PRODUCTION, CHARACTERIZATION AND IMMOBILIZATION OF THERMO-ALKALI-STABLE XYLANASE FROM *GEOBACILLUS THERMODENITRIFICANS* X1 AND ITS APPLICATION IN PAPER PULP BIO-BLEACHING

Thesis submitted in fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

RITIKA VERMA

Department of Biotechnology and Bioinformatics

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT, SOLAN, HP - 173234, INDIA

December 2019

TABLE OF CONTENTS

DECLARATION

I hereby declare that the work reported in the Ph.D. thesis entitled **"Production, characterization and immobilization of thermo-alkali-stable xylanase from *Geobacillus thermodenitrificans* X1and its application in paper pulp bio-bleaching"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Sudhir Kumar.** I have not submitted this work elsewhere for any other degree or diploma. I am fully responsible for the contents of my Ph.D. Thesis.

Ritika Verma

Enrollment no. 136559

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology, Waknaghat, India

Date:

CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Production, characterization and immobilization of thermo-alkali-stable xylanase from *Geobacillus thermodenitrificans* X1 and its application in paper pulp bio-bleaching"** submitted by **Ritika Verma (Enrollment No. 136559)** at **Jaypee University of Information Technology, Waknaghat**, **India,** is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

Dr. Sudhir Kumar

Professor and Head

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat, Solan, H.P., India – 173234

Date:

ACKNOWLEDGEMENT

At the end stage of my Ph.D. journey my heart overflows with emotions and gratitude towards people who believed in me and spend their time and energy for the same. This journey would not have been completed without the help, support, efforts and guidance of a lot of people.

*First of all I would thank the "**Almighty God"** for guiding me throughout and bestowing on me the strength to make the right decisions with dignity, to know when to hold on and when to let go and teaching me to be humane and patient at all times.*

"Guru Brahma, Gurur Vishnu, Guru Devo, Maheshwaraha, Guru Saakshat Param Brahma Tasmai Sree Gurave Namaha". I *owe my deepest thanks to my mentor **"Dr. Sudhir Kumar" Professor and Head, Department of Biotechnology and Bioinformatics.** He has always been encouraging and supportive throughout the ups and downs of the whole journey. As a mentor he was liberal in accepting my perspectives; which helped me to develop my research intellectual. I thank him for always believing in my potential and transformed me into a confident individual. He has been there patiently to always support in the hard times. For any student words would be short to express the gratitude towards his/her mentor; so is for me. I wish I could succeed in life as per his expectations.*

*I also owe my sincere thanks to JUIT administration, Vice Chancellor, **Prof. (Dr.) Vinod Kumar**, Dean, **Prof. (Dr.) Samir Dev Gupta** and Registrar **Maj. Gen. (Retd.) Rakesh Bassi** for providing all the facilities and infrastructure to accomplish this work.*

*I emphatically express my thanks to DPMC members **Dr. Ashish Kumar, Dr. Jitendraa Vashistt**,* and ***Dr. Vijay Kumar Garlapati*** *and for their invaluable guidance, positive criticism, help and kind cooperation. I would also like to express my sincere thanks to all the faculty members of Department of Biotechnology and Bioinformatics, for their help, support and guidance at various stages of this study.*

*I am also highly beholden to **Dr. Aditya Bhalla** and **Dr. Ashok Kumar** for their valuable suggestions and expertise guidance during my research work.*

*I am deeply obliged to **Dr. Bhupinder Singh Chadha**, Professor, Guru Nanak Dev University, Amritsar, Punjab. I feel myself fortunate to get research training under his supervision.*

*I am also indebted to **Dr. Rajneesh Giri**, Assistant Professor, Indian Institute of Technology, Mandi, H.P. for his help and positive support during my research work in his lab.*

*I also express my sincerest thanks to **Mr. Sanjay Chechi**, Kauntum Papers Ltd. Hoshiarpur, Punjab for his kind support during my research work in the industry.*

*I cannot forget to express heartful thanks to my constant pillar of strength and support; **Dr. Hemant Sood** right from the starting of this long journey. She has always stood by my side as a great friend, family and a mentor.*

*Also I am thankfull to **Dr. Jatashankar** for boosting my spirits by his kind and inspiring words in all the odd times and for giving valuable suggestions.*

*Among laboratory staff, I would like to thank **Mr. Baleshwar** for his generous help during tough time of my work. I also owe thanks to lab staff **Mrs. Mamta Mishra, Mrs. Somlata Sharma, Mrs. Sonika,** and **Mr. Ismail** for their kind help during study period.*

*Friends are the important part of life and without their support this journey would not be completed. My heartful thanks to my dear friends **Poonam** and **Avni** for giving me wonderful memories for life long, that we shared throughout this journey. My special thanks to **Nupur** for being a great friend and helping me out in the odd times. Iam also thankfull to many others: **Gaurav Sharma, Anil, Raman, Shraddha, Sanjay, Himanshi, Kavita, Rakesh, Deepika, Soniya, Rajneesh, Neha kondal, Varun sir, Jibesh sir, Shivani mam, Nutan mam, Swapnil sir, Ira mam, Manali mam, Archit sir, Kirti sir, Swati, Shikha, Neha, Nitin** for their constant support in this journey.*

*This acknowledgement would not be complete without thanking my lab mates for their generous help and support during entire work period. Thanks to **Anil Sharma, Pooja, Pragya, Gaurav Patial, Gorky, Shivani, Wageesha**, **Sakshi, Ashima, Kirti, Priya** and others. I also thank **Mr. Karam Das** (Project Assistant) for his help during the tenure.*

*I cannot thank enough to my family for their unconditional love and support throughout the journey. Their support has been a driving force for me to fight all the tough times. I owe my success to my mother **Mrs. Sunita Verma**, my father **Mr. Raj Kumar Verma** and my adorable sister **Rishika**. I am also thankfull to my grandparents **(Sh. Dunichand Verma, Smt. Indira, Sh. Nandlal Verma and Smt. Chandravati)** for their hearty blessings.*

Ritika

ABSTRACT

In the current study, cellulase free thermo-alkali-stable xylanase was produced through a cost effective method; followed by its immobilization and application in paper pulp bio-bleaching. A potent thermophilic xylanolytic bacteria; identified as *Geobacillus thermodenitrificans* X1 (GTX1) was isolated from Tattapani hot spring soil. The process of xylanase production from GTX1 was optimized by two methods: "one-factor-at-a-time method" (OFAT) and "response surface methodology" (RSM). Highest xylanase titer of 18.5 U/mL was observed after optimization by OFAT, whereas 1.3-fold elevation in the titer (24 ± 2 U/mL) was observed after RSM. For minimizing the production cost, commercial xylan was substituted by lignocellulosic residues. The xylanase titer achieved by utilizing lignocellulosic residues in the present study is highest in comparison to that, reported from other *Geobacillus thermodenitrificans* isolates. The crude GTX1 xylanase was further immobilized to form xylanase cross-linked enzyme aggregates (Xy-CLEAs). Various parameters influencing the preparation of Xy-CLEAs were optimized. GTX1 Xy-CLEAs rendered spherical shape with 202 nm diameter. After immobilization variation in the temperature and pH optima of xylanase was observed. Also, Xy-CLEAs rendered better stability at high temperature (70ºC) and alkaline pH (10) in comparison to free xylanase. The promising commercial potential of Xy-CLEAs was reflected through its reusability for six consecutive cycles with 53.5% retained xylanase activity and storage stability with an efficiency of 86% activity after being stored for 8 weeks at 4 ºC. Immobilization through preparation of Xy-CLEAs was observed to be advantageous in terms of enhanced functional and operational stability. The only loophole in the process was decreased xylanase activity after immobilization. Further, for exploring the potential of GTX1 xylanase as a bio-bleaching agent in the paper industry, crude xylanase was used. Bio-bleaching application of GTX1 xylanase was conducted in R&D laboratory of Kauntum Papers Ltd. situated in District Hoshiarpur, Punjab, India. Xylanase was used at a dosage of 50U/mL/g of agropulp (wheat straw-bagasse) for bio-bleaching. Application of GTX1 xylanase improved the paper properties such as freeness, breaking length and tear factor. The brightness of the paper produced was >80 ISO and there was 20% reduction in the chlorine consumption during the pulp bleaching process after xylanase pretreatment. Thus, GTX1 xylanase proved to be efficient bio-bleaching agent that could potentially cut down the consumption of harsh chemical bleaching agent, without compromising with the paper quality and environment safety.

LIST OF ABBREVIATIONS

NB	Nutrient Broth
NA	Nutrient Agar
BW	Beechwood
SP	Sodium Phosphate
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
ºC	Degree Celsius
Min	Minute
H	Hour
LAF	Laminar Air Flow
Psi	Per Square Inch
w/v	Weight By Volume
v/v	Volume By Volume
mL	Milliliter
G	Gram
Mg	Milligram
g/L	Gram per liter
L	Liter
µL	Microliter
Rpm	Revolutions Per Minute
%	Percent

M	Molar
mM	Millimolar
Nm	Nanometer
rRNA	Ribosomal Ribonucleic Acid
µmol	Micromole
U	Unit
IU	International Unit
NCBI	National Center For Biotechnology Information
BLAST	Basic Local Alignment Search Tool
OFAT	One-Factor-At-A-Time
RSM	Response Surface Methodology
CCD	Central Composite Design
ANOVA	Analysis Of Variance
Fpase	Filter Paper Activity
Cm	Centimeter
FPU	Filter Paper Unit
pNP	Para Nitro-Phenol
pNPC	4-Nitrophenyl-β-D-Cellobioside
pNPG	4-Nitrophenyl-β-D-Glucopyranoside
pNPX	4-Nitrophenyl-β-D-Xylopyranoside
pNPA	α-L-Arabinofuranosidase
HPLC	High Performance Liquid Chromatography
RI	Refractive Index
kDa	Kilodalton

SDS	Sodium Dodecyl Sulfate
PAGE	Polyacrylamide Gel Electrophoresis
GTX1	*Geobacillus Thermodenitrificans* X1
CLEAs	Cross-Linked Enzyme Aggregates
GA	Glutaraldehyde
Xy	Xylanase
DLS	Dynamic Light Scattering
SEM	Scanning Electron Microscopy
FTIR	Fourier-Transform Infrared Spectroscopy
ISO	International Organization For Standardization
R^2	Coefficient Of Determination
CMC	Para Nitro-Phenol
Xos	Xylo-Oligosaccharides
ºSR	Schopper Riegler
m^2	Meter Square
Cps	Centipoise
Kg	Kilogram
IUMB	International Union Of Biochemistry And Molecular Biology
EC	Enzyme Commission
USD	US Dollar
SmF	Submerged Fermentation
SSF	Solid State Fermentation
MTCC	Microbial Type Culture Collection
a.a	Amino Acid

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Xylanases [EC 3.2.1.8] are one of the lignocellulases that specifically act on β-1,4-xylosidic linkage, joining the two xylose moieties and hydrolyze the complex xylan structure [1]. Xylan is the second most abundant lignocellulosic component after cellulose. Thus, action of xylanases can effectively carry out hydrolysis of diverse lignocellulosic residues such as wood and forest residues, agricultural residues and landfill wastes; which constitute xylan as one of their structural ingredient. The diverse sources of xylanases constitute bacteria, fungi, arthropods, protozoa, gastropods and actinomycetes. Among various producers, bacteria and fungi are the leading sources that are exploited on commercial scale for xylanase production [2]. Especially, thermostable xylanases have extensive commercial viability with an approximate annual market to reach USD 500 million by 2023 [3].

The thermophilic microfauna thrive in geothermal areas; hot springs are the most enriched areas, bestowed with diversity of microbial thermophiles [4, 5]. So, far researchers have discovered wide variety of potential thermophiles, which have been exploited for xylanase production. Some of the thermophilic producers have been used in their wild form; whereas some have been genetically evolved to meet the desired commercial requirements. SmF and SSF are the two methods being practiced for xylanase production. However, 90% of the total xylanase production is performed through SmF because there is homogenized distribution of microbial biomass, nutrients and oxygen in the liquid medium used in SmF [6]. The production of xylanases may be intracellular or extracellular in nature [7]. Majority of xylanases being extracellular are excreted outside the cell because the large sized substrate cannot penetrate into the microbial cell. However, current belief regarding the production of xylanase is that it is induced by means of their hydrolysis products produced. Also, extracellular xylanases are commercially acceptable due to easy downstream processing. Xylanase acts on xylan via hydrolytic reaction which involves an acid-base catalyst and a nucleophile for the formation of hydrolysis products through single and double displacement mechanism [1].

For attaining maximum xylanase production from any microbial producer under specific conditions, the influencing parameters need to be optimized. Parameters such as temperature, pH, agitation speed rate, nitrogen sources, nitrogen concentration, substrate concentration etc. have influential impact on production of xylanase. Various optimization methods such as OFAT and statistical approach called RSM are opted for optimizing the influencing parameters for maximum xylanase production. OFAT is a conventional method of

optimization that takes one parameter under observation at a single given time. On the contrary, RSM is statistical method of optimization which is used to study the interactive effect of two parameters at a given time. Literature reports depict that optimization is a prerequisite step for achieving maximum enzyme production [8]. Besides enhancing the production titer of an enzyme, minimizing the cost of production process is equally significant. Especially for large scale enzyme production, cost-effective strategies need to be indulged. Using commercial xylan as substrate for large scale xylanase production does not have economical feasibility. For reducing the production cost, commercial xylan is being replaced by cheaper substrates (lignocellulosic residues) such as corn cob, wheat straw, wheat bran etc. The hemicelluloses fraction of these lignocellulosic residues is composed of majorly xylose monomers, along with small amount of accessory sugars such as arabinose, mannose, galactose and glucose. This valorization of lignocellulosic waste for xylanase production is an eco-friendly and economical approach.

