PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF XYLANASE EXPRESSED IN THERMOPHILIC *Geobacillus* sp.

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Müge ALGAN

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We approve the thesis of **Müge ALGAN**

Examining Comittee Members:

_____
Assoc. Prof. Dr. Gülşah ŞANLI-MOHAMED
Department of Chemistry, İzmir Institute of Technology

_____

Assoc. Prof. Dr. Hüseyin Çağlar KARAKAYA
Department of Molecular Biology and Genetics, İzmir Institute of Technology

_____

Prof. Dr. Şenay ŞANLIER
Department of Biochemistry, Ege University

25 December 2015

_____
Assoc. Prof. Dr. Gülşah ŞANLI
Supervisor, Department of Chemistry
İzmir Institute of Technology

_____
Assist. Prof. Dr. Mustafa KÖKSAL
Co-supervisor, Department of
Molecular Biology and Genetics
İzmir Institute of Technology

_____
Prof. Dr. Esma Volga BULMUŞ
Head of the Department of
Biotechnology

_____
Prof. Dr. Bilge KARAÇALI
Dean of the Graduate School of
Engineering and Sciences

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ABSTRACT

PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF XYLANASE EXPRESSED IN THERMOPHILIC *Geobacillus* sp.

Xylanase is an enzyme that catalyzes the degradation of the linear polysaccharide β-1,4-xylan into xylose and breaks down the hemicellulose structure of plant cell wall. The xylanolytic property of the enzyme makes it preferable for many biotechnological applications in industry. This enzyme is possibly produced by some bacterial and fungal microorganisms.

In this study, briefly, xylanase enzyme was expressed in thermophillic *Geobacillus* sp. and purified by cold acetone precipitation and gel filtration chromatography. Molecular weight of our xylanase was found as 40.1 kDa by SDS-PAGE and this protein band was verified by Native-PAGE activity staining. Finally, it was characterized using biochemical methods.

For characterization studies, K_m and V_{max} values were calculated from Lineweaver-Burk plot as 10.2 mg/ml and 31.7 U/ml, respectively. The optima temperature and pH for enzyme activity were investigated using beechwood xylan as substrate and found as 55°C and 8.0, respectively. Furthermore, effects of some metal ions, various chemical reagents and organic solvents on enzyme activity were also determined and we observed that Ca^{2+}, Mn^{2+} and Co^{2+} affected the activity positively while Zn^{2+}, Cd^{2+}, Fe^{3+}, EDTA, SDS, CHAPS and DTT shielded the activity. And only β-mercaptoethanol caused a significant change amoung organic solvents. Lastly, that the enzyme has a long shelf-life was confirmed assaying the samples taken from enzyme stocks stored at +4°C and room temperature for six weeks.

ÖZET

TERMOFİLİK *Geobacillus* sp.'den ÜRETİLEN KSİLENAZ ENZİMİNİN SAFLAŞTIRILMASI VE BİYOKİMYASAL KARAKTERİZASYONU

Ksilanaz, β-1,4-ksilan düz zincirli polisakkaritini ksiloza parçalayan ve bitki hücresi çeperinde bulunan hemiselüloz yapısını yıkan bir enzimdir. Ksilanaz, ksilan sindirme özelliği sayesinde sanayinin pek çok biyoteknolojik uygulama alanında tercih edilmektedir. Enzimi bakteriyel ve fungal çalışmalarla üretmek mümkündür.

Çalışmamızı kısaca anlatmak gerekirse; ksilanaz enzimi thermophillic *Geobacillus* sp.'den üretilerek, soğuk aseton çöktürmesi ve ardından jel filtrasyon kromatografisi ile saflaştırılmış ve SDS-PAGE yöntemi ile moleküler ağırlığı 40.1 kDa bulunmuştur. Bu protein bandının bizim enzimimize ait olduğu ise Native-PAGE'ten sonra aktivite boyaması yapılarak tasdiklenmiştir. Son olarak, biyokimyasal yöntemlerle protein çalışmaları yapılarak karakterize edilmiştir.

Karakterizasyon işlemleri sırasında, kayın kerestesi ksilanı substrat olarak kullanılmıştır. K_m ve V_{max} değerleri Lineweaver-Burk grafiğinden sırasıyla 10.2 mg/ml ve 31.7 U/ml olarak hesaplanmıştır. Enzim aktivitesi için optimum sıcaklık ve pH sırasıyla 55°C ve 8.0 olarak saptanmış, enzim stabilitesinin geniş sıcaklık ve pH aralıklıklarında 2 saate kadar sürdüğü görülmüştür. Ayrıca, bazı metal iyonlarının, çeşitli kimyasal maddelerin ve organik çözücülerin aktiviteye etkisi de araştırılmış, Ca^{2+}, Mn^{2+} ve Co^{2+} ' ın aktiviteyi olumlu yönde etkilediği, Zn^{2+}, Cd^{2+}, Fe^{3+}, EDTA, SDS, DTT ve CHAPS' ın aktiviteyi engellediği, organik çözücüler arasında ise sadece β-merkaptoetanolün kayda değer bir değişime sebep olduğu gözlemlenmiştir. Son olarak, enzimin raf ömrünün uzun olduğu da altı hafta boyunca +4°C'de ve oda sıcaklığında duran enzim stoklarından haftada bir alınan örneklerle test edilip onaylanmıştır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table **Page**

LIST OF ABBREVIATIONS

sp.	Species
M	Molar
mM	Millimolar
L	Liter
ml	Milliliter
µl	Microliter
g	Gram
mg	Milligram
µg	Microgram
nm	Nanometer
w/v	Weight per volume
v/v	Volume per volume
rpm	Revolutions per minute
UV	Ultraviolet
ppt	Precipitation
DNS	3,5-Dinitrosalicylic Acid
BSA	Bovine Serum Albumin
EDTA	Ethylenediaminetetraacetic acid
SDS	Sodium dodecyl sulfate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
β-ME	beta-mercaptoethanol
PAGE	Polyacrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine
Na-P buffer	Sodium-Phosphate buffer

CHAPTER 1

INTRODUCTION

Enzymes are the macromolecular biological catalysts which increase the rate of a biological reaction lowering the activation energy. The relationship between enzyme and its substrate is highly specific and this specificity of enzymes is based upon their unique three dimensional structures. Therefore, they are used in many industrial processes and this is pulling the scientists' attention towards enzyme researches.

1.1. Thermophiles

Temperature is one the most important factors characterizing the ecological behaviors of living organisms (Kristjansson 1989). They can be classified with regards to their biological relations with temperture as it possibly affects the ecological, physiological and genomic properties of microorganisms in many aspects. The National Center for Biotechnology Information (NCBI) Microbial Genome Project Database defines these five terms with these temperature ranges in paranthesis to categorize microorganisms according to their thermophilicity which means they grow at optimum level; where cryophiles (-30°C to -2°C), psychrophiles (-1°C to +10°C), mesophiles (+11°C to +45°C), thermophiles (+46°C to +75°C), hyperthermophiles (above +75°C). These organisms may live at ranges that overlap with more than one category (Zheng and Wu 2010). On the other hand, slightly different temperature intervals are possibly mentioned by other scientists to categorize the thermophilicity of the microorganisms according to their maximal and minimal growth temperature intervals. Baker et al. classified microorganisms like; moderate thermophiles (+35 to +70ºC), extreme thermophiles (+55 to +85ºC) and hyperthermophiles (+75 to +113ºC) (Baker et al. 2001).

The thermophiles are commonly thrive geothermally or volcanically heated hydrothermal springs, geysers and submarine vents (Grant et al. 1998). They are naturally able to live at high temperatures and the enzymes from these organisms are generally thermostable and highly active at high temperature.

1.2. Thermophilic Enzymes

Enzymes have been considerable industrial products for decades. The potential application range has been increasing rapidly. Technological developments have lead up the use of enzymes in more area and wider range of processes. New techniques in recombinant DNA technology and protein engineering enable the high yielded production of manipulated, human-designed and robust enzymes (Shuler and Kargi 2002). Such enzymes may be produced in microbial or fungal microorganisms as listed in Table 1.1.

Table 1.1. Some important industrial enzymes
(Source: Shuler and Kargi 2002)

Name	Example of Source	Application
Amylase	*Bacillus subtilis, Aspergillus niger*	Starch hydrolysis, glucose production
Glucoamylase	*A. niger, Rhizopus niveus, Endomycopsis*	Saccharification of starch, glucose production
Trypsin	Animal pancreas	Meat tenderizer, beer haze removal
Papain	Papaya	Digestive aid, meat tenderizer, medical applications
Pepsin	Animal stomach	Digestive aid, meat tenderizer
Rennet	Calf stomach/recombinant *E. coli*	Cheese manufacturing
Glucose isomerase	*Flavobacterium arborescens, Bacillus coagulans, Lactobacillus brevis*	Isomerization of glucose to fructose
Penicillinase	*B. subtilis*	Degradation of penicillin
Glucose oxidase	*A. niger*	Glucose → gluconic acid, dried-egg manufacture
Lignases	Fungal	Biopulping of wood for paper manufacture
Lipases	*Rhizopus,* pancreas	Hydrolysis of lipids, flavoring and digestive aid
Invertase	*S. cerevisiae*	Hydrolysis of sucrose for further fermentation
Pectinase	*A. oryzae, A. niger, A. flavus*	Clarification of fruit juices, hydrolysis of pectin
Cellulase	*Trichoderma viride*	Cellulose hydrolysis

Thermophiles naturally produce thermophilic enzymes which means these enzymes are thermally stable and highly active at high temperatures. That is why, they are preferentially used in many area of industry (Table 1.2). The greatest advantages of performing processes at high temperatures are enhancing substrate solubility, lowering viscosity, decreasing the risk of microbial contaminations (Bruins, Marieke E; Janssen and Boom 2001). Apart from temperature, other extreme conditions, like pH and salt concentration, may state the property of the microorganisms and make them be called extremophiles (Rothschild and Mancinelli 2001).

Table 1.2. Thermophilic enzymes used in industry
(Source: Haki and Rakshit 2003)

Enzyme	Temperature range (°C)	Bioconversions	Applications
α-Amylase (bacterial)	90–100	Starch → dextrose syrups	Starch hydrolysis, brewing, baking, detergents
α-Amylase (fungal)	50–60	Starch → dextrose syrups	Production of maltose
Pullulanase	50–60	Starch → dextrose syrups	Production of glucose syrups
Xylanase	45–65	Craft pulp → xylan + lignin	Pulp and paper industry
Chitinase	65–75	Chitin → chitobiose	Food, cosmetics, pharmaceuticals, agrochemicals
		Chitin → *N*-acetyl glucosamine (chitibiase)	
		N-acetyl glucosamine → glucosamine (deacetylation)	
		Chitin → chitosan (deacetylase)	
Cellulase	45–55	Cellulose → glucose	Cellulose hydrolysis, polymer degradation in detergents
Protease	65–85	Protein → amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30–70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry
DNA polymerase	90–95	DNA amplification	Genetic engineering/PCR

1.3. Structure of Plant Cell Wall: Cellulose, Hemicellulose and Lignin

Plant cell wall is composed of lignocellulosic structure that contains cellulose (1,4-β-glucan), hemicellulose and lignin (a hydrophobic, polyphenolic compound) (Kulkarni, Shendye, and Rao 1999). Lignin is linked to hemicellulose covalently, and so, assists the attachment of hemicellulose to cellulose microfibrils.

