

Evaluation of five *Saccharomyces cerevisiae* promoters during growth on xylose

by

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DEDICATION

I dedicate this work to my very special, courageous and supportive father Andries Muyanalo Mande who passed on during the fulfilment of this journey and my precious joy, Imelani-Zwavhudi Phathutshedzo Makulana.

DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Microbiology has not been previously submitted by me for a degree at this or any other University, that it is my work in design and execution, and that all material contained herein has been acknowledged.

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Date: _____

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ABSTRACT

S. cerevisiae has many properties which have made it the preferred host for the expression and production of a number of recombinant proteins. Xylose is the second most abundant sugar in nature and *S. cerevisiae* has been engineered to grow on this abundant sugar. Therefore, identifying *S. cerevisiae* promoters that are strongly induced during growth on xylose will be important in the production of recombinant proteins for the biofuel and other industries. Since xylose is not a native substrate for *S. cerevisiae*, it is not known how *S. cerevisiae* promoters will react during growth on xylose. The objective of the study was to evaluate the expression of a reporter gene, the *Trichoderma reesei* xylanase 2 (*XYN2*), under the control of five commonly used expression promoters (*GPD3*, *ENO2*, *PGK1*, *ADH2* and *YG100*). Five episomal expression vectors were constructed for this purpose. These vectors were transformed to a recombinant xylose utilizing *S. cerevisiae*. Xylanase activity assays were used to determine the expression level from each of these promoters. The *PGK1* promoter was observed to be the strongest promoter with average activity/OD of 130 nkat/ml/OD on both xylose and glucose. The *GPD3* promoter showed the highest average activity/OD of 150 nkat/ml, but xylanase was only produced during growth on glucose. The data presented show that xylose is not a better carbon source than glucose for recombinant protein production in terms of the *S. cerevisiae* promoters evaluated. Further research is required to obtain a yeast strain that grows well on xylose and promoters that show higher level on protein production.

Keywords: xylose, promoter, expression, recombinant, *S. cerevisiae*

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CHAPTER 1.INTRODUCTION

1.1. Background Information

In the recent years, there has been a high usage of oil and limited amount of fossil fuel, bioethanol is an alternative fuel that could be used in the transportation industry. Plant biomass is the only foreseeable renewable feedstock for sustainable production of biofuels (LYND *et al.*, 2005, KERCKHOFFS and RENQUIST, 2013) .

The production of ethanol from plant biomass such as lignocellulosic material has the potential to meet capacity demands without impacting directly on food production (MALY and DEGEN, 2001, HILL *et al.*, 2006). Lignocellulose is the main constituent in plant material and is the non-starch fibrous part of plant material. This includes polymers like, celluloses, hemicelluloses and lignin (MABEE *et al.*, 2011).

Biological conversion of biomass to biofuels involves a number of processes (LYND *et al.*, 2005, KERCKHOFFS and RENQUIST, 2013). After pre-treatment to open up the structure of the polysaccharides in plant material (cellulose and hemicellulose), biomass processing typically involves four biological steps: the production of enzymes (cellulases and hemicellulases), the hydrolysis of polysaccharides (cellulose and hemicellulose) to sugars, the fermentation of hexose sugars, and the fermentation of pentose sugars, mainly xylose. Xylose is the second most abundant sugar in nature and is available in large quantities in the biomass industry. Wild type strains of *S. cerevisiae* cannot ferment xylose, but in recent years *S. cerevisiae* has been engineered to ferment this sugar (WAHLBOM *et al.*, 2003, KUYPER *et al.*, 2004).

1.2. Research problem

The yeast *S. cerevisiae* has been used for the production of recombinant proteins for decades and the large scale fermentation technology for this yeast is well established. The best known examples of recombinant protein production are insulin precursors (LI *et al.*, 2008), growth hormones (WALSH, 2006) as well as hepatitis B vaccines (VALENZUELA *et al.*, 1982). High level expression of recombinant proteins is usually linked to the amount of biomass obtained during fermentation (FERNDAHL *et al.*, 2010b) as well as the promoter used to drive transcription. Glucose is a preferred substrate for the cultivation of *S. cerevisiae*. However since *S. cerevisiae* is crabtree-positive yeast, it producing relatively

small amounts of biomass when cultivated on glucose, since most of the carbon source is converted to ethanol. It is possible to overcome this by limiting the amount of glucose during growth in a fed-batch process, a relatively complicated step that will increase the cost of the recombinant protein of interest. Alternatively, a non-fermentable carbon source can be used. Sugars like galactose would allow higher biomass production and improved expression from a glucose repressible promoter like *ADH2*. Unfortunately, a cheap abundant source of galactose is not readily available (LA GRANGE *et al.*, 1996) .

Since *S. cerevisiae* was engineered to grow on xylose, a large cheap resource has been made available for culturing *S. cerevisiae* on industrial scale (WAHLBOM *et al.*, 2003, KUYPER *et al.*, 2004). Identifying *S. cerevisiae* promoters that are strongly induced during growth on xylose will be of great value for the production of a variety of recombinant proteins, especially proteins used in the production of biofuels, like cellobiohydrolases, endoglucanases and β -glucosidases among others (ILMEN *et al.*, 2011). Since xylose is not a natural carbon source for *S. cerevisiae*, it is not known how its promoters will react when this yeast is grown on xylose.

1.3. Aim of the study

The main aim of this study is to evaluate the expression of a reporter gene (*Trichoderma reesei* *XYN2*) under the transcriptional control of five (*PGK1*, *ENO2*, *GPD3*, *ADH2* and *YG100*) different commonly used protein expression promoters in a recombinant xylose utilizing strain of *S. cerevisiae* during growth on xylose.

1.4. Objectives of the study

The objectives of the study were to:

- Construct five episomal expression vectors with different promoters controlling the expression of the *T. reesei* xylanase reporter gene. The vectors include: pAZ4 (Phosphoglycerate Kinase 1 promoter & Phosphoglycerate Kinase 1 terminator), pAZ2 (Enolase 2 promoter & Enolase 2 terminator), pAZ3 (Glyceraldehyde 3 phosphate Dehydrogenase promoter & Glyceraldehyde 3 phosphate Dehydrogenase terminator), pDLG5 (Alcohol dehydrogenase 2 promoter & Alcohol dehydrogenase 2 terminator), pDLG23 (*YG100* (heat inducible) promoter & *ADH2* terminator) and the negative control plasmid YEp352.
- Construct a xylose utilizing strain of *S. cerevisiae*.

- Transform the expression plasmids into the xylose utilizing *S. cerevisiae*.
- Determine the expression levels of the different promoters by measuring xylanase activity during growth on xylose.
- Compare the five different promoters for gene expression on glucose and xylose.

CHAPTER 2. LITERATURE REVIEW

2.1. Introduction

Since the emergence of recombinant gene expression, there has been an increase in the production of valuable proteins across a number of industries including agriculture, medicine and energy. The technology involves the expression of cloned DNA sequences in a host of interest to produce a protein product. Because the cloned DNA can be modified or altered, this technology brought simplicity, enabling investigators to custom-design protein products to suit specific requirements. Components of a recombinant expression system include a host organism and an expression vector with the necessary elements required for maintenance and expression of a foreign gene (BERNAUDAT *et al.*, 2011).

Recombinant protein expression enables the expression of a targeted protein at high concentrations to enable an economically feasible production of the protein of interest (FERNDAHL *et al.*, 2010b). Since the development of heterologous expression systems, products such as vaccines, hormones, antibodies, growth hormones and enzymes have been produced using the technology (JORGE and HUGO, 2012).

The first hormone produced in a heterologous host was insulin. Insulin is a hormone given to diabetics to help control blood glucose levels. For many years it was purified with considerable costs from pancreases of pigs and cows. Today, insulin is produced by genetically engineered microbial cells in large quantities at very affordable prices. Another protein that has been produced using this technology is human growth hormone. This hormone is used for the treatment of dwarfism and it is the first hormone of pharmaceutical importance produced in bacteria (*Escherichia coli*) (JORGE and HUGO, 2012).

World-wide researchers are searching for organisms and enzymes that could help with the production of biofuel from lignocellulosic material. Recombinant protein production is a multibillion Rand industry especially in developed countries. This technology will provide a platform for increasing the yield and robustness of industrial enzymes such as cellulases and xylanase. Cellulases and xylanase are enzymes used in the production of liquid fuels from plant biomass. A lot of work is being done to reduce the cost of these enzymes and also increase their activity on pre-treated biomass (DOS REIS *et al.*, 2013, ZHANG *et al.*, 2013). The continuance of these improvements will help in the development of economically feasible biofuels which could someday replace fossil fuel.

Recombinant gene expression was first developed in bacteria, in particular *E. coli*, but is now routinely used in eukaryotes. This is because certain proteins require complex post-translational modifications which are not possible in *E. coli*. Furthermore, proteins purified from *E. coli* are often contaminated with highly pyrogenic substances. Therefore purification schemes are usually needed to obtain proteins with the desired purity, structure and biological activity (JORGE and HUGO, 2012). In addition, *E. coli* cannot tolerate growth conditions often used in industry. Because of these drawbacks, an alternative expression system was needed.

A eukaryotic host compatible with industrial processes and capable of taking up complex genomic DNA was of importance in recombinant protein production. The yeast *S. cerevisiae* has been widely used in industry for centuries in brewing, baking, wine making and as an expression host for recombinant protein production and recently also for biofuel productions. It has eukaryotic features that include a secretory pathway that result in correct protein processing and post-translational modifications (MADIGAN and MARTINKO, 2006, MATTANOVICH *et al.*, 2012).

2.2. Expression Hosts

Expression hosts that result in high yield of recombinant protein are often required in industry (SODOYER, 2004). There is a wide range of bacterial and yeast expression systems available for the production of different types of recombinant proteins. Several criteria are considered when choosing an expression host for industrial use. These include; efficient biomass production, tolerance to industrial conditions and meeting safety requirements. The expression system used also depends on the target protein, whether it is soluble, membrane bound, multi domain, containing disulphide bonds, as well as the use of the protein. It is therefore also important that a recombinant protein be synthesized in the same way it is synthesized by the donor of the gene of interest. This is because intracellular and secretory environments vary amongst donor and recombinant host organism (STOCKMANN *et al.*, 2009).

2.2.1 Prokaryotes as an expression hosts

Bacterial cells are the preferred expression systems for simple proteins, because they are easy to cultivate (SØRENSEN and MORTENSEN, 2005) and modify genetically. The most widely used bacterial expression system is *E. coli* (BERNAUDAT *et al.*, 2011). This is a gram-negative, rod-shaped bacterium, commonly found in the gut of endotherms (warm blooded organisms) (MADIGAN and MARTINKO, 2006). About 80% of recombinant genes have been expressed in this organism. Therefore, this makes *E. coli* the dominant expression systems and is the preferred system for laboratory investigation and initial development work (VERMASVUORI, 2009, SØRENSEN, 2010, VICENTE and MARI, 2010).

E. coli offers rapid and economical production possibilities (WALSH, 2006) through low production cost, short generation time, and easy manipulation (SØRENSEN, 2010). One drawback of this expression systems is the fact that *E. coli* produces inclusion bodies during the expression of many recombinant proteins (BANEYX, 1999, SØRENSEN and MORTENSEN, 2005). Moreover, *E. coli* also does not have a secretion mechanism for the efficient release of the proteins into the medium (JANA and DEB, 2005).

Alternative bacterial systems used for the expression of recombinant proteins include gram-positive bacteria like *Bacillus*. *Bacillus* does not form inclusion bodies, are endo and exotoxin free and are capable of secreting proteins directly into the culture media. The disadvantages of these organisms are the production of high levels of proteases that sometimes destroy the target protein (VICENTE and MARI, 2010).

Although bacterial expression hosts have many positive traits, a eukaryotic expression host was required to enable the expression of genes that cannot be expressed in *E. coli*, *Bacillus* or other prokaryotic systems. Eukaryotic expression systems are generally able to correctly fold proteins and perform post translational modifications (WARD, 2011, SU *et al.*, 2012).

2.2.2. Fungal expression system

Yeasts are single celled eukaryotes, making them good hosts for the production of certain recombinant proteins. Although there is no universal host for the production of all recombinant proteins, yeast has more favourable characteristics compared to bacterial hosts. These include high performance, producer strain stability, growth to high density, and growth in cheap and chemically defined media. In addition, yeast is able to glycosylate and

fold complex proteins including those with a high number of disulphide bonds. Furthermore, yeast has strongly regulated promoters especially methylotrophic strains such as *Pichia*, *Candida* and *Hansenula* species (NEGRUITA *et al.*, 2010).

Eukaryotic expression hosts that are widely used in industry include filamentous fungi *Aspegillus niger* (SHUSTER *et al.*, 2002), the methylotrophic yeasts *Pichia pastoris* and *Hansenula polymorpha* (CREGG *et al.*, 2000, CREGG *et al.*, 2009) as well as the baker's/brewer's yeast *S. cerevisiae* (STRAUSBERG and STRAUSBERG, 1995).

2.2.2.1 Methylotrophic organisms as expression hosts

Methylotrophic organisms are mostly yeast and bacteria that utilize C₁-compounds such as methanol and methane as carbon source. These yeasts are commonly found on the bark of trees as well as vegetables (CRAVERI *et al.*, 1976) as methanol is derived from the methoxy chains present in lignin. Their ability to use methanol as the sole carbon source is due to the presence of peroxisomes which get induced in the presence of methanol. During growth on methanol peroxisomes proliferate and this organelle consists of enzymes used in methanol metabolism. The induction of peroxisomes in turn leads to the activation of methanol metabolizing enzymes, such as dihydroxyacetone synthase (DHAS), catalase (CAT) and alcohol oxidase (AOX) (NEGRUITA *et al.*, 2010)

Most methylotrophic yeasts use a common methanol-utilizing pathway (Figure 2.1). Methanol metabolism involves important promoters that are also used in heterologous expression. One of the key enzymes is the AOX enzyme. In the cell, the AOX is localised together with DHAS and CAT. When the growth conditions are favourable for methylotrophic yeast to grow at high density the DHAS and AOX enzymes are synthesised in high amounts. The AOX promoter is a very strong and tightly regulated promoter in the presence of methanol (NEGRUITA *et al.*, 2010).

In the methanol utilization pathway (Figure 2.1), methanol is oxidised to formaldehyde and hydrogen peroxide by AOX. Both these compounds are highly toxic. Formaldehyde can either undergo dissimilation or assimilation. Formaldehyde is fixed to xylulose-5-phosphates (X5P) by DHAS producing dihydroacetone (DHA) and glyceraldehyde-3-phosphates (GAP). DHA is phosphorylated by dihydroacetone kinase (DHAK) to dihydroacetone phosphate (DHAP). DHAP and GAP are combined to produce fructose-1, 6-bisphosphate (FBP) which is then dephosphorylated to fructose-6-phosphate (F6P). The F6P then enters the pentose

phosphate pathway for the regeneration of xylulose-5-phosphates (NEGRUȚĂ *et al.*, 2010, YURIMOTO *et al.*, 2011).

