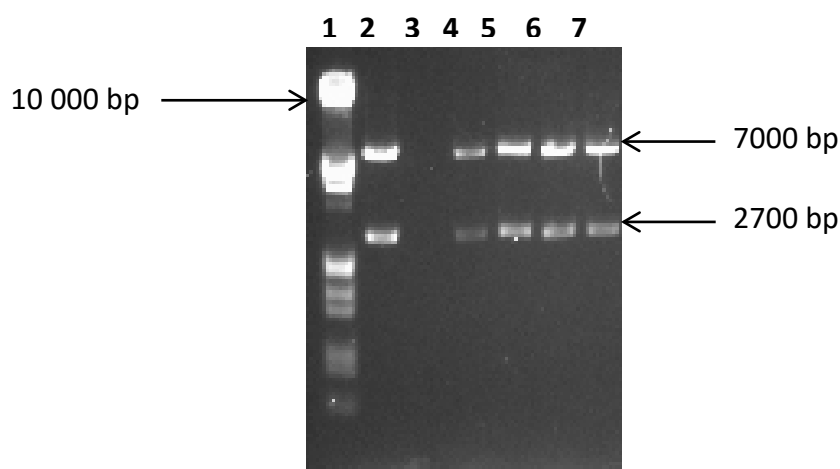


Figure 3.6: Restriction analysis of *xlnD* clones obtained by restriction digestion with *Clal* and *NotI*. Lane 1: λ DNA molecular weight marker restricted with *EcoRI* and *HindIII*. Lane 2: Positive control pDLG55 restricted with *Clal* and *NotI*. Lane 3: Control. Lane 4-7: Presumptive clones restricted with *Clal* and *NotI*.

3.3.4 Screening for Xylosidase Production

Plasmid DNA from 2540 transformants were transformed into electrocompetent *S cerevisiae* Y294. pDGL55 is a shuttle plasmid and was used for screening in both yeast and bacteria. Cells that were transformed with the plasmid in *E. coli* were identified by their ability to grow in the presence of ampicillin. However, this was not an indication of xylosidase activity. Therefore, the two thousand five hundred and forty isolates were transformed into *S. cerevisiae* and screened on uracil-deficient plates. Of the 2540 isolates transformed, 96% displayed xylosidase activity (Fig. 3.7).

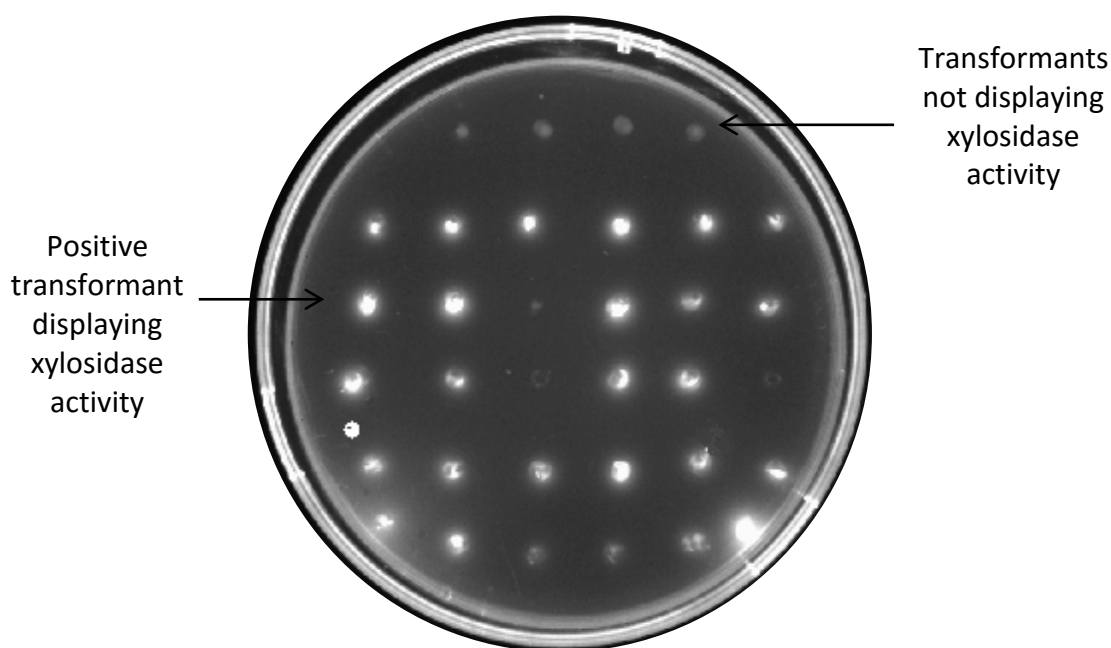


Figure 3.7: Screening plate showing positive and negative xylosidase expression in *S. cerevisiae* transformants after ep-PCR. Transformants were spotted on a uracil-deficient plate supplemented with MUX and viewed under UV light.

