EXPRESSION OF A MODIFIED XYLANASE IN YEAST

Nokuthula Peace Mchunu

Submitted in fulfillment for the requirement of a Degree of Master of Technology: Biotechnology, in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa.

FINAL COPY APPROVED FOR SUBMISSION

_____ _____

Supervisor **Date**

Professor K. Permaul

_____ _____

Supervisor **Date**

Professor S. Singh

DECLARATION

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

_____

Nokuthula Peace Mchunu

March 2009

"You are worthy, Jehovah, even our God, to receive the glory and the honor and the power, because you created all things, and because of your will they existed and were created."

Revelation 4:11

“Laws alone can not secure freedom of expression; in order that every man expresses his views without penalty there must be spirit of tolerance in the entire population.”

Albert Einstein

ACKNOWLEDGEMENTS

The author would like to extend her sincere gratitude to the following people who without their help this study would not have been successful,

Prof. K. Permaul and Prof S. Singh for their guidance, knowledge and expertise throughout the study;

Dr. Dawn E. Stephens for academic and technical support even though she was not here till the end of the study;

Dr Eva Nordberg-Karsson and Tania Pozzo for making my stay in Sweden as pleasurable as possible;

My friends, Ane, Hle and Kyle. Thank you for all the company and laughter in the lab;

Staff from the biotechnology and food technology department;

National Research Council for financial support;

My husband, Zakhe Nxumalo, and my family for moral and emotional support; and

Jehovah, almighty, for the power, protection and love he provided throughout the year without fail.

CONTENTS

ABSTRACT

Protein engineering has provided a key for adapting naturally-occurring enzymes for industrial processes. However, several obstacles have to be overcome after these proteins has been adapted, the main one being finding a suitable host to over-express these recombinant protein. This study investigated *Saccharomyces cerevisiae*, *Pichia pastoris* and *Escherichia coli* as suitable expression hosts for a previously modified fungal xylanase, which is naturally-produced by the filamentous fungus, *Thermomyces lanuginosus*. A xylanase variant, NC38, that was made alkaline-stable using directed evolution was cloned into four different vectors: pDLG1 with an *ADH2* promoter and pJC1 with a *PGK* promoter for expression in *S. cerevisiae*, pBGP1 with a *GAP* promoter for expression in *P. pastoris* and pET22b(+) for expression in *E. coli* BL21 (DE3). *S. cerevisiae* clones with the pDLG1-NC38 combination showed very low activity on the plate assay and were not used for expression in liquid media as the promoter was easily repressed by reducing sugars used during production experiments. *S. cerevisiae* clones carrying pJC1-NC38 were grown in media without uracil while *P. pastoris* clones were grown in YPD containing the antibiotic, zeocin and *E. coli* clones were grown in LB with ampicillin. The levels of xylanase expression were then compared between *P. pastoris*, *S. cerevisiae* and *E. coli*. The highest recombinant xylanase expression was observed in *P. pastoris* with 261.7 U/ml, followed by *E. coli* with 47.9 U/ml and lastly *S. cerevisiae* with 13.2 U/ml. The localization of the enzyme was also determined. In the methylotrophic yeast, *P.*

pastoris, the enzyme was secreted into the culture media with little or no contamination from the host proteins, while the in other hosts, the xylanase was located intracellularly. Therefore in this study, a mutated alkaline stable xylanase was successfully expressed in *P. pastoris* and was also secreted into the culture medium with little or no contamination by host proteins, which favours the application of this enzyme in the pulp and paper industry.

LIST OF FIGURES

LISTS OF TABLES

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Many chemical transformation processes in various industries have inherent drawbacks from a commercial and environmental point of view. This is because many of these produce chemical waste that is toxic to the environment. These problems include non-specific processes and reactions, which may result in poor product yield and high amount of waste produced. This has put a strain on industries to develop processes that are environmentally-friendly and commercially-viable.

This has seen the emergence of enzyme technology. Enzymes are biological catalysts that are produced by all living organisms. As such, they have evolved along with cells to satisfy the metabolic demands of an extensive range of cell types. In order to avoid metabolic chaos and create harmony in a cell teeming with innumerable different chemical reactions, the activity of a particular enzyme must be highly specific, both in reaction catalyzed and the substrates it targets. Because of this specificity in the reaction their catalyzed, nature has provided us with an abundance of supply of catalysts that can carry out different chemical process but with high product quality and minimal to no waste being produced.

Although biological catalysts are highly attractive for chemical synthesis, many of these biological catalysts are not applied in most industrial processes because of

various limitations. These limitations include demanding physiological parameters such as high temperature, low or high pH, high salt concentration, sluggish catalysis on non-specific substrates, low stability or little tolerance for changes in operating parameters, poor activity in non-aqueous media and many additional requirements that may increase production costs.

This has seen the development of directed evolution technology, which can improve the various characteristics of different enzymes. This technique mimics natural evolution but in a shorter period of time. This is done by introducing errors in the DNA sequence of that particular enzyme, then screening for improved variants of that protein. This creates a need for functional expression in a host suitable for production of large quantities of the improved protein. However step is, however, still a serious bottleneck for expression and production of acceptable levels of protein for an industrial process.

Many scientists have looked at using different expression systems in trying to improve production rate of the recombinant enzymes. Many of the hosts used are usually not the natural host of that particular recombinant enzyme, thus may fail to increase productivity because the enzyme is either produced at low amounts, stored where it is not easily accessible, and may require expensive downstream processing or the host being used does not have a good transport system for the export of that particular enzyme.

To overcome these problems, expression in a host that secretes most of its proteins or into a natural host would be more attractive. This is because a host that secretes the products, allows for easier purification and down stream processing. Thus, the host will be able to produce large amounts of the product, because its does not accumulate inside the cell which, in most cases, it may cause product inhibition. The advantage of natural host is that no added promoter genes or secretion signals are needed because the host is finely tuned to produce the recombinant protein because it has the natural gene biochemical pathway for expression and transportion for easy accessibility.

1.1 Enzymes

Enzymes are biological catalysts that are in involved in all biological processes. Enzymes are needed to speed up these reactions and during these reactions they will remain unchanged (Arnold, 1996). Enzymes can perform intricate regioselective and/or enantioselective chemical transformations and can accelerate reactions rates significantly (Arnold *et al*., 2001). Due to this, the wide use of enzymes for medical, industrial and environmental purposes is prevalent today and will continue to expand rapidly into the next century (Fig 1.1). This has also opened the need for enzyme bio-prospecting. The global market for industrial enzymes was estimated to be about US$ 2.3 billion in 2007 with a projected increase of 4% in annual growth rates, growing from US$ 1.5 billion in 1998 (van Beilen and Li, 2002).

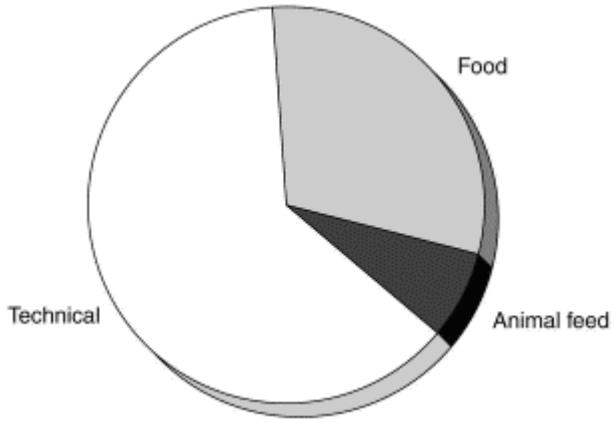

Fig 1.1 Segmentation of the industrial enzyme market (Kirk *et al.,* 2002).

Although highly attractive for bioprocessing, mesophilic enzymes are not able to perform under industrial production conditions. These limitations include sluggish catalysis on non-natural substrates, low stability and little tolerance for changes in operating conditions (Arnold *et al*., 2001). This has lead to the isolation of enzymes from extremophiles and the discovery of these organisms and their enzymes has revolutionized the enzyme industry.

1.2 Extremophiles as a source for industrial enzymes

Extremophiles are organisms that have evolved to exist in a variety of extreme environments for example deep-sea hydrothermal vents, hot springs and extreme pH environments. Extremophiles can be classified into a number of groups that includes thermophiles, acidophiles, alkalophiles, psychrophiles, barophiles and others as seen in Table 1.1 (van der Burg, 2003). As a result,

these microorganisms produce unique biocatalysts that function under conditions in which their mesophilic counterparts could not survive, permitting potential application and development of additional industrial processes (Jaenicke and Bohm, 1998). However, humans have been striving to create new uses or enhance what nature has provided. They have become more aware that the environment they live in has to be conserved and protected and in doing so, have realized the importance of enzymes. These enzymes are of great importance because they can be used for industrial processes that convert or degrade biomolecules.

Table 1.1 Different enzymes from extremophiles and their industrial application (van der Burg, 2003).

Type	Growth characteristic	Enzyme	Applications
Thermophiles	Temp > 80^{o}C, hyperthemophiles	Proteases	Detergents, hydrolysis in food and feed, brewing, baking
	Temp 60-80^{o}C, thermophiles	glycosylhydrolase (e.g. amylases, pullulanase, glucoamylase, glucosidases, cellulases, xylanases)	Starch, cellulose, chitin, pectin processing, textiles
		chitinases	Chitin modification for food and health products
		xylananse	Paper bleaching
		DNA polymerases	Molecular biology (e.g. PCR)
		dehydrogenases	Oxidase reactions
Psychrophiles	Temp <15^{o}C	proteases	Detergents, food application (e.g. dairy industry)
		amylases	Detergents and bakery
		cellulases	Detergents, feed and textiles
		dehydrogenases	biosensors
		lipases	Detergents, food and cosmetics
Halophiles	High salt, (e.g. 2-5 M NaCl)	proteases	Peptide synthesis
		dehydrogenases	Biocatalysis in organic media
Alkaliphiles	pH >9	protease, cellulases	Detergents, food and feed
Acidophiles	pH <2-3	amylase, glucoamylase	Starch processing
		protease, cellulases	Feed component
		oxidase	Desulfurization of coal
Barophiles	Pressure-loving; up to 130 MPa	To be defined	Food processing and antibiotic production

Screening from the environment has identified potentially applicable enzymes but most of these enzymes did not meet all the industrial requirements (Blanco *et al.,* 1995). However, there are a number of reports on successful application of the enzymes from extremophiles, which include the use of *Taq* polymerase from *Thermus aquaticus*. This enzyme is used in molecular biology and has contributed greatly in genetic engineering (Schiraldi and de Rosa, 2002). Today, many improved recombinant forms of this enzyme are in use in molecular biology. Although these enzymes have been isolated from nature, the culturing and mass production of these organisms or their enzymes is very difficult due to their growth requirements or conditions (Schiraldi and de Rosa, 2002).

The process of evolution in nature has provided us with many of these biocatalysts, which provide alternatives to the use of chemicals in industry. The establishment of the Kyoto protocol, which binds industrialized countries to reduce greenhouse gas emissions, has seen major industries trying to establish environmentally-friendly processes. Thus the use of enzymes has flourished as these industries are being forced to find alternatives to chemical processes. These industries are discovering that these alternatives might be even cheaper than the established chemical processes. In the last two decades, the application of enzymes for industrial and commercial purposes has increased. Major applications of enzymes in industries (Table 1.2) include the use of lactases in the dairy industry, xylanases, phytases and cellulases in animal feed, peroxidases, catalase, protease in both the detergent and dairy industry,

biosensors and bioremediation purposes and amylases in textile industry, (Coughlan, 1985; Kirk *et al*., 2002; Kumamaru *et al*., 1998; Rubingh, 1997; van Beilen and Li, 2002). Enzymes offer a viable alternative to chemical processes because as they are renewable resources. Another major application of enzymes in an industrial scale is the production of biofuel. The alternatives to petroleum-derived fuel are being sought as oil reserves dry up and the cost of petroleum-based fuel escalates. Ethanol has been identified as the most common bio-fuel. The USA alone has the resource potential to produce over 1 billion tons a year if the total available biomass can be converted, which can replace 30% of the current demand on petroleum based fuels (Gray *et al*., 2006). In order for this to be achievable, enzyme application is needed for the conversion of this biomass to bio-fuels.

This wide application of enzyme technology has directly affected other fields of biotechnology positively and has lead to the growth and development of recombinant DNA technology. Recombinant DNA technology provides tools and the means to scientists to study and alter the gene structure of living organisms. This field has become known as genetic or protein engineering. Many approaches have developed to improve proteins, either specific or random mutations and have been used for the enhancement of enzymes to make them more industrially-applicable (Tobin *et al*., 2000).

Table 1.2 Enzymes used in various industrial segments and their applications (van Beilen and Li, 2002).

Industry	Enzyme class	Application
Detergent (laundry and dishwash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Fat stain removal
	Cellulase	Cleaning and colour clarification
Starch and fuel	Amylase	Liquefaction, saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Xylanase	Viscosity reduction
	Protease	Yeast nutrition fuel
Food (including dairy)	Protease	Milk clotting, flavour
	Lipase	Cheese flavour
	Lactase	Lactose removal
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning
	Phospholipase	Dough stability and conditioning
	Protease	Biscuits and cookies
Animal feed	Phytase	Phosphorus release
	Xylanase	Digestibility
Beverage	Pectinase	Mashing and de-pectinization
	Amylase	Juice treatment, low calorie beer
	Laccase	Clarification and flavour
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Catalase	Bleach termination
	Peroxidase	Excess dye removal
Pulp and paper	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage, fibre modification
	Amylase	Starch-coating
	Protease	Biofilm removal
	Amylase	Starch-coating
	Protease	Biofilm removal

1.3 Xylanases in industry

Endo-β-1,4-xylanase belongs to the glycosyl hydrolase family 10 and 11 (formerly known as family G and H). Xylanases have the ability to degrade xylan which is a hemicellulose biopolymer found in plant tissues as a major component of the cell wall. Xylan is the second most-abundant polymer in nature and represents more than 30% of dry weight in renewable sources available on earth (Gabrielii and Gatenholm, 1998). Xylan is a complex molecule composed of β-1,4 linked xylose chains with branches containing arabinose and 4-*0*-methylgluconic acid (Bim and Franco, 2000). Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone and in the side chain. Xylan requires the action of several enzymes for its complete degradation, which include: β-1,4-endoxylanases, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterases and phenolic acid esterases. However, xylanases play a major role in degradation of the backbone of xylan.

1.4 Biotechnological application of xylanases

Xylanases, like most enzymes that have an ability to degrade certain biological molecules, have attracted great interest. The difference between the two families that xylanases belong to is that, family 10 exhibits greater catalytic versatility or lower substrate specificity than family 11 (Biely *et al*., 1997; Čepeljnik *et al*., 2004). This catalytic ability makes xylanases a potential candidate for the

application in the pulp and paper industry(fig. 1.2). The xylanases preferred for bio-bleaching applications are usually family 11 xylanases (Bajpai, 1999; Buchert *et al*. 1994). This is because the family 11 xylanases are relatively small, which makes it easy for this enzyme to penetrate the pulp fibre, so that the enzyme has contact with relatively large areas (Fig 1.2). The other reason is that family 11 lacks cellulase activity, which can affect the pulp yield and strength negatively (Gomes *et al*., 1993).

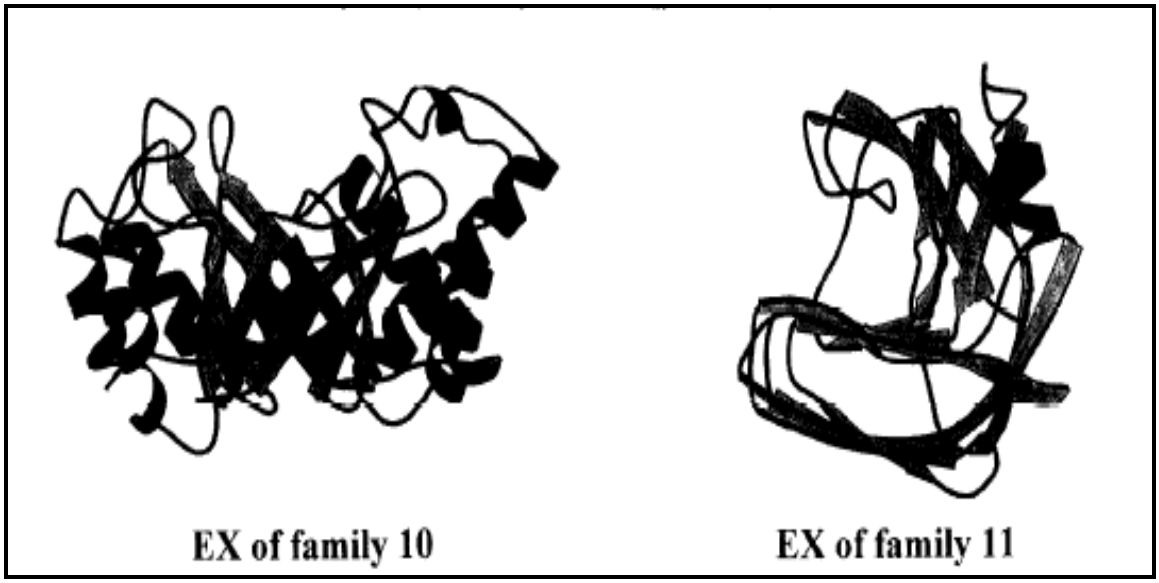

Fig 1.2 Ribbon representation endoxylanases from families 10 and 11 (Biely *et al*., 1997).

1.4.1 Xylanases in animal feed

Xylanases have also been used in animal feed to improve the digestibility of animal feed for better feed utilisation (Nortey *et al*., 2007; Uhlig, 1998). The major component of cereal grains that is used for animal feed, especially for chicken and pigs, contains high amounts of xylan. This is not of any nutritional value to these animals as it is well known that they lack the enzymes that are mostly associated with the ruminant microorganisms found in other animals (e.g., cattle) (Bedford and Classen, 1992). These ruminant microorganisms are responsible for the production of these enzymes responsible for digestion, including xylanases. When these non-ruminant animals are fed with consumer feed that contains high amounts of xylan, the feed causes high viscosity in the gut resulting in difficulties in penetration of digestive enzymes and absorption of nutrients (Bedford and Classen, 1992). The effect is that the feed is not efficiently utilised resulting in poor growth of animals, especially chickens. The addition of xylanase to the feed, with other hemicellulose degrading enzymes, helps to decrease this negetive effect of xylan (Silva and Smithard, 2002). The xylanases also help in degradation of the xylan component in the feed, thus improving nutrient availability (Bedford, 2003)

1.4.2 Xylanase application in food industry

In the baking industry, xylanases act on the xylan component of wheat. The xylanase action on the wheat improves water distribution during dough formation. This results in improving the quality of bread by improving dough rheology (Jaing *et al.,* 2005; Maat *et al*., 1992). It also achieves desirable texture and mouth feel and increases bread volume and shelf life of baked products (Courtin *et al*., 2001).

Xylanases have also found application in the clarification of liquid products (e.g., fruit juice and beer) (Biely, 1985). Debyster *et al.* (1997) found that the addition of xylanases helped in the degradation of water-soluble xylan. These water soluble xylans cause haze formation and high viscosity during fermentation which in turn makes filtering of the beer difficult. Therefore, the use of xylanase decreased viscosity, thus making the filtration step much easier and faster and reduced haze formation resulting in a clarified product which is more acceptable to the consumers.

1.4.3 Xylanases for bio-fuel production

A recent application of xylanase is in production of biofuels. It is estimated that the total energy content of global xylan and cellulose waste is equivalent to almost 640 billion tons of oil (Sa-Pereira *et al*., 2003). Xylanases are used to

convert the polymeric xylan into fermentable sugars for the production of ethanol and xylitol from plant biomass (Galbe and Zacchi, 2002). The effect of the hydrolysis of the xylan component in plant biomass is that it increases the availability of cellulose to cellulose-degrading enzymes (cellulases) thus increasing the total conversion of biomass to fermentable sugars. This ultimately increases the fermentation products from the sugars, the main product, being ethanol.

1.4.4 Xylanase application in pulp and paper industry

In the pulp and paper industries, xylanases are used to remove or loosen, most of the impurities found in paper pulp, making it less problematic to remove these impurities and leaving the paper brighter (Fig 1.3). The chemical bleaching of paper pulp is traditionally achieved by the addition of chlorine and chlorine dioxide (Tolan *et al*., 1996; Viikari *et al*., 1994; Ximenes *et al*., 1999). The chlorine and chlorine dioxide present in the effluent from the paper mill form persistent organic chemicals (usually phenolic compounds) that are toxic to organisms in the waterways close to the paper plant and may present a health risk to humans. Pre-treatment of paper pulp with xylanases can enhance the efficiency of lignin extraction and, in doing so, reduce the amount of chlorine that is required also decreasing the consumption of hydrogen peroxide and ozone (Bissoon *et al*., 2002; Srinivasan and Rele, 1999). There are numerous advantages in using xylanases for paper pulp bio-bleaching which include:

- reduction in chemicals used for bleaching,
- improved pulp properties such as brightness and strength,
- cost,
- easy adaptation to different bleaching sequences,
- improved effluent with reduced chlorinated organic content, and
- environmentally-friendly process (Christov and Prior, 1998).

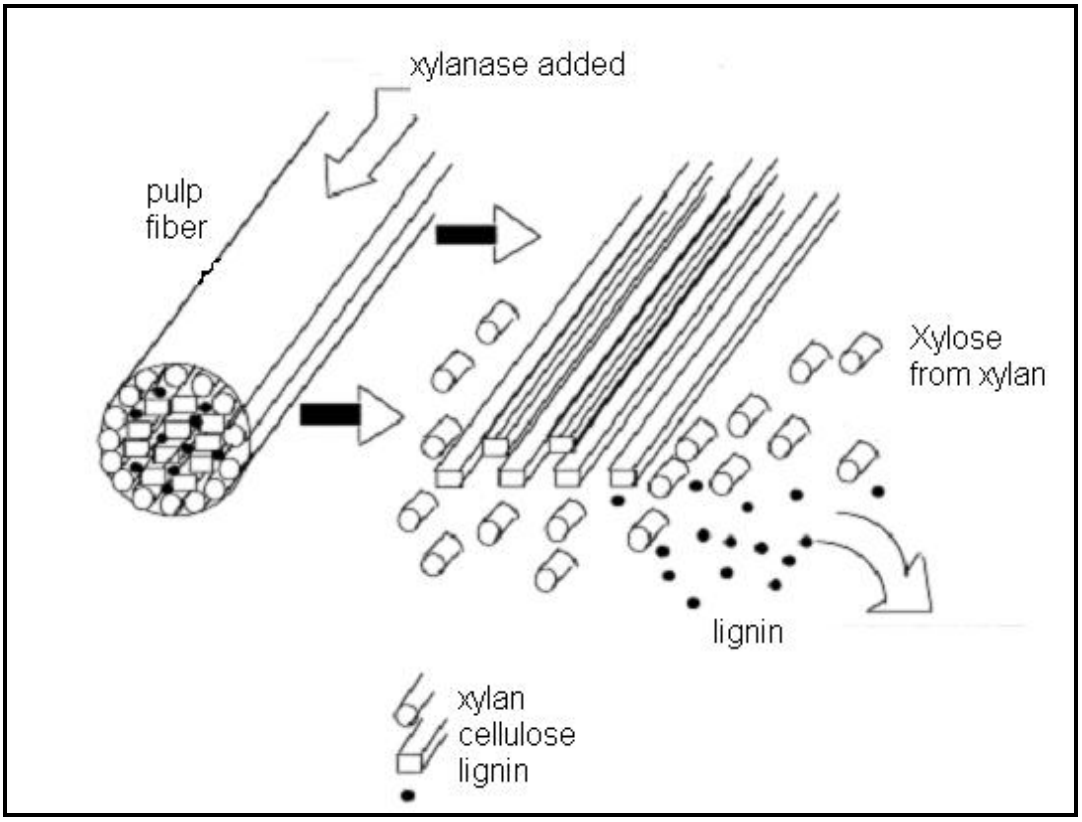

Fig 1.3 Schematic showing the effect of adding xylanase in wood and the end products that are obtained.