Hemicellulose is the name of heteropolymer including mainly xylan and other polymers like glucuronoxylan, arabinoxylan, glucomannan, and xyloglucan (Gibson 2012). Therefore degradation of this structure is only possible with a complex mixture of xylanolytic enzymes that includes xylanases (EC 3.2.1.8), α-L-arabinofuranosidase (EC 3.2.1.55), β-xylosidases (EC 3.2.1.37), acetylxylan esterase (EC 3.1.1.72) and feruloyl esterase (EC 3.2.1.73) digesting the different points of the main chain. For example, as shown in Figure 1.1, xylanase cuts the backbone of β-1,4-xylan structure into the xylose as a reducing sugar, β-xylosidases hydrolyzes the oligosaccharides releasing xylose, α-L-arabinofuranosidase degrades α-1-2, 1-3 and 1-5 arabinofuranosidic bonds and release L-arabinose as a reducing sugar (Collins, Gerday, and Feller 2005; Khandeparker and Numan 2008).

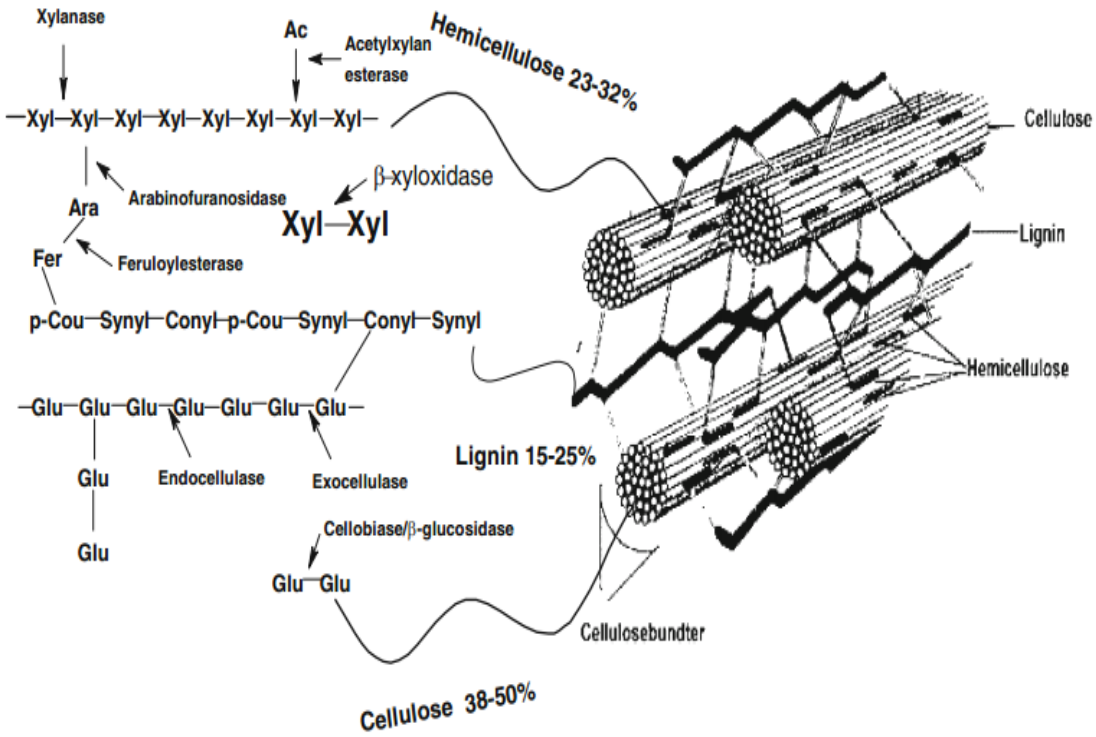

Figure 1.1. Lignocellulosic structure
(Source: Khandeparker and Numan 2008)

1.4. The Substrate: Xylan

Xylan is the second most abundant heteropolymer in nature and the main component of hemicellulose structure of plant cell wall (Biely 1985) (Schädel et al. 2010). Xylan is formed by a backbone of β-1,4-D-xylopyranosyl residues with various substitute groups on the side chain. Because of its heterogeneous structure, the biodegradation of xylan requires the accesory enzymes besides main chain degradating enzymes as shown in Figure 1.2.

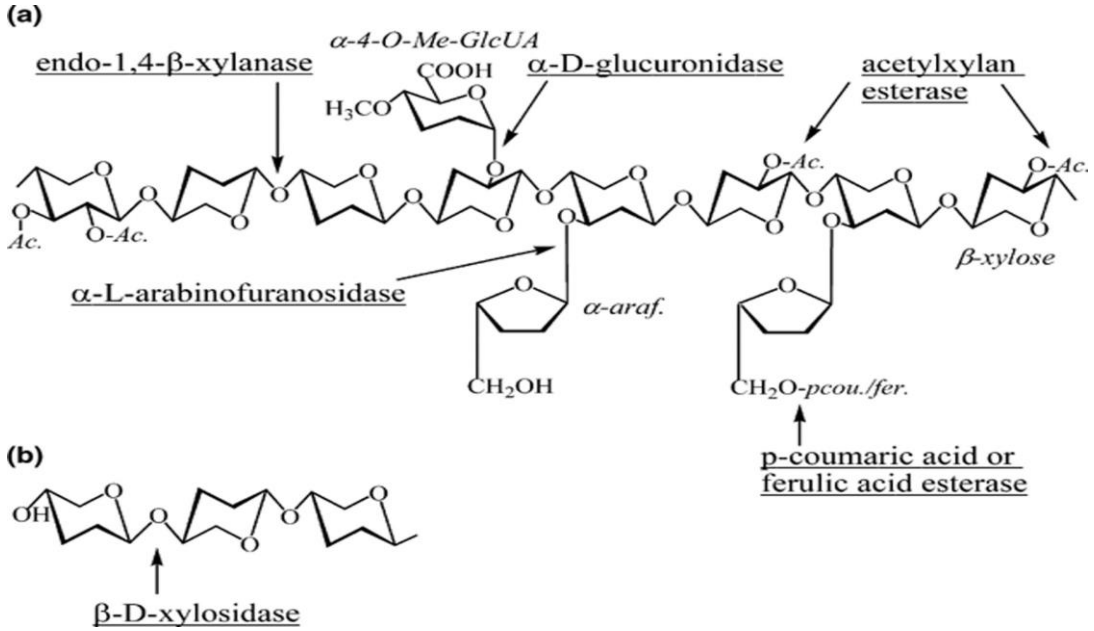

Figure 1.2. a) Xylan structure and the attack sites by xylanolytic enzymes. The backbone of the substrate is composed of 1,4- β-linked xylose residues. (b) Hydrolysis of xylo-oligosaccharide by β-xylosidase. Abbreviations: Ac. (Acetyl group), α-araf. (α-arabinofuranose), α-4-O-Me-GlcUA (α-4-O-methylglucuronic acid), pcou. (p-coumaric acid), fer. (ferulic acid) (Source: Collins, Gerday, and Feller 2005).

1.5. Xylanase

Xylanases (β-1,4-D-xylan xylanohydrolase; EC 3.2.1.8) are hydrolytic enzymes which catalyze the random cleavage of the β-1,4-glycosidic linkages and degrade the backbone of β-1,4-xylan being a part of the complex plant cell wall and produce xylose and xylo-oligosaccharides (Kamble and Jadhav 2012). Xylanases are produced by bacterial and fungal organisms as shown in Table 1.3.

Xylanases are important enzymes for industrial applications which are paper pulp bleaching in paper manufacturing for enhancing the brightness of paper (Viikari et al. 1994), increasing the digestibility of animal feed stock (Wong, Tan, and Saddler 1988) and for clarification of fruit juices (Nagar, Mittal, and Gupta 2012). Additionally, as enzymes are reusable biomolecules, it can be immobilized on magnetic nanoparticles (Soozanipour, Taheri-Kafrani, and Landarani Isfahani 2015; Shahrestani et al. 2016), chitosan (M. Liu et al. 2015) or nanofiber membranes (Kumar et al. 2013) for recovery.

Applications of xylanase avoid the use of expensive, toxic and pollutive chemical reagents (Huitro 1997).

Table 1.3. Source of microorganisms and properties of thermostable xylanases (Source: Haki and Rakshit 2003)

Organism	Enzyme properties	
	Optimal temperature (°C)	Optimal pH
Baccillus amyloliquefaciens	80	6.8–7.0
Bacillus circulans	80	6.0–7.0
Bacillus sp.	60–75	8.0–9.0
Bacillus sp. strain SPS-0	75	6.0
Bacillus subtilis	50	6.0
Clostridium abosum	75	8.5
Dictyoglomus sp. strain B_1	90	6.0–7.0
Fusarium proliferatum	55	5.0–5.5
Pyrococcus furiosus	100	6.0
Pyrococcus furiosus	102	–
Scytalidium thermophilum	65	6.0
Streptomyces sp. strain S38	60	6.0
Sulfolobus solfataricus	105	5.3
Teheromyces lanuginosus (wild and mutant)	60, 70	7.0, 6.7
Teheromyces lanuginosus-SSBP	70–75	6.5
Thermoascus aurantiacus	50	5.0
Thermotoga maritima MSB8	92	6.2
Thermotoga maritima MSB8	95	6.0–7.5
Thermotoga maritima MSB8	75	6.2
Thermotoga neapolitana	95	6.0
Thermotoga neapolitana	85	5.5
Thermotoga neapolitana	102	5.5
Thermotoga sp. strain FjSS3-B1	80	7.0
Thermotoga sp. strain FjSS3-B1	105	6.8–7.8
Thermotoga sp. strain FjSS3-B1	115	5.3
Thermotoga thermarum	80	6.6

1.6. Aim of This Study

Considering the importance of xylanase enzyme explained above, in this study, our aim was expression, purification and biochemical characterization of a xylanase from thermophilic *Geobacillus* sp.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The complete list of the chemicals used in this study is given in Appendix A and preparations of the buffer solutions are explained in Appendix B.

2.2. Methods

2.2.1. Bacterial Strain and Growth Condition

Thermophillic *Geobacillus* sp. was isolated from Balçova (Agamemnon) Geothermal Region in Izmir of Turkey (Yavuz et al. 2004) .