For industrial applicability of xylanase, its characteristic features in terms of pH optima, temperature optima, thermostability and pH stability play significant role. For commercialization, characteristic profile of xylanase needs to comply with requirements of particular industry. Besides pH and temperature profile, storage stability, easy recovery process and reusability are other parameters that have vital contribution in deciding the industrial applicability of xylanase. Enzyme immobilization is an approach to enhance the efficiency of enzymes and elevate their commercial value [9, 10].The reported techniques of immobilization (carrier bound or carrier-free) are comprised of certain loopholes. In adsorption method leakage from carrier material is observed, immobilization through binding to solid-carrier via affinity interactions involves use of recombinant tags which is unsuitable for large scale application [11]. Similarly, binding to carrier material through covalent interactions require surface modifications of carrier, which is an expensive approach. With reference to the above mentioned problems, CLEAs offer easy recovery of immobilized enzyme from the reaction medium by centrifuging or filtering the medium, preparation cost is comparatively low and renders efficient reusability, higher stability profile, easy scale-up and allow low cost preparation by directly using crude enzyme; instead of purified [12]. Also, preparation of CLEAs does not requires carrier or immobilization support, thus they can be fed into industrial reactors in higher amounts as extra space is acquired by the carrier or immobilization support used [13]. Scarce literature is available on preparation of xylanase CLEAs. In recent study by J.S. Hero and coworkers, xylanase CLEAs of *Conhella* sp. AR92

were prepared for their application in lignocellulosic biomass conversion [14]. More research on preparation of xylanase CLEAs is required to explore the potential of this immobilization strategy in lowering the cost and enhancing the applicability xylanase on industrial scale.

Xylanases serve diverse industries; namely food and feed industry, pharmaceutical industry, biofuel industry and paper industry. In food industry, xylanases aid in reducing the viscosity and increasing the clarity of juices, which enhances the juice quality [15]. Addition of xylanases in poultry diet increases the nutritional value and digestibility of feed. Xylooligosaccharides produced by hydrolyzing action of xylanases on xylan; are used as ingredient in many feed products [15]. In biofuel industry, xylanases help in lignocellulosic biomass conversion by hydrolyzing the hemicellulosic component into sugars, which could be further fermented into biofuel [16]. In paper industry, xylanases act on hemicelluloses component of paper pulp and thus breaks the bonds between hemicellulose and lignin. This makes the lignin molecules easily accessible to other bleaching agents for removal [17]. Moreover, it has been reported that use of xylanases for bio-bleaching leads to reduction in consumption of chlorine and also enhance the quality of paper.

The present study was aimed at finding a potential thermo-alkali-stable xylanase for application in paper pulp bleaching. In this context, from literature survey certain research gaps were filtered out:

Research Gaps

- To be applicable in paper industry, xylanases need to be thermo-alkali-stable and cellulase free. Application of xylanases for bio-bleaching in paper industry is limited by their pH stability in narrow range, low thermal stability and high expenditure on production process.
- The xylanases from fungi have associated cellulase activity, with pH optima in neutral or acidic range and are active at mesophilic range temperature. That is why; bacterial xylanases have been in more demand in comparison to fungal xylanases; especially in bleaching of wood pulp.
- Commercial applications require cheaper enzymes. So, cost of the process for enzyme production need to be reduced by developing better fermentation processes and recovery systems.

With respect to the above mentioned research gaps, following objectives were designed for the present study:

Objectives

- Screening of potent thermophilic xylanolytic bacteria from Tattapani hot spring of Himachal Pradesh.
- Production, parametric optimization and characterization of xylanase from *Geobacillus thermodenitrificans* X1.
- Synthesis and characterization of cross-linked enzyme aggregates (CLEAs) of xylanase.
- Application of xylanase for bio-bleaching of wood pulp in paper industry.

REVIEW OF LITERATURE

Alarming concern about the environment sustainability and security has drifted efforts towards substituting the chemical oriented practices with the environment friendly practices. In all the areas of development, there has to be replacement of fossil energy with the green energy. However, an appreciable initiative in this direction started years back when application of enzymes in various industrial processes was introduced. With time, use of enzymes as tremendous biocatalyst has gained momentum and currently they are efficiently driving diverse industrial processes. With an objective of leading to a "green future", researchers have shown keen interest in lignocellulases (cellulases, hemicellulases and lignases) in the past few decades. This class of enzyme plays vital role in valorization of the lignocellulosic residues which are an abundant and renewable source of bio-energy. About 500 Million tons of agricultural waste is generated in India, per year [18]. However, the recalcitrant composition of lignocelluloisc residues is a major hindrance and thus requires potential lignocellulases, for being converted into value-added products. Vast exploitation of lignocellulases has been done so far and is still under progress to overcome the existing research gaps. In this context, the following chapter is focused on xylanases, the type of hemicellulase which breaks down the most common form of hemicellulose i.e. xylan. Currently, thermostable xylanases have specifically grabbed much attention due to its compatability with the industrial conditions. Thus, the chapter includes discussion about the diverse sources of xylanase, its production processes, factors affecting the production of xylanase, immobilization of xylanase and its application in various industries.

2.1 Xylanase definition

Xylanase breaks down the complex backbone of polysaccharide "xylan" to release simple sugars in the form of xylose and xylooligsaccharides. Officially, xylanase is called as endo-1,4-β-xylanase. The nomenclature clearly indicates that xylanase specifically breaks 1,4-β-D-xylosidic bonds present between the xylose monomers of the linear chain of xylan (Fig. 2.1). Originally, xylanases were named as pentosanases when they were reported for the first time in 1955 [19]. Whereas, the enzyme commission number of xylanase (EC number) i.e. EC 3.2.1.8 was assigned in 1961 by the International Union of Biochemistry and Molecular Biology (IUBMB) [1].

2.2 Xylan backbone

Xylan being the major constituent of hemicellulose accounts for about one-third of all renewable organic carbon on earth [20].The backbone of xylan comprises of a linear stretch of xylose units joined together by 1,4-β-xylosidic linkage [Fig. 2.1]. Often, it is accompanied by branching at random distances due to the presence of side chain residues; intervening the continuous xylose stretch. The side chain residues are composed of arabinose, glucuronic acid (GlcA), acetyl and 4-O-methylglucoronic acid [21]. However, there are literature reports that evident plant species having xylan with no side chain residues [22, 23, 24].

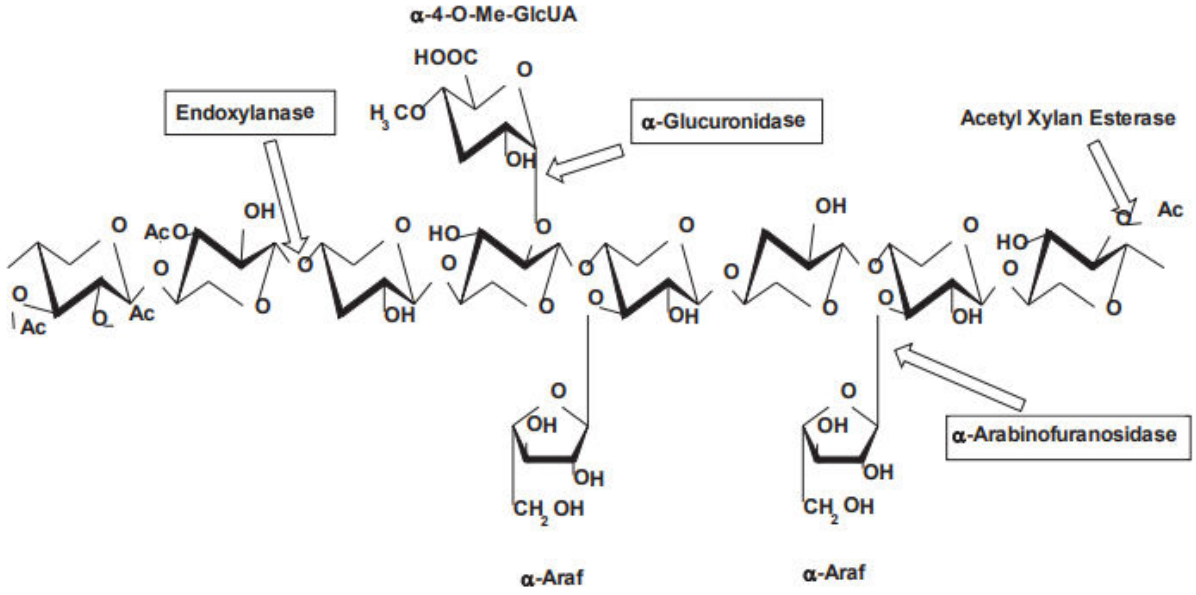
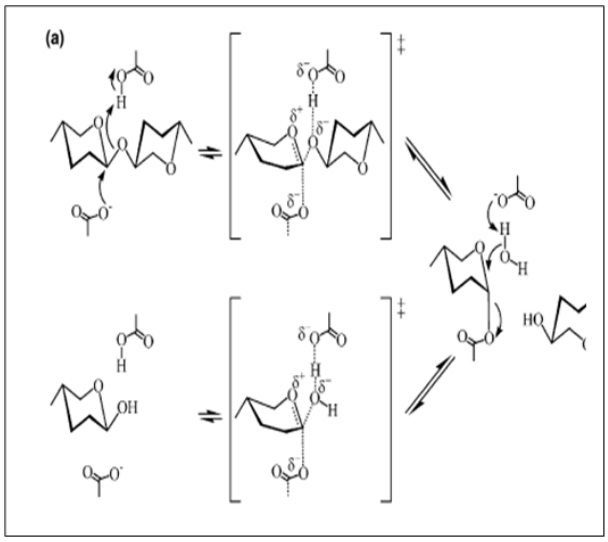


Figure 2.1: Structure of xylan and various xylanolytic enzymes involved in its degradation. Ac: Acetyl group; α-Araf: α-arabinofuranose; α-4-O-Me-GlcA: α-4-O-methylglucuronic acid [25].

2.3 Localization and mode of action of xylanase

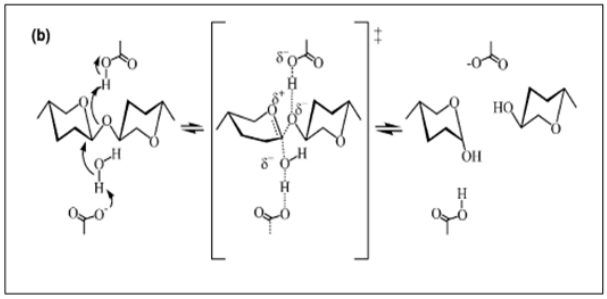
Majority of xylanases are extracellular in nature and are excreted into the environment outside the cell because the large sized substrate cannot penetrate into the cell. However, currently the production of xylanase is believed to be induced by their own products from hydrolysis [7, 25, 26]. Constitutively expressed xylanases generate xylo-oligomers which may be transported into the cell. Inside the cell intracellular xylanases and β-xylosidases conduct further degradation of xylo-oligomers [27, 28], and induction of xylanase synthesis. Mechanism of hydrolytic action of xylanases differs among xylanases from six different glycosyl hydrolase families, namely GH5, GH7, GH8, GH10, GH11 and GH43. Xylanases from families 5, 7, 10 and 11 carry out hydrolysis with retention of configuration at the anomeric center and the two glutamate molecules are the catalytic residues (Fig. 2.2 and

Table 2.1). The other mechanism involves inversion of anomeric center, glutamate and aspartate in catalytic mechanism (Fig. 2.2). This mechanism is followed by xylanases from families 8 and 43 (Table 2.1).



Retention mechanism:

- Double-displacement mechanism which involves formation of a covalent glycosyl-enzyme intermediate and subsequent hydrolysis via oxocarbenium-ion-like transition states.
- Two carboxylic acid residues of the active site are involved in the formation of the intermediate; one acts as a general acid catalyst by protonating the substrate, while the second performs a nucleophilic attack which results in the departure of the leaving group and the formation of the α-glycosyl enzyme intermediate (inversion β to α).
- In second step, the first carboxylate group now functions as a general base, abstracting a proton from a nucleophilic water molecule which attacks the anomeric carbon. This leads to a second substitution in which the anomeric carbon again passes via an oxocarbenium-ion-like transition state to give to a product with the β configuration (inversion α to β).



Inversion mechanism:

- Single displacement reaction in which one carboxylate provides for a general acid-catalyzed leaving group departure and the second functions as a general base, activating a nucleophilic water molecule to attack the anomeric carbon, thereby cleaving the glycosidic bond and leading to an inversion of the configuration at the anomeric carbon.

Figure 2.2: Mechanism of hydrolysis by xylanases (a) retention mechanism (b) inversion mechanism.

2.4 Classification of xylanases

Xylanases have been categorized on the basis of: (a) molecular weight (MW) and isoelectric point (pI) (b) crystal structure and kinetic properties and (c) substrate specificity and product profile [29]. However, complete classification of all the xylanases could not comply with the criteria based on MW and pI. So, later on classification related to structural based and comparative analysis among various catalytic domains was introduced. All the updated information regarding xylanases classification and characteristics is found in the Carbohydrate-Active Enzyme (CAzy) database. This database classifies xylanases into various glycoside hydrolase families namely 5, 7, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51 and 62. Out of these, only families 5, 7, 8, 10, 11 and 43 have fairly distinct catalytic domain with endoxylanase activity [1]. Rest of the families including 16, 51 and 62 have bifunctional nature with two catalytic domains whereas families 9, 12, 26, 30 and 44 may have residual or

secondary xylanase activity. Overall differentiating features of the families 5, 7, 8, 10, 11 and 43 have been discussed in the Table 2.1.

Table 2.1: Characteristics and mechanism of hydrolysis of xylanases classified under different families.