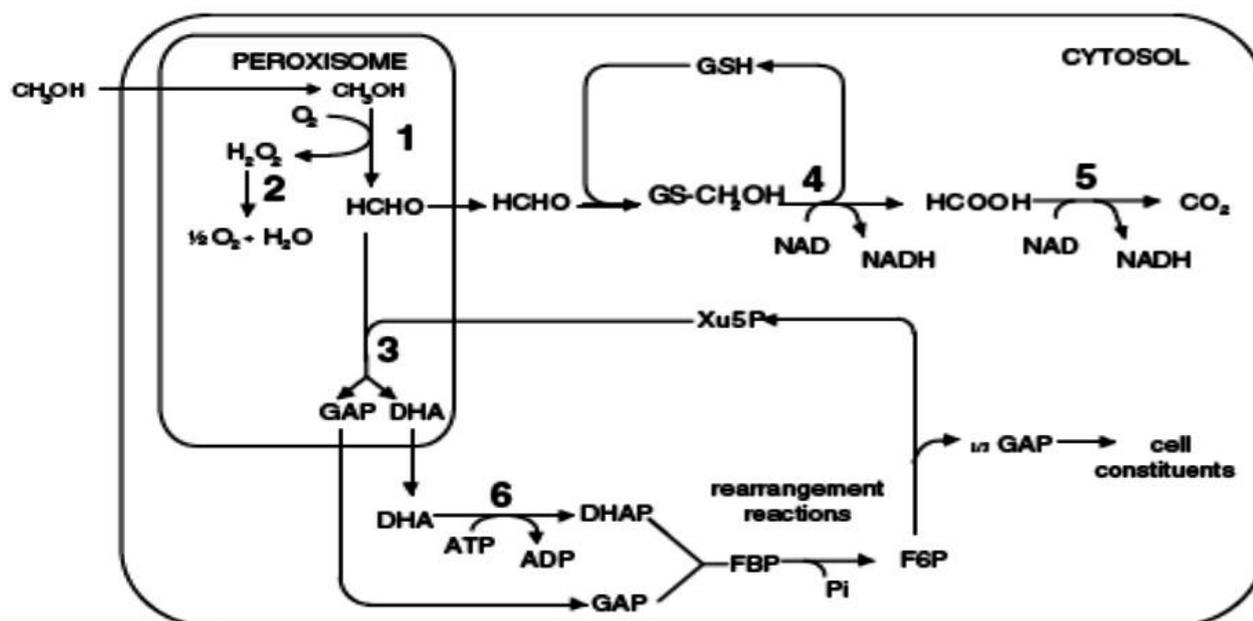


Figure 2.1. A schematic representation of the most important enzymes involved in the methanol metabolism pathway (NEGRUȚĂ *et al.*, 2010).

1 – Alcohol oxidase, 2 – Catalase, 3 – Dihydroxyacetone synthase, 4 – Formaldehyde dehydrogenase, 5 –Formate dehydrogenase, 6 – Dihydroxyacetone kinase, **CH₃OH** - Methanol, **HCHO** - Formaldehyde, **GAP** - Glycealdehyde-3-phosphate, **DHA** - Dihydroacetone, **DHAP** - Dihydroacetone phosphate, **GSH** – glutathione, **Xu5P** – xylulose-5-phosphate, **FBP** – fructose-1,6- bisphosphate, **F6P** -fructose-6-phosphate.

Pichia pastoris as an expression host

Pichia pastoris is a non-filamentous, unicellular eukaryotic organism, which grows on various simple carbon sources including glucose, glycerol, galactose, fructose, ethanol and methanol (NEGRUITA *et al.*, 2010). The ability of this yeast to use methanol as sole carbon source is a feature of industrial importance. Furthermore, *P. pastoris* is relatively easy to manipulate genetically, enabling the expression of recombinant proteins (KRAINER *et al.*, 2012). *P. pastoris* is one of the main eukaryotic host organisms used in the production of recombinant proteins. It has been used for the production of approximately 200 intracellular and extracellular recombinant proteins (CREGG *et al.*, 2000, KRAINER *et al.*, 2012). This is due to important characteristics that include fast cell growth, high cell density during fermentation and the potential of producing soluble and correctly folded recombinant

proteins that requires post-translational modification. Expression vectors in this yeast are introduced by homologous recombination. Homologous recombination results in the disruption of a gene and these results in single copy number of the expression cassette. The availability of strong tightly regulated promoters reduces cost of protein production through high product yields (FERRER-MIRALLES *et al.*, 2009, NEGRUITA *et al.*, 2010).

The AOX enzyme oxidizes methanol to formaldehyde and hydrogen peroxide in the presence of oxygen. AOX is reported to be sensitive to oxygen. Therefore, to compensate for its sensitivity to oxygen; it is synthesized in high amounts. The AOX promoter, the analogue of the methanol oxidase promoter (*MOX*) a strong and widely used promoter in the expression of heterologous proteins as it is also tightly regulated and induced by methanol (SZAMECZ *et al.*, 2005). The most often used methylotrophic yeasts are *P. pastoris* (HIGGINS, 2001) and *H. polymorpha* (RYABOVA *et al.*, 2003). Both these were isolated decades ago on media enriched with methanol (NEGRUITA *et al.*, 2010). In addition, *P. pastoris* and *H. polymorpha* are favoured because they do not harbour pyrogens, pathogens or viral inclusions (STOCKMANN *et al.*, 2009, KRAINER *et al.*, 2012), which are important criteria for host organisms for the industrial production of recombinant proteins.

Hansenula polymorpha as an expression host

Hansenula polymorpha has been used for years for the expression of recombinant proteins. Recombinant protein production in *H. polymorpha* is controlled by the strongly regulated *MOX* promoter. This promoter is from a gene that is found in the methanol utilizing pathway. The *MOX* promoter differs from the *AOX* promoter in that it is de-repressed in the environment with limited glucose. Furthermore, the *AOX* promoter is strongly and tightly regulated in the presence of methanol more than the *MOX* promoter. *H. polymorpha* enables multi-copy integration that results in high copy number of the integrated cassette. In *P. pastoris* gene expression is targeted to a single locus (*HIS* or *AOX*) through homologous recombination resulting in single copy expression. This is a limitation in *P. pastoris*, since the integration in *AOX* locus results in the disruption of the *AOX* gene (CELIK and CALIK, 2012).

H. polymorpha has been used for the production of a number of recombinant proteins, including insulin and hepatitis B vaccines, which has been approved by the United States Food and Drug Administration (USFDA) (NEGRUITA *et al.*, 2010). Recently,

H. polymorpha has been reported to have been used for the production of bioethanol from lignocellulosic sugars (DMYTRUK and SIBIRNY, 2011). This is due to the organism's thermo tolerance as well as a complete xylose metabolic pathway which is important for Simultaneous Saccharification and Fermentation (SSF) processing of biomass. Even though *H. polymorpha* can ferment a variety of sugars including glucose, xylan, xylose, cellobiose, starch and glycerol to ethanol, a bacterial xylose isomerase has been over-expressed in *H. polymorpha* to improve ethanol production from xylose (SUWANNARANGSEE *et al.*, 2012). Since major drawback when culturing methylotrophic yeasts on methanol is the hazards associated with the use of methanol in a closed and highly oxygenated environment. In addition, food-processing enzymes such as glucose-oxidase are inhibited by methanol and therapeutic products such as human serum albumin will be toxic if contaminated with methanol. Therefore, it is important that all traces of methanol from the fermenter be removed (COX *et al.*, 2000).

2.2.2.2. *Filamentous fungi* as expression hosts

Filamentous fungi are multicellular micro-organisms that have a thick cell wall. Fungi are widely used in industry for large-scale production of industrial enzymes used in food and fruit processing, in baking as well as in detergents (SU *et al.*, 2012). Filamentous fungi such as *Aspergillus niger*, and *Trichoderma reesei* and a few others are used in recombinant protein production. These species possess important characteristics, including an efficient secretion system, being able to glycosylate proteins and have a high specific growth rate (SHUSTER *et al.*, 2002, WIEBE, 2003). Filamentous fungi are known for their high production yield and rate of hydrolytic enzymes which are mainly excreted into the extracellular media. These species have been reported to secrete high amounts of homologous and heterologous protein (SHUSTER *et al.*, 2002).

Aspergillus species has been used for decades in industry and is generally regarded as safe (GRAS) by the USFDA. *Aspergillus niger* is also used in biotransformation and waste treatment. This species relies on strong constitutive promoters for recombinant protein production including the glyceraldehydes-3-phosphate dehydrogenase (*GPD3*) promoter. Widely used inducible promoters include the glucoamylase (*GlaA*) and the alcohol dehydrogenase (*alc*) promoters. The *A. nidulans alcA* promoter is repressed by glucose, but is induced by ethanol (TOEW *et al.*, 2004). The *alcA* promoter is often used for studying the function of endogenous genes in *Aspergillus*.

The glucoamylase (GlaA) promoter that is found in the *Aspergillus* species functions similar to the *CBHI* found in *T. reesei*. The *GlaA* promoter has been intensively studied and used in heterologous protein production. Although the *GlaA* promoter is a very strong promoter with the ability to produce 20g per litre of glucoamylase from a single copy of the gene, it is repressed by the sugar xylose. This promoter is induced by starch, maltose and low concentration of glucose (FOWLER *et al.*, 1990). Unfortunately, there has been medical cases such as lung infection reported in immune-compromised patients, therefore *A. niger* needs careful handling to avoid formation of spore dust (SHUSTER, 1989, SHUSTER *et al.*, 2002).

Another filamentous fungus that is widely studied and used in heterologous protein production is *Trichoderma reesei* (WIEBE, 2003, WARD, 2011). In its natural environment *T. reesei* degrades plant material in the soil. This fungus is noted for its ability to produce 100 g/L of extracellular protein (60% of secreted protein is *CBHI* and 20% *CBHI*enzymes) (DEMAIN and VAISHNAV, 2009). It is one of the best known fungi in biomass processing because it is a prolific producer of cellulases and hemicellulases. The *T. reesei CBHI* promoter is often used in industry, because it is very strong and can also be used in other recombinant hosts organisms (HUANG *et al.*, 2010, ILMEN *et al.*, 2011). This promoter has been used in the production of heterologous protein such as calf chymosin (UUSITALO *et al.*, 1991) as well as in the production of antibody fragments (NNYSSONEN *et al.*, 1993). The disadvantage of filamentous fungi is similar to that of bacteria in that they produce proteases which could degrade heterologous protein products (WARD, 2011).

2.2.2.3. *Saccharomyces cerevisiae* as expression host

Saccharomyces cerevisiae is well-studied yeast in traditional biotechnology. This yeast has been used for decades in the production of fermented food and alcoholic beverages (MADIGAN and MARTINKO, 2006, CELIK and CALIK, 2012). There are several reports on the use of *S. cerevisiae* in recombinant protein production (HARFORD *et al.*, 1987, OSTERGAARD *et al.*, 2000, MATTANOVICH *et al.*, 2012). *S. cerevisiae* has been used as an expression host for the production of recombinant vaccines that are effective against human viral infections such as hepatitis B (HARFORD *et al.*, 1987). In addition, *S. cerevisiae* has been used for the production of most of the recombinant therapeutics that are approved by the USFDA and the European Medicines Agency (EMA). This includes products such as insulin, urate oxidase, and glucagons (CELIK and CALIK, 2012). Characteristics that made *S. cerevisiae* dominant include well-developed tools for genetic

manipulation, genetics and biochemistry that have been well-studied, eukaryotic secretory pathway, eukaryotic post-translational modification pathway with *N*-linked and *O*-linked glycosylation, and *S. cerevisiae* has generally regarded as safe (GRAS) status (STRAUSBERG and STRAUSBERG, 1995). A major disadvantage of *S. cerevisiae* for recombinant protein production is its inefficient transport of proteins to the extracellular media especially high molecular weight mammalian proteins (STRAUSBERG and STRAUSBERG, 1995, FERENDAHL *et al.*, 2010b).

Saccharomyces cerevisiae is well-established in the ethanol industry. It has been and is still used for the production of bioethanol from sugar, starch and recently also from hydrolysed cellulose. In a manner similar to bacteria, (HAHN-HÄGERDAL *et al.*, 2007) *S. cerevisiae* grows fast and it is easy to modify genetically unlike methylotrophic yeasts and filamentous fungi. *S. cerevisiae* has the ability to perform post-translational modification and it also contains strongly regulated promoters (NEGRUITA *et al.*, 2010). Features that make *S. cerevisiae* a good candidate for the production of bioethanol from lignocelluloses is its ability to utilize and ferment hexose sugar efficiently in aerobic and anaerobic conditions (HAHN-HÄGERDAL *et al.*, 2007). Furthermore, it has the ability to tolerate high ethanol concentration and microbial inhibitors often found in lignocellulosic hydrolysates (KUYPER *et al.*, 2003, HAHN-HÄGERDAL and PAMMENT, 2004). These are important features to consider when selecting biofuel production organisms.

S. cerevisiae is a Crabtree positive yeast and when cultivated on glucose it converts most of the carbon in sugars to ethanol producing low amounts of biomass (DE DEKEN, 1966, PFEIFFER and MORLEY, 2014). High levels of protein production are linked to the amount of biomass that is obtained during fermentation (VERDUYN *et al.*, 1992). *S. cerevisiae* should produce high amounts of biomass when cultivated on xylose, since xylose would not result in the Crabtree effect. This yeast has many strong constitutive and inducible promoters that can increase recombinant protein production. Some of these promoters are repressed in the presence of glucose, but it is not known how they function during growth on xylose.

Unfortunately, *S. cerevisiae* can neither ferment nor use xylose as a carbon source, but it has been engineered to do so. A Dutch group was the first to report the successful expression of a xylose isomerase (XI) in *S. cerevisiae* (KUYPER *et al.*, 2003). The xylose isomerase pathway is most common in bacteria while the xylose reductase, xylitol

dehydrogenase pathway (XR/XDH) is mostly found in fungi (Figure 2.2). The XI pathway involves the use of a xylose isomerase that isomerizes D-xylose to D-xylulose in a single step without co-factors involved (PATRICK, 1968, CHEN, 1980). Xylulose is then phosphorylated by a xylulokinase enzyme (XKS) to D-xylulose-5-phosphate, which enters the pentose phosphate pathway where it is converted to glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate is converted to pyruvate during glycolysis which is ultimately converted to ethanol. The XR/XDH pathway involves the conversion of xylose first to xylitol and then to xylulose. This often leads to accumulation of xylitol and a co-factor imbalance especially under anaerobic conditions (HAHN-HÄGERDAL *et al.*, 2007)(Figure 2.2).

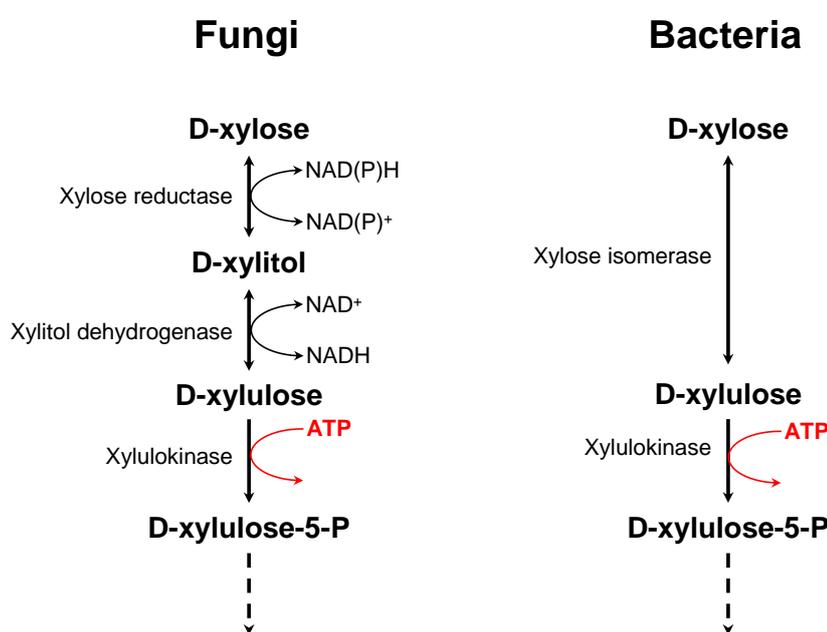


Figure 2.2. Outline of the xylose metabolism pathway for production of ethanol by fungi and bacteria (KUYPER *et al.*, 2003).