Bacterial strain was streak-plated in antibiotic-free LB-agar medium and incubated at 55ºC overnight. Then, one of the single colonies was inoculated into a liquid medium described in Appendix C and cultivated overnight at 55ºC and 200 rpm.

2.2.2. Protein Extraction

The culture was harvested by centrifugation at 5,600 x *g* for 15 minutes at 4°C. The cell pellet and the supernatant were both kept separately for further enzyme activity assays (Section 2.2.4).

The supernatant was the extracellular crude extract. Besides this, the cell pellet was resuspended in 10 ml of 50 mM sodium phosphate buffer, pH 7.0 and disrupted by discontinuous sonication in ice bath for 10 minutes. The homogenate was centrifuged at 6,000 x g, 4ºC for 20 minutes in order to remove cell debris and the supernatant was used as crude intracellular enzyme extract.

2.2.3. Protein Purification

For both intracellular and extracellular enzyme extracts, total protein precipitation by an organic solvent was applied. Double sample volume of cold (-20°C) acetone was added slowly to the protein extract, briefly vortexed and incubated at -20°C for 1 hour. They were centrifuged at 6,000 x g, 4ºC for 20 minutes, the supernatants were decanted and remaining acetone was evaporated from uncapped tubes at room temperature for 30 minutes.

Protein pellets were resuspended in 50 mM sodium phosphate buffer (pH 7.0) and crude xylanase extracts were ready for activity assays.

According to the activity assay results (Section 3.1), our xylanase was stated as a majorly extracellular enzyme and the rest of the studies were continued accordingly.

As next step, the crude enzyme was loaded to gel filtration (Sephadex G-100, Sigma) column (2.5 x 50 cm, Sigma) which had been equilibrated with sodium phosphate buffer (50 mM, pH 7.0) before use. Every 60 drops were collected as a fraction and then activities of each fraction were assayed. By highest activity, top five of the forty fractions were selected (Section 3.1), pooled and stored at -20 ºC until the following experiments.

2.2.4. Enzyme Activity Determination

The enzyme activity determination assays were performed via the DNS method (Miller 1959). The method is based upon measuring the amount of reducing sugars which form a complexation with 3,5-dinitrosalicylic acid (DNS) and give absorbance after enzymatic degradation reactions (Appendix D).

The reaction mixture (total volume 1.0 ml, in 1.5 ml mini-centrifuge tubes) containing 0.36 ml of 1% beechwood xylan (prepared in 50 mM sodium phosphate buffer, pH 7.0) as substrate and 0.04 ml enzyme sample was incubated at 55°C for 10 minutes. Then, the reaction was terminated adding 0.60 ml of DNS reagent. The tubes were placed in boiling water for 15 minutes (Bailey 1992). After cooling to room temperature, the absorbance was measured against blank (which contained all the reagents but enzyme, 50 mM sodium phosphate buffer, pH 7.0 instead) at 540 nm using Perkin Elmer Lambda Bio+ Spectrophotometer.

A series of activity assays were performed for intracellular and extracellular protein extracts before and after acetone precipitation and gel filtrated protein.

To evaluate the absorbance values we recorded, a standard curve was formed using xylose standard solutions (Appendix E). As in the method described by Bailey et al., 0.010 M xylose solution was prepared in 50 mM sodium phosphate buffer (pH 7.0) and 1:1, 1:2, 1:3, 1:5 diluted samples served as standards (Bailey 1992).

The protein concentration was determined by Bradford protein assay benefitting from a BSA standard curve (Bradford 1976). Bradford protein assay is a total protein concentration determination method based on binding the arginine, lysine primaryly and histidine aromatic residues in lower degree (Compton and Jones, 1985) (Congdon et al., 1993). The concentration data were used to calculate the total protein mass (in mg). The curve and the preparation of BSA standards were presented in detail in Appendix F.

2.2.5. Electrophoretic Studies

To determine the molecular weight of *Geobacillus* sp. xylanase and to profile the purity of the enzyme, SDS-PAGE method was performed. Moreover, by activity staining property of the Native-PAGE, it was verified that the protein band we observed positively belonged to our enzyme.

2.2.5.1. SDS-PAGE

SDS-PAGE is an electrophoretic technique to separate the proteins in an electrical field according to their size. The main principle is to denaturate the protein samples by heat and strong denaturants like SDS and β-mercaptoethanol, then to image the vertical separation of all the linearized protein content of the sample by their sizes. Smaller proteins easily move further through the pores of the polyacrylamide gel, whereas larger proteins retain. This principle provides us to image the purity of the sample and to estimate the size of the target protein by the help of a defined protein marker.

From the protein expression to obtaining the purified protein, aliquots were collected in each step for SDS-PAGE analysis (Laemmli 1970). Purified protein, total protein before and after acetone precipitation, inactive fractions of gel filtration chromatography and intracellular protein were profiled on the same gel.

Samples were diluted with the dye-containing sample buffer by a volume ratio of 1:4 and held in boiling water for 10 minutes for complete denaturation of the proteins. Starting from the first well of the gel, 5 µl of molecular weight marker and 25 µl of sample-sample buffer mixture were loaded into the wells. The gel cassette was inserted in the buffer tank (Bio-Rad Mini-PROTEAN® Tetra Cell) and it half-filled with 1X electrode (running) buffer. After electrophoretic run (100 V for 85 minutes), silver staining method was applied to dye the protein bands.

Preparation protocols of the buffer systems, gels (separationg gel 12% and stacking gel 4%) and silver staining reagents were explained in detail in Appendix G.

2.2.5.2. Native-PAGE

Native-PAGE is an electrophoretic method but the main difference with SDS-PAGE is that the proteins are separated according to their size and also charge. The aim for preparing a native gel is to separate the proteins without any denaturation to preserve the enzyme activity. Thus, the samples were not heated and while preparing the buffer systems and gels, the denaturants; SDS and β-mercaptoethanol were excluded.

For activity-staining of the desired bands, the gel was incubated in 2% xylan solution at 55°C and 60 rpm, overnight.

Preparations of the buffer systems, gels (12% separating gel and 4% stacking gel) and substrate solution were listed in detail in Appendix G.

2.2.6. Biochemical Characterization Studies

After expression and purification steps, lastly, the enzyme was characterized biochemically determining its kinetic parameters, optimum temperature and pH, thermostability, stability against pH, shelf life and response to various metal ions, chemicals and organic solvents.

2.2.6.1. Analysis of Kinetic Parameters

In this section, kinetic parameters of the enzyme, K_m and V_{max}, were investigated by Lineweaver-Burk plot method (Lineweaver and Burk 1934). The substrate concentrations of the reaction media were varied in the range of 1 to 20 mg/ml and the double reciprocal plot was drawn.

2.2.6.2. Effect of Temperature on Activity and Stability of Xylanase

The optimum temperature for the activity of the xylanase expressed in *Geobacillus* sp. was determined under the standard activity assay conditions. The only parameter changed was the incubation temperature of substrate-enzyme reaction medium ranging from 30°C to 90°C (5°C intervals).

To investigate the thermal stability of the enzyme, six enzyme aliquots were pre-incubated at varying temperatures from 30°C to 90°C (10°C intervals) for 120 minutes. From these aliquots, samples were collected after 15, 30, 60 and 120 minutes respectively. Each sample was brought to 55°C quickly and each was assayed for its residual activity at 55°C as summarized in Figure 2.1.

2.2.6.3. Effect of pH on Activity and Stability of Xylanase

The effect of pH on the activity of our xylanase was studied by performing the assays at different pH values using different buffer systems; pH 3.0, 4.0, 5.0, 6.0 (citrate buffer), pH 7.0 (sodium-phosphate buffer), pH 8.0 (Tris-HCl buffer), pH 9.0 (Glycine-NaOH buffer).

To test the pH stability of the enzyme, enzyme aliquots were pre-incubated with the buffers mentioned above for 15, 30, 60 and 120 minutes at room temperature before activity measurements as charted in Figure 2.2. The residual activity of each was calculated and evaluated as percent relative activities.

