Cloning and expression of xylanase variants in *Pichia pastoris*

NATASHA GOVINDARAJULU

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

_____	_____
Supervisor **Professor K. Permaul**	**Date**
_____	_____
Supervisor **Professor S. Singh**	**Date**

DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Applied Science, 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.

…….

Natasha Govindarajulu

TABLE OF CONTENTS

ABSTRACT

Microbial xylanases have attracted considerable research interest because of their various applications in biotechnology including the biobleaching of kraft pulp, to increase the nutritional value of foods and animal feed as well as for their potential use in the production of ethanol and methane. In the paper and pulp industry, the bleaching process involves the use of toxic chemicals and in the interim produces harmful gases that have a negative impact on the environment. The application of enzymes for this process will potentially reduce the environmental pollution by this industry. In addition, using an enzyme that is thermostable and alkali tolerant means that they will remain active under the required processing conditions. The xylanase gene, *xyn*A derived from *Thermomyces lanuginosus* DSM 5826, was previously evolved to produce a number of xylanase variants, which were further enhanced for increased thermostability and alkalinity. In this study, these variants were cloned in *Pichia pastoris* using the pBGP1 vector to achieve extracellular production of the recombinant proteins. The xylanase genes were isolated using PCR. Both vector and DNA inserts were linearized with restriction enzymes *Eco*RI and *Xba*I and ligated. Electroporation was employed to transform the yeast with the recombinant plasmids. This was followed by the expression of the enzymes in *P. pastoris* grown in yeast peptone glucose (YPD) medium. Enzyme activity was thereafter assessed and the yeast was found to produce 164, 78, 96 and 142 IU/ml of S325, S340, G41 and G53 xylanase respectively, higher levels than bacterial hosts. The enzymes were then characterized and it was established that the optimum temperatures and pH for maximum xylanase activity were, 60°C, pH 6 for S325; 40°C, pH 5 for S340; 60°C, pH 6 for G41 and 60°C, pH 7 for G53.

The pH and temperature stabilities of the respective enzymes were investigated, the S325 variant was exceptionally stable at a pH between 5 and 7 and temperature range of 40-80°C and retained a minimum of 40% of activity at higher pH and temperature after an incubation period of 90 min. The S340 variant was the least thermostable and alkali stable from all four variants, it however retained 40% of activity when subjected to conditions of pH 9, 80°C after 90 min. The G41 and G53 were highly stable under the pH and temperature conditions that they were subjected to. Thus being suitable for potential application in the pulp and paper industry. The enzymes were able to retain 80% of activity at pH 9, 80°C after 120 min. *P. pastoris* has been proven to be a more suitable protein expression vector than *E. coli* for a number of reasons, including; the ability to perform complex post-translational modifications and grow to high densities in minimal media resulting in the production of a high yield of heterologous proteins.

ACKNOWLEDGEMENTS

First and foremost I would like to thank God almighty for the wisdom, knowledge and understanding that he has bestowed upon me, nothing is impossible with you.

I wish to express my sincere gratitude and respect to my academic supervisor, Professor Kugen Permaul for his patience, advice and valuable critique of my work. I am thankful for his willingness to take the time to teach me and provide guidance whenever it was required. I would also like to thank Professor Suren Singh for his excellent input and for always making problems seem smaller than they really were throughout the project; your leadership and encouragement are most appreciated.

Thanks are expressed to the staff of the Department of Biotechnology and Food Technology at the Durban University of Technology for all the assistance they have so graciously provided and for being so generous in sharing your knowledge and expertise with me and for never turning me away however much I pestered you all. My sincere thanks to my parents and family for being patient and believing in me, your reassurance and support has meant the world to me. My husband Colin, without whom I couldn't have achieved many of my life's ambitions, you have been my strength and your support has remained unwavering through for all the time I have known you. To my darling son Callym, I dedicate this achievement to you, each day is brighter and filled with unbelievable joy because of you.

I wish to thank the National Research Foundation for financial assistance.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Pollution is defined as the introduction of contaminating materials into the environment that have the ability to cause instability, harm or alter the surrounding ecosystem i.e. physical systems and/or living organisms. There are three aspects that determine the harshness of a pollutant: its chemical nature, its concentration as well as its persistence in the environment. Increasing concern for the environment has led to the rapid development of novel technologies to reduce pollution, such as the use of enzymes to replace harsh chemicals in industrial and manufacturing processes.

Microbial enzymes have for a number of years been used in numerous industrial processes, such as α-amylase in the baking industry, β-glucanase and papain in the brewing industry and proteases in the detergent industry. These enzymes provide a suitable alternative to chemical use and improve the overall quality of products. The production of polymer intermediates, pharmaceuticals, speciality chemicals and agrochemicals is often hindered by expensive processes that have low selectivity and usually results in the production of undesirable by-products. Mesophilic enzymes are often not suitable for the harsh reaction conditions that are employed in industrial processes due to the lack of enzyme stability. It is for this reason, that the application of biocatalysts in organic reactions comprised only a small proportion of the potential industrial market previously. The industrial application of enzymes that have the ability to withstand harsh conditions has increased significantly, mainly as a consequence of the discovery of novel enzymes from extremophilic microbial sources. These enzymes have found the most extensive commercial use to date mainly due to their overall natural stability. Increasing

demands to reduce pollution caused by the paper industry has fuelled the need for biotechnological advances that aim to replace harsh chemicals with enzymes. In the paper manufacturing industry, xylanases are utilized in order to enhance the brightness of pulp, reduce impurities and retention time of pulp. In addition to reducing the toxicity of the process and making it eco-friendly, xylanases also facilitation a reduction in costs related to the production process, and the consumption of natural resources such as water, electricity and fossil fuels.

Xylanases are produced by various microorganisms; *viz;* bacteria, actinomycetes and fungi. Xylanases hydrolyse xylan which is a major component of hemicelluloses that are found predominantly in the cell walls of plants. Xylan is considered to be the second most abundant polysaccharide in nature; it is composed of a linear backbone of 1, 4-β-linked D-xylose units and often contains side chains of other sugar residues such as arabinose and glucuronic acid. Xylanases have been extracted from extremophilic sources and genetically manipulated for enhanced stability at higher temperature and pH conditions such as the one employed in this research study.

Yeasts have been genetically manipulated for the production of recombinant proteins. The methylotrophic yeast, *Pichia pastoris* has been developed over many years to be an exceptional host for the production of foreign proteins or genes since its inducible alcohol oxidase promoter was isolated and cloned. In this project the constitutive glyceraldehyde-3-phosphate promoter was used for the expression of the genetically modified xylanases and thus requires no induction for protein expression.

The xylanase gene, *xyn*A was originally isolated from *Thermomyces lanuginosus* DSM 5826. This gene was then genetically modified for increased thermostability and alkaline stability by directed enzyme evolution involving random mutagenesis and DNA shuffling techniques, the result of which was the production of a number of xylanase clones; including S325 and S340, alkaline stable and thermostable variants, G41, a thermostable variant and G53, an alkaline stable variant (Stephens *et al*., 2007). *Escherichia coli* expresses these recombinant enzymes at relatively low levels. In order to increase overall enzyme production and activity, *P. pastoris* was chosen as the xylanase expression host for this study. In addition, the conditions required for optimum growth of *P. pastoris* for the expression of the recombinant xylanases, have not yet been established.

Laboratory scale production of recombinant proteins is essential for the possible commercialisation of the product, especially for the production of industrial enzymes. Optimization of specific parameters such a temperature and pH will allow for the elucidation of a production protocol for higher expression levels of the protein of interest and hence increase the quantity and quality of the yield.

1.1. Pollution in the pulp and paper industry

Pulp and paper manufacturing is considered as part of the largest manufacturing divisions worldwide. With the advances in technology and the introduction of computers, the actual utilization of paper and associated products has been considerably reduced. Various chemicals that are used in the multiple stages of paper processing *viz*; bleaching chemicals, internal size

chemicals, coating chemicals, drainage and retention aids, starch and pulping chemicals (Frost and Sullivan, 2013).

A major resource that is consumed extensively in the paper manufacturing sector is water. It is estimated that the industry releases tens of millions of litres of water per day (Goncalves *et al*., 2008; Frost and Sullivan, 2013). The wastewater that is released during paper processing contains a number of toxic by-products of manufacturing or chemicals some of which include; terpenes, methanol, chloroform, detergents, surfactants and dyes which could be classified as volatile organic compounds (Goncalves *et al*., 2008). The resulting effluent may also contain thiols, sulphur dioxide and sulphides that can result in a strong stench in the surrounding environment. (Frost and Sullivan, 2013). In addition to these compounds, it also contains fibres, and resins, bleaching agents such as hydrogen peroxide, chlorine dioxide and caustic soda. These hazardous substances greatly reduce the quality of the water, causing irreversible damage and destruction of the fauna and aquatic flora. The chemicals present in the water have been found to accumulate in fish which are in turn consumed by birds and the chemicals find their way into the systems of higher animals in the food chain causing a phenomena termed biological accumulation (Shivakumar *et al*., 2012).

Chlorine and associated derivatives are also employed for the bleaching process in paper manufacture, and when they are allowed to react with the organic matter present in the pulp, they result is the formation of chlorinated organic compounds (Khandeparkar *et al*., 2007).

These compounds are toxic and can be detrimental to the environment, they are carcinogenic and tend to persist in nature.

In attending to all these problems associated with the production of paper authorities seek to find suitable solutions, involving the identification of strategies that will effectively deal with the growing pollution problem. One such solution is the use of enzymes for the biobleaching of pulp and xylanases have demonstrated biobleaching efficacy of bagasse pulp as one such example (Birijlall *et al*., 2011). Enzymes provide a sustainable resolution to the use of chlorine and associated chemicals and have been extensively tested for these purposes (Soltani and Shirkolaee, 2007). Various companies have developed enzymes for use in the paper industry. Novozyme is currently marketing enzymes that are proposed to have the ability of improving pulping processes with various attractive attributes and benefits, Novozyme Fibercare R which improves the papermaking performance of bleached chemical pulps, Novozyme Fibercare U which improves the paper and board making performance of unbleached chemical pulps and Novozyme Fibercare D which reduces the drying energy and increases overall papermill productivity (Novozymes report, 2008). Allzym PT is a commercial xylanase produced by Alltech for improving animal feed. Pulpzyme and Ecopulp X-200 are manufactured by Novozymes-Denmark and Primalco, respectively for cellulose treatment and pulp bleaching. SuzizymeX is a xylanase produced by Shin Nihon used specifically in the manufacture of mushroom and vegetable extracts, bread making, enzymatic peeling of cereals and animal feed improvement (Polizeli *et al*., 2005).

1.2. Enzymes in industry

Biotechnology plays a major role in modern industrial processes, particularly in the application of microbial enzymes. Biotechnological methods are used in over fifty applications and in

hundreds of products and consumer goods globally. The applications include; bread making, detergents, fuel production, food and beverage processing to name a few. These industries utilize enzymes that are produced via the large scale fermentation of microorganisms. As both rational and random methodologies for enzyme improvement are evolving, enzymes are being developed for their integration into key roles as biocatalysts in industrial processes for an ever expanding industry (Cherry and Fidantsef, 2003). The industrial enzymes market had an estimated value of USD 4.2 Billion in the year 2014 and has since been projected to grow at a rate of 7.0% from the year 2015 to 2020. (Anon., 2015; Moshelion and Altman, 2015). The enzyme industry is generally divided into three segments. The largest sector is that of technical enzymes, that contribute 65% of total sales, these include those enzymes catalysts utilized in the detergent, starch, textile, leather, pulp and paper, personal care manufacturing industries (Cherry and Fidantsef, 2003) and the highest sales being attributed to the biofuel division (Sarrouh *et al*., 2012). Food enzymes encompass the second largest segment, at 25% of the total market, these include enzymes employed in the dairy, brewing, wine and juice, fats and oils, and baking industries. Finally, feed enzymes, comprising enzymes used in animal feeds, contributes approximately 10% of the market (McCoy, 2000).

Enzymes are now the preferred choice over chemical catalysts in a number of industrial processed since they offer several advantages compared to their chemical counterparts in that they are derived from renewable resources, are biodegradable, possess the inherent ability to function under both mild and adverse conditions of pH and temperature. An additional quality is that they are able to extend a high degree of substrate specificity and selectivity in both reactant

and end-product stereochemistry (Cherry and Fidantsef, 2003; Johannes and Zhao, 2006; Wang *et al*., 2012). With the increasing population and industrialization of countries, there is a significant requirement to establish eco-friendly approaches to the production of consumer goods. Enzymes have already made their breakthrough in industry and have been applied to enhance or improve the quality of products as well as to reduce pollution. Examples include the application of proteases and cellulases to replace phosphates in household detergents. In the baking industry, chemical emulsifiers have been substituted with lipases and in the textile industry, sodium hydroxide, a well-known pollutant has been replaced with amylases and pectinases (Atomi *et al*., 2011). The list of enzyme applications is constantly expanding (Table 1.1), bringing with it a list of significant social and environmental benefits (Cherry and Fidantsef, 2003).

The standard process that is followed for the discovery of novel and useful biocatalysts for application in industrial purposes is firstly; the identification and study of microbes in various environmental circumstances based on the catalytic application of interest, ranging from mild conditions to relatively harsh conditions. Thereafter metagenomic screening is carried out such as sequence based screening (PCR or hybridization). This is followed by sequencing or mapping of the microbial genomes in order to identify specific enzyme sequences by comparison to those available in genetic databases. Random mutagenesis or directed evolution techniques (Wang *et al*., 2012; Johannes and Zhao, 2006) can then be used to create enzyme variants, these include DNA shuffling and Error-prone PCR. (Adrio and Demain, 2014). The resulting clone library is screened for enhanced and required traits or enzyme function.

Table 1.1. Applications of enzymes in industry (Kirk *et al*., 2002)

Industry	Enzyme class	Application
Detergent (laundry and dish wash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, colour clarification
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Xylanase	Viscosity reduction (fuel and starch)
Baking	Amylase	Dough softness and volume
	Xylanase	Dough conditioning
	Lipase	Dough stability (in situ emulsifier)
	Glucose Oxidase	Dough strengthening
Food (including dairy)	Protease	Milk clotting, infant formulas, flavour
	Lipase	Cheese flavour
	Lactase	Lactose removal (milk)
	Pectinase	Fruit-based products
Animal feed	Phytase	Phytate digestibility-phosphorous release
	Xylanase	Digestibility
	β-glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-glucanase	Mashing
	Acetolactate decarboxylate	Maturation (beer)
	Laccase	Clarification (juice), flavour (beer)
Pulp and paper	Lipase	Pitch control, contamination control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking
	Xylanase	Bleach boosting
	Cellulase	De-inking, fibre modification
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Fats and oils	Lipase	Trans-esterification
	Phospholipase	De-gumming, lyso-lecithin production
Leather	Protease	Unhearing, bating
	Lipase	De-pickling
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semi synthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose Oxidase	Bleaching, antimicrobial
	Peroxidase	Anti-microbial

1.3. Enzymes from extremophiles

Extremophiles have evolved to exist in a range of extreme environments and can be arranged into a number of different classes that include thermophiles, acidophiles, alkalophiles, psychrophiles, and barophiles (piezophiles). They have adapted to thrive in ecological niches such as deep-sea hydrothermal vents, hot springs, and sulphataric fields (Demirjian *et al*., 2001). As a result, these microbes possess the ability to produce distinctive biocatalysts that are capable of functioning under extreme conditions in which their mesophilic microbial counterparts would not be able to survive (Pikuta *et al*., 2007), thus permitting the requirement for the development of additional industrial processes. In previous years, extremophiles were a relatively unknown group of microorganisms, explored by a limited number of research groups worldwide. Currently however, even though they retain their unconventional status, they are routinely utilized as the sources of novel enzymatic catalysts at pharmaceutical, research-based and enzyme-discovery companies (Demirjian *et al*., 2001).

1.4. Factors affecting extremozyme stability

There has been a number of theories and extensive research that attempts to explain or single out specific factors that contribute to the stability of extremozymes in harsh conditions. Recent studies on extremophilic enzymes, particularly thermophilic enzymes, aids us in understanding the various attributes, functions and general trends that may factor into the enhanced stability of extremozymes as well as their inherent ability to survive in adverse environments (Demirjian *et al*., 2001, Kumar *et al*., 2011). Extreme environmental conditions would require optimized

interactions within a protein, at the protein–solvent boundary, or with the co-operation of external factors which may include metabolites, cofactors, and compatible solutes (Demirjian *et al.*, 2001). Structural components within the protein that are considered to be contributing factors that results in the significant stability of extremozymes include an increased number of ion pairs than in mesophilic counterparts, a considerable reduction in the size of loops, a lower number of cavities, a reduced ratio of surface area to volume, variations in the composition of specific amino acid residues , increased hydrophobic interaction at subunit interfaces, variations in solvent-exposed surface areas, increase in the degree of secondary structure conformation and truncated amino and carboxyl termini (Demirjian *et al.*, 2001; Atomi *et al*., 2011).

1.5. Xylanases

The xylanase catalysts mainly researched are either of fungal or bacterial origin and in most cases these enzymes are found to have optimal activity at, or near mesophilic temperatures (approximately 40-60°C) and neutral (particularly for bacterial xylanases) or slightly acidic pHs (fungal xylanases) (Collins *et al*., 2005, Viikari, 1990). There are however instances in which the xylanases not only display a high stability, but are extremely active at extremes ranges of temperature and pH. Xylanases have the ability to remain active at temperatures ranging from 5 to 105°C and pH ranging from 2-11 as well as NaCl concentrations as high as 30% (Collins *et al*., 2005).

Xylanases are distributed into main families of glycosyl hydrolases: Family 10 (F) and Family 11 (G) (Fig. 1.1). The structural similarity among enzymes within these families can be established

either by pairwise alignments of the protein sequences or by the basic local alignment search tool (BLAST) to distinguish sequence relatedness (Jeffries, 1996; Bosetto *et al*., 2016).

EX of family 10 **EX of family 11**

Figure 1.1: Ribbon representations of the main fold of the catalytic domains of the exoxylanases (EX) of family 10 and 11 (Davies and Henrissat, 1995)

Xylanases have numerous and widespread applications in industries (Table 1.2). In the food industry they are used for the modification of baking products, for enhancing the recovery of starch from wheat flours and assisting the extraction and clarification of fruit juices. In the animal feed industry, xylanases can be used to increase the digestibility of animal feed to allow easy absorption of nutrients and vitamins in monogastric animals. Xylanases are also being used extensively in biobleaching of pulp (Tsai *et al*., 2008).

Table 1.2. Applications of xylanases

Market	Industry	Application	Function	Reference
Food	Fruit and vegetable processing, brewing, wine production	Fruit and vegetable juices, nectars and purees, oils (e.g. olive oil, corn oil) and wines	Improves maceration and juice clarification, reduces viscosity. Improves extraction yield and filtration, process performance and product quality.	Polizeli et al., 2005
	Baking	Dough and bakery products	Improves elasticity and strength of the dough, thereby allowing easier handling, larger loaf volumes and improved bread texture.	Beg et al.,2001 Collins et al., 2006
Feed	Animal feeds	Monogastric (swine and poultry) and ruminant feeds	Decreases the content of non-starch polysaccharides, thereby reducing the intestinal viscosity and improving the utilization of proteins and starch. Improves animal performance, increases digestability and nutritive value of poorly degradable feeds, e.g., barley and wheat.	Polizeli et al., 2005 Subramaniyan and Prema, 200
Technical	Paper and pulp	Biobleaching of kraft pulps	Reduces chlorine consumption and toxic discharges	Singh et al., 2000
		Bio-mechanical pulping	Facilitates the pulping process and reduces the use of mechanical pulping methods, hence reduces energy consumption	
		Bio-modification of fibres	Improves fibrillation and drainage properties of pulp, hence improving the process efficiency and the paper strength	Khristova et al., 2006
		Bio-de-inking		Jeffries, 1996
	Starch	Starch-gluten separation	Facilitates the de-inking process and reduces the use of alkali.	Subramaniyan and Prema, 2002
	Textiles	Retting of flax, jute, ramie, hemp, etc.		Collins et al., 2005
	Bioremediation/ Bioconversion	Treatment of agricultural, municipal and food industry wastes	Reduces batter viscosity, improves gluten agglomeration and process efficiency Enzymatic retting reduces/replaces chemical retting methods. Treatment/recycling of wastes. Production of fermentable products, renewable fuel (bioethanol) and fine chemicals	Beg et al., 2001

Most of the xylanases currently being employed in industrial processing of goods are of mesophilic and/or neutrophilic origin, yet enzymes from extremophilic sources may be better suited to many biotechnological processes in the manufacturing sector. Thermophilic enzymes could be applied to replace processed that would require a cooling step. Cooling steps tend to be uneconomical during manufacture (Collins *et al*., 2005). Extremozymes can be utilized in stages where higher temperatures are required for example, to increase the bioavailability and/or solubility of substrates, to reduce viscosity of the product and/or reduce the risk of potential contamination (Viikari, 1990; Beg *et al*., 2001). Acidophilic and alkaliphilic enzymes would evidently be beneficial for processes in which extreme pH conditions are mandatory or in processes where adjustment of the pH to neutral conditions is uneconomical. On the contrary, cold-adapted xylanases would prove to be useful to those processes that are carried out at the lower temperature ranges where heating is economically counterproductive or where the maintenance of low temperatures are essential to avoid alteration of the ingredients (Harris and Ramalingam, 2010) , a discrepancy in the final quality of the product (e.g., flavour, colour etc.), to increase the shelf life of a product and/or to avoid microbial growth and fermentation as well as to avoid product denaturation. For optimum results, extremophilic enzymes which combine more than two desirable traits may generate the greatest interest for application in industry. (Collins *et al*., 2005).

Of particular interest is the application of xylanases is in the pulp and paper industry where the high temperature of between 55 and 70°C and alkaline pH of the pulp substrate involves the utilization of thermo-alkaliphilic enzymes for efficient biobleaching. Thermo-alkaliphilic or even thermo-acidophilic xylanases could also be of use in the bioconversion process where a number

of treatments, including hot water and steam blasting, alkaline, solvent or acidic pre-treatments may be used prior to or together with enzymatic treatment. Alkaliphilic xylanases can be applied or incorporated in the detergent sector of industry where processes are carried out under alkaline conditions, whilst a thermostable xylanase would be beneficial for the processing of animal feeds, where the enzymes can be added to the feedstock prior to the pelleting process, commonly carried out at temperatures between 70 and 95°C (Polizeli *et al*., 2005). For this latter application, the enzyme must be highly active at the required temperature (approximately 40°C) and pH conditions (approximately pH 4.8) of the animal digestive tract. Cold adapted xylanases, being most active at lower and intermediate temperature ranges, could offer a number of advantages over the currently utilized xylanases in industries that process goods at low to moderate temperature environments such as the food industry. For example, they would be best suited for usage in the baking sector since dough preparation and proofing is traditionally done at temperatures below 35°C and it has recently been confirmed that a cold-adapted family 8 xylanase that has proven to be more effective in baking than a commonly utilized commercial enzyme (Beg *et al*., 2001; Collins *et al*., 2005).

1.6. Substrate interaction and characterization

Xylan is an important structural polysaccharide in plant cell walls and is the second most abundant polysaccharide in nature, and it accounts for approximately a third of all renewable organic carbon on earth (Beg *et al*., 2001). Xylan constitutes the major component of hemicelluloses, a complex of polymeric carbohydrates. Xylan is found in large quantities in hardwoods (15-30% of the cell wall content) and softwoods (7-10%), as well as in annual plants

(<30%) (Singh *et al*., 2003). Its typical location is in the secondary wall but is also found in the primary cell wall particularly in monocots. A complex highly branched heteropolysacchride, it varies in structure from one plant species to another. Complete hydrolysis of xylan requires the combined catalysis by a plethora of enzymes since it has a complex and heterogeneous structure, the enzymes required include; β-1, 4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, ferulic and *ρ*-coumaric acid esterases (Fig. 1.2). All these enzymes act co-operatively to convert xylan into its constituent sugars. Endo-1, 4-β-D-xylanases randomly cleave the xylan backbone (Collins *et al*., 2005).

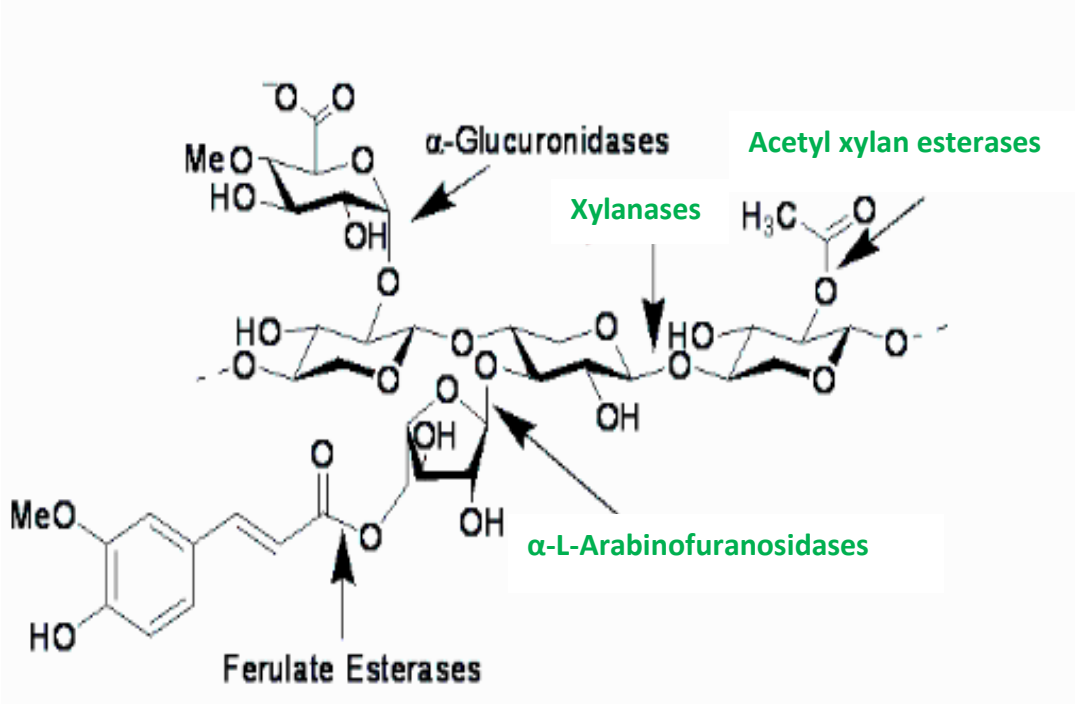

Figure 1.2. Microbial hydrolysis of xylan: Three classes of enzymes involved in the dissimilation of plant polysaccharides. (a) Xylanases hydrolyse the β1, 4 glycosidic bonds in xylan. (b) Arabinofuranosideases hydrolyse both the α-1, 2 and 1, 3 arabinofuranosyl moieties from arabinan and xylan. (c) Acetyl xylan esterases hydrolyze the O-acetyl substituents at the *O*-2 position of the xylan backbone.

1.7. Application of xylanase in the paper and pulp industry

Generally in the paper industries the residual lignin from cellulose pulp is chemically releases with the use of chlorine based compounds. Raw materials are usually cooked under conditions of high temperature and pressure in the traditional pulping process, and are the source of many problems including the amounts of energy consumed, requirement of higher volumes of chemicals and problems associated with environmental pollution. Therefore, the need for "greener" technology has promoted the search for alternative bleaching and especially ozone-free and hydrogen peroxide-free bleaching processes (Roncero and Valls, 2003; Khristova *et al*., 2006; Liu and Liu, 2008). Pulp bleaching with the addition of microbial xylanases has since been proven as an environmentally friendlier technology for the pulp and paper industries (Shirkolaee *et al*., 2008).

The biobleaching process is greatly dependent on the growth/catalytic action of the microorganisms and/or enzymes on the pulp material. Microbial xylanases that are both thermostable and cellulase-free are usually the preferred choice for biobleaching. The interest for xylan degrading biocatalyst and their applications in the pulp and paper manufacturing industry has advanced considerably over the past few decades (Shirkolaee *et al*., 2008). In this process, the bond between lignin and hemicelluloses can be removed by xylanase. Once the hemicellulose layer is removed, the lignin can be separated due to the degradative action of the ligninolytic enzymes (Duan *et al*., 2016). The use of xylanase in the biobleaching process helps to attain pulp with high brightness with a reduction in the use of bleaching chemicals by 20-25% (Soltanali and Shirkolaee, 2007; Shirkolaee *et al*., 2008).

In the kraft process, elimination of residual lignin from Kraft pulp is limited by the physical and chemical composition of hemicelluloses. Lignin has been found to link with hemicelluloses (Karlsson *et al*., 2001; Subramaniyan and Prema, 2002) and research has provided an indication of the formation of lignin-carbohydrate complexes present in the kraft pulp. The most commonly known pulping process is the Kraft process or Sulphate process which involves the cooking of wood chips in a sodium sulphide/ sodium hydroxide solution 170°C for a period of which results in the degradation and solubilisation of lignin. The pulp that is extruded from this process has a characteristic brown colour due mainly to the presence of residual lignin and lignin derivatives present in the pulp (Bajpai, 1999). The intensity of the pulp colour is an indication of the amount and chemical structure of the remaining lignin. In order to obtain pulp that has an intense brightness and brightness stability, a considerable amount or even all of the lignin in the structure should be efficiently removed from the pulp (Puls, 1997; Singh *et al*., 2000). For this purpose, chemical pulping is more effective than mechanical pulping (Subramaniyan and Prema 2002). There is however, the formation of residual lignin which has to be removed by the process of bleaching (Puls, 1997; Bian *et al*., 2013). The remaining lignin in the chemically-processed pulp is darker in colour because it has been expansively oxidized and modified during the cooking process. This residual lignin is covalently linked to hemicellulose and cellulose fibres, thus it is difficult to remove. The bleaching of the pulp can be therefore regarded as a purification process because in entails the breakdown, alteration or solubilization of the residual lignin, coloured organic matter and other undesirable residues that may be present on the fibres of the pulp (Subramaniyan and Prema, 2002).

The bleaching of chemically-processed pulp to a higher level of brightness without completely removing the lignin has not proven to be successful thus far. Conventionally, chlorine is utilized for the bleaching of kraft pulp. Chlorination of pulp does not produce any decolourising effect, incidentally, the colour of the pulp may increase with chlorination due to the oxidative mechanism which aids in the bleaching. At low pH the primary function of chlorine is chlorination and not oxidation. Thus chlorine has the ability to selectively chlorinate and degrade lignin compounds rather than the carbohydrates present in pulp *viz*; hemicelluloses and cellulose moieties (Subramaniyan and Prema, 2000; 2002). Chlorine is used in the bleaching process to chemically alter the residual lignin in the pulp to form water soluble or alkali soluble by-products. The effluents that are produced during the bleaching process, particularly those following the chlorination process and the first extraction phases are the major contributors to waste water pollution that results from the pulp paper industry. During the kraft process, Portions of the xylan are rearranged on the surface of the fibres. A substantial quantity of xylan is present in the fibres following the pulping process. Enzymatic hydrolysis of the re-precipitated and rearranged xylan molecules on the surface of the fibres may render the structure of the fibre more permeable. This increase in permeability allows for the easier movement of lignin or lignin-carbohydrate molecules to increased volumes and of higher molecular masses than in the successive chemical reactions (Subramaniyan and Prema 2000).

The application of hemicellulases was first demonstrated by Viikari (1990) which results in a significant reduction in the consumption of chlorine. Lundgren *et al.* (1994) conducted a Mill trial on TCF (total chlorine free) technology for the biobleaching of pulp with the aid of xylanase

enzymes from *Bacillus stearothermophilus* strain T6 which has displayed optimum activity at pH 6.5. There are numerous studies related to the application of microbial xylanases (Adrio and Demain, 2014) but only a limited number of them display characteristics that are applicable for the processes in the pulp and paper industry (Bhagat *et al*., 2014).