Pulp beaching is performed at high temperature and extreme alkaline conditions. The xylanases for this application would therefore have to be thermostable and alkaline stable. The potential usefulness of this enzyme in industry has motivated scientists to put considerable efforts towards finding applicable xylanases. There are two approaches that researchers have chosen to achieve this goal. The first one is searching for naturally-occurring xylanases from numerous sources in nature. Xylanases with either high temperature or high alkaline tolerance have been reported (Blanco *et al*., 1995). No naturally-occurring xylanase has been reported to possess both characteristics since it is very rare to find these conditions occuring simultaneously in nature (Nakamura *et al*., 1993; Singh *et al.,* 2003). Instead, spontaneous mutations provide microorganisms with abilities to survive and propagate in a constantly changing environment. This is why most enzymes isolated from the environment might not contain all the desired characteristics needed for an industrial process. The second approach is by engineering known xylanases or producers of xylanases to improve targeted characteristics. In this area genetic engineering is trying to fill the gap by tailor-making enzymes for specific industrial application (Turner, 2003).

1.5 Improvement of enzymes by genetic engineering

For decades scientists have been mutating or changing gene expression and protein function of microorganisms using classical methods. These classical methods include the use of chemical mutagenesis and UV light. The drawback of

these classical mutation methods is that the mutations are not target specific. These methods can interfere with any piece of DNA making it impossible to identify the mutated region or gene. In most instances the organism is either damaged or killed, and sometimes reverts back to the wild type. In the last decade, protein engineering has put mutations for protein enhancement into a whole new perspective. This is due the increase in knowledge on natural evolution and the development of new techniques. These techniques borrow from the process of natural evolution in creating enzymes suitable for industrial applications (Arnold *et al*., 2001).

There are two general molecular methods that have been developed to try to improve proteins (Chen, 2001). The first approach is 'rational design' where precise changes in amino acid sequence are introduced based on the knowledge of the protein structure, function and mechanism, usaully using site-directed mutagenesis. Site-directed mutagenesis is a molecular biology technique in which a mutation is created at a defined site in a DNA molecule. In general, site-directed mutagenesis requires that the wild-type gene sequence be known. This approach is based on studies dedicated to understanding natural evolution. This method has also provided the necessary information after the generation of a mutant using random mutagenesis. This was demonstrated in the production of an alkalophilic xylanase variant from *Neocallimas*tix *patriciarum* (Chen *et al*., 2001). It was also used in the improvement of the thermostability of a fungal

peroxidase enzyme (Cherry *et al*.,1999) and pH stability of certain enzymes like the xylanase (Lui *et al*., 2002).

The second approach originates from borrowing from the evolutionary algorithm of mutation and natural selection, known as directed evolution. New techniques have been developed which enable the directed evolution of genes (and their encoded proteins) to be more successful (Crameri *et al.,* 1997; Crameri *et al.*, 1995; Stemmer, 1994). Directed evolution techniques have allowed the generation of enzymes with greatly enhanced characteristics, and in some examples, enzymes with new and completely novel substrate specificities (Cherry *et al*., 1999; Giver *et al*., 1998; Larsson, 2003). Since the usefulness of these methods has been proven, wide application of industrial enzymes has gained more potential.

1.6 Different hosts for protein expression

1.6.1 Escherichia coli for recombinant protein expression

Although all these genetic engineering techniques developed and improved existing and newly-discovered, enzymes for large-scale application, these proteins need to be produced in sufficient quantities using cost effective production processes. Therefore, choosing a screening and production host for recombinant enzymes is very important. In some instances, one host may not be

suitable for both screening for a recombinant library and heterologous expression of a recombinant protein.

Among the many systems available for heterologous expression, *Escherichia coli* is usually the first choice for cloning and expression of heterologous proteins. This Gram negative, enteric bacterium is one of the most extensively used prokaryotic organisms for genetic manipulations and for industrial production of proteins of therapeutic or commercial interest (Banexy, 1999). Compared with other established and emerging expression systems, *E. coli* offers several advantages, including growth on inexpensive carbon sources, rapid biomass accumulation, amenability to high cell-density fermentations and relatively simple process scale-up (Lee, 1996). Although recombinant gene product accumulation at high levels in a biological active form is not guaranteed, a considerable amount of effort has been directed at improving the performance and versatility of this organism.

Due to its long history as a model system and because *E. coli* genetics are very well characterized, many tools have been developed for chromosome engineering and to facilitate gene cloning and expression. But, as a prokaryote, it is usually unable to produce heterologous proteins that require complex post-translational modifications and most eukaryotic proteins do require these modifications. There are other problems that cause production of non-functional heterologous proteins in *E. coli,*e.g.,

- if the foreign gene might contain introns, this would be a major problem as *E. coli* genes do not contain introns so it does not possess the necessary machinery for intron removal from transcripts;
- the codon usage of the gene may not be ideal for translation in *E. coli;*
- incorrect protein procession e.g., *E. coli* recombinant proteins are never glycosylated correctly;
- misfolding of recombinant protein; and
- degradation of recombinant proteins.

There are many other problems associated with this host. Among other is the utilization of the *lac* operon where most of the promoter systems are derived as shown Table 1.3 (Choi *et al*., 2006). The *lac* promoter can use isopropyl-β-D-1-thiogalactopyranoside (IPTG), which is a non-hydrolysable analog of lactose, for induction. The cost of this induction chemical and toxicity often limits the use of IPTG for production of large quantities of proteins for industrial application. These costs may, however, be overlooked in production of high value-added products. The toxicity issue of this promoter can also be overcome by the use of lactose itself as an inducer. This was demonstrated by Ramchuran *et al.* (2005a), and higher amounts of a thermostable xylanase isolated from *Rodothermus marinus* was produced. The leakiness of the *lac* promoter, may also be a problem for the production of membrane proteins or other genes toxic to the cells (Figgie *et al*., 1988).

The use of the commercialised pET system (Novagen) for gene expression has become increasingly popular. In this system, the target gene is positioned downstream of the bacteriophage T7 promoter, on a medium copy number plasmid. This strong T7 RNA polymerase leads to synthesis of high amounts of mRNA and, in most cases, the accumulation of the desired protein is very high (40-50% of total cell protein). But several problems can also occur. The high concentrations of mRNA can lead to the dissociation of ribosomes and the leaky expression may result in plasmid or expression instability (Baneyx, 1999). Also, pET vectors themselves have been reported to be toxic to the cell, even without the recombinant gene (Miroux and Walker, 1996).

Table 1.3 Different promoters and induction types used for expression of recombinant proteins in *E. coli* (Baneyx, 199

Promoter	Regulation	Induction	Problems
lac (*lacUV5*)	*lac*I, *lac*I^q	IPTG, thermal	Low level expression relative to other systems, leaky expression;
trp	*trp*	Trp, starvation of indoleacrylic acid	Leaky expression;
tac	*lac*I, *lac*I^q	IPTG, thermal	Leaky expression;
λpL	*λcIts* 857	Thermal	Induction cannot be performed at low temperature, partial induction cannot be achieved
T7	*lac*I, *lac*I^q	IPTG, thermal	Leaky expression; difficult to achieve high cell densities
PhoA	*pho*B, *pho*R	Phosphate starvation	Not titratable, limited media options
Ara	*ara*C	L-arabinose	Few vectors available
Cad	*cad*R	pH	Limited characterization, few vectors available
RecA	*lex*A	Nalidixic acid	Not titratable

Another strategy for production of recombinant proteins in *E. coli*, is the use of high cell density culture. This can be done once an expression system has been developed or selected. Studies have shown that nutrient composition, nutrient feed strategy and fermentation variables like temperature, pH, and dissolved oxygen can affect transcription, translation, proteolytic activity, secretion, production levels and stability. In two studies, Ramchuran *et al*. (2002 and 2005a) demonstrated that nutrient composition can affect levels of production where an increase levels of succinic acid and malic acid resulted in increased levels of production using a Glucose-limited fed-batch system (Lee, 1996). These two reports also demonstrated that the production of protease is caused by induction stress response which can lead to the degradation of product.

There are numerous examples of recombinant protein production in *E. coli*, and it is still going to continue being used extensively (Deng *et al*., 2005). There are also numerous strategies that have been employed for efficient production but still problems persist (Jana and Deb, 2004). These problems and other associated with expression and production of recombinant proteins in *E.coli* has lead to a renewed search in finding new hosts for expression of recombinant proteins, especially from eukaryotes.

1.6.2 Yeast as protein factories

Although *E. coli* is still the first choice for genetic manipulation and expression of heterologous proteins, yeasts also have some attractive features. Yeasts are unicellular eukaryotic microorganisms. This feature is important because it combines the ease of genetic manipulation with the ability to perform eukaryotic processing steps on the polypeptides expressed and thus constitute attractive systems for various aspects of modern biotechnology. This makes it easy to study eukaryotic proteins, especially human proteins. Yeasts have been used by humans for thousands of years for various processes. Yeasts unlike most eukaryotes, have relatively fast growth rate which makes them useful for screening large libraries

The expression of a foreign protein in yeast consists of four basic steps: firstly, cloning of a foreign protein-coding DNA sequence within an expression cassette containing a yeast promoter and transcriptional termination sequences; secondly, transformation and stable maintenance of this DNA fusion in the yeast cell; thirdly, synthesis and expression of the foreign protein under specified conditions; and finally, purification and characterisation of the heterologous protein (Cereghino and Cregg, 1999).

Proteins produced in yeast, unlike those produced in *E. coli*, lack endotoxins. In certain special cases, such as the hepatitis B core antigen, the product produced in yeast has higher activity than that produced in *E. coli*. In contrast with using *E.*

coli, several post-translational processing mechanisms available in yeast have allowed the expression of several human or human pathogen-associated proteins with appropriate authentic modifications. Such post-translational modifications include particle assembly, amino terminal acetylation, myristylation and proteolytic processing (Cereghino and Cregg, 1999). In addition, heterologous proteins secreted from specially engineered strains are correctly cleaved and folded and are easily harvested from yeast culture media. The use of either homologous or heterologous signal peptides has allowed authentic maturation of secreted products by the endogenous yeast apparatus. The importance of yeast for the production of protein products by recombinant DNA methods is illustrated by the fact that the first approved human recombinant vaccine, hepatitis B core antigen (Velanzuela *et al*., 1982), and the first recombinant food product, rennin, were produced in yeast.

1.6.3 Saccharomyces cerevisiae

S. cerevisiae has been used for thousands of years by mankind in brewing and baking. This yeast is regarded a GRAS organism (generally regarded as safe) by all countries except in Europe where its GRAS status has been removed because it has been suspected of being an opportunist pathogen (Murphy and Kavanagh, 1999). An overwhelming wealth of information on its genetics, molecular biology and physiology has been accumulated. This makes *S.*

cerevisiae the best characterized eukaryotic system because thousands of genes have been characterized and its entire genome has been sequenced.

During the last three decades, the availability of relevant structural genes and its use for genetic engineering has made it a fascinating approach in creating strains that can produce recombinant proteins in high amounts and quality. Thus, it is not surprising that the first commercialized recombinant vaccine, the hepatitis B vaccine, is a *S. cerevisiae* derivative. *S. cerevisiae* has been developed as production system for many different proteins, among others, therapeutics like insulin (Gellissen and Cornelis, 1997).

1.6.4 Methylotrophic yeasts

There is a limited number of yeast species that has the ability to grow on methanol as sole energy and carbon source. They belong to the four genera *Hansenula, Pichia, Candida and Torulopsis*. These genera share a general methanol utilization pathway. The crucial enzyme of this pathway can make up to 30% of the total intracellular protein content in methanol–grown cells (Gellissen and Cornelis, 1997).The isolation of genes encoding for these enzymes provided strong inducible promoter elements for the efficient expression of heterologous proteins. This has lead to the development of transformation procedures and the

use of these species as alternative for production of heterologous proteins in high yields.

Although a range of *P. pastoris* derived proteins have been generated, *Hansunela polymorpha* has been used for industrial applications because of its favourable fermentation characteristics. Some products from this organism have successfully passed clinical examination, with the hepatitis B vaccine produced in this yeast already available on the market (Gellissen and Melber, 1996; Lepetic *et al.,* 1996). The two ascomycetous yeast species, *Hansenula* and *Pichia*, have been used because they are the most efficient for biotechnological application.

1.6.5 Pichia pastoris for protein production

P. pastoris has emerged as one of the most popular yeast expression hosts. This popularity may be due to a number of factors: The use of the alcohol oxidase **I** (*AOX*1) promoter, which is one of the strongest and tightly regulated promoters known to man; secondly, the ability to stably integrate expression plasmids at a specific site in either single or multi-copy; thirdly, the ability to culture strains into high density fermenters; and finally; its commercialization by Invitrogen Corporation. *Pichia pastoris* can express and secrete large amounts of heterologous proteins. This, coupled with the ability to perform complex post-translational modifications, and the ability to grow to very high densities makes

this methylotrophic yeast a powerful tool for expression and production of recombinant proteins (Cereghino and Cregg, 2000; Scorer et al., 1994). Other factors for success with *P. pastoris* maybe largely attributed to:

- the availability of other strong, tightly controlled promoters like GAP and CUP;
- the relative ease, speed and cost-effectiveness with which it could be genetically modified and propagated;
- strong preference for aerobic growth – a key physiological trait that greatly facilitates culturing at high cell densities compared with the fermentative baker's yeast *S. cerevisiae;*
- growth on simple, chemically defined media and it releases comparatively few endogenous proteins release into culture media while secreting large amounts of recombinant protein; and
- subsequent ease of protein purification and downstream processing.

P. pastoris expression systems have been used to produce recombinant proteins with different application. An example for diagnostic and therapeutic application is production of a functionalized single-chain antibody fragment as described by Rahbarizadeh *et al*. (2006). The wide use of this yeast for production of therapeutics is because the correct glycosylation of proteins which makes it unlikely that it will induce an antigenic reaction if used in the bloodstream, a problem that is frequently encountered with over-glycosylation of proteins synthesized in *S. cerevisiae*.

Proteins previously produced in *P. pastoris* include, xylanase inhibitor (Fierens *et al*., 2004), thermostable family 10 xylanase (Ramchuran *et al*., 2005b), lysozyme (Masuda *et al*., 2005), aqualysin I (Olędzeka *et al*., 2003) and others. The wide variety and levels of protein production in this expression host, has made it one of the most utilized expression system in recent years.

1.7 This study

Several xylanases have been successfully engineered using directed evolution for application in the pulp and paper industry. But most of this work was done using *E. coli* as the model of choice for cloning. However, this microorganism is, however, not suitable for overproduction of all recombinant proteins. This study investigated the use at yeast, in this case *P. pastoris,* as an alternative to overcome this problem in producing a genetically modified xylanase which was obtained using directed evolution. This removes the burden caused by unavoidable decrease of activity during the process of tailor making.

Although most commercial systems use the *AOX*I promoter, this promoter requires two stages, growth and expression, with methanol induction. In this study the promoter that was used is a constitutive *GAP* promoter which has not been widely used for protein production and overexpression. The pET system was also used for comparison.

CHAPTER TWO: METHODOLOGY

2.1 Plasmids, bacterial and yeast strains

The plasmids that were used for this study are listed in Table 2.1 and include Bluescript which carried the mutated *xyn*A gene and was designated pNC38 (Fig 2.1) from a previous study (Mchunu *et al*., 2009), pET-22b(+) (Fig 2.2) for expression in *E. coli*, pBGP1 (Fig 2.3) for expression in *P. pastoris*, pJC1 and pDLG1 (Fig 2.4 and 2.5) for expression in *S. cerevisiae*. *E. coli* strains BL21 (DE3) (Novagen) and XL1 Blue (Stratagene), were maintained on LB media (10 g/l bacteriological peptone, 5 g/l yeast extract, 5 g/l NaCl and 15 g/l agar).

Pichia pastoris GS511 (Invitrogen) and *Saccharomyces cerevisiae* Y294 strain, obtained from W.H. Van Zyl, Stellenbosch University, were maintained on YPD media (10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose, 20 g/l agar). Selection and maintenance of *E. coli* was done on LB media containing 100 µg/ml of ampicillin. *Pichia* clones were selected in YPD media with 100 µg/ml of zeocin and *S. cerevisiae* clones were selected in uracil minus yeast minimal media (2 g/l of drop-out amino acid mix without uracil (US Biological), 6.7 g/l yeast nitrogen base (without amino acids) and 20 g/l dextrose, 20 g/l agar).

Table 2.1 Plasmids vectors used in this study

Plasmid	Characteristics	Source
pBluescript	Amp^R , *LacZ*	Stratagene
pNC38	Amp^R , *LacZ,* NC38 xylanase	Mchunu, 2009
pET-22b(+)	Amp^R , *LacZ*, His-Tag	Novagen
pDLG1	Amp^R *URA3*$^-$*, ADH2* promoter and termination cassettes	W. H. Van Zyl
pJC1	Amp^R , *URA3*$^-$*, PGK* promoter and termination cassettes	W. H. Van Zyl
pBGP1	Amp^R , $Zeocin^R$, *GAP* promoter and termination cassettes	Lee *et al*., 2006

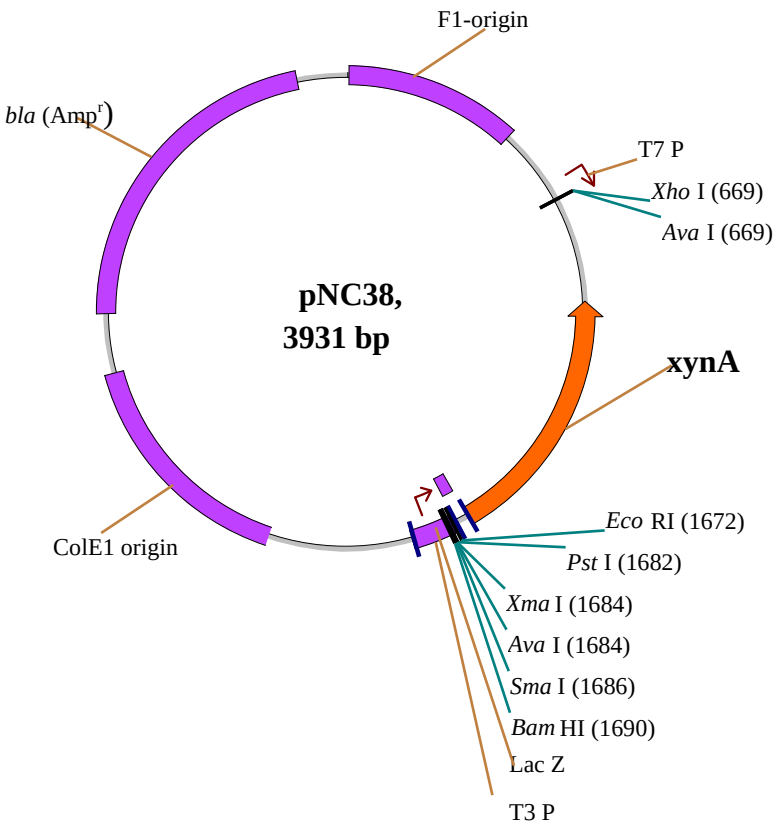

Fig. 2.1 Map of pNC38 showing location of the mutated *xynA* gene. pNC38 contains the β-lactamase (*bla*) gene, *xynA* and other regions. Restriction sites on the polylinker of the plasmid are shown. T3 P and T7 P designate the positions of the T3 and T7 promoters, respectively. *Lac*Z indicates the position of a short section of the *Lac*Z gene that was used in the cloning of *xynA* as a LacZ-fusion protein (Mchunu, 2009).

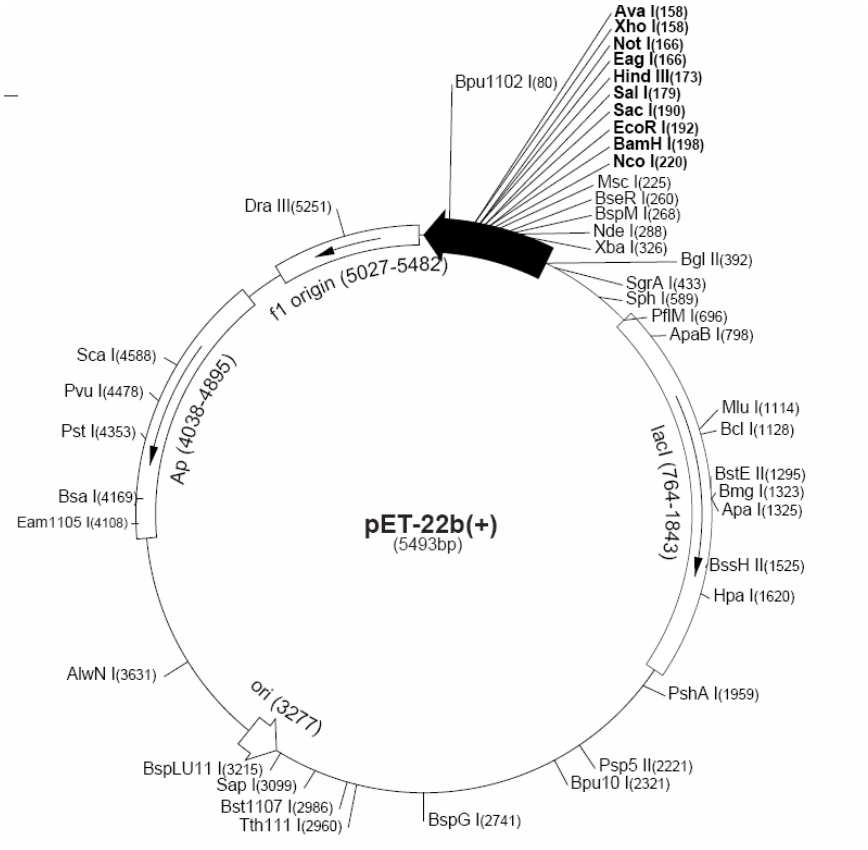

Fig. 2.2 Map of pET-22b(+) used for expression of xylanse in *E. coli* showing location of the replication region. Restriction sites on the plasmid are shown and their exact positions indicated in brackets and other regions (Novagen). The mutated xylanase gene was inserted into pET-22b(+) using the *Xho*I and *Eco*RI restrictions sites.