3.3.5 Xylosidase Expression and Extraction

Of the two thousand five hundred and forty isolates screened on MUX plates, 96% displayed fluorescence under UV illumination. Fluorescence is an indication of the functionality of the xylosidase gene. After UV analysis each positive clone was grown, and supernatant assayed for level of expression. An increase and decrease in xylosidase activity was noticed. 29% of the recombinants tested had a 2-fold increase in xylosidase activity. 54% remained the same and Clone 152 (Table 3.4) displayed an activity of 0.85 U/ml (14.2 nkat/ml). This was the highest activity obtained from all screened isolates. This activity was higher than the control which produced 0.20 U/ml (3.5 nkat/ml) xylosidase. Clones that showed an increase in xylosidase activity were tested in triplicate and then subjected to stability analysis at the optimum pH and temperature.

Table 3.4: Xylosidase activity obtained from selected recombinants

Clone	Activity (nkat/ml)
pDLG55 (control)	3.5
1	8.1
2	7.6
45	10.3
57	2.1
152	14.2
987	1.9
1156	2.8
1659	12.1
2115	0.6

3.3.6 Thermostability of Xylosidase from *S. cerevisiae*

20 recombinant clones displaying an increase in activity were assayed for thermostability and pH stability, however only variant 152 showed a noticeable difference. Thermostability of clone 152 was tested at pH 5 at 50°C (Fig. 3.10) and 60°C (Fig. 3.11) and was compared to the wild-type. At 50°C the control retained 86% activity while variant 152 retained 80% (Fig. 3.10). Xylosidase activity dropped in the first 15 minutes for both the control and variant 152. At 60°C, more than 60% activity was retained after 120 minutes for variant 152 and only 38% activity was maintained for the control (Fig. 3.11). Compared to the control, thermostability in variant 152 was maintained for 120 minutes, at 50°C and 60°C with a minimal loss in relative activity.

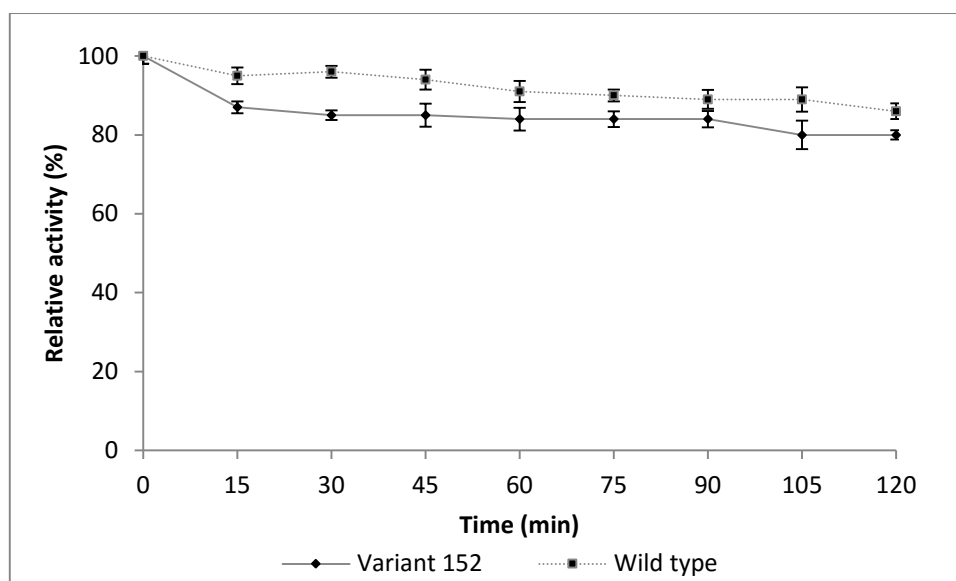


Figure 3.10: The effect of temperature stability at 50°C of the wild type *xlnD* and variant 152.

