# PARTIAL PURIFICATION AND CHARACTERISATION OF *Phialophora alba* XYLANASES AND ITS APPLICATION TO PRETREATED SUGARCANE BAGASSE

**BY**

**LETICIA MOSINA**

**Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal (Westville Campus).**

**As the candidate's supervisor, I have approved this dissertation for submission.**

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## PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences; University of KwaZulu-Natal (Westville Campus), Durban, South Africa from March 2010 to December 2012, under the supervision of Dr. R. Govinden and the co-supervision of Dr. B. Masola. These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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**TABLE OF CONTENTS**

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# ABSTRACT

Xylan is the major component of hemicellulose and its degradation can be achieved through the hydrolytic action of microbial xylanases. Xylanases have an array of applications one being bioethanol production. The lack of thermophilic xylanases has prompted the search for new enzymes with increased thermostability. Previous work on the crude enzyme of *Phialophora alba* has demonstrated optimal activity (39 U/µg) at a pH of 4 and two temperature optima of 50°C and 90°C. These desirable properties highlighted the need for further research on the purified enzyme. In the present study *P. alba* was identified as a thermophilc Ascomycete that forms conidia and chlamydospores during the asexual and sexual stages of its life cycle, respectively. The various isozymes present in the crude enzyme extract were subsequently detected by zymogram analysis. Up to six xylanase isozymes ranging from 90-210 kDa in size were detected. The crude enzyme was subsequently purified by precipitation and ion exchange chromatography (IEX). Protein precipitation methods, desalting methods, IEX resins, elution buffers and NaCl gradients were optimized. The 31-70% ammonium sulphate precipitate had the highest levels of xylanase activity. Separation of proteins with the anion exchanger, HiTrap Q sepharose fast flow column and a linear gradient of 0-2.5 M NaCl in phosphate buffer (50 mM, pH 7) yielded a partially pure xylanase isozyme with molecular weight of 210 kDa. A final yield of 1.4% and purification fold 10.6 was obtained after ion exchange chromatography. The specific activity of the xylanase was 21 IU/µg. At optimum pH (pH 4) and temperature (50°C) a combined xylanase activity of 32 IU.ml$^{-1}$ was detected. The partially pure xylanase was stable from pH 4-6 with 86% of xylanase activity retained for 90 minutes. Thermostability was observed from 40-70°C with 95% of activity retained for 90 minutes at optimum temperature. The ability of the partially pure xylanase and crude enzyme to hydrolyze untreated and pretreated

(alkali and temperature/pressure) sugarcane bagasse was tested at a constant enzyme loading rate of 15 IU/g. Overall, maximum hydrolysis was achieved with the alkali pretreatment and saccharification with the crude enzyme: approximately, 2.4 g/ml of reducing sugars were liberated over a 48 hours. The partially pure xylanase liberated a maximum amount of 2.3 g/ml reducing sugars after 48 hours. The results obtained highlight the desirable characteristics of the partially pure enzyme and its applicability to bioethanol production.

# LIST OF FIGURES

# LIST OF TABLES

## CHAPTER 1

## LITERATURE REVIEW

## 1.1. INTRODUCTION

In the forestry and agriculture industry, lignocellulosic residues or waste are highly abundant due to its accumulation after industrial processing. Lignocellulose serves as a vital starting material in the production of second-generation biofuels and various bio-based products (Lal, 2007; Deutschmann and Dekker, 2012). In the past two decades there has been a growing interest in the constituents of lignocellulose which include cellulose (45-55%), hemicellulose (25-35%) and lignin (20-30%) (Turner *et al*., 2007; Deutschmann and Dekker, 2012). All three chemical components are located within the plant cell wall and serve as its three major constituents. Hemicellulose is the second most abundant plant polysaccharide and is more complex than cellulose. Xylan, the major component of hemicellulose is composed of xylose residues linked via glycosidic bonds. Depending on the plant source, the degree of complexity and substitution with acetyl, glucoronosyl, 4-*O*-methyl-D-glucuronopyranosyl, α-l-arabinofuranosyl, feruloyl and *p*-coumaroyl groups may differ (Ebringerová and Heinze, 1999; Beg *et al*., 2001).

Cellulases, xylanases and ligninases are an essential group of enzymes involved in the degradation of the three major plant cell wall constituents. Xylanases hydrolyse the glycosidic bonds between the monomeric xylose residues to produce xylo-oligosaccharides. These glycosyl hydrolases are produced by an array of microorganisms which tend to produce different isoforms of these enzymes termed isozymes. These isozymes can be purified for application purposes using different liquid chromatography techniques. The type of techniques used depends on the properties (especially the known properties) of the enzyme. Once pure, xylanases can be applied

in the paper and pulping industry, production of biofuels, brewing industry and in the production of animal feed. However, thorough knowledge of xylanases, their enzymatic properties, catalytic mechanism and substrate specificity are vital in order to fully understand the enzyme and its applicability to different lignocellulosic residues.

## 1.2. STUCTURAL COMPOSITION OF LIGNOCELLULOSE

The three major components of the plant cell wall (cellulose, hemicellulose and lignin) represent the most abundant form of plant biomass (Ito *et al*., 2003). Cellulose and lignin are interlinked within the plant cell wall *via* the hemicellulose component. Hemicellulose is an insoluble polysaccharide that may either be branched or linear. A group of heterogenous compounds such as xylan, xylobiose, α-1,5-ʟ-arabinan, arabino-galactan, mannobiose and galacto-glucomannan constitute the hemicellulose proportion of the plant cell wall. The structure and proportion of each of these high molecular weight polymers varies amongst plant species. Therefore, the botanical source determines the diversity and complexity of xylan, which in turn is related to their functionality within the plant (Ebringerová and Heinze, 1999). Xylan, the major constituent of hemicellulose contributes 20-35% to the total dry weight of hardwoods and 8% of softwoods (Haltrich *et al*., 1996). ᴅ-Xylopyranosyl residues linked through β-1,4 xylosidic linkages make up xylan (Wesje *et al*., 2003) and the degree of substitution varies depending on the nature of the substrate (Figure 1). The main xylan polymers in hardwood and softwood are *O*-acetyl-4-ᴅ-methylglucuronoxylan and arabino-4-ᴅ-methylglucuronoxylan, respectively (Jacobs *et al*., 2001).

## 1.2.1. CELLULOSE

Cellulose is the most abundant plant polymer on earth (Mukherjee *et al*., 2011). It is the least complex plant polysaccharide (van den Brink and de Vries, 2011) composed of D-glucose subunits residues linked *via* β-1,4-glycosidic linkages to form cellobiose (dimer). Several cellobiose residues linked together by hydrogen bonds and van De Waals forces form cellulose. In nature, the association of cellulose with other polymers enhances its resistance to biodegradation. Structural cellulose consists of para-crystalline and crystalline regions with the latter being more resistant to biodegradation as a result of numerous hydrogen bonds (Howard *et al*., 2003). Each of the above mentioned regions are degraded through the synergistic action of several cellulose degrading enzymes.
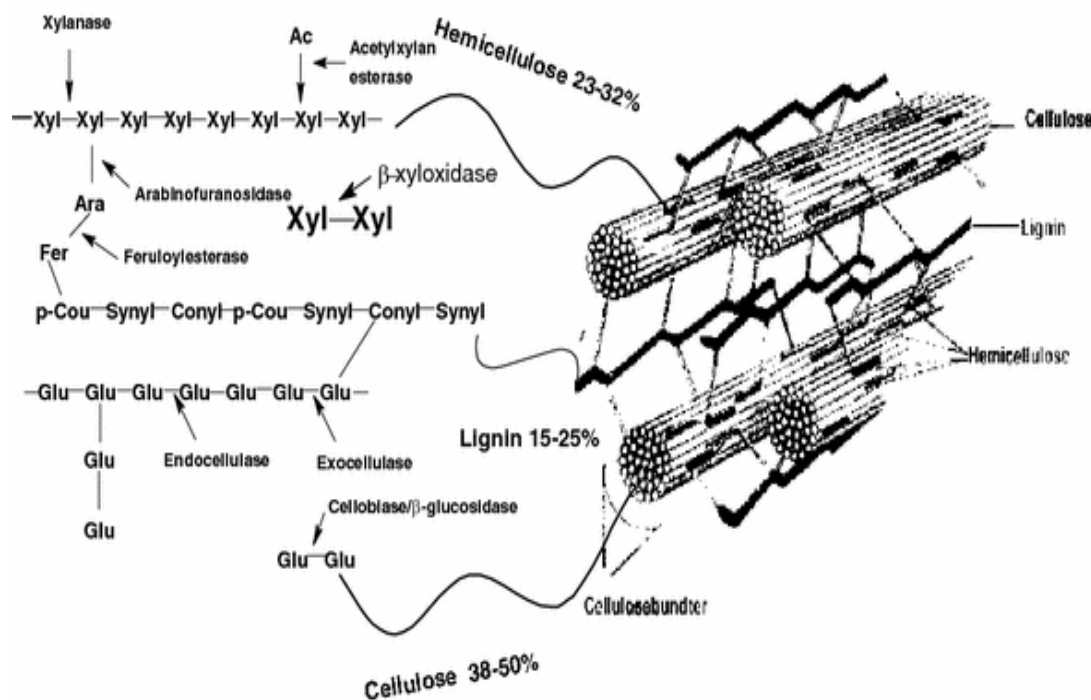


**Figure 1.1: Major components of the plant cell wall and the respective hydrolytic enzymes** (Rakhee and Numan, 2008).

## 1.2.2. LIGNIN

Lignin is a three dimensional network comprised of dimethoxylated, monomethoxylated and non-methoxylated phenyl propanoid units. These units are derived from their corresponding *p*-hydroxycinnamyl alcohols resulting in various subunits including a variety of ether and C-C bonds (Martinez, 2005). Lignin is derived from three monolignols, namely *p*-coumaroyl, coniferyl and synapyl alcohols. These monolignols differ temporally and spatially and are targeted to discrete regions within the plant cell wall. Within these discrete regions the monolignols polymerize to form biopolymers with distinct biochemical properties (Whiting and Goring, 1982; Davin and Lewis, 2005). The monomers of lignin such as *p*-coumaroyl and coniferyl are differentially targeted to the middle lamella and the secondary wall of the xylem elements (Fergus and Goring, 1970; Fukushima and Terashima, 1991; Davin and Lewis, 2005). Differential targeting has a physiological significance and permits the construction of lignified cell walls with unique biochemical properties. Lignin in the plant cell wall is essential for providing mechanical support to the aerial parts of the plant (Iiyama *et al*., 1994). The presence of lignin in the wall of conducting tissue provides resistance to the tensile forces generated during respiration (Raven, 1977; Horvath *et al*., 2010). Additional associative forces are provided for the plant by the presence of covalent cross-linking between lignin and other polysaccharides. The recalcitrant nature of lignin often inhibits the bioconversion of crops to biofuels (Brown, 1985; Howard *et al*., 2003; Chen and Dixon, 2007) and various other bio-processes.

## 1.2.3. HEMICELLULOSE

Hemicellulose is a polymeric carbohydrate that is found in association with cellulose (1,4-β-glucan) and lignin (polyphenolic compound) within the plant cell wall (Howard *et al*., 2003). Xylan, xyloglucan, glucomannans, galacto-glucomannan and arabinogalactan are the constituents of hemicelluloses (Howard *et al*., 2003). D-Xylopyranosyl residues linked β-1,4 through xylosidic linkages make up xylan (Wesje *et al*., 2003) however, in some instances the xylosidic linkage may be β-1,3. Xylan in its pure form (i.e., linear unbranched xylosyl residues) is rare amongst higher plants. Linear unbranched homoxylan has been found in esparto grass, ferns, seaweed and Chlorophyta (Ebringerová and Heinze, 1999; Beg *et al*., 2001). In *Caulerpa* sp. (green algae), homoxylan with β-1,3 linkages are known to replace cellulose in the cell wall. However, in the red seaweeds *Palmariales* and *Nemaliales* the cell walls have a mixture of β-1,3 and β-1,4 homoxylan linkages. Higher plants possess a β-1,4 linked xylan backbone with a variety of substituents and sugar residues attached (Iiyama *et al*., 1994). The main xylan polymers in hardwood and softwood are *O*-acetyl-4-D-methylglucuronoxylan and arabino-4-D-methylglucuronoxylan, respectively (Jacobs *et al*., 2001). Plants of the highest evolutionary level have a higher frequency and distribution of substituted residues (Izydorczyk and Biliarderis, 1995).

### 1.2.3.1. Xylan and its substituents

Inside the plant cell wall, xylan can be substituted with various side chains such as glucuronic acid, acetate, feruloyl, *p*-coumaroyl, L-arabinose and D-galactose. The occurrence and the number of substituents on xylan may vary depending on the botanical source which in turn is related to their functional role within the plant cell wall (Ebringerová and Heinze, 1999). Xylan

from hardwood is acetylated whereas softwood xylans contain arabinose residues. Hardwood and softwood xylan molecules contain a single 4-$O$-methyl-α-D-glucuronic acid substituent which contributes 10% and 18% to these residues, respectively. In most xylan molecules, substituents are located on the non-reducing end which may suggest that the xylosidic linkages on one side of the branch points are preferentially protected (Wong *et al*., 1988). The presence of side chains influences xylanase activity on certain substrates. These side chains may cause steric hindrance, blocking the catalytic domain and its associated catalytic modules. Reduced activity or no activity may be a direct result of steric hindrance. Hence, xylanases are specific for certain types of xylan polymers. On the other hand, substituents may play a positive role in substrate-enzyme binding thereby enhancing enzyme activity (Pai *et al*., 2012).

## 1.3. ENZYMATIC DEGRADATION OF LIGNOCELLULOSE

The enzymatic degradation of lignocellulose involves a complex interplay between microbial enzymes and accessory enzymes. Each enzyme targets a specific substrate within the plant cell wall. There are three major groups of enzymes namely cellulases, xylanases and ligninases, which are responsible for degrading the major cell wall components. Glycosyl hydrolases (GH) such as cellulases and xylanases are divided across different GH families based on differences in amino acid sequence, substrate specificity and various other biochemical properties (Wong *et al*., 1988; Collins *et al*., 2005; Puchart and Biely, 2007; Vlasenko *et al*., 2010; Chen *et al*., 2012). Microbial xylanases are of major interest as they hydrolyse the most complex polysaccharide within the cell wall, xylan.

### 1.3.1. CELLULASES

Cellulases refer to a group of enzymes responsible for the enzymatic breakdown of the polymeric substrate cellulose. Three types of enzymes are involved in the hydrolysis of cellulose; (i) endoglucanase, (ii) cellobiohydrolase and (iii) β-glucosidases (Mukherjee *et al*., 2011; van den Brink and de Vries, 2011). Endoglucanases hydrolyse the internal β-1,4-glycosidic linkage of the amorphous regions of cellulose opening up sites within the matrix for attack by cellobiohydrolases. Cellobiohydrolases (CBH/exoglucanases) hydrolyse highly crystalline cellulose from the reducing (CBH I) and non-reducing (CBH II) end by removing monomers and dimers to produce glucose and cellobiose (Esterbauer *et al*., 1991; Boisset *et al*., 2000; Howard *et al*., 2003). The cellobiose is subsequently hydrolysed to glucose by β-glucosidases (Wong *et al*., 1988; Gilbert and Hazlewood, 1993). Endoglucanases and cellobiohydrolases work in synergy to hydrolyse cellulose however, the exact mechanism has not been determined (Rabinovich *et al*., 2002).

### 1.3.2. LIGNINASES

Lignin biodegradation is achieved by a group of enzymes belonging to the family of phenol oxidases. These include lignin peroxidases (LiP), lacasses and manganese peroxidases (MnP) (Orth *et al*., 1993; Martinez *et al*., 2005; Harreither *et al*., 2009). White rot fungi (*Phanerochaete chrysosporium*, *Botrytis cinerea*, *Trametes versicolor* and *Phanerochaete ostreatus*) have been studied for their ability to completely degrade lignin (Crawford, 1978; Tien and Kirk, 1983; Dashtban *et al*., 2010). Lignin degradation involves a series of complex reactions (ligninolytic system) and forms part of the secondary metabolism of the responsible microorganism.

### 1.3.3. XYLANASES

The enzymatic breakdown of hemicellulose is achieved through the synergistic action of hemicellulases which target specific components of the polymer (Shallom and Shoham, 2003). Hemicellulases refer to a broad group of enzymes that are responsible for the hydrolysis of hemicellulose. Degradation of hemicellulose is often slow due to the insoluble nature of the plant cell wall. Endo-1,4-β-xylanase hydrolyzes the natural substrate beechwood xylan (Kulkarni *et al*., 1999) to form xylo-oligosaccharides which are further hydrolyzed to xylose monomers by exo-1,4-β-xylosidase (Saha, 2001). Xylanases are the major hemicellulases which hydrolyse the xylan backbone to xylo-oligosaccharides (Figure 2) which are further hydrolysed to xylose by β-xylosidases (Otieno and Ahring, 2012). Differences in the catalytic mechanism, enzyme activity and enzymatic properties have been observed across different xylanases. Due to these differences xylanases are classified into different GH families. Xylanases are classified into six glycosyl hydrolase (GH) families designated GH 5, GH 7, GH 8, GH 10, GH 11, GH 43 on the basis of their fold, catalytic action and substrate specificity (Puchart and Biely, 2007). The most studied xylanases belong to GH 10 and 11 (Collins *et al*., 2005). Arrays of fungi and bacteria have been identified that are capable of producing xylanases with varying activities and enzymatic properties. Xylanases have been reported in *Bacillus* sp., *Dictyoglomus* sp., *Rhodothermus marinus* and *Streptomyces* sp. (Salles *et al*., 2000; Subramaniyan and Prema, 2002; Bajaj and Singh, 2010; Buthelezi *et al*., 2011). Fungal xylanases have been reported in *Acrophialophora nianiana*, *Trichoderma viride*, *Thermomyces lanuginosus* and *Aspergillus niger* to name a few (Subramaniyan and Prema, 2002; Howard *et al*., 2003; Manimaran *et al*., 2009; Pal and Khanum, 2010).

Terminal xylose: $R^2=R^3=R^4=H$
4-xylose: $R^4$= substituent, $R^2=R^3=H$
2,4-xylose: $R^2=R^4$= substituent, $R^3=H$
3,4-xylose: $R^3=R^4$= substituent, $R^2=H$
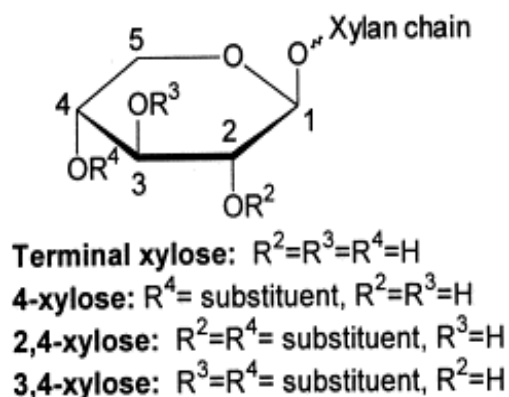
**Figure 1.2: Chemical structure of xylose residues in xylan** (Li *et al*., 2000).

### 1.3.3.1. Catalytic mechanism of endo-β-1,4-xylanases

Thus far, GHs have demonstrated two types of catalytic mechanisms (i) retention and (ii) inversion of the anomeric configuration of the substrate (Rye and Withers, 2000). Retention is a double displacement reaction in which the anomeric configuration (α or β) is retained (Vasella *et al*., 2002). This reaction is catalyzed by two glutamate residues located within the catalytic site. The first residue acts as a general acid thereby protonating the substrate. The second residue acts as a base and performs a nucleophilic attack on the substrate resulting in the departure of the leaving group. Together the catalytic residues result in the formation of a α-glycosyl intermediate. The first carboxylic acid residue then performs a nucleophilic attack on a water molecule thereby abstracting a proton and protonating the glycosyl intermediate. The α-glycosyl is subsequently hydrolyzed to a β-product *via* an oxo-carbenium ion-like transition state. In contrast, inverting β-GHs catalyze hydrolysis with the aid of glutamate and aspartate as the catalytic residues. Inversion is a single displacement reaction in which one carboxylic acid residue acts as a general acid and the other as a general base (Laitinen *et al*., 2000). Both residues result in the formation of an α-product *via* the formation of an oxo-carbenium ion-like

9

transition state. The same mechanism is followed by retaining α-GHs and inverting α-GHs except the end products possess an alpha and beta configuration, respectively.

The type of catalytic mechanism (inversion/retention) is dependent on the nature of the substrate. The source of the xylan determines whether it is linear, branched, a homopolymer or a heteropoylmer (Ebringerová and Heinze, 1999). Other than the catalytic domain additional supplementary domains have been identified such as the xylan binding domain, cellulose binding domain, dockerin domain and thermostable domain. The supplementary domains provide additional stability during substrate binding and hydrolysis (Collins *et al*., 2005). Substrate specificity is also affected by the presence of side chains. However, classification based solely on substrate specificity does not accommodate polyspecific enzymes as xylanases display broad overlapping activities (Henrissat and Davies, 1997). When used in conjunction with the above-mentioned properties such a classification system is more precise.

### 1.3.3.2. Multiplicity of xylanases

Microorganisms are capable of synthesizing an array of isozymes in order to efficiently degrade the complex xylan substrate (Wong *et al*., 1988; Gilbert and Hazlewood, 1993). *Melanocarpus albomyces* and *A. niger* are known to produce seven (Saraswat and Bisaria, 2000) and15 (Biely, 1985) extracellular xylanases, respectively. These extracellular hydrolases are modified post-translationally and partially proteolysed. The isozymes may result from multiple genes or a single gene which has been modified (Wong *et al*., 1988; Collins *et al*., 2005). The physicochemical properties, specific activities, structure and yields of the isozymes may vary (Collins *et al*., 2005). The diverse and complex nature of xylanases allows for efficient

hydrolysis of xylan (Collins *et al*., 2005). Many xylanases also exhibit cellulose activity (Gilbert and Hazlewood, 1993).

### 1.3.3.3. Thermophilic xylanases

On an industrial scale mainly *Aspergillus* and *Trichoderma* spp. are utilized for the production of xylanases (Haltrich *et al*., 1996). However, various other fungal spp. are known to produce thermophilic xylanases but they have not been fully characterized. Furthermore, many of these xylanases are not expressed at the desired level for application purposes. Unlike most bacteria, fungi are capable of releasing enzymes into the medium (Decelle *et al*., 2004) including auxillary enzymes for debranching the substituents of xylan (Haltrich *et al*., 1996). This is often advantageous in biotechnology as the cells do not have to be disrupted to release the enzyme. Thermophilic and hyperthermophilic xylanases have optimal activity at 50-80°C and >80°C, respectively (Collins *et al*., 2005). Enzyme stability at optimum temperature and temperatures greater than the optimum may vary. The presence of a thermostabilising domain (TSD) in thermophilic xylanases is a major contributing factor to thermostability. The thermostabilising domain has been closely linked to the catalytic domain, however little is known on the exact function and structure (Kulkarni *et al*., 1999). Several xylanases have been reported to have TSDs such as XynX of *Clostridium thermocellum* (Kim *et al*., 2000), XynA of *Thermoanaerobacterium saccharolyticum* (Li *et al*., 1993; Fontes *et al*., 1995) and the xylanase of a *Bacillus* sp. (Blanco *et al*., 1999). All deletion mutants that lacked the TSD demonstrated lowered thermostability when compared to the full length enzyme. Most xylanases, in particular thermophilic and hyperthermophilic xylanases belong to GH families 10 and 11. The cleavage of

xylan by GH 10 and GH 11 xylanases results in the release of large and small end products, respectively (Dodd and Cann, 2009; Zhang *et al*., 2011).

Lignocellulose degrading enzymes, including xylanases, can be used in various commercial processes to minimize the accumulaton of waste products. Protein purification is often a pre-requisite to industrial application as detailed information on the enzymatic properties is required. Xylanases have can be purified using an array of liquid chromatography techniques and have been further characterized prior to industrial application.

## 1.4. PROTEIN PURIFICATION METHODS

Preliminary purification involves concentrating and desalting of the crude extract prior to chromatographic analysis. Protein samples can be concentrated using organic polymers, organic solvents and salts to reduce the dilution effect created by subsequent purification steps. The method of choice is dependent on the nature and properties of the sample and the type of chromatographic techniques that will be used. An array of chromatographic techniques (Table 1.1) are available depending on the properties of the protein to be purified (Scopes, 1993; Ali *et al*., 2010). The various types of liquid chromatography methods used in proteomics research include ion exchange, gel filtration, reversed phase, affinity, immobilized metal ion affinity and hydrophobic interaction chromatography (Hage, 1999). A pure protein can be obtained by using a combination of chromatographic techniques which have been selected based on the known properties of the protein. Several xylanases from both bacterial and fungal origin have been purified using an array of techniques. Xylanases typically range from six to 80 kDa (Butt *et al*., 2008) in size however, there are a few report on the purification of high molecular weight

xylanases. A 350 kDa cell-associated xylanase from *Thermoanaerobacterium* sp. was purified by ion exchange chromatography, hydrophobic interaction chromatography and gel filtration (Shao *et al.*, 1995). Bergquist *et al.* (2001) reported the presence of 266 kDa xylanase from *Thermotoga maritima*.

**Table 1.1: List of chromatographic techniques and the protein properties** (Scopes, 1993)

| Chromatographic technique | Protein property |
|---|---|
| Affinity chromatography | Specific ligand recognition |
| Immobilized metal ion affinity chromatography | Metal ion binding |
| Ion exchange chromatography | Charge |
| Gel filtration chromatography | Size |
| Reversed phase chromatography | Hydrophobicity |
| Hydrophobic interaction chromatography | Hydrophobicity |

### 1.4.1. PROTEIN PRECIPITATION METHODS

Ammonium sulphate precipitation is the preferred and most commonly used method due to the affordability of the salt and its good reproducibility. This method of precipitation exploits the presence of hydrophobic amino acids on the surface of proteins (Englord and Seifter, 1990; Burgess and Deutscher, 2009). Water molecules aggregate around the hydrophobic patch to create a protein hydration shell. In the presence of ammonium sulphate this hydration shell is disrupted and the hydrophobic areas are exposed allowing them to interact with one another. Due to hydrophobic interactions precipitation or "salting out" occurs (Scopes, 1993; Voet *et al.*, 2008). Depending on the saturation point proteins will precipitate at different ammonium sulphate concentrations.