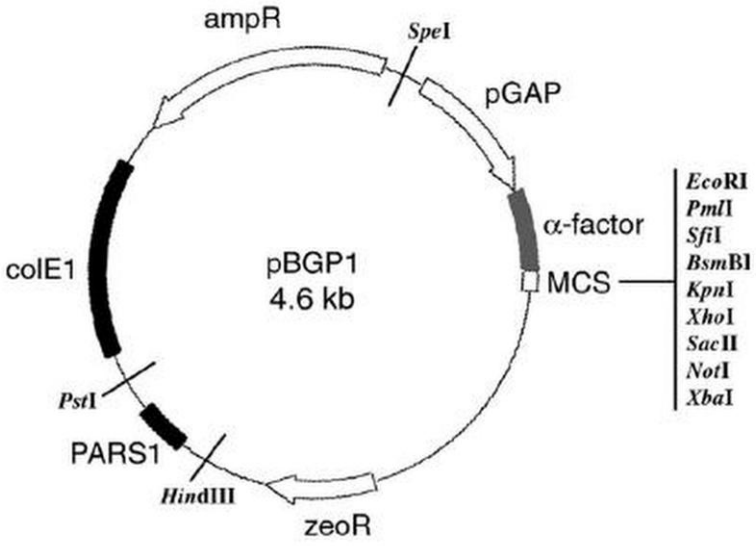

Fig. 2.3: Map of the *Pichia* vector, pBGP1, showing the location of the ampicillin resistance (Amp^R) gene, zeocin resistance (Zeo^R) gene, *GAP* promoter (pGAP), secretion signal (α-factor) and multiple cloning sites (MSC) and *E. coli* replicon (colE1) and *Pichia* autonomous replication sequence (PARS1) .

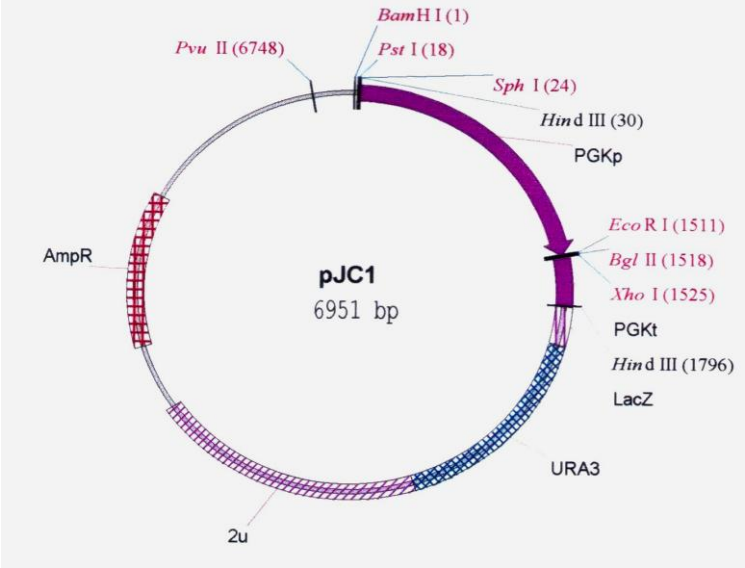

Fig. 2.4 Map of the yeast-*E. coli* shuttle vector pJC1. The vector contains an origin of replication (2μ), Amp^R gene, uracil selectable marker (URA3) and PKG*1* promoter and termination cassette.

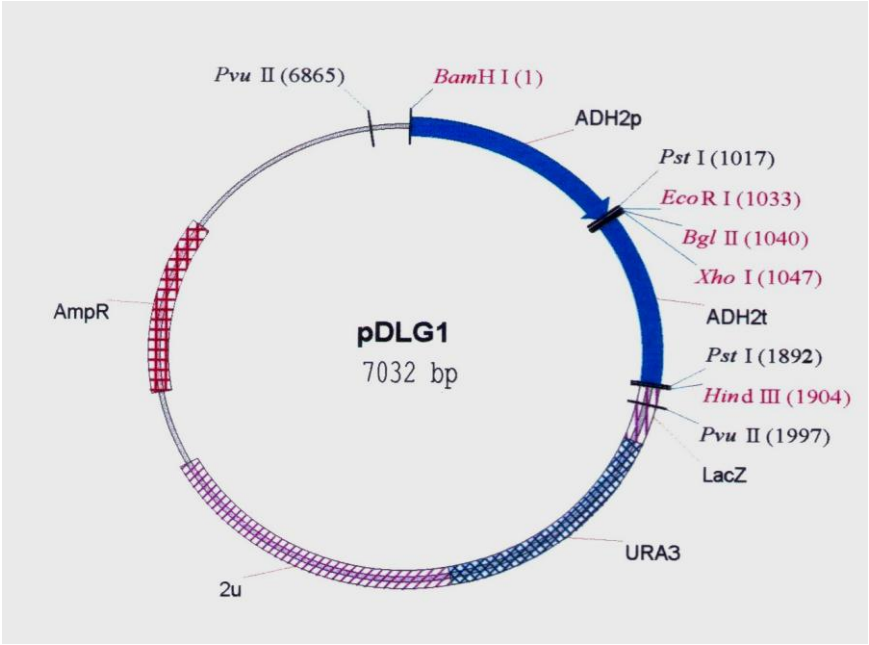

Fig. 2.5 Map of the yeast-*E. coli* shuttle vector pDLG1. The vector contains the origin of replication, Amp^R gene, uracil selectable marker (URA3) and *ADH2* promoter and termination cassette.

2.2 Plasmid isolation

Plasmid isolation from *E. coli* was done using a GeneJET Plasmid Miniprep Kit (Fermentas) according to the instruction manual. *E. coli* cultures containing plasmids were grown overnight in 5 ml LB broth with 100 μg/ml of ampicillin at 37^{o}C. The culture was then centrifuged and the pellet resuspended in 250 μl ice cold lysis solution, followed by the addition of neutralizing buffer with gentle mixing. The mixture was then incubated at room temperature for 3 min, followed by centrifugation in a microcentrifuge for 5 min at 10 000 *g*. The supernatant was transferred into spin columns. The spin columns were centrifiged for 1 min followed by two washing steps with ethanol. The plasmid DNA was eluted using 50 μl of sterile double distilled water. Five microlitres of the plasmid suspension was used to verify the presence of the plasmid DNA using agarose gel electrophoresis. The rest of the suspended plasmid solution was stored at -20^{o}C.

Plasmid isolations from yeast were done using a modified procedure of the GeneJET Plasmid Miniprep Kit (Fermentas). The yeast cells were resuspended and after addition of acidified glass beads (Sigma-400 μm), the cells were vortexed for 3 min, in order to lyse the cells. Thereafter, the isolation kit procedure was followed as mentioned above. The DNA solution obtained from this isolation was added to *E. coli* competent cells for transformation in order to produce sufficient amounts for further DNA analyses.

2.3 Agarose gel electrophoresis

Visual analysis and confirmation of plasmid DNA isolation, restriction digestion analysis, PCR analysis and DNA purification was done using agarose gel electrophoresis. The separation of DNA molecules in this technique is based on the size of the molecules. Agarose gels were prepared by microwaving 0.8% agarose in 1x TAE buffer (50x stock: 242 g Tris-base, 57.1 ml glacial acid, 100 ml of 0.5 M EDTA, pH 8) for 40 seconds at high power. The molten agarose was mixed thoroughly and poured into a casting tray fitted with well combs and allowed to set. The gel was then placed in the electrophoresis tank and covered with 1X TAE electroporation buffer prior to applying the samples. Samples were mixed with 6X gel loading buffer (0.0375 g bromophenol blue, 4.0 g sucrose, 1.5 ml 10% SDS and 3 ml 0.5 M EDTA, pH 8), in a ratio of 1:5. The DNA samples, along with a DNA molecular markers (section 2.4), were loaded into the wells of the gel and electrophoresis was conducted at a field strength of 85 V for 90 min. The gels were stained in ethidium bromide (50µg/ml) for 15-20 min and destained in distilled water for a further 15-20 min. The gels were then viewed using a UV transilluminator and the desired bands were identified by a comparison with the marker bands. The gel images were captured using a Scion Digital imaging system.

2.4 DNA molecular weight marker preparation

A DNA molecular weight marker was used to determine if the correct or expected banding patterns and DNA sizes were obtained after each DNA manipulation. The DNA marker was prepared by restricting 35 µl of λ DNA (Fermentas) with 10 µl *Eco*RI and *Hin*dIII (Fermentas), followed by the addition of 10 units of restriction enzyme buffer B and adding distilled water to a final volume of 100 µl. The mixture was incubated at 37^{o}C for 1 h, followed by deactivation of the enzymes by incubation at 65^{o}C for 10 minutes. Gel loading buffer was added at a ratio 1:5 to DNA and resulting mixture was stored at 4^{o}C. Volumes of 5-10 µl were used for analyses by agarose gel electrophoresis.

2.5 DNA purification

Purification of DNA molecules from agarose gel slices or solution was performed using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), according to the manufacturer's instructions. The DNA solution was added to spin columns containing 500 µl of capture buffer, followed by centrifugation in a microcentrifuge at 10 000 x*g* for 1 min. Gel purifications were performed by adding 1 µl capture buffer to every 1 mg agarose followed by heating at 60^{o}C for 5-10 min. The molten agarose with DNA was transferred into spin columns. The spin columns were spun for 1 min followed by one washing step with 500 µl of wash buffer. The plasmid DNA was eluted using 50 µl of sterile double distilled

water. Five microlitres of the plasmid suspension was used to verify the presence of the plasmid DNA using agarose gel electrophoresis. The rest of the DNA solution was stored at -20^{o}C.

2.6 DNA quantification

Quantification of DNA was carried out using a UV-VIS spectrophotometer based on the premise that an absorbance of 1 corresponds to 50 ng of DNA/µl (Sambrook *et al*., 1989). DNA solutions with A_{260}/ A_{280} ratio of 1.7 and above were considered to contain DNA of sufficient purity to proceed with further DNA manipulations. If the ratio was below this value, the DNA solution was further purified by precipitation with ethanol and sodium acetate as described in Sambrook *et al*., 1989.

2.7 PCR

The mutated xylanase gene was amplified in order to have adequate concentrations for further DNA manipulation. The amplification was performed using the PCR cycling conditions described in Table 2.2 and the following amplification primers (Inqaba Biotech):

T3 forward primer: 5'-ATTAACCCTCACTAAAGGGA-'3

T7reverse primer: 5'-TAATACGACTCACTATAGGG-'3

The PCR reaction mixtures contained 1 μl of DNA (50 ng/μl); 2.5 mM $MgCl_2$; 1x PCR buffer (Roche); 0.5 μM Primers, 0.1 mM dNTPs and 1 U of Taq polymerase (Fermentas).

Table 2.2 PCR cycling conditions used for DNA amplification

Steps/Cycle	Temperature	Time
Denaturation	95^{o}C	90 seconds
Primer annealing	42^{o}C	60 seconds
Extension	72^{o}C	150 seconds

PCR reactions were carried out for 30 cycles using a PCR Genius thermal cycler, (Techne). Upon proper amplification, which was determined using agarose gel electrophoresis, DNA purification was performed and followed by agarose gel electrophoresis for verification of DNA presence after purification.

2.8 Restriction and ligation of vectors and insert

Plasmid cloning vectors and NC38 PCR products were digested with endonucleases, *Eco*RI and *Xho*I (Fermentas), to create compatible sticky ends for ligation using 1unit of enzyme per 1μg of DNA (Sambrook *et al.*,1989). These enzymes were use when the xylanase gene was original cloned into Blueskript.

The resultant reaction mixtures were incubated at 37^{o}C for one hour and the reactions were terminated by incubation at 65^{o}C for 15 min.

The restriction digestion was followed by agarose gel electrophoresis using 5 µl of the reaction mixture to verify that complete restriction digestion had occurred. After verification, the DNA was purified from the mixture using the GFX PCR DNA and GEL Band Purification Kit (Amersham Biosciences) as outlined in section 2.5. An aliquot of the purified DNA was used for agarose gel electrophoresis analyses to ensure that the DNA was still present after purification. The DNA was subsequently quantified using a UV-VIS spectrophotometer. Equimolar concentrations of the vector and insert were ligated. The ligation mixture contained vector, insert, 1X ligation buffer and T4 DNA ligase (Fermentas) and the reaction was incubated at 22^{o}C overnight using a thermal heating block (BIOER). This was then transformed into SEM-competent *E. coli* cells for pET-22b(+), pJC1 and pDLG1. However for *P. pastoris* transformation with the ligation mixture was performed directly in electrocompetent yeast cells

2.9 Preparation of *E. coli* SEM-competent cells

E. coli BL21 (DE3) (Novagen) were made competent using the Simple and Efficient Method prior to transformation (Ausubel *et al.,* 1989). An overnight colony was used to inoculate 5 ml sterile SOC medium (20 g/l tryptone, 20 mM

$MgSO_4$, 5 mM KCl, 40 mM glucose, 20 mM NaCl and 5 g/l yeast extract), incubated at 37^oC and shaken overnight. One milliliter of this culture was used to inoculate 29 ml of fresh SOC medium and shaken until it reached an OD_{600} of 0.375. The resultant culture was immediately placed on ice and kept cold for the duration of the procedure. The cells were pelleted by centrifugation at 5000x *g* for 10 min and the supernatant was discarded. The cells were resuspended in 10 ml of cold 100 mM $CaCl_2$ and centrifuged at 5000x *g*, then resuspended in 10 ml of cold 100 mM $CaCl_2$. This was then incubated on ice for 20 min then centrifuged. The competent cells were resuspended in 2 ml 100 mM $CaCl_2$ containing 10% glycerol. One hundred microlitres of competent cells was dispensed into 1.5 ml tubes, kept overnight at 4^oC and then transferred to -80^oC for storage.

2.10 Transformation and screening in *E. coli*

The ligation mixture (up to 5 µl) was added to 100 µl of competent *E. coli* cells and incubated on ice for 30 minutes, followed by heat shock for 30 seconds at 42^oC. Heat shocking was followed by the addition of 900 µl SOC medium (20 g/l tryptone, 5 g/l yeast extract, 40 mM glucose, 20 mM NaCl, 20 mM $MgCl_2$, 20 mM $MgSO_4$, 5 mM KCl). The cells were then incubated at 37^oC for 1 hour while shaking at 200 rpm. Aliquots of 50 µl were plated out on to RBB-xylan plates (containing 100 µg/ml of ampicillin) and incubated at 37^oC overnight. Transformed cells were selected by identification of a zone of hydrolysis around the colonies (Biely *et al.*, 1985; Biely *et al.,* 1988). Plasmid isolation of *S.*

cerevisiae was performed for transformation in yeast cells. This was followed by expression studies and characterization of the enzyme.

2.11 Preparation of *P. pastoris* and *S. cerevisiae* competent cells

Yeast host cells were made competent by treating the cells with DTT and subsequent stabilization in sorbitol (Scorer *et al.,* 1994). Both *P. pastoris* and *S. cerevisiae* were grown overnight on YPD plates at 30^{o}C. A single colony was inoculated into 200 ml of YPD broth and the cells were further incubated overnight at 30^{o}C to an OD_{600} of approximately 1.4. The cells were harvested by centrifugation at 2750 x*g* for 10 min, cells were resuspended in 40 ml of YPD broth, 1 ml of DTT (1M) and 8 ml of HEPES buffer (1 M), pH 8 and incubated at 30^{o}C for 15 min. The cells were then sequentially washed three times by suspending and centrifugation at 2750 x*g* for 10 min, with 200 ml of sterile water, 100 ml and 8 ml of 1 M sorbitol. The cells were finally resuspended in 400 µl of 1 M sorbitol and stored in aliquots of 50 µl at -80^{o}C.

2.12 Transformation and screening in *P. pastoris* and *S. cerevisiae*

Electroporation of *P. pastoris* and *S. cerevisiae* was performed using a Gene Pulser Xcell (BioRad) under the following pulsing conditions: 1.7 kV, 200 Ω and 25 µF. Fifty microlitres of yeast competent cells were mixed with up to 5 µl of the plasmid DNA with xylanase gene for transformation in a sterile 1.5 ml tube. The

electrocompetent cells were transferred into 0.2 cm electroporation cuvettes (BioRad) that had been kept on ice, and the pulse was delivered. Ice cold sorbitol solution (1 M) was added immediately after electroporation and the cells were incubated on ice for 15 min. One milliliter of YPD was added followed by further incubation for 1 h at 30^{o}C without shaking. Aliquots (100 µl) were plated onto YPD selective plates containing 0.4% RBB-xylan supplemented with 100 µg/ml of zeocin for *P. pastoris* and uracil minus plates for *S. cerevisiae* and incubated at 30^{o}C for 3-4 days. Yeast clones able to degrade the surrounding xylan and produce a zone of hydrolysis were selected for further use (Biely *et al*, 1985). Expression studies and characterization of the xylanase variants were subsequently carried out.

2.13 Xylanase expression and extraction

The selected yeast clones showing xylanase activity were plated out on selective media and single colonies were used to inoculate 20 ml liquid media which consist of YPD with zeocin for *P. pastoris*, uracil minus media for *S. cerevisiae* and LB with ampicillin for *E. coli*. After growth, these cultures were used to inoculate 200 ml liquid media for xylanase production, containing similar media components as mentioned above for each host. Expression in *P. pastoris* was terminated after 60 h with samples taken every 3 h. Readings at 600 nm were taken for growth analysis and then samples were centrifuged at 10 000x *g* and the supernatant stored at 4^{o}C for further enzyme characterization. Expression in

S. cerevisiae lasted for 48 h with samples take every 3 h and in *E. coli* for 8 h, including a 6.5 h period of induction with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) with samples taken every 30 min for growth and enzyme activity analysis.

In *S. cerevisiae*, the enzyme extractions were carried out using Yeastbuster™ Protein Extraction Reagent (Novagen). The cells were harvested from liquid culture by centrifugation at 3 000 *g* for 10 min at 4°C. The pellet was resuspended by adding 5 ml of Yeastbuster™ reagent per gram of wet cell pellet and vortexing. The cell suspension was incubated for 20 min in a shaking incubator with low settings at room temperature. The cells were then centrifuged at 13 000 *g* for 30 min at 4°C and the supernatant was stored at 4°C for further anaylsis. *E. coli*, the enzyme was extracted using Bugbugster Protein Extraction Reagent (Novagen) according to the suppliers' manual. The *E. coli* cells were harvested by centrifugation at 10 000 *g* for 10 min at 4°C. The Bugbuster™ was added to the ratio of 1: 30. The cells were then incubated for 20 min with slow shaking at room temperature. The cells were then centrifuged at 13 000 *g* for 30 min at 4°C. The supernatant was retained as it contained the enzyme fraction and stored at 4°C for further enzyme characterization.

Bugbuster™ Protein Extraction Reagent was not used for extraction of enzyme for characterization at high pH. This is because this reagent affected the stability of the xylanase at higher pH levels. Thus, breaking buffer (6.8 g/l KH_2PO_4, 0.61

g/l $MgCl_2.6H_2O$, 0.77 g/l DTT, 0.37 g/l EDTA – pH 6.8 followed by addition of PMSF after autoclaving) was used in conjunction with sonication (1 min pulsing followed by incubation on ice for 1 min for 3 cycles with frequency of 20 kHz) for cell lysis. This was followed by centrifugation at 13 000 *g* for 30 min at 4^{o}C. The supernatant containing the enzyme fraction was stored at 4^{o}C for further enzyme characterization.

2.14 SDS-PAGE and zymogram analysis

After protein extraction, the protein samples were analyzed using SDS-PAGE (Laemmli, 1970) and zymogram analysis using a 12.5% running-gel and a 5% stacking-gel. Samples were prepared by adding equal volumes of sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 0.1% bromophenol blue and 10% glycerol) and protein sample followed by boiling for 10 min. The samples were then loaded onto the gel and allowed to run for about 2 h in running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS). The SDS-PAGE gel was stained for 2 h in the staining solution containing 0.25% Coomassie Brilliant Blue in 90 ml of methanol: water (1:1 v/v) and 10 ml glacial acetic acid in a rocking platform, then de-stained overnight in a de-staining solution containing 90 ml of methanol: water (1:1 v/v) and 10 ml glacial acetic acid. For zymogram analysis, a similar procedure to that of SDS- PAGE was followed except that the gel was not stained and the proteins in the gel were renaturated in a solution containing 1% Triton X-100 and 20 mM Tris-HCl, pH 7 for 30 min followed by washing in a 20

mM Tris-HCl solution for 1 h. The gel was then sandwiched with an agarose gel containing 0.3% xylan and incubated for 1 h at 50^{o}C. This was followed by staining with 1% congo red and destaining in 1 M NaCl. Active xylanase bands were identified by zones of hydrolysis (Beguin, 1982). A BioRad protein marker was used to estimate the sizes of the protein in the tested samples.

2.15 Partial purification of xylanase

After SDS analysis, the enzyme extractions from *E. coli* were partially purified by heat-treating the proteins at 65^{o}C for 15 min to remove native proteins. This was followed by centrifugation at 13 000 *g* for 30 min at 4^{o}C. These samples were further purified using HiTrap his-tag columns (Amersham Biosciences) and the AKTA Prime Protein Purifier (Amersham Biosciences). The composition of the buffers used was as follows: binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4), wash buffer (distilled water) elution buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4 and 0.5 M imidazole, pH 7.4). The cells were resuspended in binding buffer before sonication. Then enzyme extraction was performed as described in section 2.13. The enzyme sample port tube was placed inside the bottle containing the sample. Copper column was used for the purification as the protein contained a His-tag. The column was first standardised using the binding, running at 0.5 ml/min, and the samples that were eluted during the start of the peak to the end were collected then tested for enzyme activity and purity.

2.16 Temperature and pH stability

β-1,4-xylanase activity was assayed according to Bailey *et al*. (1992) by incubating 100 µl enzyme extract and 900 µl substrate solution of 1% birchwood xylan (Sigma) in citrate buffer (0.05 M, pH 6.5) at 50^{o}C for 5 min. The reaction was terminated by the addition of 1.5 ml dinitrosalicyclic acid (DNS) solution, and boiling for 5 min. One enzyme unit was defined as the amount of β-xylanase which liberates 1 µmol of xylose per minute.

Temperature and pH optima of the enzymes produced in the different hosts, *P. pastoris*, *S. cerevisiae* and *E. coli*, was determined by incubation of the enzyme at temperatures ranging from 40^{o}C to 90^{o}C (Giver *et al.*, 1998). The activity of the xylanase was also determined at pH levels ranging from 4.0-10.0 (pH 4-6.5, using citrate buffer; pH 7-9, using Tris-HCl buffer and pH 10.0, using glycine-NaOH, all at 0.05 M concentrations) using above mentioned assaying conditions. Thermostability of the xylanase variants was determined by incubating the enzyme at 60^{o}C, 70^{o}C and 80^{o}C for 90 min, removing samples at 15 min intervals, for storage on ice and assaying for residual xylanase activity (Bailey *et al*., 1992). Long term stability of the enzyme at alkaline pH was tested by incubating the enzyme at pH 9 and pH 10 for 90 min. During the 90 min incubation, samples were taken at 15 min intervals and assayed for residual xylanase activity (Bailey *et al*., 1992).