Two types of processes are involved in the enzymatic pretreatment of kraft pulp. The major change in the pulp is due to the breakdown of reprecipitated and readsorbed xylan and/or xylan-lignin complexes has become separated throughout the cooking process (Singh *et al*., 2000). During the enzymatic treatment process, the pulp is made more easily available to oxidation by the bleaching chemicals (chlorine). A minor difference in the pulp structure is due to the enzymatic hydrolysis of the remaining non-dissolved hemicellulose by endoxylanases. Residual lignin that is present in unbleached pulp (Kraft pulp) is linked to hemicellulose and enzymatic cleavage of this linkage will allow for the release of the lignin (Subramaniyan and Prema, 2002).

1.8. Why xylanases for improvement of pulp and paper manufacture processes?

Xylans tend to not form tightly packed structures, hence are highly accessible to hydrolytic enzymes such as xylanase. For this reason, the specific activity of xylanase is approximately two-three times higher compared to the hydrolases of other polymers like crystalline cellulose (Li *et al*., 2000). It is essential to bleach the pulp to reduce the concentration of certain elements present in the pulp such as; lignin and associated derivatives in order to obtain white and bright pulp suitable for manufacturing good quality paper products. Biobleaching of pulp is reported to be more effective with the catalytic action of xylanases compared to lignin degrading enzymes

(Shirkolaee *et al*., 2008). This is due to the fact that the lignin present in the pulp is cross-linked primarily to the hemicellulose and the hemicellulose is more easily depolymerised than the lignin component (Garg *et al*., 1996). Removal of even a minor portion of the hemicellulose can be sufficient to expose the residual lignin polymers and facilitate their removal by mild oxidants (Nagar *et al*., 2013). The most important objective of the implementation of biotechnological methods for this purpose is the achievement being able to carry out the selective removal of hemicellulose without the degradation of cellulose. Degradation of cellulose is a major hindrance associated with the conventional pulping process, which in turn affects the cellulose fibre, and thereby the quality of paper produced (Shallom and Shoham**,** 2003). Elimination of xylan from the cell walls has resulted in a significant decrease in energy demand during the process of bleaching. Therefore enzymatic treatments of pulp using xylanases have better prospect in terms of economical processing and potentially improves the overall quality of the fibre (Subramaniyan and Prema, 2002).

1.8.1. Ideal xylanases for biobleaching

For efficient processing of pulp the following characteristics are required:

i. The xylanase of interest must be cellulase-free to prevent a reduction in the quality of pulp;

ii. High yields of enzyme must be obtained at low cost;

iii. It is desirable to have a low molecular weight xylanase that is able to penetrate the pulp fibre. An enzyme must have access to the substrate in order to release lignin derived compounds;

iv. Xylanase must have an alkaline pH optima and isoelectric point since kraft pulp processes are operated at alkaline pH (8 - 10). Pulp fibres are generally negatively charged due to the presence of sugar acids, and if an enzyme has an alkaline pI, it will bind more effectively;

v. The xylanase must be stable at the temperature that is prevalent in the pulp stock (60 - 90°C) for 3 - 5 h. The combination of alkaline and thermal stability however, has been extremely difficult to obtain from natural sources and has thus been the focus of genetic engineering efforts for decades; and

vi. Xylanase should possess appropriate substrate specificity to release the chromophores and extract residual lignin (Jeffries, 1996; Techapun *et al*., 2003; Stephens *et al*., 2007).

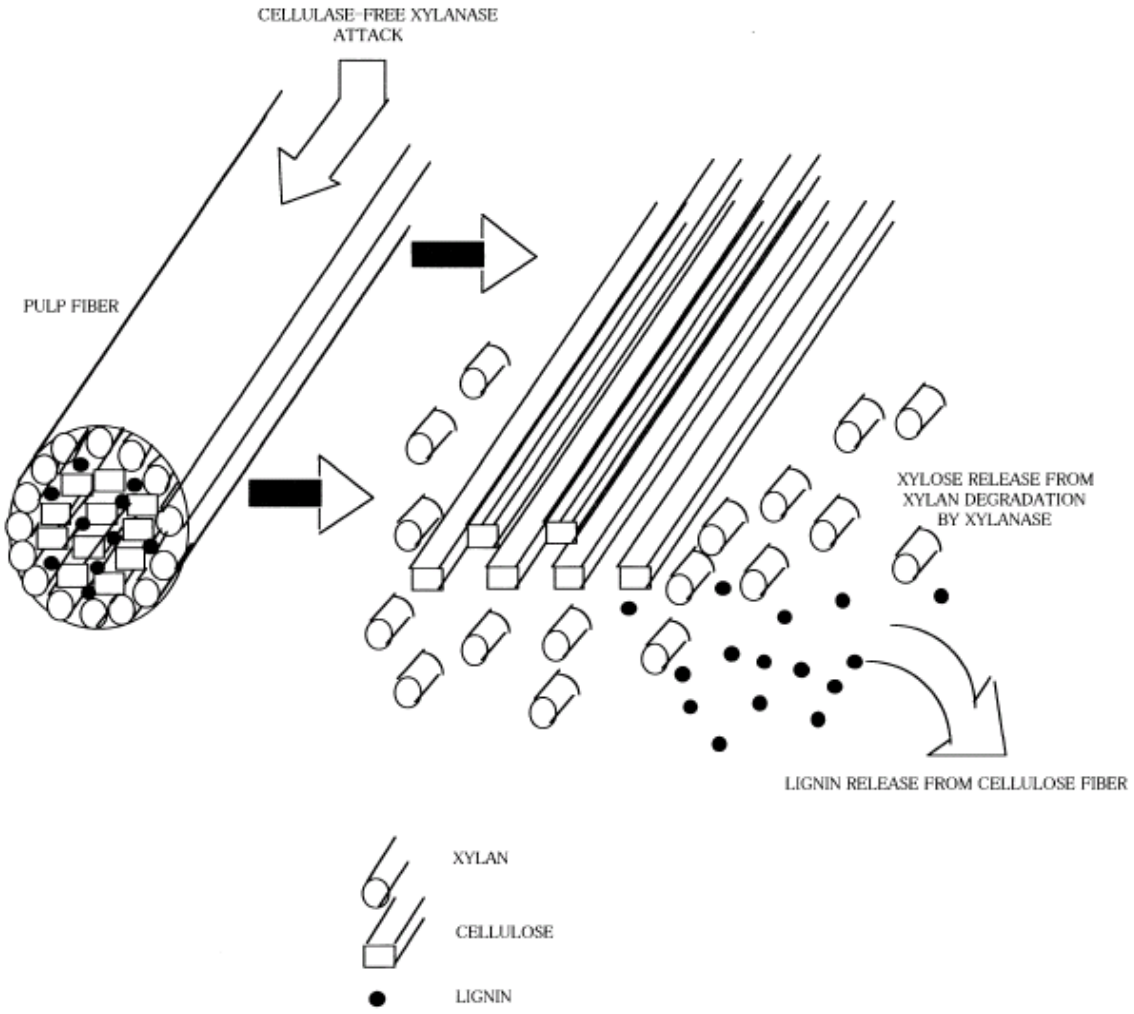

Figure 1.3. Lignin associated hemicellulose fraction removal from pulp structure by the action of cellulase-free xylanase (Techapun *et al.*, 2003).

1.9. Modification of xylanase from *T. lanuginosus* DSM 5826

Xylanases with several different properties have been studied in recent years, and several have been reported to produce xylobiose and xylooligosaccharides (Isil and Nilufer, 2005; Sun *et al*., 2007; Chutani and Sharma, 2015). Interaction of xylanases with their substrates depends upon the substitution of the xylan moiety.

Damaso *et al*. (2003) found that highly efficient production of a *T. lanuginosus* OC-4145 ß-1, 4-xylanase was achieved in *P. pastoris*. *P. pastoris* colonies expressing recombinant xylanase were screened using plate assay procedures, and their innate ability to secrete high levels of the xylanase was investigated in small-scale (shake flask) cultures. A high level of cellulase-free, thermostable xylanase production by *T. lanuginosus* DSM 5826 has been reported (Gomes *et al*., 1993; Purkarthofer *et al*., 1993) with a molecular weight of 26.0 kDa (Purkarthofer *et al*., 1993). The selectivity and specificity for xylan as a substrate, the thermostability and the catalytic activity over an expanded range of pH make this enzyme extremely beneficial for the bleaching of paper pulp (Gomes *et al*., 1993; Schlacher *et al*., 1996). Cloning of the respective gene provides the basis for optimizing the catalytic efficiency of the enzyme (Schlacher *et al*., 1996).

A study was undertaken by Stephens, D. E. (2007) to improve the thermal and alkaline stabilities of the xylanase from the fungus *Thermomyces lanuginosus* using error prone-PCR and DNA shuffling techniques. An enzyme library consisting of 960 clones was analyzed in duplicate for stability at 80°C and pH 10. Mutant G41 retained 75% of its initial activity at 80°C for 90 min whilst mutant G53 retained 93% of its activity at pH 10 for 90 min. These mutants were then recombined using a DNA shuffling method called the staggered extension process (StEP) in order to assemble both these properties into a single xylanase. The products of the StEP reaction were cloned into *E. coli* and 516 recombinants were collectively obtained and tested for alkaline and thermal stability. Recombinants S340 and S325 displayed 60% activity at pH 10 and 54% and 85% activity at 80°C for 90 min, respectively (Stephens *et al*., 2009). DNA sequencing revealed parent mutants G53 and G41 to have one and four mutations, respectively. The recombinants S340 and

S325 exhibited three and five mutations respectively. The cloned *xyn*A, its fungal counterpart, parents G41 and G53 as well as recombinants S325 and S340 were exposed along with a commercial xylanase, (Luminase), to thermophilic and alkaline pulping conditions. At higher temperatures, G41 and S325 were quite stable. First order kinetics established that these were the most stable xylanases created in the study. The mutants G41, G53 and S325 were subsequently cloned onto the plasmid Bluescript SK (Stratagene) and S340 onto the pET22b vector for expression (Wakelin, 2009).

1.10. Yeasts for protein production

As we advance in the "Biotechnology era" scientists continue to engineer yeasts to produce eukaryotic proteins. Even though these discoveries are not as revered and appreciated as their application in the bread and beer-making applications, heterologous proteins expressed in yeasts are currently being utilized for the synthesis of life-saving drugs for the pharmaceutical industry and actively assist in the unravelling of complex regulatory phenomena that form the basis of research.

The expression of heterologous genes is a subject of considerable interest for the manufacture of pharmaceutical proteins of therapeutic (i.e. interferon, interleukins, etc.) and commercial (i.e. industrial enzymes) interest (Dominguez *et al*., 1998). Initially, the commercial production of heterologous proteins was accomplished with the use of *E. coli* as an expression host. The dominance of *E. coli* in this particular area of research is a direct result of the availability of vast amounts of information relating its genetic and biochemical systems that have been collected

over many decades of research and trials. As the innate characteristics of the recombinant proteins increased in complexity and as transformation systems evolved in a greater number of species, a variety of production hosts that range from prokaryotes to transgenic animals and plants have been developed (Dominguez *et al*., 1998; Spencer *et al*., 2002). Yeasts present as a valuable expression host because they exhibit several advantages over other microbial host systems. *Saccharomyces cerevisiae*, specifically, has been used for centuries as a preferred microbe for its application in food production. There is a plethora information that is available that discusses their safety and they are considered as Generally Recognized as Safe (GRAS) by the American Food and Drug Administration (FDA). On the contrary, prokaryotic microorganisms may have toxic cell wall pyrogens (endotoxins) and mammalian cells may contain oncogenic or viral DNA. The growth and fermentation protocols of yeasts are well established and these microbes have adapted to grow rapidly on relatively simple media to high cell densities and at a lower cost compared to other fermentation systems (Dominguez *et al*., 1998; Cheng *et al*., 2005). As eukaryotic organisms, they have the natural ability to perform eukaryotic processing on the polypeptides that are expressed, such as post-translational modifications (disulphide bond formation, proteolytic maturation of prohormones, *N* and *O*-linked glycosylation, etc.), which are required for the retention of functional synthesis of many heterologous proteins, and the quality and authenticity of the protein is of a much higher standard than those produced in prokaryotic microorganisms (Eckart and Bussineaut, 1996).

The majority of recombinant proteins produced in yeast systems have been expressed using *S. cerevisiae* as the host due mainly to the ease with which it can be genetically manipulated and

modified as to the vast amounts of information that is available relating to its molecular biology and physiology (Demain and Vaishnav, 2009; Petranovic and Vemuri, 2009). In addition, the sequence of its entire genome has been extensively studied and mapped. Despite these facts, several limitations have been identified in the *S. cerevisiae* expression system. Examples include a low product yield, and inefficient secretion mechanism (many *S. cerevisiae* proteins are not released into the culture medium but instead, are retained in the periplasmic space or associated with the cell wall (Buckholz and Gleeson, 1991; Dominguez *et al*., 1998; Cregg *et al*., 2009). The limitation to this species has been bypassed the recognition and development of expression systems in other yeast species, one such yeast is the methylotrophic *P. pastoris* which has been widely used as an exceptional host for the production of foreign proteins (Mattanovich, 2012; Illias, 2014).

1.11. *P. pastoris* as a host heterologous protein production

Over the years, scientists have discovered how to identify and manipulate organisms, they have also learnt how to extract DNA, alter and manipulate genes and/or place these genes into a variety of similar organisms or a host that differs genetically to the source. A major use of recombinant organisms is to express or produce foreign proteins, such as enzymes. Since many proteins may be of immense commercial value, a number of studies have been focused on finding ways to produce them inexpensively, simply and in fully functional structure and mode.

For several years *E. coli* has been the organism of choice for the expression of various proteins, due to the fact that its genome has been fully mapped and it is relatively easy to handle. *E. coli* can be cultured rapidly; it requires an inexpensive, defined medium for growth. However, *E. coli*

is a prokaryotic organism and hence lacks intracellular organelles, such as the endoplasmic reticulum and the Golgi apparatus that are present in eukaryotes. These organelles function in the modification of proteins being produced; therefore proteins are expressed in *E. coli* in an incomplete, non-functional form because glycosylation or post-translational modifications cannot be carried out. In addition, *E. coli* lacks the ability to excrete the recombinant proteins extracellularly thereby increasing the costs of downstream processing for recovery of the foreign protein (Darby *et al*., 2012).

Table 1.3. Heterologous proteins expressed in *P. pastoris*

Type of protein	Heterologous protein	Reference
Phytase	E. coli phytase	Chen et al., 2004
Xylanase	Fungal endo-β-1,4-xylanase	Berrin et al., 2000
	Thermobifida xylanase	Cheng et al., 2005
	Chaetomium thermophilum xylanase	Andleeb et al., 2008
	Aspergillus terreus xylanase 10	Chantasingh et al., 2006
Vaccines	Vaccines	Balamurugan et al., 2007
Lipase	Rhizopus oryzae lipase	Arnau et al., 2010
	Rhizopus oryzae lipase	Resina et al., 2004
	Rhizopus chinensis lipase	Wei et al., 2009
Tannase	Aspergillus oryzae tannase	Zhong et al., 2004
Human proteins	lumbrokinase (PI239)	Ge et al., 2007
	S-adenosylmethionine	Zhang et al., 2008

The increasing popularity of the *P. pastoris* expression system can be ascribed to several factors, mainly:

i. The simplicity of molecular techniques required for the genetic manipulation of *P. pastoris* and its similarity to *Saccharomyces cerevisiae*, (an extensively studied microbial system in modern biology) ;

ii. The ability of *P. pastoris* to produce heterologous proteins at high yields, either intracellularly or extracellularly ; (Table 1.3)

iii. The ability to perform a number of eukaryotic post-translational modifications, such as glycosylation, disulphide bond formation and proteolytic processing; and

iv. The availability of the expression system as a commercially-available kit (Cereghino and Cregg, 2000).

1.12. Glyceraldehyde-3-phosphate promoter of *P. pastoris*

This promoter was specifically designed and intended for high-level, constitutive expression in *P. pastoris*. It was generated by substituting the methanol-regulated *AOX1* promoter with the constitutive, glyceraldehyde-3-phosphate dehydrogenase *(*GAP) promoter (Fig. 1.4) in the backbone of the pPICZα vector. Constitutive expression under the control of pGAPZα vector can produce greater yields than inducive expression, although the yield of any type of heterologous protein constitutively expressed in *P. pastoris* depends on the degree of toxicity of the protein to yeast. The pGAPZα vectors do not include a yeast origin of replication. Transformants can only be isolated if recombination between the plasmid and the *Pichia* genome is achieved (Zhang *et al*., 2009).

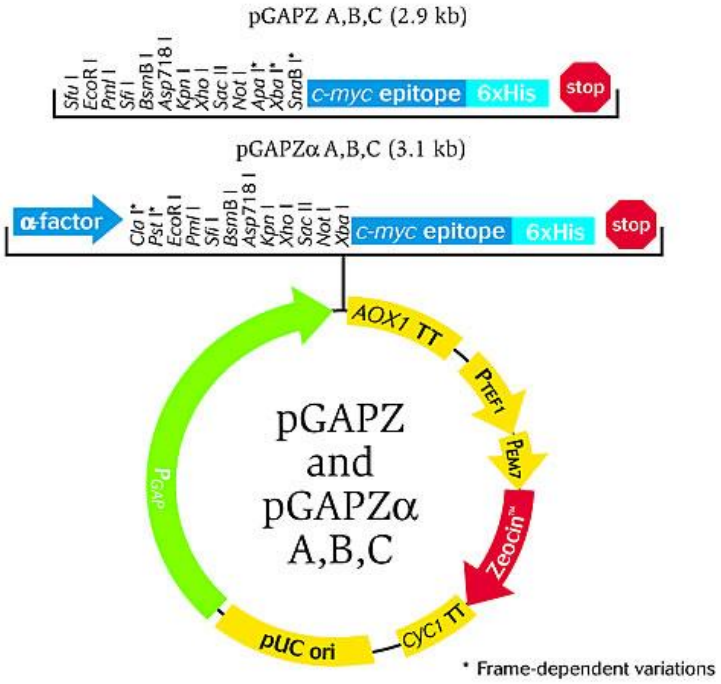

Figure 1.4. pGAPZ vector from Invitrogen allows for the constitutive expression of foreign proteins in host organisms and therefore eliminates the requirement for methanol induction for expression (Invitrogen life technologies 2002).

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme is constitutively expressed at high levels in many microbial host systems, including *P. pastoris*. The promoter of the gene (GAP) encoding the GAPDH protein has been extensively studied and displayed the ability to express recombinant proteins at high levels in *P. pastoris*, the expression levels depend also on the carbon source utilized. The level of expression with the GAP promoter (pGAP) is marginally higher than that attained with use of the AOX1 promoter (Waterham *et al*., 1997; Ilgen, 2005; Ahmad *et al*., 2014).

The GAP promoter derived expression structure has been developed into an essential expression system for the constitutive expression of foreign proteins in *P. pastoris*. In this system, the

heterologous genes will be produced simultaneously with cell growth provided that the target protein is not toxic to the cell. It does not require a washing step in order to remove non-methanolic carbon sources, and there is no requirement for accurate optimization of the culture conditions as in methanol induction phase, and the general hazard and costs related to the storage of large volumes of methanol are eradicated. The pGAP-based vectors permits the continuous production of the heterologous protein thereby avoiding the fed-batch fermentation system which uses methanol as an inducer. The pGAP expression system is therefore an asset and could add tremendous value to the development of economical methods for large-scale production of foreign recombinant proteins or the macromolecular assembly of protein complexes. A further advantageous trait is the ability to easily design expression vectors that contain multiple copies of the foreign gene insert, with an improved ability to produce higher yields of the required recombinant protein(s) in soluble form, correctly folded and suitably post-translationally modified (Zhang *et al*., 2009).

1.13. The pBGP1 vector for heterologous protein expression

A limitation to employing *P. pastoris* as an expression host to screen mutant libraries is that most *P. pastoris* expression vectors are designed for integration into the host chromosome. These vectors do not have an autonomous replication sequence and therefore lack the ability to exist as episomal (i.e., replicative) plasmids. To overcome this limitation, the vectors must be linearized, thereafter transformed into *P. pastoris*, and then incorporated into the yeast

chromosome. This particular characteristic of such vectors makes screening of the libraries tedious (Lee *et al*., 2005).

Firstly, linearized DNA does not transform as efficiently as compared to circular DNA, resulting in a fewer number of clones from the library that are available for screening. Additionally, when the clone is inserted into the host chromosome, it is a greater challenge to extract it for subsequent analysis and genetic manipulations. On the contrary, an episome could be isolated by a simple plasmid preparation procedure (Lueking *et al*., 2000; Lee *et al*., 2005).

Lee *et al*. (2005) reported the construction of a new *P. pastoris* expression vector for the screening of mutant gene libraries. The plasmid contains both bacterial and *P. pastoris* autonomous replication sequences that permit the vector to exist as an episome. A zeocin-resistance gene permits selection of the plasmid in *P. pastoris*.

The pBGP1 plasmid (Fig. 1.5) was designed with several beneficial features to serve as an episomal expression plasmid. The *P. pastoris* autonomous replicating sequence (PARS1) allows for the retention of the plasmid without the requirement of chromosomal integration in the yeast. The bacterial colE1 origin of DNA replication was also incorporated to permit the retention of the plasmid in *E. coli*. A signal sequence derived from the S. *cerevisiae* α-factor gene was included upstream of the multiple cloning site (MCS) (Lee *et al*., 2005). Genes that are cloned inframe to this sequence will code for enzymes that are secreted extracellularly into the culture medium. Cahill and colleagues also developed a series of *P. pastoris* episomal expression vectors (Lueking *et al*., 2000). However, their expression vectors did not contain a signal sequence.

Therefore a lysis procedure was required to release the enzymes prior to investigation enzyme activities using assay procedures (Lee *et al*., 2005).

Expression of the genes/genetic material cloned into pBGP1 is carried out by the GAP promoter that expresses the gene of interest constitutively. This feature differs in comparison to the inducible promoters employed by Cahill and colleagues to design their episomal expression vectors. They used the AOX (methanol inducible) and the CUP (copper inducible) promoters (Lueking *et al*., 2000).

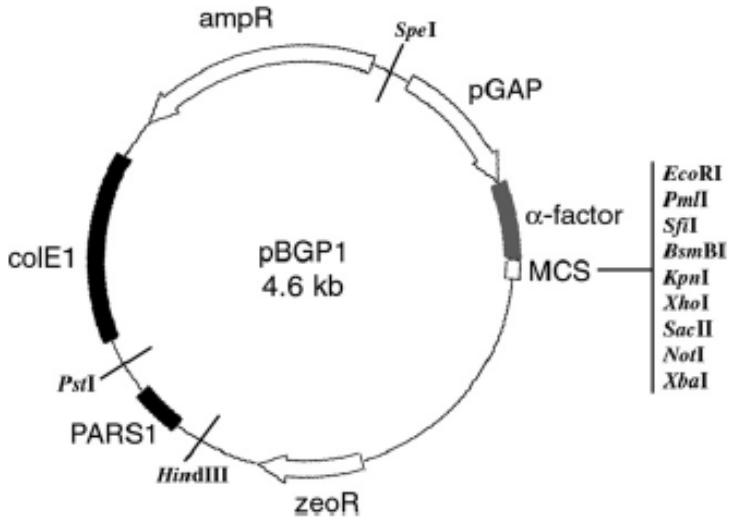

Figure 1.5. pBGP1 expression vector containing pGAP promoter; a-factor, secretion signal peptide; MCS, multiple cloning site derived from pGAPZaA plasmid; zeoR, zeocin-resistance gene; PARS1, *P. pastoris* autonomous replication sequence; colE1, bacterial replication sequence; and ampR, ampicillin-resistance gene (Lee *et al*., 2005).

In a study carried out by Lee *et al*., 2005, the pBGP1 vector was successfully used to screen a xylanase mutant gene library. In previous studies the xylanase was not active in *E. coli* or *S. cerevisiae*. Another example of xylanase production in *P. pastoris* was the expression of a

modified xylanase gene, NC38. The protein was expressed at much higher levels than in *E. coli* (Mchunu *et al*., 2009). Advantages of using the pBGP1 vector for protein expression is that the plasmid is small, it enables flexible operation; contains a zeocin resistance gene, thereby offering simplified selection procedures; by incorporation of zeocin into the media. It also allows for constitutive expression therefore does not require methanol induction. The disadvantage is that zeocin, a strong mutagen, may induce target protein mutation (Invitrogen Life Technologies protocol 2002).

1.14. *P. pastoris* for the production of heterologous proteins

The bioprocess chosen for the commercial synthesis and purification of a recombinant or heterologous protein is decided by a number of factors, such as the intrinsic biological characteristics of the protein being produced and the function for which it is required as well as the economic and marketing goals (Eckart and Bussineaut, 1996). Other criteria that need to be determined in choosing a bioprocess are product yield expectancy and process efficiency, which have a direct affect the cost of goods, the how the chosen process will impact on the quality and authenticity of the foreign protein being expressed which, for controlled biologicals, undergo rigorous characterization to ascertain biochemical equivalence to the original catalyst. Choice of host strain is usually made well in advance since it may considerably affect the downstream processing stages, the types of procedures used for testing the authenticity of the heterologous protein and regulatory filing timelines (Eckart and Bussineaut, 1996).

P. pastoris is distinctly different to *S. cerevisiae* in that it is poor fermenter, which is a major benefit in culturing this organism. In high cell density cultures, the product formed, viz; ethanol, in accumulated to toxic levels in fermentation of *S. cerevisiae*. This factor proves to be a limitation in the further fermentation and growth of the yeast as well as the directly affects heterologous protein production. *P. pastoris* prefers respiratory growth and therefore can be grown to extremely high densities (500 OD_{600} U/ml) under regulated conditions (Cereghino and Cregg, 2000; Gellissen *et al*., 2005). Fermentative culture is particularly crucial for proteins that are secreted extracellularly, since the concentration of product in the present in the culture medium is comparatively proportional to the concentration of cells (Wu *et al*., 2003; Hang *et al*., 2009). Another advantageous aspect of culturing *P. pastoris* in a fermenter system is that, the level of transcription initiated via the respective promoter can be three to five times higher in cells that are induced with methanol or glycerol in comparison with cells grown in an excess concentration of methanol or glycerol as the chosen carbon source (Cereghino and Cregg, 2000; Ruanglek *et al*., 2007; Xuan *et al*., 2009). Therefore, even in case of intracellularly expressed proteins, product yields are considerably greater from fermenter cultured organisms. In addition, carbon source (glycerol or methanol) metabolism tends to consume oxygen at a high rate, and thus production of foreign genes is adversely affected by oxygen limitation (Cereghino and Cregg, 2000).

A dominant characteristic of *P. pastoris* as a heterologous protein production system is the ease of scale-up from shake-flask to culturing them using fermenter systems (Cregg *et al*., 2009). Although some heterologous proteins have been efficiently expressed well in shake-flask culture experiments, expression levels are relatively low in comparison with fermenter cultures (Birijlall

et al., 2011). A considerable amount of research has been directed towards the optimization of foreign protein production technologies, and detailed fed-batch and continuous culture protocols have been made readily available (Boettner *et al*., 2002). The culture conditions for *P. pastoris* are well-suited for large scale and industrial production of foreign proteins, since the required medium components are relatively inexpensive and defined, are composed of pure carbon sources such as glycerol and methanol, biotin, salts, trace elements, and water. This medium is devoid of ingredients that can be potential sources of pyrogens or toxins and is thus rendered compatible with the manufacture of human pharmaceuticals (Cereghino and Cregg, 2000; Cregg *et al*., 2009).

Also, *P. pastoris* cultures can be attained using a medium with a relatively low pH level and in some cases with the addition of methanol. The *P. pastoris* expression system proposes an economical method of production, ease of genetic manipulation, the inherent ability to accomplish complex post-translational modifications and high levels of protein expression. Therefore, it is an appropriate microbial host for the recombinant synthesis of a large number of proteins that are encoded by diverse organisms (Cereghino and Cregg, 2000).

1.15. Scope of the study

E.coli has been considered the vector of choice for the production of foreign proteins for many years, but there are various limitations related to the use of this organism as a host for heterologous protein expression as mentioned earlier. In comparison, *P. pastoris* offers several advantages as an expression host and is thus the organism of choice for xylanase production in

this study. Optimization of growth conditions is further required for better expression of the recombinant xylanases by *P. pastoris*.

The focus of the present study is to transform the xylanase variants, G53, G41, S325 and S340 (Stephens *et al*., 2014) into *P. pastoris* using the pBGP1 vector for the constitutive expression of xylanase. The second step is to express these genes and investigate the parameters that allow for the optimum production of the heterologous proteins. This research was carried out using different media containing various carbon sources (Xie *et al*., 2005), incorporated with and without the antibiotic, zeocin and variation in pH and temperature conditions. It is imperative that the parameters that allow for optimum growth of *P. pastoris* and for the expression of heterologous proteins, in this case recombinant xylanases be determined in order to actively assist in improving, aiding or restructuring the methods of protein/enzyme expression in this organism by fermentation. Thereby improving the rate at which industrial production proceeds and in the process reduce costs of downstream processing and enhance the economic benefits of these applications.

In the paper and pulp industry, the bleaching process, in particular, is carried out at high temperatures, thus requiring enzymes that can remain active under these conditions. Currently, this process involves the use of toxic chemicals for the bleaching of pulp and in the interim produces harmful gases that have a negative impact on the environment. The application of these recombinant xylanases will help to reduce environmental pollution. The aim of this research project was to clone and express modified xylanases in *Pichia pastoris* and to characterize their enzymatic abilities at high temperatures and pH for the potential application on pulp.

CHAPTER 2. CLONING OF XYLANASE VARIANTS

2.1. Introduction

Gene manipulation is now considered a core technology used for a plethora of research and industrial applications. In addition to representing an extremely powerful analytical tool, it has been used to:

(i) increase the yield and quality of existing products such as proteins, metabolites or even whole cells;
(ii) improve the natural characteristics of compounds e.g., protein engineering;
(iii) produce naturally-occurring products by new routes e.g., pathway engineering; and
(iv) develop novel products not previously found in nature (Adrio and Demain, 2014).

The ability to manipulate and analyse DNA using genetic engineering techniques (Recombinant DNA Technology) was first discovered in the 1960s and was implemented in the 1970s. The technology, which has developed rapidly, evolved from a series of basic studies in the interrelated disciplines of biochemistry and microbial genetics. The advent of recombinant DNA technologies and the development of molecular methodologies has led to the possibility of analysing DNA to a resolution that was unimaginable only a few decades ago and as a consequence, the genomes of almost any organism (prokaryote or eukaryote) could be genetically modified to initiate and direct the synthesis of biological products that were usually only produced by other organisms. Genetic recombination has not only facilitated the production of certain products (proteins) at a quantity and quality that was not previously achievable, but also opened up the possibility of developing entirely new or highly-modified bioactive products.

The first objective of this study was to isolate the genes from the host organism, *E. coli* XL1-Blue and transfer these genes onto a yeast vector for expression in *P. pastoris*. This was done in order to avoid or by-pass the downstream processing that would normally be required when proceeding to obtain any protein from *E. coli*, such as purification. Since the pBGP1 plasmid

contains a constitutive promoter and signal sequence, the xylanase enzymes will be expressed extracellularly using a simple, cheap expression system.

2.2. Materials and Methods

2.2.1. Plasmids, bacterial and yeast strains

The plasmids used for this study include the plasmid Bluescript SK (pBSK) (Stratagene) which contained the mutated *xyn*A genes i.e. S325, S340, G41 and G53. pBGP1 (Lee *et al*., 2005) is a yeast vector with a constitutive GAP promoter thus was chosen to be used to transform the *P. pastoris* with the xylanase variants. *E. coli* XL1 Blue (Stratagene) was maintained on Luria Bertani media (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 12 g/l bacteriological agar). *P. pastoris* GS115 (Invitrogen) was maintained on yeast peptone dextrose media (10 g/l yeast extract, 20 g/l peptone, 20 g/l D-glucose and 12 g/l bacteriological agar). Selection and short term maintenance of *E.coli* was done on LB media containing 100 µg/ml of ampicillin. *P. pastoris* clones were selected in media containing 150 µg/ml of zeocin (Invitrogen) and stored at 4°C in a refrigerator. For long-term maintenance, cultures were stored in 30% glycerol and frozen at -70°C in a biofreezer. All plasmids were isolated and stored at -20°C for further use.