Organic solvents and organic polymers (polyethylene glycol) can also be used to precipitate proteins. Water molecules surrounding the hydrophobic areas are displaced by organic solvent molecules due to the higher solubility of these molecules. The electrostatic and dipolar van Der Waals forces result in aggregation and precipitation of proteins (Scopes, 1993; Berg *et al*., 2001). Organic polymers like polyethylene glycol allow proteins to precipitate using a similar principle. Precipitation of proteins helps eliminate unwanted proteins which may be present in the sample and concentrates the proteins of interest. The concentrated sample is desalted to remove residual salts and media components present in the crude cell extract which may interfere with subsequent purification steps.

### 1.4.2. AFFINITY CHROMATOGRAPHY

Affinity chromatography exploits the ability of proteins to bind specifically and reversibly to ligands (Varilova *et al*., 2006). Proteins migrate through a column packed with an insoluble polymer to which a specific ligand has been covalently attached and immobilized (Cuatrecasas, 1970; Hage, 1999; Voet *et al*., 2008). The success of any affinity chromatography is determined by the immobilized ligand and its compatibility to the protein of interest. Proteins which do not exhibit affinity for the bound ligand will pass through the column unretarded. However, those proteins which exhibit affinity for the ligand will be retarded through the column (Cuatrecasas, 1970; Berg *et al*., 2001). These interactions could be between an enzyme and an inhibitor or an antibody and an antigen (Hage, 1999). The sequence in which the bound proteins are eluted is dependent on the binding affinity constant under experimental conditions (Figure 1.3) (Magdeldin and Moser, 2012). The type of media selected, the type of eluant(s) selected and

changes in pH are major factors that affect the binding affinity constant. Therefore, proteins with a low affinity for the ligand will be eluted prior to those with a high affinity for the bound ligand (Winzor, 2000). Affinity chromatography has been utilized in the purification of a recombinant xylanase produced by *Streptomyces olivaceoviridis* (Ito *et al*., 2004). The recombinant xylanase, SoXyn10A, was loaded onto a lactosyl-Sepharose column and eluted in 200 mM lactose prepared in 20 mM phosphate buffer.
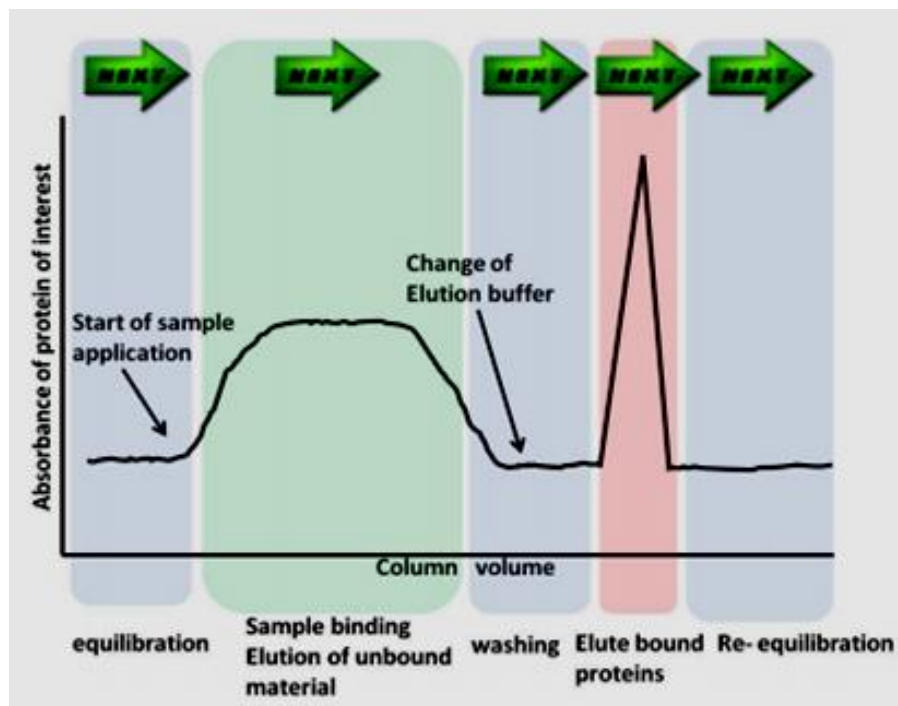


**Figure 1.3: Steps involved in typical affinity chromatography purification** (Magdeldin and Moser, 2012).

### 1.4.3. IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

Immobilized metal affinity chromatography (IMAC) is an operationally and chemically complex chromatographic technique (Gagnon, 2012). IMAC is considered a rapid method for the purification of recombinant proteins. This high-performance liquid chromatography (HPLC)

technique has the ability to differentiate a single histidine residue on the surface of the protein. IMAC requires an additional step of immobilizing the metal ion prior to chromatographic separation (Graslund *et al*., 2008). Separation by IMAC involves the surface interaction of histidine-tagged proteins with divalent metal ions that have been immobilized *via* a chelating ligand (Li and Dass, 1999). Histidine-tagged proteins bind strongly to the immobilized metal compared to other proteins which may be present in a crude sample extract. Other cell proteins will bind weakly or not at all. Therefore, proteins which have been tagged with a histidine tend to have a higher affinity in IMAC due to the multiple (six-10) histidine residues (Gagnon, 2012). An array of adsorbents can be used to immobilize the metal ion. Furthermore, a diverse group of metal ions can be selected to mediate selectivity in conjunction with an elution method of choice. Therefore, several combinations of adsorbent-metal-elution methods can be used. IMAC is ideal for the purification of recombinant proteins, including xylanases that have been expressed in bacterial, insect and mammalian cells. A recombinant xylanase isolated from goat rumen was expressed in *Escherichia coli* and purified using IMAC (Wang *et al*., 2011). The his-tagged recombinant xylanase was loaded onto a $Ni^{2+}$-NTA agarose gel column. Recently, Driss *et al*. (2012) purified a recombinant xylanase produced by *Penicillium occitanis* using a similar agarose resin which had a high degree of specificity and selectivity for the his-tagged protein. Proteins are typically eluted with a low pH buffer or with a linear gradient of imidazole (Graslund *et al*., 2008).

### 1.4.4. REVERSED PHASE CHROMATOGRAPHY

Reversed phase liquid chromatography allows for the separation of proteins based on their inter-action with a hydrophobic matrix (Scopes, 1993). The hydrophobic matrix is highly polar.

During separation proteins bind to the hydrophobic matrix in the presence of an aqueous buffer. The protein molecules are eluted sequentially using a linear gradient of an organic solvent. The matrix is composed of spherical silica beads with linear octadecane groups (C18) that are covalently attached to the surface. The porous nature of the silica beads increases the surface area available for binding. The octadecane groups are highly non-polar allowing polar molecules to bind. Therefore, charged peptides in a highly polar solvent such as water are able to bind to the octadecane groups. Reversed phase chromatography is derived from the opposite technique of normal phase chromatography. The latter involves the separation of proteins based on their interaction with a polar matrix (silica beads without octadecane groups attached) in the presence of a non-polar solvent (Scopes, 1993). The purification of xylanases is rarely conducted using reversed phase chromatography. However, Bray and Clarke (1994) successfully purified a xylanase A produced by *Schizophyllum commune* using the Ultrasphere C18 ODS column, with C18 representing the functional group. Ion exchange chromatography, gel filtration chromatography and affinity chromatography are a few of the more popular chromatographic techniques that are currently used to purify crude xylanases.

### 1.4.5. HYDROPHOBIC INTERACTION CHROMATOGRAPHY

The hydrophobic interactions between proteins and the chromatographic matrix can be exploited to purify proteins (Voet *et al*., 2008). The chromatographic matrix is highly substituted with phenyl and octyl groups. As the salt concentration increases during chromatographic separation, the non polar groups on the surface of the protein interact with the hydrophobic groups on the matrix. An eluant consisting of an aqueous buffer with decreasing salt concentrations and increasing concentrations of detergents is used to elute the bound proteins. The detergents disrupt

the hydrophobic interactions and allow for the proteins to be eluted from the column matrix at different elution times depending on the strength of the interactions. Shao *et al*. (1995) purified a thermophilic cell-associated xylanase produced by *Thermoanaerobacterium* sp. strain JW/SL-YS485 using a combination of chromatographic techniques. Hydrophobic interaction chromatography, anion and cation exchange chromatography and gel filtration were employed to purify the 250 kDa xylanase.

### 1.4.6. ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography (IEC) is a popular chromatographic technique (Marvin and Pinto, 2002) which allows for separation of proteins based on their net charge. Separations by IEC can occur in aqueous buffers and hydrophilic surfaces unlike reversed phase chromatography which requires harsh solvents and stationary phases (Marvin and Pinto, 2002). The type of ion exchange chromatography (anion, cation or zwitterionic) is dependent on the isoelectric point (pI) of the protein and the pH of its surrounding environment (Scopes, 1993). If the pH is greater than the pI the overall net charge of the protein is negative. However, if the pH is less than the pI, the overall net charge of the protein is positive. Unlike hydrophobic interaction chromatography, in ion exchange chromatography, proteins are eluted in an aqueous buffer containing increasing concentrations of salt (sodium chloride) (Figure 1.4) (Campbell and Farrell, 2007). The increase in salt concentration disrupts the ionic interaction between the protein and the column matrix. Whether sodium ions or chloride ions displace the protein is determined by the type of ion exchange chromatography, that is, anionic or cationic (Voet *et al*., 2008). Xylanases from *Aureobasidium pullulans*, *Humicola insolens*, *Streptomyces* sp. and

*Phialophora* sp. have been purified using ion exchange chromatography (Li *et al*., 1993; Düsterhöft *et al*., 1997; Deesukona *et al*., 2011; Zhang *et al*., 2011).
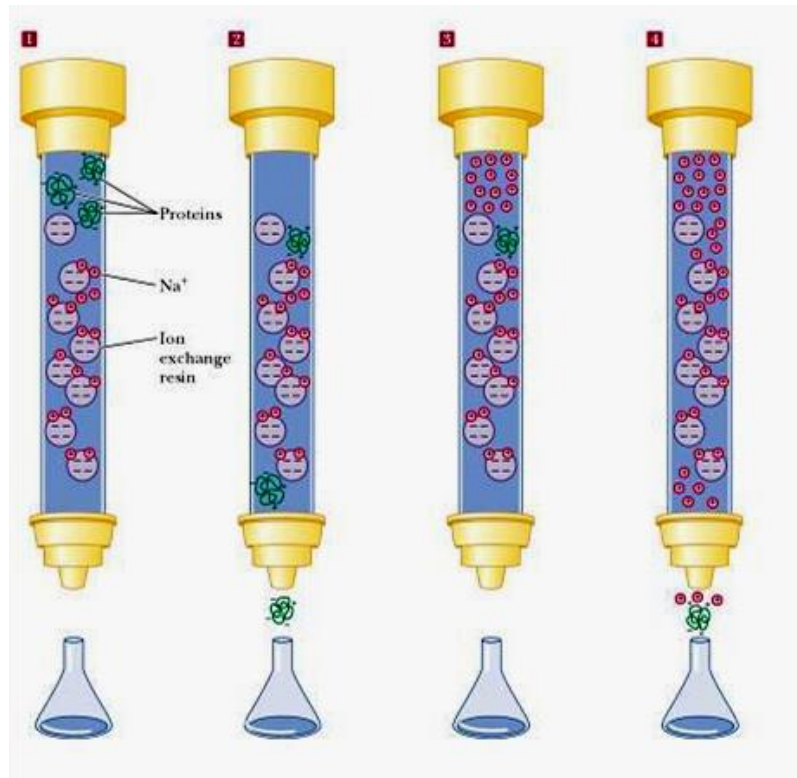


**Figure 1.4: Schematic representation of the principle of ion exchange chromatography** (Campbel and Farrell, 2007).

1.4.7. GEL FILTRATION CHROMATOGRAPHY

Gel filtration, also known as size exclusion chromatography, allows for the separation of proteins based on molecular weight (Winzor, 2000). This is one of the simpler chromatographic techniques. Separation by gel filtration can be performed in the presence of various co-factors, detergents, urea, guanidine chloride and at high or low ionic strengths (Scopes, 1993; Voet *et al*., 2008). Most importantly it can be performed at temperatures ranging from room temperature to those of the cold room or any temperature necessary for the experiment. Sample volumes are to

be kept as small as possible and highly concentrated. Large, dilute sample volumes promote axial dispersion resulting in a considerably lowered peak concentration (Winzor, 2003). Therefore, it is essential to load a concentrated sample to allow for the detection of the applied sample in the eluant (O'Fágáin *et al*., 2011).

A gel filtration medium is packed into a column to form a packed bed (Berek, 2010). The porous matrix of spherical particles is chemically and physically stable. The medium is also non-reactive and lacks adsorptive properties, therefore it is said to be inert (Scopes, 1993; Tsonev and Hirsh, 2008). Equilibration of the packed bed is important and involves filling of the pores of the matrix and spaces between the particles. Equilibration is achieved once the stationary and mobile phases are in equilibrium. The stationary phase refers to liquid inside the pores of the matrix and the mobile phase is the liquid outside the particles (Winzor, 2000). Differences in protein size are exploited by choosing gel filtration resins with a suitable pore size (Figure 1.5). Based on the pore size of the resin, proteins below a specific molecular weight move through the medium at a rate which is proportional to their molecular weight (Berek, 2010). Therefore, high molecular weight proteins are eluted prior to low molecular weight proteins during separation by gel filtration (Tsonev and Hirsh, 2008). Purified proteins may be eluted in any buffer making gel filtration suitable for proteins which are pH sensitive. Once samples have been loaded onto the packed column, they are eluted isocratically, hence there is no need for a change of buffer during separation. Separation by gel filtration is often followed by a washing step in the elution buffer to remove proteins that may have been retained on the column. The washing step also prepares the column for a new run.
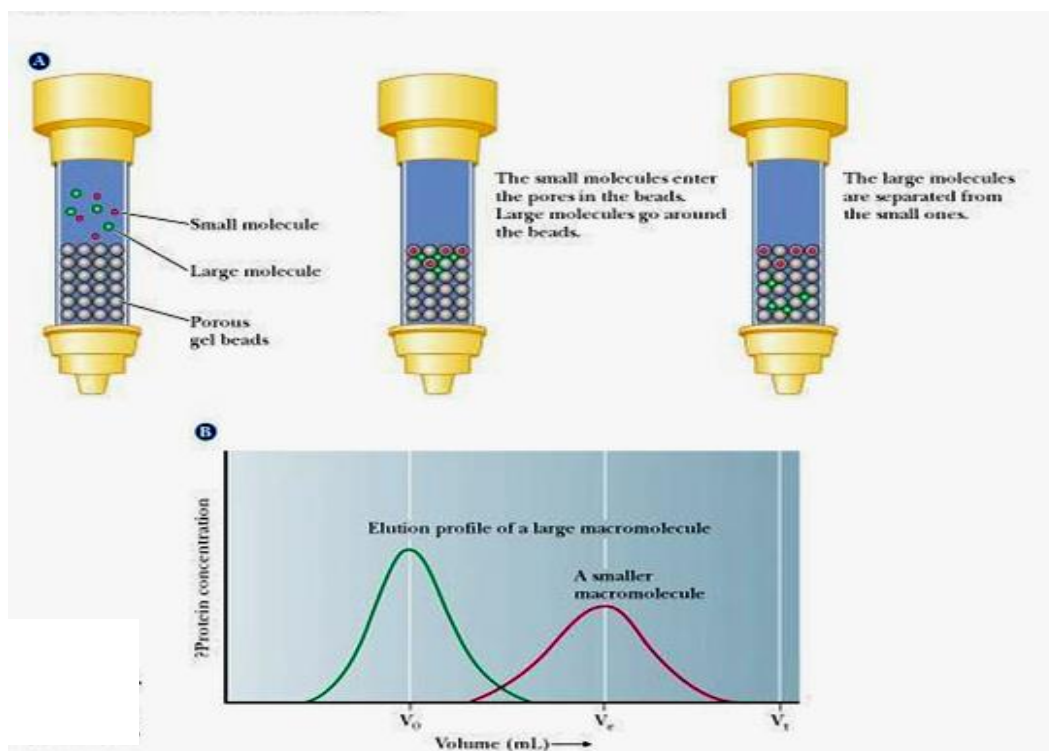
**Figure 1.5: Schematic representation of gel filtration chromatography showing a) the separation of proteins of varying size and their b) theoretical elution profile of proteins** (Campbel and Farrell, 2007).

As the most popular chromatographic technique, gel filtration has long been used for the separation of proteins based on size. Xylanases from bacterial and fungal origin have been purified or partially purified using this chromatographic technique. Faulet *et al*. (2006) purified two cellulase-free xylanases, Xyl A (63–66.1 kDa) and Xyl B (60.7–62.4 kDa), from termites (*Macrotermes subhyalinus*) using Sephacryl S-200. In conjuction with other chromatographic techniques a purification factor of 77 and 153 was obtained for Xyl A and Xyl B, respectively. *Acrophialophora nianiana*, *Aspergillus caespitosus* and *Chaetomium thermophile* xylanases have also been purified using gel filtration (Salles *et al*., 2000; Sandrima *et al*., 2005; Latif *et al*., 2006).

**1.5. USE OF XYLANASES IN INDUSTRIAL BIOPROCESSING**

Thermostable enzymes are capable of tolerating elevated temperatures without denaturing due to the presence of a thermostabilising domain. Various industrial bioprocesses demand thermostable and pH stable enzymes with elevated enzyme activities which are able to withstand the extreme conditions within the bioreactor. Xylanases display various biochemical properties and have an array of applications in biotechnology. The presence of isozymes in a single isolate indicates that xylanases have different pH and temperature optima and enzyme activities. Therefore, differences in thermostability and pH stability can be observed. The broad biochemical property of xylanases highlights their potential application in various bio-processes.

### 1.5.1. BIOCONVERSION

The conversion of lignocellulosic substrates to bioproducts involves four main steps: (i) pre-treatment, (ii) enzymatic hydrolysis of polymers to readily metabolizable sugars, (iii) use of these molecules for microbial growth or for the production of chemicals and (iv) separation and purification (Sun and Cheng, 2002; Berlin *et al*., 2006; Sánchez, 2009). The main goal of pre-treatment is to disrupt the lignin seal and crystalline cellulose (Mosier *et al*., 2005) to increase the availability of polysaccharides for enzymatic hydrolysis. Conventional pre-treatment strategies involve chemical or physical disruption. According to Mosier *et al*. (2005), the use of hot water, dilute acids, lime, ammonia fibre expansion (AFEX) or steam explosion have proven to be potential cost-effective pre-treatment methods. An effective pre-treatment method should be inexpensive, energy efficient and preserve the hemicellulose portion of the biomass. Recalcitrants like lignin can be removed by the enzymatic action of ligninases during pre-treatment (Howard *et al*., 2003). On a large scale, pre-treatment and enzymatic hydrolysis of the

polysaccharides serves as an economic barrier. A more feasible method would be to use enzymes in both steps to reduce costs. Xylanases hydrolyse the complex hemicellulose complex sandwiched between the cellulose and lignin component.

### 1.5.2. BIOFUEL PRODUCTION

The 1970s oil crisis can be marked as a turning point in white biotechnology resulting in increased activity in the production of fermentable sugars from cellulosic waste in Europe and the United States (Turner *et al*., 2007). Due to increasing levels of carbon emissions, the production of biofuels has gained popularity (Figure 1.6). Several countries have developed special programs aimed at developing biofuel production from renewable resources. The most common biofuel produced is bioethanol. The conversion of lignocellulosic substrates to bioethanol involves three main steps: (i) pre-treatment, (ii) enzymatic hydrolysis of cellulose and hemicellulose and (iii) fermentation of the liberated sugars to form ethanol (Howard *et al*., 2003; Berlin *et al*., 2006). Over the past 30 years Brazil has been producing bioethanol (Walter and Ensinas, 2009), however, several factors limit the success of bioethanol production. In order for biofuels to become competitive with fossil fuels, several bottlenecks need to be overcome. A major limitation is that most enzymes in biotechnology are from non-extremophiles and often require protein engineering (Wesje *et al*., 2003). This bottleneck has been overcome with the aid of bioprospecting. Several thermophilic xylanases have been identified and characterized to date that are suitable for bioprocessing, however, there is still a demand for thermostable and pH stable xylanases. For biofuel production xylanases which function at pH 5 and 50°C are essential. According to Himmel *et al*. (2007), cost-effective production of biofuels from biomass can be achieved by 2030 as it is generally accepted by the public.

**Figure 1.6: Overview of biofuel production and overall effect.**

(http://arstechnica.com/uncategorized/2008/09/bioengineering-bacteria-for-efficient-biofuel-production)

Pre-treatment as the initial step of biomass conversion is one of the processes which are expensive and further increase capital costs (Howard *et al*., 2003). In addition, pre-treatment involves a series of chemical or biological treatment steps. These steps weaken the lignocellulose matrix allowing the enzymes to access their specific target sites (Figure 1.7). A more feasible method would be to use enzymes for the pre-treatment and enzymatic hydrolysis to reduce costs in a process called simultaneous saccharification and fermentation (SSF). SSF has a number of advantages which include: i) increased hydrolysis rate due to the conversion of sugars that inhibit certain enzymes, ii) lower quantities of the enzyme are required and a higher yield is obtained, iii) shorter process time and iv) reduced reactor volumes (Sun and Cheng, 2002; Howard *et al*., 2003).

Recalcitrants like lignin can be removed by the enzymatic action of ligninases during pre-treatment. Xylanases with high enzyme activity that are thermostable and pH tolerant can be cloned into the yeast *Pichia pastoris*. Cloning of the xylanase gene and expression in *P. pastoris* typically results in the production of a high yield of ethanol. Depending on the type on promoter, inducible or constitutive, expression of heterologous or homologous genes may vary (Li *et al.*, 2012).
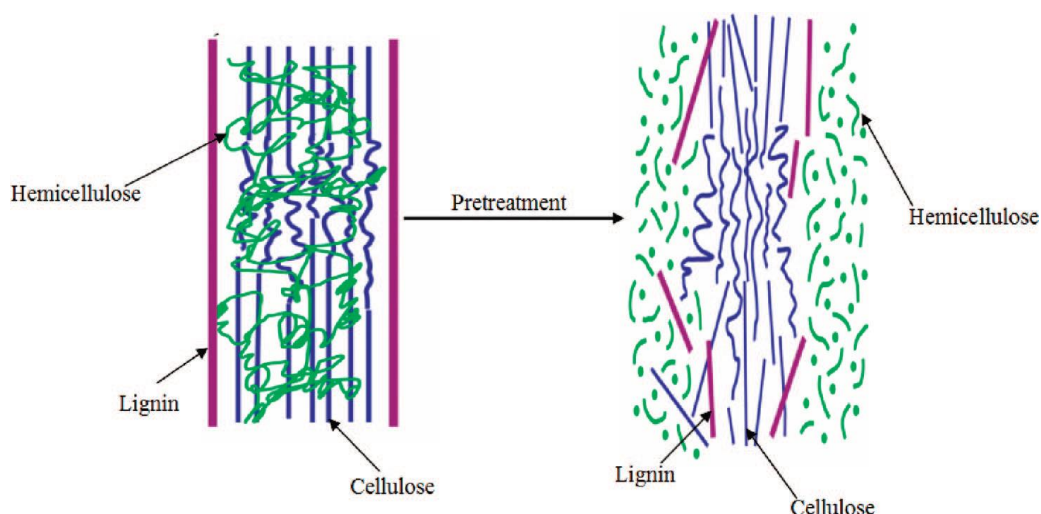


**Figure 1.7: Schematic representation of the role of pretreatment in the conversion of lignocellulose to bioethanol** (Kumar *et al.*, 2009).

### 1.5.3. BIOPULPING AND BIOBLEACHING

Biopulping involves the separation of wood fibres and lignin removal (Vieille and Zeikus, 2001) with the aid of microbial enzymes. Conventional pulping processes employ chemical hot-alkali pre-treatment and the effluents have harsh effects on the environment. Therefore, enzymatic treatment offers a safer alternative and reducing the impact the paper and pulp industry have on the environment.

Major companies like Sappi and Mondi, located in KwaZulu-Natal, South Africa produce the majority of the country's pulp and paper. The conversion of wood to pulp can be achieved *via* the Kraft process which accounts for more than 75% of the world's paper produced (Angayarkanni *et al*., 2006; Yadav *et al*., 2010). Although this process yields pulp of high strength there still remain several disadvantages with this conventional process. A high energy and capital cost, low yield, production of toxic effluent and by-products are some of the limitations of this process (Yadav *et al*., 2010). Dissolving pulp can be achieved through enzymatic treatment prior to thermochemical pulping. Biopulping improves the paper strength, reduces electrical energy consumption, and reduces the environmental impact of the production process and the pitch content.

The residual lignin remaining in the pulp can be entrapped in the pulp matrix by xylan. Bleaching of the pulp using chlorine and chloride chemicals serves to remove the residual lignin and to brighten the pulp, however, large quantities of these chemicals are required (Savitha *et al*., 2009). These compounds generate toxic and highly persistent chlorinated organic by-products which ultimately pollute the environment and surrounding water bodies (Saraswat and Bisaria, 2000; Ninawe and Kuhad, 2006). In recent years extensive research has been conducted to find biological alternatives to conventional pulp and paper industry processes (Angayarkanni *et al*., 2006). Biopulping and biobleaching are two examples in which enzymes can be used in the production of pulp and paper.

Xylanases can be used as pretreatment agents to minimize the dosage of chlorinated compounds required. The hydrolytic action of these enzymes liberates the entrapped lignin thereby further

enhancing the efficiency of the process. Furthermore, hemicellulose is sandwiched between cellulose and lignin (Figure 1.7) and is bound by covalent and non-covalent interactions (Angayarkanni *et al*., 2006; Ninawe and Kuhad, 2006). Xylanases hydrolyse the xylan polymer that traps the lignin (Pastor *et al*., 2007). Enzymatic hydrolysis of this polysaccharide leads to the dissociation of cellulose and lignin thereby allowing for the efficient removal of lignin.