2.3. *Saccharomyces cerevisiae* expression vectors

One of the most important tools that made recombinant DNA technology possible is the DNA molecule called a plasmid. Plasmid DNA is an extra-chromosomal, circular, double stranded (dsDNA) molecule that replicates independently of the chromosome. Natural plasmids contain a gene that provides a benefit to the host cells. Furthermore, a plasmid usually has a symbiotic relationship with the host (MADIGAN and MARTINKO, 2006). Plasmids act as vehicles that carry a foreign gene to a new host organism.

Most *S. cerevisiae* cloning plasmids contain common components (Figure 2.3).

Selectable marker – this can be genes that provide resistance to a certain antibiotic and is used to select for organisms carrying the vector with the gene of interest.

Origin of replication - allows the plasmid to replicate independently of the host chromosome

Multiple Cloning Sites - a region containing different restriction sites in which a DNA fragment can be inserted.

After the gene fragment has been inserted into the plasmid, the plasmid was then transformed into the *S. cerevisiae* host (ROMANOS *et al.*, 1992). The plasmid then replicates to high copy number in the host.

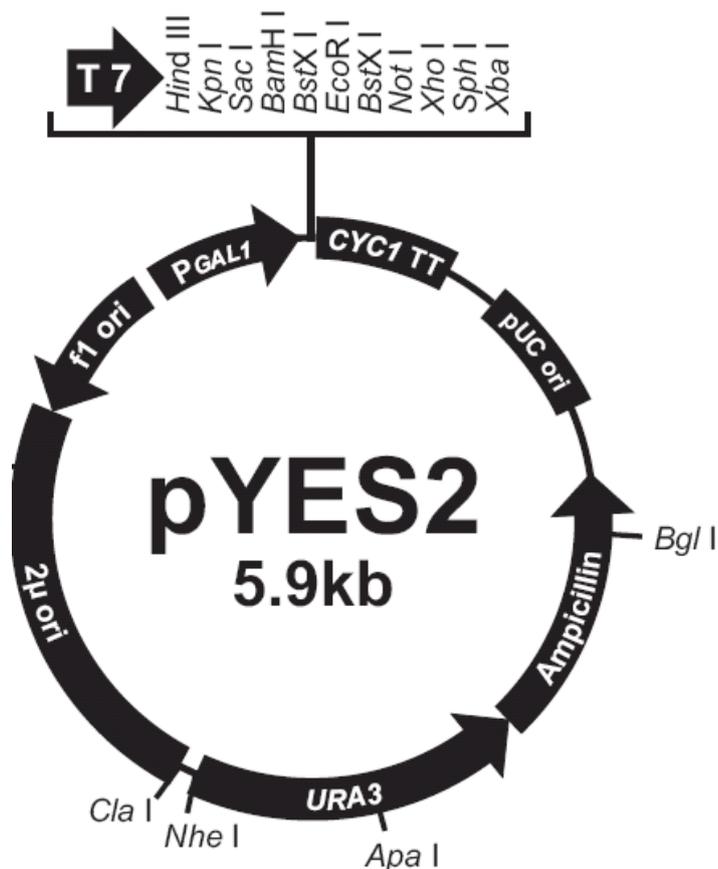


Figure 2.3. A schematic representation of a restriction map of a typical *S. cerevisiae* episomal expression vector (LODISH *et al.*, 2000).

A shuttle vector is a vector that can replicate both in bacteria (*E. coli*) and in yeast (*S. cerevisiae*). These vectors contain selectable markers for prokaryotes and for eukaryotes. In yeast, recessive markers are often used for selection of transformants. Recessive markers for *S. cerevisiae* are usually genes encoding enzymes involved in the synthesis of a particular amino acid or nucleotide. Host strains contain a mutation of the corresponding gene and is complemented by the plasmid vector (ROMANOS *et al.*, 1992, MADIGAN and MARTINKO, 2006). There are four different types of the shuttle vectors that have been developed to be used in *S. cerevisiae*. These include:

2.4.1. Episomal plasmid

Yeast episomal plasmids (YEp) are plasmid vectors derived from the 2 μ plasmid and have been engineered to replicate in both *S. cerevisiae* and *E. coli*. Some of these plasmids contain the entire 2 μ plasmid and others just the 2 μ origin of replication. The 2 μ plasmid has the ability to replicate autonomously independent of the host chromosome. These plasmids replicate autonomously using the DNA segment of the yeast 2 μ plasmid. The 2 μ segment serves as the origin of replication and results in high plasmid copy number. The 2 μ segment is also responsible for the high frequency of transformation of YEp vectors. Yeast episomal plasmids contain two copies of a 559 bases long sequence in inverted orientation. This is important for plasmid amplification. There are a number of proteins involved in replication and maintenance of the copy number. These include the Rep1 and the Rep2 proteins. Rep1 and Rep2 are required for stability, stable inheritance and also in the regulation of the copy number (Figure 2.3) (ROMANOS *et al.*, 1992).

2.4.2. Yeast Artificial Chromosome

The yeast artificial chromosome (YAC) is a shuttle vector that can propagate in *S. cerevisiae* and *E. coli*. A YAC also contains selectable markers for selection in both organisms. This plasmid vectors are circular and approximately 12 kilobases in size when manipulated in *E. coli*. This shuttle vector is linear and of very large size typically hundreds of kilobases when used in yeast (Figure 2.4). These vectors confer important features that make them attractive for use in gene cloning. This includes autonomously replicating sequence (ARS) which is necessary for independent replication of the host chromosome. Additionally, it has a centromere (CEN) for segregation at cell division and two telomeres for vector maintenance. The advantage of YACs over other vectors is its ability to accept

large DNA inserts of up to 150 kilobases (BRUSCHI and GJURACIC, 2002). This shuttle vector functions in the same way as yeast chromosomes during meiosis and mitosis (GRIFFITHS *et al.*, 1999, MADIGAN and MARTINKO, 2006). The YAC is as stable as natural chromosomes, provided the three components ARS, CEN and TEL are present and a minimum required size DNA fragment has been inserted (GRIFFITHS *et al.*, 1999).

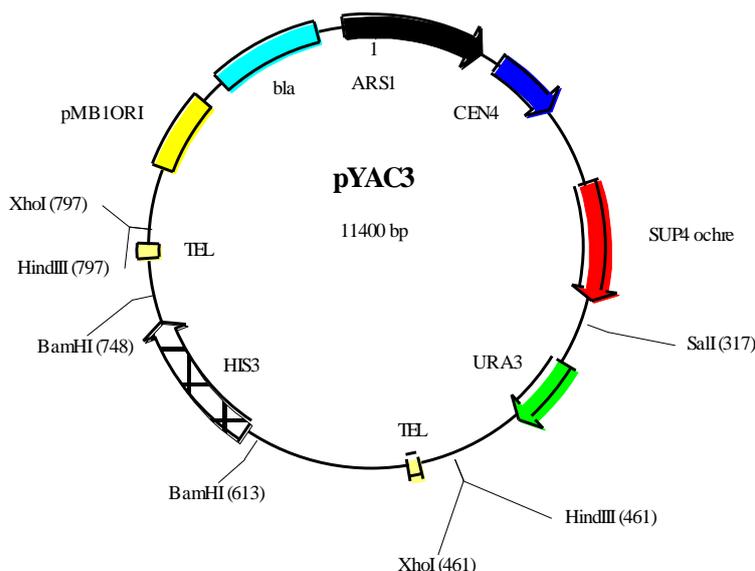


Figure 2.4. A schematic representation of a yeast artificial chromosome (YAC) vector used in recombinant DNA technology. This plasmid vector consists of an Autonomous Replicating Sequence (ARS1), a centromere (CEN4), a telomere (TEL), an origin of replication (pMB1ORI), and an auxotrophic marker (*URA3* & *HIS3*). Prior to transformation of the yeast cells, this plasmid vector is linearised to allow it to function as a yeast chromosome in the yeast cells.

2.4.3. Integrative plasmid (Ylp)

Another widely used shuttle vector is the integrative plasmid (Ylp). Ylp vectors are similar to episomal plasmids (Figure 2.5), but Ylp vectors do not have an origin of replication. Before transformation they are usually linearized and are introduced into the host cell by homologous recombination. This allows for high transformation efficiency and targets integration into the genome of the host organism (ROMANOS *et al.*, 1992). This plasmid is dependent to the yeast chromosome for replication. Ylp plasmids use the origin of

replication of the host chromosome. The plasmid is often introduced at a single locus in the cells and results in single copy of the plasmid (SIKORSKI and HIETER, 1989). Plasmid integration results in less loss of the plasmid during generation in the absence of selection (HINNEN *et al.*, 1978).

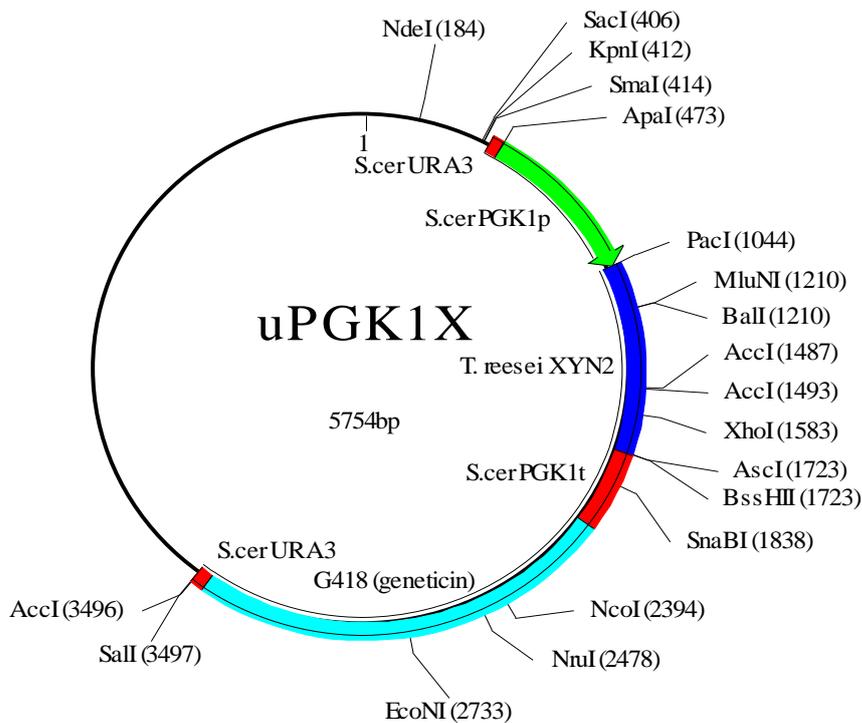


Figure 2.5. The schematic representation of the restriction map of an integrative plasmid vector. This integrative plasmid consists of a selectable marker (*URA3*) an expression cassette (*S. cerPGK1p*), *T. reesei XYN2*, *S. cer PGK1_t*, G418 –Geneticin 418.

2.5. *Saccharomyces cerevisiae* selectable markers

A very important component of all expression vectors is a selection marker that is used for selection of transformants. The use of genes as selectable marker has become one of the most important tools in recombinant DNA technology. There are two types of selectable markers, namely complementation (auxotrophic) and dominant selectable markers (ROMANOS *et al.*, 1992).

Dominant selection markers confer resistance to antibiotics or other toxic compounds and can be used either in yeast or in bacteria. The most widely used dominant markers in yeast

are G418 and cyclo-hexamide (ROMANOS *et al.*, 1992). These are added to the growth media at a certain concentration and inhibit the growth of untransformed yeast cells. The disadvantage of these markers is that they also affect the cellular function of the organisms, which has the resistance gene.

Complementation markers are genes that complement an auxotrophic mutation in the genome. The genes that are mostly used as markers in *S. cerevisiae* are *URA3*, *TRP1*, *HIS3* and *LEU2*. These auxotrophic markers are used in selection of different types of expression systems and they can be used in episomal, integrative and centromeric plasmid.

The *URA3* gene encodes orotidine-5-phosphate decarboxylase (ODCase), which catalyses one of the reactions involved in the synthesis of pyrimidine ribonucleotides in yeast RNA. The *URA3* gene can be used for negative or positive selection. Positive selection is carried out by auxotrophic complementation of the *URA3* mutation and negative selection based on specific inhibitors. This is usually done by growing the mutants on 5-fluoro-orotic acid (FOA), which inhibits the growth of the strains that has intact *URA3* gene. These strains will not survive because orotidine-5-phosphate decarboxylase will convert 5-fluorouracil to toxic compounds. Strains that carry inactive *URA3* gene can grow on the 5-FOA. The *URA3* marker is widely used in yeast episomal plasmids. Disruption of the uracil phosphoribosyltransferase (*FUR1*) gene enables auto selection of episomal plasmids (KERN *et al.*, 1990)

Other genes that are used as selective markers include the *LYS1* gene which encodes α -aminoadipate reductase which is required for biosynthesis of lysine. This gene is large and contains many restriction sites, limiting the number of unique site available for cloning. Additionally, yeast markers include *ADE1* and *ADE2* that encode for phosphoribosylamino-imidazole succinocarbozamide synthetase and phosphoribosylamino-imidazole-carboxylase which are involved in adenine synthesise (VICKERS *et al.*, 2013). These genes are both used to complement auxotrophic mutations.

2.6. *Saccharomyces cerevisiae* promoters

Promoters are DNA control elements necessary to drive and control the expression of genes under their control. A promoter is positioned upstream of each gene on the chromosome. DNA control elements usually differ in how they are regulated or

activated/deactivated. They initiate transcription by binding RNA polymerase II, which is responsible for generation of mRNA. Additionally, the strength of promoters differs and some are controlled by environmental conditions or substrates. Because of these, the choice of promoter in heterologous protein production is important (MADIGAN and MARTINKO, 2006).

When choosing a promoter it is important to consider the strength of the promoter and the gene expressed, since the gene can influence the strength of the promoter. Promoter selection is important for yield and productivity optimization. This is because all promoters behave differently under different growth conditions. Furthermore, the level of transcription is determined by the promoters' strength.

There are different types of promoters that are used in heterologous protein production in yeast. These include:

2.6.1. Constitutive promoters

Constitutive promoters are those promoters that are always active in all cells. These promoters offer simplicity (no inducers or repressors needed) and relatively constant levels of expression (DA SILVA and SRINKRISHNAN, 2012). Constitutive promoters direct expression in all tissues and are independent of the environmental and developmental factors (SHUSTER, 1989). One of the important advantages of constitutive promoters is the fact that they make stability of production easier to achieve, on the other hand strong constitutive promoters may have an impact on plasmid stability (LU, 2007). Additionally, because these promoters are always active, this can lead to toxic overproduction of the transcribed gene of interest in the cell. The most widely used promoters for heterologous protein expression are those that are involved in the glycolytic pathway such as the phosphoglycerate kinase 1 (*PGK1*) (OGDEN *et al.*, 1986), pyruvate decarboxylase 1 (*PDC1*) (KELLERMANN *et al.*, 1986) and triose phosphate isomerase 1 (*TPI1*) (ALBER and KAWASAKI, 1982). These promoters are of importance since glycolysis is the best characterised pathway in *S. cerevisiae*.