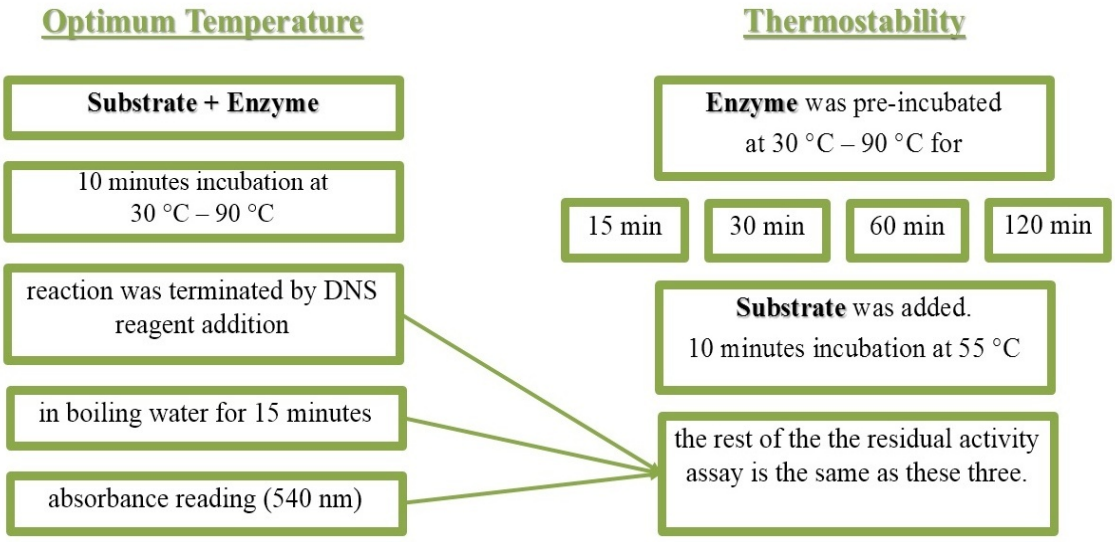

Figure 2.1. Method chart for optimum temperature and thermostability

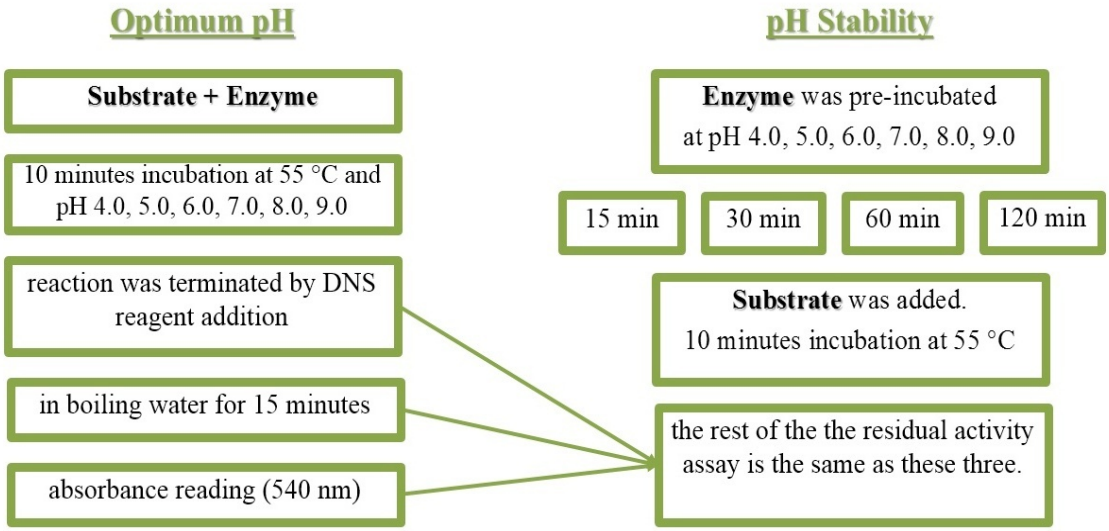

Figure 2.2. Method chart for optimum pH and pH stability

2.2.6.4. Effects of Metal Ions, Chemical Reagents and Organic Solvents On Xylanase Activity

Effects of different metal ions were determined by applying standard activity assay after incubating the enzyme with various metal ions for 10 minutes (KCl, NaCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, NaF, $FeCl_3$, $NiCl_2$, $CuSO_4$, $CdSO_4$, $CoCl_2$, $ZnCl_2$ with 1 mM and 10 mM final reaction mixture concentrations).

Similarly, the effects of EDTA, SDS, CHAPS and DTT (10 mM final reaction mixture concentrations) and DMSO, Triton X-100, Tween 20, Tween 80 (10% v/v in reaction mixture) were tested.

Lastly, how the organic solvents affect the activity was assayed by incubating the enzyme with ethanol, methanol, acetone, isopropanol, chloroform, acetonitrile and β-mercaptoethanol (10% v/v in reaction mixture) for 10 minutes before assaying the residual activity.

A sample containing no additive served as control and its activity was accepted as 100% to be compared with the activities of the other samples. Besides this, absorbance values were measured against blanks containing additives, substrate, DNS reagent and the same volume of sodium phosphate buffer (pH 7.0) instead of enzyme.

2.2.6.5. Shelf-life Determination

To investigate the shelf-life of our xylanase, one aliquot was stored at room temperature and another aliquot was stored at +4°C for 6 weeks. The activities of the enzyme samples were checked every week.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Total Protein and Purified Protein

In this study, partial purification of xylanase from thermophilic *Geobacillus* sp. by acetone precipitation and gel filtration chromatography was achieved. According to preliminary activity assays, being consistent with the literature studies, our xylanase was found out to be an extracellular enzyme. Therefore, extracellular extract was selected for following gel filtration step.

After centrifugation, the cell debris was left and the supernatant was collected. Then, acetone precipitation was applied to collect the total protein. The precipitate was resuspended in sodium phosphate buffer (50 mM, pH 7.0) and loaded to gel filtration column which separates the proteins according to their molecular sizes. Each fraction was tested for activity and the ones with highest activity were pooled (Figure 3.1). The purification steps result in 8.089 fold, but unfortunately 16.395% yield, while the specific activity of the purified *Geobacillus* sp. xylanase was calculated as 160.619 U/mg.

Table 3.1. Activity of xylanase from thermophilic *Geobacillus* sp.

Protein	**Total Activity (U)**	**Total protein (mg)**	**Specific Activity (U/mg)**	**Purification fold**	**Yield (%)**
Crude extract (before acet. ppt.)	29.395	1.480	19.857	1.000	100
Total protein (after acetone ppt.)	66.971	0.685	97.712	4.921	46.230
Purified protein (after gel filtration)	38.981	0.243	160.619	8.089	16.395
Intracellular crude protein	12.702	0.354	35.842	-	-

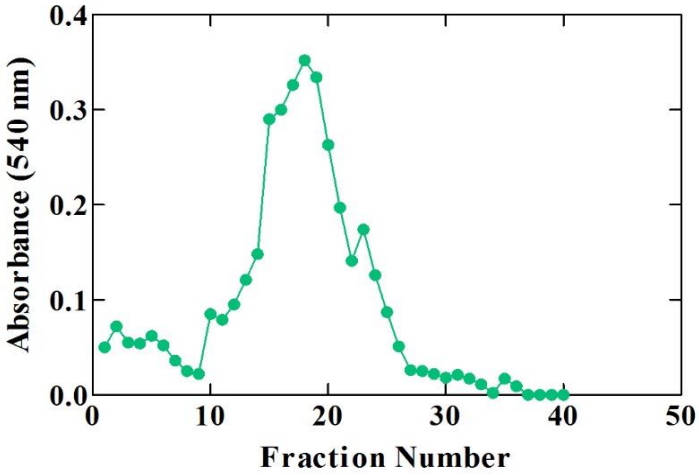

Figure 3.1: Gel filtration profile of total protein containing xylanase

3.2. Electrophoretic Studies and Activity Staining

SDS-PAGE and Native-PAGE were both run and desired bands were observed.

3.2.1. SDS-PAGE

A single distinct band was observed for the protein sample which was purified by gel filtration, while no band was observable for inactive fractions as it was supposed to be. The same band with the purified protein existed in the total protein and crude extract together with some other bands. On the other hand, intracellular protein sample gave a smear image (Figure 3.2) (Bio-Rad Molecular Imager VersaDoc™ MP 4000 System).

When we compared our protein band with the molecular weight marker (Fermentas #SM0671), we calculated the molecular weight of our enzyme was ~40.1 kDa. The size of xylanase expressed in other bacterial species vary from ~20 to ~49 kDa, fungal xylanases have relatively smaller average, but there are some exceptionally larger xylanases as well (Subramaniyan and Prema 2002). For example, xylanase from *Enterobacter* isolates is ~43 kDa (Sharma, Pujari, and Patel 2009), from a *Bacillus* sp. is ~29.8 kDa (Kamble and Jadhav 2012).

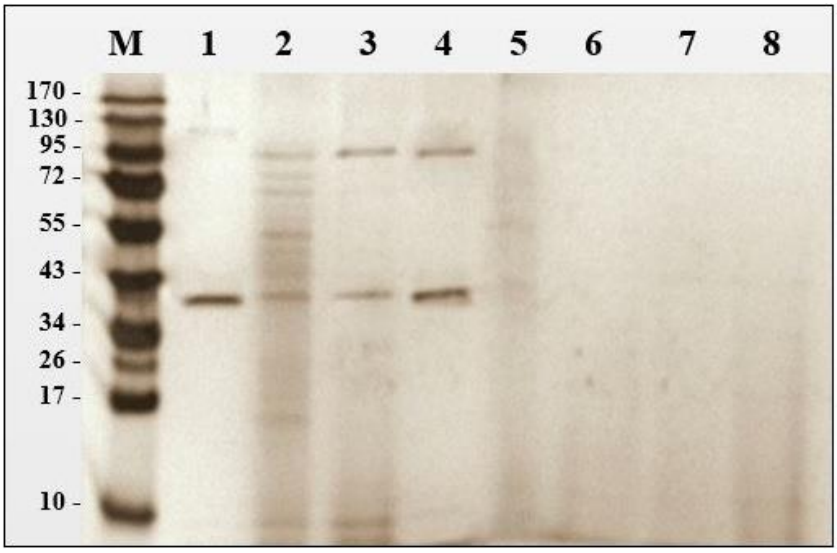

Figure 3.2. SDS-PAGE image

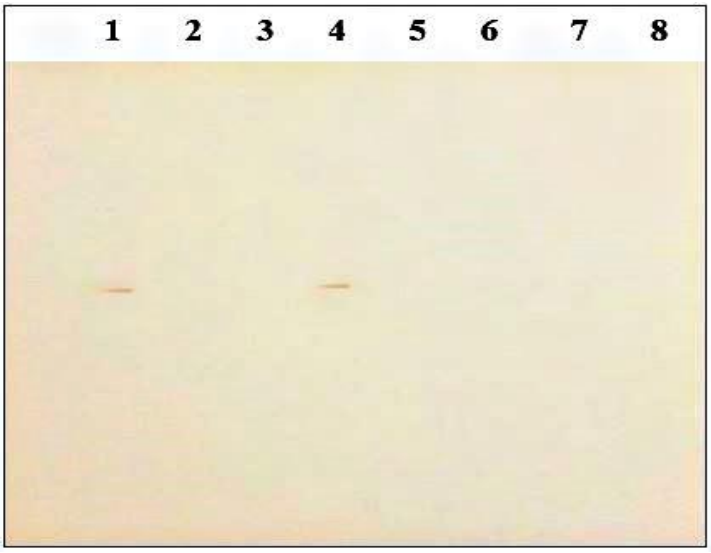

Figure 3.3. Native-PAGE image

Order of the lanes for both gels is as follows:

M – Molecular marker (Fermentas #SM0671) (only on SDS polyacrylamide gel)	
1 – Purified protein (fraction 15-20)	**5 –** Inactive fraction of gel filt. (1-9)
2 – Intracellular protein	**6 –** Inactive fraction of gel filt. (10-14)
3 – Total protein (after acetone ppt.)	**7 –** Inactive fraction of gel filt. (21-30)
4 – Crude extract (before acet. ppt.)	**8 –** Inactive fraction of gel filt. (31-40)

3.2.2. Native-PAGE

The native polyacrylamide gel was stained overnight in the substrate solution to verify that the protein band observed on SDS gel was xylanase. Only on the lane 1 and lane 4, a brown band appeared where the enzyme is due to the xylanolytic activity with the substrate (Figure 3.3).

3.3. Biochemical Characterization of Xylanase

Using the biochemical methods based upon the enzyme-substrate relations, conditional behaviours of the xylanase were investigated.

3.3.1. Kinetic Analysis

To determine the kinetic parameters, K_m and V_{max}, of the thermophilic *Geobacillus* sp. xylanase, initial reaction rates at different substrate concentrations ranging from 1 to 20 mg/ml were measured. It exhibited a hyperbolic response obeying Michaelis-Menten Kinetics. In order to obtain Lineweaver-Burk plot (Figure 3.4.), 1/Substrate concentration (1/[S]) values were plotted against 1/Reaction rate (1/V) and kinetic parameters were calculated using the trendline equation of this double reciprocal graph.