2.2.2. Plasmid isolation

Plasmid isolation from *E. coli* was done using the GeneJET Plasmid Miniprep Kit (Fermentas) according to the instruction manual. *E. coli* cultures containing plasmids were grown overnight in 5 ml LB broth containing 100 µg/ml of ampicillin at 37°C. The cultures were then centrifuged and the pellet resuspended in 250 µl ice cold lysis solution, followed by the addition of 350 µl neutralizing buffer with gentle mixing. The mixture was then incubated at room temperature for

2 min. This was followed by centrifugation in a microcentrifuge for 5 min at 13 200 × *g* and the supernatant was transferred into spin columns. The spin columns were centrifuged for 1 min followed by two washing steps with 70% ethanol. The plasmid DNA was eluted using 30 µl of elution buffer by incubation at room temperature for 2 min. Five microlitres of the plasmid suspension was used to verify the presence of the plasmid DNA using agarose gel electrophoresis. The remaining plasmid solution was stored at -20°C.

2.2.3. Agarose gel electrophoresis

A 0.8% agarose gel was used with 1 × TAE as the buffer (242 g Tris-HCl, 57.1 ml acetic acid, 100 ml of 0.5 M EDTA, pH 8) at a constant voltage of 90 V. DNA samples were mixed with 2-3 volumes of sample buffer (0.0375 g bromophenol blue, 4 g sucrose, 1.5 ml 10% SDS, 3 ml of 0.5 M EDTA dissolved in 15 ml distilled water) before loading onto the gel. Phage λ DNA (Roche Diagnostics) was restricted with *Eco*RI and *Hin*dIII (Fermentas) to produce DNA fragments of known molecular weights to serve as the molecular weight marker for all agarose gels used in this study. Gels were stained with ethidium bromide (50 µg/ml) for 20 minutes, destained with distilled water and viewed on a UV transilluminator. Gel images were captured using the Gel Doc XR+ System (Bio-Rad).

2.2.4. DNA quantification

The concentration and purity of DNA was estimated by measuring the absorbance at both 260 nm and 280 nm. Ideally, pure DNA should have a 260/280 ratio of greater than 1.8. DNA

concentration was calculated on the premise that an absorbance of 1 at 260 nm corresponds to 50 ng DNA/µl.

2.2.5. PCR

PCR was done to isolate the xylanase genes from the recombinant plasmids they were cloned in. The composition of each PCR tube was as follows: 27 µl sterile deionized water, 2 µl DNA (10 ng), 5 µl forward primer, 5 µl reverse primer (Inqaba Biotech), 5 µl dNTPs, 5 µl 10× buffer and 1 µl *Pfu* DNA polymerase enzyme (Fermentas).

Primer sequence:

Table 2.1. PCR cycling conditions for DNA amplification

Steps/Cycle	Temperature	Time
Denaturation	94°C	60 seconds
Primer annealing	58°C	60 seconds
Extension	72°C	60 seconds

PCR reactions were carried out for 30 cycles (Table 2.1.) 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. The PCR products were stored at -20°C in a biofreezer and used for subsequent experiments.

2.2.6. DNA purification

PCR products were purified using the DNA Clean and Concentrator Kit (Zymo Research) according to the manufacturer's instructions. Two volumes of DNA binding buffer was added to each volume of double-stranded DNA. The mixture was loaded into a Zymo-spin column and placed in a collection tube. This was followed by centrifugation for 30 s at 10 000 × *g* and the flow through was discarded. 200 µl of wash buffer was added to the column and centrifuged for 30 s. The wash step was repeated, after which the column was placed in a sterile Eppendorf tube and subsequently 6-10 µl of sterile deionized water was added to the column followed by centrifugation to elute the purified DNA. The DNA was stored at -20°C, until required.

2.2.7. Restriction and ligation of vectors and inserts

Restriction of DNA at particular nucleotide sequences was accomplished by restriction endonucleases that recognize and cleave double-stranded DNA at specific sequences. If the vector is linearized with the same endonuclease as the insert, the sticky ends of the insert complements the sticky ends of the vector, making ligation easier. Thus, both pBGP1 (which was used as a vector in this study) and the purified PCR products were restricted with type II endonucleases *viz*., *Eco*RI and *Xba*I (Fermentas), to create compatible sticky ends for ligation(Fig

2.1.). Restriction digestion was carried out using type II restriction endonucleases. For a standard restriction, a digestion procedure comprising of: DNA (approximately 100-300 ng), 1 µl *Eco*RI (10 U), 1 µl *Xba*I (10 U) and 2 µl Tango 10× buffer (Fermentas) was carried out in a total volume of 20 µl, containing sterile deionized water. Mixtures were incubated with the restriction enzymes at 37°C overnight. The recovery of insert DNA was accomplished using the DNA Clean and Concentrate Kit (Zymo Research). After quantification of the digested and purified target gene insert and plasmid vector, the insert was ligated to the vector. The vector and PCR products were ligated at a molar ratio of 1:3 vector to insert. Ligase mixtures containing the vector and insert DNA were heated to 65°C for 5 minutes. After rapid chilling on ice, 1× ligase buffer and 1 U of T4 DNA ligase (Roche Diagnostics) was added, to a final volume of 10 µl and incubated overnight at room temperature. The resulting ligation products were then ready for transfer into a host bacterial cell by transformation (Sambrook, 1989; Sambrook and Russel, 2001).

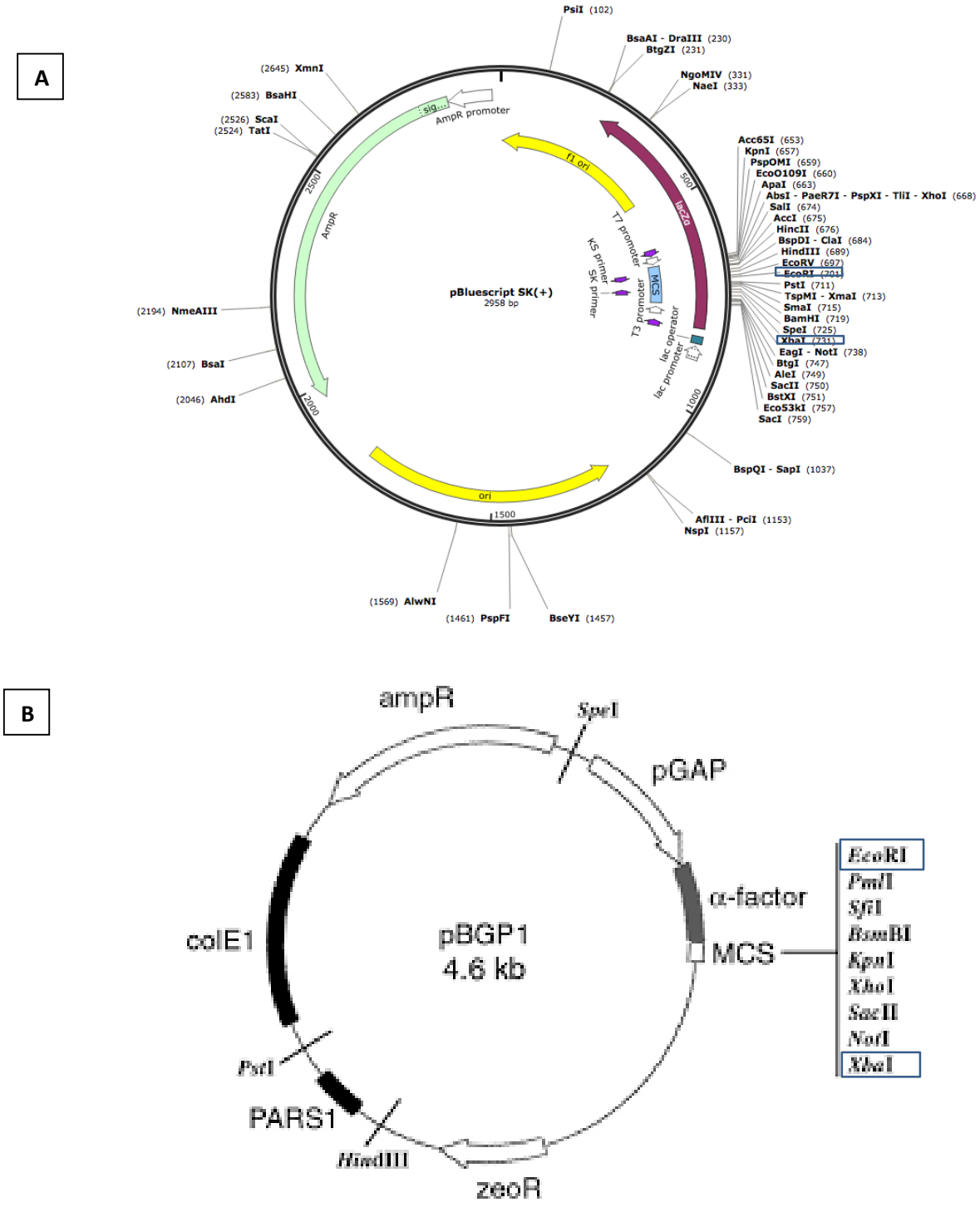

Figure 2.1. Structure of plasmids displaying the restriction sites for enzymes *Xba*I and *Eco*RI. A: Plasmid Bluescript (pBSK), B: Plasmid pBGP1

2.2.8. Preparation of *E. coli* and *P. pastoris* competent cells

Host cells were rendered artificially "competent" by exposing early log-phase cells to bivalent cations. In this study, *E. coli* XL1 Blue was used for cloning. One millilitre of an overnight culture of *E. coli* XL1 Blue was diluted to 30 ml with LB broth. The culture was incubated at 37°C until an optical density of 0.375 at 590 nm was reached. The cells were pelleted at 5000 × *g* for 10 min and the supernatant was discarded. The cells were resuspended in 10 ml ice cold 100 mM $CaCl_2$, recentrifuged and resuspended again in 10 ml cold 100 mM $CaCl_2$. Cells were incubated on ice for 20 minutes and then centrifuged. Competent cells were then resuspended in 2 ml $CaCl_2$ (100 mM), containing 10% glycerol (Ausubel *et al*., 2003). One hundred microlitres of the prepared cells were dispensed into eppendorf tubes and stored at -70°C. *P. pastoris* host cells were grown overnight on YPD plates at 30°C. A single colony was inoculated into 200 ml of YPD broth and the cells were further incubated overnight at 30°C to an OD_{600} of approximately 1.4. The cells were harvested by centrifugation at 4000 × *g* for 15 min. Cells were resuspended in 50 ml of ice-cold sterile distilled water per 50 ml of culture followed by centrifugation at 4000 × *g* for 15 min, the cells were then washed in 25 ml of ice-cold sterile distilled water. This step was followed by washing the cells with 50 ml and 25 ml of 1 M ice-cold sorbitol and centrifuged at 4000 × *g* for 15 min. The cells were finally resuspended in 400 µl of 1 M sorbitol and stored in aliquots of 80 µl at -70°C (Eppendorf protocols online 2001).

2.2.9. Transformation and screening in *E .coli* and *P. pastoris*

Transformation of the ligation mixture in *E. coli* was accomplished by adding 1 µl of the ligation reaction mixture to 50 µl of competent cells on ice, followed by gentle mixing. The mixture was incubated on ice for 20 minutes. The cells were then heat-shocked for two minutes at 42°C and returned immediately to ice and incubated for 5 minutes. One milliliter of LB broth was added to the eppendorf tubes and incubated for an hour at 37°C at 180 rpm. Thereafter, the transformation mixture was plated onto LB plates containing 100 µg/ml ampicillin and incubated at 37°C overnight (Sambrook and Russel, 2001).

Electroporation of *P. pastoris* was performed using a Gene Pulser Xcell (Bio-Rad) and the following pulsing conditions were used; 1.7 kV, 200 Ω and 25 µF. 80 µl of yeast competent cells were mixed with 20 µl of the ligation mixtures for transformation in a sterile eppendorf tube. The electrocompetent cells were transferred into 0.2 cm electroporation cuvettes (Bio-Rad) that had been kept on ice and then the pulse was delivered. Ice-cold 1 M sorbitol was added immediately after electroporation and the cells were incubated on ice for 15 min. One millilitre of YPD was added followed by further incubation for 1 h at 30°C with shaking at 200 rpm. Aliquots of 100 µl were plated onto YPD selective plates containing 0.4% RBB-xylan supplemented with 100 µg/ml of zeocin. These were incubated at 30°C for 3-4 days. Positive yeast clones containing the xylanase gene were able to degrade the surrounding xylan and transformants that produced a zone of hydrolysis on the RBB-xylan (Sigma-Aldrich) plates were selected for further use (Biely *et al*., 1988). RBB-xylan is a soluble chromogenic substrate for the assay of endo-1, 4-ß-xylanases.

This substrate was prepared by conjugating 4-O-methyl-D-glucurono-D-xylan with Remazol Brilliant Blue dye (dye content approx. 15%) (Biely *et al*., 1988).

2.2.10. Xylanase expression and extraction

The selected yeast clones displaying xylanase activity were used to inoculate 20 ml of YPD broth containing zeocin and LB broth containing ampicillin for selected *E. coli* transformants. These cultures were used to inoculate 180 ml liquid media for xylanase production. Expression in *P. pastoris* was terminated after 72 h. The culture was centrifuged and the supernatant stored at 4°C for further enzyme characterization.

2.2.11. SDS-PAGE and Zymogram analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was demonstrated using a 5% (w/v) stacking and 12% (w/v) separating polyacrylamide gels in the presence of 0.1% (w/v) SDS (Harlow and Lane, 1988) and run on a Bio-Rad Mini Protean II Dual Slab apparatus. Zymogram analysis was carried out using 5% (w/v) stacking and 12% (w/v) separating polyacrylamide gel containing 1% (w/v) Birchwood xylan. Samples were run on SDS-PAGE gels and zymograms. After electrophoresis, the SDS-PAGE gel was placed in a staining solution (2.5 g/l Coomassie Brilliant Blue R-250, 30% of methanol, 10% acetic acid and 60% distilled water) overnight (Laemmli, 1970). This was followed by destaining in a solution of 30% of methanol, 10%

acetic acid and 60% distilled water for 4-6 hours until bands were clearly visible. Following electrophoresis, the zymogram was washed using a 1% solution of Triton X100 in 0.2 M buffer (sodium citrate, pH 5 for S340; sodium phosphate, pH 6 for S325 and G41; sodium phosphate, pH 7 for G53 and glycine-NaOH,pH 9 for NC38) to remove the SDS, for 1 hour with shaking at room temperature. This was followed by washing twice in buffer for 1 hour. The gels were then submerged in buffer and placed at 50°C for 3 hours. After incubation the gels were stained in a solution of 0.1% Congo red (Sigma Aldrich) for 10-15 min. Destaining was performed in 1 M NaCl for 15 min to remove unbound dye. The stain was set using 0.05% acetic acid. Xylanase bands appeared white against a dark background.

2.3. Results and Discussion

2.3.1. PCR of xylanase variants

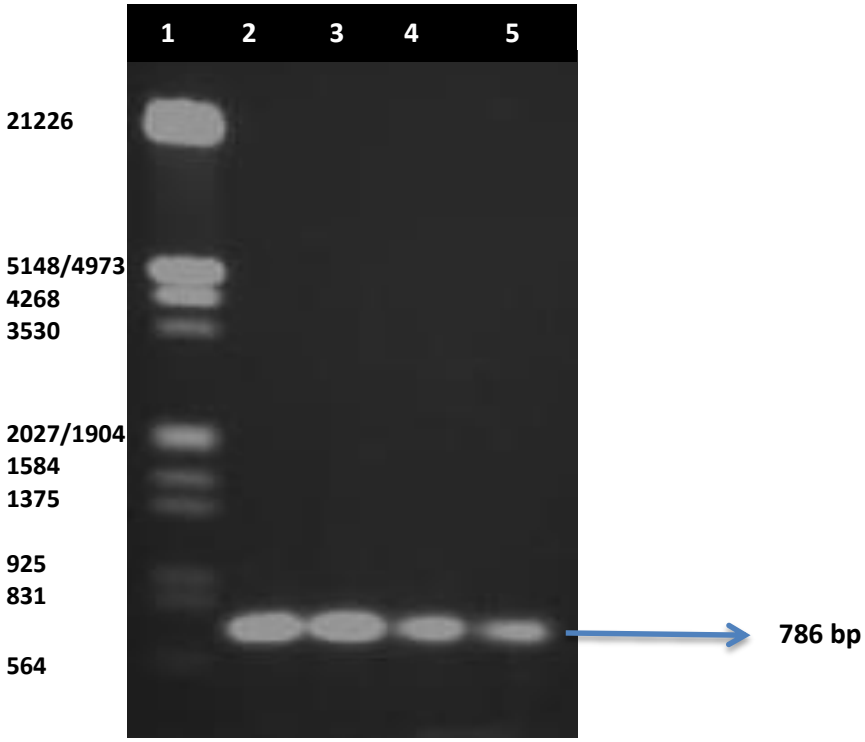

Figure 2.2. PCR of xylanase genes from *E. coli* plasmid vectors.
Lane 1: DNA molecular weight marker; Lane 2: S325; Lane 3: S340; Lane 4: G41; Lane 5: G53

PCR was carried out as described in section 2.2.5. From the research conducted by Stephens *et al*. (2007) the modified xylanases viz.; S325, S340, G41 and G53 were 786 bp in size when cloned into pBluescript. Following PCR, 5 µl of each of the PCR reactions for the respective modified xylanases were run on an agarose gel. The gel was stained in ethidium bromide and visualized using the Gel Doc XR+ System (Bio-Rad). The four genes were amplified. Single bands were clearly visible (Fig. 2.2). Sufficient quantity was amplified for further manipulation and the sizes were

corresponding to 786 bp confirming that the xylanases were the correct size and could be used for subsequent research.

2.3.2. Restricted and purified ligation reactants

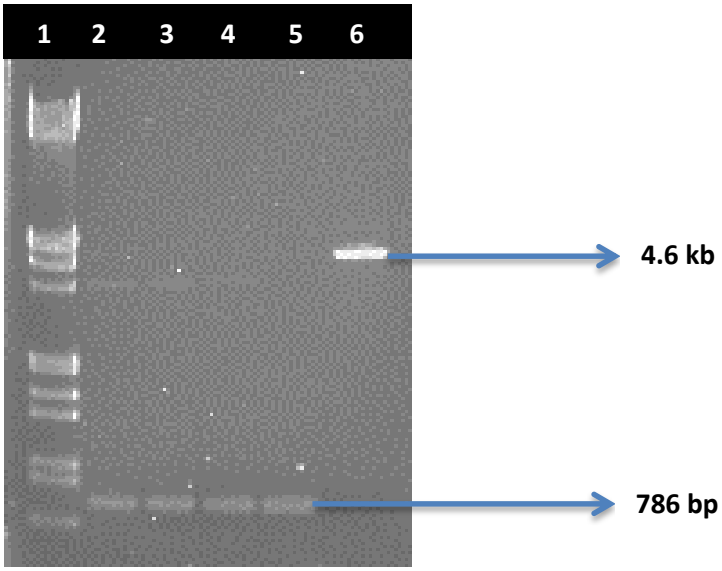

Figure 2.3. Restricted and purified ligation reactants. Lane 1: DNA molecular weight marker; Lane 2: S325; Lane 3: S340; Lane 4: G41; Lane 5: G53; Lane 6: Restricted pBGP1 plasmid

The PCR products for each of the modified xylanases were purified using the Clean and Concentrate Kit (Zymo Research). Following purification, the xylanase genes and the pBGP1 plasmid were linearized with restriction enzymes *EcoR*I and *Xba*I in a total volume of 20 µl at 37°C, for 16 h. Both the ligation reactants were run on an agarose gel to confirm the size of the fragments prior to ligation (Fig 2.3). The xylanase genes presented at 786 bp on the agarose gel. The vector presented at 4.6 kb when linearized. These reactants were ligated overnight at room temperature. The ligation reaction was stored at -20°C until required for transformation.

2.3.3. Screening in *E. coli*

The ligation mixture was used to transform *E. coli* XL1-blue cells as described in section 2.2.9. Following incubation with shaking at 37°C for 1 h, 100 µl of the transformation mixture was plated in duplicate onto LB-amp plates and incubated overnight at 37°C, the negative control containing plasmid only and positive control being the *E. coli* culture . Numerous colonies (Fig 2.4) were present on each of the respective agar plates. Positive transformants were identified as relatively large colonies indicating that they were well established and resistant to the antibiotic i.e. Ampicillin. Five colonies of each of the xylanase transformants were randomly selected and transferred to 5 ml of Luria Bertani broth containing 1 µg/ml Ampicillin. The suspensions were cultured overnight at 37°C, 180 rpm.

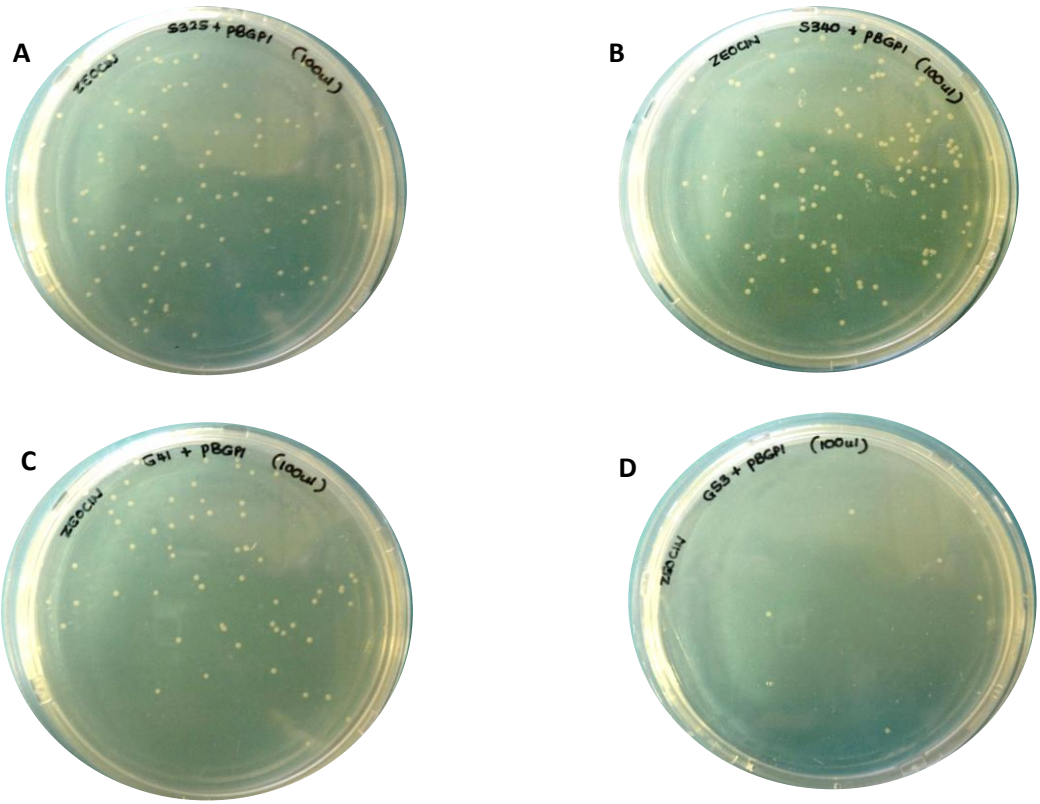

Figure 2.4. Positive *E. coli* transformants. A: S325, B: S340, C: G41, D: G53

2.3.4. Plasmids isolated from positive clones

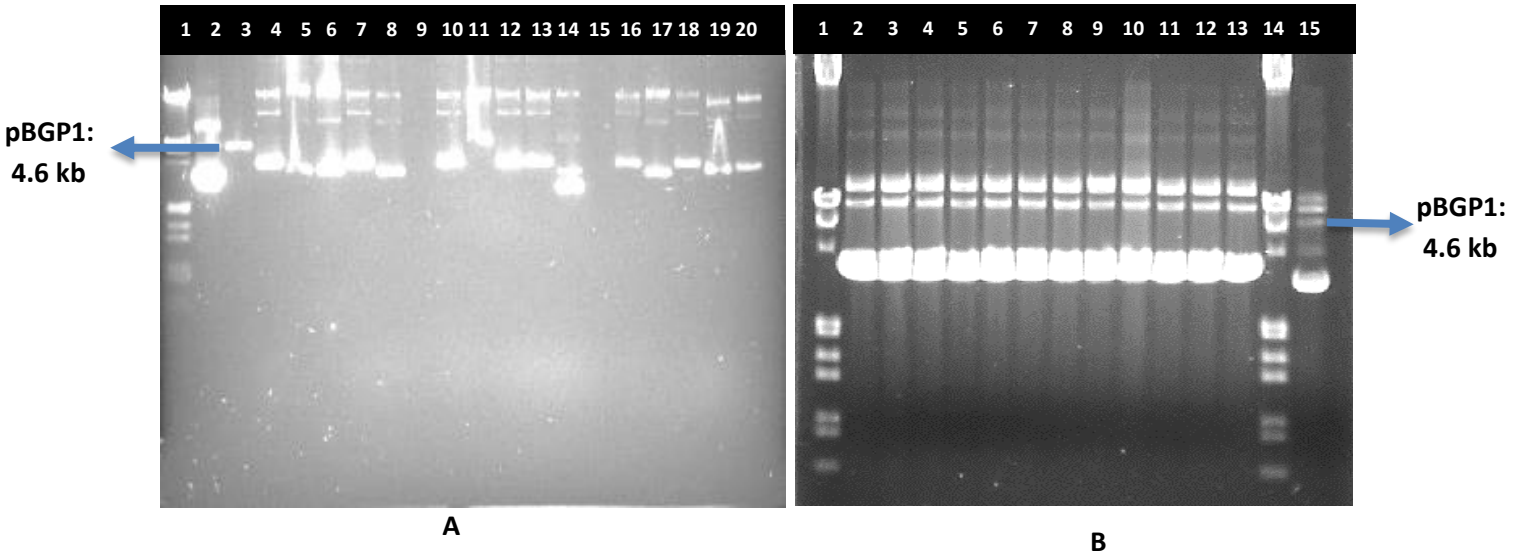

Figure 2.5. Plasmid DNA isolated from *E. coli* transformants.

A: Lane 1: DNA molecular marker; lane 2: pBGP1 (restricted); lane 3: pBGP1 (restricted); lane 4-8: S325 transformants; lane 10-14: G41 transformants; lane 16-20: G53 transformants

B: Lane 1: DNA molecular marker; lane 2-13: S340 transformants; lane 14: molecular marker lane 15: pBGP1 plasmid.

Each of the five transformants selected were cultured and thereafter the plasmids were isolated as described in section 2.2.2. 5 µl of each of the plasmid isolates in elution buffer was run on an agarose gel (Fig 2.5). Gel A, shown above indicates that the plasmid pBGP1 restricted with *EcoR*I to yield a linear fragment presents at 4.6 kb. In lanes 4, 6 and 7 which contain plasmids of the xylanase S325 on pBGP1, the linear form of the plasmid appears larger than 4.6 kb. As is the same with the plasmid isolates of S340 (Lanes 2-13 of gel B), G41 (Lanes 10, 12 and 13 of gel A) and G53 (Lanes 16, 18 and 20 of gel A). From the results on the agarose gel it could be ascertained that the transformants contained a plasmid (4.6 kb) that was now ligated to the inserts (786 bp)

with the total size being approximately 5386 bp. This was confirmed by the size of the isolated plasmids being greater than that of the plasmid itself.

2.3.5. Restricted plasmids of positive transformants

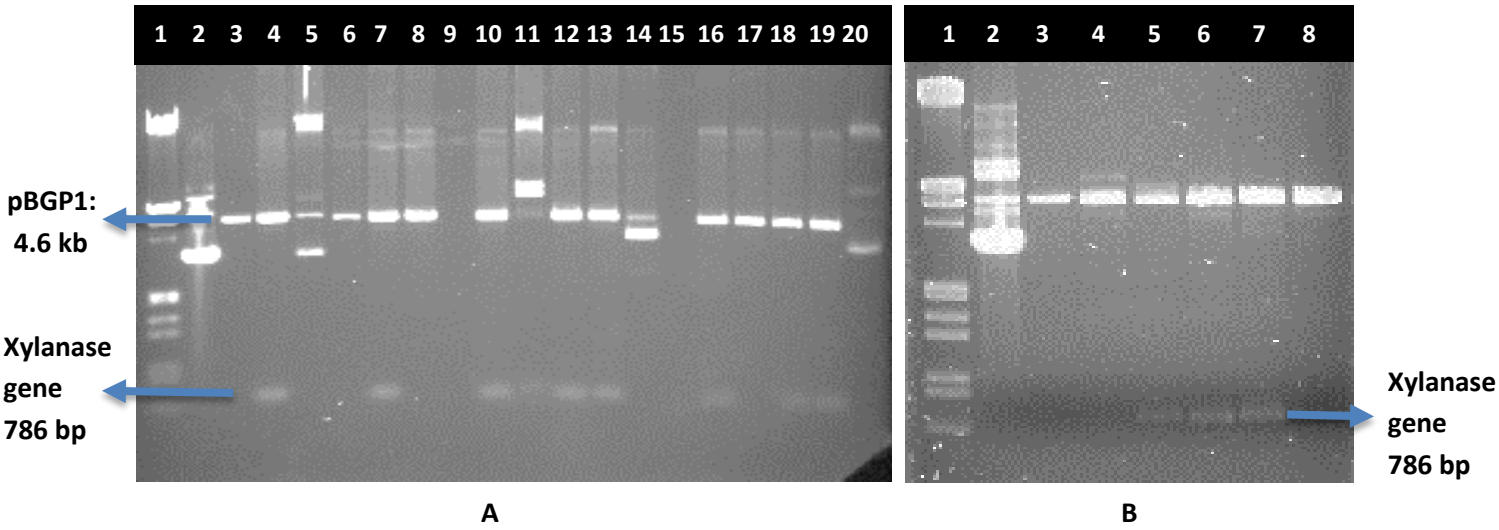

Figure 2.6. Restricted plasmid DNA from positive E.coli transformants.

A: Lane 1: DNA molecular marker; lane 2: pBGP1 (unrestricted); lane 3: pBGP1 (restricted); lane 4-8: S325 transformants; lane 10-14: G41 transformants; lane 16-20: G53 transformants.
B: Lane 1: DNA molecular marker; Lane 2: pBGP1 (unrestricted); Lane 3: pBGP1 (restricted); lane 4-8: S340 transformants.

The plasmids described in 2.3.4. above were linearized using the restriction enzymes, *EcoR*I and *Xba*1. The restriction yielded two fragments, one corresponding to the size of 4.6 kb (pBGP1) and another corresponding to 786 bp (xylanase genes). Lanes 4 and 7 in gel A indicated positive S325-pBGP1 transformants. Lanes 10, 12 and 13 of gel A indicates positive G41-pBGP1 transformants. Lanes 15, 17 and 18 of gel A indicate positive G53-pBGP1 transformants. Lanes 5, 6 and 7 of gel B indicate positive S340-pBGP1 transformants.

In gel A, lanes 4, 7, 10, 12, 13, 16, 18 and 19 indicate positive transformants since after restriction with *Eco*RI and *Xba*I the plasmid and inserts are clearly identified. Lanes 6, 8 and 17 indicates the presence of the vector only. Lanes 5, 11, 14 and 20 indicate a negative result. In gel B, lanes 5, 6 and 7 are indicative of a positive result, with lanes 4 and 8 showing the vector only. These plasmids were then used to transform *P. pastoris* cells.