Phosphoglycerate kinase 1 (PGK1) promoter

The *PGK1* promoter is one of the important strong constitutive promoters in the glycolytic pathway. During glycolysis the *PGK1* enzyme transfers a high energy phosphoryl group from the acyl phosphate of 1,3-diphosphoglycerate to ADP to produce ATP. The *PGK1*

promoter is induced by the presence of glucose (KINGSMAN *et al.*, 1990). There are three transcription factors involved in activation of transcription, namely Rap1p, Abf1p, and Reb1p (HITZEMAN *et al.*, 1980).

This promoter has been used for the expression of numerous heterologous proteins in *S. cerevisiae* (TUIE *et al.*, 1982). When *PGK1*-based vectors are constructed using a high copy number plasmid, the *PGK1* promoter can drive production of the phosphoglycerate kinase enzyme up to 30-40% of total cell protein (JEFFRIES and JIN, 2004, RAGAUSKAS *et al.*, 2006). Although there are reports of lower yields for several proteins expressed using the *PGK1* system (ARISTIDOU and PENTTILÄ, 2000) the behaviour of this promoter on xylose is unknown.

Enolase 2 promoter

Enolase is one of the enzymes present in the glycolytic pathway. Enolase (EC4.2.1.11) is a metalloenzyme and in the glycolysis pathway it is involved in the conversion of the 2-phosphoglycerate to phosphoenolpyruvate the ninth step in glycolysis and the reverse reaction during gluconeogenesis. *S. cerevisiae* has two enolase genes (*ENO1* and *ENO2*) per haploid genome. *ENO1* and *ENO2* encode very similar proteins but the expression of these genes is differentially regulated. MCALISTER and HOLLAND, (1982) reported that when the laboratory strains of *S. cerevisiae* is grown on glucose, the *ENO2p* predominates and, on non-fermentable carbon sources, such as ethanol or glycerol + lactate, there are similar amounts of the two proteins (*ENO1* and *ENO2*). This suggests that the *ENO2* gene contains regulatory regions that mediate glucose-dependent induction of gene expression (COHEN *et al.*, 1987).

Furthermore, the *ENO2* promoter is a strong constitutive promoter and is found in all organisms and tissues that are capable of fermentation and glycolysis. This promoter is expected to allow high expression level of the *XYN2* gene under its control when the strain is cultivated on glucose, but it is not known how it will function on xylose. The enolase promoters has been used in several studies for the production of recombinant proteins (MCALISTER and HOLLAND, 1986, PARTOW *et al.*, 2010, SUN *et al.*, 2012).

Glyceraldehyde-3-phosphate (*GPD3*) promoter

The glyceraldehyde-3-phosphate dehydrogenase (*GPD3*) promoter which is also called the *TDH3* promoter is a strong glycolytic promoter. This promoter is well characterised and studied in heterologous protein production (DA SILVA and SRINKRISHNAN, 2012). The

GPD3 enzyme catalyses the phosphorylation of glyceraldehyde-3-phosphate to produce 1, 3-diphosphoglycerate in the glycolysis pathway. The *GPD3* promoter is similar to *PGK1* in that it is also a strong constitutive promoter. *GPD3* is induced in the presence of glucose and the *GPD3* mRNA represent up to 5% of the total mRNA in eukaryotic cells (ROMANOS *et al.*, 1992). Most glycolytic promoters are poorly regulated (constitutively expressed) and are not suitable for the expression of toxic proteins.

2.6.2. Inducible promoters

The performance of inducible promoters is controlled by environmental conditions or external stimuli. Inducible promoters can be artificially controlled. They offer the advantage in heterologous protein production in controlling the time and the level of gene expression (DA SILVA and SRINKRISHNAN, 2012). Among the group of inducible promoters are those whose activity are determined by abiotic factors such as light, oxygen levels, heat, and cold. There are different types of inducible promoters that have been used for the expression of several genes in *S. cerevisiae*. The most tightly regulated native promoters in *S. cerevisiae* are from the galactose-pathway namely; *GAL1*, *GAL7*, and *GAL10* (DOUGLAS and HAWTHORNE, 1964). Inducible promoters are thus more suitable when expression of genes is desired at a specific stage of cell growth, or to prevent the build-up of toxic pathway intermediates (DA SILVA and SRIKRISHNAN, 2012).

Alcohol dehydrogenase II (*ADH2*) promoter

The alcohol dehydrogenase II (*ADH2*) promoter is a glycolytic promoter that is repressed in the presence of glucose. The *ADH2* promoter is not a constitutive promoter but is described as an inducible promoter because it is induced by the absence of some carbon sources such as glucose. This promoter is highly repressed in the presence of glucose and only de-repressed when glucose is depleted. When the glucose is depleted and the cells switch to fermentation phase. The transcription factor Adr1 binds to the upstream activating sequence UAS1 of *ADH2p*. This transcription factor is dephosphorylated when glucose is depleted and the cell switches to growth on ethanol. The advantage of this promoter over other inducible promoter is the fact that it does not require an inducer. Furthermore, this promoter is the preferred promoter for heterologous protein production depending on the heterologous gene being expressed (BADZIONG *et al.*, 1999). This is due to the fact that constitutive expression, performed by a range of very strong promoters like *GPD3p* (WATERHAM *et al.*, 1997) and *PGK1p* (TUIITE *et al.*, 1982) from *S. cerevisiae* is not always

preferable. This is because recombinant proteins can have a toxic effect on their host organism because of constant high level expression. Controllable gene expression can be achieved with derepressed promoters and offer more advantage over other promoters. This promoter has been widely used in recombinant protein production (LEE and DA SILVA, 2005). When this promoter is used to control the expression of heterologous protein, expression will begin once the glucose is depleted. Because this promoter is highly repressed by glucose, a carbon source that does not cause the Crabtree effect in *S. cerevisiae* might be the carbon source that can result in high protein yield when this promoter is used.

Heat inducible (YG100) promoter

The *YG100* (*SSA1p*) promoter is one of the promoters that are induced by shock conditions. *S. cerevisiae* contains 3 heat inducible genes. The *YG100* promoter is expressed at basal levels and is induced by heat shock. *S. cerevisiae* genes for the heat shock proteins are reported to be related to those in *Drosophila*. *YG100* has been characterised and found to have elevated level of transcription after heat shock. The *YG100* promoter is highly induced at 37°C and can be very interesting for use in industry (BRAZZELL and INGOLIA, 1984).

CHAPTER 3. RESEARCH METHODOLOGY

3.1. Introduction

The cell walls of plants consist of cellulose, hemicellulose and lignin. Cellulose consists of a polymer of glucose and has been used for the production of ethanol. The hemicellulose fraction consists mostly of xylan, representing up to 35% of the dry weight in certain plants (LA GRANGE *et al.*, 1996). During paper production and pretreatment for ethanol production from cellulose, hemicellulose is extracted producing slurry consisting of mostly xylose and xylooligosaccharides. This represents a significant amount of carbon, which is often treated as a waste product requiring disposal at considerable cost, especially in the paper and pulping industry.

The yeast *S. cerevisiae* has been used in industry for centuries; therefore the large scale fermentation technology is well established. *S. cerevisiae* has been used for the production of a number of recombinant proteins including hydrolases and proteins of pharmaceutical importance. High level expression of recombinant proteins is usually linked to the amount of biomass obtained during fermentation (FERNDAHL *et al.*, 2010b) as well as the promoter used to drive transcription. Glucose is the preferred substrate for the cultivation of *S. cerevisiae*, however since *S. cerevisiae* is Crabtree-positive yeast; it produces relatively small amounts of biomass when cultivated on glucose, since most of the carbon source is converted to ethanol. Sugars like galactose allow higher biomass production and improved expression from a glucose repressible promoter like *ADH2*. Unfortunately, a cheap abundant source of galactose is not readily available (LA GRANGE *et al.*, 1996).

S. cerevisiae has been genetically engineered to grow on xylose with two different metabolic pathways by two independent groups (KUYPER *et al.*, 2004). The xylose reductase (XR), xylitol dehydrogenase (XDH) pathway was introduced by a Swedish group (WALFRIDSSON *et al.*, 1997) while the xylose isomerase (XI) pathway was introduced by a Dutch group (VAN MARIS *et al.*, 2007). The XR-XDH pathway suffers from a co-factor imbalance and an accumulation of xylitol (VERDUYN *et al.*, 1985). Using this pathway in yeast is therefore not an optimal use of the carbon source available. The XI on the other hand does not require any co-factors and xylose is converted to xylulose in a single step. This allows the yeast to grow to very high biomass concentrations. Preliminary work done at the University of Stellenbosch indicated that it is possible to produce almost double the amount of biomass on xylose compared to growth on glucose when using the XI pathway in

S. cerevisiae. This should in theory lead to the production of higher levels of heterologous protein. Since xylose is not a natural substrate for *S. cerevisiae* to utilize, it is not known how *S. cerevisiae* promoters will react during growth on xylose. The aim of this study is to evaluate 5 commonly used *S. cerevisiae* promoters during growth on xylose and on glucose.

3.2. Materials and methods

3.2.1. Escherichia coli and Saccharomyces cerevisiae strains used

E. coli strain XL1 Blue MRF' (Stratagene) was used for plasmid transformation and propagation. *S. cerevisiae* strain Y294 (*MAT α leu 2-3, 112 ura 3-52, his 3, trp 1-289*) was used as the source of promoter and terminator sequences as well as for promoter evaluation.

3.2.2. Media and culture condition

Bacterial cells were routinely cultured in LB medium [0.5% yeast extract (Merck), 1% NaCl (Merck), 1% tryptone (Sigma)] supplemented with ampicillin (100mg/l)(Sigma) and *S. cerevisiae* in YPD medium [2% yeast extract, 2% Bacto peptone (Sigma) and 2% glucose (Merck)]. Engineered *S. cerevisiae* Y294 was adapted on YPX medium containing [1% yeast extract, 2% Bacto Peptone] with 2% of xylose (Sigma). YPD or YPX plus 20 g Bacteriological agar (Merck) per liter were used for solid media. *S. cerevisiae* Y294 was transformed as described by (HILL *et al.*, 1991). Transformants were selected and maintained on SC^{-ura} medium plates [0.17% yeast nitrogen base w/o amino acids (Sigma) and 0.5% (NH₄)₂SO₄ (Merck), 2% glucose/xylose, 2% agar, and supplemented with amino acids (Sigma) as required]. *FUR1* disrupted strains were maintained on SC^{-ura-leu} medium. SC medium containing 0.2% of 4-O-methyl-D-glucurono-D-xylan–Remazol Brilliant Blue R (RBB-xylan) and 2% glucose or xylose as carbon source and bacteriological agar was used to confirm xylanase activity. RBB-xylan was synthesized according to the methods described by BIELY *et al.* (1988).

3.2.3. Construction of xylose utilizing yeast strains

Plasmid pMJM121 with the xylose isomerase (XI) gene from the *Bacteroides thetaiotaomicron* and the xylulokinase (*XYL3*) from *Pichia stipitis* was integrated into the *S. cerevisiae* Y294 genome at the delta locus using zeocin as selectable marker (MERT

and VAN ZYL, 2015). The *S. cerevisiae* *GRE3* coding for a non-specific aldose reductase can lead to the accumulation of the xylitol. Primers TPRgre-L and TRPgre-R containing 40 bp extensions homologous to *GRE3* were used in polymerase chain reaction (PCR) to amplify *S. cerevisiae* *TRP1* (Table 3.2). The 1050 bp PCR product was used to replace the *GRE3* gene on the chromosome of *S. cerevisiae* Y294. The resulting transformants containing a 300 bp deletion in *GRE3* were selected on SC^{-trp}. It is important to minimize xylitol formation since xylitol is a strong inhibitor of the *B. thetaiotaomicron* xylose isomerase enzyme. After deletion of the *GRE3* gene and integration of MJM121 plasmid, the transformants, Y294 pMJM121 *gre::TRP*, were able to produce colonies with a diameter of 1 mm within 10 days. Through streaking and re-streaking on YPX media Y294 pMJM121 *gre::TRP* was adapted until cells were able to produce a 1 mm diameter colony in 3 days.

Table 3.1. PCR primers used for the isolation of the promoters and terminators use in the study. *TPR1gre3* and *LEU2fur1* were used to disrupt *GRE3* and *FUR1*, respectively. The *XYN2* primers were used to confirm the presence of the xylanase reporter gene. Primer mistakes are highlighted.

Primer name	Primer sequence (5' to 3')	Restriction sites
ENO1-L	GGATCCACTAGTCTTCTAGGCGGGTTATC	<i>Bam</i> HI <i>Spe</i> I
ENO1-M	CTAGAAGGCTTAATCAAAAGCTCTCGAGATCTCGCGA ATTCTTTGATTTAGTGTTTGTGTG	<i>Eco</i> RI <i>Nru</i> I <i>Bgl</i> II <i>Nru</i> I
ENO1-R	AAGCTTGCGGCCGCAAAGAGGTTTAGACATTGG	<i>Not</i> I <i>Hind</i> III
ENO2-L	GGATCCCGGGTATAGTACCTCCTCGCTC	<i>Bam</i> HI <i>Sma</i> I
ENO2-M	CTAATAATTCTTAGTTAAAAGCACTCGAGA T CTCGCGA ATTCTATTATTGTATGTTATAGTATTAGTT	<i>Eco</i> RI <i>Nru</i> I <i>Bgl</i> II <i>Nru</i> I
ENO 2-R	GGATCCATGCATATCACTGTGGTC	<i>Bam</i> HI
GPD-L	AAGCTTCTAGAATATGATTATCCCTGCG	<i>Hind</i> III <i>Xba</i> I
GPD-M	CATTAAAGTAACTTAAGGAGTTACTCGAGATCTCGCGA ATTCTTGTTTTGTTTGTGTTG	<i>Eco</i> RI <i>Nru</i> I <i>Bgl</i> II <i>Nru</i> I
GPD-R	GGATCCGGTACCAACGCTAAGTGATACTCC	<i>Kpn</i> I <i>Bam</i> HI
PGK1-left	CGGGATCCTTAAAGATGCCGATTTGG	<i>Bam</i> HI
PGK1-right	CGGAATTCTATTTGTTGTAAAAAGTAGATAATTAC	<i>Eco</i> RI
YG100-L	AGTCGGATCCGTCGACAAATTGTTACGTTGTGCTTTG	<i>Bam</i> HI
YG100-R	TGACGAATTCATTATCTGTTATTTACTTGAATTTTTGTTT CTTGTAATACTTG	<i>Eco</i> RI
XYN2-left	ATGGTCTCCTTCACCTCCCTCCTC	
XYN2-right	TTAGCTGACGGTGATGGAAGCAGAG	
FUR1L	ATTTCTTCTTGAACCATGAAC	
FUR1R	CTTAATCAAGACTTCTGTAG	
TRPg _{gre} -L	GCGATATGGGACTTGATTATTTAGACCTGTATTATATT CAAGTGCCACCTGACGT	
TRPg _{gre} -R	GTTTTTGCCAACTGTAAGTCCATCTCAATGAATGATTG AGGACGGATATTAGGCTGTA	

3.2.4. Promoter isolation

Total chromosomal DNA was isolated from *S. cerevisiae* Y294 using the method described by HOFFMAN and WINSTON (1987) and used as template for polymerase chain reaction (PCR) amplifications of the various promoters and terminators. The PCR primers (Table 3.1) were based on the DNA sequences of *S. cerevisiae* (Genbank). All the primers were designed with suitable restriction enzyme sites to allow for downstream cloning into vectors and the eventual insertion of the *XYN2* gene. The PCR was done in a 50 µl reaction (10 pmol of each primer, Taq reaction buffer, 1 mM MgCl₂, 200 µM each deoxynucleoside triphosphate, 2 µl of *S. cerevisiae* genomic DNA [20 ng/µl] and 2.5 U of Taq DNA

polymerase [Promega Corporation, USA]) with a Perkin Elmer GeneAmp® PCR System 2400 (The Perkin-Elmer Corporation, Norwalk, Connecticut). Overlap PCR was used to isolate the different promoters and terminators.