GH family 11 (Esteghlalian *et al*., 2008) and recently family 5 xylanases (Collins *et al*., 2005; Valls *et al*., 2010b) have demonstrated the ability to facilitate in pulp bleaching in order to minimize the production of effluents during the bleaching process (Heinzle *et al*., 1992). During alkaline cooking of wood (such as *Eucalyptus*) side chains of xylan (4-*O*-methylglucuronic acid) can be converted to their respective unsaturated hexuronic acids (HexA) (Daniel *et al*., 2003). In bleaching processes it is important to reduce the HexA content as it is known to influence the properties of the pulp and the bleaching process. According to Cadena *et al*. (2010), HexA increases the kappa number, the consumption of bleaching reagents, retaining metal ions and brightness reversion and play a role in the formation of oxalic acid (Valls *et al*., 2010a). Enzymatic treatment with xylanases allows for the removal of HexA and can be utilized for their bleach boosting capacity. Pastor *et al*. (2007) reported that biobleaching with the aid of xylanases can result in up to a 20-25% saving on chlorine-based chemicals and a 15-20% reduction in the amount of effluent (organic chlorine compounds). However, the effectiveness of the bleaching process is determined by several factors. These factors include; (i) the sequence of bleaching, (ii) type of wood species and (iii) type of pulping method (Suurnäkki *et al*., 1996; Christov *et al*., 2000). For biopulping and biobleaching enzymes which function at high temperatures and within the pH range of 6.5-7.0 are required (Wakarchuk *et al*., 1994).

### 1.5.4. ADDITIONAL APPLICATIONS

Xylanases lend themselves to various other industrial applications and can be applied at different stages of industrial processing. The enzymatic processing of biomass can be used produce sugars (glucose and xylose) that can be used in fermentation process. Alternatively, a single enzyme can be used to target a specific polysaccharide producing valuable oligosaccharides (Prade, 1995). In the animal feed industry and the food industry there is a tremendous demand for xylanases. Microbial β-1,4-endoxylanases are commonly used to supplement the non-starch polysaccharide rich diet of poultry to increase digestibility (Mourao *et al*., 2006). The addition of hydrolases to diets increases the degree of de-polymerization in polysaccharides thereby reducing the viscosity. The bioconversion of xylan to xylitol which is a low calorie sweetener and preventative agent against caries present in dental gum and toothpastes (Lee, 1997; Deutschmann and Dekker, 2012; Rafiqul and Sakinah, 2012). When used in conjunction with amylases, xylanases can be used to enhance the quality, shelf-life and volume of bread (Subramaniyan and Prema, 2002; Butt *et al*., 2008). Xylanases can also be applied in the beer brewing process to reduce the haziness and viscosity of beer and increase the filterability of the wort (Pastor *et al*., 2007). Additional applications in the food industry include the clarification of fruit juices and wines (Subramaniyan and Prema, 2002). When used in detergents, xylanases improve the stain removing ability of the detergent on grass stains, fruit, vegetables and different types of soil (Kuhad *et al*., 2006). Furthermore, textile fibres extracted from plants have a lower lignin content making the xylan more accessible to xylanases (Prade, 1995).

## 1.6. SCOPE OF THE STUDY

Xylan degrading enzymes are vital for the bioconversion of lignocellulosic residues to bioethanol. Xylanases displaying elevated activity, pH stability and thermostability are ideal for bioethanol production as they further enhance the efficiency of the saccharification process conducted at elevated temperatures. The enzymatic properties of these hydrolytic enzymes tend to differ allowing xylanases to be applied to a plethora of applications. However there is a lack of robust biocatalysts that will withstand the extremes in pH and temperature.

*Phialophora alba*, is a thermophilic fungus isolated from *Eucalyptus* spp. woodchips. Previous work (Mosina, 2010) on the crude enzyme has indicated that *P. alba* produces xylanases that are thermostable, pH stable and exhibit enzymatic activity greater than 60 $IU.ml^{-1}$. Furthermore, six different isozymes ranging from 90-210 kDa in size were produced in the crude enzyme extract. The desirable properties displayed by the crude enzyme highlighted the need for the purification and characterization of the individual isozymes in order to establish their true enzymatic properties. This is the first report of xylanases produced by *P. alba* (Mosina, 2010) highlighting the novelty of the study. Therefore, the aim of this study was to purify, characterize and apply a thermophilic xylanase from *P. alba* to pre-treated sugarcane bagasse using conditions for bioethanol production.

## 1.7. HYPOTHESIS

The lack of robust biocatalysts has proven to be a major limitation in the application of xylanases. This emphasizes the need for novel enzymes with increased thermostability and pH stability compared to the existing library of xylanases. There have been no reports or evidence that *P. alba* produces xylanases, therefore, this fungus acts as a novel source of xylanases. It is hypothesized that the purified xylanase will retain its original properties as a thermostable, pH stable and highly active enzyme. It is further hypothesized that the purified enzyme will successfully degrade pre-treated sugarcane bagasse under conditions for bio-ethanol production.

## 1.8. OBJECTIVES

1.8.1. To identify *P. alba* and its microscopic characteristics by sequencing the 18S rRNA gene and scanning electron microscopy, respectively.

1.8.2. To purify a xylanase produced by *P. alba* using ammonium sulphate precipitation and chromatographic techniques.

1.8.3. To characterize the purified xylanase by determining specific activity, pH and temperature optimum and stability.

1.8.4. To determine the ability of the partially purified xylanase and crude enzyme extracts to degrade pre-treated sugarcane bagasse.

# CHAPTER 2

## IDENTIFICATION OF *P. alba* BY DNA SEQUENCING AND SCANNING ELECTRON MICROSCOPY

### 2.1. INTRODUCTION

Early fungal classification schemes relied on the macroscopic features of fungal fruiting bodies (Liu, 2011). However, the classification scheme has since evolved and is based on differences in microscopic features and DNA sequences. DNA based taxonomy has become the most popular method of choice as it is now possible to generate and analyze homologous DNA sequences of functionally conserved genes (Weber, 2009; Gherbawy and Voigt, 2010). Sequencing and comparison of the conserved 18S gene located in the internal transcribed spacer (ITS) region of ribosomal DNA reduces variations among isolates (Babu *et al*., 2007). Differences in microscopic features are also key components of the classification system that can be used to confirm information obtained from DNA sequencing (Weber, 2009).

Ascomycota represent a group of fungi which produce asci and ascospores that are unique to this phylum (Geiser *et al*., 2006; Kavanagh, 2011). The classification of this Ascomycota is constantly evolving with the emergence of fungal isolates that have microscopic features that fall under more than a single phylum. The classification of the genus *Phialophora*, which belongs to the phylum Ascomycota, is not well defined. This genus is polyphyletic and only 16 species have been recognized with an additional 69 unassigned species (de Hoog *et al*., 2000; Liu, 2011). This genus is characterized by the presence of short conidiophores, and hyphae that are 5 μm wide (Cole and Kendrick, 1973; Liu, 2011). The hyphae are typically branched and hyaline to brown in colour. *P. alba* has been isolated from a few environmental sources however, the key microscopic features of this species have not been defined (Summerbell, 1989).

The presence of lignocellulolytic enzymes in *P. alba* has not been reported previously. The xylanase activity detected in the crude enzyme extract produced by *P. alba* was approximately 39 U/µg (Mosina, 2010). A pH optimum of 4 and temperature optima of 50°C and 90°C were detected. The crude enzyme was stable over broad pH and temperature conditions, highlighting the applicability of the crude enzyme. However, the isozyme(s) contributing to this desirable enzymatic activity are not known. Prior to protein purification and industrial application, it is essential to determine the molecular weight and the protein profile of the crude enzyme. SDS-PAGE as described by Laemmli *et al* (1970) is a simple and effective method of determining the molecular weight of proteins (Burgess and Deutscher, 2009). However, depending on the nature of the sample optimization steps may be necessary. The percentage of the PAGE gel, type of gel staining and sample preparation are a few key factors that affect the quality of results obtained in the electrophoresis of proteins (Chevalier, 2010).

Dithiotreitol (DTT) and 2-mercaptoethanol are powerful reducing agents that can be employed in the preparation of protein samples for reducing SDS-PAGE (Knight, 2004). These agents reduce the disulphide bonds present within a protein molecule or polypeptide. 2-Mercaptoethanol disrupts can be used to disrupt the tertiary and quaternary structure of proteins by breaking the disulphide bonds. Unlike 2-mercaptoethanol, DTT is a more powerful reducing agent and has a redox potential of -0.33 V at neutral pH compared to -0.26 V of 2-mercaptoethanol (Aitken *et al*., 2008). DTT reduces the disulphide bonds present in protein molecules thereby exposing the reactive cysteine residues (Garcia-Manyes *et al*., 2009). Cysteine residues provide structure, flexibility and mechanical properties to the protein macromolecules. Oxidising conditions promote the formation of stable intramolecular disulphide bonds that are easily broken under

reducing conditions (Buehler and Yung, 2009). For enzymes, zymogram analysis is effective in detecting the presence of specific enzyme and possibly different isoforms of the enzyme. However, for both techniques, SDS-PAGE and activity gels, optimization of the various run conditions is essential in order to provide a true representation of the protein profile (Bonner, 2007).

## 2.2. MATERIALS AND METHODS

### 2.2.1. MAINTENANCE AND GROWTH

*P. alba* was previously isolated from *Eucalyptus* sp. woodchips andgrown on malt extract agar (MEA) at 30°C for four days. Short term stock cultures were maintained on MEA (Merck, South Africa) at 4°C and subcultured every six to seven weeks. Long term glycerol stocks were prepared and stored at -20°C and -70°C.

### 2.2.2. IDENTIFICATION OF *P. alba* BY DNA SEQUECING AND SCANNING ELECTRON MICROSCOPY

The fungal isolates were inoculated in malt extract broth (MEB) (Sigma-Aldrich, South Africa) and incubated at 30°C for 3 days. The culture was centrifuged at 10 000×*g* for 3 minutes and the pellet was re-suspended in 200 µl of sterile distilled water. Fungal DNA was extracted using a soil microbe DNA kit (Zymo Research, USA) as per manufacturer's instructions and DNA samples were stored at -20°C. The broad range primer pair ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4R (5'-CCTCCGCTTATTGATATGCTAAG-3') were used to amplify the ITS1-5.8S-ITS2 region of the fungal rDNA genes (White *et al*., 1990). PCR products were visualized by electrophoretic analysis on a 1% agarose gel. Once the

presence of the PCR amplicon was confirmed they were sent for sequencing at Inqaba Biotech. BLAST searches of the amplified regions were conducted to obtain the identity of the isolates. The morphological characteristics of *P. alba* were evaluated by scanning electron microscopy (SEM) using fungal specimens grown on potato dextrose agar (PDA). Cultures ranging from one day and 14 days old were analysed. The fungal sample was fixed in 3% glutaraldehyde at 4°C overnight. This was followed by treatment with 1% osmium tetroxide for 1 hour and samples were subsequently dehydrated in ethanol. The fungal sample was subsequently freeze-substituted in acetone containing 2% osmium tetraxide at -80°C and subsequently embedded in epoxy resin and polymerized at 60°C for 2 days. Prior to viewing the fungal samples were mounted on copper grids.

### 2.2.3. ENZYME PRODUCTION

For xylanase production, *P. alba* was grown in medium described by Gomes *et al*. (2000) [one litre] supplemented with 1% (w/v) Birchwood xylan (Sigma-Aldrich, USA), 5 g $NH_4NO_3$, 0.05 g $CaCl_2$, 5 g $KH_2PO_4$, 0.5 g $MgSO_4.7H_2O$ and a 1 ml trace element solution [$g.l^{-1}$; $MnCl_2.4H_2O$ (0.03), $ZnSO_4.7H_2O$ (1.4), $H_3BO_3$ (0.3), $CoCl_2.6H_2O$ (0.2), $CuCl_2.2H_2O$ (0.01), $NiCl_2.6H_2O$ (0.02), $NaMoO_4.H_2O(0.0277).6H_2O$, $CoCl_2.6H_2O$]. Each flask was inoculated with a standardized inoculum (1 cm × 1 cm) of *P. alba* and incubated at 50°C for seven days on a rotary shaker at 200 rpm (New Brunswick Scientific, Innova 44, USA) (Mosina, 2010). After seven days the culture was passed through glass fibre filters to remove the mycelia and to obtain the crude enzyme filtrate. The crude enzyme and subsequent enzyme fractions were aliquoted and stored in 30% glycerol at -20°C until ready to use.

## 2.2.4. OPTIMIZATION OF SAMPLE PREPARATION FOR REDUCING SDS-PAGE

The molecular weight (kDa) of the individual isoenzymes was determined by SDS-PAGE as described by Laemmli *et al*. (1970) on a 10% denaturing polyacrylamide gel containing 0.1% SDS. A broad range molecular weight pre-stained protein ladder (Promega), was used. Due to low total protein concentration, all gels were silver stained as described by Blum *et al*. (1987). Most of the protein sample remained as a complex at the top of the gel or in the well. Therefore, to enhance the visibility of protein bands on reducing SDS-PAGE gels, two reducing agents *viz*; dithiotreitol (DTT) and 2-mercaptoethanol were tested at varying concentrations. Concentrations of DTT and 2-mercaptoethanol ranged from 30-50 mM and 10-50%, respectively. Electrophoretic analysis was conducted as described above and all gels were silver stained.

## 2.2.5. OPTIMIZATION OF ZYMOGRAM ANALYSIS

Xylanase activity was detected by zymogram analysis of reducing SDS-PAGE gels loaded with crude enzyme. Several renaturation conditions were tested and optimized in an attempt to fully renature the enzyme to its native conformation. Upon electrophoresis the gels were washed with deionized water and subsequently washed twice in 25% isopropanol containing citrate buffer (pH 5) for 30 minutes (Stålbrand *et al*., 1993). Renaturation of the protein was further carried out by washing twice with citrate buffer for 1 hour each. A 1% substrate gel containing Beechwood xylan dissolved in citrate buffer (50 mM, pH4) was prepared. The PAGE gel was placed onto the substrate gel and incubated for 1 hour at 50°C. The substrate gel was then stained for 1 hour with 0.1% Congo red and destained with 1 M NaCl. The gel was fixed by the addition of 0.5% acetic acid and subsequently visualized.

A second protocol for zymogram analysis as described by Royer and Nakas (1990) was tested and optimized. All samples were reduced a described in 2.2.4. A substrate gel was prepared as described above and sandwiched to the reducing 10% SDS-PAGE gel. The sandwich was incubated at 50°C for 1 hour. Once separated the substrate gel was immersed in 95% ethanol for 30-60 minutes and subsequently visualized.

The zymogram analysis protocol described by Ninawe *et al*. (2006) was tested. A 10% Native PAGE gel supplemented with 1% Beechwood xylan (250 µl) was cast. The crude enzyme was loaded, separated by electrophoresis and subsequently stained with Congo red for 1 hour and destained with 1M NaCl (Ninawe *et al*., 2008).

## 2.3. RESULTS

### 2.3.1. IDENTIFICATION OF FUNGAL ISOLATE

Figure 2.1 depicts the 600 base pair of 18S rRNA amplicon. The edited sequence (Appendix A) was used to determine the identity of the fungus against a database of known microorganisms which was identified as *P. alba* (HM 116755) with a 99% homology. The microscopic characteristics were determined by scanning electron microscopy (SEM) to determine the structural features of *P. alba*. Figure 2.2 (a) shows the presence of phialide structures in a 5 day old culture. Conidiogenic hyphae were present in the 5 day old culture (Figure 2.2 b). After 10 days, several oval shaped conidia and conidiogenic hyphae were observed (Figure 2.2. c). A two week old culture highlighted the presence of thick walled resistant structures termed, chlamydospores amongst a network of septate hyphae (Figure 2.2 d).

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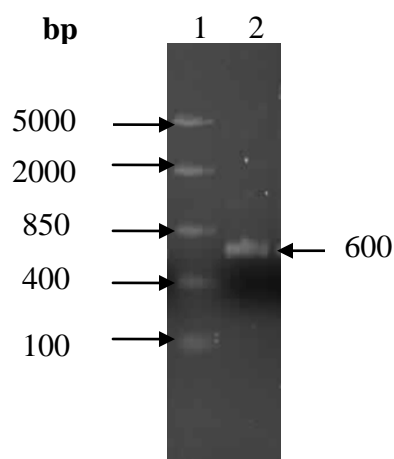
**Figure 2.1: 1% Agarose gel depicting the 18S rRNA amplicon of fungal DNA.** (Lane 1: FastRuler middle range DNA ladder, 2: 18S PCR amplicon).
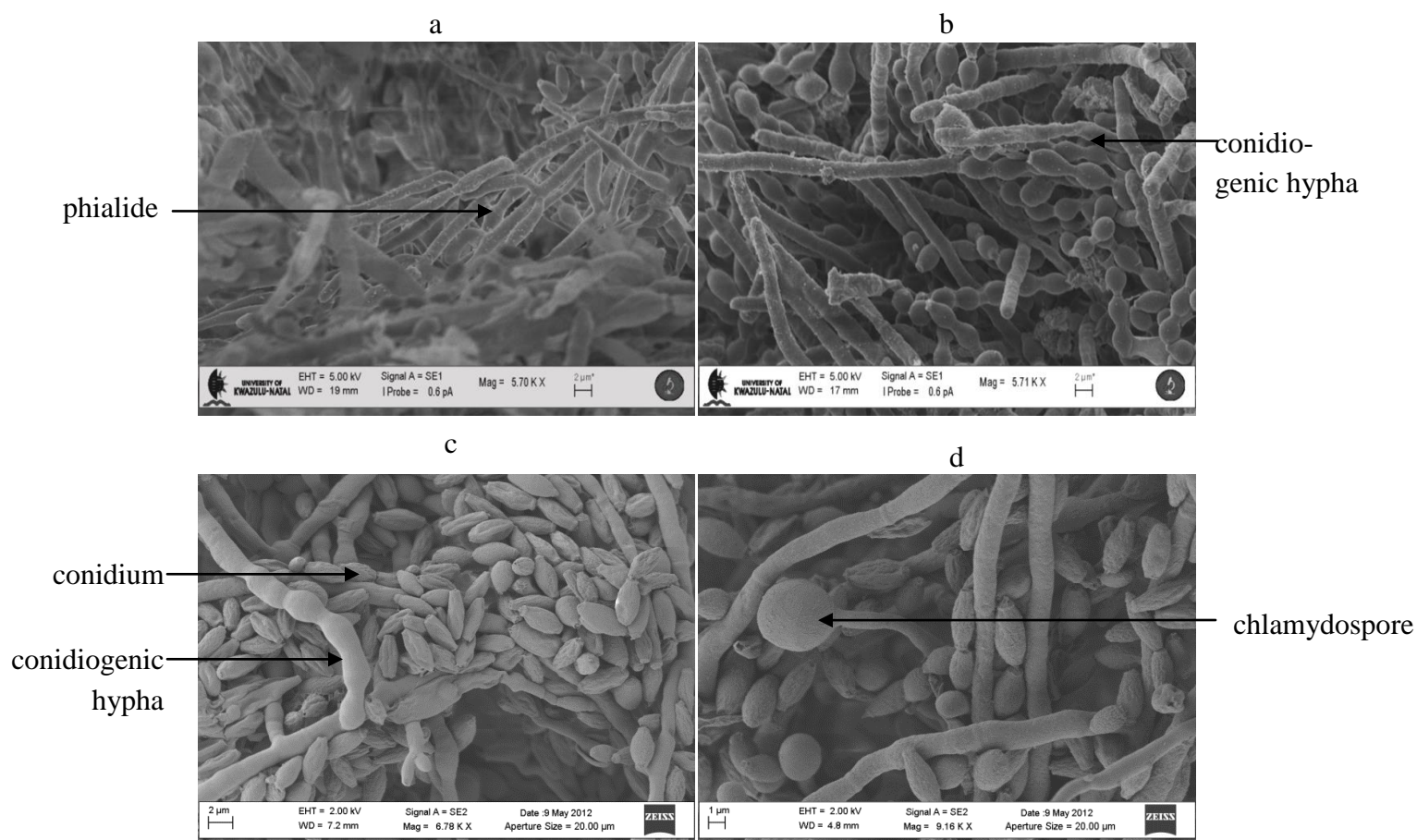


**Figure 2.2: Scanning electron microscopy images of** *P. alba* **grown on potato dextrose agar showing morphological differences in a a) 5 day old culture, b) 7 day old culture, c), 10 day old culture and d) 14 day old culture.**

37

## 2.3.2. OPTMIZATION OF SAMPLE PREPARATION FOR REDUCING SDS-PAGE

All concentrations of 2-mercaptoethanol tested either resulted in no or partially reduced crude enzyme (Figure 2.3a). A large proportion of the protein sample remained in the wells and appeared as a dark mass at the top of the gel. A considerable amount of smearing can be seen and only a few distinct bands in all crude enzyme preparations. In Figure 2.3b crude enzyme preparations were subjected to varying concentrations of DTT. At DTT concentrations below 50 mM, proteins were partially reduced and an accumulation of low molecular weight subunits can be seen on the lower end of the SDS-PAGE gel. Some of the protein sample remained at the top of the gel. The crude enzyme was successfully reduced in the presence of 50 mM DTT as distinct bands, representative of the various subunits are visible.
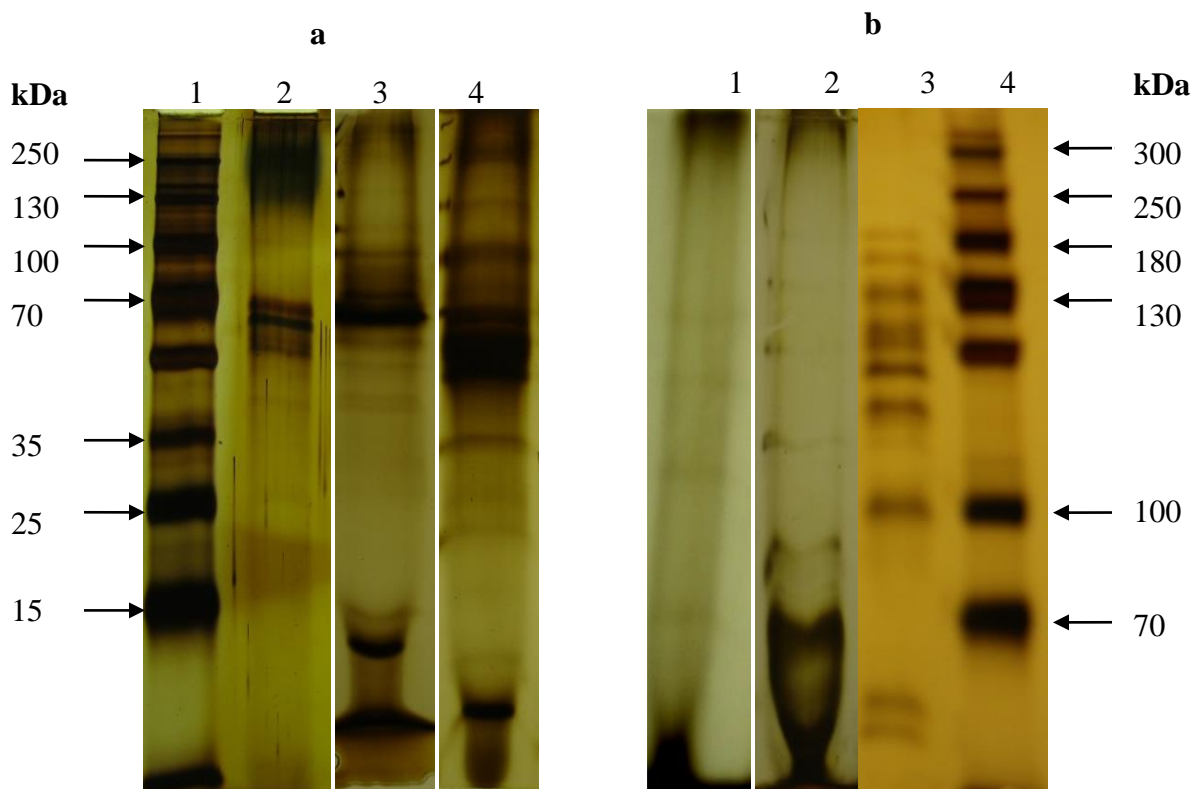


**Figure 2.3: 10% Denaturing SDS-PAGE gel showing the crude enzyme reduced with varying concentrations of (a) 2-mercaptoethanol and (b) dithiothreitol \*.** (a: Lane 1: protein ladder 2: crude enzyme (10% mercaptoethanol), 3: crude enzyme (30% mercaptoethanol), 4: crude enzyme (50% mercaptoethanol)) (b: Lane 1:

crude enzyme (10 mM DTT), 2: crude enzyme (30 mM DTT), 3: crude enzyme (50 mM DTT), 4: protein ladder). *A few representative DTT concentrations are shown.

### 2.3.3. OPTIMIZATION OF ZYMOGRAM ANALYSIS

Figure 2.4 depicts a zymogram prepared from a substrate gel. No hydrolysis zones which are indicative of the absence of xylanases were detected. A similar trend was observed in the second optimization attempt (Appendix B). The native PAGE gel supplemented with substrate was successful in showing the hydrolysis of the substrate. Figure 2.4 clearly shows the presence of six xylanases isozymes ranging from 90-210 kDa in molecular weight.
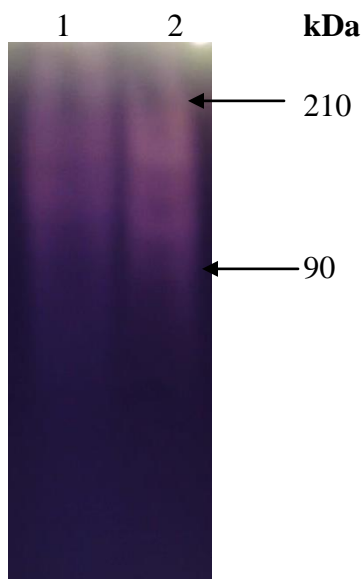


**Figure 2.4: Optimized 10% Native PAGE gel supplemented with 1% beechwood xylan showing the isozymes present in the crude enzyme.** (Lane 1: crude enzyme, 2: 10× concentrated crude enzyme).