CHAPTER THREE: RESULTS

3.1 Cloning of the modified xylanase into different plasmid vectors

3.1.1 PCR

Isolation of pNC38 which contained the modified xylanase gene was conducted using the procedure described in section 2.2 to yield DNA for PCR. Successful amplification was verified using agarose gel electrophoresis. Single, discreet bands were observed on agarose gels (Fig. 3.1). The size of the amplified DNA bands correspondes to that of the DNA fragment containing of the *xyn*A gene, at 1003 bp. The intensity of the band was sufficient for purification of PCR product for subsequent DNA restriction, ligation and transformation.

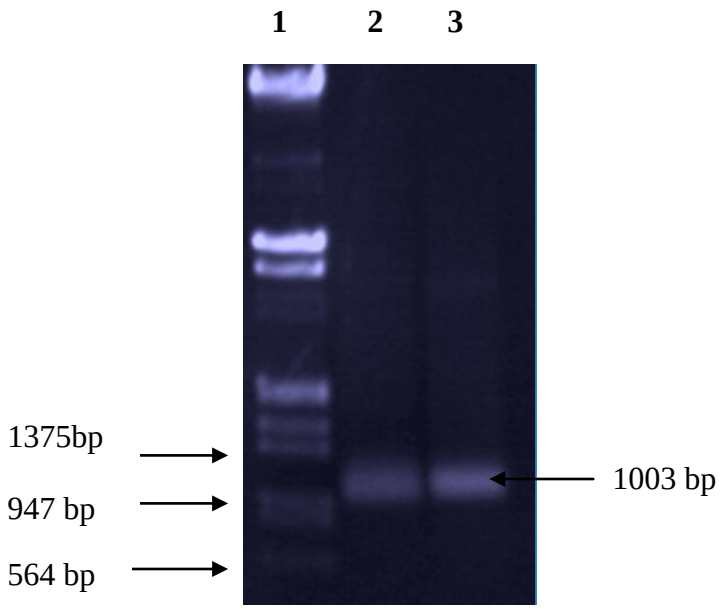

Fig 3.1 Amplification of the NC38 xylanase variant from pNC38. Lane 1 shows the DNA molecular weight marker that was used for estimation of the molecular weight of the bands produced and lane 2 and 3 are duplicate samples of 1003 base pair amplication product containing the mutated xylanase gene.

3.1.2 Screening for xylanase production

The xylanase gene was cloned into different vectors then transformed into the respective hosts. The positive transformants were selected on different selection media designed for the different expression hosts. These were RBB-xylan YPD supplemented with zeocin for *P. pastoris,* RBB-xylan uracil minus medium for *S.*

cerevisiae and RBB-xylan LB for *E. coli*. These transformants were identified by the halo around the colony which meant that the colonies were producing a xylanase which was hydrolyzing the RBB-xylan which is a blue complex into colourless degradation products (Fig. 3.2 and 3.4), halos produced by *E. coli* were small (Fig. 3.2), but sufficient to allow for their identification and selection. In *S. cerevisiae,* the zones around the colonies were very small on glucose while the pJC1-NC38 construct produced a bigger zone on galactose (Fig 3.3), compared to the pDLG1-NC38 construct. Zones produced by the pBGP1-NC38 construct in *P. pastoris* were much larger (Fig. 3.4) than those produced in *S. cerevisiae* and *E. coli*.

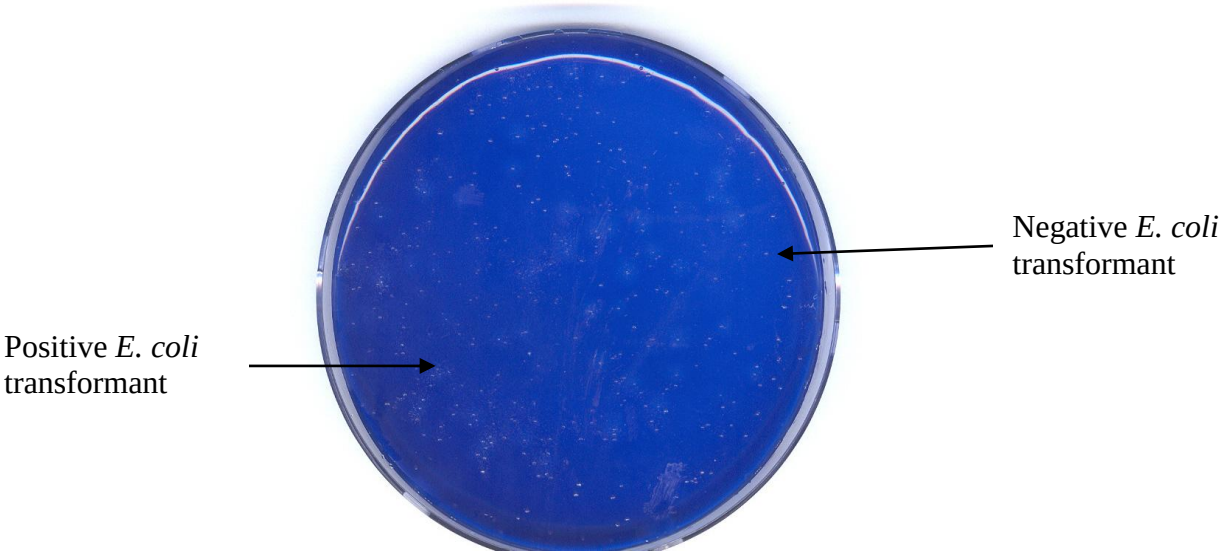

Fig. 3.2 Screening plate showing positive and negative *E. coli* BL21 transformants spotted on a plate of RBB-xylan LB with ampicillin.

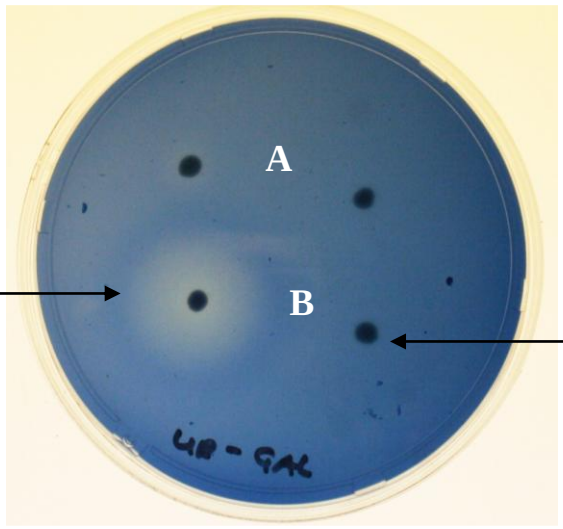

Fig. 3.3 Screening plate showing positive and negative *S. cerevisiae* transformants spotted on a plate minimal media with galactose. **A** - pDLG1 and **B** -pJC1, with non-producing strains on the right.

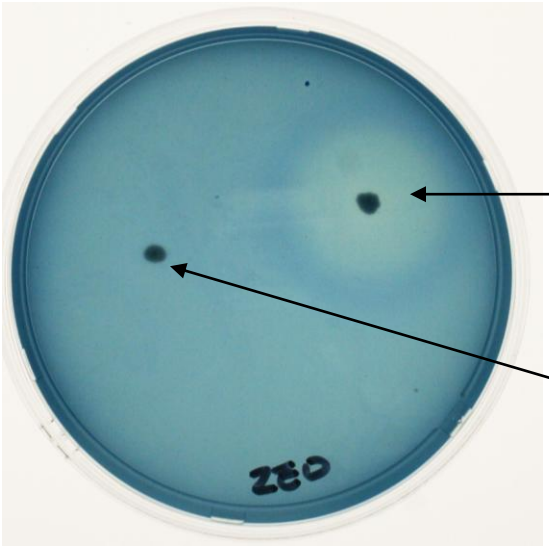

Fig. 3.4 Screening plate showing positive and negative *P. pastoris* transformants spotted on a plate RBB-xylan YPD with zeocin. The clear zone is indicative of xylanase activity**.**

3.1.3 Restriction analysis of selected clones

Restriction analysis was performed on the recombinant plasmids isolated from *E. coli*, *P. pastoris* and *S. cerevisiae* after transformation and selection of their ability to produce active xylanases. For yeast restriction analysis, plasmid isolation was first done and the DNA solution was used to transform SEM-competent cells to generate large quantities of plasmid DNA required for restriction analysis.

Restriction endonucleases *Eco*RI and *Xho*I were used for the restriction analysis as these enzymes were employed during the cloning of the insert. Two bands were produced after restriction, which corresponds to vector and insert (Fig. 3.5-3.7). This confirmed successful ligation of the modified xylanase into the different expression vectors. Fig. 3.5 shows the restriction analysis of the plasmid DNA isolated from a clone with a zone of hydrolysis. The bands that were observed corresponds to the size of the pET22b(+) vector at 5493 bp and the *xyn*A insert which is 1003 bp. A different molecular weight marker from Fermentas (Gene Ruler 1000 bp DNA Ladder) was used in this gel. Similarly, restriction analysis confirmed successful insertion of the xylanase gene in pBGP1 (Fig. 3.6). The restriction analysis was performed using plasmid DNA recovered from *P. pastoris* transformants. Two bands were observed, with one at approximately 4600 bp corresponding to the pBGP1 plasmid vector and the smaller band corresponded to the insert size of 1003 bp. Restriction analysis of plasmid DNA isolated from *S.*

cerevisiae for both vector, pJC1 and pDLG1, produce two bands corresponding to the vector with approximately 7000 bp and the insert with 1003 bp (fig. 3.7).

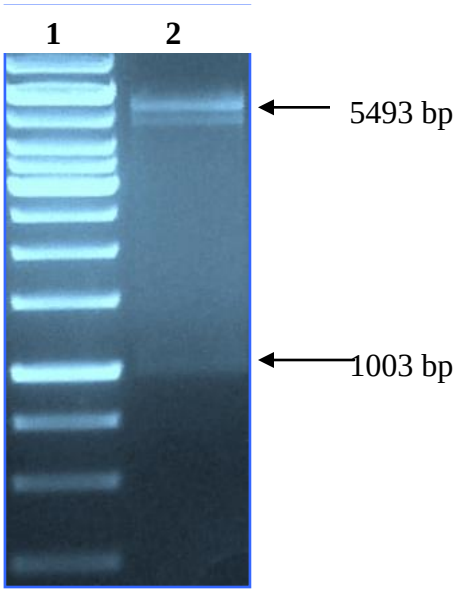

Fig. 3.5 Agarose gel electrophoresis of restriction of plasmid DNA isolated from *E. coli* BL21 for verification of xylanase gene insertion into the vector. Lane 1: Fermentas DNA marker and lane 2: two bands that were obtained after the restriction of the plasmid from the clones.

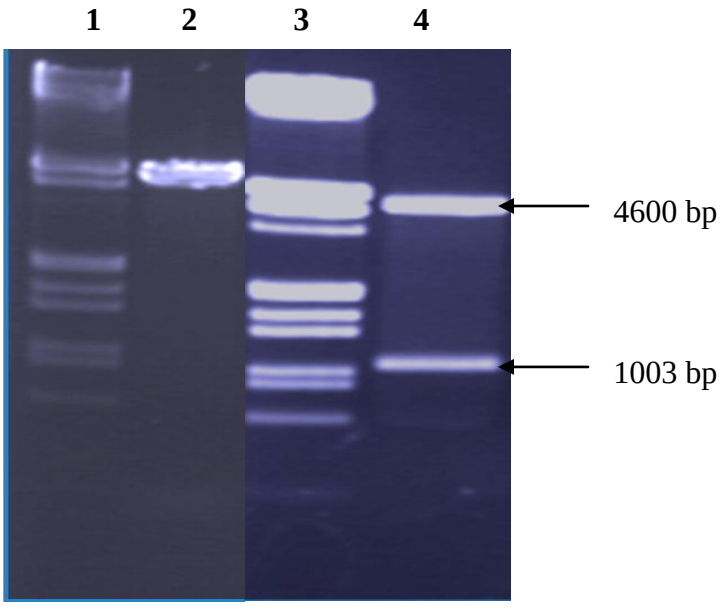

Fig. 3.6 Agarose gel electrophoresis of restriction analysis of plasmid isolated from positive *P. pastoris* transformants. Lane 1 and 3: DNA marker; Lane 2: linear *P. pastoris* vector pBGP1 and Lane 4: Two bands that were obtained after the restriction.

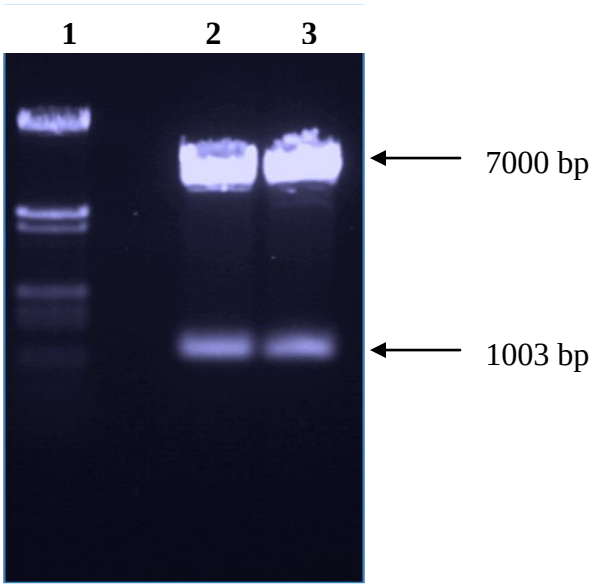

Fig. 3.7 Agarose gel electrophoresis of restriction analysis of plasmid DNA isolated from positive *S. cerevisiae* transformants. Lane 1: DNA marker; Lane 2: vector pDGLI with the *xyn*A insert and Lane 3: pJCI with *xyn*A insert.

3.2 Characterization of the xylanases from *P. pastoris* and *E. coli*

3.2.1 SDS-PAGE and zymogram analysis

In order to evaluate and compare recombinant protein production, samples obtained after extraction of the enzyme from *E. coli* and media from *P. pastoris* shake flask experiments were analysed using SDS-PAGE. The sample from *P. pastoris* showed a single protein band which was approximately 24 kDa which is

the size of this family 11 xylanase (Fig. 3.8). Samples that were obtained from lysis of *E. coli* BL21 cells had high contamination with intracellular proteins as expected. The *E. coli* sample was partially purified using heat treatment and a His-tag column, and this resulted in the gradual decrease in protein content after each purification step (Fig. 3.9). But still several bands were observed after SDS-PAGE analysis.

In some cases, although recombinant proteins are expressed, they can be inactive. Thus, an activity stain was performed using an agarose overlay. The activity stain showed that the single band that was obtained in *P. pastoris* was an active xylanase (Fig. 3.10). Using the SDS-PAGE for *E. coli*, it was not possible to be sure which of the bands represented the xylanase protein. The activity stain showed that there was a band that corresponded to the expected size of 24 kDa but other protein active protein bands positioned higher on the gel were observed.

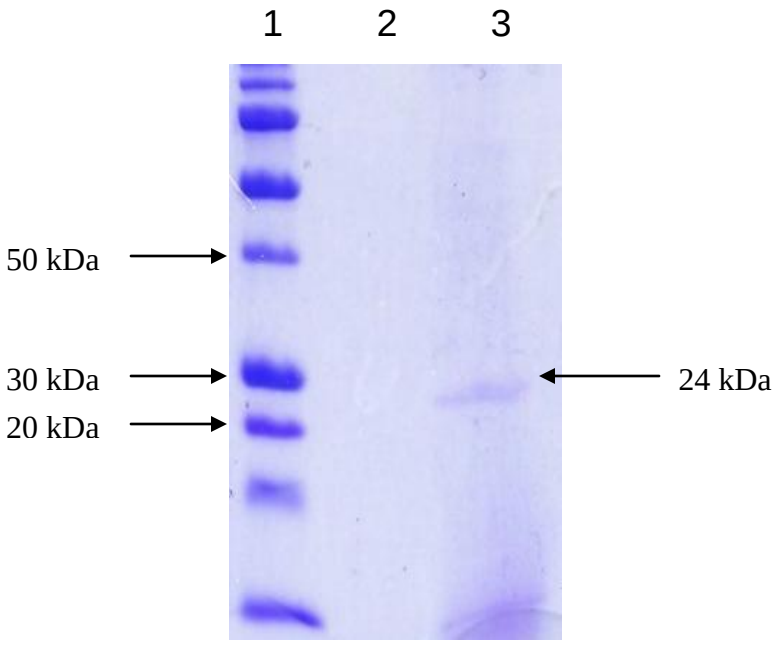

Fig. 3.8 SDS-PAGE of *P. pastoris* clones. Lane 1: Protein marker; Lane 2: Negative control and Lane 3: Sample obtained in shake flasks.

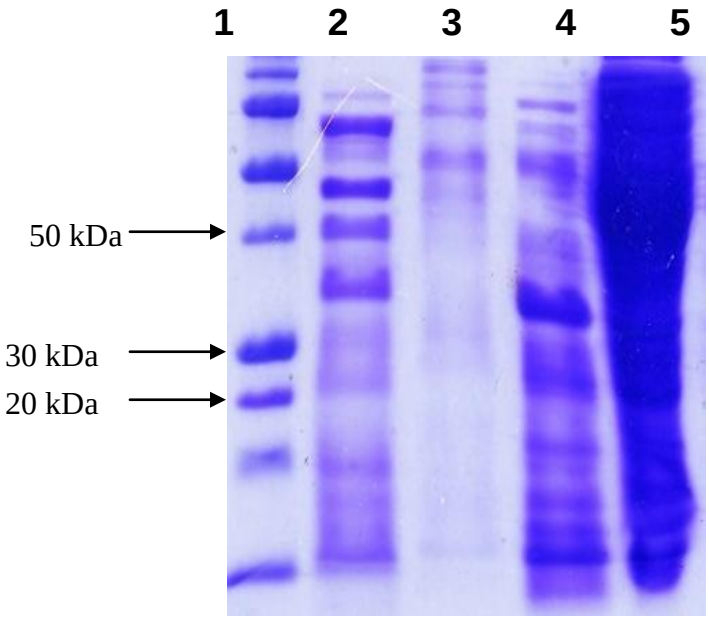

Fig. 3.9 SDS-PAGE of extraction sample from *E. coli* BL21 clones. Lane 1: Protein marker; Lane 2: Sample purified using His-tag column, Lane 3: Negative control, Lane 4: Heat treated sample at 65^{o}C for 15 min and Lane 5: Crude extract.

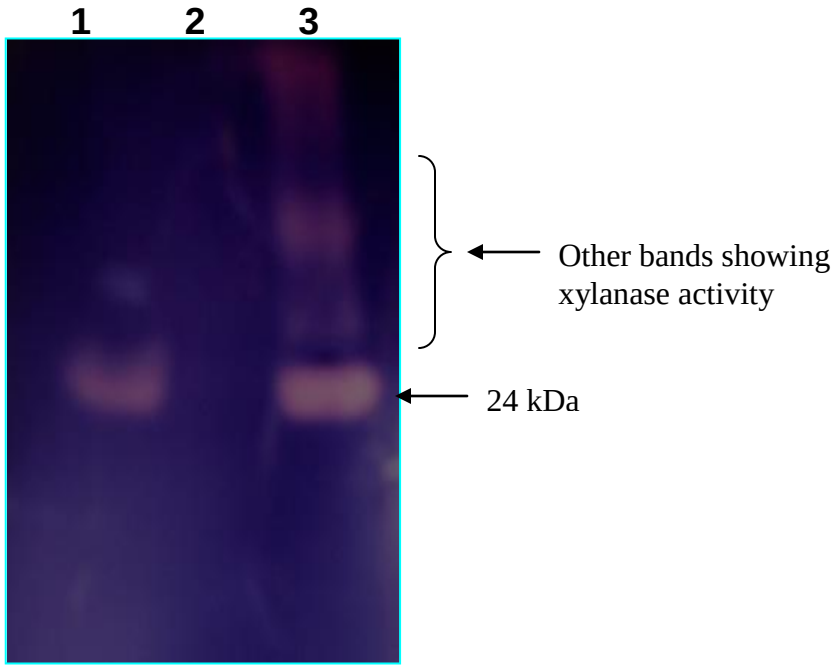

Fig. 3.10 Activity staining of *P. pastoris* fermentation sample and extraction sample from *E. coli* BL21 clones. Lane 1: *P. pastoris*, Lane 2: YPD medium, and Lane 3: *E. coli* BL21.

3.2.2 Expression of the xylanase by P. pastoris and E. coli

P. pastoris cultivations were performed using shake flasks. There was no induction required for *P. pastoris* cultivations because of the constitutive promoter in the cloning vector and the selective pressure was maintained with the antibiotic. The highest xylanase expression was obtained after 60 hours of incubation with xylanase activity of 261.7 U/ml and a final OD of 27.7 was obtained (Fig.3.11). The concentration of the xylanase reached a level of 1 mg/ml after 60 h of incubation and the xylanase produced in yeast was found to be

extracellular and almost none was detected from extraction of intracellular content. Total activity that was obtained in 200 ml was 522 400 U which was more than 500-fold higher than in *E. coli* (Table 3.1).

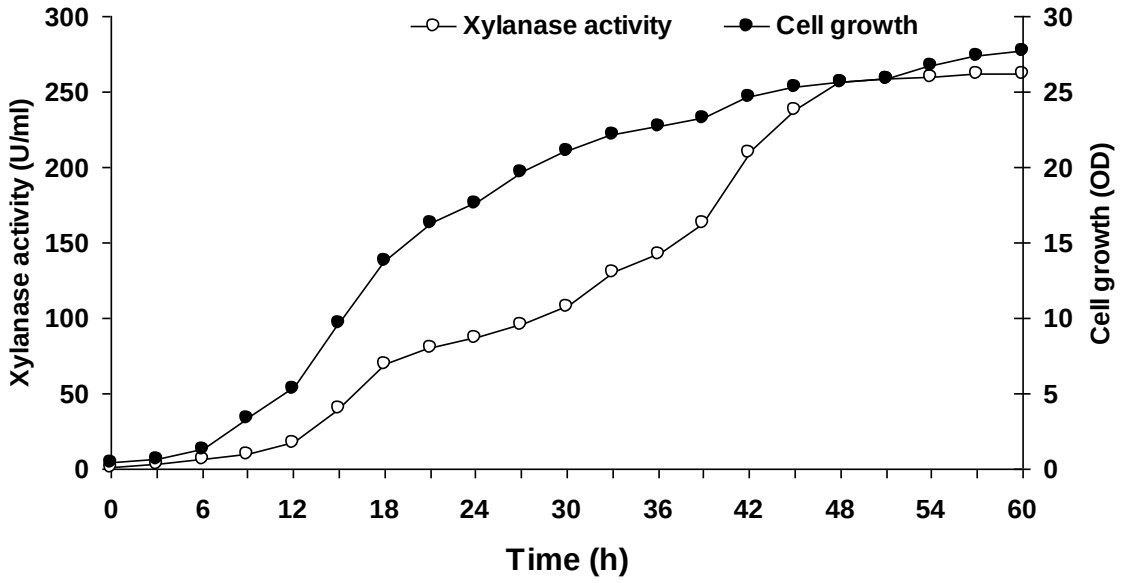

Fig. 3.11 Recombinant xylanase production expressed in *P. pastoris* in YPD broth supplemented with zeocin. Xylanase expression was performed over 60 h at 30^{o}C shaking at 250 rpm.