Glycoside Hydrolase (GH) Family	Classifying Characteristics	Hydrolysis mechanism	References
GH 5	Largest glycoside hydrolase family, only seven amino acids including nucleophile and general acid/base residue, are strictly conserved among all members, activity affected by the presence of substituent along the main chain, produces products shorter than those produced by family GH 7.	Retaining	[1]
GH 7	High molecular weight, low pI, small substrate binding sites contains approximately four subsites with catalytic site in the middle.	Retaining	[1]
GH 8	Composed of cellulases, chitosanases, lichenases and endo-1,4-β-xylanases, cold adapted xylanases, hydrolyze xylan to xylobiose and xylotetarose, have large substrate binding clefts, six xylose binding residues with catalytic site in the middle.	Inverting	[1, 30, 31]

GH 10	High molecular mass, low pI, display an (α/β)-8 barrel fold, four to five small substrate binding sites, composed of endo-1,4-β-xylanases and endo-1,3-β-xylanases, also capable of hydrolyzing the aryl β-glycosides of xylobiose and xylotriose at the aglyconic end. Exhibit greater catalytic versitality and lower substrate specificity in comparison to GH 11, activity less hampered by presence of side chains.	Retaining	[1, 26, 31]
GH 11	Low molecular weight and basic pI, small size, high substrate specificity, high catalytic efficiency, wide range of optimum values for pH and temperature, considered as true xylanases as they exclusively act on D-xylose containing substrates, can hydrolyze aryl β-glycosides of xylobiose and xylotriose at the aglyconic end but are inactive on aryl cellobiosides, have larger substrate binding clefts (minimum seven subsites), prefer cleavage of unsubstituted regions, forms consists of two large β-pleated sheets and a single α-helix that forms a structure similar to a partially closed right hand.	Retaining	[32-34]
GH 43	Display five blade β-propeller fold, have glutamate and aspartate as catalytic residues, grouped with family 62 in clan GH-F, also demonstrated in family 8 enzymes.	Inverting	[1, 31]

2.5 Sources of xylanase

Bacteria, actinomycetes, fungi, anthropods, algae, gastropods and protozoa, gastropods and anthropods are the reported xylanase producers [1]. Since, microorganisms have extremely high rates of multiplication and can be easily cultivated under controlled conditions; they are the most preferred source of enzyme producers [28]. Among the diverse microflora, bacteria and fungi are in the limelight [35], however, currently bacteria are the most desired producers of enzyme in various industrial applications. Bacterial sources put forth various advantages over fungal sources such as rapid growth rate and mass production, less space requirement, easy maintenance, and higher accessibility for genetic modifications [36]. Xylanases from bacteria have tremendous potential for industrial application as they render high thermostability and alkaline stability. Bacteria form the genus *Bacillus* and *Geobacillus* have grabbed utmost attention for industrial exploitation as they are potent producers of diverse extracellular enzymes, endowed with thermophilic activities [2, 37, 38].

2.6 Xylanase from extremophiles

Xylanases from mesophilic bacteria and fungi cannot withstand the extreme environments such as high acidity (acidophiles) or alkalinity (alkalophiles), high temperature (thermophiles) or extreme low temperature (psychrophiles) and high salinity (halophiles). Thus, extremophiles are looked upon as a reliable source of xylanases that can combat such extremities [1]. Some of the themostable xylanases produced by thermophilic bacterial and fungal sources have been listed in the Table 2.2.

Table 2.2: Thermostable xylanases from thermophilic bacterial and fungal sources.

Source	Name of microorganism	References
Bacterial	*Geobacillus* sp. WSUCEF1	[39]
	Herbivorax saccinola	[40]
	Herbinix hemicellulosilytica	[41]
	Bacillus oceanisediminis	[42]

	G. stearothermophilus KIBGE-IB29	[4]
	Rhodothermus marinus	[43]
	Geobacillus sp. DUSELR 13	[44]
Fungal	*Clostridium thermocellum*	[45]
	Melanocarpus albomyces	[46]
	T. lanuginosus DSM 10635	[47]
	Humicola insolens	[48]
	Remersonia thermophila	[49]
	Thermoascus aurantiacus	[50]
	Thermomyces lanuginosus	[7]

Industries majorly crave for thermostable xylanases, as the industrial processes are carried out under robust conditions usually not bearable by thermolabile xylanases.

2.7 Isolation of xylanase producing microbial strains

For isolation of potent microbial strains, sample collection is an indispensable crucial step. The relevant site, correct way of sample collection followed by storage (sterile conditions) and safe transportation are vital for efficient sampling. The geographical area being selected for sample collection has relevance to the category of microorganism required. If thermophilic microbial producer is the priority, high temperature areas such as hot springs, mud pots and fumaroles will be best suited for sample collection. Similarly for mesophilic microorganisms, area with an ambient temperature would be preferred. Literature reports evident collection of diversity of sample for isolation of xylanolytic microorganisms which includes soil, water, lignocellulosic residues, cow feed etc. [4, 35, 51]. For selective isolation, enrichment of sample is done to increase the fraction of desired microbial population. For isolation of xylanolytc bacteria, enrichment of the sample is done in a medium which is incorporated with xylan. Sample from the enriched medium is plated on xylan nutrient agar (NA) plates under

specific conditions. Further, the isolates are screened for xylanase activity through Congo red assay and Dnitrosalicylic acid method [52].

2.8 Xylanase production

Xylanase production is carried out by different fermentation methods which can be categorized as SSF and SmF. Both practices have been widely exploited by the researchers for xylanase production. However, 90% of the total xylanase production is performed through SmF because there is homogenized distribution of microbial biomass, nutrients and oxygen in the liquid medium [5, 6]. SmF is advantageous in terms of easy scale up and maintenance of uniform conditions throughout the liquid production medium [53]. On the other hand, SSF has a limitation that it is more appropriate only for production using fungal source as it supports better mycelia growth on the solid substrate. Thus, majorly till date, SmF is preferred over SSF for xylanase production [54, 55]. As per literature reports, several *Bacillus* and *Geobacillus* sp. render xylanase production through SmF [4, 35, 38, 56, 57]. Accordingly in the current research work, xylanase was produced through SmF using *Geobacillus thermodenitrificans* X1 (GTX1).

2.9 Parameters influencing the xylanase production

Besides the method of fermentation, there are several physiological and nutritional parameters that hold great significance in the enzyme production. Factors such as inoculume size, incubation time, temperature, pH, substrate concentration, nitrogen sources/concentration, agitation speed rate and carbon sources were taken under consideration to study their impact on xylanase production. The mentioned factors have also been explored in earlier reports [58, 59]. For efficient and maximum yield of xylanase, these influencing parameters need to be optimized with respect to the production medium.

2.9.1 Inoculum size

The inoculum size defines the initial microbial population used for initiating the xylanase production. It plays a critical role as the population has to be in synergy with available environmental conditions to avoid the depriving of nutrients and oxygen in the production medium. Any deteriorating impact on the microbial population, eventually affects the production of enzyme. Thus using an optimum inoculum size is important for attaining maximum xylanase production. Usually, inoculum size in the range of 1- 2% (v/v) has been

reported to be optimum for xylanase production in the literature reports [60, 61]. Higher inoculum is not preferred because it leads to depletion of nutrients at faster rate and thus minimizes the xylanase yield [62].

2.9.2 Incubation time

The optimum time period required for maximum xylanase production varies among different sources and strains of the microorganisms used for the production. Usually, fungal producers require longer incubation time for xylanase production in comparison to bacterial producers. In recent studies, maximum production of xylanase from *Aspergillus foetidus* through SmF was attained after incubation time of 168 h [58]. However, variations have also been observed among various bacterial producers. *Anoxybacillus kamchatkensis* NASTPD13 has been reported to produce maximum xylanase within an incubation period of 24 h [56] whereas studies by Muhammad Irfan and his coworkers, reported optimum incubation time of 48 h for production of xylanase from *Bacillus subtilis* [63]. Other factors such as feedback inhibition by accumulation of the end product, nutrient depletion and variations in the pH of the production medium have correlation with incubation time [64].

2.9.3 Temperature

Different temperature optima are exhibited by microbial producers for their growth and production of enzymes [4]. This parameter has an important influence on xylanase production and need to be optimized according to the microbial producer used in the production medium. Variations in the temperature affect the microbial protein structure and properties and eventually enzyme production [65]. Temperature below and higher to the optimum, results in decreased xylanase production as the significant metabolic pathways involved in the secretion of xylanase are negatively affected [66]. Earlier literature reports also show variations in the temperature optima for xylanase production among different microorganisms. Optimum temperature for maximal xylanase production from *Geobacillus* sp. Strain DUSELR13 was 60°C. It's been reported that temperature higher to optima is more deteriorating than the lower temperature. That is why, at 70°C, decrease in xylanase production was observed for *Geobacillus* sp. DUSELR13. Temperature beyond the optima could affect the growth of microbial culture by denaturing the essential metabolic enzymes required by cell. Eventually, due to reduced microbial cell growth, enzyme production also gets reduced [44]. Also, at temperature higher than the optimum, membrane proteins associated with the secretion of

extracellular protein are negatively affected. Thus, the production of extracellular enzyme is decreased [67]. Other thermophilc bacteria such as *Anoxybacillus kamchatkensis* NASTPD13 [56] and *Bacillus amyloliquefaciens* [68] have also shown maximum xylanase production at high temperatures of 65°C and 50°C, respectively. On the contrary, being mesophilic bacteria *Bacillus cereus* SAMRC-UFH1 and *Bacillus pumilus* VLK-1, both rendered maximum production of xylanase at 30°C [62, 69].

2.9.4 pH

pH of the production medium vastly affects the enzyme yield because of its impact on the transportation of nutrients and enzymes across the microbial cell membrane, substrate accessibility and synthesis and activity of microbial proteins [70, 71]. The internal environment of the microbial system is influenced by the pH of the external environment comprising of the production medium [4]. Thus, pH is an influential parameter to be taken under consideration while optimizing xylanase production. Literature reports have shown variations in the pH optima for different microbial producers of xylanase. This is because; every microorganism has its specific pH optima for growth, which consequently affects the enzyme production by it. In 2016, Irfan et al. observed impact of initial pH on xylanase production from *Bacillus* sp. (*subtilis* and *megaterium*) , within a range of pH 4-10. For both the *Bacillus* sp. production medium with initial pH 8 rendered maximum production of xylanase [63]. Neutral pH (7) was favored by *Bacillus vallismortis* RSPP-15 for maximum xylanase production [35]. Whereas, acidic pH was found optimum for xylanase production from *Geobacillus* sp. DUSEL13 [44], *Geobacillus stearothermophillus* KIGBE-IB29 [4] and *Bacillus altitudinis* DHN8 [72].

2.9.5 Type of Substrate and its concentration

The lignocellulosic composition of the substrate has a key role to play. Since, xylanase production is induced in the presence of respective substrate (xylan), so the hemicellulosic content of the substrate used should be high enough to induce xylanase production. Diverse carbon sources such as glucose, birchwood xylan, xylose, wheat bran, rice straw, orange peel, wheat straw, sugarcane bagasse etc. have been exploited for xylanase production. However, for cost-effective production of enzyme, birchwood xylan is usually replaced with the cheaper lignocellulosic substrates. There is variation in the hemicellulosic content of various lignocellulosic residues; 26-32% in wheat straw, 23-28% in rice straw, 19-24% in bagasse etc.

Like other parameters, choice of substrates varies among different microbial producers. Birchwood xylan resulted in maximum xylanase production from *Bacillus halodurans* MTCC 9512 [73]. No xylanase production was observed from *Geobacillus stearothermophilus* KIBGE-IB29 when maltose and xylose were fed into the production medium as sole carbon source. This result was attributed to the inducible nature of xylanase [4]. In context of combating the cost constraints of xylanase production, many cost effective lignocellulosic substrates (wheat straw, corn cob, wheat bran etc.) have been used in recent studies [74, 75]. Some pretreatment studies have also been reported to open up the recalcitrant structure of lignocellulosic substrates for increasing the accessibility to microbial culture and thus enhance the xylanase production. In a study by Bandikari and coworkers in 2014, enhanced xylanase production (2,869.8 ± 0.4 IU/g) was achieved from *Trichoderma koeningi* on using pretreated (alkaline pretreatment) corn cob [76]. Whereas, the untreated corn cob showed lower xylanase production with xylanase activity of 1,347.2 ± 0.7 (IU/g). Similarly in another study, sugarcane bagasse was pretreated and used as substrate for xylanase production from *Penicillium echinulatum* 9A02S1. Pretreated bagasse resulted in higher production in comparison to untreated one [77]. However, to avoid usage of chemicals and keep the production cost to minimum, usually mechanically treated biomass is used. Besides the choice of substrate, its concentration is a deciding factor for the amount of carbon and energy supply to the production medium for the synthesis of xylanase. At low substrate concentrations, low xylanase yield is attained due to lack of adequate energy supply for the microbial producers [78]. Whereas, high substrate concentrations decrease the xylanase yield due to substrate inhibition and non-uniform circulation of oxygen and nutrients because of the high viscosity of the medium [64, 79].

2.9.6 Nitrogen sources/concentration

The influence of nitrogen sources and their respective concentrations have also been found to vary among different microorganisms. Nitrogen sources have significant impact as they affect the availability of precursors for the synthesis of proteins required for microbial growth and enzyme production [80]. Nitrogen sources mainly used in the basal medium for xylanase production constitute peptone, beef extract, yeast extract, malt extract, gelatin, casein, urea, sodium and potassium nitrate and ammonium salts [35]. Peptone and yeast extract have growth factors and traces of nitrogen compounds that stimulate production of xylanase and microbial growth [81]. However, variations in the choice of best nitrogen source have been

reported in the literature. Xylanase production from *Bacillus subtilis* sp. BS04 [63] and *Geobacillus thermolevarans* [82] was maximum in presence of tryptone as an organic source, whereas use of tryptone (0.2%) decreased xylanase production from *Geobacillus stearothermophilus* KIBGE-IB29 [4]. Similarly, urea has been observed to show inhibitory action on xylanase production from *Bacillus vallismortis* RSPP-15 [35] and *Bacillus aerophilus* KGJ2 [71] whereas it enhanced the production in case of *Trichoderma harzianum* 1073 D3 [83] and *Aspergillus terreus* [84].

2.9.7 Agitation speed rate

Enzyme production through submerged microbial fermentation is greatly affected by the agitation speed rate. It affects the oxygen mass transfer and uniform mixing of nutrients; such that it is adequately available for microbial growth [72, 85]. An optimal rate of agitation speed is significantly required for maximal xylanase production. At lower agitation, there is improper mixing of components of the production medium and poor oxygen transfer [69] whereas high agitation has been deleterious for xylanase production because of the shearing stress subjected to the microbial cells [86, 87].