## 2.4. DISCUSSION

The thermophilic fungus in the present study was identified as *P. alba*. The thermophile produces several oval shaped conidia in its asexual state and chlamydospores in its sexual state (Figure 2.2 a-d). The production of conidia, termed conidiogenesis, involves several stages in the

fungal life cycle (Kavanagh, 2011). Over a two week period, various structural differences could be seen in *P. alba*. From the highly branched hyphal network a group of metulae-like structures that arose forming a brush-like structure (Figure 2.2 a). Further incubation resulted in the formation of conidiogenic hyphae and phialides with a pointed apex (Figure 2.2 b, c). Phialides are specialized conidiogenic cells that produce conidia. According to Cole and Kendrick (1973) *Phialophora verrucosa* phialides are borne directly on the hyphae and at the apex of short lateral branches that arise from aerial or repent hyphae. A distinct accumulation in conidia was observed in Figure 2.2 (c) after 10 days of cultivation followed by the production of chlamydospores after 14 days. The genus *Phialophora* has not been fully studied as the classification of some species including *P. alba* is incomplete. There are differences in the classification of *Phialophora* due to the polymorphic nature of the thermophile (Medlar, 1915; Heitman, 2006). Currently, all *Phialophora* species belong to the phylum Ascomycota. All reports on *Phialophora* provide evidence on the presence of septate hyphae, conidia, phialides and chlamydospores (Medlar, 1915; Lagerberg *et al*., 1927) and to date no changes in the classification of this genus have been reported. Most species have been identified as dematiaceous filamentous fungi and have been isolated from soil, decomposing plant matter, decaying food and wood pulp (Cole and Kendrick, 1973; Summerbell *et al*., 1989). The microscopic features of *P. alba* have not been documented and the present study provides the first evidence of these *Phialophora* type microscopic characteristics of *P. alba*.

The crude enzyme of preparation from *P. alba* showed the presence of high molecular weight proteins (Figure 2.3) as is evidenced by SDS-PAGE. Various conditions had to be tested in order to visualize distinct bands in a reducing SDS-PAGE gel. DTT is a very powerful reducing agent

and unlike 2-mercaptoethanol, very low concentrations are effective in reducing proteins to its subunits (Keten, 2012). Several attempts were made to renature the xylanases however as evidenced by the absence of xylanase activity on the zymograms the xylanases did not maintain their native conformation. The cysteine residues contribute to the stability of the folded protein (Keten *et al*., 2012). In the presence of reducing agents the configuration of the proteins is altered ultimately affecting the function of the enzymes (Fass, 2011). As a result, the isozymes could not be renatured to their fully active and native conformation. Therefore, the initial attempts at zymogram analysis using a reducing SDS-PAGE gel were unsuccessful. The crude enzyme in these gels was reduced with 50 mM DTT were used. A successful zymogram was obtained when samples were electrophoresed in a 10% native PAGE supplemented with 1% Beechwood xylan (Figure 2.4). As the crude enzyme migrated through the PAGE gel the incorporated xylan was hydrolysed forming distinct bands of hydrolysis which are indicate of the presence of xylanase isozymes. Six different isozymes ranging from 90-210 kDa were detected. To determine the properties of the individual isozymes, each needs to purified by chromatographic techniques

# CHAPTER 3

## PURIFICATION OF a XYLANASE PRODUCED BY *P. alba* BY AMMONIUM SULPHATE PRECIPITATION AND ION EXCHANGE CHROMATOGRAPHY

## 3.1. INTRODUCTION

Column chromatography can be employed to purify crude enzymes of bacterial and fungal origin (Burgess and Deutscher, 2009). Xylanases range from 6-80 kDa in size (Butt *et al*., 2008) and there are few reports on the purification of high molecular weight xylanases. However, there are reports of a 266 kDa and 350 kDa xylanase from *Thermotoga maritima* and *Thermoanaerobacteria* sp., respectively (Shao *et al*., 1995; Bergquist *et al*., 2001). The type of technique and columns used is entirely dependent on the known properties of the crude enzyme. A typical purification process begins with purification by precipitation. Different precipitation conditions need to be tested to determine the saturation point and most suitable method with maximum enzyme recovery (Sharma and Sanga, 2009). Ammonium sulphate and polyethylene glycol are the most reliable methods for protein precipitation (Wilson and Walker, 2010). Precipitation eliminates contaminating proteins present in the crude enzyme that do not have the same saturation point as the fractions enriched with the enzyme. The precipitated sample can be further purified using chromatographic techniques depending on the purity of the sample. Ammonium sulphate precipitation is the most commonly used precipitation method for thermophilic xylanases. El-Nasser *et al*. (2010) purified a cellulase-free xylanase from *Streptomyces rochei* by 60% ammonium sulphate precipitation in conjunction with other chromatographic techniques.

Prior to chromatography it is essential to remove salts present in the sample either by dialysis or by means of a desalting column such as Sephadex G25 (Janson, 2012). The presence of salts and

media components may interfere with separation by chromatography. There are numerous reports on the purification of xylanases however; they differ in the yield, enzyme recovered and purification factor. Typically ion exchange chromatography and gel filtration are employed for the separation of proteins. An array of materials can be used as the matrix and depending on the type of resin selected the enzyme may bind irreversibly (Heftmann, 2004). Therefore, it is essential to select the most suitable starting material that will allow for the separation of proteins.

Ion exchange chromatography exploits the differences in the net charge of proteins allowing for separation based on the isoelectric point (pI) (Burgess and Deutscher, 2009). Anion- or cation-exchange chromatography can be used based on the pI of the xylanase (Campbell and Farrell, 2007). Where the pI of the enzyme is unknown it becomes essential to test different resins (anionic/cationic). This will aid in determining whether anion or cation exchange chromatography will be conducted (Rosenberg, 2005). Once the type of ion exchange (IEX) resin has been established various optimization steps are required to ensure binding and purification of the protein of interest. Optimization of IEX may prove to be difficult as the elution buffer used may influence the binding capability of xylanases (Aguilar, 2004). Therefore, the outcome of the purification process is dependent on the type of chromatographic techniques used and the conditions under which the proteins are separated. The degree of purity required is ultimately determined by the intended application of the xylanase. Several thermophilic xylanases have been purified by ion exchange chromatography from *Melanocarpus albomyces*, *Aureobasidium pullulans, Streptomyces cyaneus* (Saraswat and Bisaria, 2000; Tanaka *et al*., 2006; Ninawe *et al*., 2008)

Developing a specific purification process for a protein requires optimization of several steps in the purification process. Therefore, each purification process needs to be tailored to suit a specific protein of interest. Protein purification can prove to be a lengthy process. Depending on the nature of the sample, some proteins can only be partially purified, and total purity may be difficult to attain. The success of any selective purification is monitored by measuring the total protein content in conjunction with SDS-PAGE and zymogram analysis as well as enzyme assays to identify the protein of interest (Janson, 2012).

Various assays can be used for the detection of protein. The Lowry method utilizes the Folin reagent which is a mixture of sodium tungstate, molybdate and phosphate together with copper sulphate to detect protein levels (Wilson and Walker, 2010). In the presence of protein the solution develops a blue-purple colour that is quantified by measuring absorbance at 660 nm (Nigam and Ayyagari, 2007). Colour development is largely due to the presence of tyrosine and tryptophan residues. However, the presence of zwitterionic buffers, EDTA and Tris may interfere with the protein assay (Waterborg, 2009). The Bradford assay is a dye based assay that is used to measure the amount of protein present in a sample (Bradford, 1976). The Coomassie brilliant blue used in the assay bind to the protein and absorbance of this complex is measured at 595 nm. The UV absorbance of the protein is measured at 280 nm and can be used to calculate the concentration of the protein using a known molar extinction coefficient (Graslund et al., 2008). A major advantage of the Bradford assay is that colour development is rapid and stable (Wilson and Walker, 2010). The dinitrosalicylic acid (DNS) assay detects reducing sugars including xylose which is liberated by the hydrolytic action of xylanases on xylan (Bailey et al.,

1992). This enzyme assay provides a suitable method of determining xylanase activity thus ensuring that fractions containing enzyme are identified.

SDS-PAGE is essential in determining the purity of the sample and the molecular weight of the enzyme (Laemmli, 1970; Bonner, 2007). The intensity of the bands is usually directly proportional to the protein concentration. Zymogram analysis is conducted in conjunction with SDS-PAGE analysis to determine the number of isozymes present in sample and their respective molecular weights. This technique uses the same principle as polyacrylamide gels. As the proteins migrate through the pores of the acrylamide matrix the xylanases hydrolyse the incorporated substrate. This allows the enzymes to band according to size forming distinctive bands of hydrolysis which are indicative of the molecular weight of the xylanase.

Although the purification of xylanases has been carried out for many years, due to vast differences in enzymatic properties these enzymes no single optimal protocol exists that can be used to purify all xylanases. The vast number of protocols and differences in enzyme properties further increases the complexity of the purification process. This chapter focuses on the method optimization for the isolation of the thermophilic high molecular weight xylanase from *P. alba*.

## 3.2. MATERIALS AND METHODS

### 3.2.1. ENZYME ASSAYS

Xylanase activity of the crude enzyme and partially pure enzyme was assayed at optimum pH (Mosina, 2010) using the DNS assay (Bailey *et al*., 1992). Enzyme activity was determined at 50°C and pH 4 (0.05 M citrate buffer) with 1% Beechwood xylan (Sigma-Aldrich, South Africa)

as the substrate. Xylanase activity was expressed in nkatals per ml (1 nkat is equivalent to the amount that is required to catalyze the transformation of 1 nmol of substrate in 1 second under the specified conditions).  Using a 0.01 M xylose solution a series of xylose concentrations were prepared and utilized as standards. The substrate alone and the inactivated enzyme were used as controls. All reactions were carried out in triplicate and the average absorbance readings (x axis) was plotted against enzyme activity (y axis). The xylanase activity was expressed as IU/ml.

### 3.2.2. PROTEIN ASSAY

Total protein concentration was determined using the Bradford assay (Bradford, 1976) and expressed in µg/ml. Specific activity of the xylanase was determined using the equation shown below and expressed in IU/µg. Specific activity = [enzyme activity (IU/ml)] / [total protein concentration (µg/ml)].

### 3.2.3. ENZYME PURIFICATION

Purification of the xylanase produced by *P. alba* was optimized using different precipitation methods, desalting methods and ion exchange columns. Ammonium sulphate precipitation was compared with polyethylene glycol precipitation. Once a suitable method of precipitation was established the sample was desalted by dialysis or a desalting column. The highly concentrated and desalted xylanase was subsequently purified by ion exchange chromatography using different resins to establish the suitable resin.

### 3.2.3.1. Ammonium sulphate precipitation

The crude xylanases were precipitated using ammonium sulphate (20-100% saturation). An appropriate amount of ammonium sulphate was added to the sample to obtain a pre-determined concentration of 20% at 4ºC (Scopes, 1993). The precipitate was collected by centrifugation at 5000 rpm (Eppendorf Centrifuge 5430R) for 15 minutes. The resulting pellet was re-suspended in citrate buffer (50 mM, pH 4) followed by dialysis against the same buffer (1:100) overnight using Snake skin pleated dialysis tubing (Thermo Scientific, USA) with a 10 kDa molecular weight cut-off. This fraction of the sample was tested for xylanase activity using the DNS assay. Ammonium sulphate was added to the remaining supernatant to obtain a concentration of 40% and allowed to settle overnight at 4°C. Samples were subsequently centrifuged, re-suspended, dialyzed and tested for enzyme activity. These steps were repeated after each saturation point to obtain concentrations of up to 100% saturation as shown in Figure 3.1. Once the saturation point of the crude enzyme was established the bulk of the crude enzyme was precipitated in a single step at the pre-determined saturation point. This represented the proportion of the sample that was most concentrated with the enzyme of interest.

### 3.2.3.2. Polyethylene glycol precipitation

Polyethylene glycol (PEG) precipitation was performed using the modified method of Polson *et al*. (1964). Different concentrations of PEG 6000 were used on the crude enzyme to determine the saturation point of the enzyme(s). The crude enzyme (0.5 ml) was mixed with 1 ml of citrate buffer (50 mM, pH 4) to which 10% PEG 6 000 was added to the crude enzyme with gentle stirring. The suspension was centrifuged (12 000 $\times$ $g$, 10 minutes, 4ºC) and the pellet obtained was dissolved in citrate buffer (50 mM, pH 4). Xylanase activity and protein concentration were

determined using the DNS assay and the Bradford assay, respectively. The above-mentioned steps were followed for different concentrations of PEG 6000 (20-80%) until the optimal recovery of the enzyme was determined.

```
                          ┌─────────────────┐
                          │  Crude enzyme   │
                          └─────────────────┘
                                   │
┌──────────────────────────┐      ▼
│ Ammonium sulphate (30%    │───────────────┐
│ saturation), 4°C, overnight.│             │
│ Centrifuge (5000 rpm, 15 min)│            │
└──────────────────────────┘               │
                     ┌────────────┐   ┌─────────┐
                     │ Supernatant│   │ Pellet  │
                     └────────────┘   └─────────┘
                          │                 │
                          ▼                 ▼
┌──────────────────────────┐        ┌─────────────────────┐
│ Ammonium sulphate (70%    │────────│ Re-suspend in citrate│
│ saturation), 4°C, overnight.│       │ buffer (pH 4). Dialysis,│
│ Centrifuge (5000 rpm, 15 min)│      │ DNS assay, Bradford │
└──────────────────────────┘        │ assay, SDS-PAGE,    │
                     ┌────────────┐  │ zymogram analysis   │
                     │ Supernatant│  └─────────────────────┘
                     └────────────┘   ┌─────────┐
                                      │ Pellet  │
                                      └─────────┘
                          │                 │
                          ▼                 ▼
┌──────────────────────────┐        ┌─────────────────────┐
│ Ammonium sulphate (100%   │────────│ Re-suspend in citrate│
│ saturation), 4°C, overnight.│       │ buffer (pH 4). Dialysis,│
│ Centrifuge (5000 rpm, 15 min)│      │ DNS assay, Bradford │
└──────────────────────────┘        │ assay, SDS-PAGE,    │
                     ┌────────────┐  │ zymogram analysis   │
                     │ Supernatant│  └─────────────────────┘
                     └────────────┘   ┌─────────┐
                                      │ Pellet  │
                                      └─────────┘
                                           │
                                           ▼
                              ┌─────────────────────────┐
                              │ Re-suspend in citrate buffer (pH 4).│
                              │ Dialysis, DNS assay, Bradford assay,│
                              │ SDS-PAGE, zymogram analysis │
                              └─────────────────────────┘
```

**Figure 3.1: Overview of the steps involved in ammonium sulphate precipitation.**

### 3.2.3.3. Dialysis

All samples resulting from ammonium sulphate precipitation were dialysed against citrate buffer (50 mM, pH 4) to remove the salt using dialysis tubing (Sigma-Aldrich, South Africa) at 4°C. Different dialysis times (4-12 hours) were tested to ensure the removal of salts.

### 3.2.3.4. Desalting column

The 70% ammonium sulphate suspension was desalted using Sephadex G25 (15×2 cm). The proteins were desalted against citrate buffer (pH 4) and eluted at a constant flow rate of 1 ml/min. Fractions (2 ml) were collected and assayed for enzyme activity and protein concentration. The fractions enriched with the enzyme were pooled and concentrated by freeze drying. The concentrated enzyme fractions were subjected to SDS-PAGE and zymogram analysis.

### 3.2.3.5. Ion exchange chromatography

A manual protein purification system (BioRad) was initially used for the purification of the desalted 70% ammonium sulphate suspension. A 5 ml DEAE column (BioRad, USA) () was used. All proteins were eluted in 50 mM citrate buffer and a linear gradient of NaCl (0.1 M). Fractions (2 ml) were collected at a constant flow rate of 1ml/min.

The manual protein purifications system was subsequently replaced with an automated system, AKTA Purifier 100. The desalted 70% ammonium sulphate suspension (57 120 µg/ml) was fractionated by ion exchange chromatography using a 1 ml ion exchange column pre-equilibrated

with 50 mM citrate buffer (pH 4). Different types of ion exchange columns (Table 3.1) were used in the optimisation process. The proteins were eluted in a 50 mM citrate buffer (pH 4) solution with a NaCl linear gradient of NaCl (0-1.0 M). All proteins were eluted in 20 column volumes (CV) of the selected buffer and 2 ml fractions were collected at a constant flow rate of 1 ml/min. Enzyme activity was tested using the DNS assay (Bailey *et al*., 1992) and total protein concentration was determined using the Bradford assay (Bradford, 1976). The active fractions were pooled, concentrated by freeze drying and dialyzed against 50 mM citrate buffer (pH 4). Once a suitable resin was selected separation was conducted as described above however, proteins were eluted in 50 mM phosphate buffer (pH 7) to enable binding. The active fractions were pooled, concentrated by freeze drying and re-suspended in citrate buffer (pH 4). The sample was then dialyzed against citrate buffer (pH 4) and used in further analysis. Subsequent analysis indicated the presence of the xylanase in the column washings. To allow for the elution of xylanase higher NaCl gradients (0-1.5 M and 0-2.5 M) were tested.

The purification fold calculated for each step was calculated by dividing the specific activity at each step by the specific activity at the initial step. The specific activity of the crude enzyme was given a value of 1. The overall yield (%) in at each purification steps was determined using the equation (Scopes *et al*., 1993) :[(Total activity of target protein)/(Total activity of target protein at initial step)]×100.

**Table 3.1: Types of anion and cation exchange HiTrap columns selected for optimization**

| HiTrap columns | Charged group | Exchanger | Size (cm) | Capacity (mg.ml$^{-1}$) |
|---|---|---|---|---|
| Q sepharose fast flow (Q FF) | $-N^+(CH_3)_3$ | Anion | $0.7 \times 2.5$ | 120 |
| Q sepharose XL (Q XL) | $-N^+(CH_3)_3$ | Anion | $0.7 \times 2.5$ | >130 |
| DEAE sepharose XL (DEAE) | $-N^+H_2(C_2H_5)_2$ | Anion | $0.7 \times 2.5$ | 110 |
| SP sepharose fast flow (SP FF) | $-CH_2CH_2CH_2SO_3^-$ | Cation | $0.7 \times 2.5$ | 70 |
| SP sepharose XL (SP XL) | $-CH_2CH_2CH_2SO_3^-$ | Cation | $0.7 \times 2.5$ | >160 |

## 3.3. RESULTS

### 3.3.1. PURIFICATION BY PRECIPITATION

#### 3.3.1.1. Ammonium sulphate precipitation

The total protein present in the crude enzyme was 28.56 µg/ml and the specific activity was 1.98 IU/µg at 50°C and pH 4. Two series of ammonium sulphate precipitation were tested and in the first series the highest xylanase activity recovery was obtained in the 20% saturated ammonium sulphate suspension (Appendix B). However, a very low percentage of the total xylanase (1.89%) was recovered with the majority of the xylanase remaining in the supernatant. Table 3.2 represents the second series of ammonium sulphate concentrations tested. More than 13% of enzyme was recovered in the 30% ammonium sulphate suspension. A significant amount of xylanase was also recovered in the 100% AS fraction and the remaining supernatant. However, the saturation point of the crude enzyme was at 70% ammonium sulphate saturation as the highest amount of xylanase activity (52%) was recovered in this fraction. The 70% ammonium sulphate suspension displayed a specific activity of 23.06 IU/µg and total protein concentration of 127.1 µg/ml.

**Table 3.2: Ammonium sulphate concentrations tested at the respective enzyme activities and protein concentrations.**

| Sample | Protein | | | Xylanase activity | | Specific activity IU/µg | % Xylanase activity recovered |
|---|---|---|---|---|---|---|---|
| | Vol (ml) | Protein (µg/ml) | Tot. prot. (µg) | Activity (IU/ml) | Total xylanase activity (IU) | | |
| **0%** | 100 | 28.56 | 2856 | 56.6 | 5660 | 1.98 | 100 |
| **0-30%** | 2.3 | 44.81 | 103.1 | 332.3 | 764.29 | 7.41 | 13.5 |
| **31-70%** | 3.4 | 37.38 | 127.1 | 862.3 | 2931.82 | 23.06 | 51.79 |
| **71-100%** | 3.6 | 7.4 | 26.64 | 163.55 | 588.78 | 22.1 | 10.4 |
| **Supernatant** | 115 | 22.4 | 2576 | 11.94 | 1373.1 | 0.533 | 24.25 |

### 3.3.1.2. Polyethylene glycol precipitation

Polyethylene glycol (PEG) 6000 was used to precipitate and concentrate the crude enzyme and the results obtained were compared to those obtained by precipitation with ammonium sulphate. Table 3.3 shows the effect of PEG at different concentrations. The highest activity of enzyme precipitated was obtained at 20% PEG. PEG concentrations of 60% and above resulted in lowered enzyme activity. The high PEG concentrations used interfered with the Bradford assay

and alternative methods for the removal of PEG were not worth exploring. Therefore, the protein concentration and specific activity could not be determined.

**Table 3.3: Enzyme activity and protein concentration of the crude enzyme subjected to precipitation by PEG.**

| %PEG | Xylanase (IU/ml) | Protein (mg/ml) |
|------|------------------|-----------------|
| 0    | 64.8             | -               |
| 10   | 77               | -               |
| 20   | 82               | -               |
| 30   | 74.4             | -               |
| 40   | 69               | -               |
| 50   | 75.8             | -               |
| 60   | 42               | -               |
| 70   | 26               | -               |
| 80   | 12.6             | -               |

### 3.3.2. OPTIMIZATION OF DESALTING METHODS

The removal of ammonium sulphate and salts present in the media is essential prior to chromatographic analysis. Table 3.4 illustrates the enzyme and protein recovered from a new batch of crude enzyme subjected to 70% ammonium sulphate precipitation. The 70% ammonium sulphate suspension was dialysed overnight and approximately 96% of enzyme activity was recovered after dialysis against citrate buffer (pH 4) and 82% using Sephadex G25.

**Table 3.4: Summary of results obtained for the removal of salts from the 70% ammonium sulphate suspension using dialysis and Sephadex G25.**

| Sample | Protein | | | Xylanase activity | | Specific activityI U/µg | % xylanase activity recovered |
|--------|---------|---------|----------------|-------------------|---------------------------|-------------------------|-------------------------------|
| | Vol (ml) | Protein (µg/ml) | Tot. prot. (µg) | Activity (IU/ml) | Total xylanase actvity (IU) | | |
| **70%** | 10 | 40.38 | 403.8 | 862.32 | 8623.2 | 21.35 | |
| **DS1** | 10 | 39.89 | 398.9 | 827.83 | 8278.3 | 20.75 | 96 |
| **DS2** | 10 | 38.27 | 382.7 | 707.1 | 7071 | 18.47 | 82 |

70%: 70% ammonium sulphate suspension, DS1: dialysed 70% pellet, DS2: 70% ammonium sulphate suspension desalted using Sephadex G25

### 3.3.3. ION EXCHANGE CHROMATOGRAPHY

#### 3.3.3.1. Purification of xylanase using DEAE column chromatography

The anion exchange resin, DEAE and manual fractionation methods produced nine protein peaks in the 35 fractions collected (Figure 3.2). Highest protein levels were detected in fraction 17. Xylanase activity was detected in fraction nine to 25 and peaked in fractions 12-18.  The protein and xylanase profiles were determined using denaturing SDS-PAGE (Figure 3.3) and zymogram (Figure 3.4), respectively. SDS-PAGE showed the presence of several contaminating proteins in the pooled fractions. Xylanase isozymes were present in the crude enzyme, 70% ammonium sulphate supension and pooled active fractions (13-18).
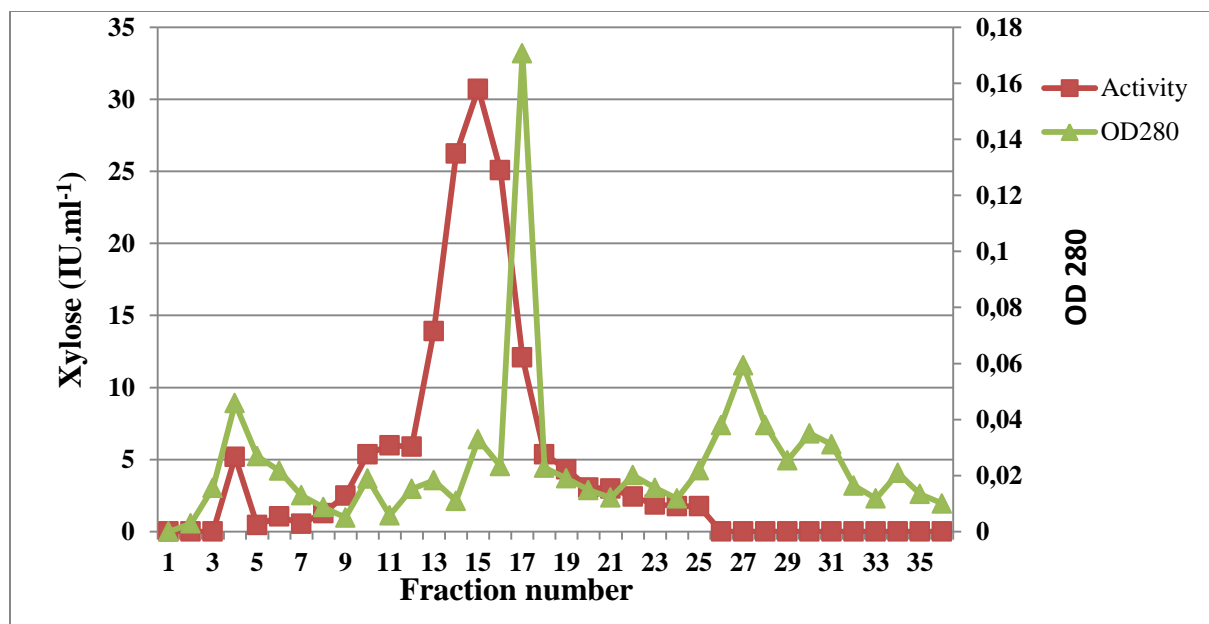
**Figure 3.2: Activity (U/ml) and absorbance at 280 nm for the fractions collected during ion exchange chromatography.**
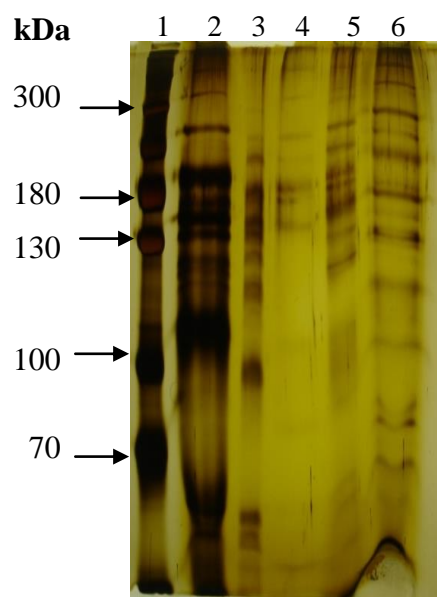


**Figure 3.3: 10% reducing SDS-PAGE of pooled ion exchange fractions that displayed xylanase activity\*.**

(Lane 1: protein ladder, 2; 70% ammonium sulphate suspension, 3:70% supernatant, 4: fractions 19-25, 5: fractions 9-12, 6: fractions 13-18). \*All samples were treated with 50 mM DTT.
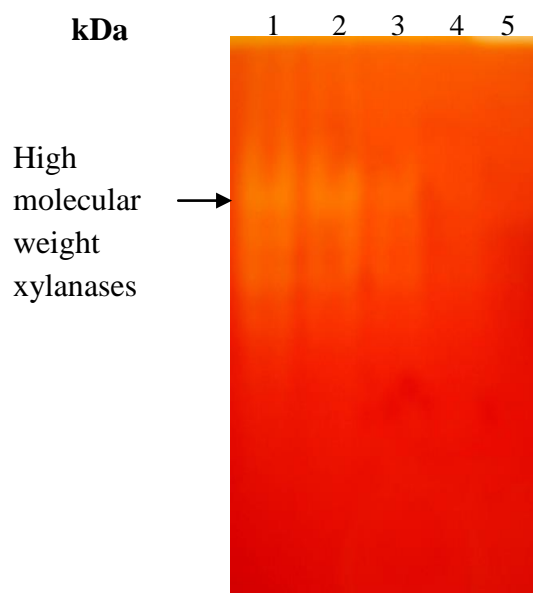
**Figure 3.4: Native PAGE supplemented with 1% beechwood xylan of the pooled ion exchange fractions enriched with xylanase.** (crude enzyme (1), 70% ammonium sulphate suspension (2), fractions 13-18 (3), fractions 9-12 (4), fractions 19-25 (5)).