2.3.6. Positive *P. pastoris* transformants

The plasmids selected as positive xylanase variant clones were used to electro-transform *P. pastoris* GS115 cells made competent as described in section 2.2.8. The plasmids were introduced into the electro-competent yeast cells by electroporation (2.7 kV, 0.2 cm cuvette). The electroporated cells were mixed with 1 ml of YPD broth and incubated at 30°C, with shaking at 200 rpm for 2 hours. 50 µl of the transformation mixture was plated out onto RBB-xylan YPD-zeocin plates in duplicate. Zeocin was used as a selective antibiotic since the pBGP1 plasmid contains the zeocin resistance gene making identification and selection of positive transformants simple. These were incubated at 30°C for 3-4 days. Positive transformants presented zones of clearing around the colony indicating the metabolism of xylan present in the media, therefore these isolates were actively expressing the respective modified xylanase genes. The plates with the xylanase variants presented with numerous colonies producing zones of clearing that were clearly visible thus displaying utilization of the RBB-xylan medium (Fig 2.7).

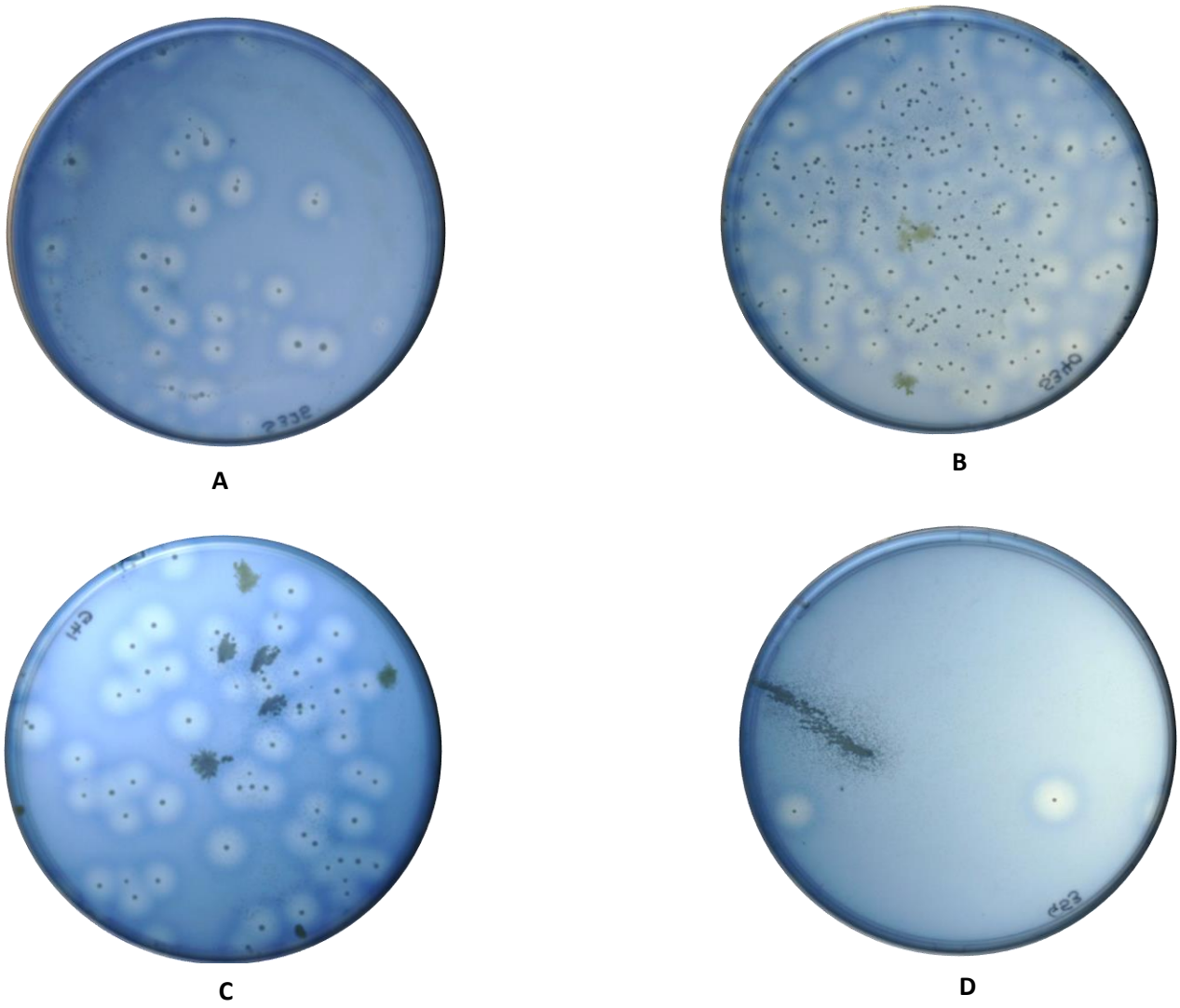

Figure 2.7. Positive *P. pastoris* transformants A: S325; B: S340; C: G41 and D: G53

2.3.7. Xylanase variants in pBGP1

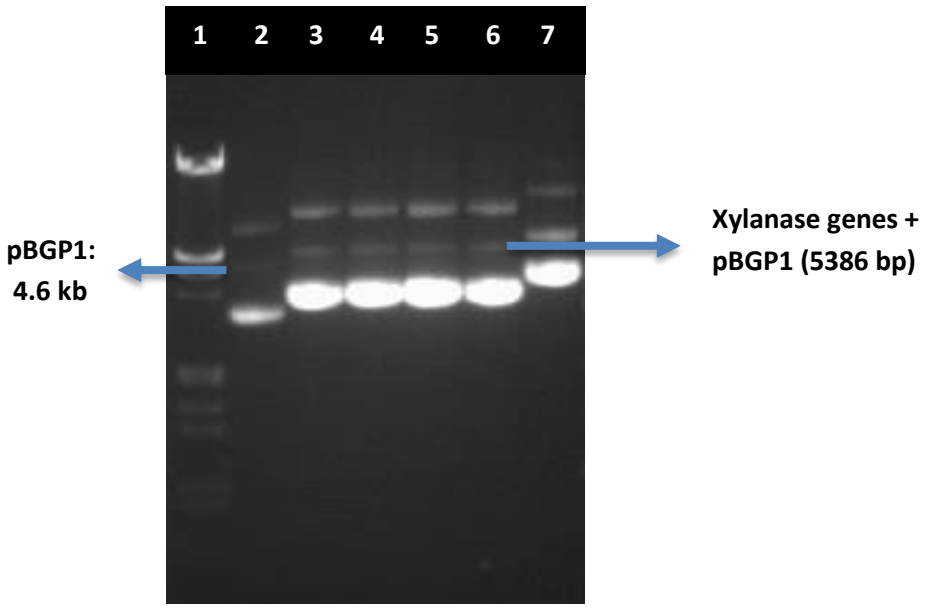

Figure 2.8. Xylanase variants on pBGP1 plasmid isolated from *P. pastoris* transformants.

Lane 1: DNA molecular marker, lane 2: pBGP1 vector (unrestricted), lane 3: S325 xylanase; lane 4:S340 xylanase; lane 5: G41 xylanase; lane 6: G53 xylanase variant; lane 7: NC38 on pBGP1.

In order to confirm the presence of the xylanase variants on the constitutive pBGP1 vector, the plasmids were isolated from *P. pastoris* cells cultured overnight at 30°C, with shaking at 200 rpm. The plasmids were then isolated as described in section 2.2.2. The plasmids were used to transform competent XL1-Blue *E. coli* cells. The plasmids were again isolated and run on the gel (Fig 2.8). The bands for the positive clones appeared at approximately 5.386 kb, whilst the NC38 xylanase (1003 bp) on pBGP1 (4.6 kb) appeared at 5.6 kb.

2.3.8. SDS-PAGE analysis of modified xylanases

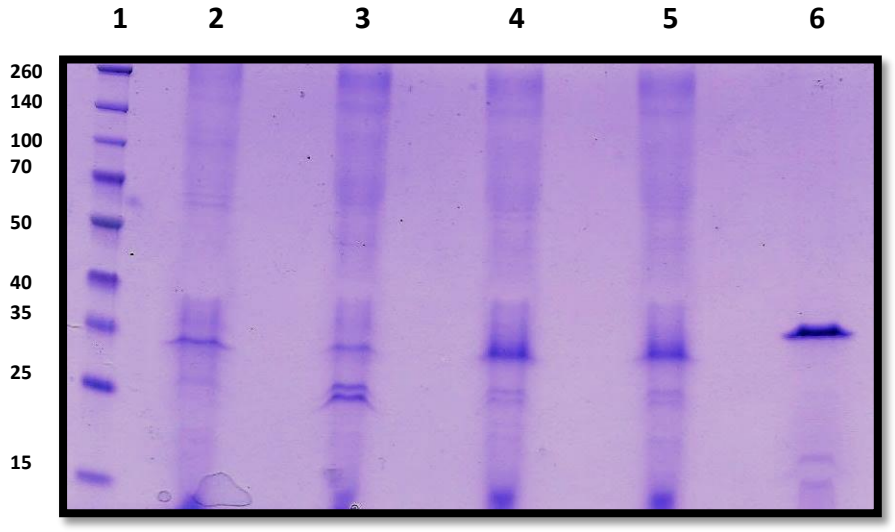

Figure 2.9. SDS-PAGE of modified xylanases. Lane 1: Protein marker (15-260 kDa); lane 2: S325 xylanase; lane 3: S340 xylanase; lane 4: G41 xylanase; lane 5: G53 xylanase, lane 6: NC38 xylanase.

SDS-PAGE analysis was carried out according to the method described by Laemlii, 1970 (Fig 2.9). The bands depicting the xylanase variants in lanes 2-5 appeared at 32 kDa. NC38 was used in this experiment as a control protein. When the xylanase genes were modified from the parent *xyn*A gene from *T. lanuginosus* DSM 5826, the flanking ends (remaining *T. lanuginosus* sequences) were restricted and removed before cloning onto the Bluescript vector (Stephens *et al*., 2007). There were three bands displayed in lanes 2, 3, 4 and 5 depicting S325, S340, G41 and G53 respectively with molecular weights of 26, 28 and 32 kDa. Some xylanases from microorganisms have been reported to be glycoproteins (Deng *et al*., 2006) and the different bands appearing on the electrophoresis gel are not likely due to a difference in the degree of glycosylation of the polypeptide chains (Deng *et al*., 2006) which is prevalent in yeast hosts.

2.3.9. Zymogram analysis of the modified xylanases

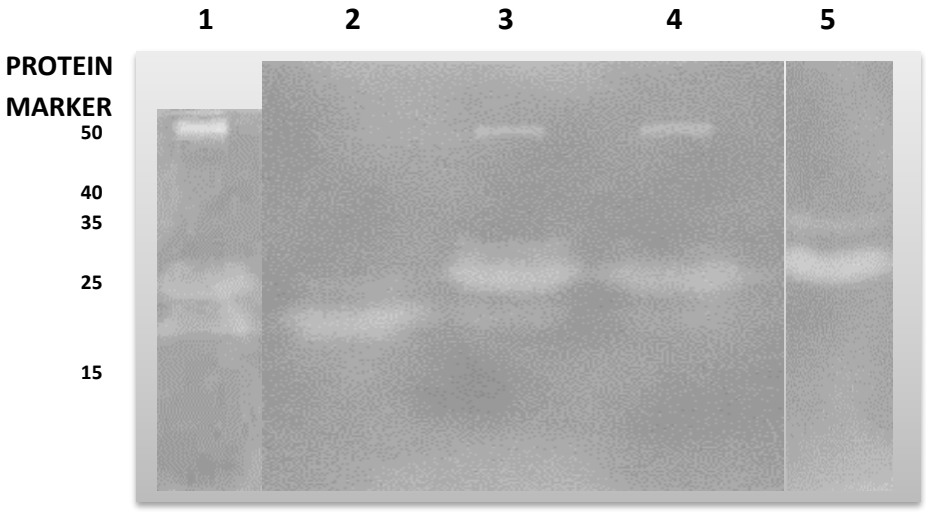

Figure2.10. Zymogram of xylanase variants. Lane 1: S325 xylanase variant; lane 2: S340 xylanase variant; lane 3: G41 xylanase variant, lane 4: G53 xylanase variant; lane 5: NC38 xylanase variant (control)

Each of the xylanase variants were run on a zymogram gel substituted with 1% (w/v) Birchwood xylan. After electrophoresis the gel was washed in a solution of Triton X-100 and buffer. The gel was then incubated for 3 hours at 50°C, followed by staining with Congo-red to visualize the hydrolysis of xylan in the gel by the xylanase variants (Fig 2.10). Each of the xylanase variants were able to hydrolyze the xylan in the gel and this was clearly visible.

CHAPTER 3. CHARACTERIZATION OF XYLANASE VARIANTS IN *P. pastoris*

3.1. Introduction

Enzymes are regarded as an important, beneficial and suitable complement to the increasing range of chemical catalysts for the creation of complex chiral molecules, the processing conditions employed in an industrial catalytic reactor are seldom ideally suited for the maintenance of a highly active, stable, resilient and robust enzyme. Natural enzymes have evolved and adapted over the centuries to function in a particular environment and even though they are extremely diverse; natural and industrial environments are considerably different (Hibbert *et al*., 2005; Strafford *et al*., 2012). Therefore, for a number of industrial processes a suitable enzyme catalyst is not readily available in nature, suggesting that there is a requirement for the discovery of novel biocatalysts. Although many complex chemical reactions can be proficiently performed by biocatalysts, as mentioned previously, industrial conditions are usually different from those in nature with respect to substrate concentrations and affinity, shearing forces, environmental aspects such as temperature and pH as well as the presence of organic solvents in many industrial processes. These factors make the implementation of enzymes for industrial processes difficult, specifically, where multiple (rather than singular) traits are required to be satisfied in order to create the ideal industrial enzymatic catalyst. Most biocatalysts found in soil and water may possess the desired activity, but are usually not suitable for industrial applications (Marrs *et al*., 1999; Otten and Quax, 2005).

Interest in thermostable enzymes has increased significantly as resistance to thermal inactivation and increased stability has become desirable properties of the enzyme catalysts that are

employed in many industrial applications (Singh *et al*., 2000). Thermostable enzymes are can be defined as those biocatalysts that display an optimum temperature above that of the maximum temperature required for growth of an organism or with exceptional stability above 50°C over an extended period of time (Gupta and Gupta, 1993; Singh *et al*., 2000). Stability is an important characteristic of a biocatalyst, this factor co-determines the economic feasibility of the application of the enzyme catalyst in industrial processes. The ability to maintain a high stability is considered an economic advantage because it subsequently results in a reduction in enzyme turnover. In addition, thermostable enzymes provide an allowance for the use of high process temperatures, which may have valuable effects on reaction rates, reactant solubility and considerably reduce the risk of microbial contamination. Enzymes are increasing in popularity for application in feed supplementation where they may be required to withstand hygienic heat treatments and harsh processing conditions such as extrusion (Eijsink *et al.*, 2004).

The xylanase variants; S325, S340, G41 and G53 were the product of directed evolution studies (Stephens *et al*., 2007). These variants were modified to withstand high temperatures and alkaline conditions, for particular use in the pulp and paper industries with the potential of replacing the use of conventional bleaching methods of pulp bleaching such as the utilization of chlorine. The xylanase variants listed above were expressed in *E. coli* with success but the yields were low and further downstream processing was required. As an alternative expression host for the modified xylanases, *P. pastoris* has several advantages over the bacterial host systems for the production of heterologous proteins. The yeast system is able to produce a glycosylated protein, in a partially purified form and there is no downstream processing required.

The purpose of this part of the study was to investigate the use of *P. pastoris* as a host system for the production of the modified xylanases and to compare the results of the study to previous research done in the bacterial host.

3.2. Materials and Methods

3.2.1. Shake flask cultivation of *P. pastoris*

P. pastoris was cultured in YPD broth containing 100 µg/ml of zeocin. The culture was grown at 30°C shaking at 200 rpm for 72 hours. A 10% inoculum was used to inoculate 180 ml of YPD-zeocin broth.

3.2.2. Extraction of xylanase

Following incubation, the culture was centrifuged at 4000 × *g* for 30 min. The pellet was discarded and the supernatant containing the xylanase protein was placed at 4°C for short-term storage and -20°C for long- term storage, until required for further analysis.

3.2.3. Concentration of proteins

The proteins present in the supernatant were concentrated using the acetone precipitation method (Thermo Fisher Scientific, 2009). The acetone required for this process was first cooled to a temperature of -20°C. The protein sample (supernatant) was placed in a plastic centrifuge tube and acetone was added in a ratio of 1:4 (supernatant: acetone). The contents of the tube

were first mixed followed by incubation at -20°C for 1 h. The protein sample was thereafter centrifuged at 13000 × *g* for 10 min. The supernatant was carefully discarded and the pellet was first air-dried and then suspended in the appropriate buffer. For the determination of protein concentration, the Folin-Lowry method was employed (Waterborg, 2009) and the concentration of the proteins were standardized for use in further experiments.

3.2.4. Quantification of xylanase activity

Xylanase activity was determined using the method described by Bailey *et al*. (1992). The extracted enzyme sample (100 µl) was added to 900 µl of the substrate (10% Birchwood xylan) and incubated for 5 min at 50°C, unless otherwise stated. Following incubation, 1.5 ml DNS was added followed by boiling at 100°C for 5 min. The resulting colour change was quantified at an absorbance of 540 nm using a spectrophotometer. Xylanase activity was determined using a xylose standard curve.

3.2.5. pH optima of xylanase variants

Crude xylanases were diluted in appropriate buffers over the pH range of 5 – 9 to determine pH optima of the respective xylanases. The buffer systems (0.2 M) used were citrate-NaOH (pH 5 and 6), sodium phosphate (pH 7 and 8) and glycine-NaOH (pH 9). Substrate (0.1% birchwood xylan, Sigma) was prepared in buffers of the corresponding pH and assayed for xylanase activity, as described in section 3.2.4.

3.2.6. Temperature optima of xylanase variants

Enzyme samples were diluted in their optimal pH buffers (pH 5 – 7) and assayed for xylanase activity from 40°C – 80°C for 5 min as described in section 3.2.4., for determination of the temperature for optimal activity of the xylanases.

3.2.7. Temperature and pH stability of the xylanase variants

Xylanases were tested for stability in the pH range (pH 5 – 9) using 0.2 M citrate-NaOH (pH 5 and 6), 0.2 M sodium-phosphate (pH 7 and 8) and 0.2 M glycine-NaOH (pH 9) from 40°C – 80°C for up to 120 min. Samples were removed every 15 min and stored at 4°C and then assayed to determine enzyme activity.

3.3. Results and Discussion

3.3.1. pH and temperature optima

3.3.1.1. pH optima

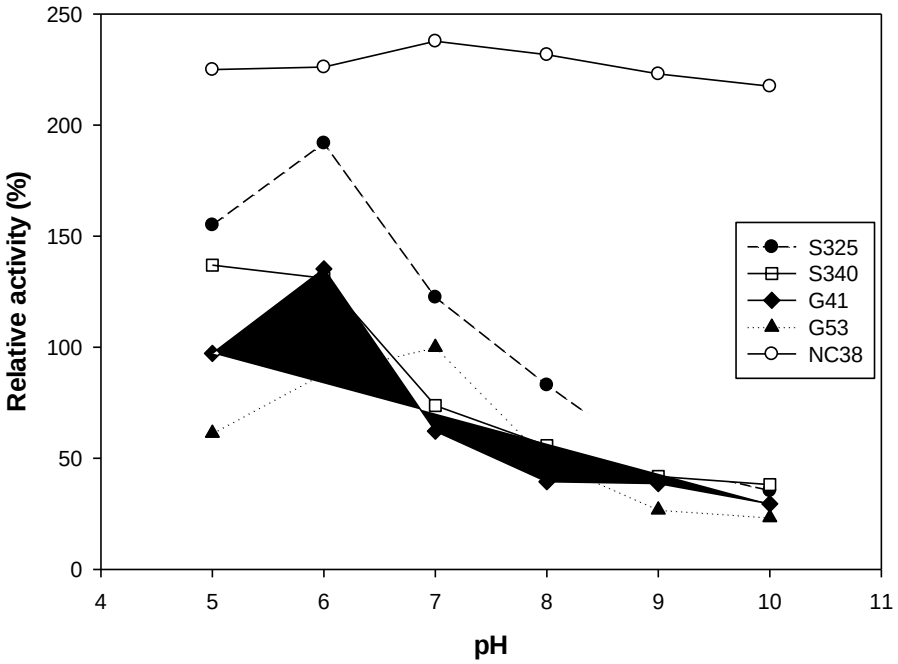

Figure 3.1. The effect of pH on the activity of xylanase variants. NC38 xylanase against xylanase variants; S325, S340, G41, and G53 from pH 5 – 10 at 50°C for 5 min. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

At pH 5, the xylanase activity for the S325 variant was 155 IU/ml, at pH 6 the xylanase activity increased to 192 IU/ml in citrate-NaOH buffered substrate. As the pH of the substrate increased and the buffer system changed, the enzyme activity decreased. At pH 7, the enzyme activity was 122 IU/ml. There was a gradual decline in enzyme activity from pH 8-10 (Fig 3.1.). The enzyme retained 18% of its activity at pH 10. For the variant S340, the enzyme activity was at the highest at pH 5, with a value of 137 IU/ml. At pH 6, the activity was 131 IU/ml, activity declined rapidly

between pH 6 and 7, however the enzyme retained over 50% activity at pH 7. There was a gradual decrease in enzyme activity from pH 8-10 with the enzyme retaining 28% of activity at pH 10. The G41 variant had an optimum activity of 135 IU/ml at pH 6. An increase of 30% in activity was recorded from the activity at pH 5. At pH 7, the enzyme activity decreased to 60 IU/ml. The enzyme retained 30% of its activity at pH 8 and 9 and gradually declined to 22% activity relative to the optimum at pH 10 with an activity of 30 IU/ml. The G53 variant displayed an activity of 84 IU/ml at pH 5. The activity increased proportionally with an increase in pH with an optimum activity of 137 IU/ml at pH 7. At pH 8, the activity decreased to 49% of its optimal activity to 67 IU/ml. At pH 9 and 10, the enzyme retained over 30% of its activity relative to the optimum. For the xylanase, NC38, the enzyme activity at pH 5 was 225 IU/ml, the activity gradually increased to 226 IU/ml at pH 6. Optimum activity was displayed at pH 7, with an activity of 237 IU/ml. At pH 8, the enzyme activity was 232 IU/ml. At pH 9, enzyme activity decreased to 223 IU/ml. At pH 10, the NC38 variant retained 91% of activity relative to the optimum value recorded. Therefore two variants (S325 and G41) had a pH optima of 6 while the variants; G53 and NC38 displayed optimal activity at pH 7. The NC38 variant had the broadest optima suggesting better pH stability.

3.3.1.2. Temperature optima

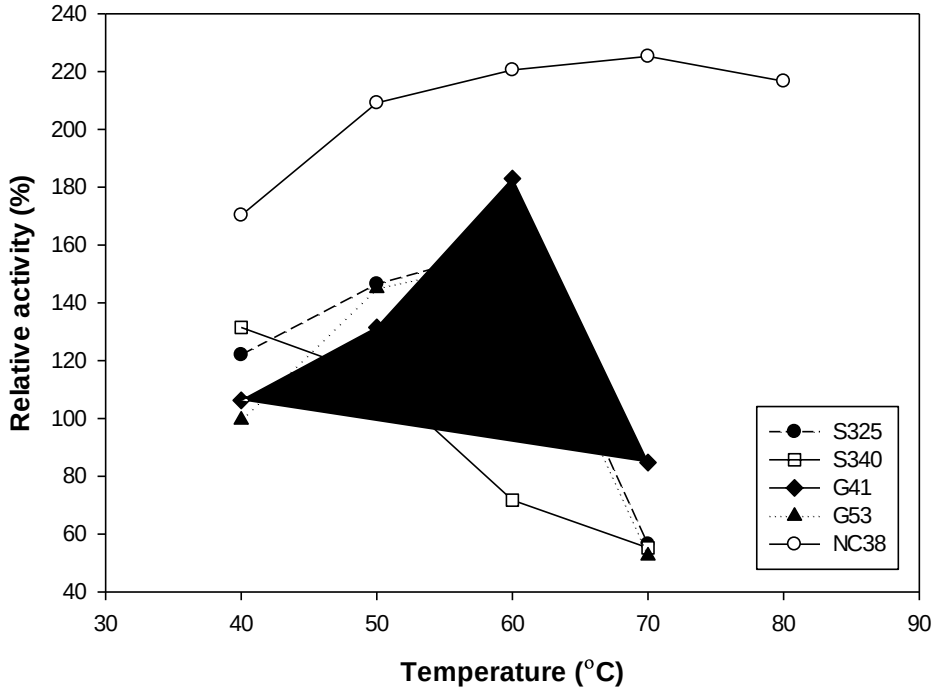

Figure 3.2. The effect of temperature on the activity of xylanase variants. NC38 xylanase against xylanase variants; S325, S340, G41, and G53 from pH 5 – 10 at 50°C for 5 min. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations

For the xylanase variant S325, an activity of 122 IU/ml was recorded at 40°C. The activity increased to 147 IU/ml at 50°C. The optimum activity was visible at 60°C with an activity of 159 IU/ml. Between 60 and 70°C there was a rapid decrease in activity retaining 35% of activity relative to the optimum. Xylanase variant S340 displayed optimum activity of 131 IU/ml at 40°C (Fig. 3.2). An activity of 114 IU/ml was obtained at 50°C. Between 50 and 60°C the enzyme activity decreased to 72 IU/ml. The variant retained 48% of activity at 70°C. The variant G41 displayed an activity of 106 IU/ml at 40°C. The activity increased to 132 IU/ml at 50°C. There was a significant increase in enzyme activity between 50 and 60°C from 132 to 183 IU/ml respectively. When the

temperature was increased to 70°C the activity decreased to 85 IU/ml, retaining 46% of activity relative to the optimum. For the xylanase variant G53, the enzyme activity was 100 IU/ml at 40°C and an activity of 145 was noted at 50°C. The optimum temperature for the variant was 60°C with an activity of 155 IU/ml. At 70°C, 34% of activity relative to the optimum was retained. At 40°C, the variant NC38 had an activity of 170 IU/ml. At 50°C the activity increased to 209 IU/ml. An increase in temperature also increased enzyme activity proportionally at 60 and 70°C. An optimum activity of 225 IU/ml was obtained at 70°C. This enzyme maintained the ability to hydrolyse the xylan substrate with a high rate of success retaining 96% of activity even after the temperature was increased to 80°C.

3.3.2. Temperature and pH Stability

3.3.2.1. pH 5, 40°C

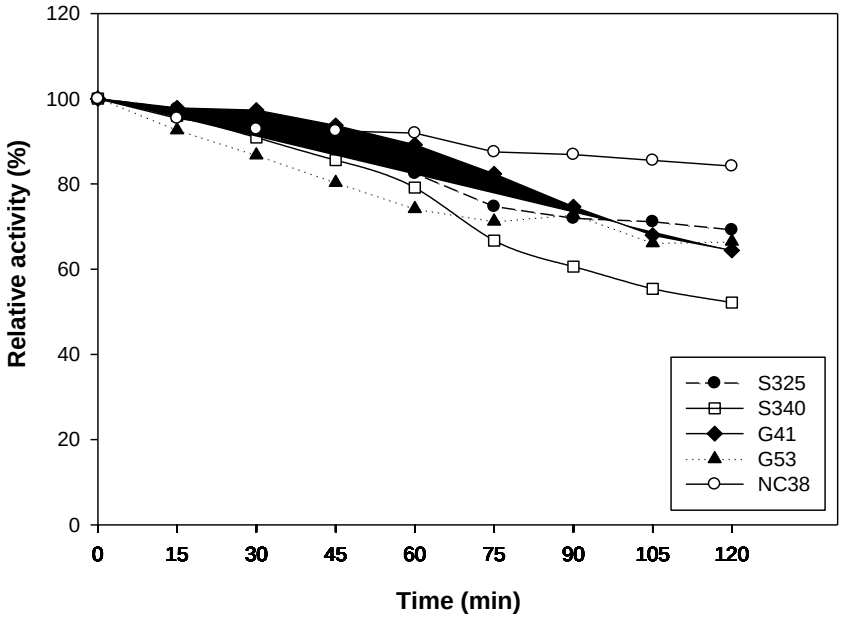

Figure 3.3. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 5, 40°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 222 IU/ml, after 30 min the activity decreased to 93%, the enzyme lost 20% of activity after a period of 60 min. Activity declined to 72% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 69% of activity. For the variant S340, the initial activity recorded was 224 IU/ml, following 30 min of incubation, the variant lost 10% of activity. After 60 min, the enzyme activity decreased to 79%. At 90 min, the activity was reduced to 61% relative to the initial activity. At 120 min, 52% of activity was retained. The activity recorded at T_0 for the G41 variant was 187 IU/ml, the activity declined to 97% after 30 min. At 60 min, the activity decreased to 89% activity. Seventy five percent and 64%

of activity was retained after a period of 90 and 120 min, respectively. The xylanase variant G53 had an initial activity of 186 IU/ml. Following 30 min of activity, the enzyme activity decreased to 87%. After 60 min of incubation at pH 5, 40°C, the enzyme activity declined to 74%. After 90 min the activity decreased by 30% and the enzyme retained 67% of activity after 120 min. The variant NC38 had an activity of 237 IU/ml at T_0. The enzyme lost 10% of activity after 60 min. This variant retained 87% and 84% activity after 90 and 120 min at pH 5, 40°C respectively (Fig 3.3). S325, G41 and G53 variants displayed similar stabilities after 120 min, retaining 70% of activity. S340 was the least stable of the variants and retained 52% of activity after the 120 min of incubation. NC38 was the most stable of the xylanases and retained more than 80% of activity at 120 min.

3.3.2.2. pH 5, 50°C

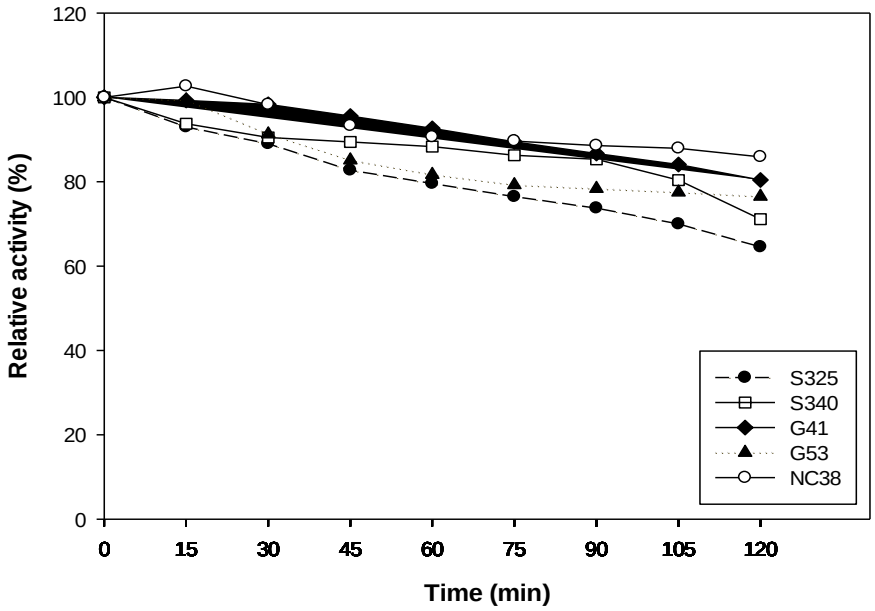

Figure 3.4. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 5, 50°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 224 IU/ml, after 30 min the activity decreased to 89%, the enzyme lost 20% of activity after a period of 60 min. Activity declined to 74% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 65% of activity. For the variant S340, the initial activity recorded was 207 IU/ml, following 30 min of incubation, the variant lost 10% of activity. After 60 min, the enzyme activity decreased to 88%. At T_{90}, the activity was reduced to 85% relative to the initial activity. After 120 min, 71% of activity was retained. The activity recorded at T_0 for the G41 variant was 162 IU/ml, the activity declined to 98% after 30 min. At 60 min, the activity decreased to 93% activity (Fig. 3.4). Eighty seven percent and 80% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53 had an initial activity of 173 IU/ml. Following 30 min of activity, the enzyme activity decreased to 91%. After 60 min of incubation at pH 5, 50°C, the enzyme activity declined to 82%. At T_{90} the activity decreased by 22% and the enzyme retained 77% of activity after 120 min. The variant NC38 had an activity of 233 IU/ml at T_0. The enzyme lost 10% of activity after 60 min. This variant retained 89% and 86% activity after 90 and 120 min at pH 5, 50°C respectively. Under these conditions the xylanase variants displayed a high level of stability. Four of the five variants retained 80% activity after 120 min and the S325 variant retained 65% activity after the duration of the experiment.