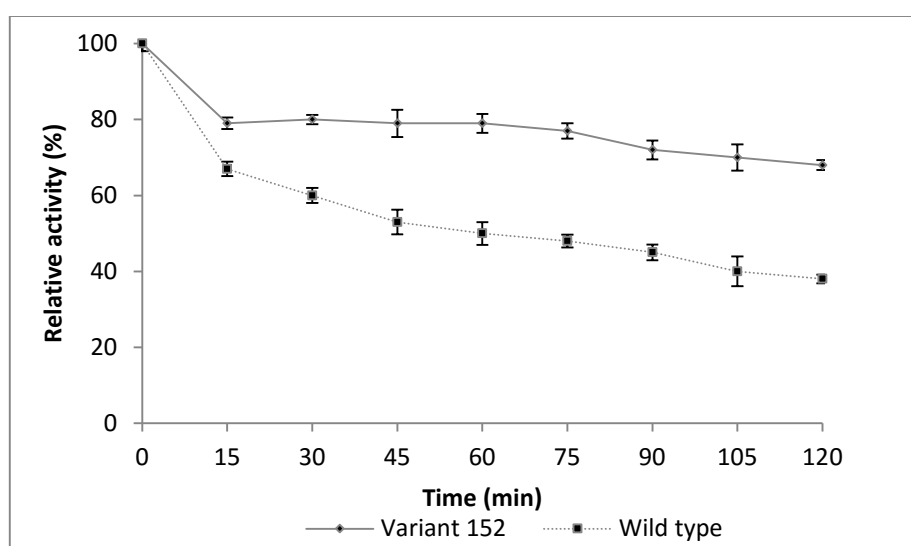


Figure 3.11: The effect of temperature stability at 60°C of the wild type *xlnD* and variant 152.

3.3.7 pH Stability of Xylosidase Variant 152 from *S. cerevisiae*

pH stability was tested at pH 4 and 5 at 50°C over 2 h for the wild-type *xlnD* and variant 152 expressed in *S. cerevisiae*. The results obtained from variant 152 were compared to the wild-type *xlnD*. At pH 4 (Fig. 3.12) both wild-type and variant retained almost 80% of their initial activities after 2 hours of incubation at 50°C.

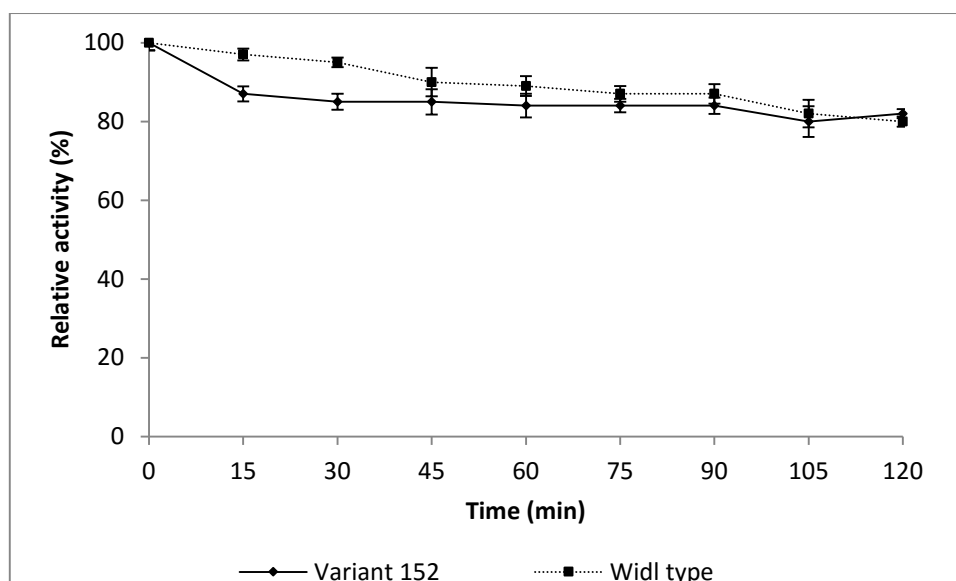


Figure 3.12: pH stability of the wild type *xlnD* and variant 152 at 50°C, pH4. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

3.3.8 Sequence Analysis of *xlnD* Variant 152 and Wild Type *xlnD*

The *xlnD* gene along with the xylosidase variant 152 and a few other variants were sequenced to determine if there were any amino acid substitutions that caused the observed differences in activity and stability under the tested conditions. The protein sequence alignment (Fig. 3.13) revealed one mutation in variant 152. Variant 152 has a single amino acid substitution at position 226. The amino acid asparagine (N) was replaced with aspartic acid (D).

```

152  HNHSRLGNDMNITQQDLSEYYTPQFHVAARDAKVH SVMCAYN AVNGVPACADSYFLQTLL 241
      |||||||||||||||||||||||||||||||||||||:|||||||||||||||
xlnD HNHSRLGNDMNITQQDLSEYYTPQFHVAARDAKVH SVMCAYN AVDGV PACADSYFLQTLL 241

```

Figure 3.13: Partial amino acid sequence alignment of variant 152 with the wild type *xlnD*. L152 change in amino acid sequence is highlighted in red.

3.4 DISCUSSION

Directed evolution has been a powerful tool for introducing diversity in the coding sequence of proteins, and has over time developed into a rapid and versatile approach to protein engineering. This chapter details the directed evolution of xylosidase using error-prone PCR. The wild-type *A. niger* xylosidase showed a maximum activity of 5.3 nkat/ml. The aim was to increase xylosidase activity and alter the pH and temperature characteristics of this enzyme. This was achieved by introducing random mutations in the xylosidase gene (*xlnD*), by mutagenic PCR. A range of mutagenic conditions were explored and conditions A, B, E and F (Table 3.2) was chosen since there was a noticeable decrease in the band intensity after amplification by *Taq* polymerase (Fig. 3.4 and 3.5). The variations in manganese and dNTP concentration affected the fidelity of *Taq* DNA polymerase, resulting in varying PCR product intensities (Eckert and Kunkel, 1990). This indicated that the *Taq* DNA polymerase was adversely affected and may have created mutations within the gene.