### 3.3.3.2. Optimization of ion exchange chromatography using the AKTA Purifier

Of the two anion and three cation exchange columns tested, only the anion exchanger HiTrap Q FF eluted active fractions with xylanase activity. With the exception of HiTrap Q FF column, the total xylanase loaded in the ion exchange columns was strictly eluted in the unbound fraction. No xylanase activity was detected in the column washings. Figure 3.5 shows the elution profile of the desalted 70% ammonium sulphate suspension loaded on HiTrap Q FF. Two proteins peaks were eluted ahead of the NaCl gradient and collected in fractions A1-A4. All active fractions were eluted with the unbound protein and xylanase activity was detected in fractions A3 (47 IU/ml), A4 (33 IU/ml) and A5 (0.72 IU/ml). Three protein peaks that were eluted in fractions C2-C10 and two protein peaks were eluted in fractions D15-D15. However, no active xylanases bound to the resin as the remaining xylanase activity was detected in the unbound fractions, A1

and A2. Xylanase activity was also detected in the washings of the anion exchange column after all proteins were eluted.

The protein profiles and zymograms of the active fractions were determined using Native PAGE (with substrate), Native PAGE and SDS-PAGE. Figure 3.6 (a) shows the isozymes present in the eluted active fractions. Two isozymes of varying sizes were observed in fraction A3 and a single high molecular weight isozyme was observed in fraction A4. Native PAGE analysis (Figure 3.6 b) indicated that the active fractions A3 and A4 are only partially pure as trace amounts of low molecular weight contaminating proteins were present. Reducing SDS-PAGE analysis (Figure 3.7) showed the presence of a dominant 90 kDa band which could be a monomer of the xylanase which was evident in the crude enzyme and the eluted fractions that had been reduced with DTT.
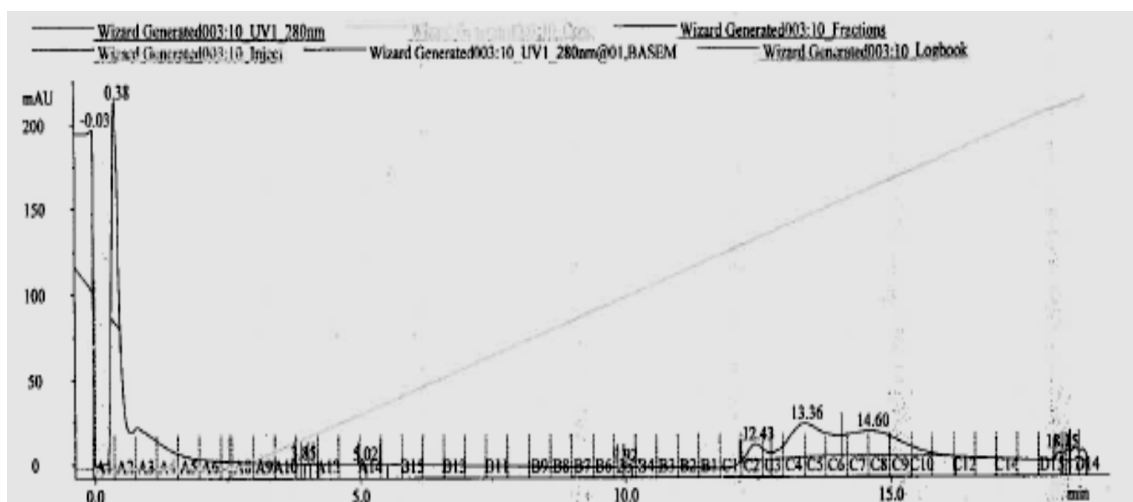


**Figure 3.5: Elution profile of the desalted 70% ammonium sulphate suspension with HiTrap Q FF sepharose using a linear gradient of 1 M NaCl.**

**Figure 3.6: a) 10% Native PAGE supplemented with 1% beechwood xylan and b) silver stained 10% Native PAGE of the ion exchange chromatography fractions enriched with xylanase.** (70% ammonium sulphate suspension (1), A1-A2 (2), A3 (3), A4 (4)).
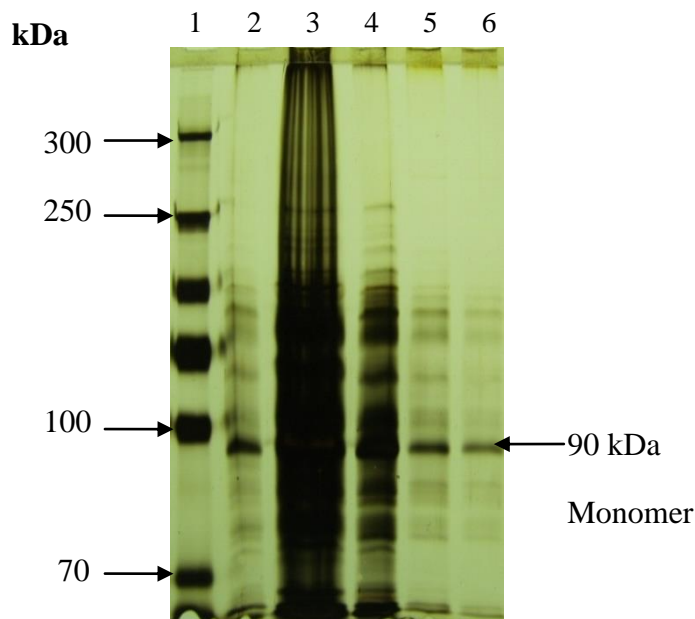


**Figure 3.7: 10% SDS-PAGE of proteins eluted by ion exchange chromatography with HiTrap Q FF sepharose against a 1 M NaCl gradient that have been reduced with 50 mM DTT.** (Prestained protein ladder (1), crude enzyme (2), 70% ammonium sulphate suspension (3), A3 (4), A4 (5), A5 (6)).

### 3.3.3.3. Optimization of ion exchange run conditions

In order to obtain a pure xylanase without any trace amounts of contaminating proteins the run conditions for the HiTrap Q FF column were further optimized. To enable binding of the isozymes detected in 3.3.3.2 above, a more suitable elution buffer, phosphate buffer (50 mM, pH 7) was used and different NaCl gradients (0-1.5 M and 0-2.5 M) were tested. Figure 3.8 illustrates the elution profile of the desalted 70% ammonium sulphate suspension against a linear gradient of 1.5 M NaCl. High levels of contaminating proteins were eluted in the unbound fraction. Three broad peaks were detected after the NaCl gradient was established and were therefore eluted at 100% 1.5 M NaCl. Xylanase activity was detected in the unbound fraction (1583 IU.ml$^{-1}$) and in fractions A13-14 (50.21IU.ml$^{-1}$), A15 (54.23 IU.ml$^{-1}$) and B15 (37.4 IU.ml$^{-1}$).



**Figure 3.8: Elution profile of the desalted 70% ammonium sulphate suspension using a linear gradient of 1.5 M NaCl and phosphate buffer (pH 7) using HiTrap Q FF sepharose.**

Native PAGE analysis (Figure 3.9 (a) and (b)) demonstrated a high degree of xylanase activity in the crude enzyme, 70% ammonium sulphate suspensionand unbound samples. The degradation of xylan substrate in these samples is visible over half the length of the Native PAGE supplemented with 1% beechwood xylan. Several different isozymes present in the active fractions in lanes 4-6; i.e fractions A13-14 and A15. Three isozymes varying in size were detected in B15. Higher concentrations of contaminating proteins were detected in the fractions eluted with 1.5 M NaCl compared to those eluted with 1 M NaCl (Figure 3.9 b). A dominant 90 kDa band was observed in all active fractions by SDS-PAGE analysis (Figure 3.10). Several other protein bands were evident as well.
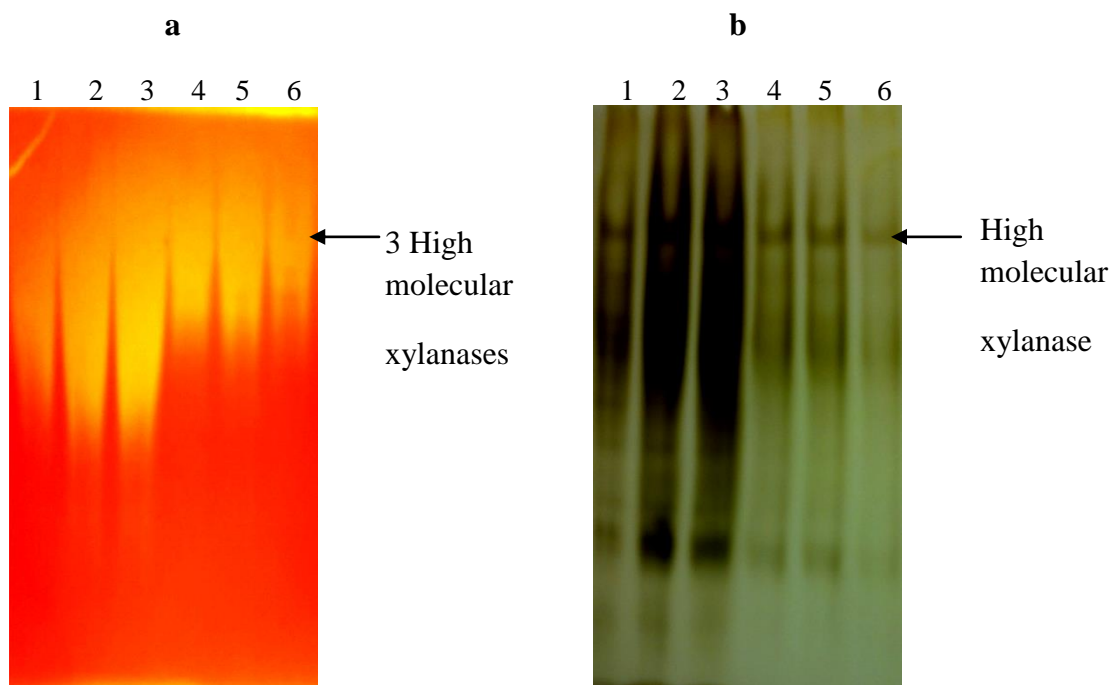


**Figure 3.9: a) 10% Native PAGE supplemented with 1% beechwood xylan and b) silver stained 10% Native PAGE of the ion exchange chromatography fractions eluted with 1.5 M NaCl.** (a: crude enzyme (1), 70% ammonium sulphate suspension (2), unbound fraction (3), A13-A14 (4), A15 (5), B15 (6)). (b: crude enzyme (1), 70% ammonium sulphate suspension (2), unbound fraction (3), A13-A14 (4), A15 (5), B15 (6)).
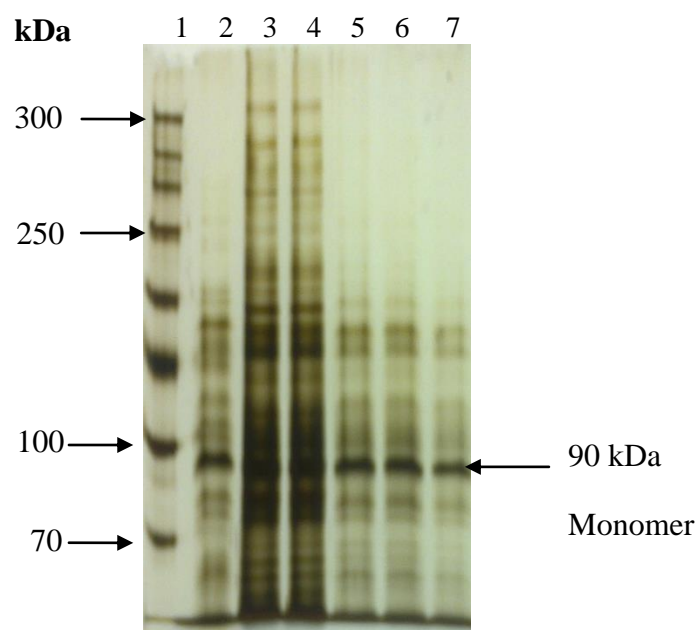
**Figure 3.10: 10% SDS-PAGE of protein eluted by ion exchange chromatography with HiTrap Q FF sepharose against a 1.5 M NaCl gradient that have been reduced with 50 mM DTT.** (Prestained protein ladder (1), crude enzyme (2), 70% ammonium sulphate suspension (3), unbound fraction (4), A13-A14 (5), A15 (6), B15 (7)).

The increased 2.5 M NaCl gradient allowed for the elution of five major protein peaks: i) one major peak corresponding to the unbound protein, ii) two peaks which displayed a cross-over effect and iii) two peaks after the NaCl gradient (Figure 3.11). Fractions A7 and A8 represent a single protein peak and were therefore combined. Xylanase activity in these combined fractions was 524 IU.ml$^{-1}$. Very high enzyme activity was detected in the unbound fraction (254 158 nkat.ml$^{-1}$) and in firaction A9 (57.34 IU.ml$^{-1}$), A10 (60 IU.ml$^{-1}$) and A11 (52.37 IU.ml$^{-1}$). The enzyme hydrolysis and native protein profiles (Figure 3.12) demonstrated the presence of several isozymes in the unbound fraction as xylan degradation was visible over half the length of the gel. High molecular weight isozymes were present in the fractions A9-11. A 210 kDa isozyme was clearly visible in the pooled active fractions (A7-8).
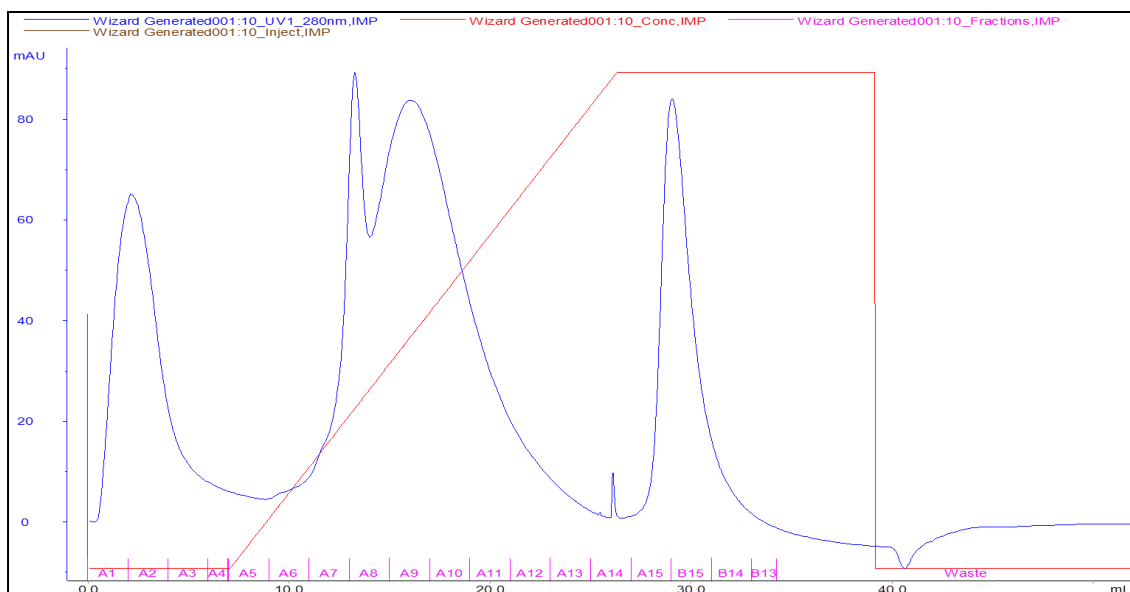
**Figure 3.11: Elution profile of the desalted 70% ammonium sulphate suspension using a linear gradient of 2.5 M NaCl and phosphate buffer (pH 7) using HiTrap Q FF sepharose.**

The protein profile of the crude extract and fractions with enzyme activity reveal the presence of a high molecular weight isozyme. A highly active xylanase with low protein concentration was purified. SDS-PAGE analysis showed only the presence of a 90 kDa subunit present in the pooled fractions (A7-8) (Figure 3.13). A final specific activity of 104.4 U/μg was obtained (Table 3.5). The purification by ammonium sulphate resulted in a yield and purification fold 52% of 79.5, respectively. Anion exchange chromatography resulted in a yield and purification fold of 1.4% and 10.6.
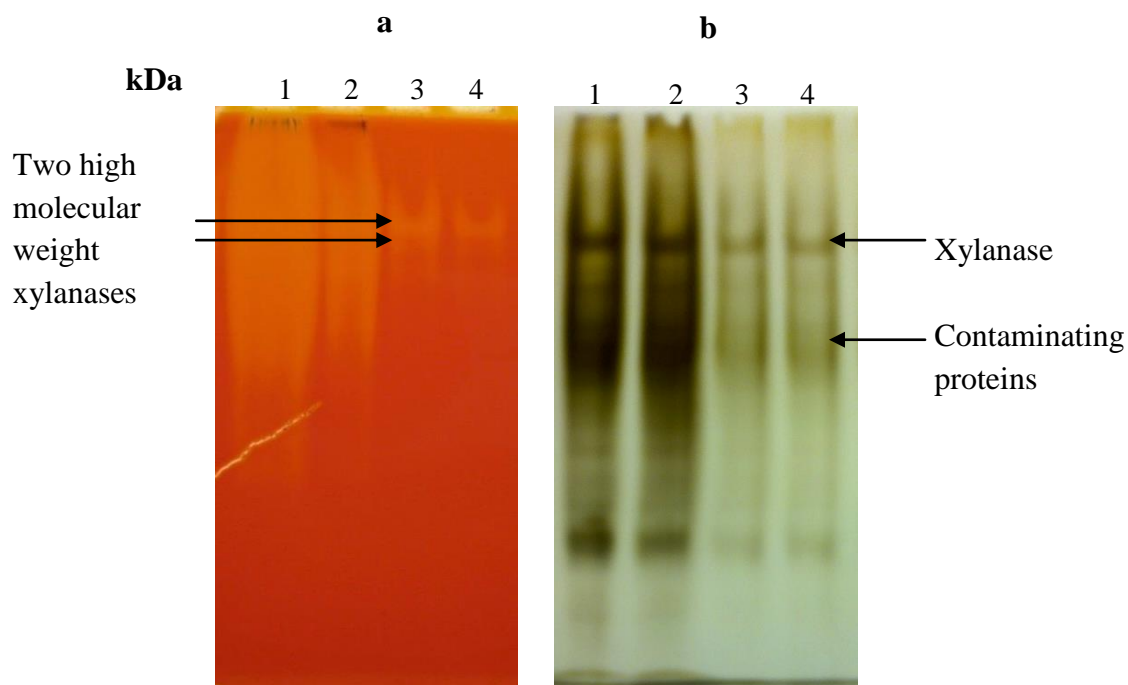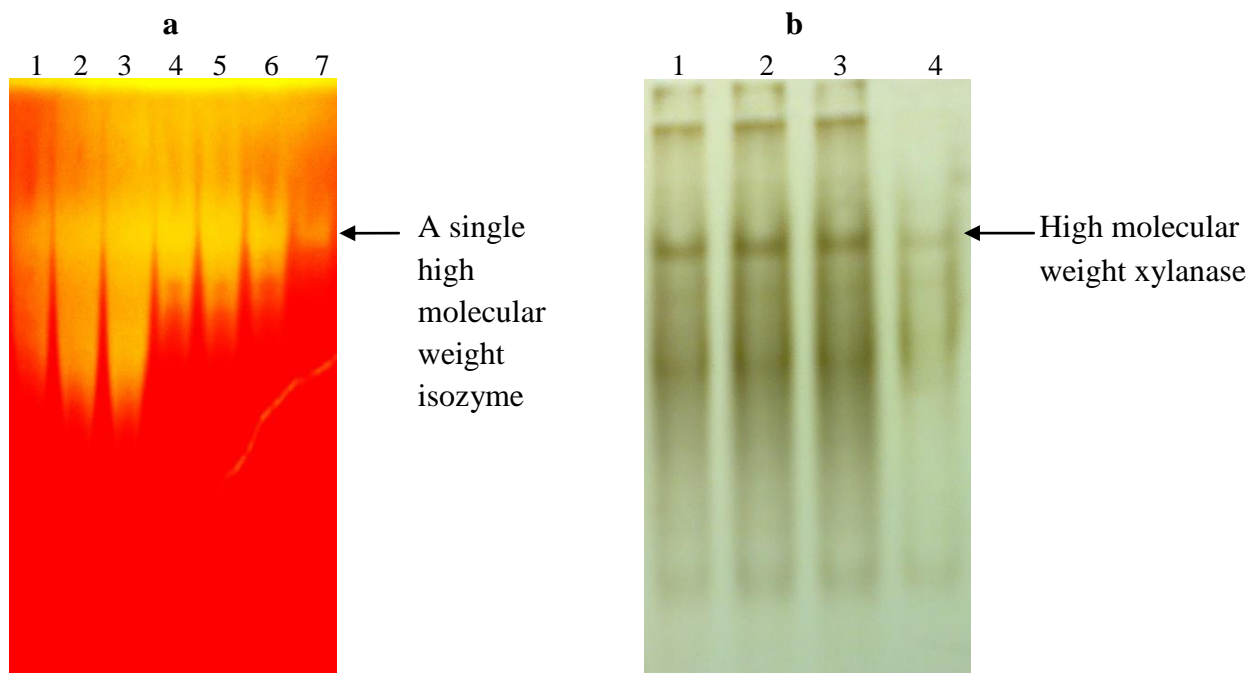
**Figure 3.12: a) 10% Native PAGE supplemented with 1% beechwood xylan and b) silver stained 10% Native PAGE of the ion exchange chromatography fractions eluted with 2.5 M NaCl[*].** (a: crude enzyme (1), 70% ammonium sulphate suspension (2), unbound fraction (3), A9 (4), A10 (5), A11 (6), A7-A8 (7)). (b: crude enzyme (1), 70% ammonium sulphate suspension (2), unbound fraction (3)A7-A8 (4)). *All samples were standardised to10 µg.ml^{-1}
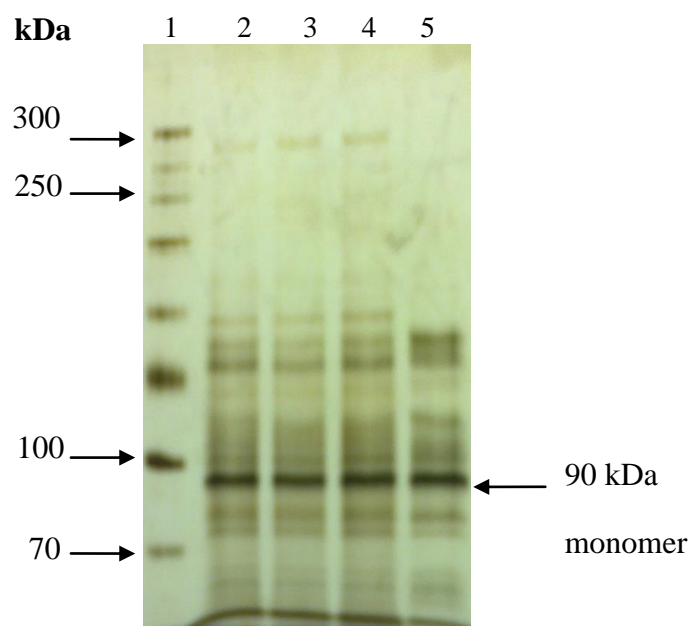


**Figure 3.13: 10% SDS-PAGE of protein eluted by ion exchange chromatography with HiTrap Q FF against a 2.5 M NaCl gradient that have been reduced with 50 mM DTT[*].** (Prestained protein ladder (1), crude enzyme (2), 70% ammonium sulphate suspension (3), unbound fraction (4), A7-A8 (5)). *All samples were standardised to10 µg.ml^{-1}

63

**Table 3.5: Summary table of purification by ammonium sulphate precipitation and anion exchange chromatography.**

| Step | Total protein (µg) | Total enzyme (IU) | Specific activity (IU/µg) | Yield (%) | Purification fold |
|------|--------------------|-------------------|---------------------------|-----------|-------------------|
| Crude enzyme | 114 240 | 226 454.7 | 1.98 | - | 1.0 |
| 70% ammonium sulphate suspension | 747.6 | 117 756.44 | 157.51 | 52 | 79.5 |
| HiTrap Q FF (pooled peak: A7-A8) | 150 | 3143.37 | 21 | 1.4 | 10.6 |

## 3.4. DISCUSSION

Two types of protein precipitation methods were tested in order to determine the saturation point of the crude xylanase. Protein precipitation with ammonium sulphate proved to be the most suitable and reproducible method in this study. Decreased levels of xylanase activity were recovered at lower ammonium sulphate concentrations. Most of the xylanase activity was recovered at very high ammonium sulphate concentrations as the saturation point of the crude xylanase was achieved at 70% ammonium sulphate. Approximately 52% of the enzyme was recovered at this ammonium sulphate concentration (Table 3.2). Less than 14% of the xylanase was recovered at 0-20, 21-40 and 41-60% ammonium sulphate (Appendix B). The results obtained suggest that the xylanases have a low number of hydrophobic residues as proteins with a large number of hydrophobic amino acid residues salt out faster (Sadasivam and Manickam, 1996; Nigam and Ayyagari, 2007). The number of hydrophobic residues present on the surface of the protein determines the concentration of ammonium sulphate at which proteins reach their

saturation point. The salting out of proteins is often influenced by pH and temperature. At neutral pH proteins have a large number of charge groups hence the solubility of the protein is altered (Campbell and Farrell, 2007). The solubility of proteins decreases with an increase in temperature hence a constant temperature of 4°C was maintained in the present study. Latif *et al*. (2006) reported the partial purification of a crude xylanase preparation from *Chaetomium thermophile* with a saturation point at 70% ammonium sulphate suspension.