3.3.2.3. pH 5, 60°C

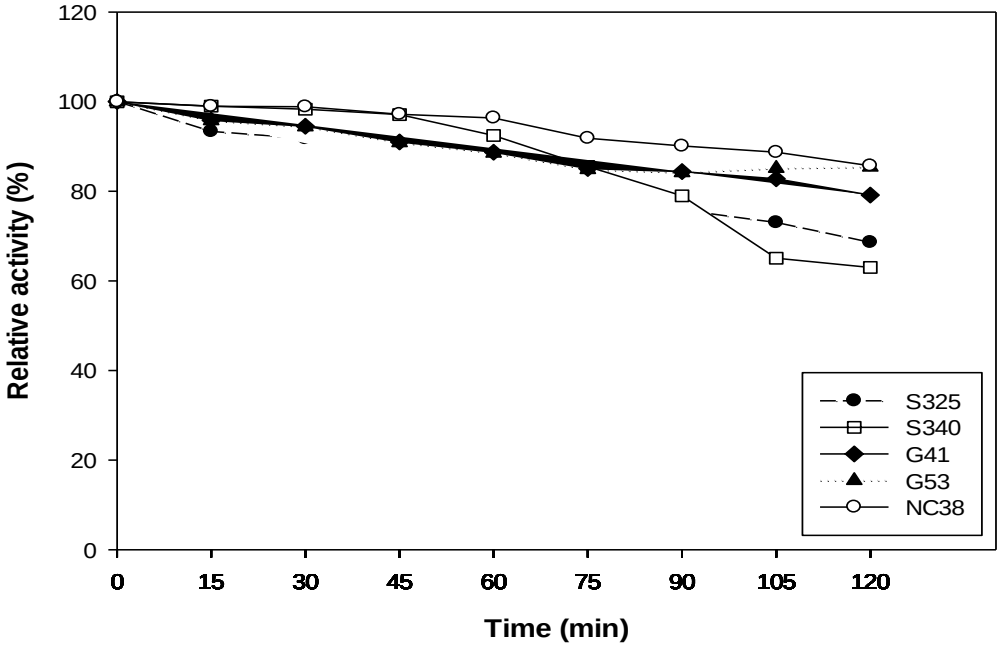

Figure 3.5. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 5, 60°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 220 IU/ml, after 30 min the activity decreased to 92%, the enzyme lost 20% of activity after a period of 60 min. Activity declined to 76% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 69% of activity. For the variant S340, the initial activity recorded was 91 IU/ml, following 30 min of incubation, the variant maintained 98% relative activity. After 60 min, the enzyme activity decreased by 10%. At 90 min, the variant lost 20% of activity. At T_{120}, 63% of activity was retained. The activity recorded at T_0 for the G41 variant was 92 IU/ml, the activity declined to 94% after 30 min. After 60 min, the activity decreased to 89% activity. Eighty four percent and 79% of activity was retained after an incubation period of 90 and 120 min respectively (Fig. 3.5). The xylanase variant

G53 had an initial activity of 91 IU/ml. Following 30 min of activity, the enzyme activity decreased to 94%. After 60 min of incubation at pH 5, 60°C, the enzyme activity declined to 89%. At 90 min the activity decreased by 15% and the enzyme retained 85% of activity after 120 min. The variant NC38 had an activity of 247 IU/ml at T_0. The enzyme retained 96% of activity after 60 min. This variant retained 90% and 86% activity after 90 and 120 min at pH 5, 60°C respectively. This enzyme maintained a high activity over 120 minutes losing only 14% of activity. After incubation for 120 min, all the variants maintained a high percentage of catalytic activity. The S340 variant displayed a sharp decline in stability after 90 min and retained the least amount of activity.

3.3.2.4. pH 5, 70°C

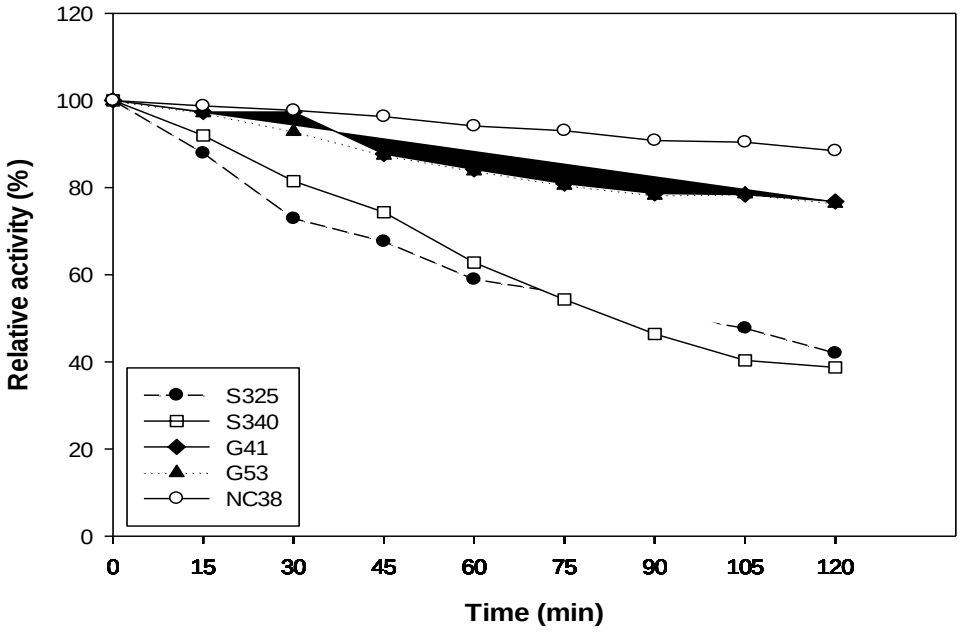

Figure 3.6. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 5, 70°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

For the xylanase variant S325 the initial enzyme activity recorded at pH 5, 70°C was 109 IU/ml. Following incubation for a period of 30 min, the activity decreased to 73%, the enzyme lost nearly 40% of activity after 60 min. A decline to 51% relative activity was noted after 90 min of incubation with a final relative activity at 120 min showing a decline of 60%. Under the same experimental conditions, the variant S340 had an initial activity of 93 IU/ml. After 30 min of incubation, the variant lost 20% of activity. After 60 min of incubation, the enzyme activity decreased to 63% of its original activity. At T_{90}, the activity was reduced less than 50% relative to the initial activity. At 120 min, 39% of activity was retained (Fig. 3.6). At $T_{0,}$ the activity noted for the G41 variant was 88 IU/ml, the enzyme lost 3% activity after 30 min, the activity declined to 84% after 60 min. Following 90 min of incubation, the activity decreased by 20%. Seventy seven percent of activity was retained after 120 min of incubation. An initial activity of 87 IU/ml was recorded for the G53 variant. Following 30 min of activity, the enzyme activity decreased to 93% of the initial activity. After 60 min of incubation at pH 5, 70°C, the enzyme activity declined to 84%. At T_{90} the activity decreased by 22% and the enzyme retained 76% of activity after 120 min. The variant NC38 had an activity of 263 IU/ml at T_0. The enzyme lost 6% of activity after 60 min. This variant retained 88% relative activity after 120 min at pH 5, 70°C. The G41 and G53 variants were able to maintain nearly 80% of their activity under these conditions and had similar characteristics under these experimental conditions. Although the S340 and S325 variants had higher initial activities, they lost 60% of activity by 120 min of incubation. NC38 xylanase displayed the highest stability of the five variants.

3.3.2.5. pH 5, 80°C

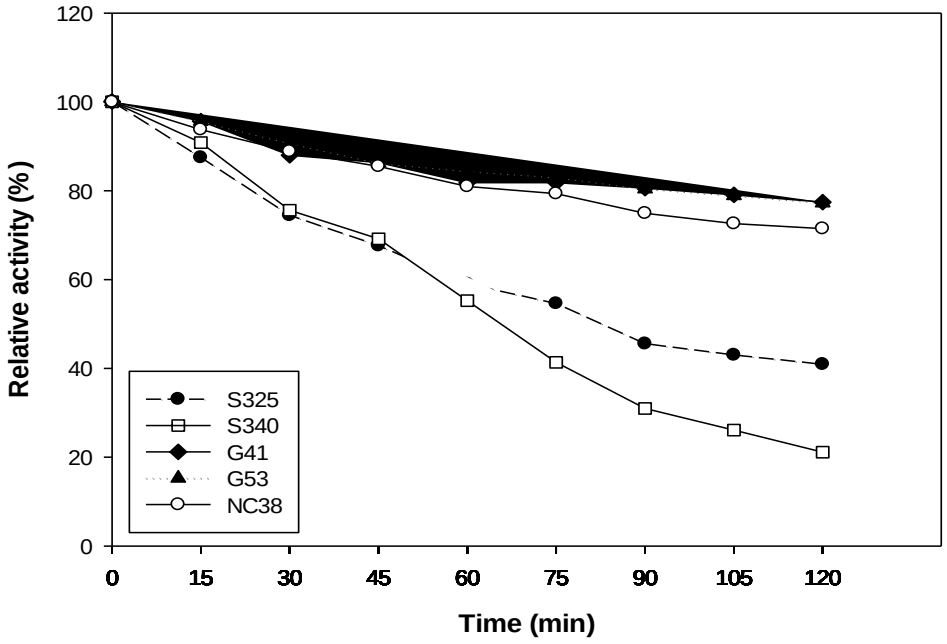

Figure 3.7. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 5, 80°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 45 IU/ml, after 30 min the activity decreased to 74%, the enzyme lost 40% of activity after a period of 60 min. Activity declined to 46% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 41% of activity. For the variant S340, the initial activity recorded was 38 IU/ml, following 30 min of incubation, the variant lost 24% of activity. After 60 min, the enzyme activity decreased to 55%. At T_{90} the activity was reduced to 31% relative to the initial activity. After 120 min, 21% of activity was retained. The activity recorded at T_0 for the G41 variant was 36 IU/ml, the activity declined to 88% after 30 min. At 60 min, the activity decreased to 82% activity. Eighty one percent and 77% of activity was retained after an incubation period of 90 and 120 min respectively (Fig. 3.7). The xylanase variant G53 had an initial activity of 36 IU/ml. Following 30 min of activity, the enzyme

activity decreased by 10%. After 60 min of incubation at pH 5, 80°C, the enzyme activity declined to 84%. After 90 min the activity decreased by 20% and the enzyme retained 77% of activity after 120 min. The variant NC38 had an activity of 243 IU/ml at T_0 which was significantly higher than the activities of the other variants. The enzyme lost 20% of activity after 60 min. This variant retained 75% and 71% activity after 90 and 120 min at pH 5, 80°C respectively. The G41, G53 and NC38 variants were able to maintain more than 70% of their respective initial activities. The S325 and S340 variants retained a relative activity above 80% after 120 min but lower than that shown at pH 5, 70°C. The stability of NC38 was lower than the G41 and G53 xylanases.

3.3.2.6. pH 6, 40°C

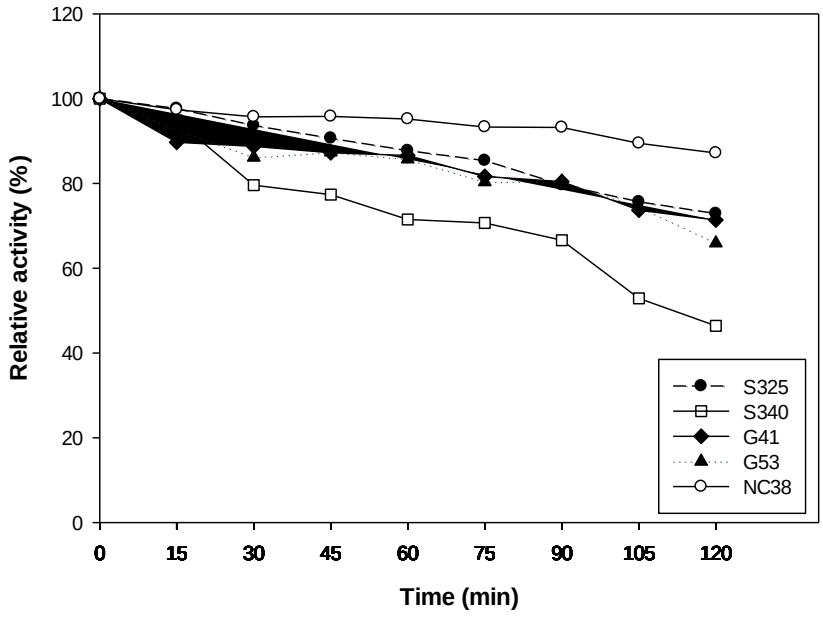

Figure 3.8. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 6, 40°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

At pH 6, 40°C, the xylanase variant S325 had an initial activity of 223 IU/ml. After 30 min of incubation the activity decline by 10%. A gradual decrease in activity from T_{60} to T_{120} was noted with the enzyme retaining 73% of activity after 120 minutes of incubation. Under these conditions the variant S340 had an activity of 212 IU/ml at T_0. At T_{30} enzyme activity decreased by 20%. After 60 min the activity decreased by 30%, following 90 min of incubation the enzyme activity 67% of the initial activity with 46% of activity being retained at T_{120}. Xylanase variant G41 had an initial activity of 182 IU/ml, after 30 min of incubation the enzyme displayed a minor loss in activity maintaining 71% of activity at T_{120}. At pH 6, 40°C, the G53 variant had an initial activity of 181 IU/ml, 86% retention of activity was attained over 60 minutes of incubation. After 90 min the enzyme lost 20% of activity and managed to retain 66% activity at 120 min of incubation (Fig. 3.8). NC38 xylanase variant had an activity of 239 IU/ml. Over a period of 90 min at pH 6, 40°C the enzyme maintained greater than 90% of activity with a final activity of 209 IU/ml (87% of initial activity.) The NC38 variant maintained high stability over the 120 min of catalysis and was the most stable of all the variants. The S340 variant was fairly stable as compared to the other variants, however this enzyme continued to lose activity at a steady rate every 15 min throughout the 120 min incubation period. The three variants (S325, G41 and G53) were able to retain over 60% of activity after 120 min.

3.3.2.7. pH 6, 50°C

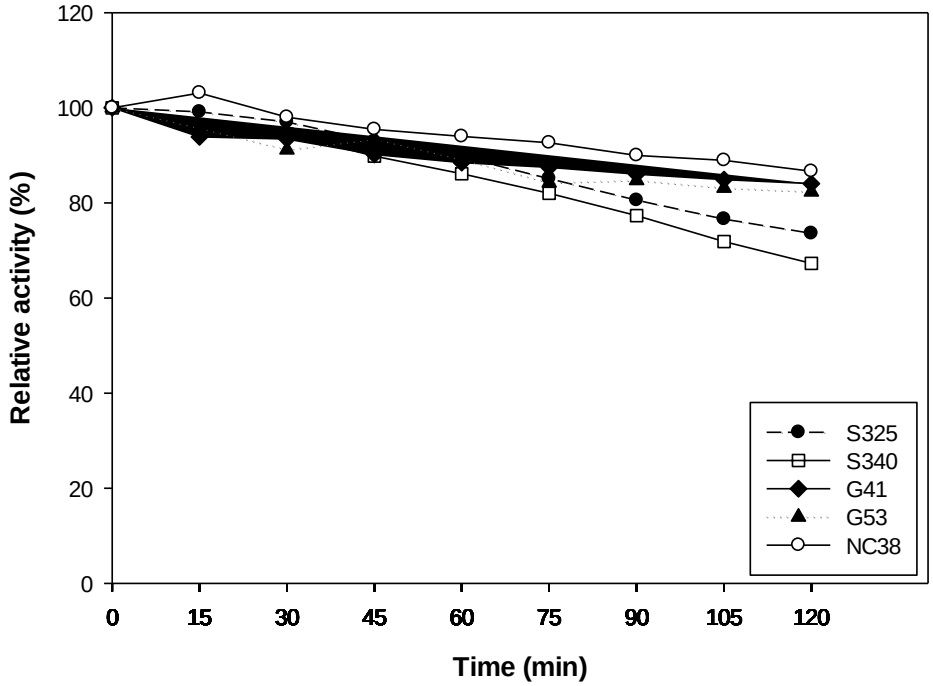

Figure 3.9. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 6, 50°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 225 IU/ml, after 30 min the activity decreased to 97%, the enzyme lost 10% of activity after a period of 60 min. Activity declined to 81% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 74% of activity. For the variant S340, the initial activity recorded was 223 IU/ml, following 30 min of incubation, the variant maintained 95% relative activity. After 60 min, the enzyme activity decreased by 15%. After 90 min, the variant lost 23% of activity (Fig. 3.9). At T_{120} 67% of activity was retained. The activity recorded at T_0 for the G41 variant was 175 IU/ml, the activity declined to 93% after 30 min. After 60 min, the activity decreased to 88% activity. Eighty six percent and 84% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53

had an initial activity of 186 IU/ml. Following 30 min of activity, the enzyme activity decreased to 91%. After 60 min of incubation at pH 6, 50°C, the enzyme activity declined to 89%. At T_{90} the activity decreased by 15% and the enzyme retained 82% of activity after 120 min. The variant NC38 had an activity of 241 IU/ml at T_0. The enzyme retained 94% of activity after 60 min. This variant retained 90% and 87% activity after 90 and 120 min at pH 6, 50°C respectively. This enzyme maintained a high activity over 120 minutes losing only 13% of activity. The G41 and G53 variants displayed good stability as well, maintaining 84% and 82% of activity respectively after 120 min. The S340 variant was least stable under these conditions losing 33% of activity. All variants retained 80% activity after 60 min. NC38 xylanase was the most stable of the five variants.

3.3.2.8. pH 6, 60°C

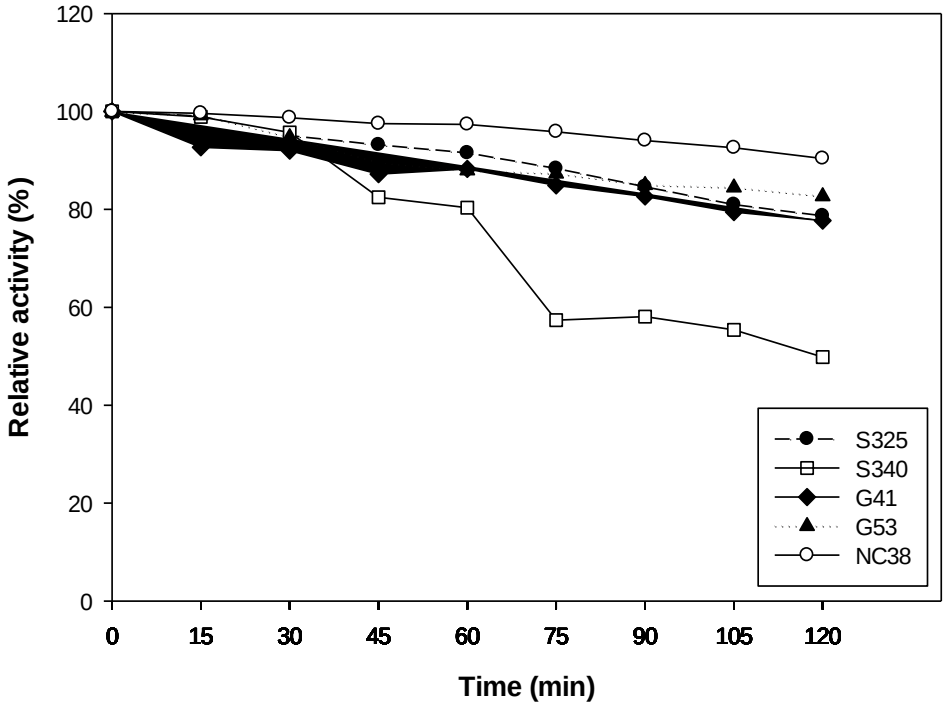

Figure 3.10. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 6, 60°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

For the xylanase variant S325 the initial enzyme activity recorded at pH 6, 60°C was 226 IU/ml. Following incubation for a period of 30 min, the activity decreased to 95%, the enzyme less than 10% of activity after 60 min. A decline to 85% relative activity was noted after 90 min of incubation with a final relative activity at 120 min showing a decline of 21%. Under the same experimental conditions, the variant S340 had an initial activity of 101 IU/ml. After 30 min of incubation, the variant lost 4% of activity. After 60 min of incubation, the enzyme activity decreased to 80% of its initial activity. From T_{60} to T_{90} the activity rapidly declined to less than 60% of its initial activity. At 120 min, 50% of activity was retained. At T_0, the activity noted for the G41 variant was 93 IU/ml, the enzyme lost 8% activity after 30 min, the activity declined to 88%

after 60 min. After 90 min, the activity decreased by 20%. Seventy eight percent of activity was retained after 120 min of incubation (Fig. 3.10). An initial activity of 92 IU/ml was recorded for the G53 variant. Following 30 min of activity, the enzyme activity decreased to 95% of the initial activity. After 60 min of incubation at pH 6, 60°C, the enzyme activity declined to 88%. Following 90 min of incubation the activity decreased by 15% and the enzyme retained 83% of activity after 120 min. The variant NC38 had an activity of 251 IU/ml at T_0. The enzyme displayed a minimum loss of activity after 60 min. This variant retained 90% relative activity after 120 min at pH 6, 60°C. All the variants were able to maintain a high level of stability under these conditions with the lowest stability noted by the S340 variant which still retained 50% activity after 120 min of incubation. The variants; S325, G41 and G53 were similar in the retention of the catalytic activities after 120 min.

3.3.2.9. pH 6, 70°C

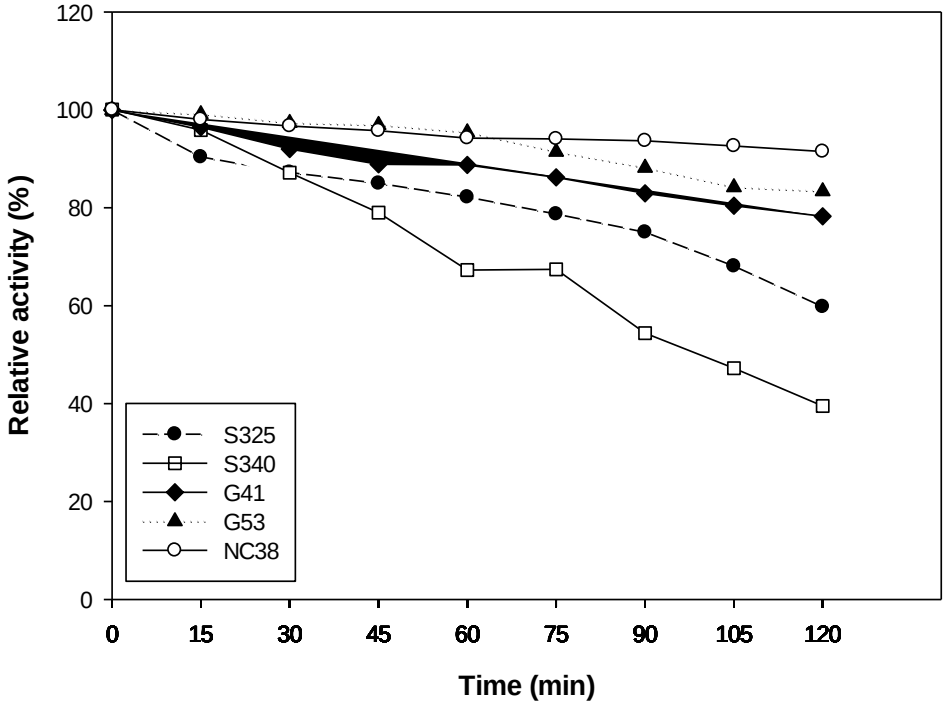

Figure 3.11. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 6, 70°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 111 IU/ml, after 30 min the activity decreased to 87%, the enzyme lost 20% of activity after a period of 60 min. Activity declined to 75% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 60% of activity. For the variant S340, the initial activity recorded was 87 IU/ml, following 30 min of incubation, the variant lost 13% of activity. After 60 min, the enzyme activity decreased to 67%. At T_{90} the activity was reduced to 54% relative to the initial activity. After 120 min, 40% of activity was retained. The activity recorded at T_0 for the G41 variant was 92 IU/ml, the activity declined to 97% after 30 min. After 60 min, the activity decreased to 89% activity. Eighty three percent and 78% of activity was retained after an incubation period of 90 and 120 min respectively (Fig. 3.11).

The xylanase variant G53 had an initial activity of 91 IU/ml. Following 30 min of activity, the enzyme activity decreased to 97%. After 60 min of incubation at pH 6, 70°C, the enzyme activity declined minimally to 95% relative activity. Following 90 min of incubation the activity decreased by 12% and the enzyme retained 83% of activity after 120 min. The variant NC38 had an activity of 263 IU/ml at T_0. The enzyme lost 3% of activity after 60 min. This variant retained 94% and 91% activity after 90 and 120 min at pH 6, 70°C respectively. This enzyme maintained a high activity over 120 minutes as did the G53 and G41 variant while the S340 variant lost 60% of its relative activity after 120 min at 70°C and the S325 variant lost 40% of activity over 120 min. The variant, S340 showed a steady decline in activity over the 120 min incubation period and was the least stable variant. The other four variants maintained over 80% of relative activity after 60 min at pH 6, 70°C.

3.3.2.10. pH 6, 80°C

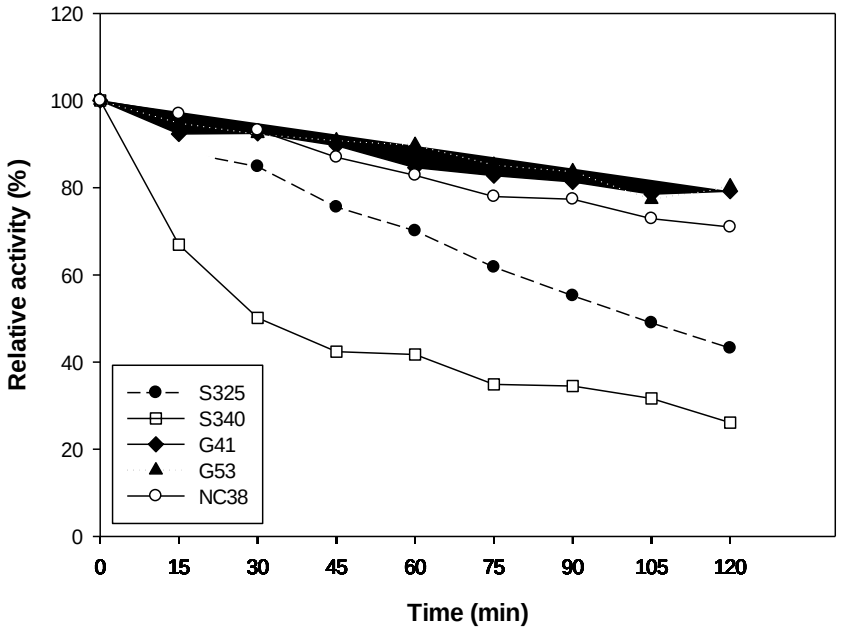

Figure 3.12. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 6, 80°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

For the xylanase variant S325 the initial enzyme activity recorded at pH 6, 80°C was 46 IU/ml. Following incubation for a period of 30 min, the activity decreased by 15%, the enzyme lost 30% of activity after 60 min. A decline to 55% relative activity was noted after 90 min of incubation with a final relative activity at 120 min showing a decline of 60% (Fig.3.12). Under the same experimental conditions, the variant S340 had an initial activity of 39 IU/ml. After 30 min of incubation, there was a significant loss in activity to 50% relative activity. After 60 min of incubation, the enzyme activity decreased to 42% of its original activity. At 90 min, the activity was reduced less than 35% relative to the initial activity. At T_{120}, 26% of activity was retained. At $T_{0,}$ the activity noted for the G41 variant was 38 IU/ml, the enzyme lost 8% activity after 30 min, the activity declined to 85% after 60 min. At T_{90}, the activity decreased by 20%. Seventy nine

percent of activity was retained after 120 min of incubation. An initial activity of 37 IU/ml was recorded for the G53 variant. Following 30 min of incubation, the enzyme activity decreased to 92% of the initial activity. After 60 min of incubation at pH 6, 80°C, the enzyme activity declined to 90%. At T_{90} the activity decreased by 16% and the enzyme retained 80% of activity after 120 min. The variant NC38 had an activity of 245 IU/ml at T_0. The enzyme lost 17% of activity after 60 min. This variant retained 71% relative activity after 120 min at pH 6, 80°C. The results obtained above are similar to those found for pH 6, 70°C, the G41 and G53 variants were the most stable under these conditions. The S325 variant displayed a steady decline in activity but retained higher catalytic ability than the S340 variant after 120 min.

3.3.2.11. pH 7, 40°C

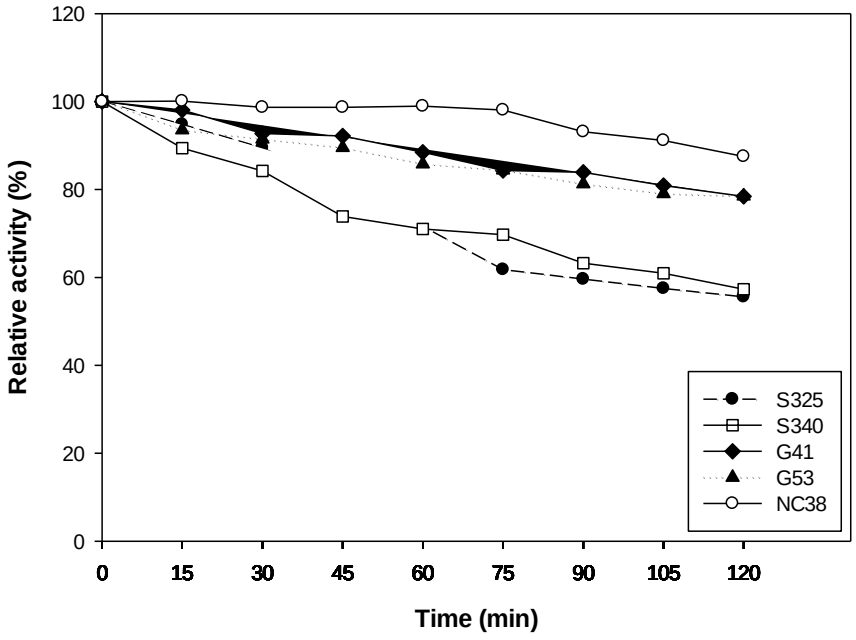

Figure 3.13. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 7, 40°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 220 IU/ml, after 30 min the activity decreased to 89%, the enzyme lost 30% of activity after a period of 60 min. Activity declined to 60% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 56% of activity. For the variant S340, the initial activity recorded was 192 IU/ml, following 30 min of incubation, the variant lost 16% of activity. After 60 min, the enzyme activity decreased to 71% (Fig. 3.13). At T_{90}, the activity was reduced to 63% relative to the initial activity. After 120 min, 57% of activity was retained. The activity recorded at T_0 for the G41 variant was 181 IU/ml, the activity declined to 93% after 30 min. After 60 min, the activity decreased to 88% activity. Eighty four percent and 78% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53 had an initial activity of 181 IU/ml. Following 30 min of catalysis, the enzyme activity decreased to 91%. After 60 min of incubation at pH 7, 40°C, the enzyme activity declined to 86%. At T_{90}, the activity decreased by 20% and the enzyme retained 79% of activity after 120 min. The variant NC38 had an activity of 247 IU/ml at T_0. The enzyme lost minimal of activity after 60 min. This variant retained 93% and 88% activity after 90 and 120 min respectively at pH 7, 40°C. This enzyme variant maintained a high activity over 120 minutes while the S325 and S340 variants lost nearly 50% of its relative activity after 120 min. NC38 variant was the most stable. The G41 and G53 xylanases retained greater than 80% of activity. The S340 and S325 variants displayed a steady decline over 120 min. The S325 variant was the least stable.