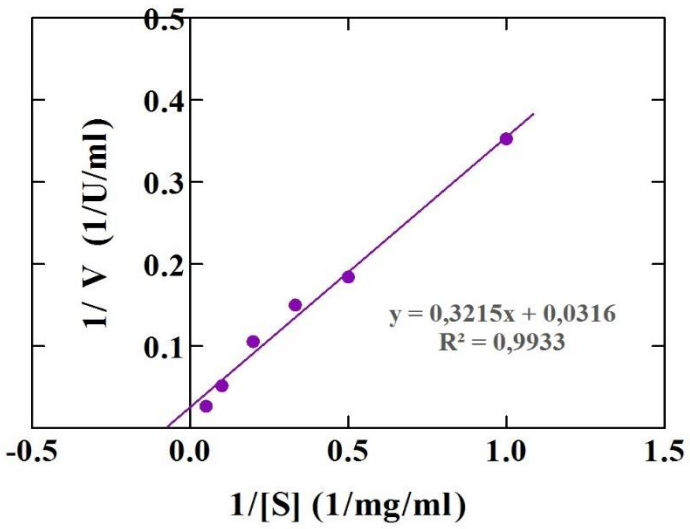

Figure 3.4. Lineweaver-Burk plot of xylanase

K_m and V_{max} values of our xylanase were determined as 10.2 mg/ml and 31.7 U/ml, respectively.

The K_m of an alkali-thermostable xylanase from *Geobacillus* sp. WBI is 0.9 mg/ml (Mitra et al. 2015). The native xylanase of *Geobacillus thermoleovorans* results a K_m value of 2.6 mg/ml (Verma and Satyanarayana 2012). The K_m value of xylanase *Bacillus arseniciselenatis* DSM 15340 acted on birchwood xylan is 5.26 mg/ml (Kamble and Jadhav 2012). Compared with the xylanases of other organisms, xylanase of *Geobacillus* sp. has a larger K_m value, which means it has a lower affinity for its substrate and requires a greater concentration of substrate to achieve its V_{max}.

3.3.2. Optimum Temperature for Enzyme Activity

Measurements of xylanase activity at variable temperatures showed that the activity never drops below 70% of the standard activity. Thus, we can pronounce our xylanase as a quite convenient and favorable enzyme for any industrial or lab-scale applications.

The optimum temperature of the purified xylanase from *Geobacillus* sp. was 55°C, the enzyme shows the highest activity at this temperature in the range of 30-90°C (Figure 3.5).

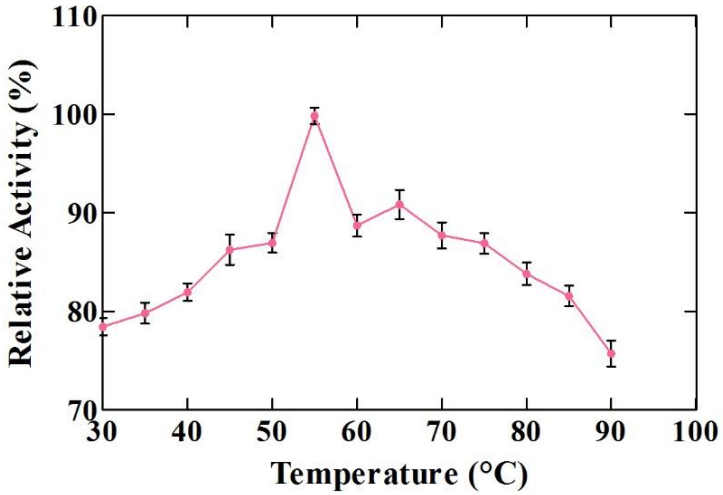

Figure 3.5. Effect of temperature on *Geobacillus* sp. xylanase activity

The optimum temperatures of xylanases from *Bacillus Subtilis* ASH (Sanghi et al. 2010) and from *Aspergillus terreus* (M. Sorgatto 2012) are 55°C as well. Closely, it is noted that thermoalkalophilic species of *Bacillus* gives the best results at 50°C (Kamble and Jadhav 2012). On the other hand, xylanase from *Streptomyces* sp. CH-M-1035 is highly thermophilic that it requires 70°C for highest activity (Huitro 1997). Similarly, the optimum temperature is 75°C for *Geobacillus* sp. TC-W7 xylanase (B. Liu et al. 2012) and even 80°C for *Nonomuraea flexuosa* and *Thermoascus aurantiacus* xylanases (Zhang et al. 2011). The xylanase we expressed in *Geobacillus* sp. has a moderate optimum temperature, whereas that it remains active in a wide temperature range makes it compensative at high temperatures.

3.3.3. Thermostability

Thermostability assays of the purified enzyme revealed that our xylanase did not lose its activity when it was incubated at high temperatures. First 30 minutes, the relative activity did not drop below 90% in any condition. After 1 hour, 85% activity was observed at 60°C and after 2 hours, the lowest residual activity reaches down to 65% at 90°C. The pattern of the graph states that the breaking point was 60°C (Figure 3.6).

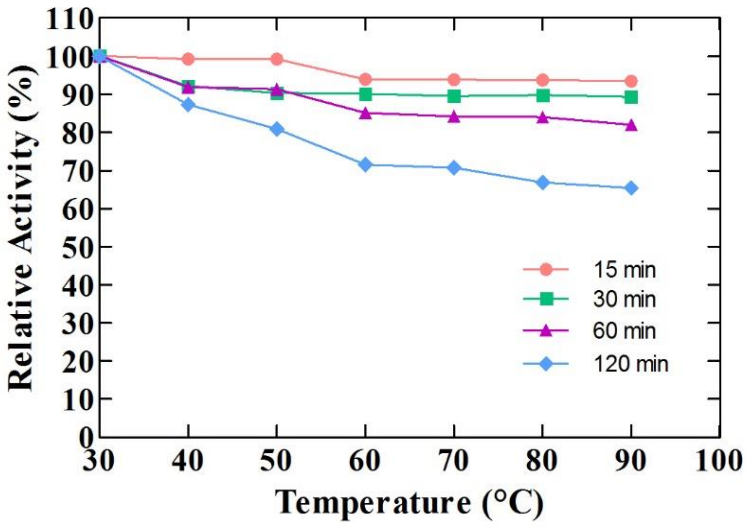

Figure 3.6. Thermostability profile of *Geobacillus* sp. xylanase

The xylanase from *Bacillus subtilis* ASH retains active until 45°C for 10 minutes incubation. Then, the relative activity drops to 50% as approching to 60°C (Sanghi et al. 2010). *Thermobifida halotolerans* YIM 90462 xylanase maintains 90% of its activity until 30 minutes when the temperature passes 70°C, but the relative activity dramatically drops to 0% after 30 minutes at 80 and 90°C. Similarly, the xylanase from *Geobacillus* sp. WBI keeps active until 65°C when incubated for 1 hour and approaches to 0% relative activity at 80°C (Mitra et al. 2015). Whereas, as Cordeiro et al. stated (Cordeiro et al., 2002.), their *Bacillus* sp. xylanase was stable for 2 hours at temperatures ranging from 30-50°C while at 60°C and 100°C, 10% and 29% of the activities were lost respectively, but it does not get below 70% even at 100°C. Compared with the other studies, xylanase expressed in *Geobacillus* sp. can be counted as a highly thermostable enzyme.

3.3.4. Optimum pH for Enzyme Activity

Based on the results of testing the effect of pH on enzyme activity, we can say that our enzyme was suitable to work in a wide pH range. The relative activity never dropped below 84%. Besides this, alkali media helped our enzyme as an activator that relative activity was the highest at pH 8.0 (Figure 3.7).

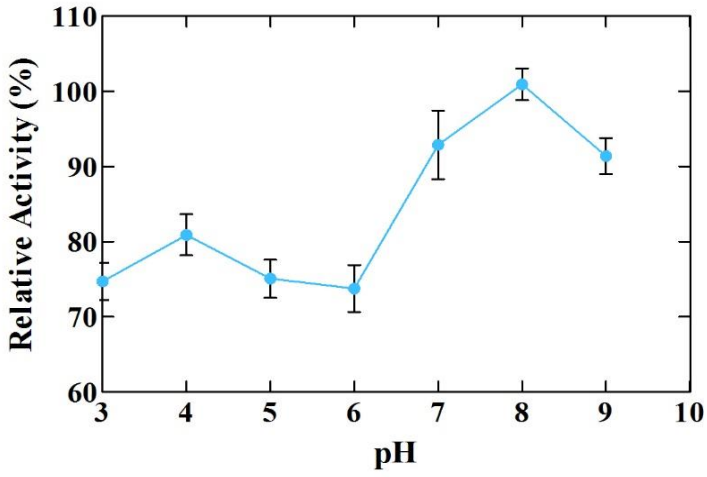

Figure 3.7. Effect of pH on *Geobacillus* sp. xylanase activity

3.3.5. pH Stability

The activity of enzyme which was not subjected to any pH treatment was accepted as 100%. Then the activities were calculated and plotted against pH values (Figure 3.8). The pattern of the graph tells us that both acidic and basic conditions were appropriate for *Geobacillus* sp. xylanase to show its activity. No significant change was observed up to pH 8.0 and relative activity remained above 80% until 60 and 120 minutes incubations of pH 9.0. The *Thermobifida fusca* xylanase is stable in the pH range 3.0-9.0 (Zhao et al. 2015), xylanase from T. lanuginosus CBS 288.54 in pH 6.5–10.0 (Li et al. 2005) and xylanase from *Bacillus amyloliquefaciens* is stable up to pH 9.0 (Breccia et al. 1998).

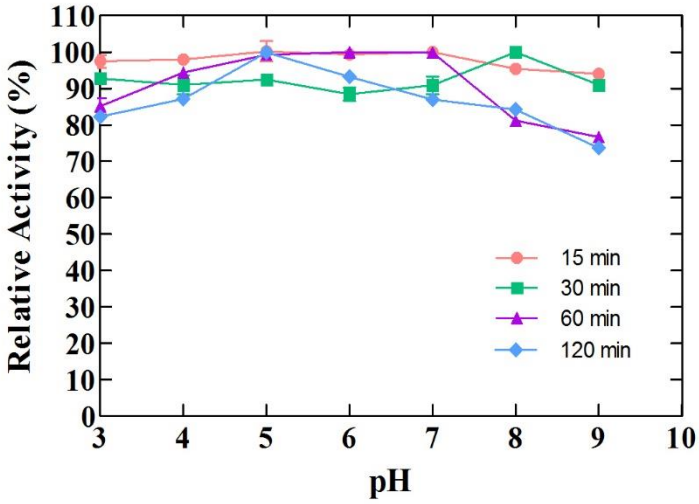

Figure 3.8. pH stability profile of *Geobacillus* sp. xylanase

3.3.6. Effects of Metal Ions on Enzyme Activity

In order to determine the effects of various metal ions on xylanase activity, the enzyme was incubated at room temperature in the presence of 1.0 mM and 10.0 mM total ion concentrations and the activity was assayed by the standard protocol. Each blank contained substrate, metal ions, buffer and DNS reagent and a sample which did not contain any metal ion was our control and accepted as hundred percent active. The effects of metal ions on enzyme activity were calculated as residual activity (Table 3.2).