2.10 Optimization studies for xylanase production

Optimization studies of various influencing factors is indispensable for attaining maximum xylanase yield from the fermentation process. OFAT is a conventional strategy opted for optimizing the parameters one by one. The method is however, time consuming and tedious, but literature reports that it is reliable in terms of increasing the xylanase yield. Xylanase yield from *Geobacillus* sp. strain DUSELR 13 was increased from 6 to 19.8 U/mL after optimization by OFAT [44]. Similarly, optimization through OFAT resulted in enhanced xylanase yield from 402 to 4986 U/mL during production from *Bacillus pumilus* VLK-1 [62]. Other than OFAT, statistical optimization through RSM is practiced for enhancing the enzyme yield and analyze interactions between two factors. Usually OFAT is initially performed to screen the significant parameters and then followed by statistical analysis of those selected parameters. By performing RSM, one can study large number of parameters with minimum number of experimental trials and also saves time and resources. In comparison to OFAT, RSM resulted in 3.7 fold increases in the xylanase production from *Bacillus tequilensis* strain ARMATI [88]. Similarly, the xylanase yield increased from 19 to 31 U/mL after RSM optimization in case of *Geobacillus* sp. strain DUSELR 13 [44]. The two

designs namely Central Composite Design (CCD) and Box-Bhenken Design (BBD) are practiced under the category of RSM [59, 89].

2.11 Purification and gene expression of xylanase

Purification is an important aspect to be considered for commercialization of an enzyme. However, it is required as per its eventual application on large scale. Food industries and pharma industries seek higly purified xylanases while industrial processes of paper pulp bleaching and biomass conversion prefer the crude form of xylanase. Various techniques such as ammonium sulphate precipitation, ion exchange chromatography, gel permeation chromatography and utrafilteration have been employed for purification of xylanases [90] [53].

Xylanase gene expression through recombinant DNA technology is significant in enhancing the xylanase production and its hydrolytic activity. The technology leads to strain improvement such that the recombinant strain has better functional properties in comparison to the native one [8]. Through these molecular techniques, xylanases with the desired industrial traits could be produced. Xylanase genes from different microorganisms vary in product length. XlnA gene from *Bacillus circulans* constitutes 185 amino acid (a.a) length whereas that from *Pseudoalteromonas haloplanktis* is of 405 a.a [91]. Many research reports have presented the expression of xylanase gene; both homologous and heterologous gene expression. *E. coli*, Yeast, *Lactobacillus* sp. and *Bacillus* sp. have been reported as efficient heterologous hosts for the expression of xylanase gene. These hosts are generally regarded as safe (GRAS); which makes them a reliable option [65]. *E.coli* has been used as an expression host for many bacterial and fungal xylanases from *Bacillus brevis* [92], *Bacillus pumilus* [93], *Aspergillus niger* [94], *Neocallimastix sp.* GMLF7 [95] etc. However, xylanases expressed in *E. coli* tend to have lower specific activity and stability [96]. In this context, *Bacillus* sp and *Lactobacillus* sp. are reliable alternatives for higher xylanase production [97]. Moreover, yeasts have ability of growing in high cell densities and secreting the protein extracellularly in fermentation medium; which makes them an attractive host too. *Pichia pastoris* is known as an efficient host for xylanase production on commercial scale because it exhibits very high expression of enzyme under its own specific promoter [98]. High xylanase activity of 1760 U/mL was attained on extracellular expression of xylanase II gene from *Aspergillus usamii* in *Pichia pastoris* [99]. Similarly, xylanase gene PoXyn2 from *Penicillium occitanis* Pol6 was expressed in *P. pastoris* [100].

2.12 Characterization of xylanase

The characteristic features of an enzyme justify its potential for industrial exploitation. For characterization, various parameters such as optimum pH and temperature, thermostability, pH stability, metal ion effect, isoelectric point, molecular weight, Km/Vmax and solvent tolerance are determined. Xylanases from various sources and their respective characteristics have been summarized in the Table 2.3.

Table 2.3: Biochemical characterization of xylanases from different sources.

Organism	Xylanase activity (U/mL)	Optimum pH/ temperature	Km/Vmax (mg/mL)/ (µM/min/ mg)	Molecular weight (kDa)	References
Bacillus amyloliquefaciens	42.5	9/50°C	5.6/433	50	[68]
Geobacillus thermodenitrificans C5	994.5	6/60°C	NA	~44	[102]
Geobacillus thermodenitrificans TSSA1	2.8	7.5/70°C	2.85/45.45	43	[57]
Melanocarpus albomyces	415	6.6/65°C	0.30/311	38	[46]
Malbranchea flava MTCC 4889	164	9.0/70°C	1.25/1666	25.2	[103]
Aspergillus fumigates R1	208	5/50°C	11.66/87.6	24.5	[104]
Bacterium-OKH	28.14	9/37°C	4.2/0.31	55	[105]
Penicillium chrysogenum	4.56	6.5/45°C	2.3/731.8	22.1	[106]

For estimating molecular weight of xylanases; techniques such as SDS-PAGE and PAGE-Zymography are being used [44, 63]. Metal ions also influence the activity of xylanase; either inhibitory or stimulating (co-factors). In 2018, Bibra et al reported increased xylanase activity under the influence of metal ions Cu^{+2}, Zn^{+2}, K^{+}, Fe^{+2}, Ca^{+2}, Mg^{+2}, and Na^{+} [44]. Similarly, activity of xylanase from *Bacillus amyloliquefaciens* also increased in presence of Ca^{+2}, Mn^{+2}

and Fe^{+2} [68]. Whereas some studies reported strong inhibition of xylanase activity by metal ions such as Mn^{+2}, Hg^{+2}, Fe^{+2} and Pb^{+2} [101]. Hydrolysis profile of xylanases has also been studied by many researchers to estimate the biomass conversion efficiency of xylanase. For this, end products (xylose and xylooligosaccharides) from substrates hydrolyzed by xylanase have been analyzed [39, 57].

2.13 Immobilization of xylanase

The application of enzymes at industrial scale is restricted by factors such as lack of operational and functional stability, tedious recovery and poor reusability [107]. Immobilization of enzyme has appeared as a reliable method to overcome the limitations confronted for industrial application of enzymes. On the same line, for increasing the commercial and industrial viability of xylanases, various kinds of immobilization methods (carrier bound or carrier-free) have been followed in the recent years for the immobilizing xylanases. In 2006, Pal and coworkers immobilized xylanase from *Aspergillus terreus* using barium alginate entrapment method [108]. Similarly, Chang and co-workers reported entrapment of xylanase as an efficient immobilization method to enhance its efficiency for industrial applications [109]. Also, there are literature studies reporting immobilization of xylanase on supports such as Eudragit TM L-100, Eudragit L-100, Eudragit S-100, polysulfone membranes, amine active poly (ethylene glycol) monoacrylate (PEGMA) and many other matrices [110-113]. Kapoor and coworkers carried out comparative studies among different methods for immobilization of xylanase. They practiced physical adsorption on chitin, entrapment using gelatin, covalent binding by HP-20 beads and ionic binding with Q-sepharose [114]. However, certain disadvantages are also associated with the immobilization methods such as leakage from the carrier material used for adsorption, poor interactions between the reactive groups of carrier support and enzymes, high expenses of modifications of the carrier support etc. [11, 115]. Many of these limitations have been overcome by preparation of xylanase cross-linked enzyme aggregates (CLEAs).

2.14 Xylanase CLEAs (Xy-CLEAs)

Application of CLEAs for industrial applications is a cost-effective approach as the process of their preparation is very simple, could be prepared by directly using the crude form of enzyme and do not occupy much space in the reactors, which is otherwise being occupied by the immobilization support [116]. Similarly, for enhancing the efficiency of xylanases in

industrial processes like biomasss conversion, xylo-oligosaccharide production and paper pulp bleaching, preparation of Xy-CLEAs have been practiced by many researchers. In recent study, J.S. Hero and coworkers, prepared cross-linked aggregates of xylanase and exploited it for the bioconversion of lignocellulosic biomass [14]. For the similar application, another group reported preparation of Magnetic-CLEAs (M-CLEAs) and Calcium-magnetic CLEAs of xylanase [117]. In 2015, Bhattacharya and Pletschke, synthesized combi-CLEAs of xylanase-mannase for its application in carrying out hydrolysis of lignocellulosic biomass [118]. The concept of modifications in CLEAs such as M-CLEAs and porous CLEAs (p-CLEAs) is attributed to provide ease of separation of CLEAs and overcome mass-transfer limitations, respectively. Through magnetic decantation, there is easy separation of M-CLEAs and also it prevents squeezing of CLEAs which promotes better mass transfer [12, 119]. Despite various advantages offered by CLEAs certain challenges are faced while using them. The size of the aggregates formed cannot be controlled, desired stability and flexibility cannot be achieved. Majorly, it is the loss in activity that has been observed after cross-linking [120]. Structural alterations after cross-linking have been considered as the reason for affecting the activity of the enzyme. These alterations are not under control; rather use of some additives as Bovine Serum albumin, cations (sodium and calcium ions), surfactants and ionic polymers has been reported as an alternative to overcome the activity loss [121-124].

2.15 Preparation of Xylanase CLEAs

Two main steps are involved in the preparation of CLEAs as shown in Fig. 2.3, (a) Formation of enzyme aggregates through precipitation ;using precipitating agents such as salts, organic solvents etc. (b) Formation of CLEAs with the aid of cross-linker (bifunctional: glutaraldehyde or multifunctional: dextran polyaldehyde) [125]. The cross linkers react with amino groups on the enzyme surface. Various factors play significant role in the preparation of CLEAs. That is why; optimization of such factors is a prerequisite necessity to synthesize CLEAs with the desired efficiency.

2.16 Factors affecting CLEA preparation

Various factors affecting the preparation of CLEAs include cross-linker, precipitating agent, cross-linking time, concentration of precipitating agent, protein concentration etc. In order to have maximum CLEA recovery for the respective enzyme, optimization of the above mentioned factors is an indispensable step in the process of CLEA preparation. These factors

eventually influence the characteristics and functional properties of CLEAs formed. These significant factors and their impact on CLEA preparation has been discussed as follows:

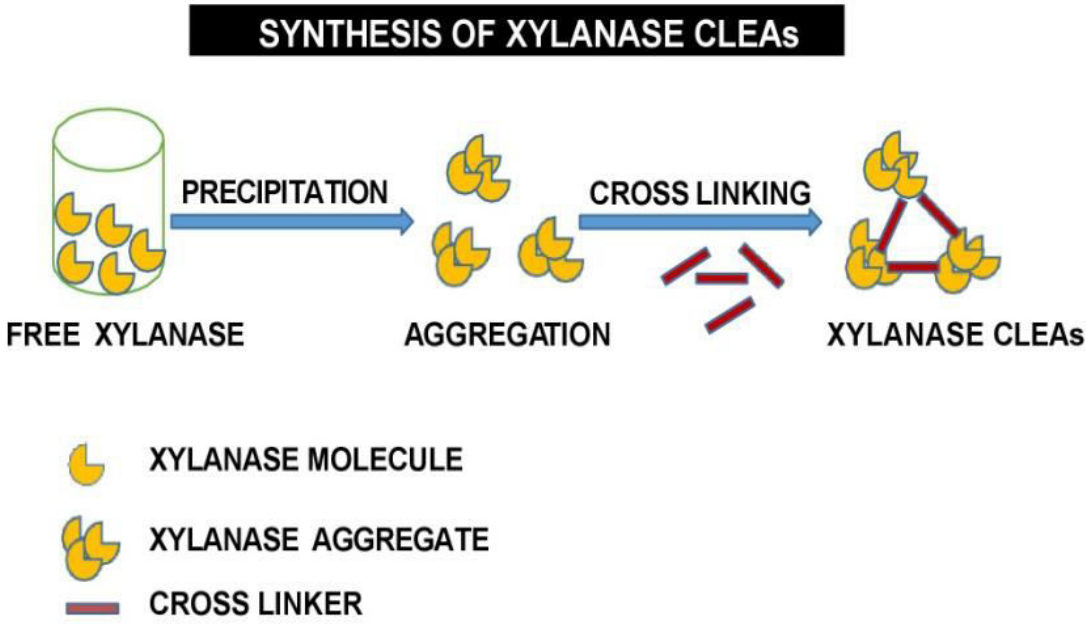


Figure 2.3: Synthesis of xylanase Cross-linked Enzyme Aggregates (CLEAs) by carrier free immobilization using cross-linker.

2.16.1 Type of precipitating agent and its concentration

The type of precipitant being used for precipitation of enzyme, affects the catalytic activity of CLEAs. Different precipitants induce the conformational alterations in the protein structure of the enzyme aggregates in a different manner [126]. That is why, for every enzyme, different precipitants need to be evaluated for their consequences on the catalytic activity. For evaluating the type of precipitant, the aggregates formed by it are assayed for the catalytic activity after being re-dissolved in the buffer [127]. Various precipitating agents used are organic solvents (acetone, isopropanol, ethanol, acetonitrile etc.), non-ionic polymers (poly-ethylene glycol) and neutral salts (ammonium sulphate) [125, 128]. For the synthesis of Xylanase CLEAs, precipitation by ammonium sulphate resulted in better activity recovery in comparison to isopropanol [14]. In another study, highest activity was retained with acetone after precipitation but after cross-linking, eventually the CLEAs formed by xylanase aggregates precipitated by ammonium sulphate showed better results [118]. The addition of precipitants does not harm the tertiary structure of protein and form aggregated supramolecular structures through non covalent bonding. The aggregation and precipitation

by the precipitant is induced by changing the hydration state of the enzyme molecule or creating alterations in the dielectric constant of the solution [129]. From commercial point of view, precipitant used should be inexpensive and should not react with the enzyme or buffer used for synthesis of CLEAs. Furthermore, the concentration of precipitant is also impactful for maximum CLEA recovery. Literature reports that a high concentration of precipitant promotes maximum retention of CLEA activity. This is because at high concentrations, precipitation rate is so fast that that chances of denaturation of enzyme gets fairly reduced due to the competition between denaturation and aggregation of enzyme molecules [130]. Increase in the retention of CLEA activity with increasing concentration of ammonium sulphate has been observed during immobilization of lipase [131], invertase [132] and acetyl xylan esterase [133]. Similar effect was observed on increasing concentration of acetone and PEG600 for lipase [134] and lipozyme [135], respectively.