3.2.5. Construction of the pGEM_{PT} vectors.

After obtaining PCR products for the *ENO2* promoter and terminator (*ENO2_{PT}*) and *GPD3_{PT}*, the fragments were cloned into the pGEM-T easy vector system (Promega Corporation, USA) creating pGEM-*ENO2_{PT}* and pGEM-*GPD3_{PT}*, respectively (Table 3.2). The nucleotide sequence of the promoter and terminator cassettes were verified by amplifying DNA fragments with the Big Dye Terminator Cycle sequencing Reader with Amplitaq DNA polymerase F5 (Applied Biosystems kit) using fluorescently labeled nucleotides, and reaction mixtures were subjected to electrophoresis on an Applied By systems automatic DNA sequencer (model ABI Prism™ 377). Sequence data was analysed by using the PC/GENE software package (IntelliGenetics, Inc. Mountain View, California).

Table 3.2. Microbial strains and plasmids sequence and names.

Strain or plasmid	Relevant genotype	Source or reference
Strains:		
<i>S. cerevisiae</i> Y294	<i>MATα leu 2-3, 112 ura 3-52, his 3, trp 1-289</i>	This laboratory
<i>E. coli</i> XL1-Blue MRF'	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB lacI^qZΔM15 Tn10 (Tet^r)]</i>	ZAP-cDNA Synthesis kit Stratagene
Plasmids:		
pGEM-T easy	<i>bla</i>	Promega
YEp352	<i>bla URA3</i>	HILL <i>et al.</i> (1986)
YEp352*	<i>bla URA3 (EcoRI site destroyed)</i>	This laboratory
pDF1	<i>bla fur1::LEU2</i>	LA GRANGE <i>et al.</i> (1996)
pJC1	<i>bla URA3 PGK1_P-PGK1_T</i>	CROUS <i>et al.</i> (1995)
pDLG1	<i>bla URA3 ADH2_P-ADH2_T</i>	LA GRANGE <i>et al.</i> (1996)
pDLG5	<i>bla URA3 ADH2_P-XYN2-ADH2_T</i>	LA GRANGE <i>et al.</i> (1996)
pDLG82	<i>bla URA3 PGK1_P-XYN2-ADH2_T</i>	This work
pGEM- <i>ENO2</i> _{PT}	<i>bla ENO2_P-ENO2_T</i>	This work
pGEM- <i>GPD3</i> _{PT}	<i>bla GPD3_P-GPD3_T</i>	This work
pDLG1- <i>ENO1</i> _{PT}	<i>bla URA3 ENO1_P-ENO1_T</i>	This work
YEp352*- <i>ENO2</i> _{PT}	<i>bla URA3 ENO2_P-ENO2_T</i>	This work
pDLG1- <i>GPD3</i> _{PT}	<i>bla URA3 GPD3_P-GPD3_T</i>	This work
pAZ2	<i>bla URA3 ENO2_P-XYN2-ENO2_T</i>	This work
pAZ3	<i>bla URA3 GPD3_P-XYN2-GPD3_T</i>	This work
pAZ4	<i>bla URA3 PGK1_P-XYN2-PGK1_T</i>	This work
pDLG23	<i>bla URA3 YG100_P-XYN2-ADH2_T</i>	This work

3.2.6. Construction of the YEp_{PT} vectors

The *ADH2*_{PT} fragment was removed from pDLG1 (LA GRANGE *et al.*, 1996) by restriction enzyme digestion with *Bam*HI and *Hind*III. The *GPD3*_{PT} fragment was isolated from their corresponding pGEM_{PT} vectors and cloned into the *Bam*HI and *Hind*III sites of the pDLG1 vector creating pDLG1-*GPD3*_{PT}. The *ENO2*_{PT} fragment was isolated from the pGEM-*ENO2*_{PT} vector and cloned into the *Bam*HI site of the Yep352* vector resulting in Yep352*-*ENO2*_{PT} (Table 3.2).

3.2.7. Construction of the XYN2 expression vectors

The *XYN2* gene was obtained from plasmid pDLG5 as a 780-bp fragment by digesting with *Bgl*II and *Eco*RI. This fragment was subsequently cloned between the promoter and terminator sequences, into the corresponding sites of *Yep352**-*ENO2*_{PT} resulting in plasmid pAZ2 (Table 3.2). Sequence analysis of the *GPD3*_{PT} clones revealed a T missing in the *Bgl*II site of the overlap primer GPD-M. Therefore, to construct pAZ3, consisting of pDLG1-*GPD3*_{PT} with the *XYN2* gene, pDLG1-*GPD3*_{PT} was digested with *Xho*I and pDLG5 with *Bgl*II. Both these 5' overhangs were filled with the Klenow enzyme to create blunt ends. Both vectors were subsequently digested with *Eco*RI, the *XYN2* fragment was isolated and cloned into the corresponding sites of pDLG1-*GPD3*_{PT}. To create pAZ4, the *PGK1* promoter was isolated from *S. cerevisiae* genomic DNA using PCR primers PGK1-left and PGK1-right. The PCR product was digested with *Bam*HI and *Eco*RI and cloned into the corresponding sites of pDLG5 to create pDLG82. pJC1 plasmid was digested with *Bgl*II and *Hind*III and cloning the resulting 278 bp *PGK1*_T fragment into the *Bgl*II and *Hind*III sites of pDLG82, thereby replacing the *ADH2*_T with the *PGK1*_T in pDLG82 to create pAZ4. The *YG100* promoter was also isolated from *S. cerevisiae* genomic DNA using PCR primers YG100-left and YG100-right. The 570 bp PCR product was digested with *Bam*HI and *Eco*RI and cloned into the corresponding sites of pDLG5 to create pDLG23. The constructed plasmid vector was confirmed using DNA sequencing, Sequence data was analysed by using the PC/GENE software package (IntelliGenetics, Inc. Mountain View, California). Restriction enzymes used were purchased from Thermo scientific and used as recommended by the supplier.

3.2.8. Transformation and confirmation of transformants

The five expression vectors constructed were transformed into the adapted xylose utilizing strain. The expression cassettes were transformed using the method described by HILL *et al.*, (1991). The transformants were grown on SC^{-ura} media with 2% glucose and 2% bacteriological agar. Genomic DNA was isolated from the transformants using the method by HOFFMAN and WINSTON (1987). PCR primers XYN2-left and XYN2-right (Table 3.1) were used to confirm the presence of the 780 bp xylanase reporter gene. Positive transformants were further confirmed by spotting on RBB-xylan plates containing 2% glucose or xylose as carbon source. β -Xylanase cleaves RBB-xylan into small fragments

which can diffuse through the agar, producing clearing zone around colonies secreting an active xylanase (BIELY *et al.*, 1988).

3.2.9. Construction of Auto-selective strains

After transformation of the *URA3* based promoter plasmids into the xylose utilizing *S. cerevisiae* Y294, the strains were made auto-selective by disrupting the *FUR1* gene. The *FUR1* gene of all the promoter evaluation strains was disrupted to ensure maintenance of the episomal plasmid under non-selective growth conditions. This was done using a *fur1::LEU2* allele isolated from plasmid pDF1 (LA GRANGE *et al.*, 1996). Plasmid pDF1 was digested with restriction enzymes, *NciI* and *NcoI* to isolate the *fur1-LEU2-1* disruption cassette. This cassette was transformed to all the yeast strains with different plasmids that have different promoters in order to disrupt the uracil phosphoribosyltransferase (*FUR1*) gene, thereby creating auto-selective strains. Yeast transformants were selected on SC^{-ura-leu} medium. To confirm the *fur1* gene disruption, total genomic DNA was isolated from all strains using the method described by HOFFMAN AND WINSTON (1987). The genomic DNA was used as the PCR template to amplify the *FUR1* region using primers FUR1L and FUR1R (Table 3.1) The disrupted *fur1* allele produce a 2568 bp band and the undisrupted allele a 590 bp band.

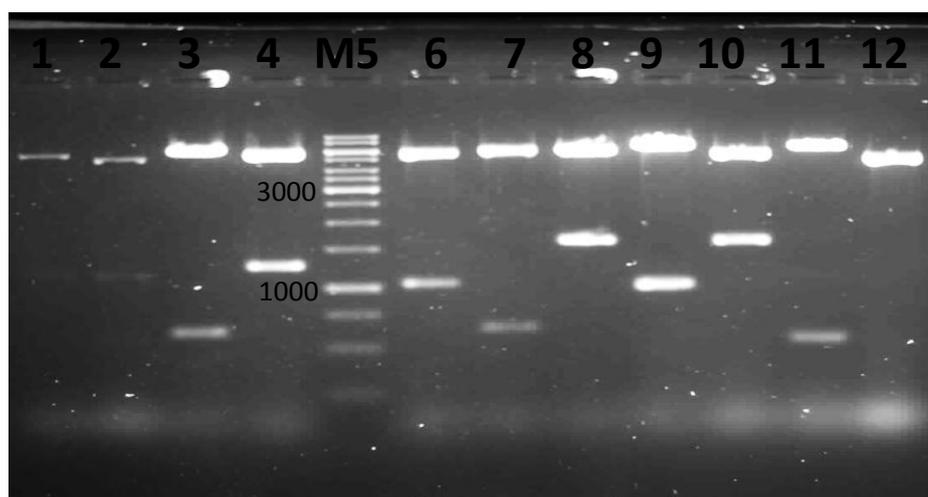
3.2.10. Determination of xylanase of activity

Growth and xylanase activity were monitored over a 312 hour period. The experiment was done in triplicate in 250 ml Erlenmeyer flasks containing 50 ml YPD or YPX. Assay flasks were inoculated to an OD_{600nm} of 0.1. Overnight cultures were used to inoculate YPD medium while YPX was inoculated with 96 hour old precultures in YPX. Flasks were incubated on a rotary shaker at 200 rpm and at 30°C. YPX cultures were sampled every 24 hours while glucose cultures were sampled every 12 hours. Xylanase activity determinations were done in 50 mM sodium-citrate buffer at pH 5 and 50°C for 5 min as described by BAILEY *et al.* (1992). The culture supernatant was used as source of β -xylanase. All activities were expressed in katal/ml, were 1 katal is the amount of enzyme needed to produce 1 mol of reducing sugar (D-xylose equivalent) from beechwood xylan per second.

3.3. Results

3.3.1. Confirmation of the constructed expression cassettes

The plasmids constructed for evaluation of promoters included pAZ2 (*ENO2p&ENO2t*), pAZ3 (*GPD3p&GPD3t*), pAZ4 (*PGK1p &PGK1t*), pDLG5 (*ADH2p&ADH2t*) and pDLG23 (*YG100p &ADH2t*) and yEP352. The above mentioned plasmids were confirmed by DNA sequencing of the function between promoter and *XYN2* and restriction enzyme digestion were used to confirm plasmids constructions.



	1	2	3	4	5	6	7	8	9	10	11
Plasmid	pAZ2	pAZ2	pAZ3	pAZ3	pAZ4	pAZ4	DLG5	DLG5	DLG23	DLG23	YEp352
Restriction enzymes	E/H	E/B	E/H	E/B	E/H	E/B	E/H	E/B	E/H	E/B	E/H
Promoter	<i>ENO2</i>	<i>ENO2</i>	<i>GPD</i>	<i>GPD</i>	<i>PGK</i>	<i>PGK</i>	<i>ADH</i>	<i>ADH</i>	<i>YG100</i>	<i>YG100</i>	Control
Bands expected	5729 1196	5186 1166 573	6428 619	5774 1273	5814 1058	6214 660	6798 1644	6798 1032	5619 1631	6680 570	5181

Key : E/H – EcoRI/HindIII
 E/B – EcoRI/BamHI
 M - 1 Kb DNA ladder molecular mass marker

Figure 3.1. Restriction digest of the six plasmids constructed for the study. Plasmids were digested with restriction enzymes and the fragments separated on a 1% agarose gel. The table below the gel image indicates the expected band sizes.

3.3.2. Confirmation of plasmid transformation

Once the xylose utilizing *S. cerevisiae* Y294 strain was transformed with pAZ2 (*ENO2p-XYN2-ENO2t-URA3*), pAZ3 (*GPD3p-XYN2-GPD3t-URA3*), pAZ4 (*PGK1p-XYN2-PGK1t-URA3*), pDLG5 (*ADH2p-XYN2-ADH2t-URA3*) and pDLG23 (*YG100p-XYN2-ADH2t-URA3*)

or YEp352 (HILL *et al.*, 1986), the genomic DNA was isolated from selected transformants. The genomic DNA was used as template for polymerase chain reaction (PCR) to amplify the *XYN2* open reading frame using primers *XYN2*-left and *XYN2*-right (Figure 3.2). The expected band after the PCR was 780 bp.

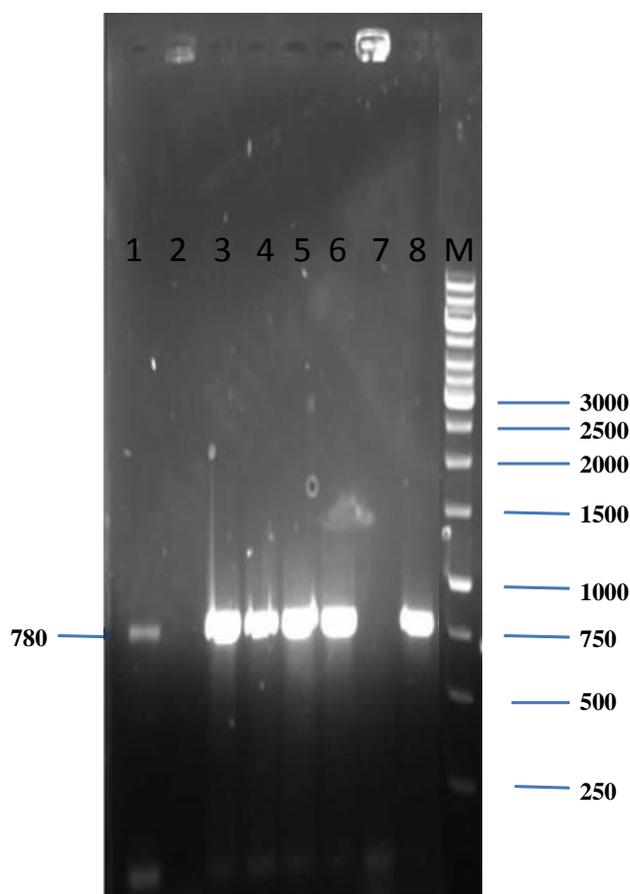


Figure 3.2. Confirmation of the presence of the *XYN2* gene in all the *S. cerevisiae* yeast strains transformed with an expression vector. (Lane 1) Positive control, (Lane 3) Y294 *gre3::TRP1* pAZ2 (*ENO2p-XYN2-ENO2t*), (Lane 4) Y294 *gre3::TRP1* pAZ3 (*GPD3p-XYN2-GPD3t*), (Lane 5) Y294 *gre3::TRP1* pAZ4 (*PGK1p-XYN2-PGK1t*), (Lane 6). Y294 *gre3::TRP1* pDLG23 (*YG100p-XYN2-ADH2t*), (Lane 8) Y294 *gre3::TRP1* pDLG5 (*ADH2p-XYN2-ADH2t*), (Lane M) 1 kb gene ruler.