3.3.2.12. pH 7, 50°C

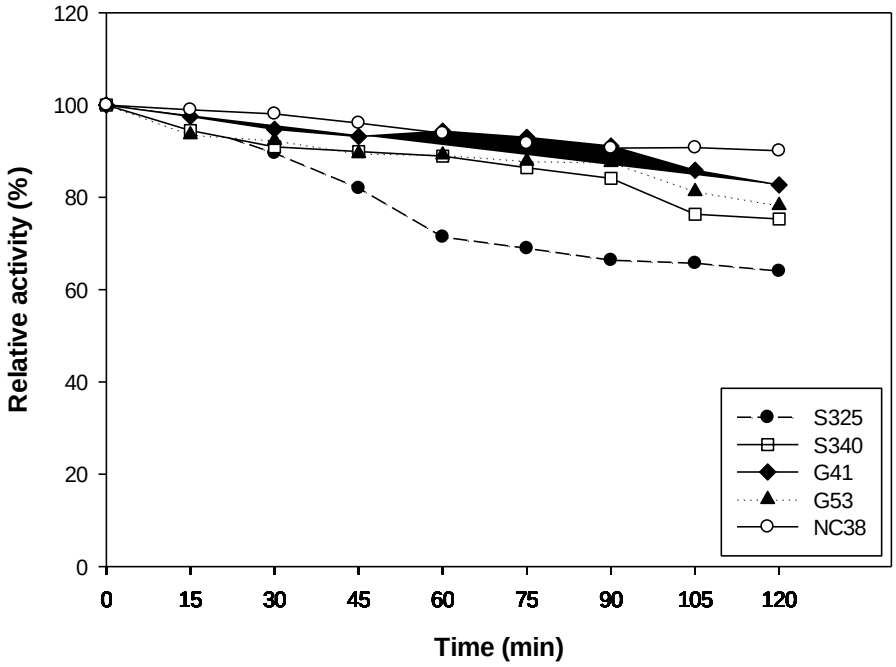

Figure 3.14. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 7, 50°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 222 IU/ml, after 30 min the activity decreased to 90%, the enzyme lost 30% of activity after a period of 60 min. Activity declined to 66% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 64% of activity. For the variant S340, the initial activity recorded was 198 IU/ml, following 30 min of incubation, the variant lost 10% of activity. After 60 min, the enzyme activity decreased to 89%. At 90 min, the activity was reduced to 84% relative to the initial activity (Fig. 3.14). At 120 min, 75% of activity was retained. The activity recorded at T_0 for the G41 variant was 171 IU/ml, the activity declined to 95% after 30 min. At 60 min a relative activity of 94% was maintained. Ninety one percent and 83% of activity was retained after an incubation period of 90 and 120 min

respectively. The xylanase variant G53 had an initial activity of 182 IU/ml. Following 30 min of activity, the enzyme activity decreased to 92%. After 60 min of incubation at pH 7, 50°C, the enzyme activity declined to 89%. At 90 min the activity decreased by 12% and the enzyme retained 78% of activity after 120 min. The variant NC38 had an activity of 249 IU/ml at T_0. This xylanase variant retained high activity over the 120 min incubation period retaining 90% of activity at the end of the experiment. All the variants retained more than 60% of their initial activities. S325 xylanase was the least stable of the five variants. The other xylanases (S340, G41, G53 and NC38) displayed a high rate of stability.

3.3.2.13 pH 7, 60°C

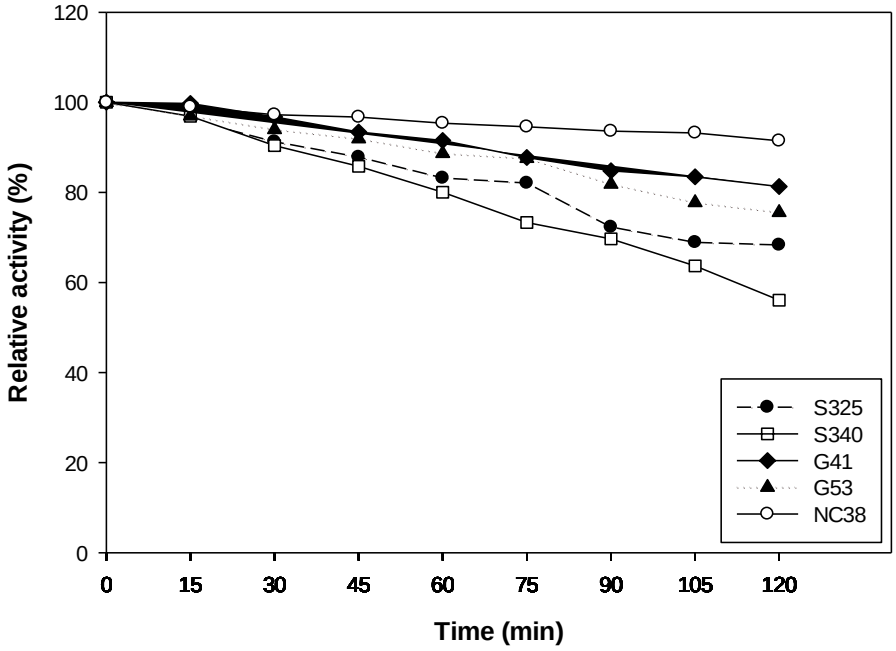

Figure 3.15. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 7, 60°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 219 IU/ml, after 30 min the activity decreased to 91%, the enzyme lost 17% of activity after a period of 60 min. Activity declined to 72% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 68% of activity. For the variant S340, the initial activity recorded was 100 IU/ml, following 30 min of incubation, the variant maintained 90% relative activity. After 60 min, the enzyme activity decreased by 20%. At T_{90}, the variant lost 30% of activity. At T_{120}, 56% of activity was retained. The activity recorded at T_0 for the G41 variant was 92 IU/ml, the activity declined to 97% after 30 min. At T_{60}, the activity decreased to 92%. 85% and 81% of activity was retained after an incubation period of 90 and 120 min respectively (Fig. 3.15). The xylanase variant G53 had an initial activity of 91 IU/ml. Following 30 min of incubation, the enzyme activity decreased to 94%. After 60 min of incubation at pH 7, 60°C, the enzyme activity declined to 89%. At T_{90} the activity decreased by 18% and the enzyme retained 76% of activity after 120 min. The variant NC38 had an activity of 253 IU/ml at T_0. This enzyme retained 95% of activity after 60 min. This variant retained 93% and 91% activity after 90 and 120 min respectively at pH 7, 60°C. NC38 maintained a high activity over 120 minutes losing only 10% of activity, the G41 variant displayed good stability as well, maintaining 81% of activity after 120 min. The S340 variant was least stable under these conditions, with a loss of 50% relative activity. All the xylanase variants maintained their xylan-degrading abilities over the 120 min period. NC38 was the most stable xylanase under the above conditions and S340 was the least stable of the five variants.

3.3.2.14. pH 7, 70°C

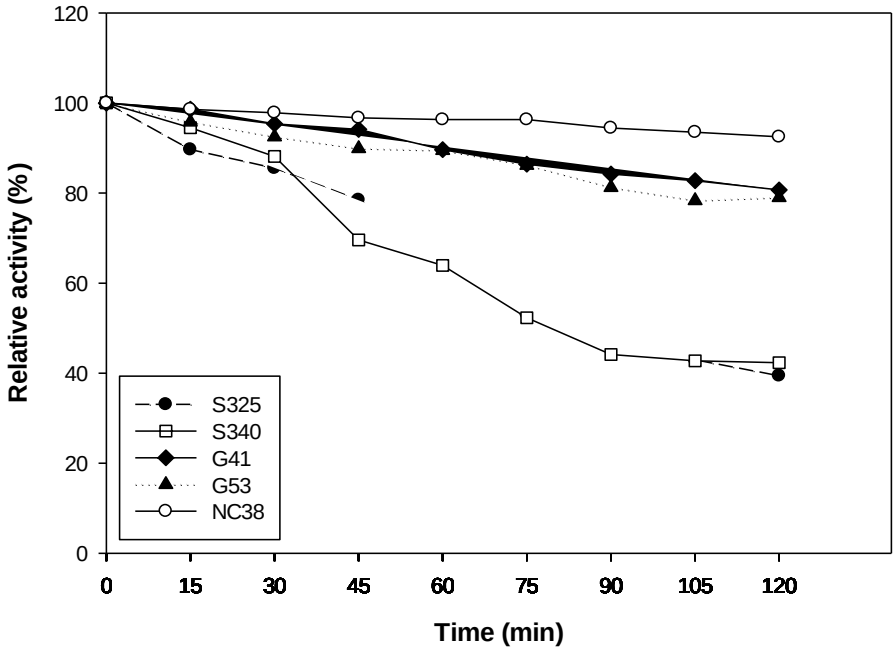

Figure 3.16. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 7, 70°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

For the xylanase variant S325 the initial enzyme activity recorded at pH 7, 70°C was 109 IU/ml. Following incubation for a period of 30 min, the activity decreased to 86%, the enzyme lost 33% of activity after 60 min. A significant decline to 48% relative activity was noted after 90 min of incubation with a final relative activity at 120 min showing a decline of 60%. Under the same experimental conditions, the variant S340 had an initial activity of 90 IU/ml. After 30 min of incubation, the variant lost 12% of activity. After 60 min of incubation, the enzyme activity decreased to 64% of its original activity. At 90 min, the activity was reduced to 44% activity relative to the initial activity. At 120 min, 42% of activity was retained (Fig. 3.16). At $T_{0,}$ the activity noted for the G41 variant was 92 IU/ml, the enzyme lost 5% activity after 30 min, the activity declined to 90% after 60 min. At 90 min, the activity decreased by 16%. Eighty one percent of

activity was retained after 120 min of incubation. An initial activity of 90 IU/ml was recorded for the G53 variant. Following 30 min of activity, the enzyme activity decreased to 92% of the initial activity. After 60 min of incubation at pH 7, 70°C, the enzyme activity declined to 89%. At 90 min the activity decreased by 19% and the enzyme retained 79% of activity after 120 min. The variant NC38 had an activity of 264 IU/ml at T_0. The enzyme lost 4% of activity after 60 min. This variant retained more than 90% relative activity after 120 min at pH 7, 70°C. The G41 and G53 variants were able to maintain 80% of their activity under these conditions. Although the S340 and S325 variants had higher initial activities, they lost 60% of activity by 120 min of incubation. The enzymes displayed similar stabilities after 30 min. Thereafter the S325 and S340 variants rapidly lost stability under these conditions. NC38 was the most stable variant.

3.3.2.15. pH 7, 80°C

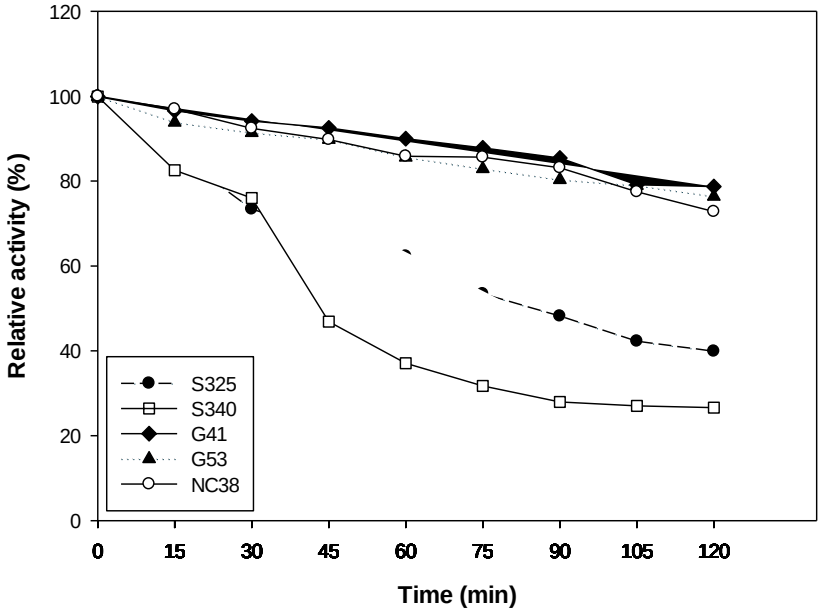

Figure 3.17. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 7, 80°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 44 IU/ml, after 30 min the activity decreased to 73%, the enzyme lost 38% of activity after a period of 60 min. Activity declined to 48% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 40% of activity. For the variant S340, the initial activity recorded was 38 IU/ml, following 30 min of incubation, the variant lost 24% of activity. After 60 min, the enzyme activity decreased to 37% (Fig. 3.17). At T_{90}, the activity was reduced to 28% relative to the initial activity. At T_{120}, less than 30% of activity was retained. The activity recorded at T_0 for the G41 variant was 37 IU/ml, the activity declined to 94% after 30 min. After 60 min, the activity decreased to 90% activity. Eighty five percent and 79% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53 had an initial activity of 37 IU/ml. Following 30 min of activity, the enzyme activity decreased by 9%. After 60 min of incubation at pH 7, 80°C, the enzyme activity declined to 86%. At T_{90} the activity decreased by 20% and the enzyme retained 76% of activity after 120 min. The variant NC38 had an activity of 246 IU/ml at T_0 which was significantly higher than the activities of the other variants. The enzyme lost 15% of activity after 60 min. This variant retained 83% and 73% activity after 90 and 120 min respectively at pH 7, 80°C. The G41, G53 and NC38 variants were able to maintain more than 70% of their respective initial activities whilst the S325 and S340 variants displayed a rapid decline in activity over the 120 min period of incubation. The results obtained were similar to those found at 7, 0°C. The NC38 variant lost more activity than the G41 and G53 variants.

3.3.2.16. pH 8, 40°C

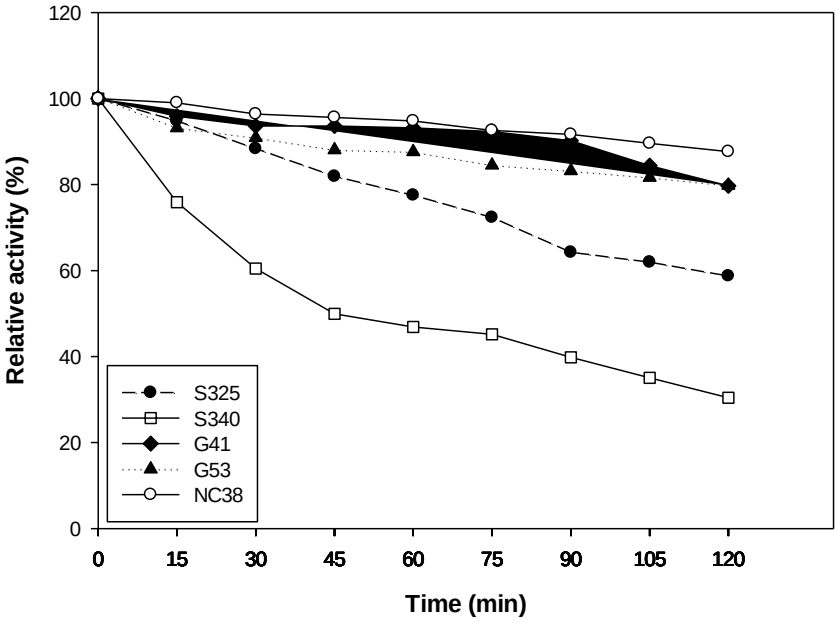

Figure 3.18. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 8, 40°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

At pH 8, 40°C, the xylanase variant S325 had an initial activity of 216 IU/ml. After 30 min of incubation the activity decline by 12%. A gradual decrease in activity from T_{60} to T_{120} was noted with the enzyme retaining 59% of activity (Fig.3.18) after 120 minutes of incubation. Under these conditions the variant S340 had an activity of 172 IU/ml at T_0. After 30 min, there was a rapid decline with the variant losing 40% of activity. Following 60 min of incubation, the activity decreased by 50%. At T_{90}, the enzyme retained 40% of the initial activity with 30% of activity being retained at T_{120}. Xylanase variant G41 had an initial activity of 175 IU/ml, after 30 min of incubation the enzyme displayed 94% of initial activity, and maintained a minor loss in activity retaining 80% of activity at T_{120}. At pH 8, 40°C, the G53 variant had an initial activity of 175 IU/ml, 88% retention of activity was attained over 60 minutes of incubation. After 90 min the enzyme

lost 17% of activity and managed to retain 80% activity at 120 min of incubation. NC38 xylanase variant had an activity of 248 IU/ml. Over a period of 90 min at pH 8, 40°C the enzyme maintained greater than 90% of activity with a final retention of 87% initial activity. The S325, G41, G53 and NC38 variants all displayed similar stabilities after 30 min, thereafter their relative activities differentiated. The NC38 and G41 and G53 variants thrived in the above conditions maintaining 80% activity over the 120 min period of incubation. The S325 variant was relatively stable with a retention of 60% activity and the S340 variant was the least stable losing over 60% of its initial catalytic ability.

3.3.2.17. pH 8, 50°C

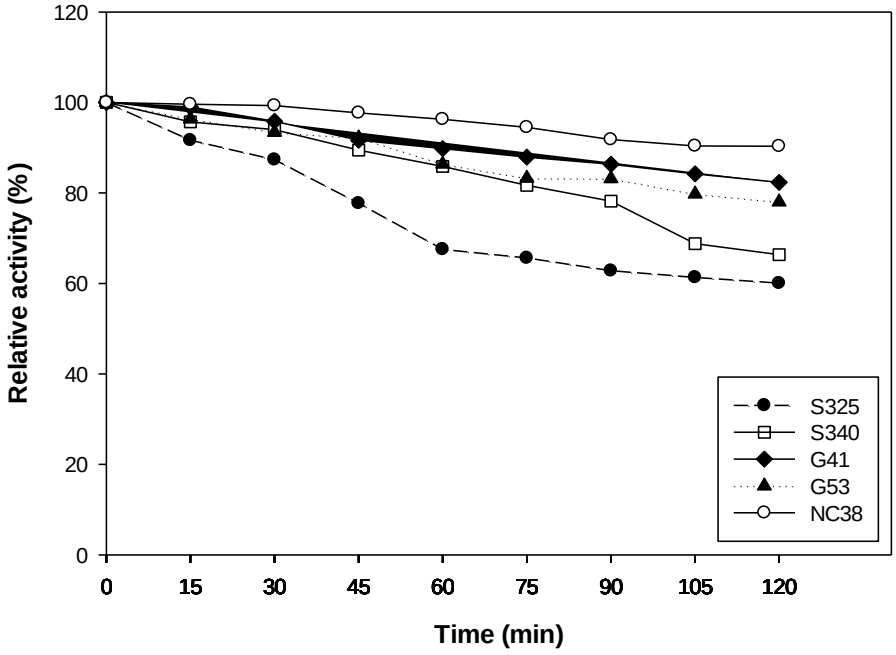

Figure 3.19. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 8, 50°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 220 IU/ml, after 30 min the activity decreased to 87%, the enzyme lost 14% of activity after a period of 60 min. Activity declined rapidly to 63% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 60% of activity. For the variant S340, the initial activity recorded was 214 IU/ml, following 30 min of incubation, the variant maintained 94% relative activity. After 60 min, the enzyme activity decreased by 14%. At T_{90}, the variant lost 22% of activity (Fig. 3.19). After 120 min of incubation, 66% of activity was retained. The activity recorded at T_0 for the G41 variant was 173 IU/ml, there was a minimal reduction in activity after 30 min. After 60 min, the variant lost 10% enzymatic activity. Eighty six percent and 82% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53 had an initial activity of 184 IU/ml. Following 30 min of activity, the enzyme activity decreased slightly to 93%. After 60 min of incubation at pH 8, 50°C, the enzyme activity declined to 86%. After 90 min the activity decreased by 17% and the enzyme retained 78% of activity after 120 min. The variant NC38 had an activity of 249 IU/ml at T_0. The enzyme retained 96% of activity after 60 min. This variant retained 92% and 90% activity after 90 and 120 min at pH 8, 50°C respectively. This enzyme maintained a high activity over 120 minutes losing only 10% of activity, the G41 and G53 variants displayed good stability as well, maintaining 82% and 78% of activity respectively after 120 min. From the results above it can be determined that the S325 and S340 variants are more stable at 50-60°C. The S325 variant was the least stable.

3.3.2.18. pH 8, 60°C

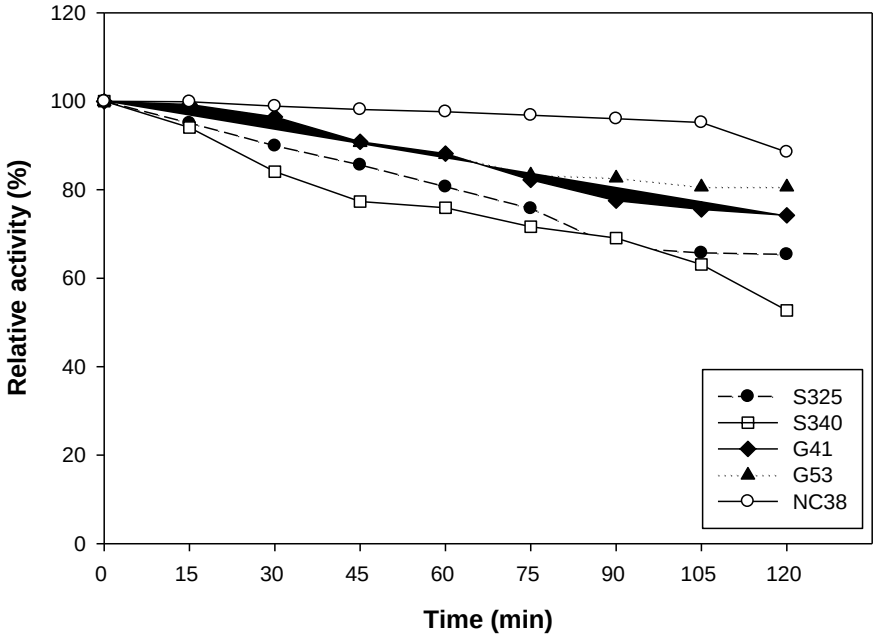

Figure 3.20. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 8, 60°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

For the xylanase variant S325 the initial enzyme activity recorded at pH 8, 60°C was 218 IU/ml. Following incubation for a period of 30 min there was a steep decline in activity to 67%. After 60 min the variant retained 61% activity. A significant decline to 45% relative activity was noted after 90 min of incubation with a final relative activity at 120 min showing a decline of 62%. Under the same experimental conditions, the variant S340 had an initial activity of 89 IU/ml. After 30 min of incubation, the variant lost 29% of activity (Fig. 3.20). After 60 min of incubation, the enzyme activity decreased to 65% of its initial activity. From T_{60} to T_{90} the activity rapidly declined to less than 60% of its initial activity. At 120 min, 41% of activity was retained. At $T_{0,}$ the activity noted for the G41 variant was 93 IU/ml, the enzyme lost minimal catalytic activity after 30 min, the activity declined to 88% after 60 min. At 90 min, the activity decreased by 22%. Seventy four

percent of activity was retained after 120 min of incubation. An initial activity of 91 IU/ml was recorded for the G53 variant. Following 30 min of activity, the enzyme activity decreased to 93% of the initial activity. After 60 min of incubation at pH 8, 60°C, the enzyme activity declined to 83%. At 90 min the activity decreased by 20% and the enzyme retained 75% of activity after 120 min. The variant NC38 had an activity of 265 IU/ml at T_0. The enzyme displayed a minimum loss of activity after 60 min. This variant retained 92% relative activity after 120 min at pH 8, 60°C. The variants, G41, G53 and NC38 were able to maintain a high level of stability under these conditions with the lowest stability noted by the S325 variant which only retained 38% activity at the end of the 120 min incubation period. As explained in the previous section, S325 and S340 variants are able to retain a high level of stability at 50-60°C. All enzymes retained greater than 60% activity after 90 min.

3.3.2.19. pH 8, 70°C

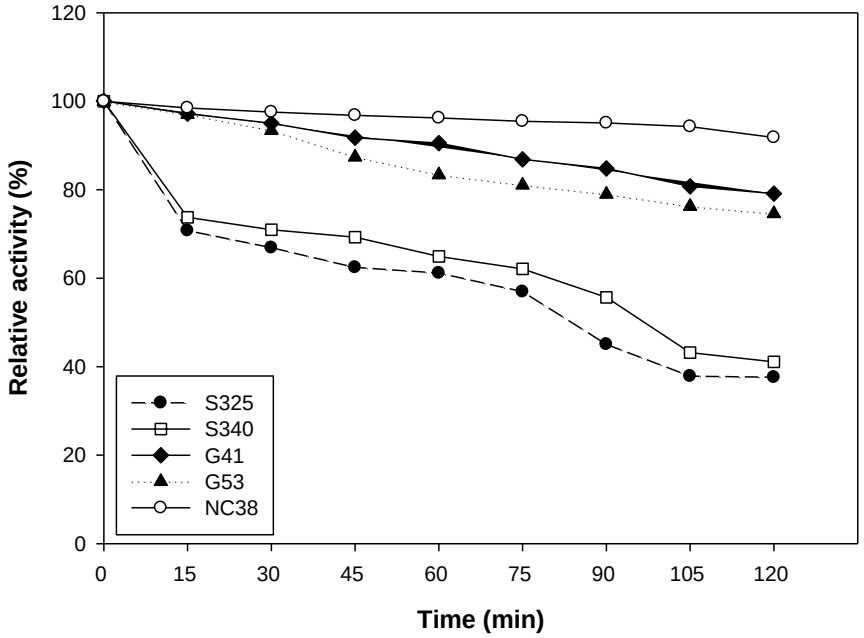

Figure 3.21. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 8, 70°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 109 IU/ml, after 30 min the activity rapidly decreased to 67%, the enzyme lost 40% of activity after a period of 60 min. Activity declined to 45% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 38% of activity. For the variant S340, the initial activity recorded was 89 IU/ml, following 30 min of incubation, the variant lost 30% of activity. After 60 min, the enzyme activity decreased to 65%. At 90 min, the activity was reduced to 56% relative to the initial activity. At 120 min, 40% of activity was retained. The activity recorded at T_0 for the G41 variant was 92 IU/ml, the activity declined to 95% after 30 min. At 60 min, the activity decreased to 91% activity. This variant retained 85% and 79% of activity after an incubation period of 90 and 120 min respectively. The

xylanase variant G53 had an initial activity of 90 IU/ml. Following 30 min of activity, the enzyme activity decreased to 93%. After 60 min of incubation at pH 8, 70°C, the enzyme activity declined minimally to 83% relative activity. At 90 min the activity decreased by 21% (Fig. 3.21) and the enzyme retained 75% of activity after 120 min. The variant NC38 had an activity of 265 IU/ml at T_0. The enzyme lost 4% of activity after 60 min. This variant retained 95% and 92% activity after 90 and 120 min respectively at pH 8, 70°C. This enzyme maintained a high activity over 120 minutes as did the G53 and G41 variant while the S325 and S340 variants lost 60% of their relative activities after 120 min. S325 and S340 variants displayed a gradual loss in stability at 15 min intervals. The G41 and G53 variants displayed similar characteristics retaining greater than 80% of activity after 120 min. The NC38 variant was the most stable.

3.3.2.20. pH 8, 80°C

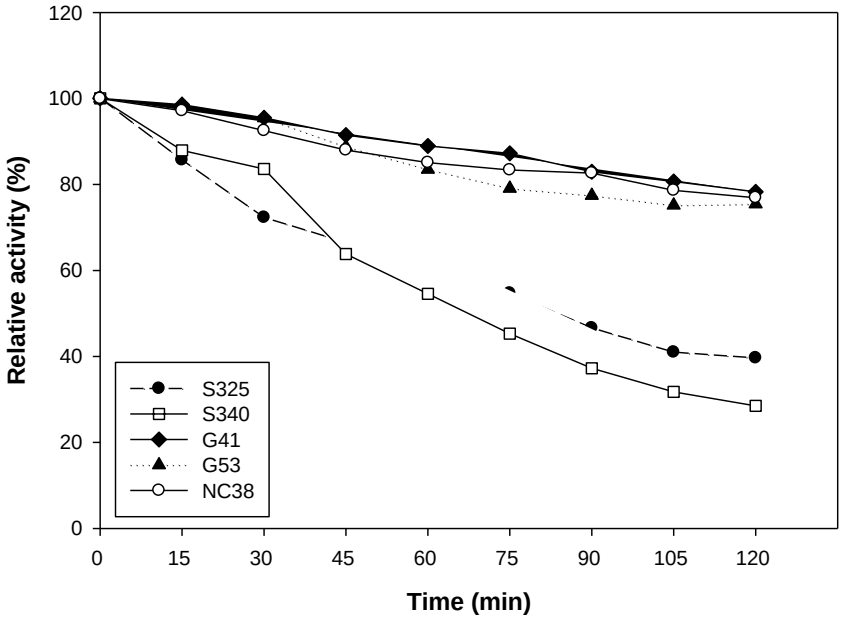

Figure 3.22. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 8, 80°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

For the xylanase variant S325 the initial enzyme activity recorded at pH 8, 80°C was 44 IU/ml. Following incubation for a period of 30 min, the activity decreased by 28%, the enzyme lost 40% of activity after 60 min. A decline to 47% relative activity was noted after 90 min of incubation with a final relative activity at 120 min showing a decline of 60%. Under the same experimental conditions, the variant S340 had an initial activity of 38 IU/ml. After 30 min of incubation, activity decreased to 84% of the initial activity. After 60 min of incubation, the enzyme activity decreased to 55% of its initial activity. At 90 min, the activity was reduced less than 40% relative to the initial activity. At 120 min, 29% of activity was retained. At $T_{0,}$ the activity noted for the G41 variant was 37 IU/ml, the enzyme lost 4% activity after 30 min, the activity declined minimally to 89% after 60 min. At T_{90}, the activity decreased by 17%. Seventy eight percent of activity was retained after

120 min of incubation. An initial activity of 37 IU/ml was recorded for the G53 variant. Following 30 min of incubation, the enzyme activity decreased to 95% of the initial activity. After 60 min of incubation at pH 8, 80°C, the enzyme activity declined to 84%. At T_{90}, the activity decreased by 23% and the enzyme retained 75% of activity after 120 min (Fig. 3.22). The variant NC38 had an activity of 247 IU/ml at T_0. The enzyme lost 15% of activity after 60 min. This variant retained 77% relative activity after 120 min at pH 8, 80°C. The results obtained under these conditions were similar to those obtained at pH 7, 80°C. The G41 and G53 variants were able to maintain nearly 80% of their activity under these conditions. The S325 and S340 variants were able to retain greater than 60% of catalytic activity after 60 min but thereafter activity declines steadily.