The pDLG55 plasmid harbours the ADH2 promoter, which is highly-repressed in the presence of glucose. Since glucose concentration was inhibitory, the *xlnD* recombinant was analysed using different concentrations of glucose (Fig. 2.20). 1% glucose concentration was found to be optimal for xylosidase expression.

Amplification with the primer set highlighted in Table 3.2 produced a distinct 2700 bp band for all the reactions tested. A control PCR was used as a reference for all mutagenic conditions tested. Examination of the bands revealed a decrease in band intensity. Conditions A, B, (Fig. 3.4) and E, and F (Fig. 3.5) were significantly less-intense than bands obtained using the control conditions and were brighter than conditions C, D and G. Mutagenic conditions were chosen based on the assumption that these conditions potentially hampered enzyme activity. *Taq* polymerase induces mutations randomly along DNA sequences and the addition of Mn^{2+} is found to be mutagenic to the *Taq* polymerase enzyme, thereby increasing the chances of a mutagenic events (Cadwell and Joyce, 1992). The brightness of the bands viewed can be indicative of the extent to which the enzyme was hampered. A faint band indicated the enzyme had been hampered due to the addition of manganese and variable nucleotide

concentrations therefore these reactions were not amplified to the same extent as the control. A brighter band can indicate that the enzyme was not hampered as much since the concentration of mutagen was too low. This can also mean the number of mutations per gene are fewer than that of the lighter PCR bands. Low concentration of the mutagen in the reaction resulted in more positive transformants, indicating the enzyme was less hampered and therefore more functional copies of the genes were produced. Since all the mutagenic conditions caused random mutations, the more variants screened increased the chances of finding a suitable mutation within the gene. Conditions C, D and G also produced fewer colonies, as expected, than conditions A, B, E and F. Since less DNA was amplified during the ep-PCR process, the degree of transformants decreased.

The *xlnD* gene was expressed with a fungal promoter system, and growth of *E. coli* transformants on ampicillin merely verified positive transformants but did not indicate the presence of the xylosidase gene. Mini-preparation plasmid isolation produced sufficient quantities of DNA for restriction analysis. Restriction digestion was performed on all positive transformants, and 96% of transformants contained the xylosidase gene. This was verified by the presence of the 2700 bp fragment after agarose gel electrophoresis (Fig. 3.6).

The most challenging aspect of mutagenic experiments is to create a screening procedure suitable and stringent enough to detect successful mutants. A screening procedure for enzyme activity could not be employed for *E. coli* transformants, since the cloning vector could not express the *xlnD* gene in *E. coli*. This meant all positive transformants were first restricted to confirm the presence of the *xlnD* gene. The only way to search the mutant library for variants was to transform all positive recombinants harbouring the *xlnD* gene into *S. cerevisiae* host cells and screen transformants for xylosidase production. This double screening process using both *E. coli* and *S. cerevisiae* was time-consuming. However, in *S. cerevisiae* the plate screening method was employed to screen xylosidase producers using MUX. This was useful and efficient since only the recombinants producing xylosidase produced a fluorescence on the plate. The hydrolysis of MUX by the action of β -xylosidase activity, resulted in the release of 4-methylumbelliferone (MU), which was visualised under UV illumination as fluorescence. Only those recombinants that produced a functional

xylosidase gene produced fluoresced. This screening method did not select variants but only xylosidase-expressing recombinants. This helped to differentiate transformants that had a functional xylosidase gene from those that did not. However, depending on the mutagenic reaction, the gene can in some cases, be severely mutated and these mutations are more likely to destroy enzyme activity rather than enhance activity.

A total of two thousand five hundred and forty isolates were obtained from all mutagenic conditions. 66% of these clones were obtained from condition G. In some studies, thousands of clones are screened in order to obtain the most novel and robust protein (Arnold, 1996). A screening method for any random mutagenesis experiment should be directed towards the desired change as closely as possible. This will aid in minimizing unwanted DNA or amino acid changes (You and Arnold, 1994). A disadvantage in the screening method used in this study was the need to test enzyme variants individually for a property of interest, which was extremely time consuming. Therefore, alternative screening methods for future studies should be directed towards a specific change. One method can be the plating of enzyme supernatant onto an agarose plate and incubated at either higher or lower temperatures to rule out unfavourable recombinants (Stephens *et al.*, 2007)