Unlike ammonium sulphate precipitation, precipitation with PEG did not yield desirable results as the protein concentration could not be determined. However, a significant amount of xylanase was detected using the DNS assay. An increase in enzyme activity was detected at 20% PEG, however, at PEG concentrations greater than 60% (Table 3.3) lower amounts of enzyme were detected. This was possibly due to less enzyme being precipitated or denaturation of enzyme in that precipitate. As a result the specific activity, protein concentration and total enzyme recovered during precipitation by PEG could not be determined. PEG can be used in sequential additions similar to ammonium sulphate and precipitates proteins by reducing solvent availability (Fahie-Wilson and Halsall, 2008). A major drawback is that PEG can also be very difficult to remove and may affect subsequent analysis (Deutscher, 1990). Dialysis, capillary electrophoresis and ion exchange chromatography are examples of a few methods that can be used to remove PEG from protein samples (Shen and Buko, 2002; Zhao and O'Connor, 2007). However, where there are high concentrations of PEG the carryover of this polymer to the sample fraction is more likely. The likelihood of contaminating liquid chromatography columns is increased.  Therefore, the 70% ammonium sulphate suspension was used for further analysis.

Two types of desalting methods were tested using the 70% ammonium sulphate suspension. Dialysis and a desalting column, Sephadex G25, were tested to determine the most suitable method for the removal of salts (Table 3.4). A greater percentage of the enzyme was recovered by dialysis compared to the desalting column. Approximately 96% of activity in the AS fraction remained after dialysis against citrate buffer (pH 4). A slightly lower amount of enzyme (82%) was recovered by Sephadex G25. Both desalting methods have been proven to be suitable methods for the removal of salts (Nigam, 2007). However, the results obtained indicate that dialysis overnight effectively removes salts such as ammonium sulphate and media salts present in the sample which will interfere with liquid chromatography. Several factors influence enzyme recovery after dialysis. These include the pore size of the dialysis membrane, the duration of the dialysis, time required for buffer change, temperature and the volume of dialysis buffer used (Burgess *et al*., 2009). All factors were optimized in the present study to ensure maximum recovery of a stable product. Most importantly, repeated changes in dialysis buffer reduces the salt concentration to negligible levels that do not interfere with subsequent analysis (Goel, 2007).

Initial attempts of ion exchange chromatography were conducted using a manual purification system. The xylanase activity detected in the pooled fractions was due to the presence of a range of isoenzymes with a dominant isozyme. However, several contaminating proteins were detected in these fractions by SDS-PAGE which is indicative of the partial purification of the 70% desalted ammonium sulphate suspension.

To ensure reproducibility of results, the manual purification system was upgraded to an automated FPLC system. Of the ion exchange resins selected, HiTrap Q FF eluted fractions

enriched with xylanase. However, xylanase activity was only detected in the unbound fraction (fractions A3-A6) of proteins eluted with a linear gradient of 1M NaCl (citrate buffer, pH 4) using HiTrap Q FF. Xylanase activity was detected in fractions A3-A6 (Figure 3.5). Xylanase activity of 30 IU.ml$^{-1}$ and 15 IU.ml$^{-1}$ was detected in fractions A4 and A5, respectively. The elevated activity in A4 was due to the presence of two high molecular weight isozymes as compared to a single high molecular weight isozyme present in A5 (Figure 3.6). The pI of a protein represents the pH at which the protein has no net charge (Campbell and Farrell, 2007). Above or below the isoelectric point, proteins carry a net negative charge or a net positive charge, respectively. Therefore, pI of a protein is of great significance in protein purification especially ion exchange chromatography which separates proteins based on their net charge. The pI of a protein affects the solubility of a protein at a given pH. The pH which corresponds to the pI of a protein provides minimal solubility and therefore, proteins precipitate. Due to the inability of the reduced form of crude xylanase and partially pure xylanase to renature, the isoelectric point (pI) could not be determined. For this reason several different optimization steps were carried out to ensure proper binding of the xylanase to the resin.

Several factors affect the binding affinity of proteins during ion exchange chromatography (Prasad, 2012). These major factors include the elution buffer selected and NaCl gradient (Simpson, 2012). The binding affinity of a protein is stronger at a pH far from the pI (Heftmann, 2004). Considering that a change in pH of a solution alters the net charge of a protein and its ability to bind to a given resin (Sharma and Sanga, 2009), in the present study HiTrap Q FF was selected as the most suitable column for ion exchange chromatography. This anion exchanger requires specific buffers within a given pH range in order to enable proper binding. Previous ion

exchange fractions were eluted at optimum pH (pH 4) for xylanase activity (Mosina, 2010) to ensure the recovery of active fractions. The elution buffer was subsequently changed from citrate buffer (50 mM, pH 4) to phosphate buffer (50 mM, pH 7) which is a recommended buffer for anion exchange chromatography (HiTrap IEX selection kit, GE Lifesciences). The change in pH of the elution buffer positively influenced binding of the protein. Three quarters of the total protein bound to the anion exchange resin with elution with 2.5 M NaCl (Figure 3.11). Approximately half the total protein did not bind and a quarter of the total protein was eluted after the NaCl gradient was established. The NaCl gradient was also increased from 1 M to 1.5 M NaCl, to ensure the removal of strong binding proteins. Salt ions compete with protein molecules for opposite charges on the surface of the resin. Higher concentrations of NaCl ensure the removal of strongly bound proteins during separation (Aguilar, 2004; Heftmann, 2004).

Using the optimized conditions proved to be effective in eluting active fractions within the NaCl gradient as two protein peaks enriched with the enzyme were obtained (Figure 3.8). The partially purified xylanase contained three high molecular weight isozymes(Figure 3.9). To enable elution of these isozymes NaCl gradients were tested. This showed that a change in elution buffer and an increase in NaCl concentration enabled the enzyme to bind to the resin (Sharma and Sangha, 2009). However, the active fractions were eluted after the NaCl gradient was established. This indicates that the anionic enzyme binds strongly to the cationic surface of the resin as high concentrations of sodium ion were required to displace the enzyme from the column (Heftmann, 2004; Janson, 2012). To attempt to isolate a single isozyme the NaCl was increased in the subsequent anion exchange chromatography. A higher sample load was used in subsequent anion exchange chromatography. Although a substantial amount of enzyme still appeared in unbound

fraction the higher NaCl concentration produced a narrow peak (A7-8) and a broad peak (A9-13). All the enzyme was eluted in these two peaks, the broad peak contained several isozymes (Figure 3.11). The narrower peak was eluted earlier in the NaCl gradient a partially pure isozyme was obtained. A dominant 90 kDa monomer of the xylanase was detected by SDS-PAGE analysis as a result of treatment with the reducing agent, DTT. Specific activity of 349 U/µg was detected in the partially pure xylanase. A yield and purification fold of 1.4% and 10.6 was obtained (Table 3.5). This indicates that the crude enzyme contained a very high concentration of contaminating proteins. Although low levels of protein were isolated in the narrow peak, elevated enzyme activity was detected. This suggests that the highly active isozymes are produced in low levels (µg/ml) thus increasing the difficulty of the purification process.

# CHAPTER 4

## EFFECTS OF pH AND TEMPERATURE ON THE ENZYME ACTIVITY AND STABILITY OF THE PARTIALLY PURIFIED XYLANASE OF *P. alba*

## 4.1. INTRODUCTION

Bacteria and fungi produce xylanases which vary in the enzymatic properties depending on the environmental source. Extremophilic xylanases have are currently in high demand due to their increased stability during commercial processing (Kulkarni *et al*., 1999). However, the lack of xylanases of this nature places an increasing demand for the identification of novel thermophilic, acidophilic and alkaliphilic xylanases. Thermophilic xylanases have optimal activity at 50-80°C and hyperthermophilic xylanases are those which are active at temperatures >80°C (Collins *et al*., 2005). A typical xylanase has an optimum temperature of 50°C however, xylanases with elevated temperature optima and stability have been identified (Subramaniyan and Prema, 2002).

The thermostable xylanase produced by the actinomycete, *Streptomyces* sp. SU9, displayed an optimum temperature of 80ºC (Bajaj and Singh, 2010). The enzyme remained reasonably stable at 60ºC for one hour retaining 95% of activity for 30 minutes. Xylanase BII from *Chaetomium thermophile* exhibited a temperature optimum of 70ºC with 100% of activity at 55 ºC for a minimum of 15 minutes (Latif *et al*., 2006). Recently, acidophilic thermophilic xylanases, XynSW2A and XynSW2B, from *Streptomyces* sp. SWU10 were purified and characterized (Deesukona *et al*., 2011). Both enzymes exhibited pH and temperature optima of 6 and 60ºC with 80% stability at 60ºC (XynSW2A) and 80ºC (XynSW2B). More than 80% of activity was retained between pH 3-9 (XynSW2A) and pH 2-9 (XynSW2A) after 16 hour incubation period at 4ºC. Acidophilic xylanases have enormous application in the production of bioethanol, xylitol, bread and biscuit making and the liquefaction fruit to improve the yield of fruit juice (Beg *et al*.,

2001; Polizeli *et al*., 2005; Rafiqul and Sakinah, 2012). Bioethanol production is conducted under acidic conditions and elevated temperatures which demand stable acidophilic thermophilic xylanases for the hydrolysis of xylan to its monomeric sugars. Unlike bacteria, fungi xylanases generally have a narrower pH optimum ranging between 3-8. Therefore, there are few reports of alkaliphilic fungal xylanases. Azeri *et al*. (2010) reported the production of cellulase free xylanases by *Bacillus* strains Ag 12, 13, 20 and 32 with maximum activity at pH 9 and 60ºC. Alkaliphilic xylanases have great potential for application in the paper and pulp industries (Kulkarni *et al*., 1999) which require enzymes to hydrolyse the alkaline pulp with minimal production of waste (Valls *et al*., 2010a).

The greater specific activity observed in fungal xylanases makes them more suitable for industrial application. Most industrial bioprocesses are conducted at extremes in pH and temperature (Kulkarni and Rao, 1996). The elevated temperatures and extreme changes in pH make it difficult for most enzymes to remain stable. A major limitation in the application of most microbial xylanases is the lack of pH- and thermo-stable xylanases (Kuhad *et al*., 2006). With the growing demand for thermal resistant xylanases, several different approaches have been taken to increase their availability for industrial application. The first approach involves improving the thermostability of existing purified and characterized xylanases. This can be achieved by chemical modification, crosslinking, immobilization and treatment with additives. The most popular technique is protein engineering which is often achieved by rational design or directed evolution (Antikainen & Martin, 2005). A major drawback with this approach is that thorough knowledge of the molecular aspects of the enzyme is required. The third approach exploits the natural habitat of xylanase producing microorganisms. Thermophilic

microorganisms are capable of producing enzyme with elevated thermal resistance compared to their mesophilic counterparts (George *et al*., 2001). Advances in increasing the diversity of thermostable xylanases have been made over the past few years as many of these enzymes have been purified, characterized and successfully applied. In order to determine the suitability of a xylanase to a given application it is essential to characterise the enzyme. Xylanase characterisation provides essential information on the pH and temperature properties of the enzyme and most importantly, whether the enzyme is pH and stable and/or thermostable. The purpose of this chapter was to determine such properties prior to application of the partially pure xylanase to pretreated sugarcane bagasse.

## 4.2. MATERIALS AND METHODS

The biochemical properties of the pure xylanase were determined in order to establish its suitability for industrial applications. The pH and temperature optimum, pH and temperature stability and size were determined.

### 4.2.1. pH AND TEMPERATURE OPTIMUM

The pH optima were determined by dissolving 1% Beechwood xylan (Sigma-Aldrich, South Africa) in buffers (0.05 M) ranging from pH 4-9 (sodium acetate (3.5), citrate buffer [4-6], phosphate buffer [7-9]). The enzyme was prepared in appropriate buffers and appropriate dilutions were made. All enzyme assays were conducted as described by Bailey *et al*. (1992). The temperature optima were determined by incubating the assays at different temperatures ranging from 40-90°C at the optimum pH (Gomes *et al*., 2000). Appropriate dilutions of the

enzyme were prepared and residual sugars were measured using the DNS assay as described in 3.2.1.

### 4.2.2. pH AND TEMPERATURE STABILITY

pH stability was determined over a two hour period at 15 minute time intervals after pre-incubation in buffers ranging from pH 3-9 at 50°C temperature (Gomes *et al.*, 2000). Thermostability was determined at 40-90°C at the optimum pH at 15 minute intervals over a two hour period. All enzyme assays were conducted using the DNS assay and appropriate dilutions were made. Residual sugars were measured as previously described (3.2.1).

## 4.3. RESULTS

Optimum pH of the xylanase of *P. alba* was determined within the pH range of 3-9. The pH profile shown in Figure 4.1 below displayed a peak in xylanase activity at pH 4. At the optimum pH, maximum activity of 32.4 $IU.ml^{-1}$ was observed. No activity was detected at pH 3 however, 10.85 $IU.ml^{-1}$ xylanase activity was detected at pH 3.5. At pH 5, there was a reduction in enzyme activity to 30-$IU.ml^{-1}$. At higher pH xylanase activity dropped to 12.68 $IU.ml^{-1}$ at pH 6. At neutral to alkaline conditions xylanase activity was below 1$IU.ml^{-1}$.
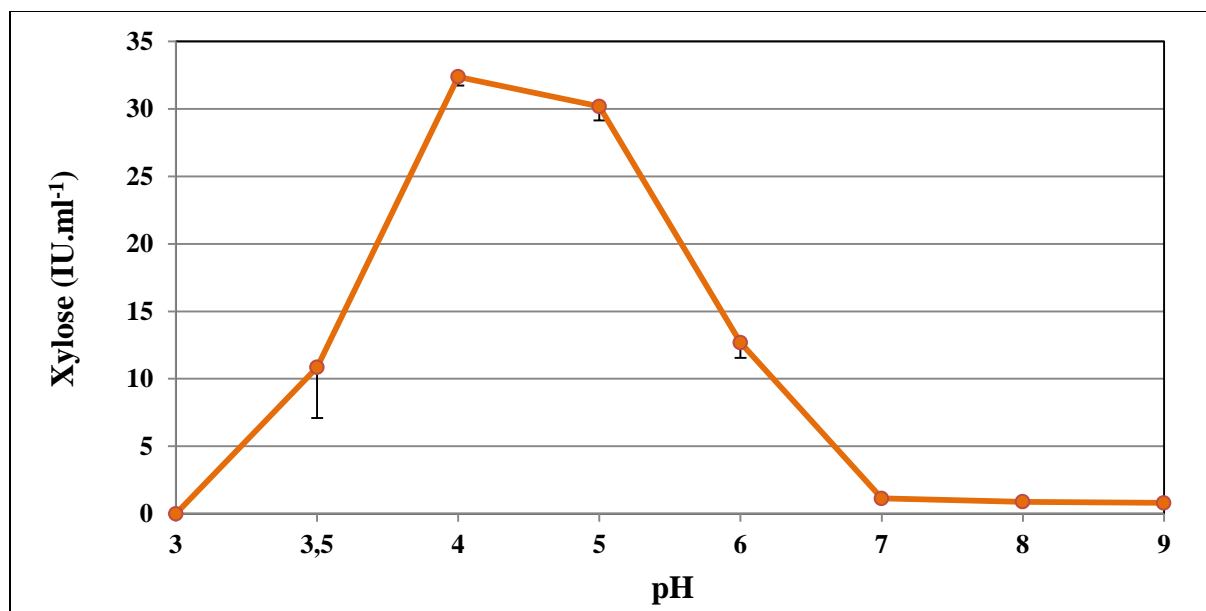
**Figure 4.1: Effect of pH on the enzyme activity of xylanase partially purified from *P. alba*.**

The optimum temperature of the partially pure xylanase was determined from 40 to 90ºC. Figure 4.2 shows the temperature profile of this enzyme. At mesophilic (40ºC) and hyperthermophilic temperatures (80 and 90ºC) minimal xylanase activity <3 IU.ml$^{-1}$ was detected. However, at thermophilic temperatures, 50-70ºC, xylanase activity was greater than 420 IU.ml$^{-1}$. At optimum temperature maximum xylanase activity was 32 IU.ml$^{-1}$. A steady decrease in activity from 50-70ºC occurred followed by a drastic decrease at temperatures >70ºC.
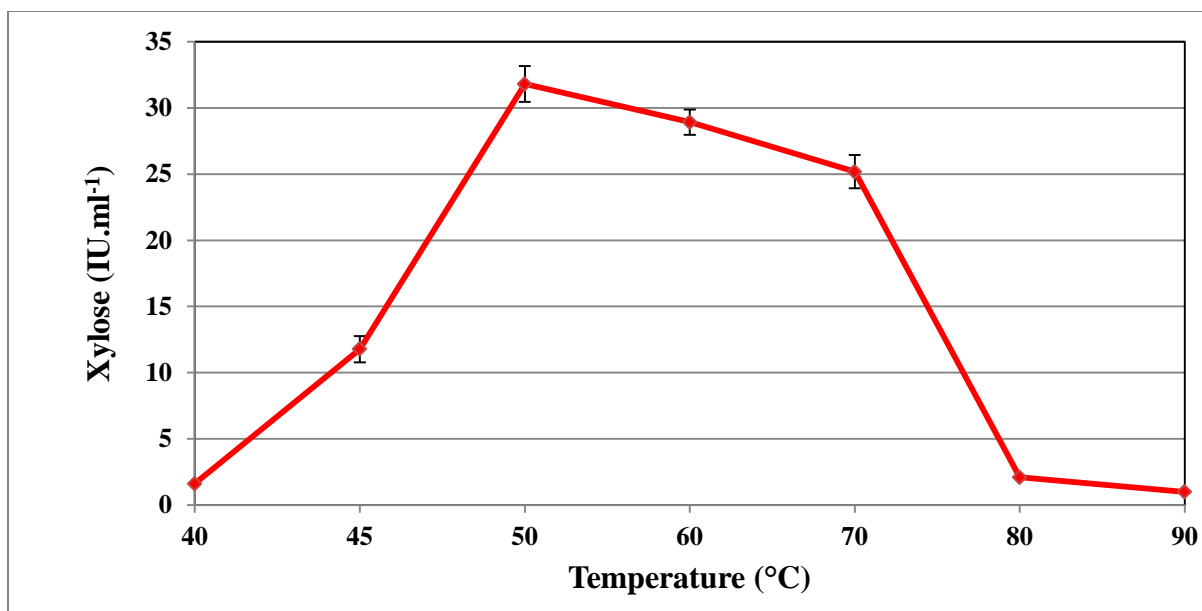
**Figure 4.2: Effect of temperature on the enzyme activity of xylanase partially purified from *P. alba*.**

pH Stability was tested over a 2 hour period from pH 4-9. At optimum pH, pH 4, 90% of activity was retained for 90 minutes (Figure 4.3). Thereafter, enzyme activity gradually decreased to 60% after 2 hours of incubation. At pH 5 and 6, 86% and 68% of activity remained after 90 minutes, respectively. Past the 90 minute incubation period, a gradual decrease in enzyme activity was seen with 56% and 45% activity after 120 minutes. Under neutral conditions, initial activity decreased to 52% after 30 minutes. Subsequently, enzyme activity remained below 50%, reaching 45% after incubation for 2 hours. Alkaline conditions between pH 8 and 9, provided minimal stability for the enzyme as a dramatic decrease in activity was observed after 15 minutes.
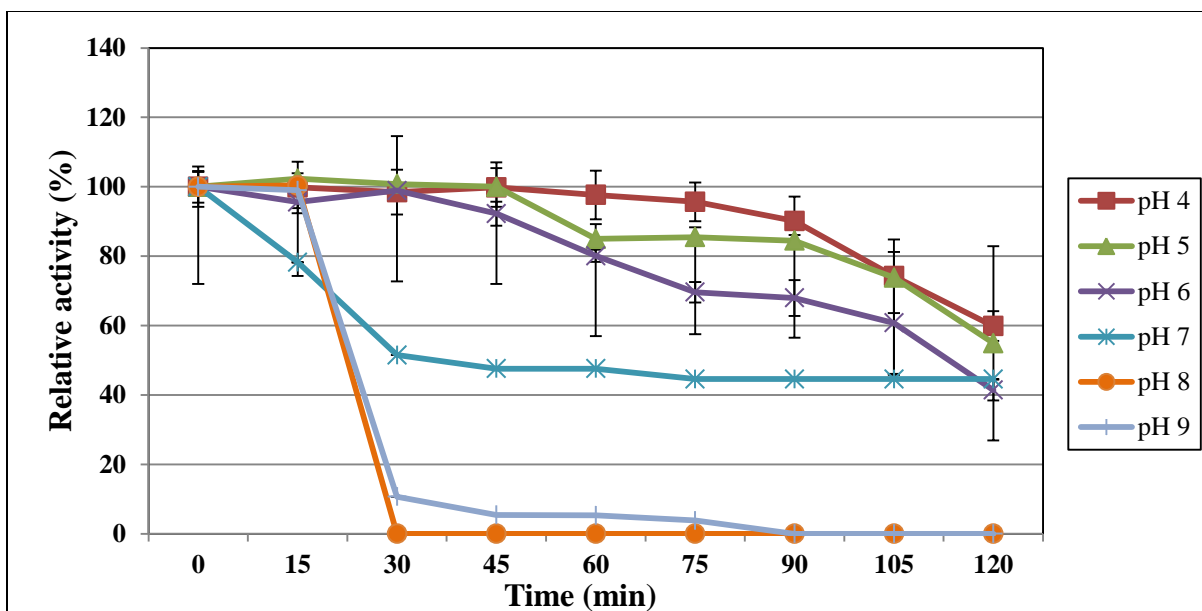
**Figure 4.3: pH stability profile of the purified xylanase incubated for 2 hours at pH ranging from 4-9.**

Under mesophilic conditions (40℃) 96% of activity remained after 75 minutes of incubation, followed by a decrease in activity to 56% at 120 minutes (Figure 4.4). Thermophilic conditions (50-80℃) had varying effects on enzymatic activity. The temperature optimum of 50℃, 95% of activity was retained after 90 minutes. This was followed a steady reduction in activity to 58% after 120 minutes. A similar trend can be seen at 60℃, however at 90 minutes 90% activity was retained. At 70℃, a step-wise reduction in activity can be seen after 45 and 90 minutes, with 100% and 65% activity retained, respectively. After 120 minutes, activity diminished to 17%. Minimal stability can be seen at 80℃ as enzyme activity dropped to 42% after 15 minutes and reached 23% after 120 minutes. Under hyperthermophilic conditions (90℃), enzyme activity diminished rapidly to 23% after 120 minutes of incubation.
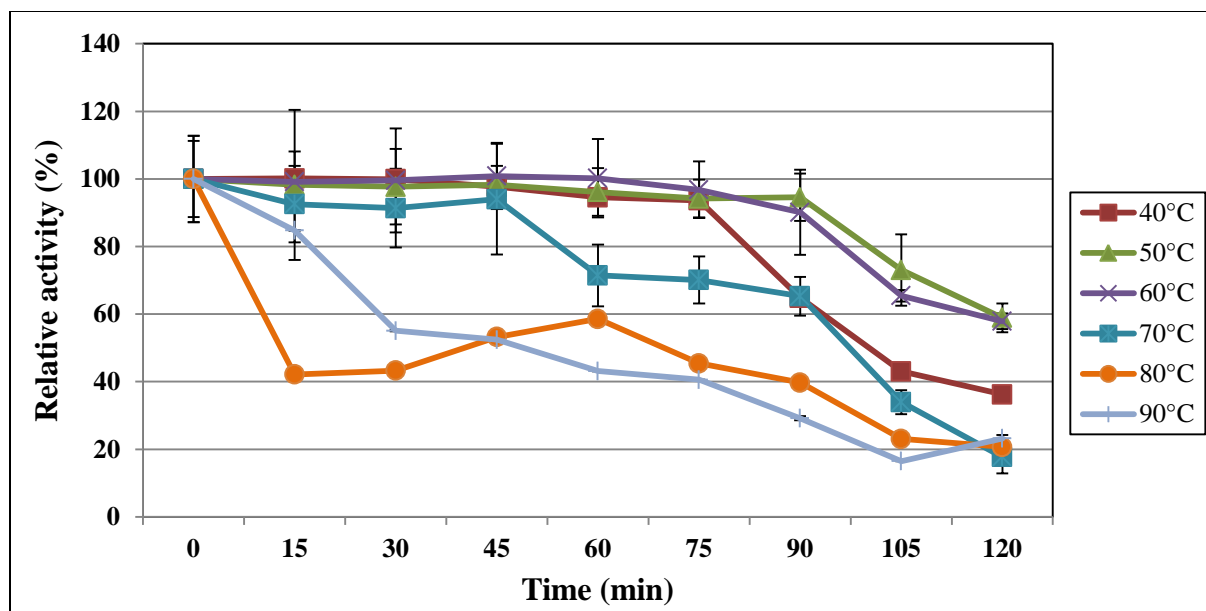
**Figure 4.4: Thermostability profile of the purified xylanase over a 2 hour period at temperatures ranging from 40-90 ºC.**

## 4.4. DISCUSSION

The present study reports a thermoactive acidophilic xylanase from *Phialophora alba*. The optimum pH for xylanase activity was pH 4 and substantial activity was observed at mildly acid pH (5 and 6) (Figure 4.1). Xylanases are active under neutral, acidic and alkaline conditions, however, fungal xylanases are typically active at pH 5 (Kulkarni *et al*., 1999; Subramanian and Prema, 2002; Beg *et al*., 2001). The majority of fungal xylanases have been reported to have maximum activity under mildly acidic conditions ranging from pH 5-7 (Subramaniyan and Prema, 2000). Similar to the present study, Zhang *et al*. (2011) reported the production of an acidophilic recombinant xylanase with an optimum pH of 4 in *Phialophora* sp. G5. In addition to this, *Thermoanaerobacterium* sp. xylanase exhibited a pH optimum of 6.2 (Shao *et al*., 1995).