3.3.3. Screening for β -xylanase activity

β -Xylanase activity was further confirmed in the xylose utilising Y294 strains after transformation of the yeast cells, with plasmids expressing the *XYN2* under the control of the *ENO2p*, *GPD3p*, *YG100p*, *PGK1p* and *ADH2p* promoters. Colonies were spotted on SC^{-ura} media containing RBB-xylan with either glucose or xylose as carbon source. All

recombinant *S. cerevisiae* strains with the *XYN2* produced clearing zones. This indicates their ability to degrade xylan during growth on glucose and xylose. *S. cerevisiae* Y294 with the control plasmid, YEp352 plasmid, did not produce clearing zone.

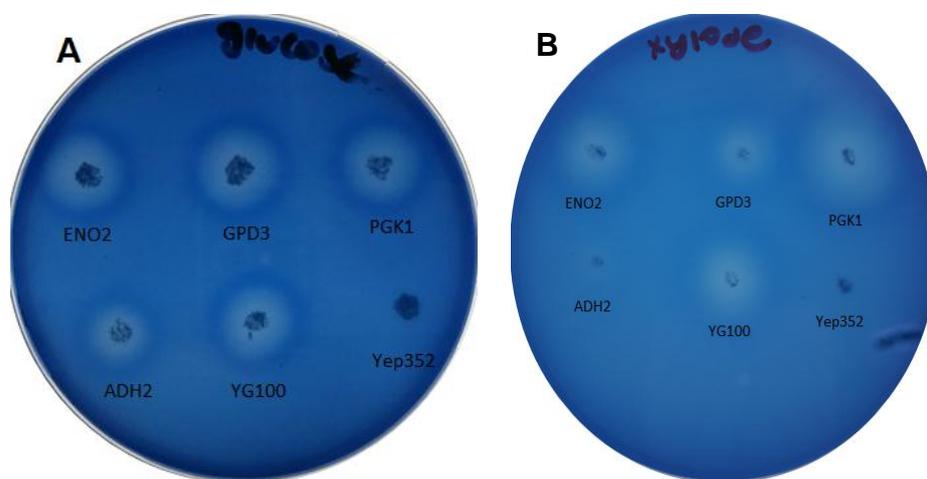


Figure 3.3. Confirming the presence of an active xylanase gene in all the *S. cerevisiae* *gre3::TRP1*pMJM121 transformants. Cells were spotted on SC^{-ura} plates with RBB-xylan as indicator of xylanase activity. Plates contained either A) glucose or B) xylose as carbon source. Transformants expressed the *XYN2* under the control of *PGK1*, *ENO2*, *GPD3*, *ADH2* or *YG100* promoter. Plates were incubated at 30°C, and photographed after 24 h (glucose) and 48 h (xylose).

3.3.4. Confirmation of the *FUR1* gene disruption.

Some of the xylose utilizing strains grew poorly on SC^{-ura} medium while some did not grow at all. Auto-selection of the *URA3* based plasmids was used in order to allow cultivation of transformants on YPD and YPX. The *FUR1* gene in the recombinant *S. cerevisiae* was disrupted as described in the materials and methods section and the disruption on the genome was confirmed by PCR. Successful disruption resulted in a 2568bp PCR fragment (Figure 3.4).

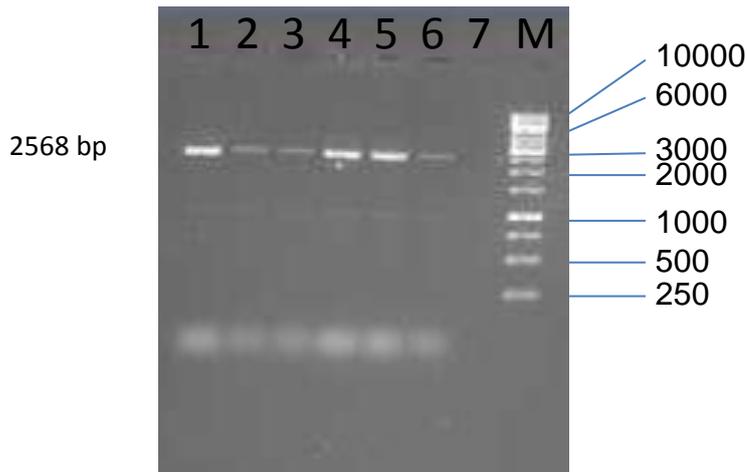


Figure 3.4. Image of a 1% agarose gel depicting the fragments obtained after PCR to amplify the *fur1-LEU2-fur1* cassette from each of the promoter evaluation strains. (Lane 1) Y294 *gre3::TRP1* pAZ2 (*ENO2p-XYN2-ENO2t*), (Lane 2) Y294 *gre3::TRP1* pAZ3 (*GPD3p-XYN2-GPD3t*), (Lane 3) Y294 *gre3::TRP1* pAZ4 (*PGK1p-XYN2-PGK1t*), (Lane 4). Y294 *gre3::TRP1* pDLG23 (*YG100p-XYN2-ADH2t*), (Lane 5) Y294 *gre3::TRP1* pDLG5 (*ADH2p-XYN2-ADH2t*), (Lane 6) YEp352 control, (Lane M) 1 kb gene ruler.

3.3.5. Determination of xylanase activity

Xylanase activity was measured as an indicator of the strength of each promoter during growth on xylose compared to growth on glucose as carbon source (BAILEY *et al.*, 1992). All graphs were scaled the same to aid comparison of data.

All *S. cerevisiae* Y294 *gre3::TRP* pMJM121 strains produced on average, half the amount of biomass on xylose when compared to biomass on glucose (Figures 3.5 to 3.10). This was unexpected since unlike glucose, abundant amount of biomass was expected on xylose. Except for the *ADH2p* (figure 3.10) all promoters produced approximately half the amount of xylanase on xylose compared to glucose. Surprisingly, the *YG100p* (figure 3.9) produced fairly high xylanase activity, without induction at an increased temperature.

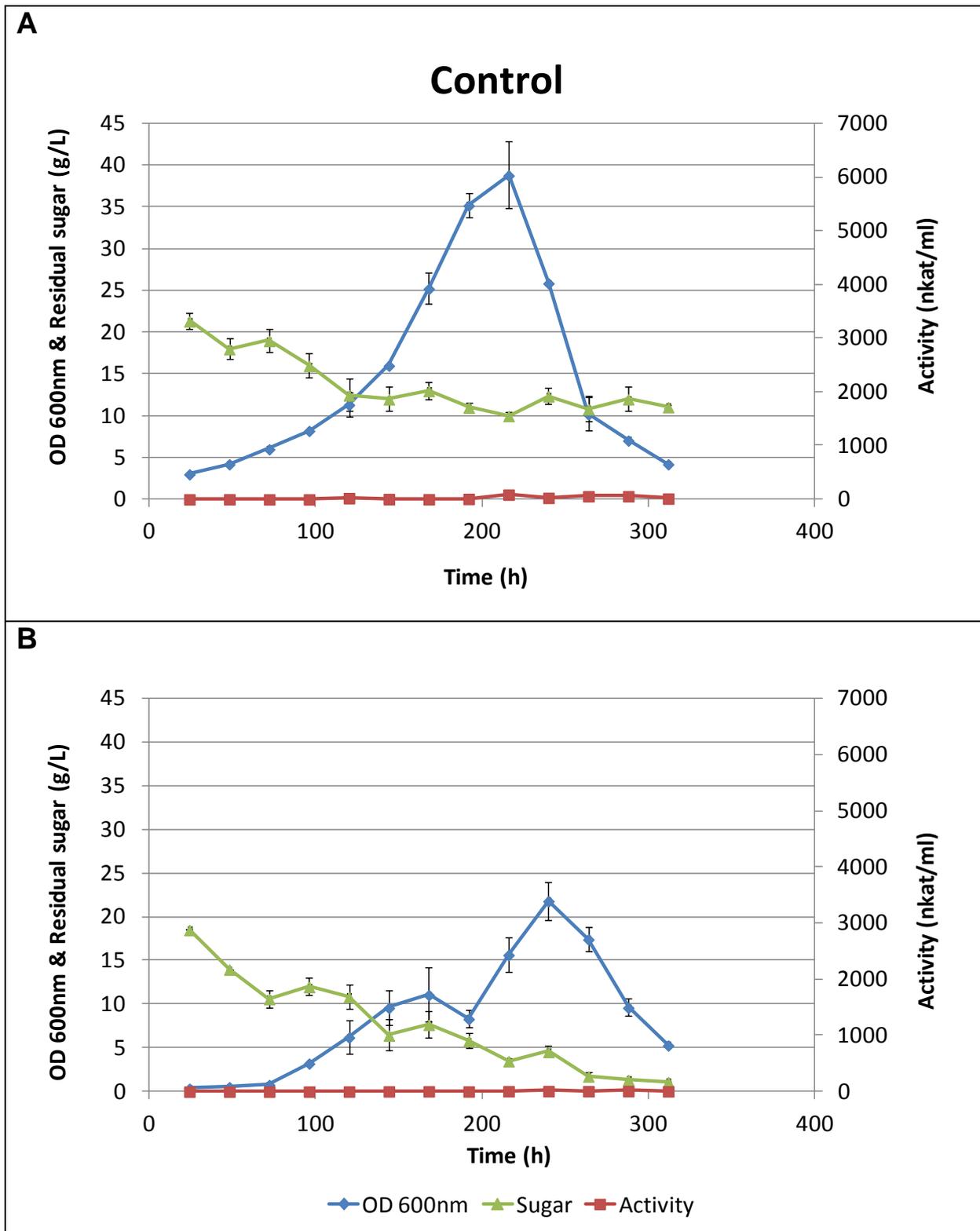


Figure 3.5. Time course of β -xylanase activity, residual sugar and cell growth of *S. cerevisiae* Y294 *gre3::TRP1* pMJM121 YEp352 (control strain without *XYN2*) during growth on (A) glucose and (B) xylose. Samples were collected every 24 hours on both glucose/xylose.

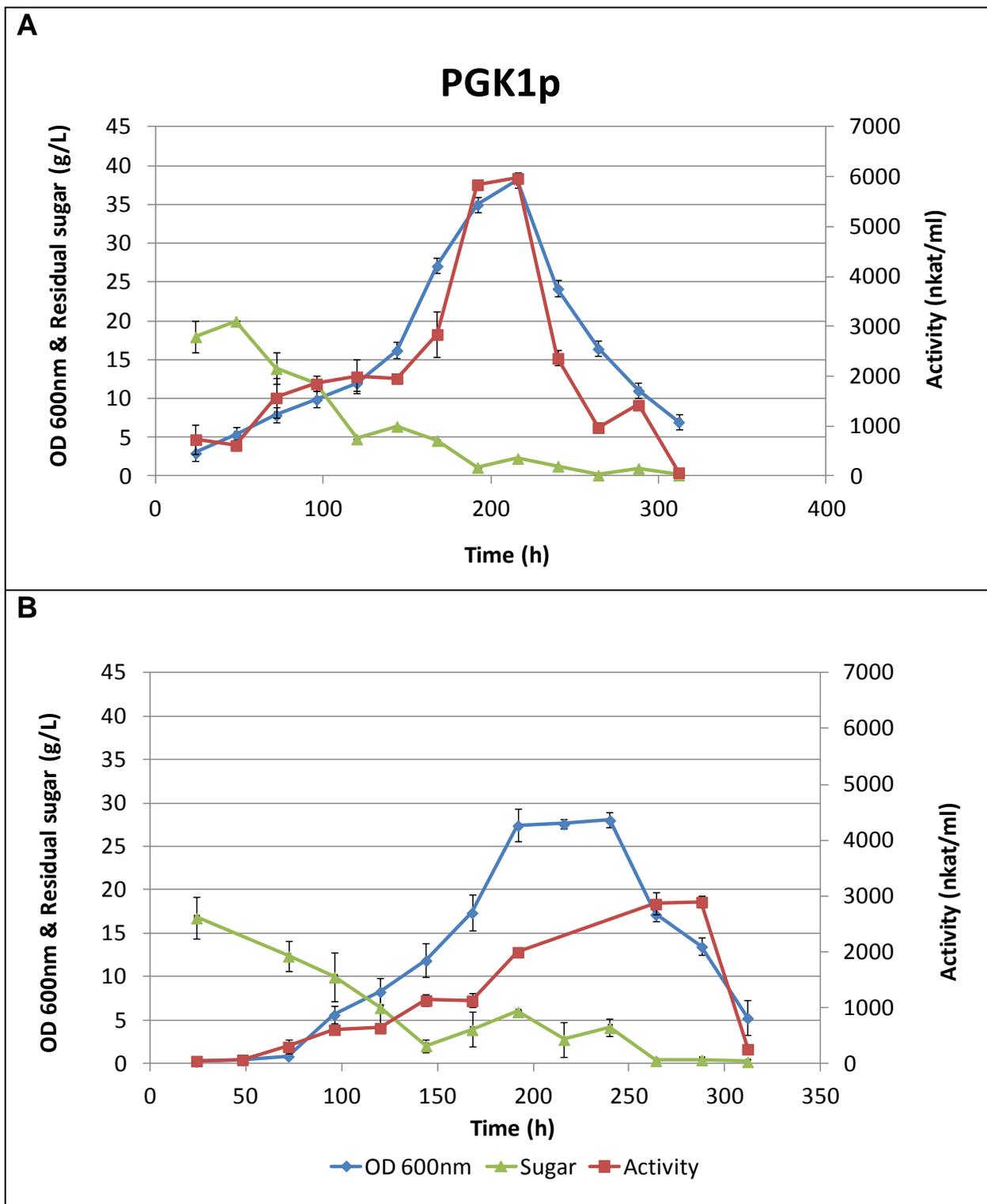


Figure 3.6. Time course of β -xylanase activity, residual sugar and cell growth of *S. cerevisiae* Y294 *gre3::TRP1* pMJM121 *PGK1p-XYN2-PGK1t* during growth on (A) glucose and (B) xylose. Samples were collected after every 24 hours on both glucose/xylose.