Table 3.2. Effects of metal ions on *Geobacillus* sp. xylanase activity

Metal Ion	Residual Activity (%) (1.0 mM)	Residual Activity (%) (10.0 mM)
None	100.0	100.0
KCl	100.5	89.8
NaCl	109.4	100.1
$CaCl_2$	142.1	177.7
$MgCl_2$	109.0	81.0
$MnCl_2$	135.8	184.3
NaF	109.3	105.4
$FeCl_3$	62.4	17.0
$NiCl_2$	137.7	89.4
$CuSO_4$	124.6	95.0
$CdSO_4$	79.6	73.9
$CoCl_2$	148.7	126.6
$ZnCl_2$	86.6	68.1

Ca^{2+}, Mn^{2+} and Co^{2+} ions with the concentration of 1 mM and 10 mM increased the activity, whereas Mg^{2+}, Ni^{2+}, Cu^{2+} affected the activity positively only when the total concentration was 1 mM. Besides these, Zn^{2+}, Cd^{2+} and especially Fe^{3+} shielded the activity considerably.

3.3.7. Effects of Various Chemical Reagents on Enzyme Activity

Surfactants may cause denaturations in the three dimensional structures of the enzymes by disrupting their non-covalent bonds. An enzyme that has lost its native conformation loses its activity. Similarly, reagents with thiol groups bring about activity losts by reducing the disulphide bonds and consequently damage enzyme structure. Moreover, DMSO addition results in protein unfolding due to preferential binding of sulphoxide to hydrophobic and aromatic side chains. Also, DMSO decreases the water solubility of the proteins as it is a strong polar aprotic solvent (Arakawa, Kita, and Timasheff 2007). In here, SDS and DTT both caused remarkable activity fall down to ~55%.

Table 3.3. Effects of various chemical reagents on *Geobacillus* sp. xylanase activity

Reagent	**Residual Activity (%)**
None	100.0
EDTA (10 mM)	88.2
SDS (10 mM)	55.8
CHAPS (10 mM)	67.0
DTT (10 mM)	55.2
DMSO (10%)	91.8
TritonX-100 (10%)	88.8
Tween 20 (10%)	94.1
Tween 80 (10%)	86.3

3.3.8. Effects of Organic Solvents on Enzyme Activity

Solvents are supposed to affect the hydrophobic interactions on the protein structure and result in change of the protein solubility in water and conformational structure. The *Geobacillus* sp. xylanase activity was not strongly affected by organic solvent additions, except β-mercaptoethanol which dropped the activity to ~65%.

Table 3.4. Effects of common organic solvents on *Geobacillus* sp. xylanase activity

Organic Solvent	**Residual Activity (%)**
None	100.0
Hexane (10%)	93.4
EtOH (10%)	97.4
MeOH (10%)	91.9
Acetone (10%)	104.7
Isopropanol (10%)	86.7
Chloroform (10%)	103.7
Acetonitrile (10%)	91.2
β-ME (10%)	65.3

3.3.9. Shelf-life Determination

The activities of the enzymes are supposed to change in time. Thus, we tested the shelf-life of our protein when stored in refrigerator and at room temperature. Both the total protein and the purified enzyme remained active at room temperature and +4°C for first 3 weeks, then slight changes was observed by fourth week espeacially for room temperaure storages (Table 3.5.) Sanghi et al. states that *Bacillus subtilis* ASH had not lost its activity for six weeks but therafter, a decline had been observed for +4°C storage. Whereas, at room temperature, the enzyme had stayed active first three weeks and then lost half of its activity up to 10 weeks (Sanghi et al. 2010).

Table 3.5. Shelf-life determination for total protein and purified protein

Time (%)	**Total protein at +4°C**	**Total protein at room temp.**	**Purified protein at+4°C**	**Purified protein at room temp.**
Week-1	99.5	99.8	99.1	99.3
Week-2	98.2	99.1	97.0	97.5
Week-3	98.4	99.4	97.5	96.1
Week-4	92.5	92.2	96.1	88.9
Week-5	90.0	91.0	94.0	88.0
Week-6	88.6	89.4	92.5	86.5

CHAPTER 4

CONCLUSION

In our study, we mainly aimed to isolate, purify and characterize a xylanase expressed in thermophilic *Geobacillus* sp.

After isolation, acetone precipitation and gel filtration chromatography techniques were followed, electrophoretic studies were completed and the molecular weight was found as ~40.1 kDa. Then, a series of biochemical characterization assays were performed.

Initially, K_m and V_{max} values were estimated from varying xylan concentrations against reaction rate double reciprocal plot as 10.2 mg/ml and 31.7 U/ml, respectively. Secondly, temperature and pH optima values for highest enzyme activity were detected. Optimum temperature was found as 55˚C and pH as 8.0. Additionally, effects of temperature and pH on enzyme stability were investigated. According to our results, the enzyme shows high resistance against temperature and pH and is able to work any condition up to 2 hours, but only when it was incubated over 80°C for 120 minutes, the residual activity drops below 70%. Also, we have tested the effects of metal ions, some specific chemicals and common organic solvents on enzyme activity. Briefly; Ca^{2+}, Mn^{2+} and Co^{2+} act as strong activators whereas Zn^{2+}, Cd^{2+}, Fe^{3+}, EDTA, SDS, CHAPS and DTT inhibit the activity. None of the organic solvents, but β-mercaptoethanol affect the activity significantly. Lastly, shelf-lives at +4˚C and room temperature were tested and no remarkable falling observed up to 6 weeks for purified protein. It drops below 90% resiudual activity only for room temperature storage.

In conclusion, the enzyme was purified and some general biochemical characteristics were determined. Further purification steps may be applied to obtain higher activities and different biotechnologies (like recombinant technologies and/or enzyme engineering techniques) may be prefered for higher yields during expression.

REFERENCES

Arakawa, Tsutomu, Yoshiko Kita, and Serge N. Timasheff. 2007. "Protein Precipitation and Denaturation by Dimethyl Sulfoxide." *Biophysical Chemistry* 131 (1-3): 62–70. doi:10.1016/j.bpc.2007.09.004.

Bailey, Michael J.; Biely, Peter; Poutanen, Kaisa. 1992. "Interlaboratory Testing of Methods For Assay of Xylanase Activity." *J. Biotechnol.* 23:257-270

Baker, G C, S Gaffar, D a Cowan, and a R Suharto. 2001. "Bacterial Community Analysis of Indonesian Hot Springs." *FEMS Microbiology Letters* 200 (1): 103–9.

Biely, Peter. 1985. "Microbial Xylanolytic Systems." *Trends in Biotechnology* 3 (11): 286–90. doi:10.1016/0167-7799(85)90004-6.

Bradford, M M. 1976. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." *Analytical Biochemistry* 72: 248–54. doi:10.1016/0003-2697(76)90527-3.

Breccia, Javier D, Faustino Siñeriz, Mario D Baigorí, Guillermo R Castro, and Rajni Hatti-Kaul. 1998. "Purification and Characterization of a Thermostable Xylanase from Bacillus Amyloliquefaciens." *Enzyme and Microbial Technology* 22 (1): 42–49. doi:http://dx.doi.org/10.1016/S0141-0229(97)00102-6.

Bruins, Marieke E; Janssen, Anja E. M;, and Remko M. Boom. 2001. "Thermozymes and Their Applications." *Appl Biochem Biotechnol.* 90: 155–86. doi:10.1385/ABAB:90:2:155.

Collins, Tony, Charles Gerday, and Georges Feller. 2005. "Xylanases, Xylanase Families and Extremophilic Xylanases." *FEMS Microbiology Reviews* 29 (1): 3–23. doi:10.1016/j.femsre.2004.06.005.

Compton, S. J. and Jones, C. G. 1985. Mechanism of Dye Response and Interference in the Bradford Protein Assay. *Analytical Biochemistry.* 151: 369–374.

Congdon, R. W., Muth, G. W., and Splittgerber, A. G. 1993. The Binding Interaction of Coomassie Blue with Proteins. *Analytical Biochemistry.* 213: 407–413.

Cordeiro, C.a.M., M.L.L. Martins, a.B. Luciano, and R.F. Silva. n.d. "Production and Properties of Xylanase from Thermophilic Bacillus Sp." *Brazilian Archives of Biology and Technology* 00045 (4): 413–18. doi:10.1590/S1516-89132002000600002.

Gibson, L. J. 2012. "The Hierarchical Structure and Mechanics of Plant Materials." *Journal of The Royal Society Interface* 9 (76): 2749–66. doi:10.1098/rsif.2012.0341.

Gomori, G. 1955. *Preparation of Buffers for Use in Enzyme Studies.* Edited by Kaplan, *Methods in Enzymology*. New York Academic Press.

Grant, K. H. a. W. D. 1998. *Extremophiles – Microbial Life in Extreme Environments*: Wiley-Liss, New York.

Haki, G. D., and S. K. Rakshit. 2003. "Developments in Industrially Important Thermostable Enzymes: A Review." *Bioresource Technology* 89 (1): 17–34. doi:10.1016/S0960-8524(03)00033-6.

Huitro, C. 1997. "B-Xylosidase and Xylanase Characterization and Production by Streptomyces Sp . CH-M-1035." *Canadian Journal of Microbiology*, 410–16. doi:10.1046/j.1472-765X.1997.00149.x.

Kamble, Rajashri D., and Anandrao R. Jadhav. 2012. "Isolation, Purification, and Characterization of Xylanase Produced by a New Species of Bacillus in Solid State Fermentation." *International Journal of Microbiology* 2012. doi:10.1155/2012/683193.

Khandeparker, Rakhee, and Mondher Th Numan. 2008. "Bifunctional Xylanases and Their Potential Use in Biotechnology." *Journal of Industrial Microbiology and Biotechnology* 35 (7): 635–44. doi:10.1007/s10295-008-0342-9.

KRISTJANSSON, J. 1989. "Thermophilic Organisms as Sources of Thermostable Enzymes." *Trends in Biotechnology* 7 (12): 349–53. doi:10.1016/0167-7799(89)90035-8.

Kulkarni, Neeta, Abhay Shendye, and Mala Rao. 1999. "Molecular and Biotechnological Aspects of Xylanases." *FEMS Microbiology Reviews* 23 (4): 411–56. doi:10.1016/S0168-6445(99)00006-6.

Kumar, Pankaj, Ashish Gupta, Sanjay R. Dhakate, Rakesh B. Mathur, Sushil Nagar, and Vijay K. Gupta. 2013. "Covalent Immobilization of Xylanase Produced from Bacillus Pumilus SV-85S on Electrospun Polymethyl Methacrylate Nanofiber Membrane." *Biotechnology and Applied Biochemistry* 60 (2): 162–69. doi:10.1002/bab.1072.