The stability of the enzyme was tested over a broad pH range in the present study. More than 90% of activity was retained at optimum pH for 90 minutes (Figure 4.3). A substantial amount of activity was retained at pH 5 (86%) and 6 (68%). A progressive decline in active was observed after 90 minutes with greater than 45% activity remaining after 120 minutes at both pH. As highlighted in the pH profile, neutral to alkaline conditions had an adverse effect on enzyme activity and stability. At pH 8 and 9, 0% and 10% activity remained after 30 minutes. According to Wong *et al*. (1988) a conserved evolutionary relationship between the molecular weight and isoelectric point (pI) of xylanases exists. As a result, low (< 22 kDa) and high (> 43 kDa) molecular weight xylanases are considered basic and acidic proteins, respectively. The latter applies to the present study as the partially purified xylanase (> 190 kDa) has an acidic pH optimum. The alkaline conditions provided by pH 8 and 9 did not provide a suitable environment for the enzyme to remain active. This could be attributed to a change in protein conformation as a result of the alkaline conditions. The pH of a solution may alter the ionization of acidic and basic proteins, that is, the acid (carboxyl side chain) and basic amino acids (amine side chain) (Scopes, 1993; Voet *et al*., 2008). Therefore, a change in pH not only alters the conformation of the enzyme but the net charge. Alterations in amino acids that contribute to the tertiary structure can result in enzyme inactivation and altered substrate recognition (Berg *et al*., 2001).

The effect of temperature on enzyme activity was studied at different temperatures ranging from 40-90°C. Maximum activity of 32 IU.ml$^{-1}$ was observed at 50°C, however, at 60°C and 70°C reasonable activity was observed of 28.93 IU.ml$^{-1}$ and 25 IU.ml$^{-1}$, respectively (Figure 4.2). Similarly, at 70°C activity of 25 IU.ml$^{-1}$ can be seen. A rapid decrease in enzyme activity was observed at 80 and 90°C, with activity reaching below 3 IU.ml$^{-1}$. Microbial xylanases typically

have a temperature optimum of 50°C (Yang *et al*., 1995; Virupakshi *et al*., 2005). This is evidenced by the present study as a similar temperature optimum was obtained. Studies by Bajaj *et al*. (2011) describe a *Penicillium* sp. SS1 xylanase with an optimum temperature of 50°C and substantial activity at 60°C. *Aspergillus caespitosus* xylanases, Xyl I and Xynl II, displayed optimal activity at 50 and 55°C, respectively (Sandrima *et al*., 2005).

At optimum temperature 95% and 58% of activity was retained for 90 and 120 minutes, respectively. Temperatures below 50°C, displayed a similar trend after 120 minutes as 56% of activity was retained at 40°C. Incubation of the enzyme at 60 and 70°C for 90 minutes showed that 90% and 65% of activity was retained, respectively. Therefore, the thermostability profile indicates that the enzyme is most stable from 40-70°C as minimal stability was observed from 80-90°C. Bajaj *et al*. (2011) reported that at optimum temperature, 100% of activity was retained for 60 minutes, however, incubation periods longer than 90°C caused a rapid decline in activity. At 55°C the enzymes had a half-life of 27.3 (Xyl I) and 90 minutes (Xyl II). An increase in temperature increases the level of kinetic energy of molecules within a system. This increase in kinetic energy ultimately increases the chemical potential energy between two molecules that collide resulting in an elevated reaction rate (Berg *et al*., 2001). Therefore, an increase in temperature increases the number of collisions between enzyme (xylanase) and substrate (xylan). A significant increase in the chemical potential energy may be sufficient to break the weak bonds between amino acids that contribute to changes of the tertiary structure of the enzyme (Voet *et al*., 2008). This results in the denaturation of the enzyme and subsequent inactivation and loss in enzyme activity (Scopes, 1993).

The pure xylanase of *P. alba* displayed properties suitable for application in bioethanol production, that is, the saccharification of pretreated lignocellulosic residues such as sugarcane bagasse. Xylanases used in bioethanol production must be active under acidic conditions (pH) and efficiently hydrolyse the xylan component. Furthermore, stability under acidic conditions and elevated temperatures (> 50°C) are essential. The abovementioned conditions are satisfied by *P. alba* xylanase thus further highlighting it potential for application.

# CHAPTER 5

## ENZYMATIC HYDROLYSIS OF PRETREATED SUGARCANE BAGASSEBY PARTIALLY PURIFIED XYLANASE AND CRUDE ENZYME EXTRACT OF *P. alba*

## 5.1. INTRODUCTION

Fossil fuels are the main source of energy and are currently in great demand. Considering that oil reserves are becoming depleted it is expected that the production of crude oil will decline (Nehring, 2009). The continuous use of fossil fuels has resulted in a tremendous environmental impact. The emission of greenhouse gases has greatly contributed to global warming (Lal, 2007). This has prompted the search for alternative and renewable energy sources. According to Nehring (2009) the reliance on alternative energy such as bioethanol will increase. Second-generation bioethanol are produced from lignocellulosic waste such as sugarcane bagasse, husks and non-food crops providing a possible solution to this problem (Cardona and Sanchez, 2007; Sanchez and Cardona, 2008).

Sugarcane bagasse (SCB) is considered an inexpensive substrate that can be readily converted to fermentable sugars for bioethanol production. A constant supply of sugarcane bagasse is available in the sugar industry and in South Africa approximately eight million tons (dry weight) of SCB is produced every year (Leibbrandt *et al*., 2011). This lignocellulosic residue is composed of cellulose (35-45%), hemicellulose (26.2-35.8%), lignin (11.4-25.2%) and cell components (2.9-14.4%) (Canilha *et al*., 2011). Pretreatment of lignocellulose increases the availability of xylan for enzymatic attack (Chandra *et al*., 2007). This process disrupts the hemicellulose component that is sandwiched between cellulose and lignin. The disruption of the lignin-carbohydrate complex allows for the liberation and removal of the highly recalcitrant lignin. Several types of pretreatment methods have been studied for lignocelluloses pretreatment

(Mosier *et al*., 2005; Alvira *et al*., 2010). Chemical, physical, physico-chemical and biological processes are often used as methods of pretreatment. Physical methods typically involve milling of the lignocelluloses to particles of a specific size that are easier to handle and have a greater surface/volume ratio. Milling is usually the initial step in most pretreatment processes and therefore, can be used in combination with other techniques (Kumar *et al*., 2009). The average size of agricultural wastes after milling at an industrial scale is 0.8-3.2 mm which is sufficient to minimise the energy input for mechanical comminution (Sun and Cheng, 2002; Sarkar *et al*., 2012).

Biological treatment involves the growth of delignifying microorganisms or white rot fungi on the lignocelluloses (Chandel *et al*., 2012). Microbial enzymes are produced that target specific components of lignocelluloses. Alternatively, steam explosion which is the most common method of physico-chemical pretreatment can be used. This technology involves the treatment of biomass with high-pressure (0.69-4.83 MPa) steam at temperatures ranging from 160-260°C followed by a gradual reduction in pressure (Sun and Cheng, 2002). Steam explosion promotes the hydrolysis of hemicellulose and alters the structure of lignin. This process is terminated by the explosive decompression of the biomass. Mild steam explosion conditions can be performed under standard laboratory conditions using an autoclave (Jedvert *et al*., 2012).

Chemical processes include treatment with acid (HCl, oxalic acid and formic acid) and alkali (NaOH, $NH_4OH$) (Sun and Cheng, 2002; Kootstra *et al*., 2009). Acid treatment converts the hemicellulose component into its monomeric sugars (xylose, glucose, mannose, glucose and galactose) and inhibitors of fermentation (furfurals and phenolic compounds) (Chen and Dixon,

2007). Unlike acids, alkali is primarily involved in delignification of lignocellulose, leaving the cellulose and hemicellulose portion intact (Sun and Cheng, 2002). According to Sun *et al*. (1995), the pretreatment of wheat straw with 1.5% NaOH for 144 hours at room temperature liberated 60% and 80% lignin and hemicellulose, respectively. Lower temperatures and pressure are required for alkaline pretreatment compared to other pretreatment conditions (Sarkar *et al*., 2012). No toxic intermediates or products that inhibit microbial growth are produced by alkaline pretreatment.

Prior to fermentation, the polysaccharides in lignocellulosic biomass are hydrolysed to fermentable reducing sugars by microbial enzymes (Margeot *et al*., 2009). The complete hydrolysis of polysaccharides requires the synergistic action of cellulases, hemicellulases and auxillary enzymes (Horn *et al*., 2012). However, the complete hydrolysis of hemicellulose is more complex compared to cellulose. An array of enzymes such as endo-β-1,4-xylanases and β-xylosidases which target xylan and xylo-oligosaccharides, respectively (Levin *et al*., 2008). Additional enzymes such as acetyl xylan esterase, α-glucoronosidases and α-L-arabinofuranosidases are also required (van den Brink and de Vries, 2011). The utilization of crude enzyme and pure xylanase preparations in the hydrolysis of raw xylan have been reported (Adsul *et al*., 2005; Singh *et al*., 2013). In KwaZulu-Natal (KZN) SCB is a readily available lignocellulosic residue that is found in abundance. Previous experiments indicate that the partially pure xylanase displays desirable properties for bioethanol production. In this study the partially pure xylanase and crude enzyme extract from *P. alba* was applied to pre-treated SCB.

## 5.2. MATERIALS AND METHODS

### 5.2.1. SAMPLE COLLECTION

Sugarcane bagasse was collected from Huletts (Tongaat, South Africa) and transported to the University of KZN (Westville). All samples were used immediately and if necessary stored at 4°C.

### 5.2.2. PRETREATMENT OF SUGARCANE BAGASSE

The SCB was dried in an oven overnight at 60°C. The dried samples were milled to particles of 0.5-1 cm in size as described by Azeri *et al*. (2010) and passed through 0.5 mm and 1 cm screens. Two different pretreatment conditions: alkaline as well as temperature and pressure were used in the enzymatic hydrolysis studies (Damaso *et al*., 2004). Due to the production of intermediates that are toxic in fermentation resulting from acid pretreatment, this method was not utilized in this study.

The milled SCB (20 g) was mixed with 450 ml of distilled water and served as no pretreatment control. A second experiment was set up in which the milled SCB was pre-treated with alkali by adding 20 g of the milled substrate  to an Erlenmeyer flask containing 450 ml of 2N NaOH. The flask was incubated at 30°C for 20 hours followed by several washing steps with distilled water to neutralize the pH of the preparation. Thermal and pressure pretreatment were conducted simultaneously by autoclaving (121°C, 0.103 MPa) an Erlenmeyer flask containing 20 g of substrate and 450 ml of distilled water for 90 minutes. All preparations (untreated,

thermal/pressure and alkali) were filtered through 0.45 µm filters to remove the liquid phase. The remaining SCB was used in subsequent hydrolysis studies.

### 5.2.3. ENZYMATIC HYDROLYSIS OF PRETREATED SUGARCANE BAGASSE

Enzymatic hydrolysis was performed using 1% (w/w) substrate in 50 mM citrate buffer (pH 5) with a final reaction volume of 50 ml. An enzyme loading rate of 15 IU/g was used. The thermally inactivated crude enzyme and partially pure xylanase served as a control for each pretreatment process and the untreated SCB (Adsul *et al*., 2005). Hydrolysis of the pretreated SCB was carried in duplicate for each type of treatment. All flasks were incubated at 50°C with shaking at 150 rpm. Samples (1 ml) were withdrawn after 0, 24 and 48 hours. All samples were filtered using 0.45 µm filters and the supernatant assayed for residual sugars using the DNS assay (Bailey *et al*., 1992). Triplicate readings were used to determine the amount of reducing sugars liberated during hydrolysis (g/ml).

## 5.3. RESULTS

The enzymatic hydrolysis of SCB was tested using a partially purified xylanase and the crude enzyme preparations from *P. alba*. The milled SCB was subjected to two different types of pretreatment conditions prior to enzymatic saccharification (alkali and temperature/pressure). The reducing sugars liberated after pretreatment with alkali and high temperature and pressure was difficult to determine as the high NaOH concentrations in the alklali-treated SCB interfered with the DNS assay. Reducing sugar concentrations after pretreatment and copious washing steps, at 0 hours was determined. It was below 0.1 g/ml for both treated samples and the

untreated control. Sugar concentrations below 0.4 g/ml were present after 24 and 48 hours in the untreated control in the absence of enzyme. The addition of either partially purified or crude enzyme preparation to the control produced tenfold higher reducing sugar levels, with higher levels observed with the crude enzyme preparation after 48 hours.

Reducing sugar levels of 0.22 g/ml and 0.3 g/ml were detected after 24 and 48 hours, in the thermal and pressure pretreatment controls, respectively. The addition of the partially pure enzyme produced 1.4 g/ml and 1.7 g/ml of reducing sugars after 24 and 48 hours of incubation, respectively (Fig. 5.1). A slightly lower amount of sugars was detected with the crude enzyme preparation after 24 (1.2 g/ml) and 48 (1.6 g/ml) hours. After pretreatment with alkali, baseline reducing sugar levels of 0.1 g/ml and 0.36 g/ml sugar were detected after 24 and 48 hours, respectively in the absence of enzyme. In the presence of the partially purified xylanase, approximately 1.8 g/ml of reducing sugars was produced after 24 hours. Over a 48 hour period a total of 2.3 g/ml sugar was liberated.

Overall, the highest amount of reducing sugars were liberated from SCB by the crude enzyme in conjunction with alkali pretreatment. Approximately, 1.9 g/ml sugar was produced over a 24 incubation period. After 48 hours of incubation, 2.4 g/ml reducing sugars were liberated.
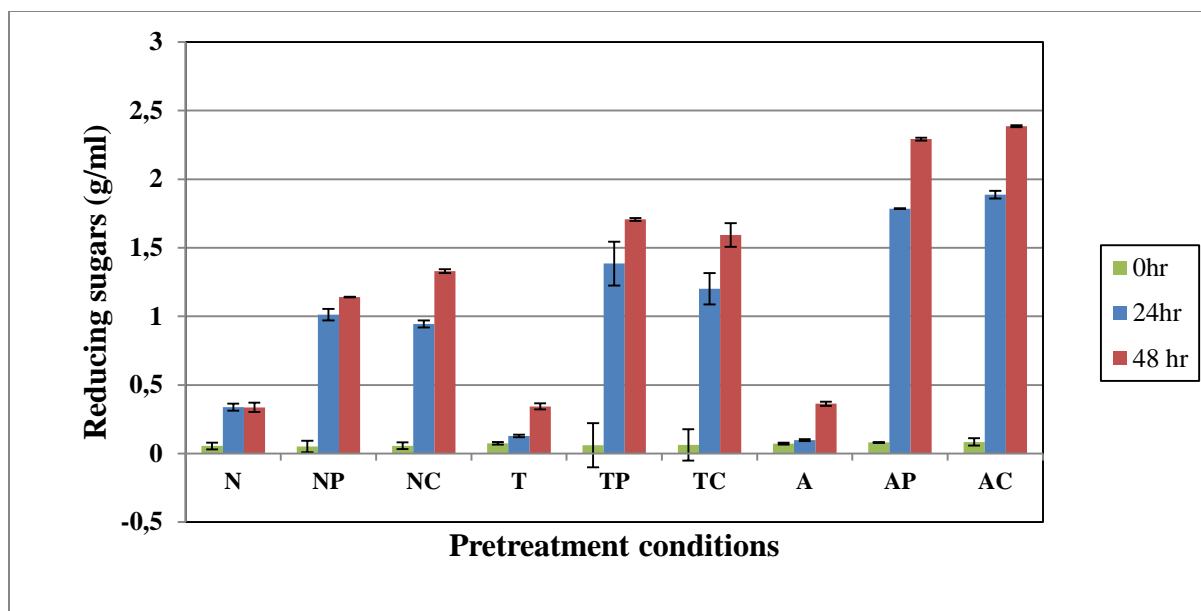
**Figure 5.1: Effect of enzyme hydrolysis using a partially pure xylanase and crude enzyme (15 IU/g) from *P. alba* on sugarcane bagasse subjected to no treatment, pre-treatment with alkali and temperature/pressure.** (N: untreated+buffer; NP: untreated+partially pure xylanase; NC: untreated+crude enzyme; T: thermal treatment+buffer; TP: thermal treatment+partially pure xylanase; TC: thermal treatment+crude enzyme; A: alkaline treatment+buffer; AP: alkaline treatment+partially pure xylanase; AC: alkaline treatment+crude enzyme).

## 5.4. DISCUSSION

In the present study the effect of two pretreatment strategies (viz., high temperature and pressure and alkali pretreatment) of SCB in conjunction with enzymatic hydrolysis was determined. The effect of pretreatment alone on SCB could not be established as the high NaOH concentration interfered with the DNS assay and precluded determination of sugars released. After several washing steps, baseline reducing sugars were similar in both pretreated SCB samples. High temperature and pressure promote the hydrolysis of hemicelluloses and alters the lignin component of SCB and several authors reported higher levels of reducing sugars with this pretreatment method (Sun and Cheng, 2002; Jedvert *et al*., 2012). In this study this determination was not possible. Pretreatment with alkali merely delignifies the SCB, leaving the

cellulose and hemicelluloses intact. The additive effect of enzyme treatment (partially pure xylanase and crude enzyme preparation) with pretreatment strategies was assessed (Figure 5.1). Milling was responsible for some degradation of the SCB as low baseline levels of reducing sugars were detected in all samples at 0 hours. Both enzyme preparations had a moderate effect on the milled SCB as less than 0.35 mg/ml reducing sugars were produced after 48 hours. There are several chemical and physical barriers in lignocellulosic residues that limit the accessibility of hydrolytic enzymes to the polysaccharides. According to Corrales *et al*. (2012), the pretreatment of lignocellulosic residues, including SCB is essential in order to achieve efficient hydrolysis to monomeric sugars. The chemical interactions between the chemical components of SCB are highly complex. Several interpolymer and intrapolymer interactions are present which reduce the accessibility of hydrolytic enzymes to their respective substrates (Faulon *et al*., 1994). Therefore, untreated SCB represents the fully intact polymer complex of lignocelluloses. da Silva *et al*. (2010) reported that milling decreased the crystallinity of the lignocelluloses thereby favouring saccharification.

For all pretreatment strategies and untreated samples reducing sugar concentrations were below 0.1 g/ml at 0 hours. This could be due to the washing step during preparation of the SCB. Reducing sugars liberated during the pretreatment processes could have been reduced after washing. Under thermal pretreatment conditions, the crude enzyme produced the maximum amounts of reducing sugars after 48 hours with sugar concentrations of 1.2 g/ml and 1.6 g/ml detected at 24 and 48 hours, respectively. Thus the reaction time determines the amount of reducing sugars liberated. Saores *et al*. (2011) assessed the amount of reducing sugars from steam pretreated SCB that were liberated over a 72 hour period. They found that maximum

reducing sugar production was obtained after 48 hours. The partially purified xylanase hydrolysed the substrate more efficiently than the crude enzyme over a 48 hour period. Approximately 1.4 g/ml and 1.7 g/ml sugars were produced at 24 and 48 hours, respectively. Steam explosion results in the degradation of hemicellulose and removal of lignin from SCB. Several factors affect the results obtained by steam explosion including particle size, temperature and residence time. In order to improve the efficiency of this pretreatment process chemicals such as acids and alkali have been added (Stenberg *et al*., 1988; Zimbardi *et al*., 2007). Endo-acting xylanases cleave the internal linkages and exo-acting xylanases cleave the polysaccharide from the termini (McKee *et al*., 2012). The accessibility of these sites may be hindered by the presence of side chains in the xylan polymer. Side chains promote steric hindrance thus reducing the accessibility of the substrate to the enzyme ultimately resulting in lowered reducing sugar levels (Gilbert, 2010). This further supports the results obtained in the study. As a substantial amount of sugars were produced when coupled with enzyme hydrolysis, however, greater sugar concentrations where obtained with alkali treated SCB.

The final pretreatment process tested under the same conditions as the high pressure and temperature and untreated SCB was .the use alkaline conditions which resulted in the production of up to 0.36 g/ml reducing sugars after 48 hours. Reducing sugar concentrations were two fold higher using the partially purified enzyme preparation compared to the untreated samples after 24 and 48 hours. This represented the sample preparation with the highest reducing sugar concentration across all pretreatment strategies. Alkaline pretreatment is effective in delignifying SCB thereby increasing the accessibility of the polysaccharides (Singh *et al*., 2011).

Accessibility is further increased by the removal of the various side chains (acetyl and uronic acid) present on the surface of hemicellulose (Chang and Holtzapple, 2000).

In the present study alkaline pretreatment coupled with enzyme hydrolysis proved to be the most effective SCB preparation resulting in the highest production of reducing sugars. A similar study by Hernandez-Salas *et al*. (2009) who used a cocktail of enzymes showed a higher concentration of reducing sugars with alkali treated SCB compared to either acid hydrolysed or steam pretreated bagasse. Approximately 11-20% reducing sugars were generated over a 4 hour period at 55°C in that study.

The hydrolysis of SCB to reducing sugars is influenced by several factors. The particle size, type of pretreatment process, enzyme loading rate and the composition of the SCB are major determining factors. Therefore, several parameters need to be optimized in order to obtain a high yield of reducing sugars that can be fermented to bioethanol. Using an enzyme preparation composed of a cocktail of enzymes that target all the polymeric substrates will further enhance degradation of SCB.

# CHAPTER 6

## CONCLUSIONS AND PERSPECTIVES

The present study provided a detailed report on the microscopic features and xylanases produced by *P. alba*. Conidiogenic hyphae, condia and chlyamdospores were the key features identified by SEM. Optimization of denaturing and native PAGE electrophoretic analysis indicated the presence of six different isozymes in the crude enzyme extract together with a high concentration contaminating proteins. Purification of the xylanase isozymes highlighted the difficulty in purifying a high molecular weight enzyme that was difficult to renature from this novel xylanase-producing fungus. Several properties of the crude xylanase were unknown thus increasing the complexity of the purification process. Protein purification required several optimization steps at every stage of the process. Electrophoretic analysis, protein precipitation, desalting and ion exchange chromatography were optimized but only allowed for the partial purification of high molecular weight isozyme

Desirable levels of enzyme activity (~30 IU.ml$^{-1}$) were obtained in the partially purified xylanase. The optimum pH and temperature of the partially pure xylanase was pH 4 and 50°C, respectively. The partially purified enzyme remained stable from 50-70°C and pH 4-6. These properties of the xylanase highlighted its applicability to bioethanol production. Temperature and pressure-treated SCB and alkali treated SCB when used simultaneously with enzyme treatment proved to be the most effect methods of pretreatment. No significant difference in the level of reducing sugars liberated was observed in between the two pre-treatment methods. However a slightly higher hydrolysis was obtained with alkaline pretreated SCB and the crude enzyme extract.

Future studies on *P. alba* should focus on purifying, characterizing and applying all xylanase isozymes. Several different chromatographic techniques may be used to obtain pure xylanases that could be fully characterized and the pI and amino acid sequence could be determined. The thermostability of the pure xylanases could be further improved by protein engineering using several approaches such as site-directed mutagenesis. The genome of *P. alba* could be sequenced and the sequencing data could be used to design primers specific for xylanase genes and other lignocelluloses degrading enzymes. This would aid in cloning the isozymes and expressing them in various bacterial and fungal hosts. Application studies could focus on different lignocellulosic substrates and various pretreatment processes and enzyme loading rates that could be tested. Other than bioethanol production, the xylanase could be applied in the baking of bread, clarification of juices and the production of xylitol. Various parameters may also be altered in the various biotechnological applications. This is the first report of a thermophilic xylanases from *P. alba*. The present study has provided a foundation for a plethora of studies that could be conducted on the crude enzyme extract of *P. alba*.