Clones were initially tested for an increase in enzyme activity (Table 3.3). Variant 152 (14.2 nkat/ml) and 1659 (12.1 nkat/ml) showed the highest increase in activity compared to the control. Based on the increase in activity, clone 152 was further tested for any increase in temperature and pH stability. Thermostability was tested at 50°C and 60°C at pH 5 in *S. cerevisiae*. These results showed a change in stability when compared to the wild-type *xlnD*. At 60°C, clone 152 retained 68% activity after 2 h, compared to the *xlnD* control that maintained only 38% activity. At 50°C, clone 152 maintained 80% activity after 2 h. A loss of 60% activity after 15 minutes was recorded and a complete loss of activity after 2 h. These results were performed in triplicate and indicated a reproducible change in stability and activity of variant 152 and was suggestive of a possible mutation.

Sequence analysis verified whether a mutation had occurred, and to identify the amino acid substitutions that could have contributed to the change in enzyme characteristics. Sequence alignments of variants showed similarity between the recombinants and the wild-type *xlnD*.

A single mutation occurred for recombinant 152 at residue 226. The amino acid aspartic acid (D) replaced asparagine (N). Our understanding of protein structure hasn't reached the level where we are able to predict the effect of change of individual amino acids on enzyme characteristics. The phenotypic difference observed in variant 152 in activity and stability results is most likely due to the change of one amino acid in the recombinant. A possible confirmatory test could be a site-directed mutation of the parent strain to deliberately mutate the gene at position 226. This can potentially confirm the altered enzyme characteristics.

Error-prone PCR mutation rates depend on both the protein and the mutagenic protocol. Directed evolution using random mutagenesis has been proven to improve catalytic activity and efficiency, modulate substrate specificity, and improve stability over a wide range of conditions. Research studies by Stephens *et al.* (2007) and Mchunu *et al.* (2009) have successfully used random mutagenesis as a means to introduce mutations in the *xynA* gene. High mutation rates as noted by Drummond *et al.* (2005) produce mostly unique sequences, but few retain their functionality.

In a study carried out by Arase *et al.* (1993), 60 000 mutant genes were screened. Of these only four heat-resistant mutants were obtained after random mutagenesis, with only 1 mutant showing an increase in temperature stability. In another study by Kohno *et al.* (2001) of the 60 000 clones obtained only 100 proved positive for lipase activity, with only 1 clone showing an increase in optimum temperature. In comparison, less than 0.01% of the mutant library obtained by Arase *et al.* (1993) and in this research produced mutants.

The mechanistic understanding of how an enzyme functions determines a suitable mutagenesis strategy (Siloto and Weselake, 2012). Random mutagenesis has proven to be a practical approach for improving enzyme stability and other characteristics (Arase *et al.*, 1993). The challenge faced lies in screening this library. The number of possibilities that can be created is so vast and their functions so unique that a good screening procedure needs to be implemented to make sure the correct clones are isolated. Random mutations of amino acid sequences are likely to introduce a change in enzyme characteristics directed by selection pressure controlled by the researcher. Directed evolution in contrast to rational design has

allowed the engineering of enzymes with novel functions and features and can be applied when not enough information can be supplied about an enzymes structure or mechanism. Site directed mutagenesis of specific residues can be used when residue specific data is readily available (Nannemann *et al.*, 2011)

CHAPTER 4 : GENERAL DISCUSSION

The β -xylosidase used in this study was isolated from *A. niger* 90216 (La Grange *et al.*, 2001) and cloned together with the *xynA* gene isolated from *Thermomyces lanuginosus*. The xylosidase was expressed in *S. cerevisiae* and *P. pastoris*. This xylosidase, under the control of ADH2 promoter exhibited an activity of 5.3 nkat/ml (La Grange *et al.*, 2001). An aim of this research was to change the characteristics of xylosidase using directed evolution. Error-prone PCR randomly mutated the *xlnD* gene, by using sub-optimal concentrations of nucleotides and manganese chloride. A mutant library containing 2700 recombinants was created. 2540 isolates contained the *xlnD* gene, of this 2540 isolates, 96% displayed xylosidase activity. Since the enzyme expressed by *S. cerevisiae* was cell-bound, whole cell enzyme extractions was performed on the entire mutant library and assayed for xylosidase activity. This proved cumbersome and time consuming.

Two clones that showed highest increase in activity were further analysed and found to produce 14.2 nkat/ml and 12.1 nkat/ml xylosidase. These activities were found to be higher than the wild-type. Further analysis also proved an increase in stability when compared to the wild-type xylosidase. At 60°C, clone 152 maintained 68% activity, whilst the wild-type retained only 38%. Whilst enzyme activity assays showed an increase in activity and stability, sequence analysis showed one nucleotide change. The amino acid asparagine was replaced by aspartic acid at residue 226.