3.3.2.21. pH 9, 40°C

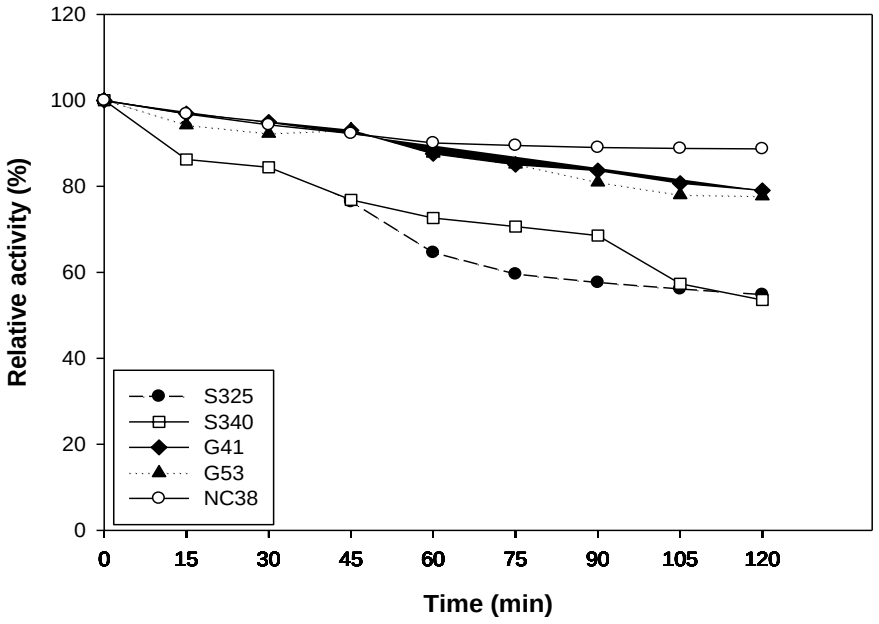

Figure 3.23. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 9, 40°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 214 IU/ml, after 30 min the activity decreased to 86%, the enzyme lost 35% of activity after a period of 60 min. Activity declined to less than 60% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 55% of activity. For the variant S340, the initial activity recorded was 164 IU/ml, following 30 min of incubation, the variant lost 16% of activity. After 60 min, the enzyme activity decreased to 73%. At T_{90}, the activity was reduced to 69% relative to the initial activity. At 120 min, 54% of activity was retained (Fig. 3.23). The activity recorded at T_0 for the G41 variant was 178 IU/ml, the activity declined minimally to 95% after 30 min. At T_{60}, the activity decreased to 88%. Eighty four percent and 79% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53 had an initial activity of 178 IU/ml. Following 30 min of activity, the catalytic activity decreased to 92%. After 60 min of incubation at pH 9, 40°C, the enzyme activity declined to 88%. At 90 min the activity decreased by 20% and the enzyme retained 78% of activity after 120 min. The variant NC38 had an activity of 253 IU/ml at T_0. The enzyme lost 10% of activity after 60 min. This variant retained 89% OF activity after 120 min at pH 9, 40°C. This enzyme variant maintained a high rate of reactivity over 120 minutes. The S340 xylanase displayed a sharp decline in activity after 90 min, these variants lost nearly 50% of its relative activity after 120 min. The G41 and G53 variants retained greater than 80% of activity after 120 min.

3.3.2.22. pH 9, 50°C

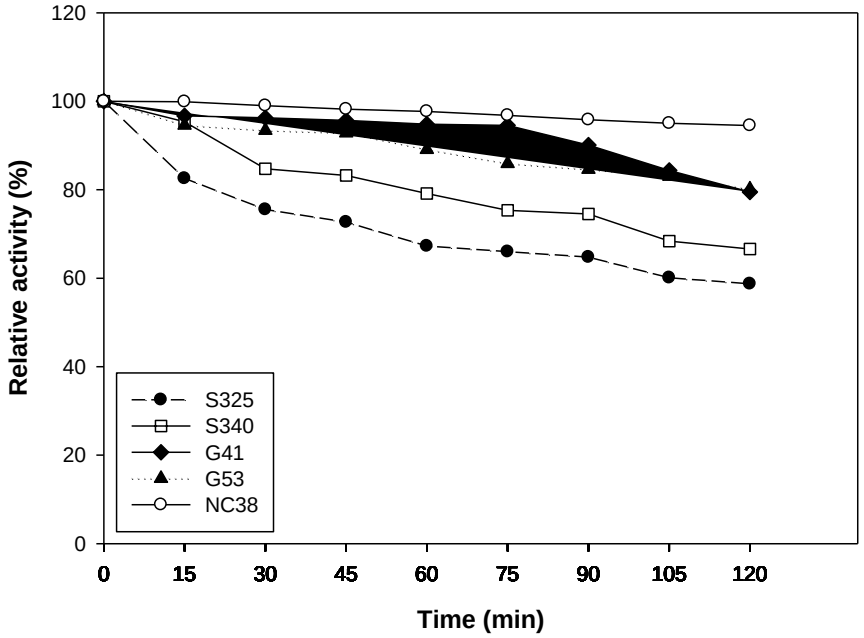

Figure 3.24. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 9, 50°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 219 IU/ml, after 30 min the catalytic activity decreased significantly to 76%, the enzyme lost 33% of activity after a period of 60 min. Activity declined to 65% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 59% of activity. For the variant S340, the initial activity recorded was 183 IU/ml, following 30 min of incubation, the variant lost 15% of activity. After 60 min, the enzyme activity decreased to 79%. At 90 min, the activity was reduced to 75% relative to the initial activity. At 120 min, 67% of activity was retained. The activity recorded at T_0 for the G41 variant was 173 IU/ml, the activity declined to 96% after 30 min. At T_{60} a relative activity of 95% was maintained. Ninety percent and 79% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53 had an initial activity of 172 IU/ml. Following 30 min of

activity, the enzyme activity decreased to 93%. After 60 min of incubation at pH 9, 50°C, the enzyme activity declined to 89%. At T_{90} the activity decreased by 15% (Fig. 3.24) and the enzyme retained 80% of catalytic activity after 120 min. The variant NC38 had an activity of 244 IU/ml at T_0. This xylanase variant retained high activity over the 120 min incubation period with minimum loss in enzymatic activity, retaining 95% of activity at the end of the experiment. Under these conditions, all the variants were able to maintain above 60% of their initial catalytic activities. The S325 and S340 variants were more stable under the conditions than at 40°C. G41 and G53 variants retained greater than 80% of activity after 120 min.

3.3.2.23. pH 9, 60°C

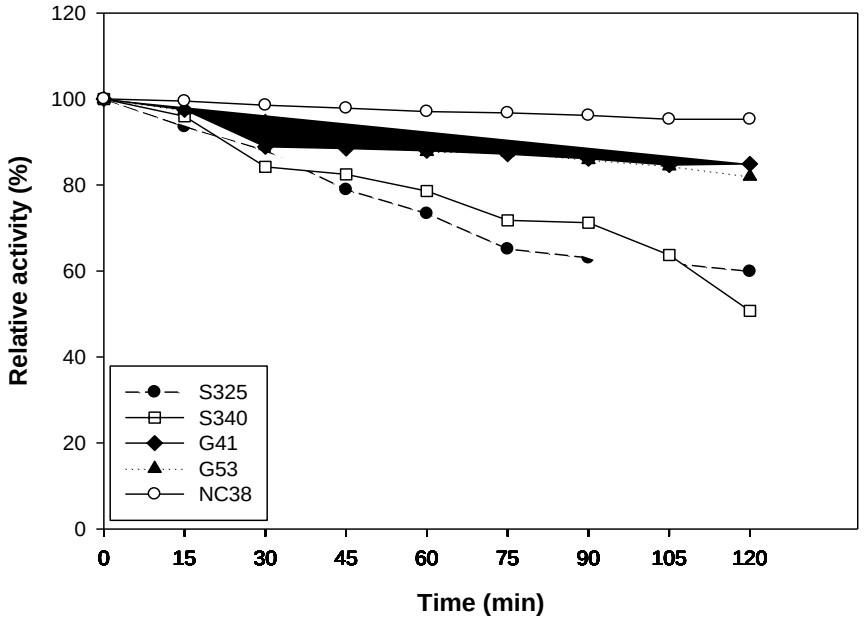

Figure 3.25. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 9, 60°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 218 IU/ml, after 30 min the activity decreased to 88%, the enzyme lost 27% of activity after a period of 60 min. Activity declined to 63% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 60% of activity. For the variant S340, the initial activity recorded was 93 IU/ml, following 30 min of incubation, the variant maintained 84% relative activity. After 60 min, the enzyme activity decreased by 21%. At T_{90}, the variant lost 30% of activity (Fig. 3.25). After 120 min, 51% of activity was retained. The activity recorded at T_0 for the G41 variant was 94 IU/ml, the activity declined to 89% after 30 min. At T_{60}, the activity decreased to 88% activity. Eighty six percent and 85% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53 had an initial activity of 93 IU/ml. Following 30 min of activity, the enzyme activity decreased to 95%. After 60 min of incubation at pH 9, 60°C, the enzyme activity declined to 88%. At T_{90} the activity decreased by 14% and the enzyme retained 82% of activity after 120 min. The variant NC38 had an activity of 251 IU/ml at T_0. The enzyme retained 97% of activity after 60 min. This variant retained 96% and 95% activity after 90 and 120 min respectively at pH 9, 60°C. The NC38 xylanase variant maintained a high activity over 120 minutes losing only 5% of activity, the G41 and G53 variants displayed good enzymatic stability , maintaining greater than 80% of activity after 120 min. The S340 variant was least stable under these conditions as compared with the other variants was still able to retain 80% of activity after 90 min after which there was a decline in activity. The S325 xylanase showed a steady decline in activity after each 15 min interval after 30 min.

3.3.2.24. pH 9, 70°C

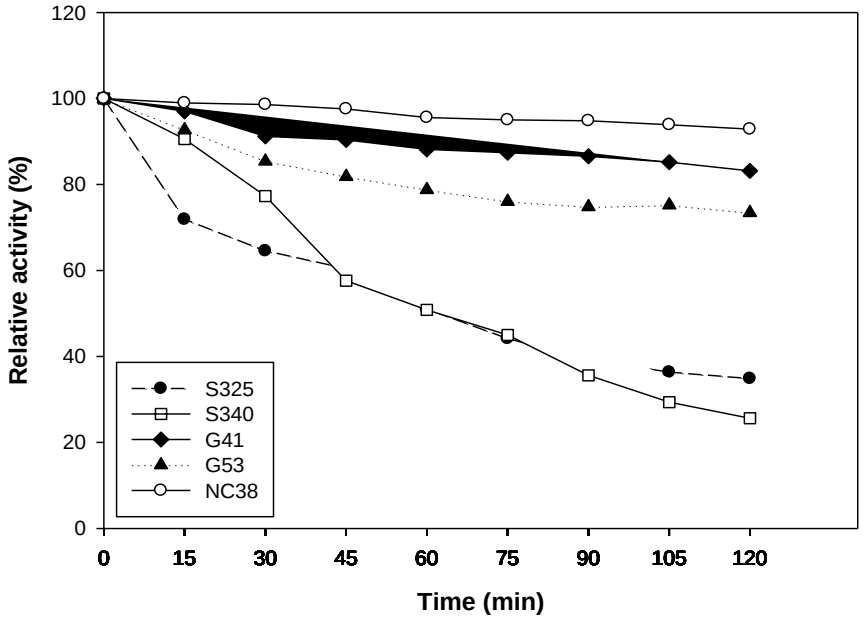

Figure 3.26. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 9, 70°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

For the xylanase variant S325 the initial enzyme activity recorded at pH 9, 70°C was 111 IU/ml. Following incubation for a period of 30 min, there was a 35% decrease in catalytic activity, the enzyme lost 50% of activity after 60 min. A significant decline to 39% relative activity was noted after 90 min of incubation with a final relative activity after 120 min showing a decline of 65%. Under the same experimental conditions, the variant S340 had an initial activity of 89 IU/ml. After 30 min of incubation, the variant lost 23% of activity. After 60 min of incubation, the enzyme activity decreased to 50% of its original activity. At T_{90}, the activity was reduced to 36% activity relative to the initial activity. At T_{120}, 26% of activity was retained (Fig. 3.26). At $T_{0,}$ the activity noted for the G41 variant was 89 IU/ml, the enzyme lost 10% activity after 30 min, the activity declined to 88% after 60 min. At T_{90}, the activity decreased by 13%. Eighty three percent of

activity was retained after 120 min of incubation. An initial activity of 88 IU/ml was recorded for the G53 variant. Following 30 min of activity, the enzyme activity decreased to 85% of the initial activity. After 60 min of incubation at pH 9, 70°C, the enzyme activity declined to 79%. At T_{90} the activity decreased by 25% and the enzyme retained 73% of activity after 120 min. The variant NC38 had an activity of 255 IU/ml at T_0. The enzyme lost 4% of activity after 60 min. This variant retained more than 90% relative activity after 120 min at pH 9, 70°C. The G41 and G53 variants were able to maintain levels higher than 70% of their activity under these conditions. Although the S340 and S325 variants had higher initial activities, they lost more than 65% of activity by 120 min of incubation. The NC38 variant was the most stable under these conditions with a loss of only 10% in activity.

3.3.2.25. pH 9, 80°C

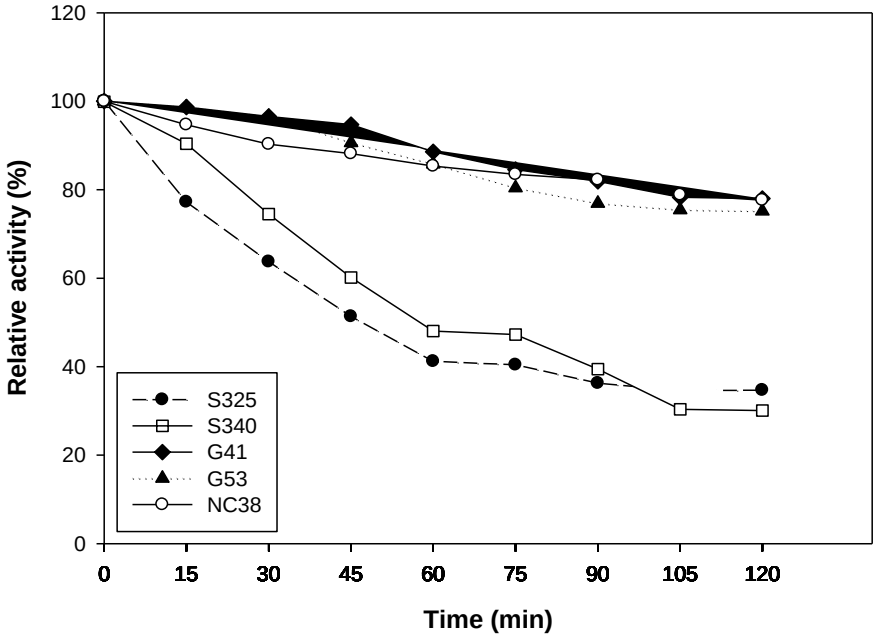

Figure 3.27. The pH and temperature stability of NC38 against xylanase variants; S325, S340, G41 and G53 at pH 9, 80°C. The remaining activities were expressed as percentages of the original activities with each point representative of duplicate determinations.

The initial enzyme activity of the variant S325 was 42 IU/ml, after 30 min the activity decreased to 64%, the enzyme lost 60% of activity after a period of 60 min. Activity declined to 36% relative activity after 90 min and when the enzyme was incubated for 120 min, it retained 35% of activity. For the variant S340, the initial activity recorded was 37 IU/ml, following 30 min of incubation, the variant lost 26% of activity. After 60 min, the enzyme activity decreased to 48%. At T_{90}, the activity was reduced to 39% relative to the initial activity. At T_{120} min, 30% of activity was retained. The activity recorded at T_0 for the G41 variant was 34 IU/ml, the activity declined to 94% after 30 min. At T_{60}, the activity decreased to 89%. Eighty two percent and 78% of activity was retained after an incubation period of 90 and 120 min respectively. The xylanase variant G53

had an initial activity of 35 IU/ml. After 60 min of incubation at pH 9, 80°C, the enzyme activity declined to 86%. At T_{90} the activity decreased by 23% (Fig. 3.27) and the enzyme retained 75% of activity after 120 min. The variant NC38 had an activity of 241 IU/ml at T_0 which was significantly higher than the activities of the other variants. The enzyme lost 15% of activity after 60 min. This variant retained 82% and 78% activity after 90 and 120 min respectively at pH 9, 80°C. The G41, G53 and NC38 variants were able to maintain more than 70% of their respective initial activities whilst the S325 and S340 variants displayed a rapid decline in activity over the 120 min period of incubation. The above conditions were the harshest that the variants were subjected to in this study. From the above result it can be deduced that the S325 and S340 variants are more stable at temperatures between 50 and 60°C and at a lower pH range. The G41 and G53 variants were able to withstand the changes in temperature and pH with little deviation in stability are thus are well suited to be utilized in industrial processes. The NC38 xylanase variant was the most stable overall of all the variants characterized and has proven to be both highly alkali stable and thermostable.

CHAPTER 4. GENERAL DISCUSSION

The worldwide industry requirement for xylanases has considerably increased in the recent decades. The practical and industrial applications of these enzyme catalysts cannot be achieved unless they are available in adequate quantities and are produced cost-effectively. The natural production of these enzyme catalysts fails to meet the high demand, mainly due to low yields and unsuitability of the conventional industrial fermentation processes with the growth requirements of many microorganisms. Therefore, heterologous protein expression is considered to be a vital strategy for the production of xylanases at the industrial level (Sibtain *et al*., 2009).

E. coli does not provide sufficient expression of xylanases. It has however, been found to be a suitable host for the cloning of fungal xylanase genes and will therefore continue to be used for the in-depth study and investigation of the xylanase gene structure and for the functional enhancement of the enzymes by protein engineering techniques. Filamentous fungi have also been found to be proficient production systems for xylanases both by heterologous and homologous gene expression using their native promoters to express the enzymes at high concentrations (Sibtain *et al*., 2009). The limiting factors for their usage are that the rate of enzyme synthesis depends on the type of solid substrates and the rate of hydrolysis of these substrates and that the production of enzyme occurs after several days. (Cesar and Msar, 1996). *S. cerevisiae* secretes high amounts of xylanases into the culture medium. Since it has already been well-established as a microorganism suitable for industrial application, it can be used opportunely for the economical production of xylanases at an industrial production level (Sibtain

et al., 2009). In addition, the absence of contaminating cellulases makes it an attractive host for the production of xylanases for application in paper and pulp industry.

As an alternative to bacterial and fungal hosts, *P. pastoris* was selected in this study since it has emerged as an exceptional host for the commercial production of xylanases, due to extremely high expression levels under the AOX1 promoter. However, one of the widely-used promoters has limitations for use in large scale production due to health and fire hazards of methanol which is used as an inducer of its promoter. This setback can easily be overcome by employing a constitutive promoter. Thus in this study the pBGP1 vector with the GAP promoter was employed.

The xylanase variants (S325, S340, G41, G53) (Stephens *et al*., 2009; 2014) were isolated from *E. coli* by molecular methods and with the use of a vector containing a constitutive promoter. They were then transformed into *P. pastoris* for extracellular expression. Following cloning of the xylanases, these enzymes were expressed in *P. pastoris* using YPD media which is a relatively inexpensive medium and results in high cell densities within 2-3 days.

The xylanase genes and the pBGP1 vector were linearized using *Eco*RI and *Xba*I endonucleases to create compatible DNA sequences for ligation. The vector and insert gene were then ligated with T4 DNA ligase using standard molecular techniques (Sambrook, 1989). The ligation mixture was then used to transform competent *E. coli* cells and positive transformants were selected on the basis of growth after incubation on Luria Bertani agar plates containing zeocin. Plasmids were isolated from the positive transformants that were transferred to selective media for growth. A fraction of the isolated plasmids were restricted using *Eco*RI and *Xba*I and analysed by

electrophoresis to confirm vector-insert ligation. This was followed by transforming electrocompetent *P. pastoris* cells with the plasmids that were isolated from the *E. coli* cells and screening on selective media (YPD plates containg RBB-xylan and zeocin). Following incubation, positive transformants were identified on the basis of the production of zones of hydrolysis around the colonies present. Numerous colonies presented with clearing around them and these were then isolated and grown in selective media for further experiments. From the results obtained for the SDS-PAGE and zymogram experiments it was determined that the xylanase variants displayed a high level of affinity for the xylan substrate and were able to degrade the xylan effectively and efficiently.

The development of several microorganisms and eukaryotic cell lines as hosts for heterologous gene expression has led to a growing number of options for the production of recombinant proteins. For industrial applications the system of choice should produce an optimal amount of authentic bioactive material within a short time period in a reproducible and stable production process (Cereghino and Cregg, 1999). Therefore, expressions systems have to meet several preconditions to allow such an efficient and economical production of a recombinant protein. The cells should be robust and easy to handle, and they should grow to high cell densities on low-cost media containing cheap energy and carbon sources. Adequate expression vectors must be available and the host must be capable of synthesizing the desired protein either intracellularly or, if certain eukaryotic modifications are required, extracellularly (Cregg *et al*., 2009). If it fails to assure a correct folding, laborious and cost-intensive in vitro refolding and modification processes might have to be applied.

As unicellular eukaryotic organisms, yeasts combine the ease of genetic manipulation with the ability to perform eukaryotic processing steps on the polypeptides expressed. When the traditional *S. cerevisiae* system was used for heterologous gene expression, disadvantages such as strain instability during the production process, low productivity, and an inappropriate glycosylation were encountered in several cases. Therefore a search was initiated to find alternative non-*Saccharomyces* yeasts suitable as hosts for recombinant protein production.

Methylotrophic yeasts meet the characteristics generally desired for an efficient industrial host, including rapid growth on low cost media, stringent control of a reproducible production process, high expression levels of the desired product using both intracellular and a secretory expression mode, proper folding, and appropriate modifications. Appropriate glycosylation was observed in several recombinant proteins that were found to be over-glycosylated when secreted from a *S. cerevisiae* host. This indicates that the methylotrophs are able to express structures similar to the mammalian high mannose type of N-linked glycosylation. The low amounts of genuine contaminating proteins and other compounds provide for easy purification of secreted recombinant products. Since virus particles, pyrogens, pathogens, or cell-derived components with an oncogenic potential are not observed in these yeast species, reliable expression systems are at hand that assure a high safety standard for products considered for administration to humans (Smith, 1994).

Several xylanases have been expressed in *P. pastoris*. Zhou *et al*. (2008) have reported a xylanase activity of 49.1 IU ml^{-1} in *E. coli*. Yin *et al*. (2008) have also observed a maximum xylanase activity of 47.1 IU ml^{-1} in *E. coli.* Stephens *et al*. (2014) reported expression of the xylanase in *E. coli* was

197 nkat/ml for the G53 clone which was the highest of activity obtained for all mutants. Wakelin (2009) demonstrated that xylanase activity for S340 was increased by 14.9% to 921.8 nkat/ml, equivalent to 55 IU/ml, which is significantly lower compared to xylanase expression in *P. pastoris* for all clones in this study.

Enzymes produced in *P. pastoris* are partially purified and have little or no accessory proteins attached to them, therefore the extracted and concentrated enzyme could be utilized for characterization. For functional characterization, an unpurified enzyme was used and results indicated that even the non-purified enzyme shows highly specific xylanase activity. Therefore, *T. lanuginosus xyn*A produced from *P. pastoris* has an advantage of showing xylanase activity that is practically free of other activities (Zhao *et al*., 2011).

The xylanase variants were characterized to determine their optimal pH and temperature as well as their respective stabilities under varying conditions of pH (5-9) and temperature (40-80°C). The NC38 xylanase was used as a positive control to confirm the accuracy of methods since this recombinant enzyme has shown to be alkali stable and thermostable (Birijlall *et al*., 2011) with high expression levels. It was determined that the S325 variant displayed optimal activity at pH 6, 60°C; the S340 variant utilized the substrate optimally at pH 5, 40°C. The G41 variant had optimal activity at pH 6, 60°C; the G53 xylanase had a pH and temperature optima of pH 7, 60°C and the NC38 xylanase variant displayed optimal activity at pH 7, 70°C. All the variants were able to retain catalytic activity even at pH 10 and 60°C.

The pH and temperature stabilities of the respective enzymes were investigated, the S325 variant was exceptionally stable at a pH between 5 and 7 and temperature range of 40-80°C and retained

a minimum of 40% of activity at higher pH and temperature after an incubation period of 90 min. The S340 variant was the least thermostable and alkali stable from all four variants, it however retained 40% of activity when subjected to conditions of pH 9, 80°C after 90 min. The G41 and G53 were highly stable under the pH and temperature conditions that they were subjected to. The enzymes were able to retain 80% of activity at pH 9, 80°C after 120 min.

The xylanases from thermophilic fungi are reported to have good stability at 20°C, pH 8 (Gomes *et al*., 1993) and 70°C, pH 6.5 (Singh *et al*., 2000), in a study conducted by Stephens *et al*., 2009, it was observed that this inherent stability was significantly reduced when the pH of the growth media and the temperature was simultaneously increased. At 60°C (pH 8) the native xylanase retained 50% of activity after 180 min of incubation and at pH 9 the enzyme retained 45% activity. Their stability steadily declined with an increase in temperature conditions (70°C - 90°C) and when the alkalinity of the surrounding media was elevated (pH 10).

In the same study, it was discovered that the recombinant xylanases displayed the following characteristics when transformed in *E*. *coli* as an expression host. Xylanase G41 retained 75% of activity after 90 min at 80°C compared to 80% retention of activity at pH 9, 80°C in *P*. *pastoris* after incubation for 90 min. The xylanase G53, retained 93% activity at pH 10 for 90 min while it retained 80% of activity at pH 9, 80°C in *P. pastoris.* S340 and S325 xylanases retained 54% and 85% activity respectively at 80°C and 60% at pH 10 after 90 min in *E. coli* while 40% of activity was retained at pH 9, 80°C after 90 min for both variants.

Unknown factors in the crude enzyme preparations such as ingredients of culture media and *E. coli* proteins or cell metabolites may have interacted with the xylanase and in doing so, may

have affected enzyme stability positively but in contrast, the enzymes produced in P. *pastoris* have little or no attached cell components and therefore extraction of required metabolic products such as the enzymes require minimal downstream processing making the process more cost efficient.

These variants maintained their catalytic efficacy at the higher ranges in temperature and pH for the length of the incubation period and their stabilities were comparable to the NC38 xylanase as well as industrial enzymes such as Luminase (Stephens *et al*., 2007; BASF, 2014) and *T. reesei* xylanase (Goncalves *et al*., 2015) presently being utilized in the pulp and paper industry for the improvement of the process as well as to meet with the environmentally-friendly approach to paper manufacturing that is now highly recommended by governments and environmental organizations.

Optimal temperature, pH stability and kinetics of xylanases as biocatalysts play a crucial role for their elective usage in industrial processes (Pillay, 2007; Sibtain *et al*., 2009). The modified xylanase enzymes produced by the methylotrophic yeast and fungal strains have displayed the ability to possess the equivalent of or even better catalytic characteristics than the native enzymes. Thermostable enzymes are an essential requirement in various industrial applications; but unfortunately thermophilic microbes are not easily propagated at large scale due to extreme fermentation conditions that are required for their culture (Ahmed *et al*., 2009; Sibtain *et al*., 2009). *P. pastoris* and *T. reesei* (de Faria *et al*., 2002) expressed the genes for thermostable xylanases efficiently with high secretion levels. Likewise, the xylanase genes from anaerobic

microbial sources are expressed effectively with success in such hosts that can be utilized in the fermentation industries.

There is a potential in the exploration of novel fungal hosts that are capable of producing recombinant xylanases in an economical manner and at high yields. Further technological advancements in the improvement and development of fungal expression systems by genetic engineering techniques will be of considerable aid in the hyper-expression of heterologous proteins in large scale production for potential application in industrial processes. Application of the enzymes for industrial processes will be established in subsequent studies.

REFERENCES

1. **Adrio, J. L. & Demain, A. L.** 2014. Microbial enzymes: tools for biotechnological processes. *Biomolecules* **4:** 117-139.

2. **Ahmad, M., Hirz, M., Pichler, H. and Schwab, H.** 2014. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Applied Microbiology and Biotechnology* **98**: 5301-5317.

3. **Ahmed, S., Riaz, S. & Jamil, A.** 2009. Molecular cloning of fungal xylanases: an overview. *Applied Microbiology and Biotechnology* **84:** 19-35.

4. **Andleeb, S., Latif, F., Afzal, S., Mukhtar, Z., Mansoor, S. and Rajoka, I.** 2008. Cloning and expression of *Chaetomium thermophilum* xylanase 11-A gene in *Pichia pastoris*. *The Nucleus* **45**: 75-81.

5. **Anon.** 2015**.** Industrial enzymes market worth 6.2 billion USD by 2020**. India**. Viewed 12-02-2016.
http://www.marketsandmarkets.com/PressReleases/industrial-enzymes.asp

6. **Arnau, C., Ramon, R., Casas, C. and Valero, F.** 2010. Optimization of the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. *Enzyme and Microbial Technology* **46**: 494–500.

7. **Atomi, H., Sato, T. & Kanai, T.** 2011. Application of hyperthermophiles and their enzymes. *Current opinion in biotechnology* **22:** 618-626.

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

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

10. **Balamurugan, V., Reddy, G.R. and Suryanarayana, V.V.S.** 2007. *Pichia pastoris*: A notable heterologous expression system for the production of foreign proteins—Vaccines. *Indian Journal of Biotechnology* **6**: 175-186.

11. **BASF** (Badische Anilin und Soda Fabrik) product catalogue 2014: Luminase PB100 and Luminase PB 200 Pulp and Paper Technology, Germany.

12. **Beg, Q.K., Kapoor, M., Mahajan, G. and Hoondal, S**. 2001. Microbial xylanases and their industrial applications: a review. *Applied Microbiology and Biotechnology* **56**: 326-338.

13. **Berrin, J., Williamson, G., Puigserver, A, A., Chaix, J., MacLauchlan, W.R. and Judge, 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.

14. **Bhagat, D., Dudhagara, P. and Desai, P**. 2014. Production and characterization of thermo-alkalistable xylanase from geothermal spring isolate *CIB Tech Journal of Biotechnology* **3**: 36-45.

15. **Bian, J., Peng, F., Peng, X.-P., Peng, P., Xu, F. and Sun, R.C.** 2013. Structural features and antioxidant activity of xylooligosaccharides enzymatically produced from sugarcane bagasse. *Bioresource Technology* **127:** 236-241.

16. **Biely, P., Mislovicova, D**. **and Toman, R.** 1988. Remazol Brilliant Blue-xylan, a soluble chromogenic substrate for the assay of endo-1, 4-β-xylanases. *Methods in Enzymology* **160**: 536-541.

17. **Birijlall, N., Ayyachamy, M., Kuttanpillai S. K., Permaul, K. and Singh, S.** 2011. High level expression of a recombinant xylanase by *Pichia pastoris* NC38 in a 5 L fermenter and its efficiency in biobleaching of bagasse pulp. *Bioresource Technology* **102**: 9723-9729.

18. **Boettner, M., Prinz, B., Holz, C., Stahl, U. and Lang, C**. 2002. High-throughput screening for expression of heterologous proteins in the yeast *Pichia pastoris*. *Journal of Biotechnology* **99**: 51-62.

19. **Bosetto, A., Justo, P. I., Zanardi, B., Venzon, S. S., Graciano, L., Dos Santos, E. L. and Simão, R. D. C. G.** 2016. Research progress concerning fungal and bacterial β-xylosidases. *Applied Biochemistry and Biotechnology* **178:** 766-795.

20. **Buckholz RG and Gleeson MAG.** 1991. Yeast systems for the commercial production of heterologous proteins. *Bio/Technology* **9**:1067–1072.

21. **Cereghino, J. L. and Cregg, J.M.** 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews* **24**: 45-66.

22. **Cesar, T. and Mrsa, V**. 1996. Purification and properties of the xylanase produced by *Thermomyces lanuginosus*. *Enzyme and Microbial Technology* **19**: 289-296.

23. **Chantasingh, D., Pootanakit, K., Champreda, V., Kanokratana, P. and Eurwilaichitr, L.** 2006. Cloning, expression, and characterization of a xylanase 10 from *Aspergillus terreus* (BCC129) in *Pichia pastoris*. *Protein Expression and Purification* **46**: 143-149

24. **Chen, C., Wu, P. Huang, C. and Cheng, K.** 2004. A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of an *Escherichia coli* phytase. *Enzyme and Microbial Technology* **35**: 315–320.

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

26. **Cherry, J.R. and Fidantsef, A.L.** 2003. Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology* **14**: 438–443.

27. **Chutani, P. and Sharma, K. K**. 2015. Biochemical evaluation of xylanases from various filamentous fungi and their application for the deinking of ozone treated newspaper pulp. *Carbohydrate Polymers* **127:** 54-63.

28. **Collins, T., Gerday, C. and Feller, G.** 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol Review* **29**: 3-23.

29. **Collins, T., Hoyoux, A., Dutron, A., Georis, J., Genot B., Dauvrin, T., Arnaut, T., Gerday, C. and Georges, F**. 2006. Use of glycoside hydrolase family 8 xylanases in baking. *Journal of Cereal Science* **43**: 79–84.

30. **Cregg, J. M., Tolstorukov, I., Kusari, A., Sunga, J., Madden, K. and Chappell, T**. 2009. Expression in the yeast *Pichia pastoris*. *Methods in Enzymology* **463:** 169-189.