Laemmli, U K. 1970. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4." *Nature* 227 (5259): 680–85. doi:10.1038/227680a0.

Li, X, Z Jiang, L Li, S Yang, W Feng, J Fan, and I Kusakabe. 2005. "Characterization of a Cellulase-Free, Neutral Xylanase from CBS 288.54 and Its Biobleaching Effect on Wheat Straw Pulp." *Bioresource Technology* 96 (12): 1370–79. doi:10.1016/j.biortech.2004.11.006.

Lineweaver, H., and D. Burk. 1934. "The Determination of Enzyme Dissociation Constants." *Journal of the American Chemical Society* 56 (3): 658–66. doi:10.1021/ja01318a036.

Liu, Bin, Ningning Zhang, Chao Zhao, Baixue Lin, Lianhui Xie, and Yifan Huang. 2012. "Characterization of a Recombinant Thermostable Xylanase from Hot Spring."

Journal of Microbiology and Biotechnology 22 (10): 1388–94. doi:10.4014/jmb.1203.03045.

Liu, Ming-qi, Wen-kang Huo, Xin Xu, and Ding-feng Jin. 2015. "An Immobilized Bifunctional Xylanase on Carbon-Coated Chitosan Nanoparticles with a Potential Application in Xylan-Rich Biomass Bioconversion." *Journal of Molecular Catalysis B: Enzymatic* 120. Elsevier B.V.: 119–26. doi:10.1016/j.molcatb.2015.07.002.

M. Sorgatto. 2012. "Purification and Characterization of an Extracellular Xylanase Produced by the Endophytic Fungus, Aspergillus Terreus, Grown in Submerged Fermentation." *African Journal of Biotechnology* 11 (32): 8076–84. doi:10.5897/AJB11.2686.

Mitra, Suranjita, Bidhan Chandra Mukhopadhyay, Anisur Rahaman Mandal, Ananta Prasad Arukha, Kuheli Chakrabarty, Gourab Kanti Das, Pran Krishna Chakrabartty, and Swadesh Ranjan Biswas. 2015. "Cloning, Overexpression, and Characterization of a Novel Alkali-Thermostable Xylanase from *Geobacillus* Sp. WBI." *Journal of Basic Microbiology* 55 (4): 527–37. doi:10.1002/jobm.201400495.

Miller, G. L. 1959. Use of Dinitrosaiicyiic Acid Reagent for Determination of Reducing Sugar. *Anal. Chem.* (3):426–428.

Nagar, Sushil, Anuradha Mittal, and Vijay Kumar Gupta. 2012. "Enzymatic Clarification of Fruit Juices (apple, Pineapple, and Tomato) Using Purified Bacillus Pumilus SV-85S Xylanase." *Biotechnology and Bioprocess Engineering* 17 (6): 1165–75. doi:10.1007/s12257-012-0375-9.

Rothschild, L J, and R L Mancinelli. 2001. "Life in Extreme Environments." *Nature* 409 (6823): 1092–1101. doi:10.1038/35059215.

Pearse, A. G. E. 1980. *Histochemistry: Theoretical and Applied*. Churchill Livingstone, Edinburgh.

Sanghi, Ashwani, Neelam Garg, V. K. Gupta, Ashwani Mittal, and R. C. Kuhad. 2010. "One-Step Purification and Characterization of Cellulase-Free Xylanase Produced by Alkalophilic Bacillus Subtilis ASH." *Brazilian Journal of Microbiology* 41 (2): 467–76. doi:10.1590/S1517-838220100002000029.

Schädel, Christina, Andreas Richter, Andreas Blöchl, and Günter Hoch. 2010. "Hemicellulose Concentration and Composition in Plant Cell Walls under Extreme Carbon Source-Sink Imbalances." *Physiologia Plantarum* 139 (3): 241–55. doi:10.1111/j.1399-3054.2010.01360.x.

Shahrestani, Hourasa, Asghar Taheri-Kafrani, Asieh Soozanipour, and Omid Tavakoli. 2016. "Enzymatic Clarification of Fruit Juices Using Xylanase Immobilized on 1,3,5-Triazine-Functionalized Silica-Encapsulated Magnetic Nanoparticles." *Biochemical Engineering Journal* 109. Elsevier B.V.: 51–58. doi:10.1016/j.bej.2015.12.013.

Sharma, Anjana, Rajesh Pujari, and Pratibha Patel. 2009. “Characterization of Thermoalkalophilic Xylanase Isolated from Enterobacter Isolates.” *Indian Journal of Biotechnology* 8 (1): 110–14.

Shuler, Michael L., and Fikret Kargi. 2002. “Bioprocess Engineering: Basic Concepts.” *Journal of Controlled Release*, 293. doi:10.1016/0168-3659(92)90106-2.

Soozanipour, Asieh, Asghar Taheri-Kafrani, and Amir Landarani Isfahani. 2015. “Covalent Attachment of Xylanase on Functionalized Magnetic Nanoparticles and Determination of Its Activity and Stability.” *Chemical Engineering Journal* 270. Elsevier B.V.: 235–43. doi:10.1016/j.cej.2015.02.032.

Subramaniyan, S, and P Prema. 2002. “Biotechnology of Microbial Xylanases: Enzymology, Molecular Biology, and Application.” *Critical Reviews in Biotechnology* 22 (1): 33–64. doi:10.1080/07388550290789450.

Verma, Digvijay, and T. Satyanarayana. 2012. “Cloning, Expression and Applicability of Thermo-Alkali-Stable Xylanase of Geobacillus Thermoleovorans in Generating Xylooligosaccharides from Agro-Residues.” *Bioresource Technology* 107. Elsevier Ltd: 333–38. doi:10.1016/j.biortech.2011.12.055.

Viikari, Liisa, Anne Kantelinen, Jorma Sundquist, and Matti Linko. 1994. “Xylanases in Bleaching: From an Idea to the Industry.” *FEMS Microbiology Reviews* 13 (2): 335–50. http://www.sciencedirect.com/science/article/pii/0168644594900906.

Wong, K K, L U Tan, and J N Saddler. 1988. “Multiplicity of Beta-1,4-Xylanase in Microorganisms: Functions and Applications.” *Microbiological Reviews* 52 (3): 305–17.

Yavuz, E., H. Gunes, S. Harsa, and a. Fazil Yenidunya. 2004. “Identification of Extracellular Enzyme Producing Thermophilic Bacilli from Balcova (Agamemnon) Geothermal Site by ITS rDNA RFLP.” *Journal of Applied Microbiology* 97 (4): 810–17. doi:10.1111/j.1365-2672.2004.02374.x.

Zhang, Junhua, Matti Siika-Aho, Terhi Puranen, Ming Tang, Maija Tenkanen, and Liisa Viikari. 2011. “Thermostable Recombinant Xylanases from Nonomuraea Flexuosa and Thermoascus Aurantiacus Show Distinct Properties in the Hydrolysis of Xylans and Pretreated Wheat Straw.” *Biotechnology for Biofuels* 4 (1). BioMed Central Ltd: 12. doi:10.1186/1754-6834-4-12.

Zhao, Longmei, Jiang Geng, Yaoqi Guo, Xiudong Liao, Xuhui Liu, Rujuan Wu, Zhaojun Zheng, and Rijun Zhang. 2015. “Expression of the Thermobifida Fusca Xylanase Xyn11A in Pichia Pastoris and Its Characterization.” *BMC Biotechnology* 15 (1): 1–12. doi:10.1186/s12896-015-0135-y.

APPENDIX A

CHEMICALS USED IN THE EXPERIMENTS

- Bacto Agar
- Tryptone
- Yeast Extract
- Sodium chloride (NaCl)
- Ammonium nitrate (NH_4NO_3)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)
- Manganese (II) sulfate monohydrate ($MnSO_4.H_2O$)
- Sodium hydrogen phosphate (Na_2HPO_4)
- Sodium dihydrogen phosphate (NaH_2PO_4)
- Monosodium citrate
- Citric acid
- Tris Base
- Hydrogen chloride (HCl)
- Sodium hydroxide (NaOH)
- Glycine
- EDTA
- SDS
- CHAPS
- DMSO
- DTT
- Triton X-100
- Tween 20
- Tween 80
- Hexane
- Ethanol
- Methanol

- Acetone
- Isopropanol
- Chloroform
- Acetonitrile
- β-mercaptoethanol
- 3,5-Dinitrosalicylic acid
- Potassium sodium tartrate
- Acrylamide
- N'N'-bis-methylene-acrylamide
- Bromophenol blue (CBB G-250)
- Phosphoric acid
- Glycerol
- Ammonium persulfate ($(NH_4)_2S_2O_8$)
- TEMED
- Sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$)
- Acetic acid
- Formaldehyde
- Silver nitrate ($AgNO_3$)
- Potassium carbonate (K_2CO_3)

APPENDIX B

BUFFER SOLUTIONS

I. Citrate Buffer (pH 3.0, pH 4.0, pH 5.0, pH 6.0)

Citrate buffer (Gomori 1955) stock solutions:

- 0.1 M citric acid (21.01 g in 1.0 L)
- 0.1 M sodium citrate (29.41 g in 1.0 L)

Following buffer systems were prepared using these stock solutions and diluting them to 100.0 ml with 50.0 ml dH_2O. In the end, they were diluted 1:2 to obtain 50.0 mM concentration.

Volume of 0.1 M Citric acid (ml)	Volume of 0.1 M Sodium citrate (ml)	Desired pH
46.5	3.5	3.0
33.0	17.0	4.0
20.5	29.5	5.0
9.5	41.5	6.0

II. Sodium Phosphate Buffer, 0.1 M (pH 7.0)

Below listed amounts of stock solutions were mixed and diluted 1:2 to obtain 50.0 mM buffer system at pH 7.0.

Volume of 1.0 M Na_2HPO_4 (ml)	Volume of 1.0 M NaH_2PO_4 (ml)	Desired pH
57.7	42.3	7.0

III. Tris-Cl Buffer, 1.0 M (pH 8.0)

121.1 g of Tris-base was dissolved in 700.0 ml of dH_2O. pH was adjusted to 8.0 using concentrated HCl. The solution was filled up to 1.0 L with dH_2O. Finally, the stock solution was diluted to 50.0 mM with dH_2O.

IV. Glycine-NaOH Buffer (pH 9.0)

First, 0.2 M glycine and 0.2 M NaOH were prepared and mixed as listed below in the table. Then, it was diluted to 100.0 ml with dH_2O (Pearse 1980).

Volume of 0.2 M Glycine (ml)	Volume of 0.2 M NaOH (ml)	Desired pH
25.0	4.4	9.0

Buffers used for electrophoretic studies are listed in Appendix D.