Shake flasks cultivations were also carried out in order to assess the levels of the recombinant proteins produced in the *E. coli* BL21 strain. The xylanase induction was achieved by introducing 0.1 mM IPTG after 90 min at an OD of 4.7 and growth tapered off after 7 h. Xylanase activity was observed in the pre-induction stage but at a very low level of 1.3 U/ml. Xylanase activity levels measuring up to 47.9 U/ml was achieved after 7.5 hours with at an OD of 3.4 (Fig. 3.12). There

was very little of the xylanase released extracellularly by *E. coli*. Most of the xylanase was found in the intracellular fraction. Total activity obtained for *E. coli* was 958 U (Table 3.1).

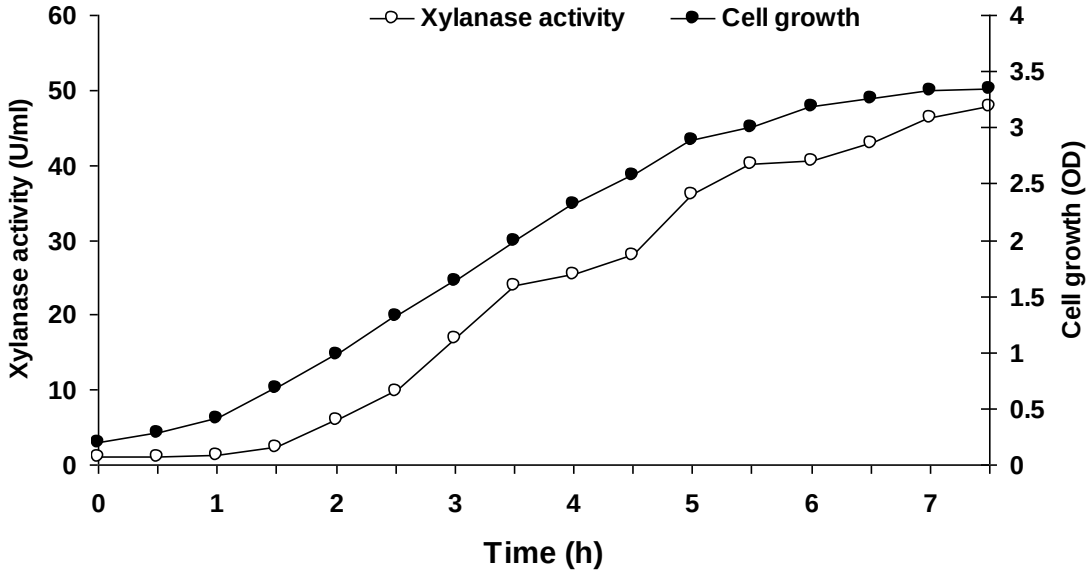

Fig. 3.12 Recombinant xylanase activity expressed in *E. coli* BL21 in LB broth supplemented with ampicillin. The *E. coli* culture was induced with IPTG after 90 min and incubation continued until 7.5 h at 30^{o}C, with shaking at 200 rpm.

Table 3.1 Summary of expression levels and localization of the recombinant xylanase produced by *P. pastoris*, *E. coli* BL21 and *S. cerevisiae*

Expression Host	Localization	Total Activity (U)	Expression Levels (U/ml)
E. coli BL21	Intracellular	958*****	47.9
S. cerevisiae	Intracellular	268	13.4
P. pastoris	**Extracellular**	**522400**[#]	**261.7**

* Total activity for *E. coli* and *Saccharomyces* was calculated as follows: U/ml x Suspension volume (20 ml). Total production volume was 200 ml

[#]Total activity for *Pichia* was calculated as follows: U/ml x Total Volume (200 ml).

3.2.3 Determination of pH and temperature optima

Enzyme characterization was performed for *P. pastoris* and *E. coli* BL21 clones only as *S. cerevisiae* showed relatively low levels of xylanase expression. The pH and temperature optima of xylanase was tested between pH 4.0-10.0 and 40-90^{o}C. Both xylanases had an optimum of pH 6.5, although the activity was stable between pH 5.0-9.0. Interestingly, these xylanases retained more than 50% of the overall activity at pH 9, with the yeast xylanase showing 62% and bacterial xylanase showing 58% of residual activity (Fig. 3.13).

The temperature optimum of these xylanase was also similar in that, the highest activity was obtained at 70^{o}C for both enzymes produced by these different

hosts. It was also observed that between 50-80^{o}C, these xylanases showed thermotolerance, with the yeast enzyme showing 61.8% activity at 50^{o}C and 79% at 80^{o}C. The bacterial xylanase had lower residual activity than the yeast enzyme with 48.5% at 50^{o}C and 58.8% at 80^{o}C. The activity values at 90^{o}C were very low for both enzymes with yeast enzyme still showing a higher residual activity of 26.8% while bacterial enzyme had residual activity of 21.8% (Fig. 3.14).

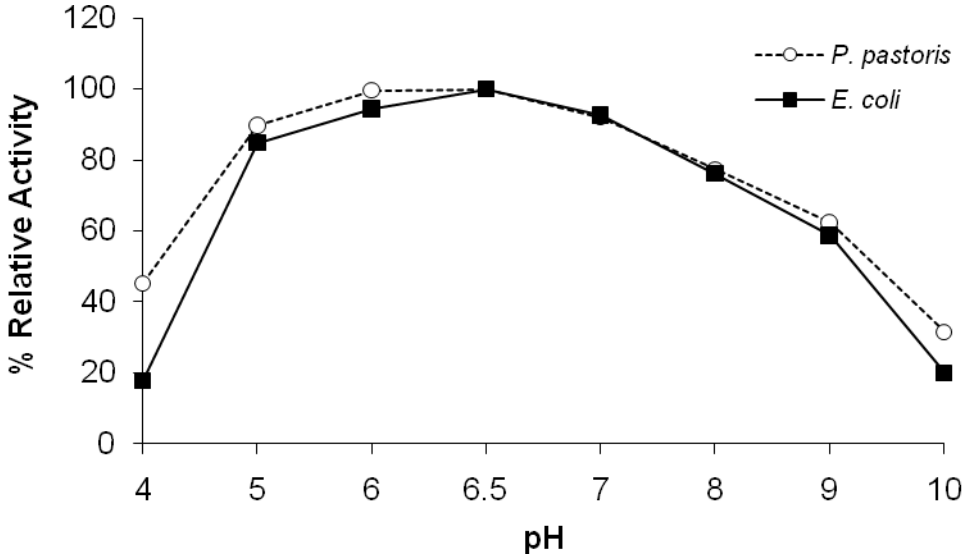

Fig. 3.13 pH profile of the xylanase from *P. pastoris* and *E. coli* BL21. The pH ranged from 4.0-10.0 over 5 min at 50^{o}C. The remaining activity was expressed as percentage of original activity with each point representing an average of duplicate sample analysis.

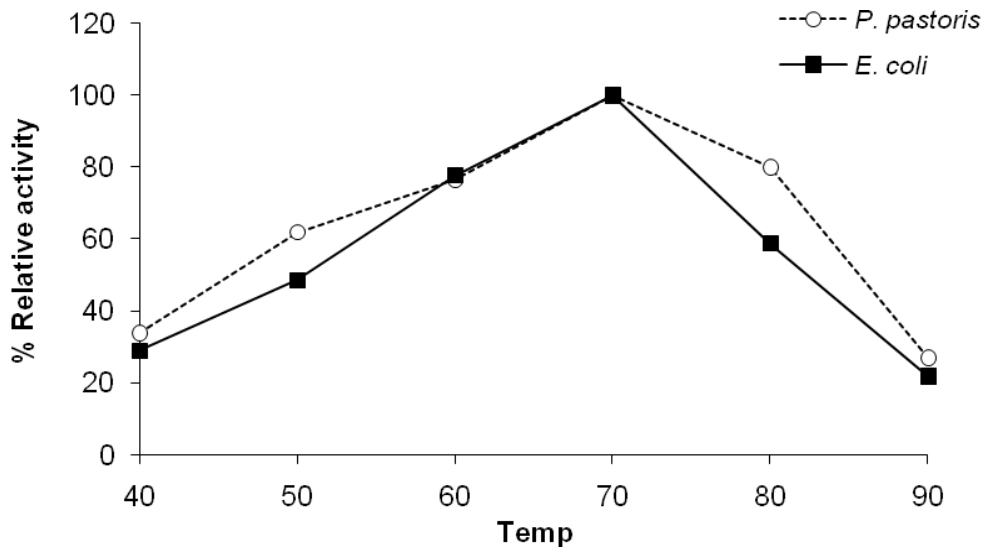

Fig. 3.14 Temperature profile of the xylanase from *P. pastoris* and *E. coli* BL21. Temperatures ranged from 40-90^{o}C for 5 min, pH 6.5. The remaining activity was expressed as percentage of original activity with each point representing an average of duplicate sample analysis.

3.2.4 Alkaline stability of xylanases from different hosts

The stability of these xylanases was tested using alkaline conditions, pH 9 and 10, because the modified xylanase used for this study was engineered for enhanced stability at high pH (Mchunu *et al*., 2009). This NC38 variant produced similar stability around neutral pH range as the wild type *T. lanuginosus* xylanase, but showed an improved performance at high pH. The temperature profile of both the wild type and NC38 was the same. In this study, the performance of this variant was again analyzed at pH 9 and 10, and at high

temperature to determine if the xylanase expressed in *E. coli* BL21 and *P. pastoris* showed any differences.

3.2.4.1 Alkaline stability at 50^{o}C

Alkaline stability showed by these xylanases at 50^{o}C at both pH 9 and 10 was high. The enzymes lost around 10% of overall activity at pH 9. The xylanase produced in yeast had 92.3% residual activity after exposure for 90 min, while the bacterial xylanase had 89.4% residual activity after 90 min (Fig 3.15). At pH 10, the yeast xylanase still had 90% of residual activity after 90 min, while the other xylanase showed less stability that at pH 9 with 79.5% residual activity after 90 min (Fig. 3.16).

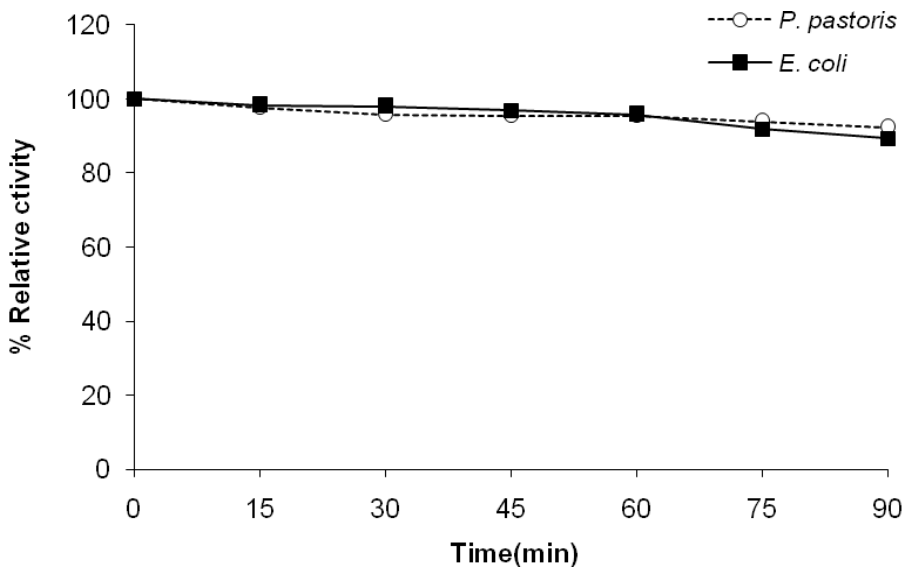

Fig. 3.15 Alkaline stability of xylanases at 50^{o}C and pH 9. The remaining activity was expressed as percentage of original activity with each point representing an average of duplicate sample analysis.

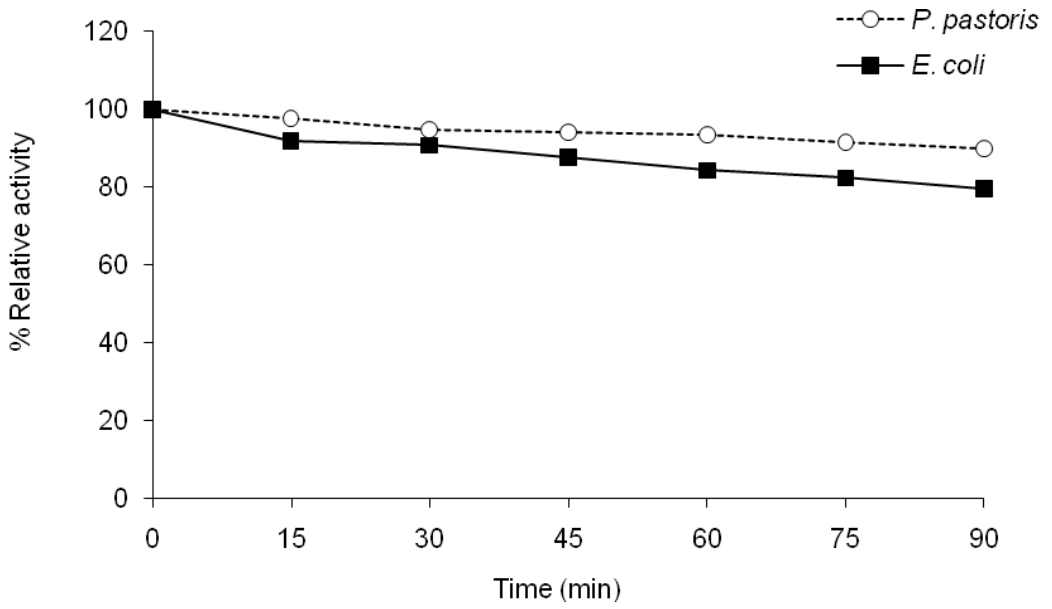

Fig. 3.16 Alkaline stability of xylanases at 50°C and pH 10. The remaining activity was expressed as percentage of original activity with each point representing an average of duplicate sample analysis.

3.2.4.2 Alkaline stability at 60°C

At 60°C, the two xylanases also maintained their overall stability. The yeast xylanase at both pH 9 and 10 had 89.2% and 84.8% residual activity after 90 min, respectively (Fig. 3.17 and 3.18). The xylanase expressed in *E. coli* also showed good activity, retaining 83.6% at pH 9 and 75.1% at pH 10 after 90 min of exposure.

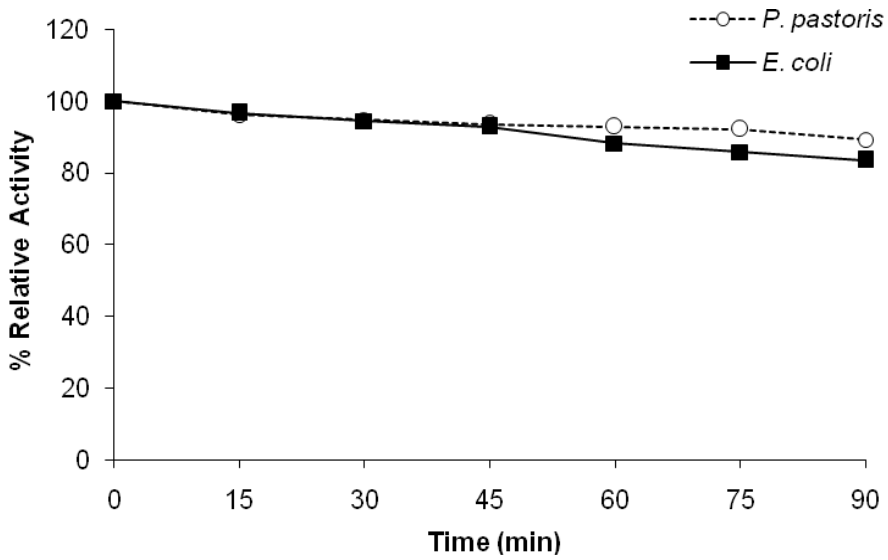

Fig. 3.17 Alkaline stability of xylanases at 60°C and pH 9. The remaining activity was expressed as percentage of original activity with each point representing an average of duplicate sample analysis.

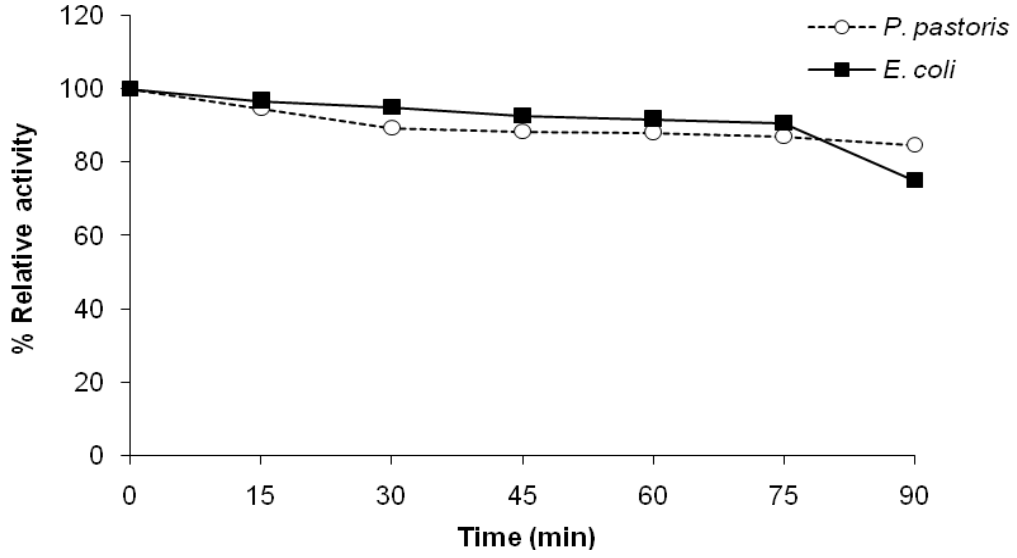

Fig. 3.18 Alkaline stability of xylanases at 60°C and pH 10. The remaining activity was expressed as percentage of original activity with each point representing an average of duplicate sample analysis.

3.2.4.3 Alkaline stability at 70°C

The stability profile of these two xylanase at 70°C was significantly lower than that showed at 50°C and 60°C. The bacterial xylanase showed better stability than that of the yeast, retaining 68.7% of its activity after 90 min at pH9 while the yeast xylanase retained 41.7% activity (Fig. 3.19). At pH 10, both enzymes had low residual activity with the bacterial enzyme still slightly higher with 30% residual activity after 90 min and the yeast showing 23% residual activity respectively (Fig. 3.20).

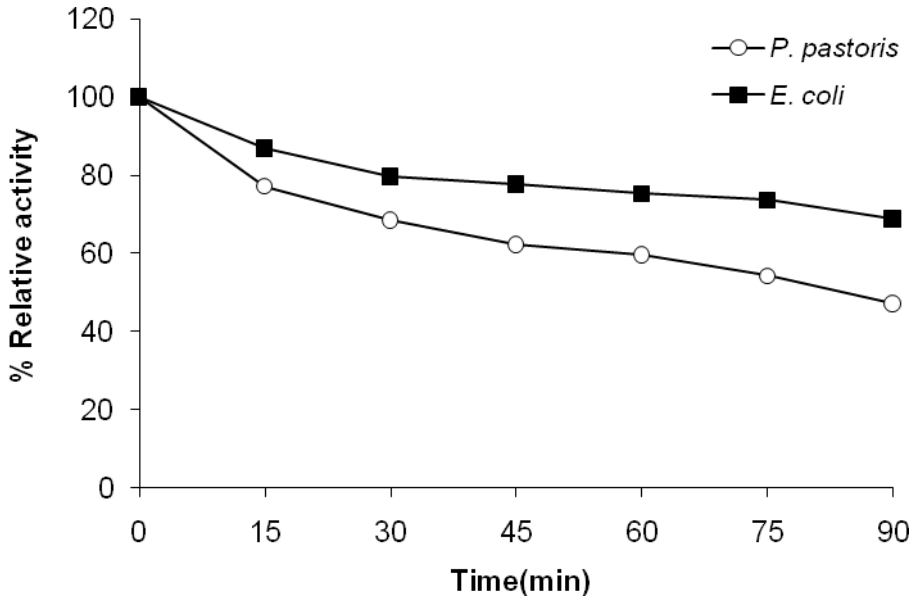

Fig 3.19 Alkaline stability of xylanases at 70°C and pH 9. The remaining activity was expressed as percentage of original activity with each point representing an average of duplicate sample analysis.

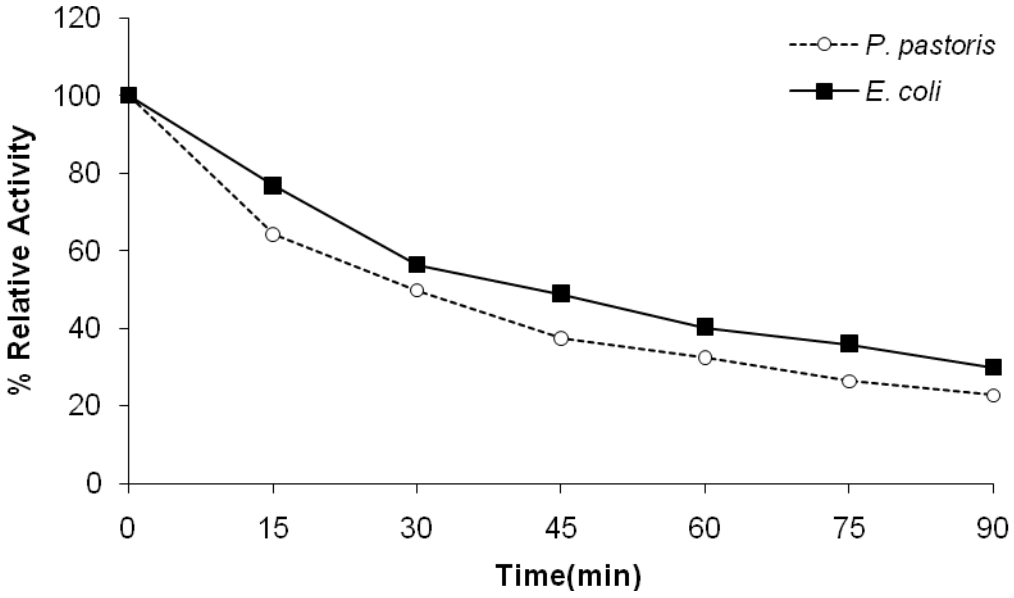

Fig 3.20 Alkaline stability of xylanases at 70^{o}C and pH 10. The remaining activity was expressed as percentage of original activity with each point representing an average of duplicate sample analysis.