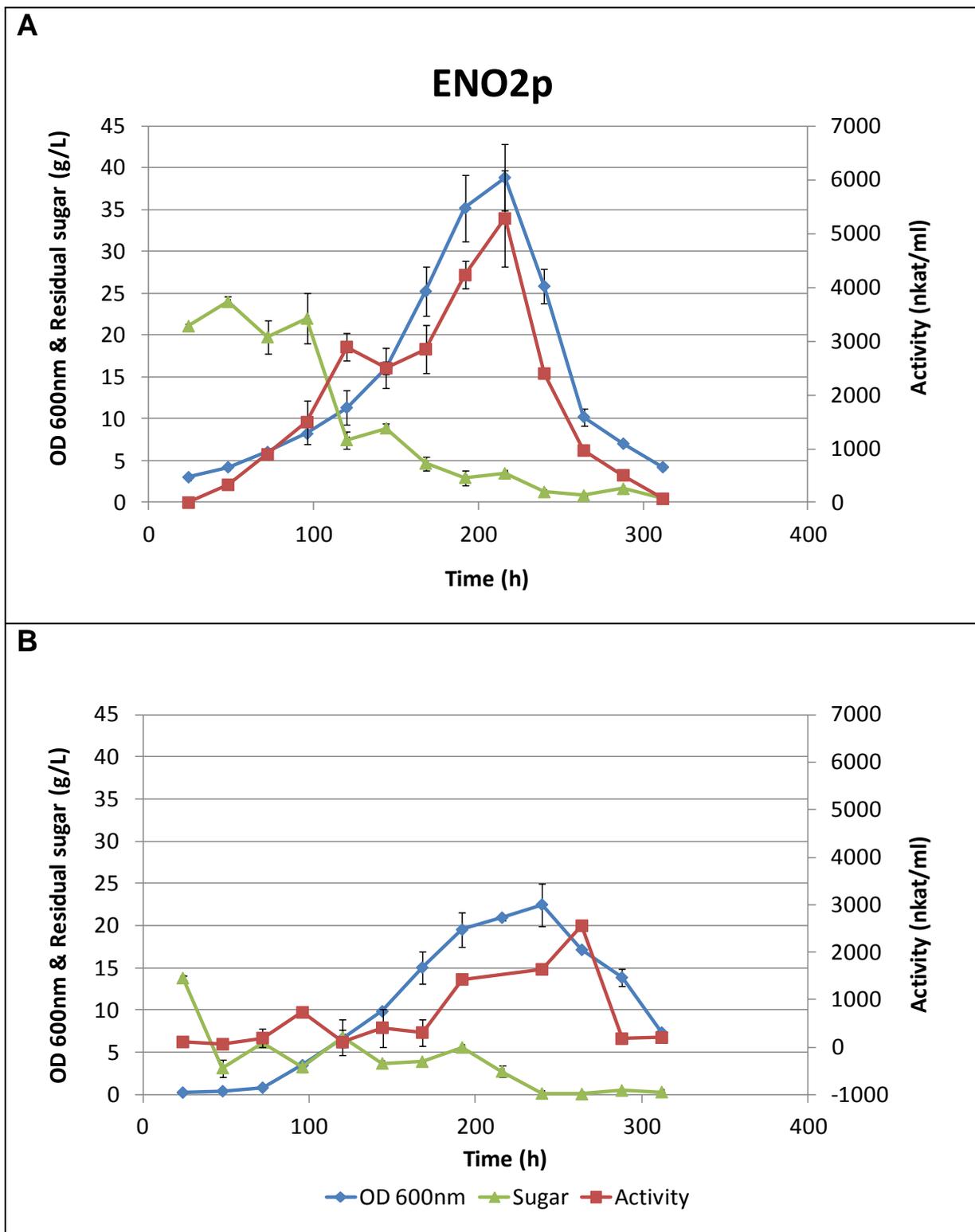


Figure 3.7. Time course of β -xylanase activity, residual sugar and cell growth of *S. cerevisiae* Y294 *gre3::TRP1* pMJM121 *ENO2p*-*XYN2*-*ENO2t* during growth on (A) glucose and (B) xylose. Samples were collected after every 24 hours on both glucose/xylose.

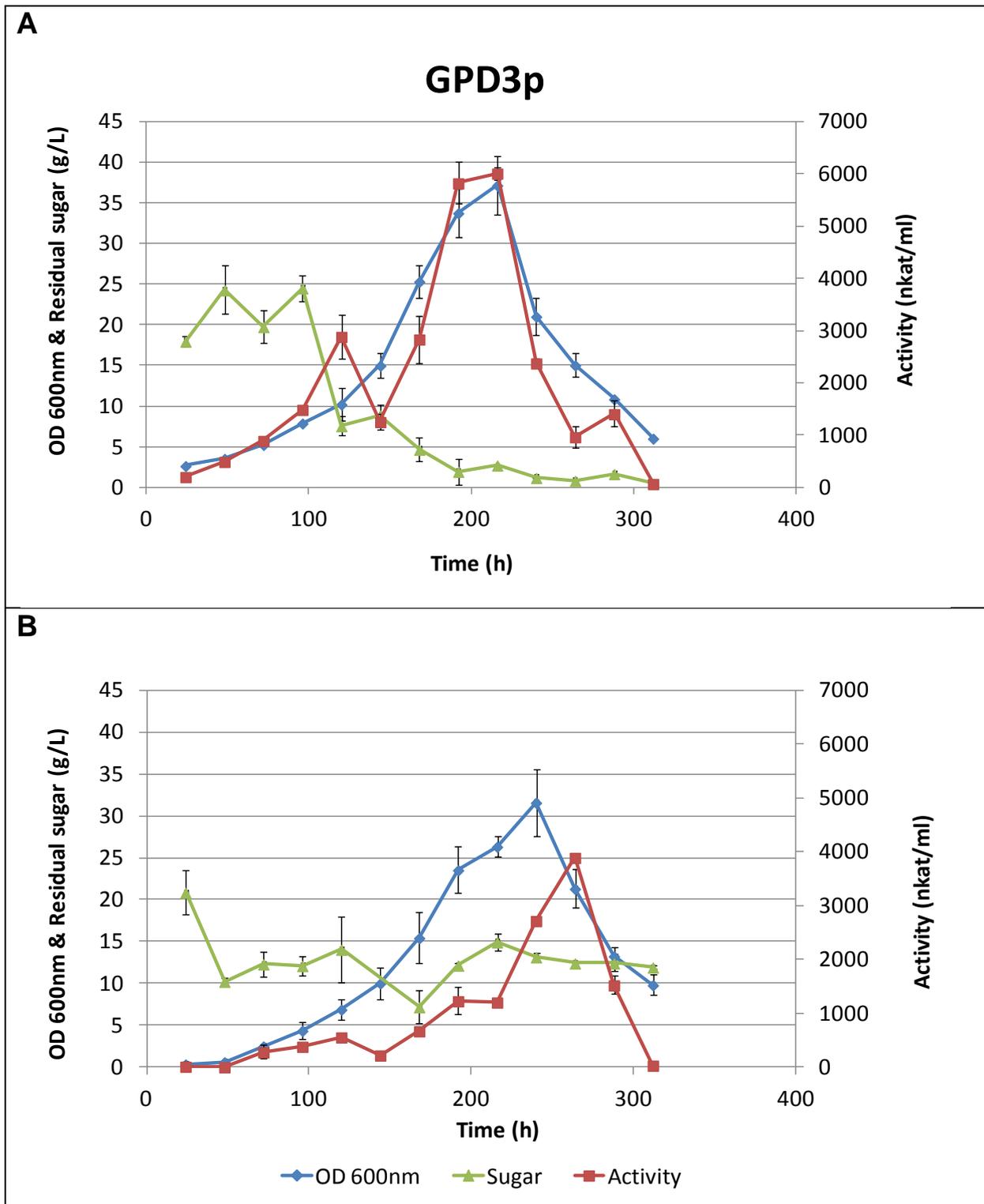


Figure 3.8. Time course of β -xylanase activity, residual sugar and cell growth of *S. cerevisiae* Y294 *gre3::TRP1* pMJM121 *GPD3p-XYN2-GPD3t* during growth on (A) glucose and (B) xylose. Samples were collected after every 24 hours on both glucose/xylose.

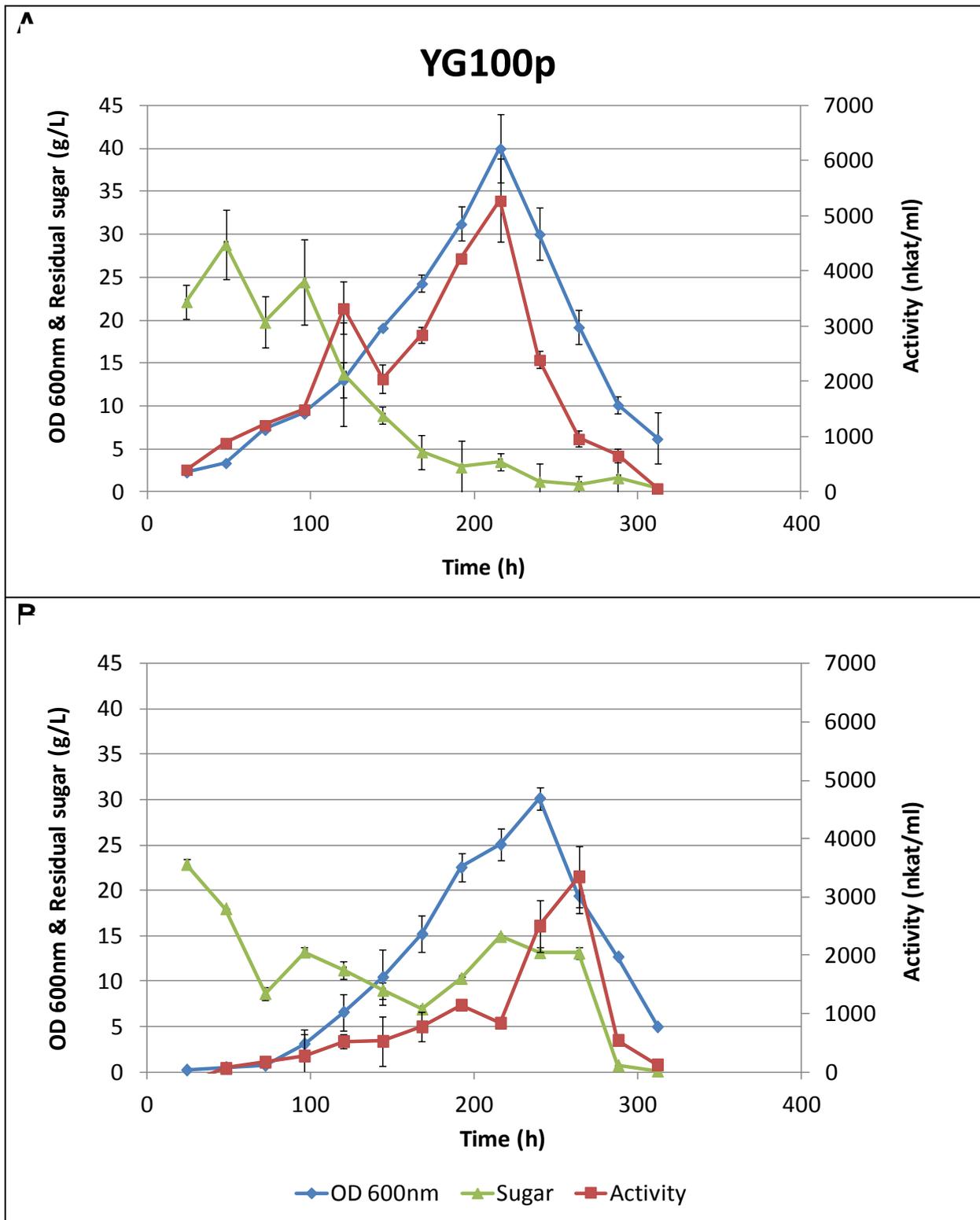


Figure 3.9. Time course of β -xylanase activity, residual sugar and cell growth of *S. cerevisiae* Y294 *gre3::TRP1* pMJM121 YG100p-XYN2-YG100t during growth on (A) glucose and (B) xylose. Samples were collected after every 24 hours on both glucose/xylose.

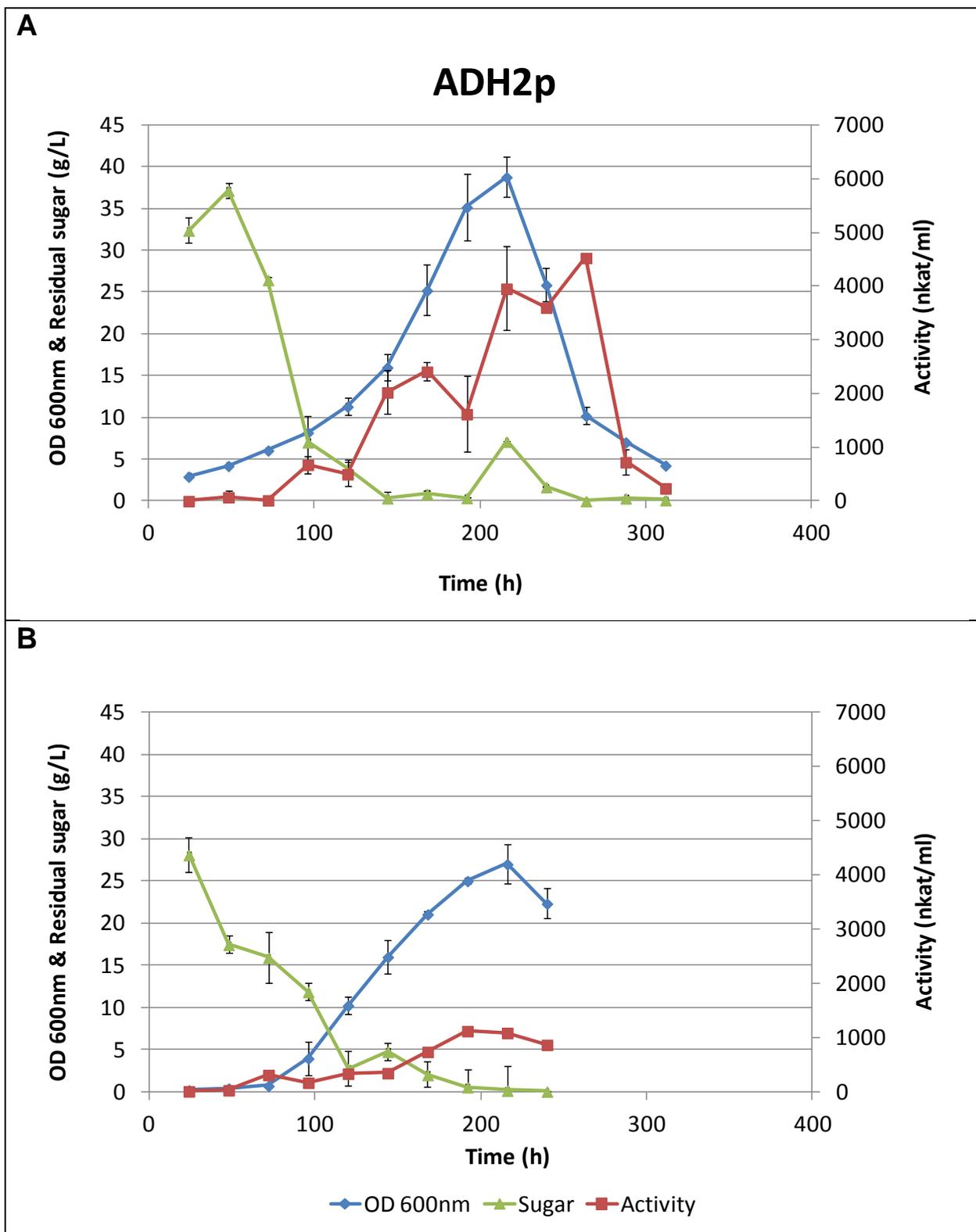


Figure 3.10. Time course of β -xylanase activity, residual sugar and cell growth of *S. cerevisiae* Y294 *gre3::TRP1* pMJM121 *ADH2p-XYN2-ADH2t* during growth on (A)

glucose and (B) xylose. Samples were collected after every 24 hours on both glucose/xylose.

Table 3.3. The maximum enzyme activity and the biomass present at the maximum enzyme activity time point. The average activity/OD was taken from several time points on the growth curve during growth of each strain on glucose.

	Time (hr)	OD600nm	Activity (nkat/ml)	Average Activity/OD
Control	216	38	2	2.05
<i>PGK1</i>	216	38	6000	127.70
<i>ENO2</i>	220	39	5300	104.76
<i>GPD3</i>	216	38	6100	148.26
<i>YG100</i>	216	40	5300	142.58
<i>ADH2</i>	264	10	4500	88.41

Table 3.4. The maximum enzyme activity and the biomass present at the maximum enzyme activity time point. The average activity/OD was taken from several time points on the growth curve during growth of each strain on glucose.