APPENDIX C

MEDIUM

Liquid Medium, 100 ml (pH 7.0)

- Beechwood xylan, 1%
- Tryptone, 2%
- Yeast extract, 0.25%
- NH_4NO_3, 0.2%
- KH_2PO_4, 0.2%
- $MgSO_4.7H_2O$, 0.1%
- $MnSO_4$, 0.005%

The solid components above were diluted to 100.0 ml with deionized water and autoclaved.

APPENDIX D

PREPARATION OF DNS REAGENT

- 1.0 g 3,5-dinitrosalicylic acid
- 20.0 ml 2N NaOH

were dissolved together.

- 30.0 g potassium sodium tartrate (also know as Rochelle salt)

was added and completed to a final volume of 100.0 ml with dH_2O.

For 2N NaOH;

- 8.0 g NaOH

was completed to 100.0 ml with dH_2O.

APPENDIX E

XYLOSE STANDARD CURVE

I. Preparation of Xylose Stock Solution (0.01 M)

- 0.0375 gram xylose

was dissolved in 25.0 ml Na-P buffer (50.0mM, pH 7.0) (stored at -20°C).

Dilution ratio	Concentrations
1:1	10.00 µmol/ml
1:2	5.00 µmol/ml
1:3	3.33 µmol/ml
1:5	2.00 µmol/ml

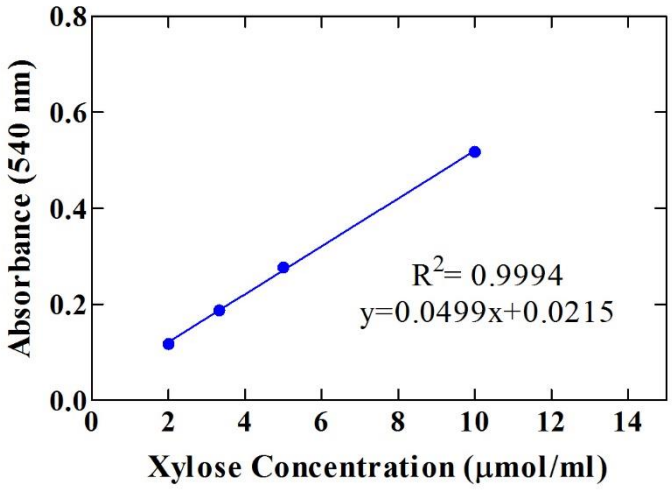

Figure E.1. Xylose standard curve

APPENDIX F

BRADFORD ASSAY AND BSA STANDARD CURVE

I. Preparation of 5X Bradford Reagent

- 50.0 mg Coomassie Brilliant Blue G-250
- 25.0 ml 95% ethanol
- 50.0 ml 85% phosphoric acid

were dissolved within the order above and brought to 100.0 ml with ultra pure water and filtered through Whatman Grade 1 qualitative filter paper (stored at 4ºC).

The Bradford reagent was diluted to 1X before use.

II. Preparation of BSA Stock Solution (0.2 mg/ml)

0.02 gram BSA was dissolved in 1.0 ml distilled water. 10 µl was taken from this solution and diluted to 1.0 ml with distilled water.

III. Preparation of BSA Standards and The Standard Curve

Standard	BSA (µl)	dH_2O (µl)	Bradford Reagent (µl)
Blank	0.0	800.0	200.0
1 µg/ml	5.0	795.0	200.0
2 µg/ml	10.0	790.0	200.0
4 µg/ml	20.0	780.0	200.0
6 µg/ml	30.0	770.0	200.0
8 µg/ml	40.0	760.0	200.0

Standard samples were prepared as in the table above and incubated at the room temperature for 5 minutes. Absorbances were measured (595 nm) using Perkin Elmer Lambda Bio+ Spectrophotometer and standard curve was plotted (Figure F.1.).

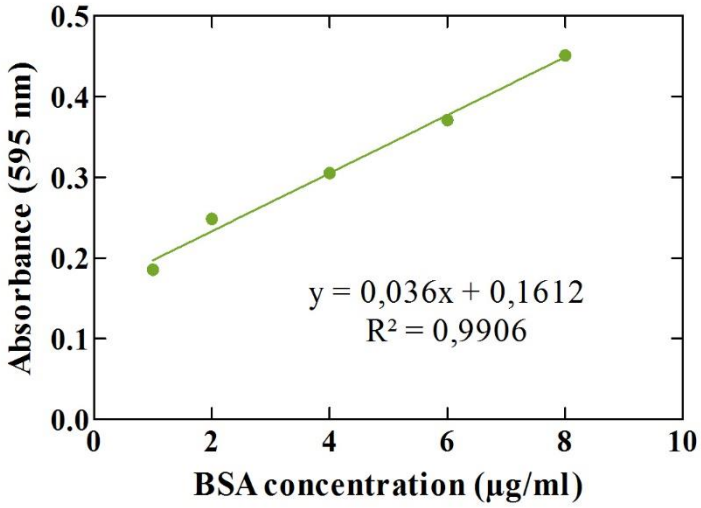

Figure F.1. BSA standard curve

APPENDIX G

PREPARATIONS FOR SDS-PAGE AND NATIVE-PAGE

I. Stock Solutions for SDS-PAGE

A. 30% Acrylamide Mixture

- 87.6 g acrylamide
- 2.4 g N'N'-bis-methylene-acrylamide

were diluted to 300.0 ml with deionized water (stored at 4°C).

B. 1.5M Tris-HCl (pH 8.8)

- 27.23 g Tris Base

was dissolved in 120.0 ml deionized water, pH was adjusted to 8.8 with 6N HCl. Then, it was diluted to 150.0 ml with deionized water (stored at 4°C).

C. 0.5M Tris-HCl, pH 6.8

- 6.0 g Tris Base

was dissolved in 80.0 ml deionized water, pH was adjusted to 6.8 with 6N HCl. And, it was diluted up to 100.0 ml with deionized water (stored at 4°C).

D. 10% SDS

- 10.0 g SDS

was dissolved in 90.0 ml deionized water by gentle stirring and brought to 100.0 ml with deionized water (stored at room temperature).

E. Sample Buffer

- 3.55 ml deionized water
- 1.25 ml 0.5M Tris-HCl (pH 6.8)
- 2.5 ml Glycerol
- 2.0 ml 10% (w/v) SDS

- 0.2 ml 0.5% (w/v) bromophenol blue (CBB G-250)
- 50.0 µl β-mercaptoethanol

were mixed together (stored at room temperature).

F. 10X Running Buffer (pH 8.3)

- 30.3 g Tris Base
- 144.0 g Glycine
- 10.0 g SDS

were dissolved in 800.0 ml deionized water and brought up to 1.0 L. Required amount was diluted to 1X just before use (stored at 4°C).

G. 10% Ammonium persulfate (APS)

- 0.1 g APS

was dissolved in 1.0 ml deionized water just before gel preparation as it is required to be fresh.

II. Gel Preparation for SDS-PAGE

Separating Gel (12%) and Stacking Gel (4%) were prepared as in the table.

Reagents	12% SDS-PAGE separating gel	4% SDS-PAGE stacking gel
Acrylamide/Bis (30%)	4.00 ml	1.30 ml
Deionized water	3.35 ml	6.10 ml
1.5M Tris-HCl (pH 8.8)	2.50 ml	-
0.5M Tris-HCl (pH 6.8)	-	2.50 ml
10% SDS	1.00 ml	10.00 µl
10% APS	50.00 µl	50.00 µl
TEMED	5.00 µl	10.00 µl
Total volume	10.90 ml	9.97ml

III. Silver Staining for SDS-PAGE

A. Fixer Solution

- 150.0 ml methanol
- 36.0 ml acetic acid
- 150.0 µl 37% formaldehyde

were mixed and completed to 300.0 ml with deionized water.

B. 50% Ethanol

- 600.0 ml absolute ethanol
- 600.0 ml deionized water

C. Pretreatment Solution

- 0.08 g $Na_2S_2O_3.5H_2O$

was dissolved in 400.0 ml deionized water.

D. Silver Nitrate Solution

- 0.8 g silver nitrate
- 400.0 ml deionized water
- 300.0 µl 37% formaldehyde

E. Developing Solution

- 400.0 ml deionized water
- 9.0 g potassium carbonate
- 8.0 ml from pretreatment solution above
- 300.0 µl 37% formaldehyde

F. Stop Solution

- 200.0 ml methanol
- 48.0 ml acetic acid

were mixed and completed to 400 ml with deionized water.

All were stored at room temperature.

IV. Stock Solutions for Native-PAGE

Stock solutions for Native-PAGE were prepared quite similarly to solutions of SDS-PAGE. Only difference was the absence of denaturating reagents like SDS and β-mercaptoethanol.

A. 30% Acrylamide Mixture

- 87.6 g acrylamide
- 2.4 g N'N'-bis-methylene-acrylamide

were diluted to 300.0 ml with deionized water (stored at 4°C).

B. 1.5M Tris-HCl (pH 8.8)

- 27.23 g Tris Base

was dissolved in 120.0 ml deionized water, pH was adjusted to 8.8 with 6N HCl. Then, it was diluted to 150.0 ml with deionized water (stored at 4°C).

C. 0.5M Tris-HCl, pH 6.8

- 6.0 g Tris Base

was dissolved in 80.0 ml deionized water, pH was adjusted to 6.8 with 6.0N HCl. And, it was diluted up to 100.0 ml with deionized water (stored at 4°C).

D. Sample Buffer

- 5.55 ml deionized water
- 1.25 ml 0.5M Tris-HCl (pH 6.8)
- 3.00 ml Glycerol
- 0.20 ml 0.5% (w/v) bromophenol blue (CBB G-250)

were mixed together (stored at room temperature).

E. 10X Running Buffer

- 30.3 g Tris Base
- 144.1 g Glycine

were diluted to 1.0 L with deionized water (stored at 4°C).

F. 10% Ammonium persulfate (APS)

- 0.1 g APS

was dissolved in 1.0 ml deionized water just before gel preparation as it is required to be fresh.

V. Gel Preparation for Native-PAGE

Separating Gel (12%) and Stacking Gel (4%) were prepared as in the table.

Reagents	12% Native-PAGE separating gel	4% Native-PAGE stacking gel
Acrylamide/Bis (30%)	4.00 ml	1.30 ml
Deionized water	4.35 ml	6.20 ml
1.5M Tris-HCl (pH 8.8)	2.50 ml	-
0.5M Tris-HCl (pH 6.8)	-	2.50 ml
10% APS	50.00 µl	50.00 µl
TEMED	5.00 µl	10.00 µl
Total volume	10.90 ml	10.06ml

VI. Substrate Solution for Activity Staining of Native-PAGE

For 2% substrate solution, 4.0 gram of beechwood xylan was dissolved in 200.0 ml deionized water by boiling and then cooled. The gel was incubated in this solution overnight at 55 °C, 60 rpm.