CHAPTER FOUR: DISCUSSION

The endo-xylanase that was used in this study is produced by thermophilic, filamentous fungus, *Thermomyces lanuginosus* DSM 5827 and was cloned into plasmid Bluescript by Schlacher *et al*. (1996). This xylanase was evolved using directed evolution (Mchunu, 2009). The strategy that was used was error-prone PCR and the conditions that were used to create the mutations in this variant were higher concentrations of individual nucleotides and magnesium chloride than what was required for standard amplifying conditions described at section 2.7 of the methodology chapter. The NC38 variant created was chosen for its enhanced alkaline stability over the wild type enzyme. But the overall production of this enzyme in *E .coli* using plasmid Bluescript was very low, reaching a maximum level around 12 U/ml if the enzyme was not concentrated. This is insufficient for the intended application, bio-bleaching of paper pulp.

In this study, other strategies were investigated to increase the level of xylanase expression. This meant finding a new expression strain and vectors for *E. coli* and using yeasts as alternative expression hosts. For *E. coli*, the pET system was selected using the *E. coli* BL21 strain from Novagen, which uses a tightly controlled promoter system. The highly processive T7 RNA polymerase system is encoded in the production host chromosome via insertion of a prophage (λDE3) encoding the enzyme under the control of IPTG-inducible lacUV5

promoter (Banexy, 1999). For yeast expression, *Pichia pastoris* and the plasmid vector pBGP1 and *S. cerevisiae* with the vectors pJC1 and pDLG1, were used. To express this modified xylanase in the above mentioned hosts, the gene had to be re-cloned into all the respective vectors.

Although cloning of the desired gene into an expression vector can be done successfully, screening is crucial to any expression research. An appropriate strategy for selection desired clones is needed or else the exercise can become very cumbersome or even fail.

In this study, the selection and screening of active xylanase producers was done using RBB-xylan (Biely *et al*., 1985). This chromogenic substrate is prepared by coupling xylan which is a substrate for xylanases and Remazol Brilliant Blue dye. When incorporated into growth media, this complex colours the media blue. But when this complex is attacked by xylanases from a colony of cells, the area around it turns clear. Therefore, those colonies that have zones of clearing will be the ones that are producing the enzyme. For *E. coli,* RBB-xylan LB plates containing ampicillin were used for screening. Ampicillin was used to select for the clones that had taken up the plasmid but it could not be used solely for screening as this marker could not discriminate between colonies that had the plasmid with the xylanase gene and those that had plasmid only. The clones that contained active genes were identified by the zone of clearing as shown in Fig. 3.2, while the ones that had taken up the plasmid only without the gene had no

zone. Similar results were obtained for *P. pastoris* and *S. cerevisiae,* when RBB-xylan was used in yeast media containing antibiotics.

Comparison between the yeast clones revealed that *P. pastoris* produced the largest zone, followed by the *S. cerevisiae* clone containing pJC1-NC38 and the smallest zone was the clone with containing pDLG1-NC38 (Fig. 3.3 and 3.4). It was initially thought that one of the possible reasons could be because *Pichia* was grown in complex media while *S. cerevisiae* was grown in minimal media. But the shake flask analysis showed a large difference in enzyme activities that could not be attributed to the media only.

When the two *S. cerevisiae* clones were grown in minimal media with glucose as carbon source, the containing pDGL1-NC38 showed no xylanase activity even though the restriction digestion analysis showed that the plasmid carried the xylanase gene. This result corresponded to the findings of La Grange *et al.* (1996) where xylanase from *Trichoderma reesei* was expressed in *S. cerevisiae*. But when glucose was substituted with galactose as a carbon source, xylanase activity was observed on RBB-xylan plates. This is because the pDGL1 vector contains an alcohol dehydrogenase II (*ADH2*) promoter which is highly repressed in the presence of glucose. The other *S. cerevisiae* clone contained the pJC1 vector which has a 3-phosphoglycerate kinase (*PGK1*) promoter which is a strong constitutive promoter which can express recombinant protein at 4-10% of the total soluble proteins depending on culture conditions (Hitzeman *et al*., 1982).

This explains why the construct containing plasmid pJC1 had a larger zone than the one with pDGL1 plasmid (Fig. 3.3).

The expression of proteins in hosts especially in *E. coli*, other than the native organism has always been followed by difficulties. The biggest downfall being that the levels of expression of the recombinant protein are usually lower than in the natural host (Banexy, 1999). This was the same case with this xylanase. The filamentous fungus, *T. lanuginosus* produces around 40 000 nkat/ml (Singh *et al*., 2003) which is more than a 100-fold higher than what was reported when this xylanase was produced in *E. coli* by Stephens *et al*. (2007). Another problem is incorrect processing and protein folding, especially if the protein is of eukaryotic origin and the expression host is prokaryotic. This is because prokaryotic organisms are unable to perform post-translational modification of proteins (Baneyx and Mujacic, 2004).

The NC38 enzyme produced by *E. coli* BL21, *P. pastoris* and *S. cerevisiae* (pJC1 clone) were analysed using SDS-PAGE to determine if production of the expected size recombinant xylanase had occurred. The clone which contained plasmid pDGL1-NC38 construct was not used for further analysis as it showed little or no active when grown in liquid media with glucose or galactose. The other clone with pJC1 showed some activity but enzyme localization was intracellular and did not produce enough levels of enzyme to warrant characterization. So the

remaining analyses were performed on xylanase from *E. coli* BL21 and *P. pastoris*, only.

P. pastoris was found to release most of the recombinant xylanase into the culture media because there was negligible amount of enzyme activity when intracellular fractions were tested. Secretion of xylanase into the media was validated by SDS-PAGE analysis and activity stain of the culture media after cultivation (Fig. 3.8). The SDS-PAGE analysis showed the presence of a single protein band. In most studies when *P. pastoris* was used as an expression host, the recombinant protein was also found in the culture media and relatively pure. But in other studies there were one or two extra bands identified on SDS-PAGE (Berrin *et al*., 2000, Ramchuran *et al*., 2005b). Cloned proteins are at higher concentrations than normal cellular proteins as they are located on the plasmid.

The other problem associated with production of thermostable recombinant proteins in *P. pastoris* is that sometimes, the protein is not active until is treated at high temperature as described by Ramchuran *et al.* (2005b). However, this was not observed in this study. The size of the xylanase protein was similar to that of the native protein produced by *T. lanuginosus,* which is about 24 kDa (Singh *et al*., 2000b). This was also confirmed to be active enzyme band using activity staining (Fig. 3.10)

E. coli BL21 was also tested see if any enzyme is released into the medium. A very low amount was detected. But the intracellular fraction was found to contain most of the enzyme. This is consistent with general expression and localization of recombinant proteins in *E. coli* (Baneyx, 1999; Baneyx and Mujacic, 2004; Choi *et al*., 2006). When a recombinant protein is over-expressed in *E. coli*, they are often produced in the form of inclusion bodies.

SDS-PAGE analysis of enzyme extracted from *E. coli* showed high contamination with other intracellular proteins. This was expected because in order for xylanase extraction to take place the cells had to be lysed. When using this crude extract it was not possible to identify the protein band corresponding to the xylanase protein (Fig 3.9). Therefore, the crude extract was treated with heat at 65^{o}C for 30 min. This removed most of the host proteins which are not as thermostable as the recombinant protein. Because the pET vector contains a His-tag, the heat-treated sample was passed through a purification column but it was not possible to obtain a purified protein. This reinstated the difficulties that are associated with production and purification of recombinant proteins in *E. coli* with downstream processing being amongst the biggest stumbling block because this could be affected by a number of factors. But it could also be that the gene sequence was not in the same reading frame as the His-tag sequence therefore not incorporated as part of the polypeptide chain. The activity stain was then done to identify the active xylanase band (Fig. 3.10), which would be recognized by a zone of clearing. The activity stain showed that there was an active

xylanase at the expected molecular weight of 24 kDa, but there were other bands positioned higher than the expected band. This could be due to improper folding of the protein in a prokaryotic host (Baneyx, 1999).

Production of a recombinant protein in an expression host may alter biochemical characteristics of the recombinant protein. This was the case when this xylanase was clone in *E. coli* by Schlacher *et al.* (1996). Other enzymes have shown similar behaviour, with Shalom *et al*. (2008) reporting alteration in temperature optimum of the α-glucanase from *Penicillium purpurogenum*. Thus, the biochemical characterization of the xylanases expressed by different hosts was performed.

The pH optimum for both the yeast and the bacterial xylanases was observed to be between at pH 5 and 7 (Fig. 3.11). This optimum was different than ones reported in other studies, Cesar and Mrša, (1996) reported an optimum of pH 7 while other xylanases from *T. lanuginosus* have been shown to have an optimum pH of 6.5 (Singh *et al*., 2003). But this was expected because this xylanase had undergone two rounds of random mutagenesis. The enzymes produced by *Pichia* and *E. coli* displayed a broad pH spectrum, with both the xylanases retaining more that 60% of total activity at pH 9. When the native enzyme produced by *T. lanuginosus* was tested, only 40% of its maximum activity was observed at pH 9 (Cesar and Mrša, 1996). The high tolerance at higher pH

ranges of the xylanase expressed in this study was expected as this enzyme was evolved to be more alkaline stable.

The temperature optimum of *P. pastoris* and *E. coli* xylanase was observed to be 70^{o}C (Fig. 3.14). This was similar to that of the native enzyme with a reported pH optimum between 60-70^{o}C. The xylanase enzyme tested in this study was active over a wide range of temperature between 50-80^{o}C, which is comparable to the native xylanase (Singh *et al.,* 2000a). The yeast enzyme maintained more than 80% of its activity at 80^{o}C, while the bacterial enzyme had around 60% residual activity. This could be attributed to enzyme being correctly folded in *P. pastoris,* compared to *E. coli*. This is in agreement with the results obtained during activity staining analysis which showed that the xylanase expressed in *E. coli* had several active conformations. These other forms may not be as stable as the 24 kDa form, accounting for the 20% difference in stability between bacterial and yeast enzymes.

The biochemical characterization of these xylanases was carried out at pH 9 and 10. This was done since the previous study was aimed at creating an alkalophilic xylanase variant (Mchunu *et al*., 2009). The tolerance of the xylanase to alkaline conditions was tested in combination with a variation in temperature. These stability studies were done at 50^{o}C-70^{o}C using pH 9 and 10 for 90 min. When the enzymes were tested at 50^{o}C and pH 9, they were stable, maintaining almost 100% residual activity for 60 min and decreasing only slightly (10%) after 90 min.

This was similar to other reports, except that the pH was much lower at pH 6.5 (Singh *et al.,* 2000a). The analysis done at pH 10 for 50^{o}C followed a similar trend to that at pH 9. The yeast xylanase showed higher residual activity than the bacterial xylanase (90% compared to 80%) after 90 min (Fig. 3.13 and Fig. 3.14). This was not surprising as the bacterial xylanase had a lower temperature profile than the yeast enzyme. This again might be related to the problems associated with the production of eukaryotic proteins in a prokaryotic system because of their inability to perform post-translation modification which can lead to misfolding and lost of stability (Baneyx and Mujacic, 2004).

The 60^{o}C exposure revealed that the yeast enzyme again showed better stability than the bacterial enzyme. When these xylanases were exposed to 60^{o}C and pH 9 conditions, the enzymes were still stable. Both yeast and bacterial enzymes retained more than 90% stability after 60 min. After 90 min of testing, the values that were obtained were 89.2% residual activity for yeast and 83.6% for bacteria (Fig. 3.17). The pH trend was similar at pH 10 but a gradual loss of activity concurrent with the increase in pH was observed. The yeast enzyme had a residual activity of 84.8% after 90 min while the *E. coli* xylanase had 75.1% activity left (Fig. 3.18). This was similar to the mutants that were obtained by Stephens (2007), produced using error-prone PCR and DNA shuffling.

The results of stability tests at 70^{o}C showed a drastic loss of activity at pH 9 and 10, respectively. The yeast enzyme had 41.7% remaining activity and the

bacterial enzyme had 68.7%. This was pronounced decrease from the test at 60^{o}C where both the enzymes had over 75% residual activity after 90 min (Fig. 3.19). Also, the bacterial xylanase was more stable that yeast xylanase, reversing the trend observed at 50^{o}C and 60^{o}C. An increase in pH to 10 also caused a further drop in stability with the bacterial enzyme having 30% residual activity and the yeast enzyme having 23% residual after 90 min (Fig. 3.20). This trend at 70^{o}C might to due to the protective effect by native proteins being more pronounced at high temperature or temperature close the optimum. Singh *et al*. (2000a) also observed a drop of xylanase stability at 70^{o}C when testing the native xylanase. It must also be kept in mind that the xylanase that was expressed in *E. coli* was only partially purified which could have resulted in higher stability values compared to the relatively pure enzyme from yeast because of the interference of native *E. coli* proteins.

Industrial application of a recombinant protein is highly dependent on expression levels and easy of production of that particulate protein as these factors determine the availability and the cost of the protein product. The levels of expression and the localization of the xylanase in their artificial hosts were assessed.

In both these hosts (*E. coli* and *P. pastoris*) lag phase was reduced by adding an inoculum that was approaching late log phase. Inoculum preparation in *Pichia* required 20-24 h while in *E. coli* it was around 12 h. The lag time in yeast was between 3-6 h, because the OD obtained after 6 h showed that the cells we entering log phase (Fig. 3.11). In *E. coli* the lag time for BL21 is 3 h (Ramchuran

et al., 2005b), but in this study induction was started after 90 min (Fig. 3.20), half the usual time. This is very important because protein production time is reduced which would reduce production costs for application.

P. pastoris has gained a reputation over that last decade as the organism of choice for expression of recombinant proteins and over 300 proteins have been expressed using this system (Cereghino and Cregg, 2000). In this study, *P. pastoris* was able to produce 261.7 U/ml compared to 47.9 U/ml produced by *E. coli*. This was achieved after 60 h of incubation, without induction. Damaso *et al.* (2003) also expressed a *T. lanuginosus* xylanase in *P. pastoris* where levels of 360 U/ml of protein was observed but the time that required to reach that expression level was 96 h of induction, not considering the time needed to obtain high cell density. In comparison with this study, Damaso *et al*. (2003) had 36 h more fermentation time. In the same study, the initial OD for production was 10, which was significantly higher than in this study which was 0.45 (Fig. 3.11).

Total activity obtained in *P. pastoris* was much higher, reaching levels of 522 400 U in 200 ml while in *E. coli* by comparison this figure was 958 U (Table 3.1). This represented a 545-fold difference between the two hosts. This is due to the protein produced in *Pichia* being secreted into the culture media (each millimeter contained 261 U), while in *E. coli* the enzyme is intracellular, thus needing extraction first. Each purification step reduced the total enzyme recovered. The xylanase from bacteria was partially purified and protein purification usually reduces total yield by 90% (Ito *et al*., 2004).

Most *P. pastoris* expression systems use *AOX* promoters to drive expression. The downfall of this promoter is that it is highly repressed by the presence of glucose. Therefore, biomass production and recombinant protein expression is separate and the latter requires continuous induction with methanol. This makes expression very laborious and long. In contrast, the constitutive promoter, *GAP*, which was used in this study allows for simultaneous expression and growth. This fact was also demonstrated by Vassilave *et al.* (2001), where hepatitis B surface antigen was produced. The AOX clone required 96 h to accomplish the same level of production as the clones that were carrying the *GAP* promoter in 24 h. The “strength” of these promoters, however, is reported to be relatively similar (Koller *et al.,* 2000).

The expression levels of this xylanase were compared to other xylanases produced in *P. pastoris*. A xylanase from *Aspergillus niger* was expressed at a level of 175 U/ml after 96 h (Lui *et al*., 2006), one from *Thermobifida* 324.2 U/ml after 144 h (Cheng *et al.*, 2005), which was more than double the time in this study. Another xylanase produced from *Bacillus* 122.9 U/ml (Lui *et al*., 2008) and two xylanase from *A. niger* 15.6 U/ml (Berrin *et al*., 2000) and 62 U/ml (Deng *et al*., 2006). All these were lower than the production levels of NC38 xylanase by *Pichia* when also taking production time into consideration. There were other xylanase which had higher expression than the levels reported in this study. An *A. niger* xylanase produced 3676 U/ml and 15 000 U/ml (Ruanglek *et al.*, 2007, Wang *et al*., 2007). It must be noted though that both these studies were carried out using a bioreactor in a fed-batch system. Cell density levels were

approximately 200 OD units before induction and the time needed was over 120 h. In this study, only shake flask experiments were conducted, which limited oxygen supply and dissolved oxygen levels.

The secretion of the xylanase into the culture media was an added advantage compared with *E. coli* and *S. cerevisiae* (Table 3.1). Thus, the production of this xylanase for the target industry (pulp and paper) will be easier because the downstream processing which includes extraction and purification will be minimal. Although *E. coli* grows faster than yeasts, the extraction and purification is laborious which reduces yield and increases production costs (Ito *et al*., 2004).

P. pastoris as an expression host in conjunction with a suitable vector has shown itself to be able to meet the demands of being a good factory for heterologous xylanase. This was achieved by using a vector system which did not require complicated induction steps and huge biomass production. Enzyme production at 261.7 U/ml, compared favourable to reported literature. This represents the first study on expression of a genetically-modified xylanase from *T. lanuginosus* DSM 5826 in *P. pastoris*.

The biochemical characterization of this study did not reveal changes to the xylanases expressed in *P. pastoris* and *E. coli* BL21. SDS-PAGE analysis revealed that expression of the xylanase in bacterial hosts might be cumbersome due to several steps being required for enzyme purification. The activity stain

indicated to improper folding of the recombinant protein which has an impact on downstream processing and enzyme stability.

The expression levels in this yeast can be further increased using high cell density fermentation which can increase production exponentially since biomass is directly proportional to expression levels. The use of an integrative plasmid can also be employed to increase the production of the xylanase. This study has demonstrated that *P. pastoris* is useful for over-expression of engineered proteins which appear to inherently lose activity after genetic alteration to improve industrial fitness.

REFERENCES

Arnold, F.H. 1996. Directed evolution: creating biocatalysts for the future. *Chemical Engineering science* **51**: 5091-5102.

Arnold, H.F., Wintrode, P.L., Miyazaki, K. and Gershenson, A. 2001. How enzymes adapt: lessons from directed evolution. *Trends in Biochemical Sciences* **26**: 100-106.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Siedaman, J.G., Smith, J.A. and Struhl, K. 1989. Current protocols in molecular biology, Vol. 2. Greene Publishing and Associates, Wesley- Interscience, New York.

Bailey, M.J., Beily, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology* **23**: 257-270.

Bajpai, P. 1999. Application of enzymes in the pulp and paper industry. *Biotechnology Progress* **15**: 147-157.

Baneyx, F. 1999. Recombinant protein expression in *Escherichia coli*. *Current opinion in Biotechnology* **10**: 411-421.

Baneyx, F. and Mujacic, M. 2004. Recombinant protein folding and misfolding: *Escherichia coli*. *Nature Biotechnology* **22**: 1399-1408.

Bedford, M.R. and Classen, H.L. 1992. The reduction of intestinal viscosity through manipulation of dietary rye and pentosanase concentration is effected through the carbohydrate of the intestinal aqueous phase and results in improved growth rate and feed conversion. *Journal of Nutrition* **112**: 560-569.

Bedford, M.R. 2003. Factors influencing the use of enzymes in cereal based diets. Recent advances in Enzymes in grain processing. Proceedings from the 3rd European Symposium on Enzymes in grain processing, Katholieke Universiteit Leuven, Lueven.

Beguin, P. 1982. Detection of cellulase activity in polyacrylamide gels using congo red-stained agar replicas. *Analytical biochemistry* **131**: 333-336.

Berrin, J., Williamson, G., Puigserver, A., Chaix, J., McLauchlan, W.R. and Juge, N. 2000. High-level production of recombinant fungal endo-β-1,4-xylanase in the methylotrophic yeast *Pichia pastoris*. *Protein Expression and Purification* **19**: 179-187.

Biely, P. 1985. Microbial xylanotic systems. *Trends in Biotechnology* **11**: 286-290.

Biely, P., Mislovicova, D. and Toman, R. 1988. Remazol brilliant blue xylan: a soluble chromogenic substrate for xylanase. *Methods in Enzymology* **160**: 536-541.

Biely, P., Vršanská, M., Tenkanen, M. and Kluepfel, D. 1997. Endo-β-1,4-xylanase families: differences in catalytic properties. *Journal of Biotechnology* **57**: 151-166.

Bisson, S., Christov, L. and Singh, S. 2002. Bleach boosting effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp. *Process biochemistry* **37**: 567-572.

Bim , M.A. and Franco, T.T. 2000. Extraction in aqueous two-phase systems of alkaline xylanase produced from by *Bacillus pumilus* and its application in kraft pulp bleaching. *Journal of Chromatography and Biochemistry* **743**: 349-356.

Blanco, A., Vidal, T., Colom, J.F. and Pastor, F.I.J. 1995. Purification and properties of xylanase A from alkali-tolerant *Bacillus sp.* BP 23. *Applied and Environmental* , 4468-4470.

Buchert, J., Tenkanen, M., Kantelinen, A. and Viikari, L. 1994. Application of xylanase in pulp and paper industry. *Bioresource technology* **50**: 65-72.

Cereghino, G.l. and Cregg, J.M. 1999. Applications of yeast in biotechnology: protein production and genetic analysis. *Current opinions in biotechnology* **10**: 422-427.

Cereghino, G.l. and Cregg, J.M. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS microbiology reviews* **24**: 507- 510.

Čepeljnik, T., Križaj, I. and Marinšek-Logar, R. 2004. Isolation and characterization of the *Psuedobutyrivibro xylanivorans* Mz5T xylanase XynT-the first family 11 endoxylanase from rumen *Butyrivibro*-related bacteria. *Enzyme and Microbial Technology* **34**: 219-227.

Cesar, T. and Mrša, V. 1996. Purification and characterization of the xylanases produced by *Thermomyces lanuginosus*. *Journal of Enzyme Microbial Technology* **19**: 289-296.

Chen, R. 2001. Enzyme engineering: rational redesign versus directed evolution. *Trends in Biotechnology* **19**: 13-14.

Chen,Y.L., Tang, T.Y. and Cheng, K.J. 2001. Directed evolution to produce an alkalophillic variant from a *Neocollimastix patriciarum* xylanase. *Canadian Journal of Microbiology* **47**: 1088-1094.