2.16.2 Type of cross-linker and its concentration

The cross-linker creates a network between the enzyme aggregates by covalently binding to the lysine residues present on the enzyme surface. The bifunctional cross-linker glutaraldehyde is most commonly used for cross-linking due to its eaem of availability on large scale and low price. Cross-linking using glutaraldehyde as cross-linker occurs via schiff's base recation [125, 136]. However, complete loss of catalytic activity after cross-linking with glutaraldehyde has been observed for certain immobilized enzymes. The small size of glutaraldehyde renders its easy penetration into the internal structure of enzyme molecules, where it may react with the catalytically essential amino residues. Binding of the cross-linker to such amino residues, eventually has negative impact on the catalytic activity of CLEAs. Using large size cross-linker instead, such as dextran-polyaldehyde has been reported as an alternative. For cross-linking of nitrilases aggregates, Mateo et al reported 10-90 times higher activity with polyaldehyde as cross-linker in comparison to glutaraldehyde [137]. Similarly, β-mannanase CLEAs formed using dextran-polyaldehyde showed 16 times higher activity than those prepared using glutaraldehyde as cross-linker [138]. Besides this, other cross-linkers such as *p*-benzoquinone, polyethylenimines and L-lysine have also been explored in some studies for the preparation CLEAs [139-141]. However, for attaining maximum activity in CLEAs, optimization of the cross-linker concentration is equally important as the choice of cross-linker. Very low concentration may not be sufficient to form CLEAs while at very high concentration of cross-linker there is loss of flexibility of enzyme

structure which minimizes the catalytic activity of CLEAs. Due to the rigid protein structure, the substrate molecules could not enter through the CLEAs and react with the catalytically active amino residues on the enzyme surface [142-144]. The required amount of particular cross-linker varies according to the available amino residues for cross-linking on the enzyme surface. Study by Bhattacharya and coworkers found optimum concentration of glutaraldehyde to be 150 mM for preparation of X6-CLEAs and 100mM for X7-CLEAs [118].

2.16.3 Cross-linking pH, time and temperature

Maximum cross-linking is achieved at an optimum pH, as it depends on the acid-dissociation constant of the lysine residues present on the enzyme surface and also on the reactivity of glutaraldehyde at a particular pH. Generally glutaraldehyde has been found to show highest reactivity and thus maximum CLEA recovery at around neutral or slightly alkaline pH [145]. Also, glutarladehyde exists in either monomeric or polymeric form in accordance with the pH and also results in different end product, respectively [136]. The duration of cross-linking is also significant for the efficient recovery and activity of CLEAs. Cross-linking reaction conducted for very short period leads to inadequate cross-linking which causes operational and functional loss of CLEAs recovered. Whereas, prolonged cross-linking is also detrimental as it causes rigidity in the structure which results in reduced or complete loss of activity [107]. Some studies report that on increasing cross-linking time, an increased thermostability and reusability of CLEAs was observed but at the cost of great activity loss (50-100%) [146]. Another significant parameter that influences the recovery of CLEAs is temperature during cross-linking. Xylanase-mannanase combi-CLEAs were prepared at 37°C [118] whereas, Combi-CLEAs of glucose oxidase-versatile peroxidase [147] and lipase-protease [148] were prepared at 30°C and 4°C, respectively. Usually low temperature (4°C) is preferably the choice during cross-linking to prevent the denaturation of enzyme due to high temperature [149, 150]. However, at very low temperature it may require long cross-linking time.

2.16.4 Protein concentration and additives

Protein concentration directly affects the amount of lysine residues available for efficient cross-linking. If the concentration is too low, the cross-linked network would not be formed by the cross-linker due to lack of sufficient binding to the amino residues. Whereas very high concentration results in activity loss of enzyme aggregates due to lack of structural mobility,

caused by overcrowding of the enzyme molecules [151]. When there is scarcity of sufficient lysine residues for cross-linking, certain additives such as BSA, surfactants and ionic polymers are used to cause co-aggregation and increase the number of available lysine residues. Also these additives protect the enzyme active sites from the deteriorating effects of cross-linkers. Lipase CLEAs with BSA as an additive retained 100% activity in comparison to that without BSA, which retained only 0.4% activity [121]. Similar, results were found for preparation of laccase and amino-acylase in presence of the additive [152, 153]. However, the catalytic activity of CLEAs is not significantly improved after using additives, as observed in case of amylase CLEAs prepared alongwith BSA [154].

2.17 Morphology and characteristics of Xy-CLEAs

Morphology and size of CLEAs has significant impact on the mass transfer and tolerance towards shear stress [155, 156]. Xy-CLEAs reported in literature are known to exhibit both type I (~ 1µm diameter) and type II (< 0.1µm diameter) morphology [14, 117]. Xy-CLEAs from *Conhella* sp. AR92 rendered two sizes of CLEAs; clustered aggregates of CLEAs with 300-400 nm diameter as well as detached spherical structures with 100-200 nm diameter. Also some exposed cavities were observed in the close packed structure of Xy-CLEAs after SEM analysis [14]. Such cavities have been reported to be beneficial in increasing surface area and facilitating better accessibility to substrate molecules [157]. So far the literature survey depicts that the immobilization of xylanase in the form of CLEAs have characteristic improvement in terms of thermostability, reusability and storage stability. CLEAs of xylanase from *Conhella* sp. AR92 showed 25% increase in relative activity at 50°C in comparison to free xylanase [14]. Similarly, half life of xylanase activity in a commercial preparation (Pectinex™ Ultra SP-L) was increased to 82 min from 22 min at 60°C after CLEAs formation [158]. Mag-CLEAs and calcium-mag-CLEAs from *Bacillus geletanii* ABBP-1 rendered 1.35 fold increased xylanase activity as compared to free xylanase. Also the half lives were increased from 52 h (free xylanase) to 136 h, 214 h and 310 h for CLEAs, mag-CLEAs and calcium-mag-CLEAs, respectively [117]. Reusability is another characteristic which undergoes tremendous improvement after CLEA formation. Xylanase CLEAs from *Conhella* sp. AR92 exhibited 50 % relative activity after five consecutive cycles. Whereas, 100% xylanase activity was observed for CLEAs from Pectinex™ Ultra SP-L after three consecutive cycles [14, 158].

2.18 Industrial applications of xylanases

Xylanases serve diverse industries including the sectors related to paper, food, feed, biofuel and pharmaceutical (Fig. 2.4).

Figure 2.4: Application of xylanases in different industries.

Several xylanases have been commercialized and are applied in various industrial processes. Some of the commercial xylanases and their respective producers have been summarized in Table 2.4.

Figure 2.4: Application of xylanases in different industries.

Commercial Xylanase	Producer	Application
Bleachzyme F	Bicon, India	Paper industry
Pulpzyme	Novozymes, Denmark	Paper industry
Ecopulp	Rohn Enzyme 0Y, Primlaco, Finland	Paper industry
Ecozyme	Thomas Swan, UK	Paper industry

Rholase 7118	Rohm, Germany	Food industry
Sanzyme X	Sankyo, Japan	Food industry
Ceremix	Novo Nordisk, Denmark	Food industry
Multifect XL	Genencor, Netherlands	Food industry
Ecosane	Biotech, Thailand	Feed industry
Biofeed	Novo Nordisk, Denmark	Feed industry
Allzym PT	Alltech, America	Feed industry
Amano 90	Amano, Japan	Pharmaceutical industry

However, researchers are still looking for xylanases with better functionality and stability, with respect to the robust industrial processes. Moreover, efforts are being made to lower down the production cost of enzyme to make their applications on large scale; an economical one.

2.18.1 Food industry

Xylanases are used for clarification of juice in combination with amylases, pectinases and cellulases. They hydrolyze the turbidity causing substances and reduce the viscosity for clearing the juices [6]. In bakery, they are used for increasing the bread volume and softness of the dough by breaking the hemicellulose content of wheat flour [159]. They also improve the nutritional properties of food and prevent starch retrogradation [160]. Cereals containing arabinoxylans are given xylanase pretreatment to reduce the viscosity of their water extract. This avoids problems of slow filtration rate and haze formation during brewing [161]. Xylanses are also applied for separating gluten from wheat starch [162].

2.18.2 Feed industry

Xylanases are used in feed industry to increase the nutritional value of feed. Addition of xylanases in poultry diet is helpful in increasing the feed conversion efficiency and weight gain of the chicks [15]. Xylooligosaccharides (XOs) produced from hydrolyzed xylan are used in many feed products [163]. Improvement in absorption of nitrogen and fiber and food

transmit time has been reported by Babalola and coworkers [164]. Usually, xylanase is used in combination with phytase and β-glucanase as poultry diet supplement.

2.18.3 Pharmaceutical industry

Xylanases are helpful in improving health due to their prebiotic effects. They stimulate the growth and activity of beneficial microflora inhabiting the colon. XOs produced by xylanases have numerous pharmaceutical applications. Micro or nanoparticles for drug delivery are prepared from XOs [165]. Likewise XOs have many other properties which constitutes of anti-cancerous and anti-microbial effect, growth regulation, immunomodulation, antioxidant, ant-allergic, anti- inflammatory and anti-hyperlipidaemic activity etc [166].

2.18.4 Biofuel industry

For generation of second generation biofuels, efficient recovery of fermentabe sugars from the lignocellulosic biomass is big challenge. Lignocellulases play significant role in bioconversion of the lignocellulosic biomass for biofuel production. Xylanases in combination with other hydrolytic enzymes (cellulases and laccases) are used in the biomass conversion process [29, 167]. After delignification, xylanases hydrolyze the hemicelluloses content and increase the porosity and swelling of the lignocellulosic biomass. This makes the cellulose content easily accessible to cellulases for conversion into sugars and further fermentation into ethanol (biofuel) [16].

2.18.5 Paper industry

Application of xylanases for bio-bleaching of pulp has significnatly minimized the usage of chemical bleaching agents in the the paper industry. Consequently, the chemical load (hazardous chlorine derivatives) on the effluents being released into the environment has also reduced [17]. Xylanases are mainly used for their pre bleaching effect, which means that they are not directly involved in the removal of lignin form the pulp. Schematic presentation of xylanase application as bio-bleaching agent has been shown in Fig. 2.5. The residual lignin is responsible for imparting the brown color to the pulp. Thus its maximum removal is necessary for achieving a quality paper with satisfactory brightness and whiteness [168]. Xylanases work by hydrolyzing the xylan and thus breaking its linkage with the lignin. The free lignin is thus easily accessible for the other bleaching agents to act upon and remove it. However, xylanases to be used in pulp bleaching processs must comply with certain

charactersistics such as thermotability, alkaline stabilty and cellulase free. Redution in chlorine dosage after biobleaching of pulp using xylanase has been reported in the litertaure. Pretreatment of wheat straw pulp by xylanase from *C. cellulans* CKMX1 resulted in 12.5% chlorine reduction [8].

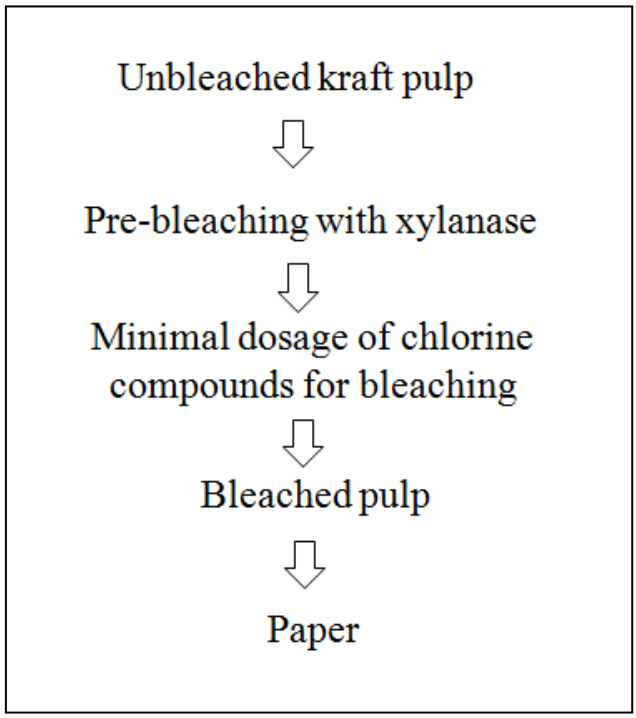


Figure 2.5: Schematic presentation of xylanase application in bio-bleaching of pulp during paper manufacturing.

Similalry, 16% chlorine reduction was observed after bio-bleaching of pulp using xylanase from *Thielaviopsis basicola* [169]. Besides chlorine reduction bio-bleaching of paper pulp with xylanase also has impact on the strength properties of the paper generated. Properties such as tear factor, burst index, breking length, freeness etc. decide paper quality. The impact of xylanase pretreatment on paper varies; depending on the dosage and quality of pulp used for paper making. Usually, pretreatment with xylanase either enhances or has neutral effect on the paper properties. Decreae in the strength properties of paper after xylanase pretreatment is only in the case where xylanase has associated cellulase activity. This implies that the xylanase to be used for bio-bleaching necessarily has to be cellulase free, to prevent deterioration of the cellulosic structural integrity of paper. Thus, cellulase free thermo-alkali-stable xylaanases are in great demand by the paper industry, as it is an eco-friendly approach and has promising potential to completely substitute the chemical bleaching agents. In context of such industrial applications of xylanase, efforst are being made to screen efficient sources

of xylanases for maximum xylanase production in cost-effectiev manner. This will elevate the viability of xylanases to be used on large scale.

MATERIALS AND METHODS

3.1 Media

3.1.1 Nutrient broth (NB)

Peptone : 0.5%

NaCl : 0.5%

Meat Extract : 0.15%

Yeast Extract : 0.15%

pH : 7.0 ± 0.2

3.1.2 Minimal media

Table 3.1: Composition of minimal media for xylanase production.