	Time (hr)	OD600nm	Activity (nkat/ml)	Average Activity/OD
Control	24	23	0	1.06
<i>PGK1</i>	288	12	2900	93.74
<i>ENO2</i>	264	13	2700	37.76
<i>GPD3</i>	264	23	4000	64.27
<i>YG100</i>	264	18	3400	55.97
<i>ADH2</i>	192	25	1200	37.24

The constructed recombinant *S.cerevisiae* strain in this study shows inconsistency in the way this strains uses the xylose and glucose sugar. This is unexpected since all the strains were constructed the same way and were expected to grow at the same rate on glucose or xylose. The only expected difference was in the xylanase activity since the promoters used are different.

The promoters strength on glucose is *GPD3*>*PGK1*>*ENO2*=*YG100*>*ADH2* (Table 3.3). This was as expected since the *GPD3* and *PGK1* promoters are strong constitutive promoter and are highly active on glucose. Furthermore, the results showed by the *ADH2* promoter were also expected since the *ADH2* promoter is repressed in the presence of glucose and xylanase activity was detected after glucose was depleted (LA GRANGE *et al.*, 1996,

BADZIONG et al., 1999). The *YG100* which is induced at elevated temperatures showed high activity even though it was not induced.

Since this is the first study evaluating the *GPD3*, *PGK1*, *ENO2*, *YG100* and *ADH2* promoters during growth on xylose, it was not known how these promoters will perform on xylose. The promoters' strength on xylose were as follows *GPD3*>*YG100*>*PGK1*>*ENO2*>*ADH2* (Table 3.4). From this the *GPD3* also showed high xylanase activity on xylose and the inducible *YG100* promoter, although not induced showed higher activity than the strong glycolytic *PGK1* promoter (KINGSMAN et al., 1990). In terms of the time, the *ADH2* promoter is a strong promoter during on xylose. The maximum activity on xylose was reached after 192 hours as compare to 264 hours on glucose. The promoter is repressed to the same level by xylose as by glucose.

The recombinant *S. cerevisiae* strain with the strong glycolytic promoter, *GPD3*, produced slightly more biomass on glucose (OD600nm = 37), compared to xylose (OD600nm = 32) (Figure 3.8). The *GPD3* resulted in 34% lower xylanase activity during growth on xylose than on glucose, while the *ENO2* promoter produced almost double the amount of biomass on glucose and this is reflected in the enzymes activity which is also double (5300 versus 2700 nkat/ml) (Figure 3.7). The *PGK1* strain produced 26% less biomass on xylose, but the activity was more than 50% lower than on glucose (6000 versus 2900 nkat/ml) (Figure 3.5). Additionally, the recombinant *S. cerevisiae* strain with the heat inducible *YG100* promoter produced 25% less biomass on xylose but xylanase activity decreased by 35% (5300 versus 3400 nkat/ml)(Figure 3.9). The total activity on both substrates would probably be higher if the *YG100* were induced at an elevated temperature. The yeast strain with the reporter gene under the control of the *ADH2* produced 30% less biomass on xylose, but the activity decreased by more than 70% (4500 versus 1200)(Figure 3.10). The *PGK1* promoter showed higher xylanase activity on both xylose and glucose, but not higher than that of the *GPD3* promoter that showed xylanase activity of 6100nkat/ml after 220 hrs on glucose and 4000nkat/ml after 280 hrs on xylose. These results depict that *GPD3* promoter is a proper promoter to be used during recombinant protein production on xylose.

CHAPTER 4. DISCUSSION

Xylose is an abundant sugar found in lignocellulosic biomass. The yeast commonly used to produce ethanol, *S. cerevisiae* cannot utilize xylose as carbon source, but it has been engineered to do so (KUYPER *et al.*, 2003). Unlike glucose, xylose should not result in the Crabtree effect (DE DEKEN, 1966). During aerobic growth on xylose, most of the carbon is converted to biomass, resulting in significantly more biomass compared to growth on glucose, which then leads to the production of ethanol (MERT and VAN ZYL, 2015). Since the amount of recombinant protein is often related to the amount of biomass, it should be possible to obtain higher concentrations of heterologous proteins, during growth on xylose. This is assuming the *S. cerevisiae* promoters perform the same on xylose as on glucose.

In this study, five commonly used *S. cerevisiae* promoters were tested during growth on xylose to determine if xylose can be used as a substrate for the production of heterologous proteins. This was done by using the xylanase gene from *T. reesei* as a reporter gene. The xylanase reporter gene is a small (21 kDa) protein produced at high activity in *S. cerevisiae* (LA GRANGE *et al.*, 1996). Prior to the transformation of the expression cassettes with the different promoters to be evaluated, a *S. cerevisiae* xylose utilizing strain had to be constructed. This was done by integrating the *B. thetaiotaomicronxylose* isomerase (*XI*) gene and the *P. stipitis* xylulokinase gene (*XYL3*) on the chromosome of a laboratory strain of *S. cerevisiae* Y294. The *XI* enabled *S. cerevisiae* to convert xylose to xylulose, while the *XYL1* gene product facilitates phosphorylation of xylulose to xylulose-5-phosphate. To prevent the formation of xylitol, a strong inhibitor of the *B. thetaiotaomicronXI*, the non-specific aldose reductase of *S. cerevisiae*, the *GRE3* gene, was deleted. This resulted in a *S. cerevisiae* strain capable of growing on xylose, but at a very slow rate. Several steps have been reported to be rate limiting during growth on xylose, including xylose uptake, xylose consumption rate, slow conversion of xylose to xylulose and limited flux through the pentose phosphate pathway (KUYPER *et al.*, 2003). Several studies have shown that microorganisms can adapt to different carbon sources, temperature and some inhibitors through evolutionary engineering (SCALCINATI *et al.*, 2012). The growth of the recombinant xylose utilising, *S. cerevisiae* strain used in this study was therefore optimised to grow on xylose through 15 cycles of adaptation. After adaptation the best strains started to grow on xylose within three days compared to the parental un-adapted strains typically taking more than 10 days to grow on xylose.

Five promoters commonly used for the expression of recombinant proteins in *S. cerevisiae*, namely the phosphoglycerate kinase 1 (*PGK1*) promoter, the heat inducible *YG100* promoter, the alcohol dehydrogenase 2 (*ADH2*) promoter, glyceraldehyde-3-phosphate dehydrogenase (*GPD3*) promoter and the enolase 2 (*ENO2*) promoters, were selected to evaluate expression of a reporter enzyme during growth on xylose. The expression plasmids used were 2 μ based episomal plasmids with *URA3* auxotrophic selection. These enable high level multi-copy expression of the gene of interest. Unfortunately, these plasmids are unstable in the absence of selection. Furthermore, the strength of the promoter can also have an impact on plasmid stability and copy number (SUN *et al.*, 2012). Single copy, integrated expression cassettes might be better suited for the evaluation of promoters as these eliminate copy number variations. However, it is sometimes difficult to determine the activity of an enzyme with only a single copy of the gene integrated on the genome, especially when working with a weak promoter. The site of integration may also affect transcription adversely.

The production of recombinant proteins in synthetic medium is not ideal, since it is not possible to obtain high cells densities under these conditions. To allow growth in complex medium without the risk of losing the episomal plasmids, auto-selective strains were constructed (KERN *et al.*, 1990, LA GRANGE *et al.*, 1996). The *FUR1* gene of *S. cerevisiae* encodes uracil phosphoribosyltransferase, which catalyzes the conversion of uracil to uridine 5-phosphate in the pyrimidine salvage pathway (KERN *et al.*, 1990). The disruption of the *FUR1* gene leads to a *S. cerevisiae* Y294 that would not be able to utilize uracil from the extracellular medium and would therefore not be viable. For these cells to be viable without the *FUR1* gene, they must possess a complementing functional *URA3* gene to synthesize uridine 5-phosphate de novo. In this case, the *URA3* gene is the yeast selectable marker on plasmid used for evaluation of different promoter. After disruption of the *FUR1* gene the strains were able to grow on both YP media with xylose or glucose as carbon source.

All the strains used in the study produced large clearing zones within 24 hours of incubation on the RBB-plate with glucose as carbon source (Figure 3.3). Even the strain with the *XYN2* reporter under the control of the *ADH2* promoter, which is known to be strongly repressed by glucose, produced a clearing zone during growth on glucose.

Growth on the xylose containing RBB-plate was very poor. These colonies were spotted from active growing cultures on YPX plate. The clearing zone noticeable on xylose containing plate is probably due to enzyme carried over from the YPX plates. Surprisingly, the *ADH2* promoter did not function well on xylose as a carbon source as only a very faint zone is visible.

During cultivation of all the promoter evaluation strains in liquid YP medium with either glucose or xylose as carbon source, the amount of xylanase activity in relation to the amount of biomass produced, was significantly lower on xylose (Figure 4.1). From this it is clear that the xylanase activity did not only decrease as a result of a decrease in biomass. All the promoters evaluated, perhaps with the exception of *PGK1*, produced less xylanase activity on xylose compared to glucose.

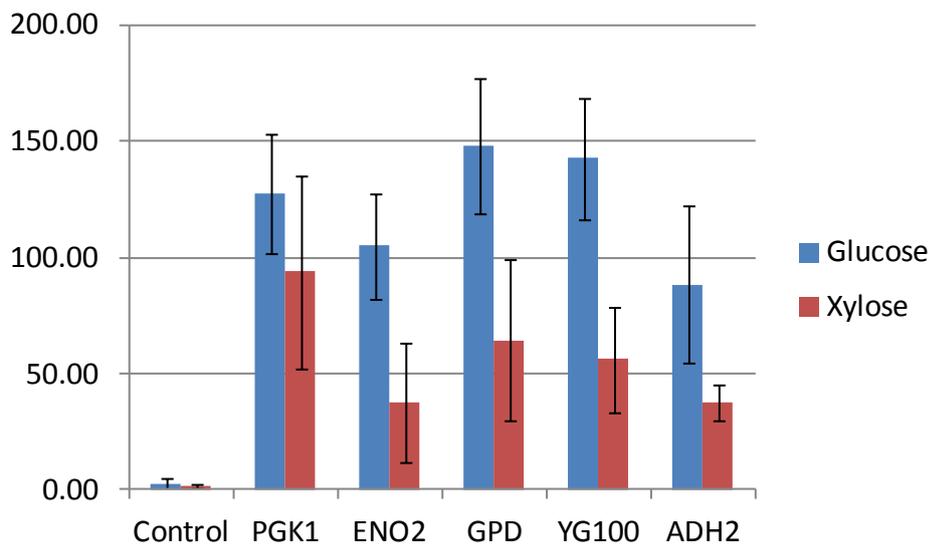


Figure 4.1. The representation of the average level of extracellular xylanase activity in relation to the amount of biomass during growth on glucose and xylose (Activity/OD).

In a previous study, *S. cerevisiae* with the xylanase gene under the control of the *ADH2* produced 8 times more extracellular xylanase activity during growth on galactose, a non-fermentable carbon source, compared to cells grown on glucose (LA GRANGE *et al.*, 1996). This difference compared might be attributed to the fact that *S. cerevisiae* naturally is able to grow on galactose, unlike xylose. Another *S. cerevisiae* strain constructed by Mert and Van Zyl (2015), Figure 4.2, produced almost 3 times more biomass on xylose compared to glucose. On glucose, this yeast reached its maximum OD600nm of 7.5 in approximately 30 hours, while it took more than 140 hours to obtain an OD600nm of 18 on

xylose. Although this recombinant xylose utilizing strain was able to produce more biomass on xylose, the activity/OD for glucose was higher (207 nkat/ml/OD) compared to xylose (186 nkat/ml/OD). This correlate with the data obtained in this study.

The results obtained in this study show that if xylose is used as a carbon source for recombinant protein production, the preferred promoter to use is *PGK1*. The *PGK1* showed the highest activity/OD on xylose (Figure 4.1).

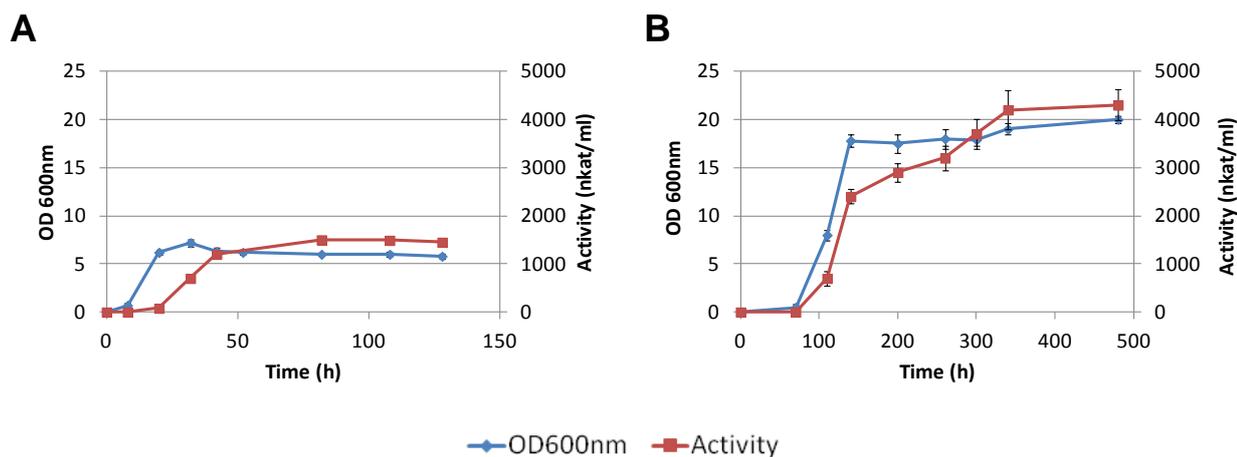


Figure 4.2. Graph indicating biomass and xylanase activity produced by *S. cerevisiae* under the control of the *ADH2* promoter on (A) glucose and (B) xylose (MERT and VAN ZYL, 2015).

CHAPTER 5. CONCLUSION

Xylose is not naturally consumed as a carbon source by *S. cerevisiae*, however a number of scientific papers report the construction of xylose utilising strains (KUYPER *et al.*, 2003, HAHN-HÄGERDAL *et al.*, 2007). In this study, the *B. thetaiotaomicron* xylose isomerase was used to enable *S. cerevisiae* Y294 to grow on xylose.

High levels of recombinant proteins are often linked to the amount of biomass (FERNDAHL *et al.*, 2010a). All the strains evaluated, including the control strain without the xylanase gene, produced significantly less biomass during growth on xylose, which translated into lower levels of enzyme secreted. The amount of enzyme in relation to the biomass produced was lower on xylose than on glucose. This is consistent with the study reported by MERT & VAN ZYL, (2015). The difference in the amount of biomass obtained in this study compared to that of (MERT and VAN ZYL, 2015) , could be due to prolonged adaptation of the engineered *S.cerevisiae* strain on xylose.

Xylose is a cheap and abundant sugar which can be used for the production of a number of industrial products, including xylitol and ethanol. *S. cerevisiae* is crabtree positive yeast and produces fairly low amounts of biomass during growth on glucose, since a significant amount of carbon is converted to ethanol, even under aerobic conditions. In theory, growing *S. cerevisiae* on a non-fermentable carbon source, like xylose, should result in higher biomass. The *S. cerevisiae* strain used in this study did not produce more biomass during growth on xylose; however (MERT and VAN ZYL, 2015) obtained 3 times more biomass on xylose than on glucose.

The amount of biomass does not necessarily translate into more secreted protein on xylose compared to glucose. Generally, the amount of protein per unit biomass is approximately 50% less on xylose than on glucose. However, if a *S. cerevisiae* capable of good growth on xylose is used, it is possible to produce more protein per gram xylose than glucose.

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