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# APPENDIX A

5'-GTAYCCTMCTGATCCGAGGTCACCTTAGAAAAATAAAGTTGGGTGTCGGCTGGCGCCGGC
CGGGCCTACARAGCAGGTGACAAAGCCCCATACGCTCGAGGACCGGACGCGGTGCCGCCGC
TGCCTTTCGGGCCCGTCCCCCGGGAGAGGGGGACGGGGGCCCAACACACAAGCCGTGCTTG
AGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGT
TCAAAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTC
TTCATCGATGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTGATTACGATAATCAAC
TCAGACTGCATACTTTCAGAACAGCGTTCATGTTGGGGTCTTCGGCGGGCGCGGGCCCGGGG
GCGCAGGGCCTCCCCGGCGGCCGTCGAAACGGCGGGCCCGCCGAAGCAACAAGGTACGATA
GACACGGGTGGGAGGTTGGACCCAGAGGGCCCTCACTCGGTAATGATCCTTCCGCAGGTTCA
CCTACGGAAACCTTGTTACRACTTTTTAMWTCMA-3'

**Figure 1A: 18S rRNA gene sequence of *P. alba*.**
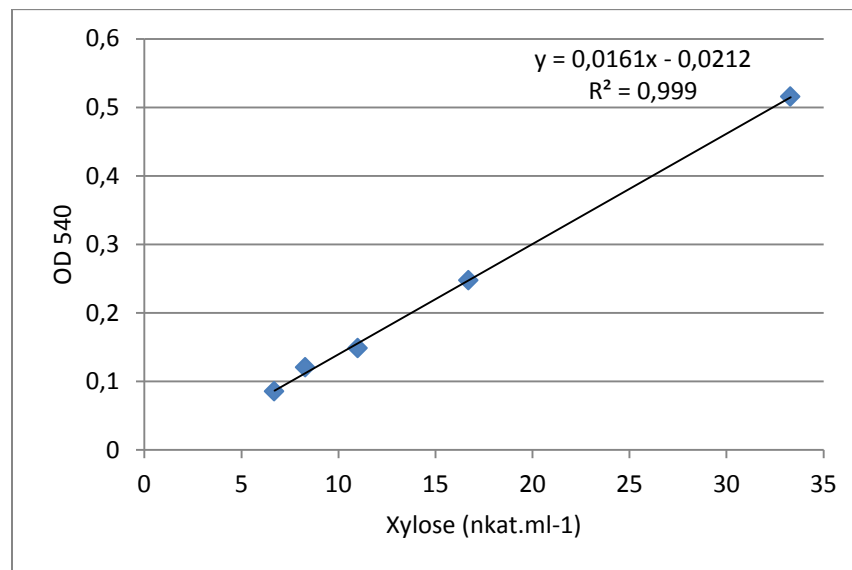


**Figure 2A: Xylose standard curve for pH 4 for the determination of xylanase pH stability.**
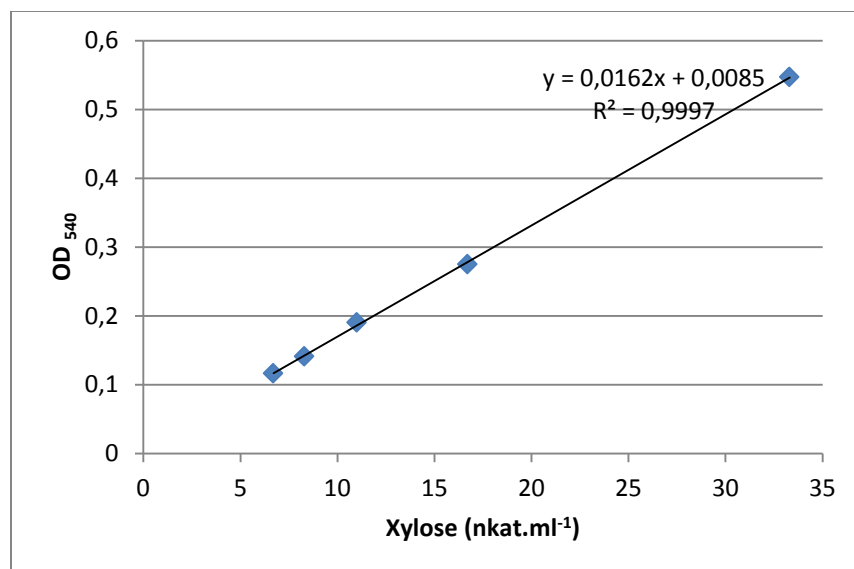
**Figure 3A: Xylose standard curve for pH 5 for the determination of xylanase activity.**



**Figure 4A: Xylose standard curve for pH 6 for the determination of xylanase activity.**
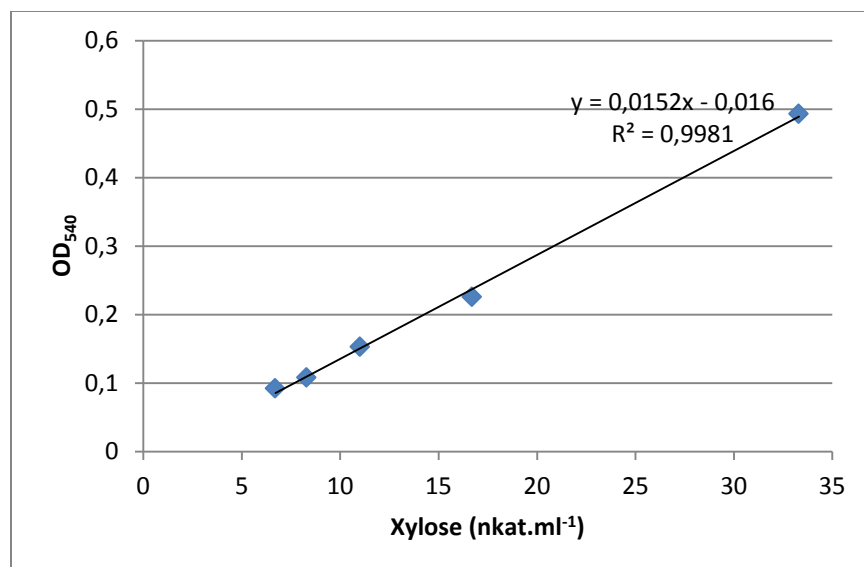
**Figure 5A: Xylose standard curve for pH 7 for the determination of xylanase activity**
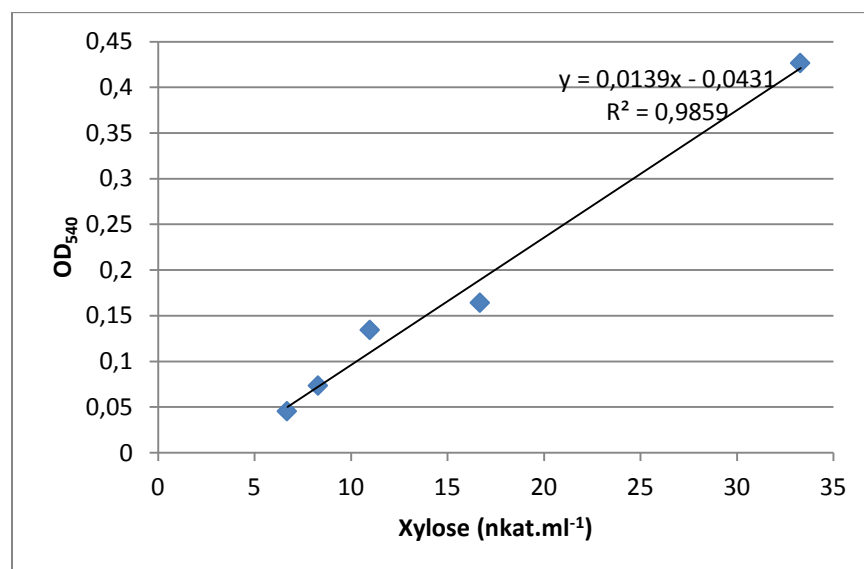


**Figure 6A: Xylose standard curve for pH 8 for the determination of xylanases activity.**
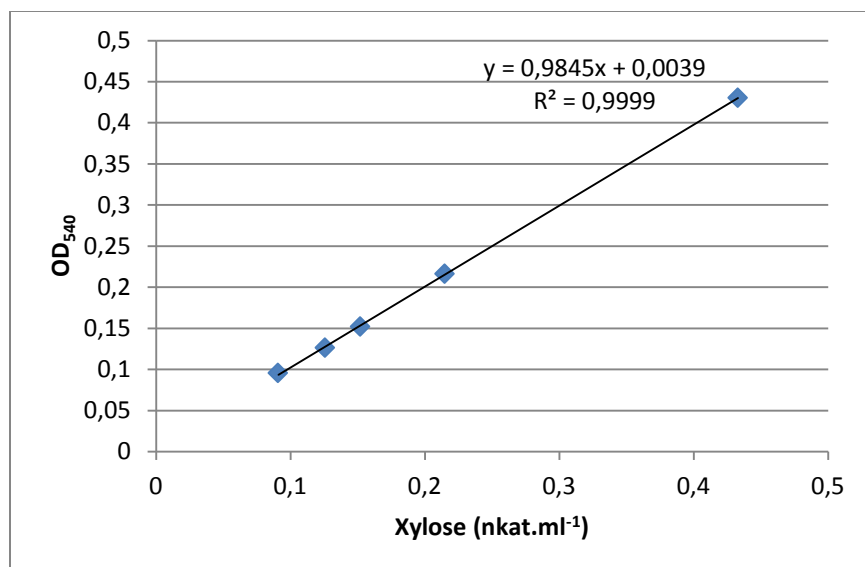
**Figure 7A: Xylose standard curve for pH 9 for the determination of xylanase activity.**



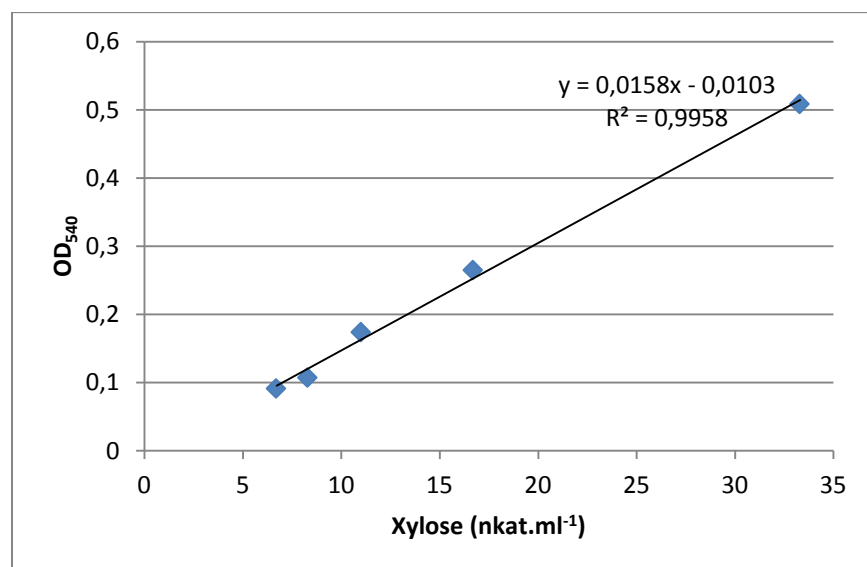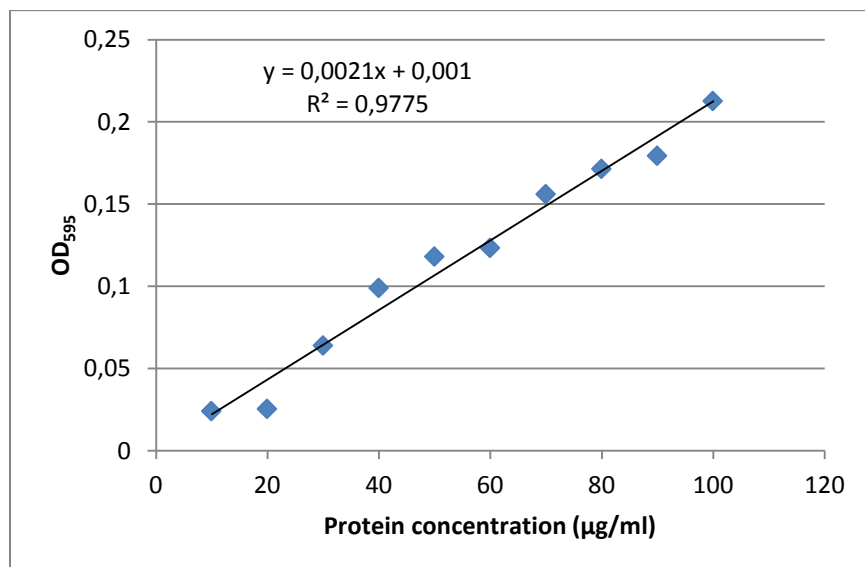**Figure 8A: Bovine serum albumin (BSA) standard curve for the determination of total protein.**
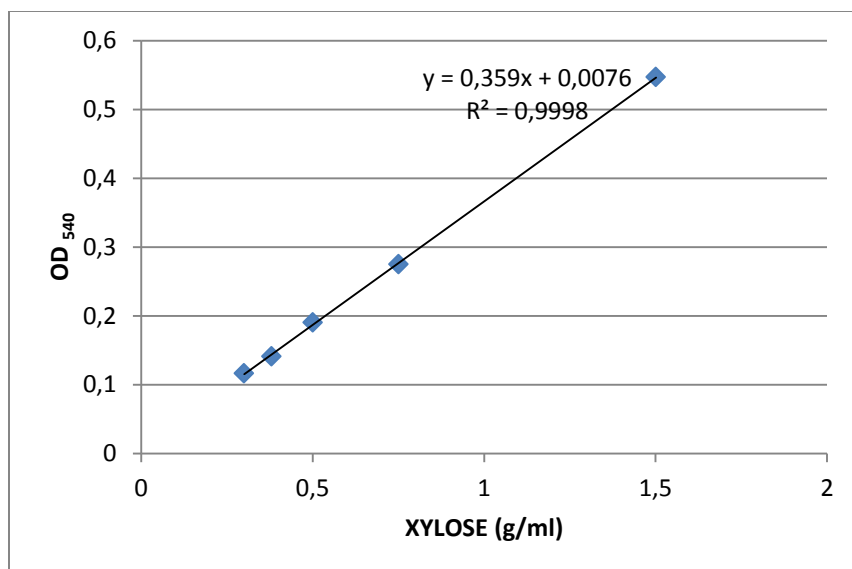
**Figure 9A: Xylose standard curve at pH 5 for the determination of reducing sugar concentrations in enzymatic treatment of pretreated and untreated SCB.**

**Figure 1B: Optimization of zymogram analysis of the crude enzyme using two different renaturation protocols.** (a: Lane 1: crude enzyme); (b: Lane 1: crude enzyme).

**Table 1B: Initial ammonium sulphate concentrations tested and the corresponding enzyme activities and protein concentrations.**

| Sample | Protein | | | Enzyme activity | | U/µg | % enzyme recovered |
|---|---|---|---|---|---|---|---|
| | Vol (ml) | Protein (µg/ml) | Tot. prot. (µg) | Activity (U/ml) | Tot enzyme (U) | | |
| 0% | 100 | 28.56 | 2856 | 943.75 | 94 375 | 33 | |
| 20% | 2 | 863 | 1726 | 706.2 | 1412.4 | 8183 | 1.89 |
| 40% | 2.3 | 262.6 | 604 | 134.4 | 309.12 | 4869.6 | 0.42 |
| 60% | 2.3 | 158.7 | 365 | 106 | 243.8 | 6666.7 | 0.33 |
| Supernatant | 116 | 1.39 | 161 | 796.5 | 92 393.1 | 1.92 | 97.9 |

**Figure 2B: Elution profile of the 70% desalted ammonium sulphate suspensionagainst a 0-1 M NaCl gradient using HiTrap SP FF.**
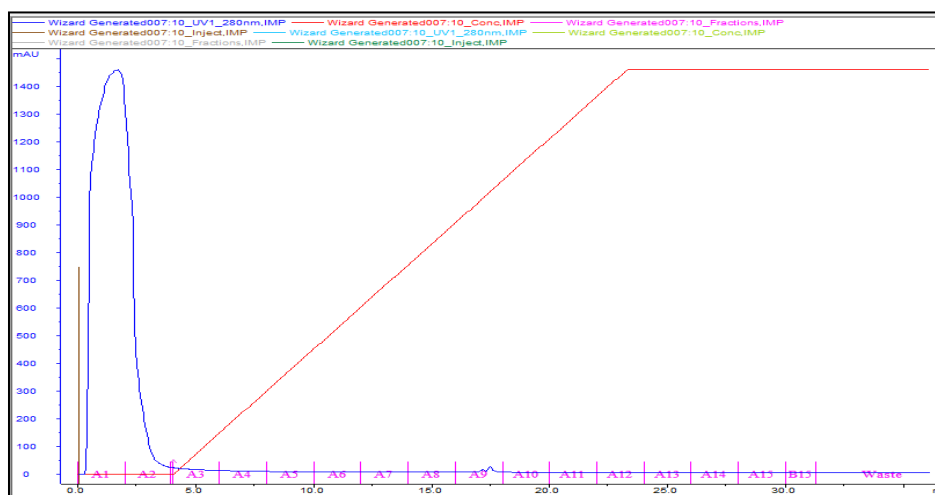


**Figure 3B: Elution profile of the 70% desalted ammonium sulphate suspensionagainst a 0-1 M NaCl gradient using HiTrap SP XL.**

**Figure 4B: Elution profile of the 70% desalted ammonium sulphatesuspension against a 0-1 M NaCl gradient using HiTrap DEAE.**

# APPENDIX C

**Table 1C: Triplicate absorbance readings and xylanase activity (U) for determination of optimum pH within the pH range of 4-9 and temperature optimum within the range of 40-90°C.**

| pH | | $OD_{540}$ | | Temperature | | $OD_{540}$ | |
|---|---|---|---|---|---|---|---|
| 4 | 0.15 | 0.149 | 0.156 | 40 | 0.4 | 0.419 | 0.404 |
| 5 | 0.135 | 0.147 | 0.138 | 50 | 0.15 | 0.14 | 0.156 |
| 6 | 0.039 | 0.049 | 0.052 | 60 | 0.135 | 0.127 | 0.138 |
| 7 | 0.305 | 0.309 | 0.319 | 70 | 0.109 | 0.109 | 0.122 |
| 8 | 0.245 | 0.256 | 0.235 | 80 | 0.552 | 0.526 | 0.533 |
| 9 | 0.22 | 0.231 | 0.22 | 90 | 0.265 | 0.233 | 0.227 |

**Table 2C: Triplicate absorbance readings and xylanase activity (U) for determination of pH stability within the pH range of 4-9.**

| | | pH 4 | | | | pH 5 | | | | pH 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Time | | $OD_{540}$ | | Time | | $OD_{540}$ | | Time | | $OD_{540}$ | |
| 0 | 0.15 | 0.149 | 0.146 | 0 | 0.135 | 0.137 | 0.138 | 0 | 0.049 | 0.05 | 0.052 |
| 15 | 0.149 | 0.15 | 0.145 | 15 | 0.139 | 0.14 | 0.14 | 15 | 0.048 | 0.047 | 0.048 |
| 30 | 0.147 | 0.143 | 0.147 | 30 | 0.132 | 0.14 | 0.141 | 30 | 0.05 | 0.049 | 0.05 |
| 45 | 0.15 | 0.146 | 0.148 | 45 | 0.137 | 0.139 | 0.134 | 45 | 0.045 | 0.045 | 0.047 |
| 60 | 0.147 | 0.142 | 0.144 | 60 | 0.117 | 0.116 | 0.119 | 60 | 0.039 | 0.038 | 0.038 |
| 75 | 0.143 | 0.141 | 0.139 | 75 | 0.117 | 0.118 | 0.119 | 75 | 0.032 | 0.033 | 0.031 |
| 90 | 0.134 | 0.132 | 0.129 | 90 | 0.116 | 0.117 | 0.117 | 90 | 0.029 | 0.032 | 0.032 |
| 105 | 0.109 | 0.103 | 0.102 | 105 | 0.105 | 0.1 | 0.104 | 105 | 0.021 | 0.029 | 0.03 |
| 120 | 0.08 | 0.082 | 0.079 | 120 | 0.069 | 0.089 | 0.078 | 120 | 0.014 | 0.016 | 0.015 |
| | | Ph 7 | | | | pH 8 | | | | pH 9 | |
| Time | | $OD_{540}$ | | Time | | $OD_{540}$ | | Time | | $OD_{540}$ | |
| 0 | 0.019 | 0.018 | 0.019 | 0 | 0.205 | 0.204 | 0.205 | 0 | 0.23 | 0.231 | 0.23 |
| 15 | 0.012 | 0.011 | 0.011 | 15 | 0.205 | 0.207 | 0.203 | 15 | 0.227 | 0.228 | 0.229 |
| 30 | 0.002 | 0.002 | 0.003 | 30 | 0 | 0 | 0 | 30 | 0.028 | 0.027 | 0.027 |
| 45 | 0.001 | 0.001 | 0.001 | 45 | 0 | 0 | 0 | 45 | 0.019 | 0.012 | 0.015 |
| 60 | 0.001 | 0.001 | 0.001 | 60 | 0 | 0 | 0 | 60 | 0.017 | 0.015 | 0.013 |
| 75 | 0 | 0 | 0 | 75 | 0 | 0 | 0 | 75 | 0.012 | 0.012 | 0.011 |
| 90 | 0 | 0 | 0 | 90 | 0 | 0 | 0 | 90 | 0.001 | 0.001 | 0.01 |
| 105 | 0 | 0 | 0 | 105 | 0 | 0 | 0 | 105 | 0 | 0 | 0 |
| 120 | 0 | 0 | 0 | 120 | 0 | 0 | 0 | 120 | 0 | 0 | 0 |

**Table 3C: Triplicate absorbance readings and xylanase activity (U) for determination of temperature stability within the range of 40-90°C.**

| | 40°C | | | Time | 50°C | | | Time | 60°C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time** | | **OD$_{540}$** | | | | **OD$_{540}$** | | | | **OD$_{540}$** | |
| 0 | 0.47 | 0.441 | 0.464 | 0 | 0.15 | 0.147 | 0.156 | 0 | 0.135 | 0.129 | 0.138 |
| 15 | 0.457 | 0.46 | 0.46 | 15 | 0.157 | 0.142 | 0.145 | 15 | 0.134 | 0.135 | 0.129 |
| 30 | 0.467 | 0.471 | 0.436 | 30 | 0.151 | 0.143 | 0.147 | 30 | 0.128 | 0.133 | 0.139 |
| 45 | 0.438 | 0.447 | 0.457 | 45 | 0.15 | 0.146 | 0.148 | 45 | 0.139 | 0.132 | 0.135 |
| 60 | 0.432 | 0.423 | 0.442 | 60 | 0.147 | 0.142 | 0.144 | 60 | 0.131 | 0.133 | 0.139 |
| 75 | 0.423 | 0.433 | 0.428 | 75 | 0.143 | 0.141 | 0.139 | 75 | 0.129 | 0.126 | 0.132 |
| 90 | 0.29 | 0.29 | 0.289 | 90 | 0.144 | 0.142 | 0.139 | 90 | 0.119 | 0.114 | 0.123 |
| 105 | 0.186 | 0.185 | 0.185 | 105 | 0.109 | 0.103 | 0.102 | 105 | 0.234 | 0.231 | 0.233 |
| 120 | 0.152 | 0.153 | 0.153 | 120 | 0.08 | 0.082 | 0.079 | 120 | 0.201 | 0.205 | 0.204 |

| | 70°C | | | Time | 80°C | | | Time | 90°C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time** | | **OD$_{540}$** | | | | **OD$_{540}$** | | | | **OD$_{540}$** | |
| 0 | 0.114 | 0.117 | 0.122 | 0 | 0.552 | 0.526 | 0.533 | 0 | 0.265 | 0.233 | 0.227 |
| 15 | 0.105 | 0.112 | 0.105 | 15 | 0.216 | 0.213 | 0.214 | 15 | 0.202 | 0.201 | 0.202 |
| 30 | 0.109 | 0.107 | 0.101 | 30 | 0.22 | 0.221 | 0.221 | 30 | 0.121 | 0.127 | 0.123 |
| 45 | 0.116 | 0.105 | 0.107 | 45 | 0.276 | 0.276 | 0.276 | 45 | 0.117 | 0.116 | 0.117 |
| 60 | 0.223 | 0.221 | 0.236 | 60 | 0.306 | 0.307 | 0.305 | 60 | 0.092 | 0.092 | 0.093 |
| 75 | 0.215 | 0.227 | 0.224 | 75 | 0.233 | 0.233 | 0.231 | 75 | 0.086 | 0.086 | 0.085 |
| 90 | 0.201 | 0.211 | 0.204 | 90 | 0.2 | 0.201 | 0.201 | 90 | 0.063 | 0.061 | 0.043 |
| 105 | 0.207 | 0.219 | 0.217 | 105 | 0.109 | 0.107 | 0.107 | 105 | 0.023 | 0.023 | 0.021 |
| 120 | 0.107 | 0.107 | 0.092 | 120 | 0.097 | 0.09 | 0.097 | 120 | 0.03 | 0.06 | 0.03 |

**APPENDIX D**

**Table 1D: Average triplicate absorbance readings from the pretreated and untreated SCB hydrolysis studies.**

| Treatment | OD$_{540}$ | | | | Treatment | OD$_{540}$ | | | | Treatment | OD$_{540}$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NT | 0.046 | 0.059 | 0.062 | 0.055 | NT | 0.119 | 0.129 | 0.139 | 0.310306 | NT | 0.133 | 0.113 | 0.139 | 0.349304 |
| NP | 0.035 | 0.057 | 0.063 | 0.052 | NP | 0.352 | 0.382 | 0.38 | 0.959331 | NP | 0.418 | 0.416 | 0.417 | 1.143175 |
| NC | 0.063 | 0.051 | 0.06 | 0.057 | NC | 0.335 | 0.352 | 0.353 | 0.911978 | NC | 0.481 | 0.491 | 0.483 | 1.318663 |
| T | 0.074 | 0.077 | 0.073 | 0.075 | T | 0.058 | 0.051 | 0.052 | 0.14039 | T | 0.121 | 0.137 | 0.135 | 0.315877 |
| TP | 0.057 | 0.059 | 0.068 | 0.061 | TP | 0.478 | 0.458 | 0.578 | 1.310306 | TP | 0.62 | 0.616 | 0.625 | 1.70585 |
| TC | 0.057 | 0.064 | 0.063 | 0.063 | TC | 0.492 | 0.417 | 0.408 | 1.349304 | TC | 0.572 | 0.617 | 0.549 | 1.572145 |
| A | 0.075 | 0.068 | 0.074 | 0.072 | A | 0.04 | 0.043 | 0.045 | 0.090251 | A | 0.138 | 0.132 | 0.144 | 0.363231 |
| AP | 0.078 | 0.086 | 0.079 | 0.081 | AP | 0.648 | 0.649 | 0.649 | 1.783844 | AP | 0.834 | 0.826 | 0.832 | 2.30195 |
| AC | 0.087 | 0.085 | 0.083 | 0.085 | AC | 0.674 | 0.685 | 0.696 | 1.856267 | AC | 0.861 | 0.867 | 0.865 | 2.377159 |