Minimal Media (per liter)	
K_2HPO_4	1.5 g
KH_2PO_4	3.0 g
$MgSO_4.7\,H_2O$	0.3 g
$CaCO_3.2\,H_2O$	0.05 g
NaCl	0.5 g
$NaHCO_3$	0.5 g
NH_4Cl	1.0 g
$FeSO_4.7H_2O$	1mg
NH_4Fe(III) citrate	10 mg
$MnSO_4.H_2O$	5 mg
$CoCl_2.6H_2O$	1mg
$ZnSO_4.7H_2O$	1 mg
$CuSO_4.5H_2O$	0.1 mg
H_3BO_4	0.1 mg
$Na_2MoO_4.2H_2O$	0.1 mg
$Na_2SeO_3.5H_2O$	0.1 mg
Nicotinic acid	2.5mg

Cyanocobalamin	2.5mg
p-aminobenzoic acid	2.5mg
Calcium pantothenate	2.5mg
Thiamine-hydrochloride	2.5mg
Riboflavin	2.5mg
Lipoic acid	2.5mg
Folic acid	0.1 mg
Biotin	0.1 mg
Pyridoxine-hydrochloride	0.1 mg
Yeast extract	5 mg
L-cystein	0.1 g

3.2 Chemicals, reagents, glassware and plastic ware

Analytical grade chemicals and reagents employed in the study were obtained from Hi-Media, Sigma-Aldrich, and Merck. Molecular grade chemicals and products were procured from Promega, and Thermo Scientific. The glassware used was purchased from Borosil, and the plasticware was purchased from Eppendorf and Tarsons.

3.3 Sterilization

Glasswares were sterilized at a temperature of 180ºC for one hour using hot air oven. All the culturing and production media were sterilized through autoclaving at 121°C, 15 psi for 20 min (minutes). All the microbiological techniques such as like culturing, inoculation, sampling etc., were performed under aseptic conditions using laminar air flow (LAF) chamber.

3.4 Isolation and screening of xylanolytic bacteria

3.4.1 Sampling and collection site

Hot springs are known to harbor diversity of potent thermophiles. Thus, in the present study Tattapni hot spring situated in Districtt. Mandi, Himachal Pradesh, India (Fig. 3.1) was selected as the site for sample collection. Soil sample was collected in sterile container and sealed. The sample was then stored at 4°C in laboratory before further processing.

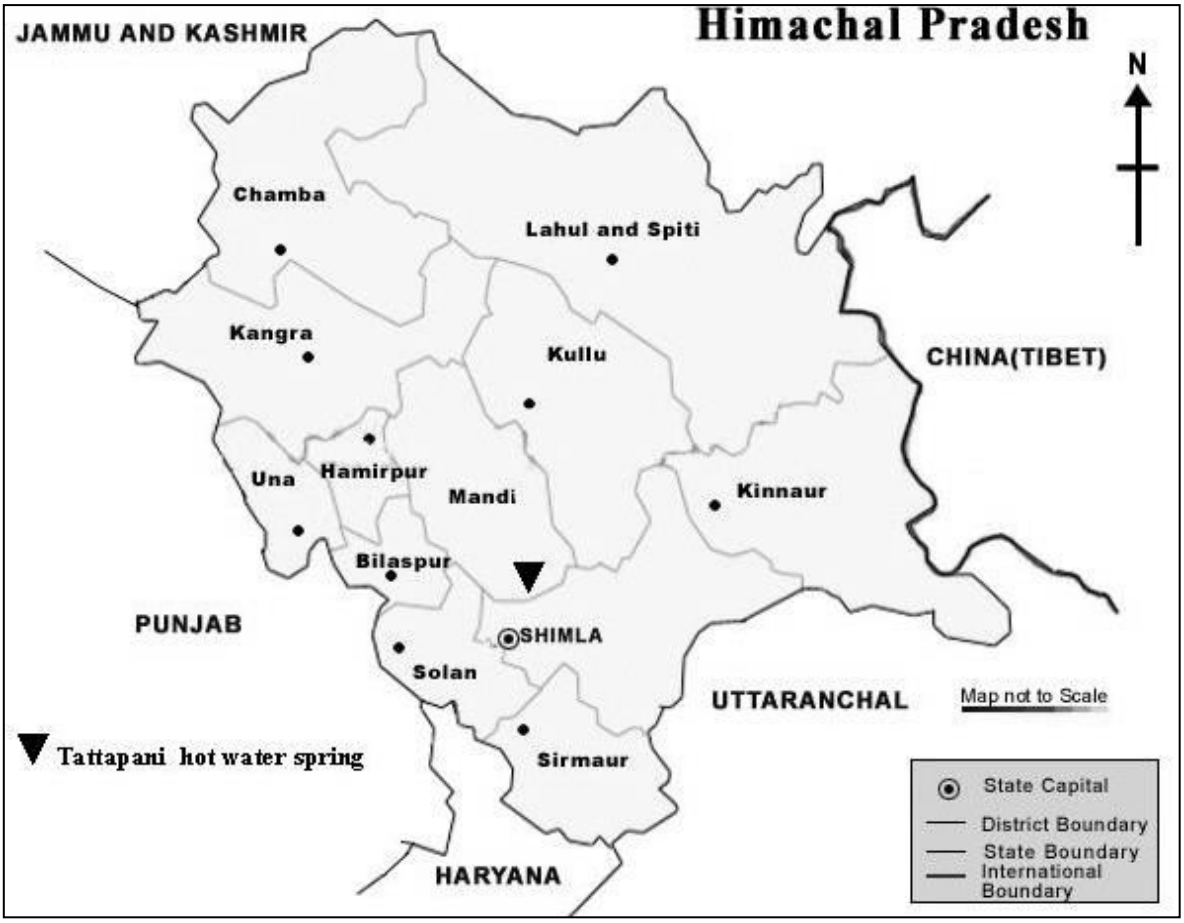


Figure 3.1: Location of Tattapani hot spring [5].

3.4.2 Media enrichment and isolation of thermophilic xylanolytic bacteria

For isolation of thermophilic xylanolytic bacteria from the hot spring soil sample; an enrichment culture technique was followed. For enrichment, one gram soil sample was inoculated into 100 mL minimal media (pH 7) contained in an Erlenmeyer flask. BW xylan (BW) at a concentration of 0.1%, w/v was an additional component in the minimal media; provided as the carbon source. The flask with enrichment culture was incubated at 60°C and 150 rpm of agitation rate. After 72 h, 1 mL of the enriched culture was inoculated into another flask with same media and incubation was carried out under same conditions for second enrichment. In similar way, four such sequential transfers were done to eventually get the culture enriched with xylanolytic thermophiles (Fig. 3.2). From the final enrichment flask, 1mL of culture was taken and serially diluted with distilled water. Different dilutions were spread on NA plates containing 0.1% BW xylan. The xylan NA plates were then kept overnight at 60°C and the thermophilic bacterial colonies formed were observed next day.

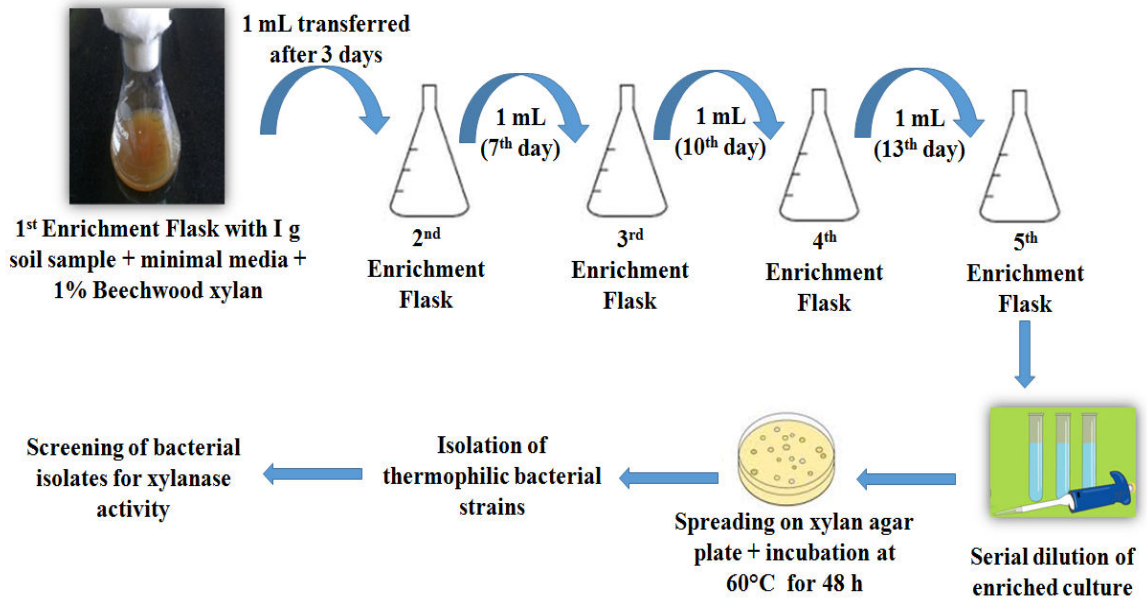


Figure 3.2: Diagrammatic representation of enrichment and isolation of thermophilic xylanolytic bacterial strains from hot spring soil sample.

3.4.3 Screening of thermophilic xylanolytic bacteria

The diverse thermophilic bacterial population obtained after the enrichment technique was screened for the potent strains with xylanase activity. For this primary and secondary screening was performed as follows:

3.4.3.1 Primary screening

It was done through Congo Red Assay [170]. Cultures of different thermophilic bacterial strains were grown in NB at 60°C with continuous agitation at 150 rpm. After 24 h, each culture was centrifuged and supernatant was collected. Wells were punctured on xylan NA plates containing 0.5% (w/v) BW xylan and filled with supernatant of different bacterial strains. The plates were then incubated for 48 h at 60°C; followed by staining with Congo red solution (0.1% Congo red dye in distilled water) and 15 min incubation at room temperature. Afterwards, plates were destained by flooding them with 1M NaCl and intermittent manual shaking. The washing with NaCl was ceased after the zone of hydrolysis was visible.

3.4.3.2 Secondary screening

BACTERIAL cultures were grown in minimal medium (pH 7; 0.1% w/v BW xylan) at 60°C with agitation at 150 rpm. After 48 h, each bacterial culture was centrifuged (10,000 rpm/10 min) to get supernatant for xylanase activity assay.

3.5 Xylanase activity assay

For activity assay bacterial culture supernatant was used as crude enzyme and 0.5% w/v BW xylan in SP buffer (100 mM, pH 7) was used as substrate. Reaction mixture of substrate (0.5 mL) and enzyme (0.5 mL) was given incubation in water bath for 5 min 70°C [171]. After incubation, the reaction was halted by adding 1.5 mL DNS and 10 min boiling. Afterwards the samples were cooled on ice for stabilization of the color produced. The optical absorbance of the xylose sugar produced was read at 540 nm. Xylanase unit activity was defined as "the amount of enzyme required to release 1 μmol of xylose-equivalent reducing groups per min under assay conditions".

3.6 Identification of xylanolytic bacterial isolate

Morphological characterization was done through Gram staining and KOH string test. To reveal the species identity, molecular characterization was practiced through 16S rRNA sequencing. For 16S rRNA gene amplification, genomic DNA of the isolate was extracted using PROMEGA DNA isolation kit. Universal primers 27F (5′-AGA GTT TGATCMTGG CTCAG, where M is A or C) and 1492R (5-ACC TTG TTA CGA CTT) were used for amplification under optimized conditions [172]. The sequencing of PCR product was performed by Xcelris Labs Ltd. Ahmedabad. The comparative and phylogenetic analysis of the sequence obtained was done using NCBI BLAST and MEGA 4, respectively [173].

3.7 Production of xylanase

Exploiting the xylanolyitc bacterial isolate, xylanase production was carried out. Minimal medium with 0.5% (w/v) BW xylan was used as the production medium. Overnight grown bacterial culture was inoculated (1% v/v) into 100mL production medium; contained in 500 mL Erlenmeyer flask. Conditions for xylanase production were 60°C temperature, 48 h incubation time and agitation speed rate of 150 rpm. Afterwards the production medium was centrifuged (10,000 rpm/10 min) and supernatant and cell pellet were collected for assaying extracellular and intracellular xylanase activity, respectively.

3.8 Localization and induction of xylanase

In order to localize the production of xylanase, its extracellular and intracellular activity was estimated. For detecting the extracellular activity, xylanase activity assay was peformed with supernatant of the production medium. For estimating intracellular xylanase activity, the cell

pellet was subjected to ultrasonication. The cell debris (settled at the bottom) obtained after ultrasonication were discarded, whereas the cell lysate was assayed by DNS method for xylanase activity. To analyze inducible nature of xylanase, its production was carried out at above mentioned conditions by using the production medium (50 mL, pH 7) containing xylose (0.1% w/v) as carbon substrate in place of BW xylan. Control of the experiment was xylanase production being carried out under same conditions with BW xylan (0.1% w/v) as carbon source. Production was followed by activity assay for xylanase.

3.9 Parametric optimization of xylanase production by OFAT

Optimization is an imperative process for attaining maximum xylanase production under given physio-chemical conditions. OFAT method was followed for optimization of parameters influencing xylanase production. Using this method, one variable is optimized at a time and that optimized value is inculcated in the succeeding step. Various parameters such asinoculum size (0.5-2%, v/v), temperature (40-60°C), rate of agitation speed (50-200 rpm), pH (4-10), incubation time (24-120 h), nitrogen sources: peptone, urea and beef extract (0.005 g/L), nitrogen concentration (0.005-5 g/L) and substrate concentration (0.5-3%) were optimized. Also, targeting the cost-effective production of xylanase, abundantly available cheap substrates such as wheat straw, wheat bran, corn cob and rice straw were valorized for xylanase production under the optimized conditions. Optimization study was initiated by using 1% (v/v) inoculum (~3.7×10^7CFU/mL) for xylanase production under SmF for 48 h at 60°C and 150 rpm. After that, production medium was centrifuged (10,000 rpm/10 min) and subjected to xylanase activity assay.