Cheng, Y., Yang, C. and Lui, W. 2005. Cloning and expression of *Thermobifida* xylanase gene in the methylotrophic yeast *Pichia pastoris. Enzyme and Microbial Technology* **37**: 541-546.

Cherry, J.R., Lamsa, M.H., Schnieder, P., Vind, J., Svendsen, A., Jones, A. and Pedersen, A.H. 1999. Directed evolution of a fungal peroxidase. *Nature biotechnol*ogy **17**: 379-384.

Choi, J.H., Keum, k.C. and Lee, S.Y. 2006. Production of recombinant proteins by high density culture of *Escherichia coli*. *Chemical Engineering Science* **61**: 876-885.

Christov, L.P. and Prior, B.A. 1998. Research in biotechnology for the pulp and paper industry in South Africa. *South African Journal of Science* **94**: 195-200.

Crameri, A., Roullard, S., Bermudez, E. and Stemmer, W.P.C. 1997. DNA shuffling of a family of genes from diverse species accelerates directed evolution *Letter to Nature*: 287-289.

Crameri, A., Whitehorn, E.A., Tate, E. and Stemmer, W.P.C. 1995. Improved Green flourescent protein by molecular evolution using DNA shuffling. *Nature Biotechnology* **14**: 289-292.

Coughlan, M.P. 1985. Cellulases: production, properties and application *Biochemical Society Transactions* **13**: 405-406.

Courtin, C.M., Roelants, A. and Delcour, A.J. 2001. Use of two endo-xylanases with deferent substrate selectivity for understanding arabinoxylans in wheat flour breadmaking*. Cereal chemistry* **78**: 564-571.

Damaso, M.T.C., Almeida, M.S., Kurtenbach, E., Martins, O.B., Pereira, N., Andrade, C.M.M.C. and Albano, R.M. 2003. Optimized expression of a thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris. Applied and Environmental Microbiology* **69**: 6064-6072.

Debyster, W., Derdelinckx, G. and Delcour, A.J. 1997. Arabinoxylans solubilization and inhibition of the barley malt xylanolitic system by wheat during brewing with wheat wholemeal adjunct: evidence of a new class of enzymes inhibitors. *Journal of American society for brewing chemistry* **55**: 153-156.

Deng, M.D., Severson, D.K., Grund, A.D., Wassink, S.L., Burlingame, R.P., Berry, A., Running, J.A., Kunesh, C.A., Song, L., Jerrell, T.A. and Rosson R.A. 2005. Metabolic engineering of *Escherichia coli* for industrial production of glucosamine and *N*-acetylglucosamine. *Metabolic Engineering* **7**: 201-214.

Deng, P., Li, D., Cao, Y., Lu, W. And Wang, C. 2006. Cloning a gene of an acidophilic endo-β-1,4-xylanase obtained from *Aspergillus niger* CGMCC1067 and constitutive expression in *Pichia pastoris*. *Enzyme and Microbial Technology* **39**: 1096-1102.

Fierens, K., Geudens, N., Brijs, K., Courtin, C.M., Gerbruers, K., Robben, J., Van Campenhout, S., Volckaert, G. and Delcour, J.A. 2004. High-level expression, purification, and characterisation of recombinant wheat xylanase inhibitor TAXI-I secreted by the yeast *Pichia pastoris*. *Protein Expression and Purification* **37**: 39-46.

Figge, J., Wright, C., Collins, C.J., Roberts, T.M. and Livingston, D.M. 1988. Stringent regulation of stable integrated chloramphenicol acetyl transferase genes by *Escherechia coli lac* repressor in monkey cells. *Cell* **52**: 713-722.

Gabrielii, I. and Gatenholm, P. 1998. Preparation and properties of hydrogels based on hemicelluloses. *Journal of Applied Polymer Science* **69**: 1661-1667.

Galbe, M. and Zacchi, G. 2002. A review of the production of ethanol from softwood. *Journal of Applied Microbiology and Biotechnology* **59**: 618-628.

Gellissen, G. and Cornelis, P. H. 1997. Production of recombinant proteins by methylotrophic yeast. *Current Opinions in Biotechnology* **8**: 554-560.

Gellesin, G. and Melber, K. 1996. Methylotrophic yeast *Hansenula polymorpha* as production organism for recombinant pharmaceuticals**.** *Drug Research* **46**: 943-948.

Giver, L., Gershens on, A., Freskgard, P.O. and Arnold, F. H. 1998. Directed evolution of a thermostable esterase. *Proceedings of the National Academy of Science*, USA. **95**: 12809-12813.

Gomes, J., Purkarthofer, H., Hayn, M., Kapplműller, J., Sinner, M. and Steiner, W. 1993. Production of high level cellulase-free and thermostable xylanase by a wild strain of *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. *Applied Microbiology and Biotechnology* **39**: 700-707.

Gray, K.A., Zhao, L. and Emptage, M. 2006. Bioethanol. *Current Opinions in Chemical Biology* **10**: 141-146.

Hitzeman, R.A., Hagie, A.F., Hayflick, J.S., Chen, C.Y., Seerburg, P.H. and Derynck, Y. 1982. the primary structure of *Saccharomyces cerevisiae* gene for 3-phosphoglycerate kinase. *Nucleic acids Resources* **10**: 7791-7808.

Jana, S. and Deb, J.K. 2004. Strategies for efficient production of heterologous proteins in *Escherichia coli*. *Journal of Applied Microbiology and Biotechnology* **67**: 289-298.

Jaenicke, R. and Bohm, G. 1998. The stability of proteins in extreme environments. *Current opinions in Structural Biology* **8**: 738-748.

Jiang, Z.Q., Yang, S.Q., Tan, S.S., Li, L.T. and Li, X.T. 2005. Characterization of a xylanase from newly isolated thermophilic *Thermomyces lanuginosus* CAU44 and its application in bread making. *Letters in Applied Microbiology* **41**: 69-76.

Ito, S., Kuno, A., Suzuki, R., Kaneko, S., Kawabata, Y., Kusakabe, I. and Hasegawa, T. 2004. Rational affinity of a native *Streptomyces* family 11 xylanase. *Journal of biotechnology* **110**: 137-142.

Kirk, O., Borchet, T.V. and Fulglsang, C.C. 2002. Industrial enzyme applications. *Current opinions in Biotechnology* **13**: 345-351.

Koller, A., Valesco, J. and Subramani, S. 2000. The *CUP*I promoter of *Saccharomyces cerevisiae* is inducible by copper in *Pichia pastoris*. *Yeast* **16**: 651-656.

Kumamaru, T., Suenanga, H., Mitsuoka, M., Watanabe, T. and Furukawa, K. 1998. Enhanced degradation of polychlorinated biphenyls by directed evolution of biphenyl dioxygenase. *Nature Biotechnology* **16**: 663-666.

La Grange, D., Pretorius, I.S. and van Zyl, W.H. 1996. Expression of a *Trichoderma reesei* β-xylanase Gene (XYN2) in *Saccharomyces cerevisiae*. *Journal of Applied and Environmental Microbiology* **62**: 1036-1044.

Larsson, A. 2003. Directed enzyme evolution of Theta class Glutathione Transferase. Comprehensive summaries of Uppsala dissertations from the faculty of Science and technology p. 878-888.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature* **227**: 680-685.

Lee, C.C., Williams, T.G., Wong, D.W.S. and Robertson, G.H. 2005. An episomal expression vector screening mutant libraries in *Pichia pastoris*. *Plasmid* **54**: 80-85.

Lee, S.Y. 1996. High cell density cultivation of *Escherichia coli*. *Trends in Biotechnology* **14**: 98-105.

Lepetic, A., Seigelchifer, M., Arduino, R.C., lazvski, J., Nacinovich, F., Sturba, E. and Stambou-lian, L. 1996. Novel recombinant HB vaccine produced by a high-level expression *Hansenula polymorpha* yeast system**.** *Clinical Infectious Diseases* **23**: 276.

Lui, M. and Lui, G. 2008. Expression of recombinant *Bacillus licheniformis* xylanase in *Pichia pastoris* and xylooligosaccharides released from xylans by it. *Protein Expression and Purification* **57**: 101-107.

Lui, M., Weng, X. and Sun, J. 2006. Expression of recombinant *Aspergillus niger* xylanase in *Pichia pastoris* and its action on xylan. *Protein Expression and Purification* **48**: 292-299.

Lui, X., Qu, Y., You, F. and Lui, Y. 2002. Studies on the key amino acid residues responsible for the alkali-tolerance of the xylanase by site-directed and random mutagenesis. *Journal of Molecular catalysis* **18**: 307-310.

Maat, J., Roza, M., Verbakel, J., Stam, H., Santos da Silva., M.J., Bosse, M., Egmond, M.R., Hagemans, M.L.D., v. Gorcom, R.F.M., Hessing, J.G.M., van Der Hondel, C.A.M.J.J., and van Rotterdam, C. 1992. Xylanase and their application in bakery: In xylan and xylanases. Edited by J. Visser, M.A. kusters.

Masuda, T., Ueno, Y. and Kitabatake, N. 2005. High yield secretion of the sweet-tasting protein lysozyme from the yeast, *Pichia pastoris*. *Protein Expression and Purification* **39**: 35-42.

Mchunu, N.P., Permaul, K. and Singh, S. 2009. Expression of an alkalo-tolerant fungal xylanase enhanced by directed evolution in *Pichia pastoris* and *Escherichia coli*. *Journal of biotechnology* **141**: 26-30.

Miroux, B. and Walker, E. 1996. Over-Production of protein in *Escherichia coli*: mutant hosts that allow synthesis of some membrane protein and globular proteins at high levels. *Journal of molecular biology* **260**: 289-298.

Murphy, A. and Kavanagh, K. 1999. Emergence of *Saccharomyces cerevisiae* as a human pathogen: Implications for biotechnology. *Enzyme and Microbial Technology* **25**: 551-557.

Nakamura, S., Wakabayashi, K., Nakai, R., Aono, R. and Horokoshi, K. 1993. Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus sp*. Strain 41M-1. *Applied Environmental Microbiology* **59**: 2311-2316.

Nortey, T.N., Patience, J.F. and Zijlstra, R.T. 2007. Xylanase supplementation improves energy digestibility of wheat by-products in grower pigs. *Livestock Science* **109**: 96-99.

Olędzeka, G., Dąbrowski, S. and Kur, J. 2003. High-level expression, and purification of the thermostable aqualysin I from *Thermus aquaricus* YT-1 in *Pichia pastoris*. *Protein Expression and Purification* **29**: 223-229.

Rahbarizadeh, F., Rasaee, M.J., Forouzandeh, M. and Allameh, A. 2006**.** Over expression of anti-MUC1 single-domain antibody fragments in the yeast *Pichia pastoris . Molecular Immunology* **43**: 425-436.

Ramchuran, S.O., Nordberg-Karsson, E., Velu, S., de Maré, L. and Holst, O. 2002**.** Production of heterologous thermostable hydrolases and the presence of host-cell proteases in substrate limited fed- batch cultures of *Escherichia coli* BL21(DE3**).** *Journal of Applied Microbiology and Biotechnology* **60**: 408-416.

Ramchuran, S.O., Holst, O. and Nordberg-Karsson, E. 2005a. Effect of postinduction nutrient feed composition and use of lactose as inducer during production of thermomstabe xylanse in *Escherichia coli* glucose-limited fed-batch cultivations. *Journal of Bioescience and Bioengineering* **99**: 477-484.

Ramchuran, S. O., Mateus, B., Holst, O. and Nordberg-Karlsson, E. 2005b. The methylotrophic yeast *Pichia pastoris* as a host for the expression and

production of thermostable xylanase from the bacterium *Rhodothermus marinus*. *FEMS Yeast Research* **5**: 839-850.

Ruanglek, V., Sriprang, R., Ratanaphan, N., Tirawongsaroj, P., Chantasigh, D., Tanapongpipat, S., Pootanakit, K. and Eurwwilaichtr, L. 2007. Cloning, expression and high cell-density production of recombinant endo-β-1,4-xylanase from *Aspergillus niger* in *Pichia pastoris*. *Enzyme and Microbial Technology* **41**: 19-25.

Rubingh, D.N. 1997. Protein engineering from bio-industrial point of view. *Current options in Biotechnology* **8**: 417-422.

Sambrook, J., Fritsch, E.F. and Maniantis, T. 1989. Molecular cloning: a laboratory manual .2nd edition. Cold Spring Harbor Laboratory, Cold Spring, New York.

Sambrook, J., Fritsch, E.F. and Maniantis, T. 2001. Molecular cloning: a laboratory manual. 3rd edition. Cold Spring Harbor Laboratory, Cold Spring, New York.

Sa-Pereira, P., Paveia, H., Costa-Ferreira, M. and Aires-Barros, M. 2003. A new look at xylanases: An overview of purification strategies. *Journal of Molecular Biotechnology* **24**: 257-281.

Silva, S.S.P. and Smithard, R.R. 2002. Effect of enzyme supplementation of rye-based diet on xylanase activity in the small intestine of broilers, on intestinal cryp cell proliferation and on nutrient digestibility and growth performance of the birds. *Journal of Poultry Science* **43**: 274-282.

Schiraldi, C. and de Rosa, M. 2002. The production of biocatalysts and biomolecules from extremophiles. *Trends in Biotechnology* **20**: 515-521.

Schlacher, A., Holzmann, K., Hayn, M., Steiner, W. and Schwab, H. 1996. Cloning and characterization of the gene for the thermostable xylanase *xynA* from *Thermomyces lanuginosus*. *Journal of Biotechnology* **49**: 211-218.

Singh, S., Madlala, A. and Prior B.A. 2003. *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *FEMS Microbiology Reviews* **27**: 3-16.

Singh, S., Reddy, P., Haarhoff, J., Biely, P., Janse, B., Pillay, B., Pillay, D. and Prior, B.A. 2000a. Relatedness of *Thermomyces lanuginosus* strains producing thermostable xylanase. *Journal of Biotechnology* **81**: 119-128.

Singh, S., Pillay, B., Pillay, D. and Prior, B.A. 2000b. Thermostability of β-xylanase produced by different *Thermomyces lanuginosus* strains. *Enzyme and microbial technology* **26**: 502-508.

Shalom, G., Pratten, J., Wilson, M. and Nair, S.P. 2008. Cloning, heterologous gene expression and biochemical characterization of the α-1,3-glucanase from the filamentous fungus *Penicillium purpurogenum. Protein Expression and Purification* **60**: 170-175.

Scorer, C.A., Clare, J.J., McCombie, W.R., Ramanos, M.A. and Sreekrina, K. 1994. Rapid selection using G418 of a high copy number transformants of *Pichia pastoris* for high level foreign gene expression. *Biotechnology* **12**: 181-184.

Srinivasan, M.C. and Rele, M.V. 1999. Microbial xylanase for paper industry. *Current science* **77**: 137-142.

Stemmer, W.P.C. 1994. DNA shuffling by random fragmentation reassembly: *In vitro* recombination for molecular evolution. *Proceedings of the national Academy of Science*, **91**: 10747-10751.

Stephens, D.E., Permaul, K., Prior B.A and Singh, S. 2007. Directed evolution of the thermostable xylanse from *Thermomyces lanuginosus*. *Journal of biotechnology* **127**: 348-354.

Tobin, M.B, Gustafsson, C and Huisman, G.W. 2002. Directed evolution: the 'ration' basis for irrational design. *Current opinions in Structural Biology* **10**: 421-427.

Tolan, J.S., Olson, D. and Dines, R.E. 1996. Survey of mill usage of xylanase. In: pulp and paper processing. Pp. 25-35. Jeffries, T.W. and Viikari, L. American Chemical Soicety , Washington, D.C.

Turner, N.J. 2003. Directed evolution of enzymes for applied biocatalysts. *Trends in Biotechnology* **21**: 474-478.

Uhlig, **H**. 1998. Industrial enzymes and their applications. John Wiley and Sons Inc., New York. pp.394.

Valenzuela, P., Medina, A., Rutter, W.J., Ammerer, G. and Hall, B.D. 1982. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* **298**: 347-350.

Van Beilen, J.B. and Li, Z. 2002. Enzyme technology: an overview. *Analytical biochemistry* **13**: 338-344.

Van der Burg, B. 2003. Extremophiles as a source of novel enzymes. *Current Opinions in Microbiology* **6:** 213-218.

Vassileva, A., chugh, D.A., Swaminath, S. and Khanna, N. 2001. Expression of a hepatitis B surface antigen in methylotrophic yeast *Pichia pastoris* using the *GAP* promoter. *Journal of Biotechnology* **88**: 21-35.

Viikari, L., Kantelinene, A., Sunquist, J and Linko, M. 1994. Xylanase in bleaching: from an idea to the industry. *FEMS Microbiological Reviews* **13**: 335-350.

Wang, Y., Zhang, H., He, Y., Luo, H. and Yao, B. 2007. Characterisation, gene cloning, and expression of a novel xylanase XYNB from *Streptomyces Olivaceoviridis* A1. *Aquaculture* **267**: 328-334.

Ximenes, F.A., Sousa, M.V., Puls, J., Silva, F.G. and Filho, E.X.F. 1999. Purification and characterisation of a low-molecular-weight xylanase produced by *Acraphialophora nainiana*. *Current Microbiology* **38**: 18-21.

APPENDIX

Journal of Biotechnology 141 (2009) 26–30

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Expression of an alkalo-tolerant fungal xylanase enhanced by directed evolution in *Pichia pastoris* and *Escherichia coli*

Nokuthula Peace Mchunu*, Suren Singh, Kugen Permaul

Department of Biotechnology and Food Technology, Durban University of Technology, 41/43 Centenary Road, P.O. Box 1334, Durban 4001, South Africa

ARTICLE INFO

Article history:
Received 7 December 2008
Received in revised form 20 February 2009
Accepted 26 February 2009

Keywords:
Xylanase
Directed evolution
Yeast expression
Pichia pastoris

ABSTRACT

The alkaline stability of the xylanase from *Thermomyces lanuginosus* was further improved by directed evolution using error-prone PCR mutagenesis. Positive clones were selected by their ability to produce zones of clearing on pH 9 and 12 xylan agar plates. Variant NC38 was able to withstand harsh alkaline conditions retaining 84% activity after exposure at pH 10 for 90 min at 60 °C, while the parent enzyme had 22% activity after 60 min. The alkaline stable variant NC38 was cloned into pBGP1 under the control *GAP* promoter and pET22b(+) for expression in *Pichia pastoris* and *Escherichia coli* BL21, respectively. Best extracellular expression of the recombinant xylanase was observed in *P. pastoris* (261.7 ± 0.61 U ml^{-1}) whereas intracellular activity was observed in *E. coli* (47.9 ± 0.28 U ml^{-1}) was low. Total activity obtained in *P. pastoris* was 545-fold higher than *E. coli*. The mutated alkaline stable xylanase from *P. pastoris* was secreted into the culture medium with little or no contamination by host proteins, which favours the application of this enzyme in the pulp and paper industry.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Xylan represents more than 20–40% of plant biomass. Xylan is a complex molecule composed of β-1,4 linked xylose chains with branches containing arabinose and 4-*O*-methylgluconic acid. Although xylan requires the action of several enzymes for its complete degradation, β-1,4 endoxylanases play a major role in degradation of xylan by catalyzing the random hydrolysis of β-1,4 xylosidic linkages in xylan. Due to this ability, xylanases have potential application in pulp and paper industry (Buchert et al., 1994; Bajpai, 1999). Xylanases have also been added to animal feed to improve digestibility and better feed utilization, and used in baking and brewing industries to improve the quality of bread and to clarify juice (Biely, 1985).

Pre-treatment of paper pulp with xylanases (bio-bleaching) can enhance the efficiency of lignin extraction and so reduce the amount of chlorine that is required (Gomes et al., 1993). Because kraft bleaching employs high temperatures and alkaline conditions, the xylanases used for this application should be thermophilic and alkaline stable (Buchert et al., 1994; Bajpai, 1999). Potential usefulness of this enzyme in industry has spurred considerable research efforts towards producing more thermophilic and alkalophilic xylanases by screening for naturally-occurring xylanases. A second approach is to explore genetic engineering for producing a thermoalkalophilic xylanase. Researchers have used different approaches to improve proteins. 'Rational design' involves precise preconceived amino acid sequence changes, which are based on the knowledge of the protein structure, function and mechanism. This was demonstrated by production of an alkalophilic xylanase variant from *Neocallimastix patriciarum* (Chen et al., 2001) and improving the thermostability of a fungal peroxidase (Cherry et al., 1999). However, the relationship between structure and function is rarely available, making random mutagenesis an attractive option (Chen, 2001). By borrowing from the evolutionary algorithm of mutation and natural selection, directed evolution techniques have been developed which have allowed the generation of enzymes with greatly enhanced characteristics (Kuchner and Arnold, 1997).

Most of these genetic manipulations are done in *Escherichia coli*, which is ideal for over-expression of recombinant proteins, while *Pichia pastoris* is able to express and secrete large amounts of heterologous proteins. This coupled with the ability to perform complex posttranslational modifications and the ability to grow to high densities, makes this methylotrophic yeast a powerful tool for expression and production of recombinant proteins (Cereghino and Cregg, 2000).

In this paper, we report the use of the error-prone PCR technique to enhance the alkaline stability of the xylanase from the thermophilic filamentous fungus, *Thermomyces lanuginosus* DSM 5826. Many of the research studies previously reported focused on the improvement of thermostability of xylanases, whereas improvement of alkaline stability has received scant attention. Furthermore, *P. pastoris* was shown to be an appropriate host for high level expression of this xylanase.

* Corresponding author. Tel.: +27 31 373 5370; fax: +27 31 373 5351.
E-mail address: nokuthula@dut.ac.za (N.P. Mchunu).

0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.02.021

2. Materials and methods

2.1. Growth and maintenance of cultures

E. coli D2 mutant was used as the starting point for error-prone PCR (Stephens et al., 2007; Schlacher et al., 1996). Luria-Bertani (LB) medium was used to grow *E. coli* cultures (XL1 Blue and BL21) in broth and agar plates at 37 °C. Ampicillin (100 μg ml^{-1}) was added to the medium to maintain the plasmids. The STET method, a variation of the boiling lysis method of Sambrook et al. (1989), was used to isolate plasmid DNA, with minor modifications to the original protocol. The plasmids pET22b(+) for *E. coli* BL21 strain (Novogen) and pBGP1 (Lee et al., 2005) for *P. pastoris* GS511 (Invitrogen) were used for expression. *P. pastoris* cultures were maintained on YPD medium (10 g l^{-1} yeast extract, 20 g l^{-1}, peptone and 20 g l^{-1}) containing 100 μg ml^{-1} of zeocin for plasmid maintenance.