31. **Damaso,M.C.T.,Almeida,M.S.,Kurtenbach,E.,Martins,O.B.,Pireira,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.

32. **Darby, R. A., Cartwright, S. P., Dilworth, M. V. & Bill, R. M**. 2012. Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris* (review). *Recombinant Protein Production in Yeast: Methods and Protocols* **14:** 11-23.

33. **De Faria, F., Te'o, V., Bergquist, P., Azevedo, M. & Nevalainen, K.** 2002. Expression and processing of a major xylanase (XYN2) from the thermophilic fungus *Humicola grisea var. thermoidea* in *Trichoderma reesei*. *Letters in Applied Microbiology* **34:** 119-123.

34. **Demain, A. L. & Vaishnav, P.** 2009. Production of recombinant proteins by microbes and higher organisms. *Biotechnology advances* **27:** 297-306.

35. **Demirjian, D. C., Moris-Veras, F. and Cassidy, C.S.** 2001. Enzymes from Extremophiles. *Current Opinion in Chemical Biology* **5**: 144-151.

36. **Deng, P., Li, D., Cao, Y., Lu, W. and Wang, C.** 2006. Cloning of a gene encoding an acidophilic endo-1,4-xylanase obtained from *Aspergillus niger* CGMCC1067 and constitutive expression in *Pichia pastoris*. *Enzyme and Microbial Technology* **39**: 1096-1099.

37. **Dominguez, A., Ferminan, L., Sanchez, M., Gonzalez, F. J., Perez-Campo, F.M., Garcia, S., Herrero, A.B., San Vicente, A., Cabello, J., Prado, M., Iglesias, F.J., Choupina, A., Burguillo, F.J., Fernandez-Lago, L. and Lopez, M.C.** 1998. Non-conventional yeasts as hosts for heterologous protein production. *International Microbiology* 1: 11-142.

38. **Duan, C., Verma, S. K., Li, J., Ma, X. and Ni, Y.** 2016. Combination of mechanical, alkaline and enzymatic treatments to upgrade paper-grade pulp to dissolving pulp with high reactivity. *Bioresource Technology* **200:** 458-463.

39. **Eckart, M.R. and Bussineaut, C.M.** 1996. Quality and authenticity of heterologous proteins synthesized in yeast. *Current Opinion in Biotechnology* **7**:525-530.

40. **Eijsink, V.G.H., Bjork, A., Gaseidnes, S., Sirevag, R., Synstad, B., Van den Burg, B. and Vriend, G**. 2004. Rational engineering of enzyme stability. *Journal of Biotechnology* **113**: 105-120.

41. **Eppendorf protocols online.** 2001**.** *Saccharomyces cerevisiae* transformation protocol. Protocol No. 4308 915.531 – 12/2001. Hamburg, Germany.

42. **Frost and Sullivan.** 2013**.** 360 degree perspective on the global pulp and paper water and wastewater treatment market. Viewed 25-04-2014. http://www.frost.com.

43. **Frost and Sullivan.** 2013**.** Analysis of the global pulp and paper market. Viewed 25-04-2014. http://www.frost.com.

44. **Garg, A.P., McCarthy, A.J. and Roberts, J.C.** 1996. Biobleaching effect of *Streptomyces thermoviolaceus* xylanase preparations on birchwood kraft pulp. *Enzyme and Microbial Technology*. **18**:261-267.

45. **Ge, T., Fu, S., Xu, L., Tang, Q., Wang, H., Guan, K. and Liang, G.** 2007. High density fermentation and activity of a recombinant lumbrokinase (PI239) from *Pichia pastoris*. *Protein Expression and Purification* **52**: 1-7.

46. **Gomes, J., Purkarthofer, H., Hayn, M., Kapplmiller, 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.

47. **Goncalves, A.R., Moriya, R.Y., Oliveira, L.R.M. and Saad, M.B.W.** 2008. Xylanase recycling for the economical biobleaching of sugarcane bagasse and straw pulps. *Enzyme and Microbial Technology* **43**: 157-163.

48. Goncalves, G. A., Takasugi, Y., Jia, L., Mori, Y., Noda, S., Tanaka, T., Ichinose, H. and Kamiya, N. 2015. Synergistic effect and application of xylanases as accessory enzymes to enhance the hydrolysis of pretreated bagasse. *Enzyme and Microbial Technology* **72:** 16-24.

49. **Gupta, S and Gupta, M.N.** 1993. Mechanisms of irreversible thermo-inactivation and medium engineering. pp. 114-122 ***In:*** Gupta, M.N. (ed.) Thermostability of enzymes. Narosa Publishing House, New Delhi.

50. **Hang, H., Ye, X., Guo, M., Chu, J., Zhuang, Y., Zhang, M. and Zhang, S.** 2009. A simple fermentation strategy for high-level production of recombinant phytase by *Pichia pastoris* using glucose as the growth substrate. *Enzyme and Microbial Technology* **44**: 185–188.

51. **Harris, A. D. & Ramalingam, C.** 2010. Xylanases and its application in food industry: a review. *Journal of Experimental Sciences:* **1 (7)**: 1-11

52. **Hibbert, E.G., Baganz, F., Hailes, H.C., Ward, J.M., Lye, G.J., Woodley, J.M. and Dalby, P.A.** 2005. Directed evolution of biocatalytic processes. *Biomolecular Engineering* **22**: 11-19.

53. **Invitrogen life technologies** 2002. pGAPZα *Pichia* expression vectors for constitutive expression of recombinant proteins. Invitrogen Life Technologies.

54. **Ilgen, C., Lin-Cereghino, J. and Cregg, J. M.** 2005. *Pichia pastoris*, pp: 143-162 ***In***: Gellissen, G (ed). Production of recombinant proteins–*Novel microbial and eukaryotic expression systems*. Wiley, Germany.

55. **Illias, R., Ramli, A. N. M., Low, K. O., Muhammad Mahadi, N., Abdul Murad, A. M. & Rabu, A.** 2014. Heterologous Expression of Proteins from Cold-Adapted Yeasts in Suitable Hosts: Methods and Applications, pp:481-495. ***In*:** Buzzini, P. & Margesin, R. (eds.) *Cold-adapted Yeasts: Biodiversity, Adaptation Strategies and Biotechnological Significance.* Springer, Berlin Heidelberg.

56. **Isil, S. and Nilufer, A.** 2005. Investigation of factors affecting xylanase activity from *Trichoderma harzianum* 1073 D3. *Brazilian Archives of Biology and Technology* **48**: 187-193.

57. **Jeffries, T. W.** 1996. Biochemistry and genetics of microbial xylanases. *Current Opinion in Biotechnology* **7**: 337-342.

58. **Johannes, T. W. & Zhao, H.** 2006. Directed evolution of enzymes and biosynthetic pathways. *Current opinion in microbiology* **9:** 261-267.

59. **Karlsson, O., Pettersson, B. and Westermark, U.** 2001. Linkages between residual lignin and carbohydrates in bisulphite (manefite) pulps. *Pulp and Paper Science* **27**: 310.

60. **Khandeparkar, R. and Bhosle, S.** 2007. Application of thermoalkophilic xylanase for *Arthrobacter sp*. MTCC5214 in biobleaching of kraft pulp. *Bioresource Technology* **98**: 897-903.

61. **Khristova, P., Kordsachia, O., Patt, R., Karar, I. and Khider, T.** 2006. Environmentally friendly pulping and bleaching of bagasse pulp. *Industrial Crops and Products* **23**: 131-139.

62. **Kirk, O., Borchert, T.V. and Fuglsang, C.C**. 2002 Industrial enzyme application. *Current Opinion in Biotechnology* **13**: 345-351.

63. **Kumar, L., Awasthi, G. & Singh, B.** 2011. Extremophiles: a novel source of industrially important enzymes. *Biotechnology* **10:** 121-135.

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

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

66. **Li, K., Azadi, P., Collins, R., Tolan, J., Kim, J.S. and Eriksson, K.L.** 2000. Relationships between activities of xylanases and xylan structures. *Enzyme and Microbial Technology.* **27**: 89–94.

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

68. **Lueking, A., Holz, C., Gotthold, C., Lehrach, H. and Cahill, D.** 2000. A System for Dual Protein Expression in *Pichia pastoris* and *Escherichia coli. Protein Expression and Purification* **20:** 372–378.

69. **Lundgren, K.R., Bergkvist, L., Hogman, S., Joves, H., Eriksson, G., Bartfai, T., Laan, J. V. D., Rosenberg, E. and Shoham, Y.** 1994. TCF Mill Trial on softwood pulp with Korsnas thermostable and alkaline stable xylanase T6*, FEMS Microbiology Review*. **13**: 365-341.

70. **Marrs, B., Delagrave, S. & Murphy, D.** 1999. Novel approaches for discovering industrial enzymes. *Current opinion in microbiology* **2:** 241-245.

71. **Mattanovich, D., Branduardi, P., Dato, L., Gasser, B., Sauer, M. & Porro, D.** 2012. Recombinant Protein Production in Yeasts, pp 329-358. **In:** Lorence, A. (ed.) *Recombinant Gene Expression.* Humana Press, Totowa, NJ.

72. **McCoy, M.** 2000. Novozymes emerges. *Chemical Engineering News.* **19**:23-25.

73. **Moshelion, M and Altman, A.** 2015**.** Current challenges and future perspectives of plant and agricultural biotechnology. *Trends in Biotechnology* **33**: 337-342.

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

75. **Nagar, S., Jain, R., Thakur, V. V. & Gupta, V. K.** 2013. Biobleaching application of cellulase poor and alkali stable xylanase from *Bacillus pumilus* SV-85S. *3 Biotechnology* **3:** 277-285.

76. **Novozymes.** 2008. The Novozyme annual report.

77. **Otten, L.G. and Quax, W.J.** 2005. Directed evolution: selecting today's biocatalysts. *Biomolecular Engineering* **22**: 1-9.

78. **Petranovic, D. and Vemuri, G.N.** 2009. Impact of yeast systems biology on industrial biotechnology. *Journal of Biotechnology* **144**: 204–211.

79. **Pikuta, E. V., Hoover, R. B. & Tang, J.** 2007. Microbial extremophiles at the limits of life. *Critical reviews in microbiology* **33:** 183-209.

80. **Pillay, S.** 2007**.** Improvement of thermostability of a fungal xylanase using error-prone polymerase chain reaction. M. Tech thesis, Durban University of Technology, Durban.

81. **Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A. and Amorim, D.S.** 2005. Xylanases from fungi: properties and industrial applications. *Applied Microbiology and Biotechnology* **67**: 577-591.

82. **Puls, J.** 1997. Chemistry and biochemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis, *Macromolecules Symposium* **120:** 183.

83. **Purkarthofer, H., Sinner, M. and Steiner, W.** 1993. Cellulase-free xylanase from *Thermomyces lanuginosus*: optimization of production in submerged and solid-state culture. *Enzyme Microbiology and Technology* **15**: 677-682.

84. **Resina, D., Serrano, A., Valero, F. and Ferrer, P**. 2004. Expression of a *Rhizopus oryzae* lipase in *Pichia pastoris* under control of the nitrogen source-regulated formaldehyde dehydrogenase promoter. *Journal of Biotechnology* **109**: 103-113.

85. **Roncero, B. and Valls, C**. 2009. Using both xylanase and laccase enzymes for pulp bleaching. *Bioresource Technology* **100**: 2032-2039.

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

87. **Sambrook, J., Fritsch, E.F. and Maniatis, T.** 1989. Molecular cloning: A laboratory manual (Second edition). Cold Spring Harbor Laboratory Press: New York.

88. **Sambrook J. and Russel D. W.** 2001. *Molecular Cloning* (3rd edition.) Cold Spring Harbor Laboratory Press: New York.

89. **Sarrouh, B., Santos, T. M., Miyoshi, A., Dias, R. & Azevedo, V**. 2012. Up-to-date insight on industrial enzymes applications and global market. *Journal of Bioprocessing & Biotechniques* **2012**: 1-10.

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

91. **Shallom, D. and Shoham, Y.** 2003. Microbial hemicellulases. *Current Opinion in Microbiology* **6**: 219–228.

92. **Shirkolaee, Y., Talebizadeh, A. and Soltanali, S.** 2008. Comparative study on application of *T. lanuginosus* SSBP xylanase and commercial xylanase on biobleaching of non-wood pulps. *Bioresource Technology* **99**: 7433-7437.

93. **Shivakumar, C.K., Krishnappa, M. and Thippeswamy, B.** 2012. Bioaccumulation potential of *Aspergillus niger* and *Aspergillus flavus* for removal of heavy metals from paper effluent. *Journal of Environmental Biology* **3**: 1063-1068.

94. **Sibtain, A., Saba, R., Amer, J.** 2009. Molecular cloning of fungal xylanases: an overview. *Applied Microbial Biotechnology* **84**: 19-35.

95. **Singh, S., Pillay, B. and Prior, B.A.** 2000. Thermal stability of β-xylanases produced by different *Thermomyces lanuginosus* strains*. Enzyme and Microbial Technology* **26**: 502–508.

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

97. **Smith, A**. 1994, *Gene expression in recombinant microorganisms: Bioprocess Technology.* Marcel Dekker, Inc., New York.

98. **Soltanali, S. and Shirkolaee, Y**. 2007. Biobleaching of bagasse pulp with xylanase enzymes and hydrogen peroxide. *Iranian Journal of Biotechnology* **5**: 3-5.

99. **Spencer, J.F.T., Ragout D.E., Spencer, A.L. and Laluce, C.** 2002. Non-conventional yeasts. *Applied Microbiology and Biotechnology* **58**: 147–156.

100. **Stephens, D.E.** 2007. Protein engineering of a fungal xylanase. D. Tech Thesis, Durban University of Technology, Durban.

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

102. **Stephens, D.E., Singh, S. and Permaul, K.** 2009. Error-prone PCR of a fungal xylanase for improvement of its alkaline and thermal stability. *FEMS Microbiology Letters* **293**: 42-47.

103. **Stephens, D.E., Khan, F.I., Singh, P., Bisetty, K., Singh, S., and Permaul, K.** 2014. Creation of thermostable and alkaline stable xylanase variants by DNA shuffling. *Journal of Biotechnology* **187**: 139-146.

104. **Strafford, J., Payongsri, P., Hibbert, E. G., Morris, P., Batth, S. S., Steadman, D., Smith, M. E., Ward, J. M., Hailes, H. C. and Dalby, P. A.** 2012. Directed evolution to re-adapt a co-evolved network within an enzyme. *Journal of Biotechnology* **157:** 237-245.

105. **Subramaniyan, S. and Prema, P.** 2000**.** Cellulase-free xylanases from *Bacillus* and other microorganisms, *FEMS Microbiology Letters* **183**: 11-15.

106. **Subramaniyan, S. and Prema, P.** 2002. Biotechnology of microbial xylanases: Enzymology, Molecular biology and application. *Critical Reviews in Biotechnology* **22 (1)**: 33-46.

107. **Sun, J., Lui, M., Weng, X., Qian, L. and GU, S.** 2007. Expression of recombinant *Thermomonospora fusca* xylanase A in *Pichia pastoris* and xylooligosaccharides released from xylans by it. *Food Chemistry* **104**: 1055-1064.

108. **Techapun, C., Poosaran, N., Watanabe, M. and Sasaki, K.** 2003. Thermostable and alkaline tolerant microbial cellulase- free xylanases produced from agricultural wastes and the properties required for use in pulp bleaching bioprocesses: A review. *Process Biochemistry* **38**: 1327-1340.

109. **Thermo Fisher Scientific.** 2009. Acetone precipitation of proteins TR0049.1, USA.

110. **Tsai, T.C. and Huang, C.** 2008. Overexpression of *Neocallimastix frontalis* xylanase gene in the methylotrophic yeast *Pichia pastoris* and *Pichia methanolica*. *Enzyme and Microbial Technology* **42**: 459-465.

111. **Viikari, L., Kantelinen, A., Poutanen, K. and Ranua, M.** 1990. Characterization of pulps treated with hemicellulolytic enzymes prior to bleaching, pp. 145-151. ***In***: Kirk, T.K., Chang, H.M. (Eds) *Biotechnology in pulp and paper manufacture*. Butterworth-Henemann, Stoneham, Mass, USA.

112. **Wakelin, K.** 2009**.** Overexpression and partial characterization of a modified fungal xylanase in *Escherichia coli*. M. Tech thesis, Durban University of Technology, Durban.

113. **Waterborg, J. H.** 2009. The Lowry method for protein quantitation, pp 7-10. ***In***: Walker, J.M. *The protein protocols handbook*. Humana Press, USA.

114. **Waterham, H.R., Digan, M.E., Koutz, P.J., Lair, S.V. and Cregg, J.M.** 1997. Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* **186**: 37-44.

115. **Wang, M., Si, T. & Zhao, H.** 2012. Biocatalyst development by directed evolution. *Bioresource technology* **115:** 117-125.

116. **Wei, X., Wang, L. and Xu, Y.** 2009. *Rhizopus chinensis* lipase: Gene cloning, expression in *Pichia pastoris* and properties*. Journal of Molecular Catalysis B: Enzymatic* **57**: 304-311.

117. **Wu, J., Chieng, L., Hsub, T. and Lee, C.** 2003. Sequential expression of recombinant proteins and their separate recovery from a Pichia *pastoris* cultivation. *Biochemical Engineering Journal* **16**: 9–16.

118. **Xie, J., Zhou, Q., Du, P., Gan, R and Ye, Q.** 2005. Use of different carbon sources in cultivation of recombinant *Pichia pastoris* for angiostatin production. *Enzyme and Microbial Technology* **36**: 210-216.

119. **Xuan, N.T., Hang M.T. and Thanh, N.V.** 2009. Cloning and over Expression of an *Aspergillus niger* XP Phytase Gene (phyA) in *Pichia pastoris. World Academy of Science, Engineering and Technology* **56:** 116-119.

120. **Yin, E., Le, Y., Pei, J., Shao, W. and Yang, Q.** 2008. High-level expression of the xylanase from *Thermomyces lanuginosus* in *Escherichia coli*. *World Journal of Microbiol Biotechnology* **24**:275–280.

121. **Zhang, J., Wang, X., Su, E., Fang, G., Ren, Y. and Wei, D.** 2008. A new fermentation strategy for *S*-adenosylmethionine production in recombinant *Pichia pastoris*. *Biochemical Engineering Journal* **41**: 74-78.

122. **Zhang, A., Luo, J., Zhang, T., Pan, Y., Tan, Y., Tan, F. and Fu, C.** 2009. Recent advances on the GAP promoter derived expression system in *Pichia pastoris*. *Molecular Biology Report* **36**: 1611-1619.

123. **Zhao, N., Guo R., Yu H., Ke, X., Jia, Y. and Bai, Y.** 2011. Expression and Characterization of a Thermostable Xylanase Gene *xyn*A from a Thermophilic Fungus in *Pichia pastoris. Agricultural sciences in China* **10(3)**: 343-350

124. **Zhong, X., Peng, L., Zheng, S., Sun, Z., Ren, Y., Dong, M. and Xu, A.** 2004. Secretion, purification and characterization of a recombinant *Aspergillus oryzae* tannase in *Pichia pastoris*. *Protein Expression and Purification* **36**: 165-169.

125. **Zhou, C., Bai, J., Deng, S., Wang, J., Zhu, J., Wu, M. and Wang, W.** 2008. Cloning of a xylanase gene from *Aspergillus usamii* and its expression in *Escherichia coli*. *Bioresource Technology* **99**:831–838.

APPENDIX A

Enzyme production levels in P. *pastoris*

pH 5, 40°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	222.1	224.1	186.6	186.1	237.3
15	216.9	215.2	182.6	172.1	226.4
30	207.0	203.7	181.6	161.2	220.4
45	195.5	191.8	174.9	149.3	219.4
60	183.1	177.4	166.4	137.8	218.2
75	165.9	149.5	153.7	132.3	207.7
90	159.7	135.8	139.3	135.3	206.2
105	158.0	124.1	126.9	122.9	203.0
120	156.2	116.9	120.1	123.6	199.8

pH 5, 50°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	224.1	206.7	162.4	173.4	233.1
15	213.9	193.8	161.2	172.1	239.3
30	204.7	187.1	159.7	158.2	229.1
45	190.3	184.8	155.2	147.5	217.4
60	183.1	182.6	150.5	141.5	211.2
75	175.9	178.4	145.3	137.3	209.0
90	169.7	176.4	140.8	135.8	206.5
105	160.9	166.2	136.6	134.3	205.0
120	148.5	147.0	130.6	132.6	200.2

pH 5, 60°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	220.1	91.2	92.4	91.2	247.3
15	205.5	90.3	88.6	87.3	244.8
30	202.0	89.7	87.3	86.1	244.5
45	193.5	88.6	84.1	82.8	240.3
60	183.1	84.3	82.0	80.7	238.3
75	175.6	78.0	78.6	77.4	227.1
90	166.9	72.0	78.0	76.7	222.9
105	160.7	59.3	76.5	77.6	219.4
120	151.0	57.5	73.1	77.9	211.9

pH 5, 70°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	108.7	92.5	87.9	86.7	262.7
15	95.5	85.1	85.6	84.3	259.5
30	79.2	75.4	85.6	80.6	256.7
45	73.5	68.8	77.1	75.9	253.0
60	64.1	58.1	74.0	72.8	247.3
75	60.6	50.2	71.1	69.9	244.5
90	55.6	42.9	69.2	67.9	238.6
105	51.9	37.3	68.9	68.2	237.6
120	45.6	35.8	67.5	66.3	232.3

pH 5, 80°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	45.1	37.9	36.3	36.0	243.3
15	39.5	34.4	34.7	34.4	228.1
30	33.6	28.7	31.9	32.6	216.2
45	30.5	26.2	31.3	31.0	208.0
60	26.6	20.9	29.7	30.4	197.0
75	24.6	15.7	29.7	29.9	193.0
90	20.5	11.7	29.3	29.0	182.3
105	19.4	9.9	28.7	28.4	176.6
120	18.5	8.0	28.1	27.8	173.9

pH 6, 40°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	222.6	212.2	181.6	181.1	239.1
15	217.4	201.2	162.9	167.4	232.8
30	208.5	168.9	161.2	155.7	228.9
45	201.7	164.2	158.5	158.0	229.1
60	195.3	151.7	157.2	155.0	227.6
75	190.0	150.0	148.3	145.3	223.1
90	177.4	141.3	146.0	145.5	222.9
105	168.4	112.2	133.8	134.6	213.9
120	162.2	98.5	129.6	119.2	208.5

pH 6, 50°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	225.1	222.9	174.6	185.6	240.8
15	223.1	214.2	163.9	177.6	248.3
30	218.4	210.7	163.2	169.2	236.1
45	209.2	200.2	157.5	173.4	229.9
60	202.0	192.0	154.5	165.4	226.4
75	191.5	182.8	152.7	156.2	223.1
90	181.3	172.4	150.2	157.2	216.7
105	172.4	160.2	148.3	154.2	214.2
120	165.7	150.0	146.8	152.7	208.7

pH 6, 60°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	226.1	100.6	93.0	91.8	250.7
15	223.9	99.5	86.2	91.2	249.8
30	214.9	96.3	85.6	87.1	247.5
45	210.7	83.0	81.1	82.3	244.5
60	207.0	80.8	82.1	80.8	244.0
75	199.8	57.7	79.0	80.2	240.3
90	191.3	58.5	76.9	78.1	235.8
105	183.1	55.7	73.9	77.6	232.1
120	177.9	50.1	72.3	76.0	226.6

pH 6, 70°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	111.1	86.9	92.2	90.9	262.9
15	100.4	83.3	88.9	90.2	257.7
30	96.9	75.7	84.8	88.6	254.2
45	94.4	68.7	82.0	88.2	251.7
60	91.3	58.5	81.8	86.8	247.8
75	87.4	58.6	79.5	83.2	247.3
90	83.3	47.3	76.5	80.2	246.3
105	75.6	41.0	74.1	76.6	243.5
120	66.4	34.3	72.1	75.9	240.5

pH 6, 80°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	45.5	38.5	37.5	37.2	245.0
15	40.2	25.8	34.6	35.3	237.6
30	38.6	19.3	34.7	34.4	228.4
45	34.4	16.3	33.6	33.8	213.2
60	31.9	16.1	31.7	33.4	203.0
75	28.1	13.4	31.0	31.7	191.0
90	25.1	13.3	30.5	31.2	189.6
105	22.3	12.2	29.5	28.8	178.6
120	19.7	10.0	29.7	29.9	173.9

pH 7, 40°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	219.9	192.3	181.1	180.6	247.3
15	208.5	171.9	177.6	168.7	247.5
30	196.8	161.9	167.9	164.9	244.0
45	177.6	142.0	166.9	161.4	244.0
60	157.5	136.6	160.2	154.7	244.8
75	135.8	134.1	152.7	152.2	242.5
90	131.1	121.6	152.0	146.5	230.3
105	126.4	117.2	146.5	142.5	225.4
120	122.1	110.2	142.0	141.5	216.4

pH 7, 50°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	221.9	197.5	170.9	181.8	248.8
15	212.2	186.6	166.7	170.1	246.3
30	198.8	179.6	161.9	167.9	244.0
45	181.8	177.6	159.2	162.7	239.1
60	158.5	175.6	161.2	162.2	233.6
75	153.0	170.6	159.0	159.7	228.1
90	147.3	166.2	155.7	159.2	225.6
105	145.8	150.7	146.8	147.8	225.9
120	142.0	148.8	141.3	142.3	224.1

pH 7, 60°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	219.2	199.3	184.8	182.3	253.0
15	210.7	193.0	184.3	176.9	250.5
30	200.0	180.1	178.9	171.4	246.0
45	192.5	170.9	172.6	167.7	244.8
60	182.3	159.5	169.2	161.7	241.3
75	179.9	146.0	162.2	159.7	239.3
90	158.5	138.8	156.7	149.3	236.8
105	151.0	126.9	154.2	141.8	235.8
120	149.8	111.7	150.2	137.8	231.3

pH 7, 70°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	109.2	89.9	91.7	90.4	263.9
15	97.9	85.0	90.4	86.7	260.2
30	93.4	79.2	87.4	83.7	258.2
45	85.7	62.6	86.3	81.3	255.2
60	72.8	57.5	82.2	81.0	254.2
75	58.0	47.0	79.2	78.0	254.2
90	52.1	39.7	77.2	73.5	249.3
105	46.9	38.4	75.9	70.9	246.8
120	43.0	38.1	74.0	71.5	244.0

pH 7, 80°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	44.4	38.1	37.1	36.8	246.3
15	38.5	31.4	35.8	34.5	238.8
30	32.6	29.0	34.9	33.6	227.6
45	30.0	17.9	34.3	33.0	221.1
60	27.7	14.1	33.4	31.5	211.4
75	23.7	12.1	32.6	30.5	210.9
90	21.4	10.6	31.7	29.6	204.7
105	18.8	10.3	29.4	29.1	190.8
120	17.7	10.1	29.2	28.1	179.4

pH 8, 40°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	215.7	172.4	175.4	174.9	248.0
15	204.5	130.8	168.2	162.7	245.5
30	190.5	104.2	164.2	158.7	239.1
45	176.6	86.1	164.2	153.7	237.1
60	167.2	80.8	163.4	153.0	235.1
75	156.0	77.9	161.9	147.5	229.6
90	138.6	68.7	158.2	145.3	227.4
105	133.6	60.4	148.0	142.5	222.1
120	126.6	52.5	139.8	139.3	217.4

pH 8, 50°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	219.9	214.4	173.4	184.3	249.0
15	201.5	205.2	171.6	177.6	248.0
30	192.0	201.5	166.2	172.1	247.3
45	170.9	191.8	159.0	169.9	243.3
60	148.5	184.1	155.7	159.2	239.8
75	144.3	175.1	152.5	153.5	235.3
90	138.1	167.7	149.8	153.2	228.6
105	134.8	147.5	146.0	147.0	225.1
120	132.1	142.3	142.8	143.8	224.9

pH 8, 60°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	217.7	96.0	92.7	91.4	253.5
15	207.0	90.3	92.0	90.8	253.2
30	195.8	80.7	89.4	88.2	250.7
45	186.3	74.3	84.2	83.0	248.8
60	175.6	72.9	81.7	80.5	247.5
75	164.9	68.8	76.2	76.2	245.5
90	145.8	66.3	71.9	75.6	243.5
105	143.0	60.6	70.0	73.8	241.3
120	142.3	50.6	68.8	73.8	224.4

pH 8, 70°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	108.8	88.7	91.5	90.3	264.7
15	77.0	65.4	88.9	87.7	260.7
30	72.8	62.9	86.9	84.5	258.2
45	67.9	61.4	84.0	79.0	256.2
60	66.5	57.6	82.8	75.4	254.7
75	61.9	55.1	79.5	73.3	252.7
90	49.0	49.4	77.6	71.4	251.7
105	41.2	38.3	73.9	68.9	249.5
120	40.9	36.4	72.4	67.4	243.0

pH 8, 80°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	44.3	38.2	37.3	37.0	246.8
15	38.0	33.6	36.8	36.5	239.8
30	32.0	31.9	35.6	35.3	228.4
45	29.5	24.4	34.1	32.8	217.2
60	26.9	20.8	33.2	30.9	210.0
75	24.2	17.3	32.5	29.3	205.7
90	20.6	14.2	30.9	28.7	204.0
105	18.2	12.1	30.1	27.8	194.0
120	17.6	10.9	29.2	27.9	189.8

pH 9, 40°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	214.2	164.4	178.4	177.9	258.2
15	200.7	141.8	172.9	167.4	250.0
30	185.1	138.8	169.4	163.9	243.5
45	163.7	126.4	165.9	165.4	238.3
60	138.3	119.4	156.2	155.7	232.6
75	127.6	116.2	151.7	151.2	231.1
90	123.4	112.7	149.3	143.8	229.9
105	120.1	94.3	144.0	138.6	229.4
120	117.4	88.1	141.0	138.1	229.1

pH 9, 50°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	219.4	182.6	173.4	172.4	249.0
15	181.1	174.1	167.7	162.9	248.8
30	165.7	154.7	166.9	160.9	246.5
45	159.5	152.0	165.9	160.0	244.5
60	147.5	144.5	164.4	153.5	243.3
75	144.8	137.6	163.9	148.0	241.0
90	142.0	136.1	156.2	145.8	238.6
105	131.8	124.9	146.3	143.0	236.6
120	128.9	121.6	137.8	138.1	235.3

pH 9, 60°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	219.4	92.9	93.9	92.7	253.2
15	205.2	89.2	91.5	90.3	252.0
30	193.0	78.2	83.5	87.8	249.5
45	173.1	76.6	83.1	83.7	247.8
60	160.9	73.0	82.6	81.3	245.8
75	142.8	66.7	81.8	81.2	245.0
90	138.3	66.2	80.8	79.6	243.5
105	135.6	59.2	79.5	78.2	241.3
120	131.3	47.1	79.7	76.0	241.3

pH 9, 70°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml

0	108.5	89.8	91.4	90.2	264.7
15	78.0	81.3	88.7	83.7	261.9
30	70.0	69.4	83.3	77.1	260.9
45	65.5	51.7	82.6	73.9	258.2
60	55.3	45.6	80.6	71.1	253.0
75	47.9	40.4	79.9	68.7	251.5
90	42.7	32.0	79.1	67.5	251.0
105	39.4	26.4	77.9	67.9	248.5
120	37.8	23.0	76.0	66.3	245.8

pH 9, 80°C	S325	S340	G41	G53	NC38
Time	IU/ml	IU/ml	IU/ml	IU/ml	IU/ml
0	44.2	38.2	37.3	37.0	248.0
15	34.1	34.5	36.8	36.5	234.8
30	28.2	28.5	36.0	35.7	223.9
45	22.7	23.0	35.3	33.5	218.7
60	18.2	18.4	33.0	31.7	211.7
75	17.9	18.1	31.5	29.8	207.0
90	16.0	15.1	30.5	28.5	204.0
105	15.3	11.6	29.2	27.9	195.5
120	15.3	11.5	29.1	27.8	192.5