Even though genetic techniques has forged the way for engineering altered proteins, there is still a gap in understanding and creating simpler mutant libraries that are less redundant in functionality and characteristic. Hibbert and Dalby (2005), suggested a more direct way of screening by focussing random mutagenesis to regions of enzymes more likely to cause the desired effect. Computational approaches provide an alternative as starting points for creating a smarter mutant library containing fewer redundant enzyme variants, guaranteeing a less time-consuming process and higher possibility of variation. Experimental studies by Reyes and Kollman (2000) verified mutations that abolish and improve binding specificity of U1A-RNA by computational approaches and suggest this as an inexpensive tool for

investigating and predicting the effects of site specific mutagenesis. Other strategies used to increase xylosidase activity included different strains and vectors to amplify expression (Den Haan and Van Zyl, 2003).

For *S. cerevisiae*, the vector pJC1 was compared to the pDLG55 expression vector previously used by La Grange *et al.* (2001). Expression with pJC1 produced 8 U/ml xylosidase after 48 h, while studies by La Grange *et al.* (2001) produced only 3.5 nkat/ml in *S. cerevisiae*. For expression in *P. pastoris* GS115, vectors pBGP1 (Lee *et al.*, 2005) and pHLS1 (Invitrogen) were compared. Expression with the GAP promoter in *P. pastoris* produced a maximum of 70 U/ml after 72 h and 0.5 U/ml after 48 h under the AOX promoter. According to literature, the maximum recorded β -xylosidase activity thus far in *P. pastoris* under the GAP promoter system was produced by the fungal xylosidase isolated from *T. lanuginosus* by Gramany *et al.* (2015), this recombinant expressed a maximum of 60 U/ml β -xylosidase after 96 hours.

The development of constitutive promoters has given great variability to yeast expression systems. These expression systems are now in use, simply due to the lower yield of product obtained from bacterial systems, and difficulty in obtaining high-level transcription of foreign genes (Romanos *et al.*, 2004). The GAP promoter system has shown comparable expression levels on glucose and glycerol media. Some studies have shown comparable results with the AOX and GAP systems (He *et al.*, 2008). The AOX promoter however is highly repressed by glucose and other carbon sources, and although essential for maximum production levels, methanol poses a potential fire hazard and may prove inappropriate for the production of food products (Cereghino and Cregg, 1999). Similar to the GAP promoter, the PGK promoter system is the most powerful glycolytic promoter used in *S. cerevisiae*, compared to the highly repressed ADH2 promoter. *xlnD* gene expression in both *P. pastoris* and *S. cerevisiae* was screened using MUX. MUX was incorporated in the growth media and formed a fluorescence under UV illumination to indicate the release of 4-methylumbelliferone (MU) by the action of xylosidase activity. Nannemann *et al.* (2011) classified selection methods into two categories: selection-based methods and biochemical screening based approaches. In selection-based methods, all mutants are assayed for the desired biochemical activity and screening based methods make use of colorimetric or fluorometric measurements. In some cases,

colorimetric screens can be adapted and applied to colony screening on an agar plate. Colonies displaying a desired colour within a certain time are selected as active. The agar plate assay is used as a primary screen to identify enzyme variants with functional turnover. Subsequently, a secondary- and sometimes tertiary-activity assay is generally performed in a 96-well microtiter plate with biochemical assay to confirm and identify the most active clones. The MUX screen used in this study is indicative of this and is less time-consuming when compared to the manual screening used in *E. coli* to screen for positive clones.

xlnD expression with the glucose-dependant ADH2 promoter showed repressed expression levels with a 2% glucose concentration, and increased production of 3.5 nkat/ml, using 1% glucose. Constitutive production in *S. cerevisiae* produced a maximum of 8 U/ml after 48 h. The change in expression systems used in *S. cerevisiae* resulted in an increase in production levels. Glycolytic genes from yeasts have an increased expression of 65% or more of soluble protein, with one of these promoters being PGK. This promoter induces a level of expression 4 – 10% of the soluble protein depending on growth media (Hitzeman et al., 1982).

P. pastoris expression vectors pBGP1 and pHLS1 highlighted a change in enzyme production. *P. pastoris* is capable of integrating multiple copies of transforming DNA via recombination into the genome at sites of sequence homology. Constructs were linearized to favour recombination at the *HIS4* locus. Expression of xylosidase was strongly induced by methanol. Expression however in BMMY medium with integrative vector pHLS1 and 0.5% methanol induction produced 0.5 U/ml xylosidase after 72 h. This activity is similar to production levels obtained using expression vector pDLG55 in *S. cerevisiae*. The *P. pastoris* recombinant pBGP1-*xlnD* cultivated in YPD media containing the antibiotic zeocin, expressed xylosidase activity levels up to 70 U/ml after 72 h. The *xlnD* gene was previously used in a study by Den Haan and Van Zyl (2003) expressed under transcriptional control of the constitutive *Pichia stipitis* transketolase (TKL) gene promoter. This system reached a maximum activity of 132 nkat/ml after 90 h.