3.10 Statistical optimization through response surface methodology

The significant parameters in terms of having maximum influence on the xylanase activity were selected from the OFAT optimization. The selected parameters were then subjected to statistical optimization (RSM) to study the mutual effect of the parameters on xylanase production. CCD was opted for performing RSM optimization with selected influential parameters. Each parameter was analyzed at five coded level -α, -1, 0, +1 and +α (Table 3.2). Under CCD, experimental trials were generated, using Design Expert 10 software. The experimental trials comprised of axial points, factorial points and central point.

Table 3.2: Range of variables at different levels used in central composite design.

Independent variables	**Units**	**Range and Level**				
		-α	-1	0	+1	+α
A: Temperature	degree Celsius	29.8	40	55	70	80.2
B: Incubation time	hours	12.7	40	80	120	147.3
C: Agitation	rpm	65.9	100	150	200	234.1

During statistical optimization, value of only selected parameters was varied; while media components (minimal medium) and other optimized parameters were constant. ANOVA was used for statistical analysis and data interpretation and plotting of response surfaces was done using Design Expert 10 software.

3.11 Model validation

Validation of the RSM model was done by carrying out xylanase production under the optimum conditions predicted by the model for maximum response.

3.12 Acessory cellulases and hemicellulases

Filter paper activity (FPase) and endo-1,4-β-D-glucanase (EC 3.2.1.4) activity was assayed for complete cellulase activity [174]. For FPase activity, crude GTX1 xylanase was appropriately diluted with Tris-HCl (pH 8, 50mM) to make 1mL reaction mixture. Afterwards, whatman filter paper no. 1 (1.0×6.0 cm) was immersed in the mixture and was given incubation for for 1 h at 60°C; followed by DNS method for sugar estimation. Filter paper unit (FPU) activity was defined as "the amount of enzyme releasing 1 µmol of reducing sugar from filter paper per mL per min". Endoglucanse activity was determined by 30 min

incubation of 0.5 mL crude xylanase with 0.5 mL of 1% (w/v) carboxymethylcellulose (pH 6, 50 mM SP) at 60°C. Reducing sugars produced were determined by DNS method. Endoglucanase unit activity was defined as "the amount of enzyme releasing 1 μmol glucose per min under the specified assay conditions". For assay of EC 3.2.1.91; exo-1,4-β-D-glucanase, EC 3.2.1.21; β-D-glucosidase, β-D-xylosidase, α-L-arabinofuranosidase, feruloyl esterase and acetyl xylanesterase activities were assayed using substrates (3mM) namely pNPC, pNPG, pNPX, pNPA, pNP ferulate and pNP acetate for microtiter plate assay. Activity assay for all enzymes was carried out for 30 min at 60°C with the reaction mixture of diluted enzyme (25 μL), pNP substrate (25 μL) and 50 mM SP buffer of pH 6 (50 μL). The reaction was terminated by addition of 100 μL glycine-NaOH buffer (0.4 M, pH 10.8) [175]. The color of released para nitro-phenol (pNP) was read at 405 nm. One unit of enzyme activity was defined as "the amount of enzyme capable of releasing 1 μmol pNP from the pNP linked substrates per min".

3.13 Characterization of GTX1 xylanase

pH optima of GTX1 xylanase activity was estimated at diverse pH range of 3-10. Different buffers were used for respective pH range; at a concentration of 100mM. For pH 3–5; sodium citrate, pH 6–7; sodium phosphate, pH 8-9; Tris-HCl and for pH10; glycine–NaOH buffer was used. For determining the optimum temperature, activity assay was performed at varying temperatures of 30°C to 100°C at an optimum pH. Thermostability of the GTX1 crude xylanase was estimated at 60°C and 70°C through its pre-incubation for 4h in 100 mM Tris-HCl (pH 8). It was followed by determination of relative xylanase activity at standard assay conditions for xylanase mentioned before. A control was run in parallel without any pre-incubation. For studying the pH stability, the crude enzyme was pre-incubated for 4h at 60°C in different pH buffers (3-10) whereas crude xylanase without pre-incubation was referred as a control. After that, estimation of relative xylanase activity was done by the standard DNS method. Effect of various metal ions was studied by preincubating the crude xylanase with a metal ion (10mM) at 60°C for 4 h; no metal ion was added in control. Afterwards relative activity was estimated under standard conditions.

Hydrolysis profile of BW xylan was analyzed by incubating 20 U xylanase/g xylan [17] in 100mM Tris HCl buffer (pH 8) at 60°C with agitation at 150 rpm for 48 h. No xylanase was added in the control for the experiment. After 48 h, products from hydrolysis were analyzed through HPLC using RI detector and Aminex HPX-87P column (300 X 7.8

mm; column). 20 μL of sample was injected using Milli-Q water as the mobile phase and the analysis was done at column temperature of 80°C with 0.6 mL/min flow rate.

Gene length and molecular weight of xylanase from *Geobacillus thermodenitrificans* X1 (GTX1) was also determined. Reverse and forward primers were designed using Primer 3 tool for amplification of xylanase gene from GTX1. For molecular weight determination, zymogram of GTX1 xylanase was performed. For, zymogram crude xylanase was concentrated using Amicon Ultra-15-Millipore with a cut off of 10 kDa in the membrane. Concentrated GTX1 xylanase was treated with SDS sample buffer; followed by heating for 5 min at 95°C, prior to loading on gel. Sample were run on 12% SDS PAGE gel, followed by cutting the gel such that the lane containing protein ladder is separated from the gel containing crude xylanase. The ladder containing gel was stained with coomassie dye. On the other hand, for zymogram the gel with crude xylanase was given three successive washings of 20 min each with 20% isopropanol. After that gel was immersed for 1 h incubation at 60°C, in SP buffer (50 mM, pH 7) containing 1% BW xylan; followed by congo red assay.

3.14 Immobilization of xylanase

3.14.1 Preparation of Xy-CLEAs

For precipitating xylanase molecules; chilled acetone (100%, v/v) was added as precipitating agent into 1.67 mg/mL of crude GTX1 xylanase (10 mL) (Fig. 3.3). The process was continued for 2 h at 4°C with continuous stirring at 150 rpm. Afterwards, glutaraldehyde (25% v/v) was added as cross-linker at 0.02% (v/v) final concentration for the formation of Xy-CLEAs. The cross-linking process was conducted for 4 h at 4°C and continuous agitation (150 rpm). For recovery of CLEAs, the reaction mixture was centrifuged (10,000 rpm; 5 min) at 4°C. After that, three times washing was given to the CLEAs using SP buffer (50 mM, pH 7) for removing unbound xylanase molecules and residual GA [39]. CLEAs were resuspended in the same buffer (50 mM; pH 7); followed by activity assay for xylanase.

3.14.2 Optimization of parameters affecting preparation of Xy-CLEAs

For recovery of maximum activity of Xy-CLEAs, influencing parameters need to be optimized. Therefore, for preparation of Xy-CLEAs; various parameters comprising of protein concentration (0.8-3.3 mg/mL), type of precipitant, protein: precipitant ratio (1:5-1:25), precipitation time (30-150 min), cross-linker concentration (0.005-0.05% v/v) and

reaction time (2-10 h) were optimized by OFAT method. Relative xylanase activity was estimated for analyzing the effect of these parameters.

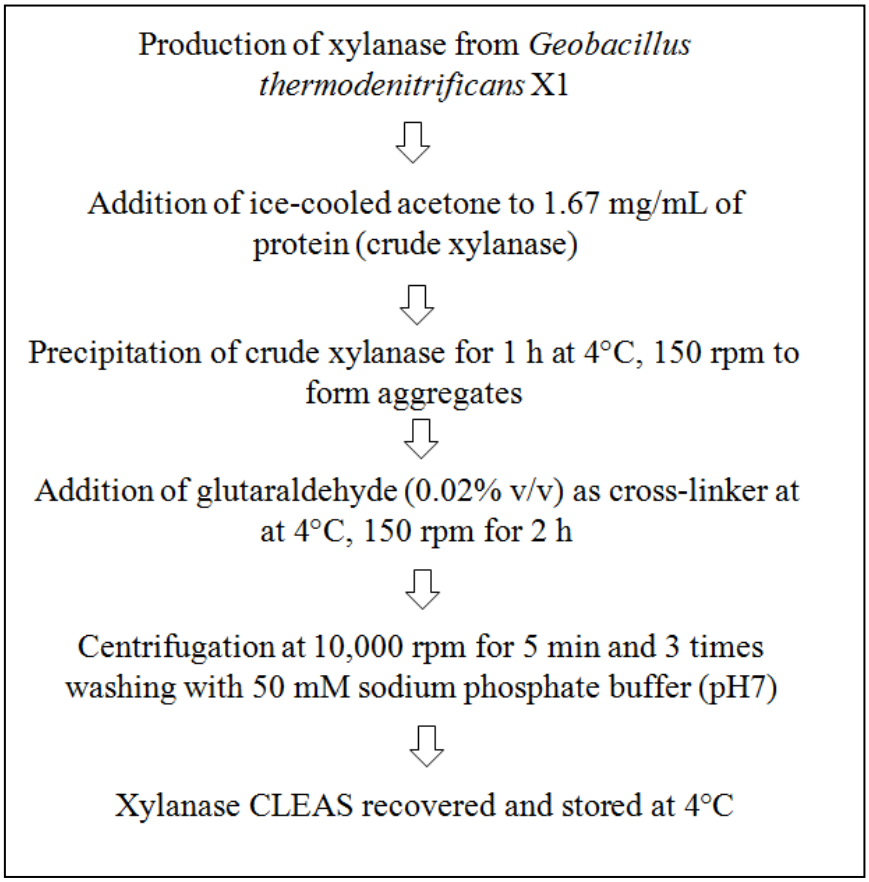


Figure 3.3: The schematic representation of preparation of thermosatble xylanase CLEAs from *Geobacillus thermodenitrificans* X1.

3.14.3 Characterization of Xy-CLEAs

3.14.3.1 Morphological analysis

Morphology of Xy-CLEAs (lyophilized) was studied by SEM analysis using FEI (Thermo Fisher) Nova Nano SEM-450. For determining CLEA particle radius, Dynamic light scattering (DLS) analysis was performed at room temperature using Malvern Zeta Sizer. An average of three runs was considered for estimation of the size of Xy-CLEAs.

3.14.3.2 Chemical characterization

Amendments in the structure of protein after formation of Xy-CLEA were determined through FTIR analysis performed by following KBr pellet method [14]. Transmittance a spectrum (wave number range of 4000 cm-1 to 500 cm-1) of free xylanase was recorded as a control for comparative analysis with respect to spectra peaks of Xy-CLEAs.

3.14.3.3 Biochemical characterization

3.14.3.3.1 Optimum pH and temperature

pH optima for activity of free and immobilized GTX1 xylanase was estimated at varied pH range of pH 3-10. Different buffers were used for respective pH range; at a concentration of 100mM. For pH 3–5; sodium citrate, pH 6–7; sodium phosphate, pH 8-9; Tris-HCl and for pH10; glycine–NaOH buffer was used. For determining the optimum temperature, activity assay was performed at varying temperatures of 30°C to 100°C at pH 7 (100 mM SP).

3.14.3.3.2 Thermostability and pH stability

For estimation of thermostability, free GTX1 xylanase and its CLEAs were pre-incubated for 4 h at 60°C and 70°C in Tris-HCl (pH 8; 100 mM); followed by estimation of relative xylanase activity at standard assay conditions. Control reaction mixture (100% relative activity) was not given any incubation. Thermal inactivation was calculated as per the equation: $\ln A/A_0 = K_d t$, where

A_0: initial activity without pre-incubation

A: residual activity after thermal exposure

K_d: thermal inactivation rate constant (h^{-1})

t: exposure time (h).

Half-life ($t_{1/2}$, h) was calculated as per the equation: $t_{1/2}=\ln 2/K_d$. pH stability was studied by incubating free and immobilized xylanase for 4 h at 60°C in different pH range (3-10) whereas xylanase without incubation was the control; considered to have 100% relative activity.

3.14.3.3.3 Reusability and storage stability

Xy-CLEAs were stored for 8 weeks at 4°C in SP buffer (pH 7; 50 mM). After every week, relative xylanase activity (%) was measured with respect to initial activity (100%) referred as control. Reusability was estimated by reusing Xy-CLEAs for repetitive six cycles of xylanase assay. Supernatant was subjected to xylanase assay whereas CLEAs in the pellet were given two times washings with SP buffer (50 mM; pH 7) before being reused. Relative activity of the first cycle was considered as 100%. After completion of last cycle; CLEAs were washed and lyophilized for SEM analysis to observe morphological changes after consecutive usage.

3.15 Application of crude GTX1 xylanase for paper pulp bio-bleaching

3.15.1 Pretreatment of pulp with crude xylanase

The agropulp (blend of 85% wheat straw and 15% bagasse) was collected from Kauntum Papers Limited situated in district Hoshiarpur, Punjab. The agropulp was distributed into three parts (100 g each) comprising of two test samples (T1 and T2) and one control sample (Fig. 3.4). Each Test sample was pretreated with xylanase dosage of 50 U/mL per one gram pulp; followed by incubation at 70°C for a retention period of 90 min with intermittent kneading. In contrast, the control sample (100 g) was not given any xylanase pretreatment.