2.2. Error-prone PCR, cloning and transformation

The xylanase gene was amplified and mutated by varying the concentration of dNTPs, $MgCl_2$ and addition of $MnCl_2$ during PCR (Chen et al., 2001). The PCR conditions that were used were as follows: 95 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min. The PCR reactions were carried out using a PCR Genius (Techne) thermal cycler for a total of 30 cycles. Vector pBSK and PCR products were restricted with endonucleases EcoRI and XhoI to create compatible sticky ends for ligation. DNA was then purified using the GFX PCR DNA and GEL Band Purification Kit (Amersham Biosciences). The purified gene was then ligated to pBSK. Ligated DNA was added to $CaCl_2$ competent *E. coli* cells, followed by heat shocking the cells. The cells were then incubated at 37 °C for 1 h in a shaking incubator. Aliquots were plated out on to Remazol Brilliant Blue-xylan LB medium containing 100 μg ml^{-1}of ampicillin and incubated at 37 °C overnight. Positive xylanase-producing colonies were selected by identification of a zone of hydrolysis around the colonies (Beguin, 1982).

2.3. Screening for mutants that produce pH tolerant xylanases

Positive mutants were detected by their ability to produce a zone of hydrolysis on acid and alkaline RBB-xylan plates. Potential mutants were replica plated on 0.1% xylan plates made with different buffers at pH 5 (citrate), pH 9 (Tris–HCl) and pH 12 (glycine–NaOH) at 50 °C overnight. Those clones that exhibited larger zones than the control were further tested at pH 4–12. The mutants were grown overnight in LB broth which was followed by enzyme extraction using a breaking buffer (6.8 g l^{-1} KH_2PO_4, 0.61 g l^{-1} $MgCl_2{\cdot}6H_2O$, 0.77 g l^{-1} DTT, 0.37 g l^{-1} EDTA – pH 6.8 followed by addition of PMSF after autoclaving) and sonication using the following conditions: 1 min pulsing followed by incubation on ice for 1 min for 3 cycles with frequency of 20 kHz. After extraction, the enzyme was diluted buffers ranging from pH 3 to 9 and incubated at 50 °C for 30 min, then assayed for residual activity (Chen et al., 2001; Bailey et al., 1992). Long term stability of the enzyme at alkaline pH and thermostabilty was tested by incubating the enzyme at 60 °C at pH 10, for 90 min (Bailey et al., 1992).

2.4. Construction of expression clones and transformation

The xylanase gene variant showing alkaline stability (NC38) was amplified using cycling conditions similar to error-prone PCR. Plasmids pET22b(+), pBGP1 and PCR products were restricted with endonucleases EcoRI and XhoI. DNA was then purified from agarose gels followed by ligation. Ligated DNA was added to $CaCl_2$ competent *E. coli* cells, followed by heat shocking the cells. Electrocompetent *P. pastoris* cells were transformed in 0.2 cm cuvettes using preset electroporation conditions (1.5 kV, 200 Ω, 25 μF). Aliquots were plated out onto RBB-xylan LB medium with ampicillin (100 μg ml^{-1}) for *E. coli* and RBB-xylan YPD medium with zeocin (100 μg ml^{-1}) for *P. pastoris*. Transformants were identified by a zone of hydrolysis around the colonies.

2.5. Xylanase expression and extraction

Single colonies were used to inoculate 20 ml YPD medium with zeocin for *P. pastoris* and LB with ampicillin for *E. coli*. Subsequently, cultures were inoculated into 200 ml liquid media for xylanase production. Expression in *P. pastoris* was terminated after 60 h and in *E. coli* after 8 h, which included 4 h of induction with 1 mM IPTG. Enzyme extraction in *E. coli* was carried out using Bugbuster Protein Extraction Reagent (Merck Biosciences). This was followed by centrifugation at 10,000 × *g* for 30 min at 4 °C. The supernatant containing the enzyme was stored at 4 °C for further studies.

2.6. SDS-PAGE and zymogram analysis

The protein samples were analysed using SDS-PAGE (12.5% running gel and 5% stacking gel (Laemmli, 1970). The gel was allowed to run for 3 h at 85 V, followed by staining with Coomassie Brilliant Blue R250. To perform zymogram analysis, the procedure for SDS-PAGE was followed except that the gel was not stained. The proteins were renaturated in a solution containing 1% Triton X-100 and 20 mM Tris–HCl, pH 7 for 30 min and washed in 20 mM Tris–HCl solution for 1 h. The gel was then sandwiched with an agarose gel containing 0.3% xylan and incubated for 1 h at 50 °C. This was followed by staining with 1% Congo Red.

3. Results and discussion

3.1. Screening for alkaline stable mutants

In this study, directed evolution was employed in an attempt to enhance the alkaline tolerance of a xylanase from *T. lanuginosus* DSM 5826. This protein expressed by the wild type strain has high activity and a optimum pH of 6.5 (Singh et al., 2000). One round of error-prone PCR of the *xynA* gene yielded the xylanase variant D2 having high activity but poor thermostability (Stephens et al., 2007). D2 variant was used for further error-prone PCR and 35 mutants were obtained which exhibited a larger zone of hydrolysis than the parent strain on xylan plates at pH 5, 9 and 12. Two variants from *E. coli*, NC38 and NC39 had a larger zones than the other 35 mutants, were selected for pH profiling. Surprisingly, the pH optima of the xylanases expressed by the two mutant genes was pH 5 compared to pH 6 for the parent enzyme, D2, but both variants maintained greater activity in the alkaline pH range, exhibiting 43% of its maximum activity at pH 9 (Fig. 1).

3.2. Alkaline stability

The NC38 xylanase was able to withstand harsh alkaline conditions, retaining 95% of its activity at pH 10 after 45 min and 84% after 90 min at 60 °C. In contrast, the parent lost 70% of its maximal activity after 45 min and only 22% enzyme activity was retained after 1 h (Fig. 2). On the other hand, the NC39 xylanase was less alkalo-tolerant than NC38 but more tolerant than the parent. In some studies, a high stability and high activity have been negatively correlated (Stephens et al., 2007). The explanation most commonly offered for the trade-off between high catalytic activity and stability is that during natural evolution, enzyme structure has adjusted to optimise the balance between rigidity (for stability) and flexibility (for activity) at their physiological relevant temperature (Arnold

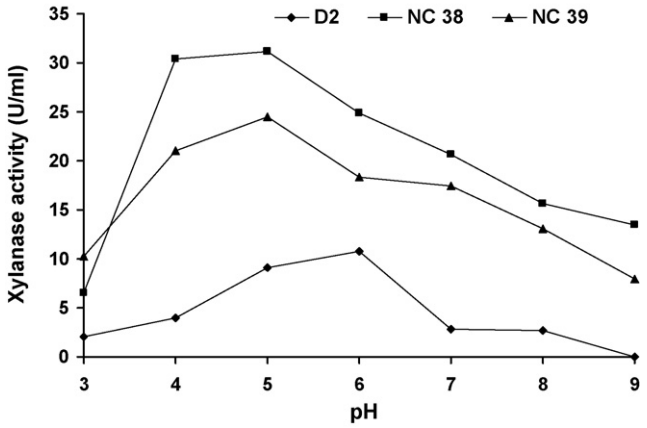

Fig. 1. pH optima profiles of xylanase variants using crude enzymes at pH 4–10 over 30 min at 50 °C for NC38, NC39 and D2 (parent strain). Each point represents a mean of triplicate determinations with a standard deviation range of 0.001–0.01 U ml^{-1}.

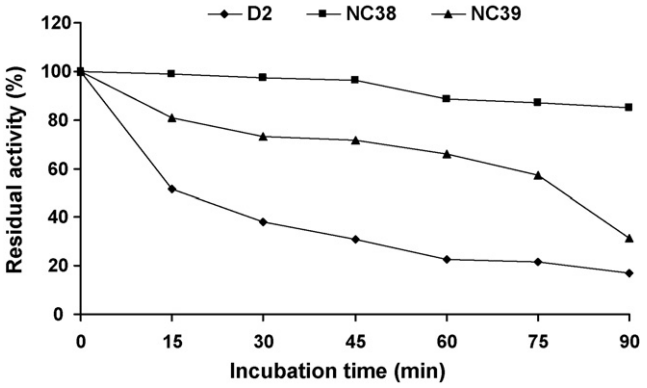

Fig. 2. Stability determination of the variants under alkaline conditions. The crude enzymes were incubated at pH 10 for 90 min at 60 °C. Each point represents a mean of triplicate determinations with a standard deviation range of 0.001–0.11 U ml^{-1}.

et al., 2001). It is possible that natural selection avoids highly stable, highly active enzymes. However, the NC38 xylanase exhibited high activity (31.1 ± 0.11 U ml^{-1}) and alkaline stability indicating that both stability and activity can co-evolve, as reported in other studies (Giver et al., 1998; Van den Berg et al., 1998). Sequence analysis showed two amino acid changes had occurred in the mutant NC38 gene, E93K and F186S (Fig. 3). Glutamic acid and lysine are similar amino acids but the second change from phenylalanine to serine led to a substitution by an unrelated amino acid. The mutations causing the stabilising effect were located on the β-sheet and the α-helix, far from the active site (Gruber et al., 1998). There was a slight increase in enzyme activity (Fig. 1).

3.3. Xylanase expression in P. pastoris and E. coli BL21

Although enhancement of alkaline stability for this enzyme was achieved, low production of the enzyme and purification costs would hinder its application in the targeted industry. Industrial application of a recombinant protein is dependent on expression levels and ease of production of the protein because these factors determine the availability and the cost of the protein product. The levels of expression and the localization of the xylanase were assessed.*P. pastoris* has become the organism of choice for expression of recombinant proteins and over 300 proteins have been expressed using this system (Cereghino and Cregg, 2000). In this study, *P. pastoris* was able to produce 261.7 ± 0.61 U ml^{-1} of enzyme secreted into the medium while *E. coli* which produced 47.9 ± 0.28 U ml^{-1}, which was located intracellular. Damaso et al. (2003) also expressed a *T. lanuginosus* xylanase in *P. pastoris* and activity levels of 360 U ml^{-1} were obtained but only after more than double the incubation period and a much higher inoculum was used. *P. pastoris* was previously used to express a number of xylanase from other organisms. The levels of production in some reports (Berrin et al., 2000; Deng et al., 2006; Lui and Lui, 2008; Lui et al., 2006) were similar to levels obtained in this study .There are other studies where expression was higher (Cheng et al., 2005; Ruanglek et al., 2007; Wang et al., 2007) but these studies were carried out using bioreactors in a fed batch system, high cell density cultivations and longer incubation times.

Total activity obtained in *P. pastoris* was 545-fold higher than in *E. coli*. This is because the protein produced in *Pichia* is secreted into the culture medium while in *E. coli* the enzyme is located intracellularly. Purification step reduces the total enzyme. Protein purification usually reduces yield considerably, in order to reach acceptable purity levels (Ito et al., 2004). Most *P. pastoris* expression systems use the *AOX* promoter to drive expression. The downfall of this promoter is that it is highly repressed by the presence of glucose, so biomass production and expression is separate and production also requires continuous induction with methanol (Cereghino and Cregg, 2000). In contrast the constitutive *GAP* promoter used in this study allows for simultaneous expression and growth. Although the strength of these promoters was shown to be similar (Koller et al., 2000), a study by Vassileva et al. (2001) shows that the *GAP* promoter was better when it was used to produce a hepatitis B surface antigen. In this comparison, the AOX clone required 96 h to accomplish the same level of antigen produced as the clones that were carrying the *GAP* promoter after 24 h.

```
xynA    VGFTPVALAALAATGAIAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTNL 60
NC38    VGFTPVALAALAATGAIAFPAGNATELEKRQTTPNSEGWHDGYYYSWWSDGGAQATYTNL 60
        ************************************************************

xynA    EGGTYEISWGDGGNLVGGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLVEYYIV 120
NC38    EGGTYEISWGDGGNLVGGKGWNPGLNARAIHFKGVYQPNGNSYLAVYGWTRNPLVEYYIV 120
        ********************************:***************************

xynA    ENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGTV 180
NC38    ENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPSIDGTQTFDQYWSVRQDKRTSGTV 180
        ************************************************************

xynA    QTGCHFDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG 224
NC38    QTGCHSDAWARAGLNVNGDHYYQIVATEGYFSSGYARITVADVG 224
        ***** ************************************
```

Fig. 3. Amino acid sequence alignment of the wild type D2 and the NC38 variant. The alignment was done using the CLUSTALW alignment program on the GenomeNet server. '*' indicates positions which have a single fully conserve residue, ':' indicates a strong conservation with a single group of amino acids and the absence of an alignment character implies that an unrelated amino acids was substituted.

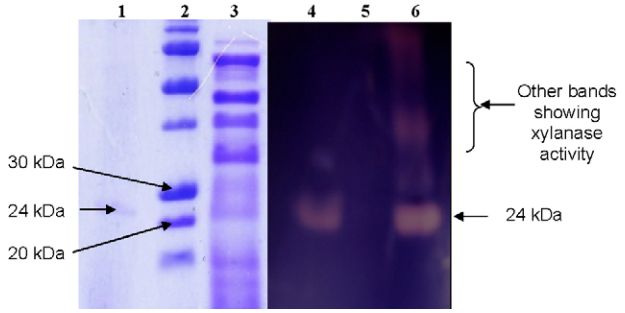

Fig. 4. SDS-PAGE and zymogram analysis of xylanase produced in *P. pastoris* and *E. coli*. Lanes 1–3 were for SDS-PAGE and 4–6 for zymogram. Lane 1 xylanase from *Pichia* (extracellular), Lane 2: Molecular Weight Marker, Lane 3: xylanase from *E. coli* BL21 (intracellular), Lane 4: xylanase in *Pichia* (extracellular), Lane 5: control (media only), and Lane 6: xylanase from *E. coli* BL21 (intracellular).

3.4. SDS-PAGE and zymogram analyisis

The enzyme produced by *E. coli* BL21 and *P. pastoris* and were analysed using SDS-PAGE to determine if correct folding of the recombinant xylanase had occurred. Secretion of this xylanase by *P. pastoris* into the media was confirmed by SDS-PAGE analysis and zymogram of the culture media after cultivation (Fig. 4). The SDS-PAGE analysis showed the presence of a single protein band. In other studies where *P. pastoris* was used with the AOX promoter, the recombinant protein was also found in the culture media and was relatively pure except for one or two extra bands identified by SDS-PAGE (Berrin et al., 2000; Ramchuran et al., 2005). The other problem sometimes associated with production of thermostable recombinant proteins in *P. pastoris* is that the protein is not active until it is treated at high temperature (Ramchuran et al., 2005). The size of the xylanase protein was identical to that of the native protein produced by *T. lanuginosus* which is about 24 kDa (Singh et al., 2000). This band was also confirmed to be the xylanase using zymogram (Fig. 4).

E. coli BL21 was also tested for enzyme release into the culture medium. A very low amount of less than a unit was detected, but the intracellular fraction was found to contain most of the enzyme. This is consistent with general expression and localization of recombinant proteins in *E. coli* (Baneyx, 1999; Baneyx and Mujacic, 2004; Choi et al., 2006). When a recombinant protein is over-expressed in *E. coli*, they are often produced in the form of inclusion bodies. The SDS-PAGE showed contamination with other intracellular proteins. This was expected and it was not possible to identify the protein band corresponding to the xylanase protein due to the background of host proteins (Fig. 4). The activity stain showed that there was an active xylanase at the expected molecular weight of 24 kDa, but there were other active bands positioned much higher than the expected band. These could be due to improper folding of the protein in a prokaryotic host (Baneyx, 1999).

P. pastoris, in conjunction with a suitable vector, has proven to be a suitable expression system for production of heterologous xylanase. This was achieved by using a vector system which did not require complicated induction steps and biomass production. The level of production of 261.7 U ml^{-1} was comparable to other literature reports. This, coupled with the ability to secrete recombinant protein with acceptable purity, is useful for a number of industrial applications. This is the first study demonstrating expression of a genetically modified xylanase in *P. pastoris*. Enzyme characterisation did not show any changes to the xylanase variant expressed in *P. pastoris* and *E. coli* BL21. The expression levels in this yeast can be further increased using high cell density fermentations.

Acknowledgements

NRF, South Africa, for providing financial support.

References

Arnold, F.H., Wintrode, P.L., Miyazaki, K., Gershenson, A., 2001. How enzymes adapt: lessons from directed evolution. Trends Biochem. Sci. 26, 100–106.
Bailey, M.J., Biely, P., Poutanen, K., 1992. Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23, 257–270.
Bajpai, P., 1999. Application of enzymes in the pulp and paper industry. Biotechnol. Prog. 15, 147–157.
Baneyx, F., 1999. Recombinant protein production in *Escherichia coli*. Curr. Opin. Biotechnol. 10, 411–421.
Baneyx, F., Mujacic, M., 2004. Recombinant protein folding and misfolding: *Escherichia coli*. Nat. Biotechnol. 22, 1399–1408.
Beguin, P., 1982. Detection of cellulase activity in polyacrylamide gels using congo red-stained agar replicas. Anal. Biochem. 131, 333–336.
Biely, P., 1985. Microbial xylanotic systems. Trends Biotechnol. 11, 286–290.
Berrin, J., Williamson, G., Puigserver, A., Chaix, J., McLauchlan, W.R., Juge, N., 2000. High level production of recombinant fungal endo-β-1,4-xylanase in the methylotrophic yeast *Pichia pastoris*. Protein Exp. Purif. 19, 179–187.
Buchert, J., Tenkanen, M., Kantelinen, A., Viikari, L., 1994. Application of xylanase in pulp and paper industry. Biores. Technol. 50, 65–72.
Cereghino, J., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 507–510.
Chen, R., 2001. Enzyme engineering: rational redesign versus directed evolution. Trends Biotechnol. 19, 13–14.
Chen, Y.L., Tang, T.Y., Cheng, K.J., 2001. Directed evolution to produce an alkalophilic variant from a *Neocallimastix patriciarum* xylanase. Can. J. Microbiol. 47, 1088–1094.
Cheng, Y., Yang, C., Lui, W., 2005. Cloning and expression of *Thermobifida* xylanase gene in the methylotrophic yeast *Pichia pastoris*. Enzyme Microbial Technol. 37, 541–546.
Cherry, J.R., Lamsa, M.H., Schnieder, P., Vind, J., Svendsen, A., Jones, A., Pedersen, A.H., 1999. Directed evolution of a fungal peroxidase. Nat. Biotechnol. 17, 379–384.
Choi, J.H., Keum, K.C., Lee, S.Y., 2006. Production of recombinant proteins by high density culture of *Escherichia coli*. Chem. Eng. Sci. 61, 876–885.
Damaso, M.T.C., Almeida, M.S., Kurtenbach, E., Martins, O.B., Pereira, N., Andrade, C.M.M.C., Albano, R.M., 2003. Optimized expression of a thermostable xylanase from *Thermomyces lanuginosus* in *Pichia pastoris*. Appl. Microbiol. Biotechnol. 69, 6064–6072.
Deng, P., Li, D., Cao, Y., Lu, W., Wang, C., 2006. Cloning a gene of an acidophilic endo-β-1,4-xylanase obtained from Aspergillus niger CGMCC1067 and constitutive expression in *Pichia pastoris*. Enzyme Microbial Technol. 39, 1096–1102.
Giver, L., Gershenson, A., Freskgard, P.O., Arnold, F.H., 1998. Directed evolution of a thermostable esterase. Proc. Natl. Acad. Sci. 95, 12809–12813.
Gomes, J., Purkarthofer, H., Hayn, M., Kapplmůller, J., Sinner, M., Steiner, W., 1993. Production of high level cellulase-free and thermostable xylanase by a wild strain of *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. Appl. Microbiol. Biotechnol. 39, 700–707.
Gruber, K., Klintschar, G., Hyna, M., Schlacher, A., Steiner, W., Kratky, C., 1998. Thermophilic xylanase from *Thermomyces lanuginosus*: high resolution x-ray structure and modeling studies. Biochemistry 37, 13475–13485.
Ito, S., Kuno, A., Suzuki, R., Kaneko, S., Kawabata, Y., Kusakabe, I., Hasegawa, T., 2004. Rational affinity of a native *Streptomyces* family 11 xylanase. J. Biotechnol. 110, 137–142.
Koller, A., Valesco, J., Subramani, S., 2000. The *CUP1* promoter of *Saccharomyces cerevisiae* is inducible by copper in *Pichia pastoris*. Yeast 16, 651–656.
Kuchner, O., Arnold, F.H., 1997. Directed evolution of enzyme catalysis. Trends Biotechnol. 15, 744–749.
Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of head of bacteriophage T4. Nature 227, 680–685.
Lee, C.C., Williams, T.G., Wong, D.W.S., Robertson, G.H., 2005. An episomal expression vector screening mutant gene libraries in *Pichia pastoris*. Plasmid 54, 80–85.
Lui, M., Lui, G., 2008. Expression of recombinant *Bacillus licheniformis* xylanase A in *Pichia pastoris* and xylooligosaccharides released from xylans by it. Protein Exp. Purif. 57, 101–107.
Lui, M., Weng, X., Sun, J., 2006. Expression of recombinant *Aspergillus niger* xylanase A in *Pichia pastoris* and its action on xylan. Protein Exp. Purif. 48, 292–299.
Ramchuran, S.O., Mateus, B., Holst, O., Nordberg-Karlsson, E., 2005. The methylotrophic yeast *Pichia pastoris* as a host for the expression and production of thermostable xylanase from the bacterium *Rhodothermus marinus*. FEMS Yeast Res. 5, 839–850.
Ruanglek, V., Sriprang, R., Ratanaphan, N., Tirawongsaroj, P., Chantasigh, D., Tanapongpipat, S., Pootanakit, K., Eurwilaichtr, L., 2007. Cloning, expression and high cell-density production of recombinant endo-β-1,4-xylanase from Aspergillus niger in *Pichia pastoris*. Enzyme Microbial Technol. 41, 19–25.
Sambrook, J., Fritsch, E.F., Maniantis, T., 1989. Molecular Cloning: A Laboratory Manual, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring, New York.

Schlacher, A., Holzmann, K., Hayn, M., Steiner, W., Schwab, H., 1996. Cloning and characterization of the gene for the thermostable xylanase *xynA* from *Thermomyces lanuginosus*. J. Biotechnol. 49, 211–218.

Stephens, D.E., Permaul, K., Prior, B.A., Singh, S., 2007. Directed evolution of the thermostable xylanse from *Thermomyces lanuginosus*. J. Biotechnol. 127, 348–354.

Singh, S., Reddy, P., Haarhoff, J., Biely, P., Janse, B., Pillay, B., Pillay, D., Prior, B.A., 2000. Relatedness of *Thermomyces lanuginosus* strains producing thermostable xylanase. J. Biotechnol. 81, 119–128.

Vassileva, A., Chugh, D.A., Swaminath, S., Khanna, N., 2001. Expression of a hepatitis B surface antigen in methylotrophic yeast *Pichia pastoris* using the *GAP* promoter. J. Biotechnol. 88, 21–35.

Van den Berg, B., Vriend, G., Veltman, O.R., Venema, G., Eijsink, V.G., 1998. Engineering an enzyme to resist boiling. Proc. Natl. Acad. Sci. 95, 2056–2060.

Wang, Y., Zhang, H., He, Y., Luo, H., Yao, B., 2007. Characterisation, gene cloning, and expression of a novel xylanase XYNB from *Streptomyces olivaceoviridis* A1. Aquaculture 267, 328–334.