Manzanares *et al.* (1999) studied xylosidase production from yeast strains belonging to *Hanseniaspora* and *Pichia*. *Pichia anomala* showed xylosidase activity of 0.1 U/ml. Activities

obtained from all strains tested ranged from 0.01 to 0.24 U/ml. The xylosidase activity produced from the *Pichia* strains reflected above proved to be insignificant when compared against the expression obtained when using the GAP promoter. Mchunu *et al.* (2009) obtained 261.7 U/ml xylanase using the GAP system, whilst only 13.4 U/ml using the PGK system, once again reiterating different results obtained using these two constitutive systems.

Whilst *P. pastoris* is the favourable choice for heterologous protein production in yeast, this β -xylosidase has been isolated and characterized from various fungi, such as *Aureobasidium pullulans* (Dobberstein and Emeis, 1991), *Aspergillus sydowi* (Ghosh and Nanda, 1993), *Trichoderma reesei* (Herrmann *et al.*, 1997), *Aspergillus japonicas* (Wakiyama *et al.*, 2008) and *Sporotrichum thermophile* (Katapodis *et al.*, 2006). Amongst the bacterium species, *Bacillus thermantarticus* characterized by Lama *et al.* (2004) produced 80 U/ml extracellular xylosidase. The yeast like fungus *Aureobasidium pullulans* revealed an activity of 0.35 U/ml and *Penicillium sclerotiorum* 17.53 U/ml. Even though, other expression hosts might be more or less productive, *P. pastoris* still remains the simplest to use.

The enzyme obtained from *S. cerevisiae* and *P. pastoris* was analysed by SDS-PAGE analysis of culture supernatant after cultivation. SDS-PAGE analysis showed the presence of a single protein band, which corresponded on the zymogram under UV illumination (Fig. 2.18). SDS-PAGE of *S. cerevisiae* supernatant showed contamination with other intracellular proteins. This is expected since the cells were lysed first, and the use of crude extract would be of little value since the protein is cell-bound. The activity stain highlighted the position of the xylosidase band on the zymogram under UV illumination (Fig. 2.19). This analysis confirmed protein size as 120 kDa from both hosts. This falls within the commonly observed range of 90 – 130 kDa for other *Aspergillus* β -xylosidases (Rodionova *et al.*, 1983). However the 120 kDa protein is larger than the estimated molecular mass of 85.1 kDa reported by La Grange *et al.* (2001). The protein was partially purified using the Amicon 50k filter and desalted. Most purified β -xylosidase exhibit molecular masses more than 100 kDa (Herrmann *et al.*, 1997; Matsuo *et al.*, 1998; Rizzatti *et al.*, 2004). Some fungal xylosidase however have higher molecular masses and are composed of two identical subunits (Wakiyama *et al.*, 2008). Juturu

and Wu (2012), isolated a β -xylosidase from thermophilic fungus *Paecilomyces thermophile* with a predicted mass of 39.31 kDa from its amino composition, but when expressed in *P. pastoris* showed a single band on SDS-PAGE with an estimated molecular mass of 52.3 kDa.

Aspergillus species have gained interest due to their thermotolerance and production of thermostable enzymes (Pedersen *et al.*, 2007). Recombinant protein expression can alter biochemical characteristics. Therefore, these characteristics were tested in both hosts. The enzyme was optimum at pH 4 and 60°C, exhibiting highest activity at these conditions and proved more stable for a longer period when expressed in *P. pastoris* compared to *S. cerevisiae* (Fig. 2.25 - 2.26). A common trend between the hosts was stability at extreme acidic conditions. At pH 3, the enzyme expressed by *P. pastoris* and *S. cerevisiae* showed a decline in stability more rapidly than at pH 4 and pH 5. Temperature stability also showed a similar trend. Results obtained with *S. cerevisiae* were similar to that obtained by La Grange *et al.* (2001). Though more stable in *P. pastoris*, as temperature increased, stability of the enzyme decreased. At 70°C, the enzyme expressed in *P. pastoris*, retained activity for 60 minutes, whilst *S. cerevisiae* retained activity for just 30 minutes. β -xylosidases of fungal origin usually show optimal activity at pH values from 4.0 to 6.0 and most favourable at temperature 55°C, 60°C, and 65°C (Knob and Carmona, 2012).

In a study carried out by Pedersen *et al.* (2007), *A. niger* was one amongst the twenty two strains evaluated to produce a thermostable β -xylosidase, with a pH and temperature optima of 5 and 75°C respectively, and retained 99% activity after 1 hour at 60°C but decreased to 44% at 75°C. The pH optima for the *Aspergillus* xylosidase was pH 5, with equal activity at pH 3 and pH 4 at 40°C, whilst *Aspergillus japonicas* was optimum at pH 6, having the same activity as *A. niger* at pH 5, proving the biochemical characteristics observed in *P. pastoris*. Xylanolytic enzymes have found use in many industrial applications. The complete enzymatic hydrolysis of xylan into its constituents requires the synergistic action of a consortium of xylanolytic enzymes (Nel, 2002). The β -xylosidase produced in *P. pastoris* under the control of the GAP promoter, was co-expressed with a fungal xylanase. Co-expression of these enzymes maintained the original activity of one whilst producing a 10-fold decrease of the other. In one instance, co-expression yields were 136 U/ml xylanase and 7 U/ml xylosidase. Xylan

degradation using supernatant containing both enzymes, produced 1.8 g/l xylose after 48 h from a 1% birchwood xylan substrate. Xylan hydrolysis with 100 U xylanase and 20U xylosidase released 7.2 g/l, confirmed by HPLC analysis. Due to co-expression of these enzymes, bioethanol production from xylose was achieved.

Ethanol fermentation has received more attention in the last few years due to concerns over the rising fuel costs that has led towards the production of alternative fuel sources. (Jeffries, 2006). These alternatives aim to reduce the dependence on oil, and have a positive effect on air pollution and climate change (Mussatto *et al.*, 2012). Several approaches have been employed to investigate production of ethanol from various sources, organism and the potential for commercial fermentation of xylose to ethanol.

Mussatto *et al.* (2012) investigated the production of ethanol from different yeast strains using waste hydrolysates from the coffee industry. Dubey *et al.* (2012) researched bioethanol production from waste paper acid pre-treated hydrolysate. 200 different species of yeasts were tested by Toivola *et al.* (1984) for their ability to ferment D-xylose. In other studies researchers sequenced the *P. stipitis* genome to provide insight on how the native xylose fermenting yeast regulates its redox balance while efficiently fermenting xylose under micro-aerobic conditions (Jeffries *et al.*, 2007)

A study by Lee *et al.* (1986) investigated the ability of yeast growing on xylose to degrade xylan. Of the 250 strains tested, *P. stipitis* and *Cryptococcus* were among the 19 strains that yielded a positive result. Some strains of the *P. stipitis* are able to degrade xylan, however the natural xylanolytic ability of this yeast is very weak (Den Haan and Van Zyl, 2003). Lee *et al.* (1986) thought it would be advantageous if yeasts could accomplish both hydrolysis of xylan and bioconversion of the monosaccharides to ethanol.

P. stipitis (*Scheffersomyces stipitis*) is known to be one of the best D-xylose fermenting yeasts and was used as the organism of choice since *P. pastoris* and *S. cerevisiae* are unable to utilize xylose. *S. cerevisiae* is unable to metabolise xylose due to the lack of xylose reductase (XR) and xylitol dehydrogenase (XDH) enzymes, which catalyse the first two steps in the xylose

metabolising pathway in yeasts. Native transporters in *Saccharomyces* are poorly regulated to facilitate xylose uptake and many studies have aimed to improve assimilation by cloning and expressing the *XYL1* and *XYL2* genes in *S. cerevisiae* (Den Haan and Van Zyl, 2003; Meinander *et al.*, 1999).

A simultaneous saccharification and fermentation was carried out using xylan as a substrate. The xylose released due to hydrolysis of xylan by co-expressed enzymes, was used as a carbon source for ethanol production by *P. stipitis* in a micro aerobic environment. After 72 hours, ethanol production peaked at 0.7%. However more work is still needed to optimize hydrolysis conditions and ethanol production levels. Efficient fermentation of sugars is critical for bioconversion to ethanol and proper uptake of sugars can improve fermentation if other metabolic steps are not rate limiting. For commercial production, bioconversion must occur at a high rate, in good yield and to concentrations that are economically recoverable (Van Vleet and Jeffries, 2009).

In summary, the β -xylosidase from *A. niger* was successfully expressed in *P. pastoris* and *S. cerevisiae* under the control of constitutive GAP and PGK promoter, and inductive *AOX1* and *ADH2* promoters. The highest expression level of 70 U/ml was obtained with the glycolytic GAP promoter. This β -xylosidase was most active at pH 4 and 60°C and showed great stability at 40°C and 50°C and at pH 3, 4 and 5. The β -xylosidase was successfully cloned and co-expressed with a *Thermomyces* xylanase. Xylan hydrolysis with this resulted in the simultaneous saccharification and fermentation of the xylose produced by *P. stipitis* to produce bioethanol. This co-expression of xylanolytic enzymes and subsequent xylose hydrolysis highlights the use of yeasts for the commercial fermentation to bioethanol, in light of the rising fuel prices. Continued research efforts in this field should aim to fulfilling the goal of engineering a yeast capable of complete fermentation of sugars to ethanol.

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