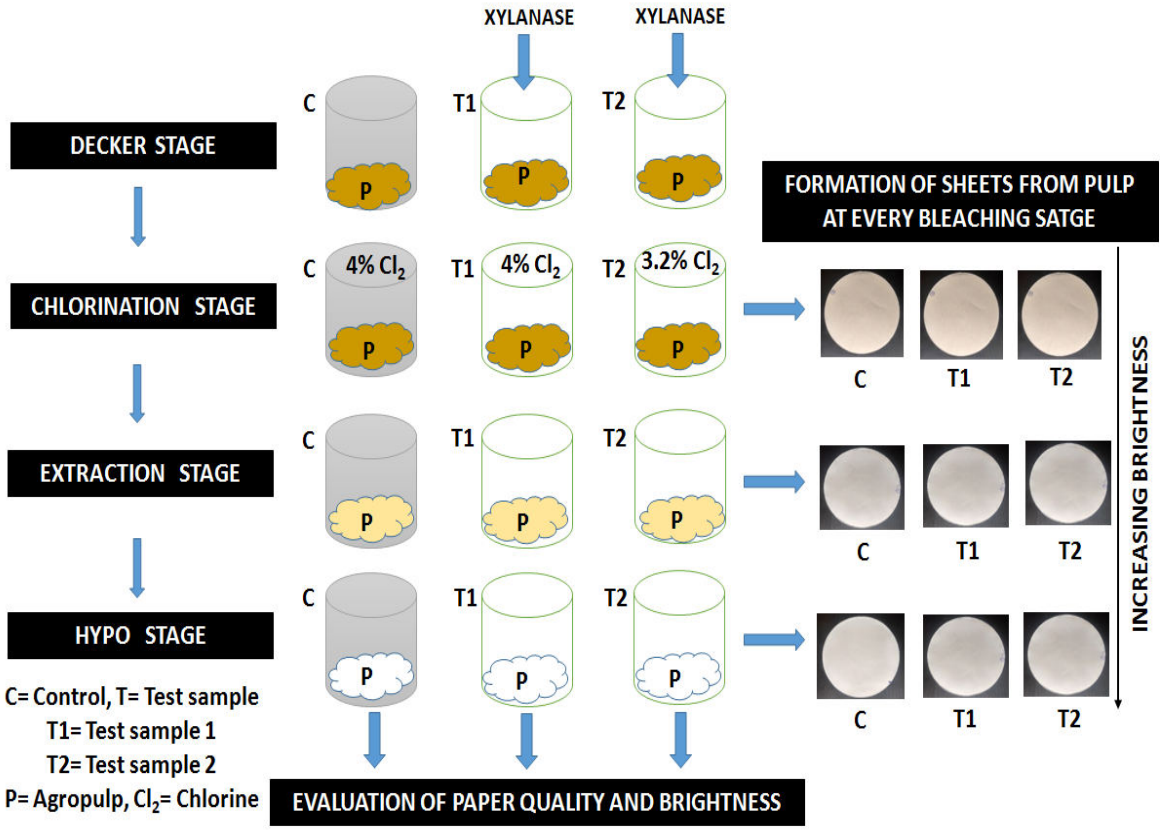


Figure 3.4: Diagrammatic representation of paper pulp bio-bleaching by xylanase.

After incubation, pulp from test and the control sample was examined for pH, kappa no. and brightness, followed by washing with fresh water. It was calculated by Tappi test method T 236 om-85. Brightness was determined using CM-3630 spectrophotometer (Konica Minolta) at 457 nm following Tappi Test method T 452 om-87. The washed pulp was appropriately filtered before the succeeding bleaching stages: Chlorination, Alkali Extraction and Hypo stage.

3.15.2 Chlorination stage

For chlorination, the pulp consistency was maintained at 3%. The chlorine dosage was varied among the pulp samples. T1 and the control sample were treated with 4% chlorine while T2 sample was treated with 3.2% chlorine. This 20% chlorine reduction for T2 sample was done

to evaluate the potential of xylanase to reduce the chlorine consumption in the bleaching process. Chlorination process was carried out at ambient temperature for 45 min, followed by washing with fresh water. Afterwards, pulp was screened for brightness, initial and final pH. A fraction of pulp from all the samples (T1, T2 and control) was pressed into sheets using automatic sheet making machine and dried by sheet dryer.

3.15.3 Extraction stage

Chlorinated pulp was treated with 2% caustic soda and hydrogen peroxide (1.32%) at a consistency of 10%. It was then followed by examination of initial pH and incubation at 70°C for 120 min. Afterwards pulp was given washing with excess of fresh water and filtered for further analysis. Kappa no. and brightness of the pulp was determined after extraction stage and also sheets were prepared.

3.15.4 Hypo stage

The pulp (consistency 10.5%) was given treatment of sodium hypochlorite solution (available chlorine 2.5%) and 0.1% sulphamic acid for 120 min at 45°C. Pulp was examined for changes in brightness and pH before and after treatment. Afterwards, sheets were prepared and examined for comparative analysis of the properties of the paper from different samples (T1, T2 and control).

3.15.5 Properties of paper

Paper sheets prepared from all samples were evaluated for quality properties in terms of viscosity, basis weight, bulk weight, breaking length, tear factor, and burst factor. Freeness measures the drainability of pulp suspension. Viscosity was measured using Viscometer (Cannon fenske) according to Tappi test method T 230 om-82. Bulk and basis weight were determined to evaluate the print quality and heaviness of paper, respectively. Bulk indicates thickness or volume in relation to weight. Basis weight gives the weight per unit area and thus decides that how much area of paper the customer will get for given weight. For determining the strength of paper, properties such as breaking length, tear factor, and burst factor were assessed. Breaking length was analyzed by following Tappi test method T 452 om-87. Tear factor was calculated using internal tearing resistance tester (Fibretic instrument Roorkee, 247667) following Tappi test method T 414. It is defined as the maximum force required tearing a paper in a direction perpendicular to the direction of stress (tearing tester). Bursting

factor of paper was determined by using digital bursting strength tester (in compliance with ISO 9001). This factor determines the stress that can be tolerated by paper before it ruptures when an external pressure is applied to it.

RESULTS AND DISCUSSION

4.1 Isolation, screening and identification of xylanolytic bacteria

Four thermophilic isolates were obtained from the enrichment culture; named as X1, X2, X3 and X4. Primary screening of isolates revealed isolate X1 as best xylanase producer as it showed maximum zone of hydrolysis (30 mm) shown in Fig. 4.1a. Similarly, secondary screening by DNS method also showed X1 isolate to be the most potent xylanolytic bacteria with xylanase activity of 2.3 U/mL. Morphological characteristics of isolate were irregular shape, off-white, viscous, entire margin, rough and flat (Fig. 4.1b). Also, X1 isolate rendered positive KOH string test and was Gram-positive (Fig. 4.1c). After analysis of 16S rRNA sequence through NCBI blast and phylogenetic tool; X1 isolate was found similar to *Geobacillus thermodenitrificans* (NR 116998) shown in Fig. 4.2. 16S rRNA partial sequence of X1 isolate (KT 899095) has been submitted to NCBI.

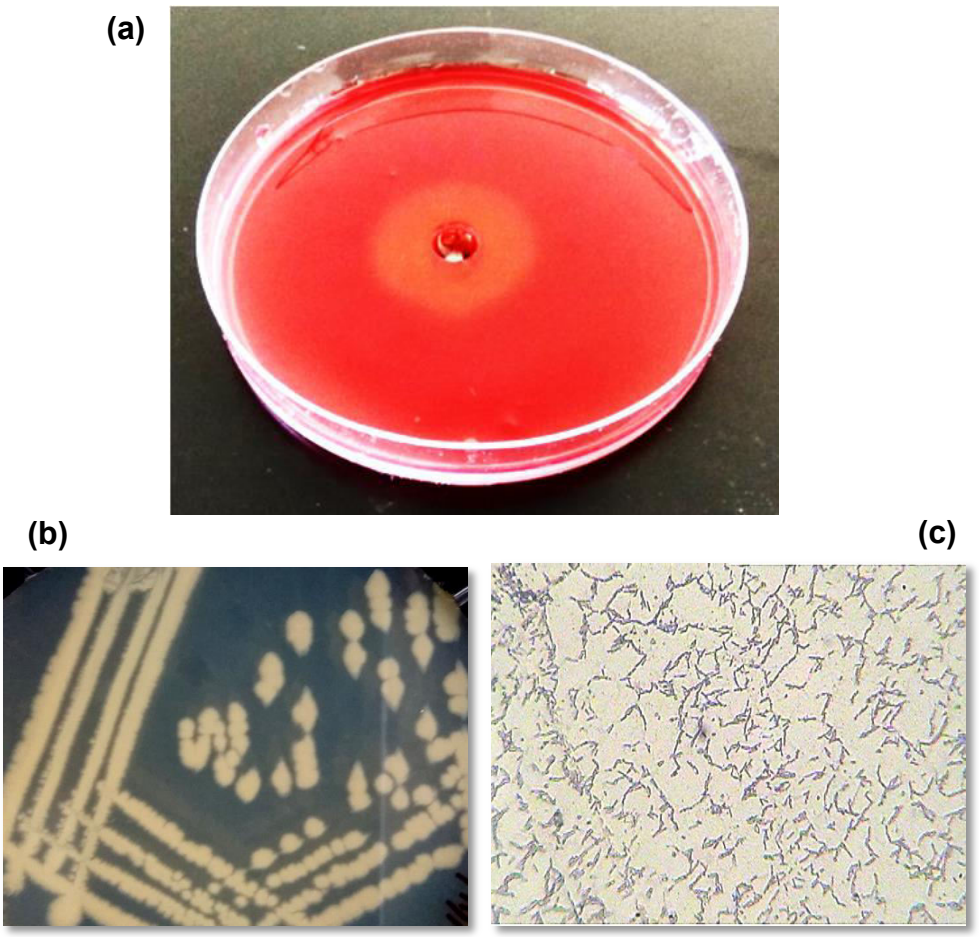


Fig. 4.1: (a) Congo red assay for qualitative estimation of xylanase produced by *Geobacillus thermodenitrificans* (b) Colony morphology (c) Gram staining

4.2 Localization and induction of xylanase

Xylanase from *Geobacillus thermodenitrficans* X1 (GT X1) was found extracellular in nature as the crude supernatant (2.38 U/mL) showed higher activity in comparison to cell lysate (0.145 U/mL) obtained after sonication of bacterial pellet. Also, inductive nature of xylanase was observed as higher xylanase activity (0.4 U/mL) was attained on using BW xylan (acting as an inducer) in the production medium. Whereas, using xylose as sole carbon source resulted in comparatively lower xylanase activity (0.03 U/mL).

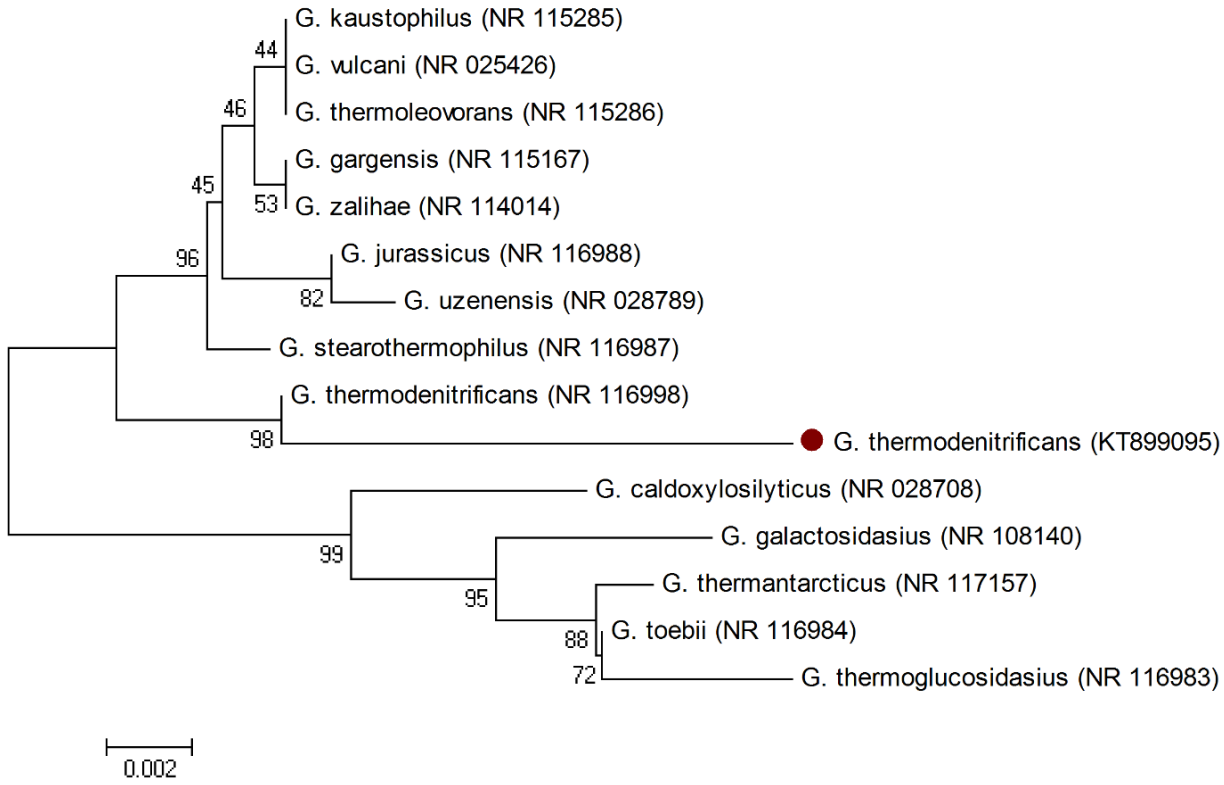


Figure 4.2: The phylogenetic tree of thermophilic bacterial isolate *Geobacillus thermodenitrificans* (KT899095), isolated from Tattapani hot spring soil. The Neighbor- joining tree was constructed with MEGA 4 software using partial 16S rRNA sequences of the other *Geobacillus* isolates retrieved from NCBI and the partial 16S rRNA of the isolate obtained in the present study (highlighted with dark red circle). The scale bar represents 0.002 substitutions per nucleotide position. Figure in the parenthesis () represents accession number of the respective bacterial isolate.

4.3 OFAT optimization for xylanase production

4.3.1 Inoculum size and incubation time

Maximum xylanase activity (2.8 U/mL) was attained with 1.5% (v/v) inoculum. Earlier reports depict that under submerged fermentation, 1.0 to 2.0% (v/v) inoculum gives maximum xylanase production [60, 61, 177]. Xylanase production from GTX1 with 0.5% inoculum lead to 35% decline in activity (Fig. 4.3a). Similarly, using higher inoculums (2%), also lead to

decreased activity. Xylanase activity was also influenced by incubation time and maximum activity (7.84 U/mL) was observed after 96 h.

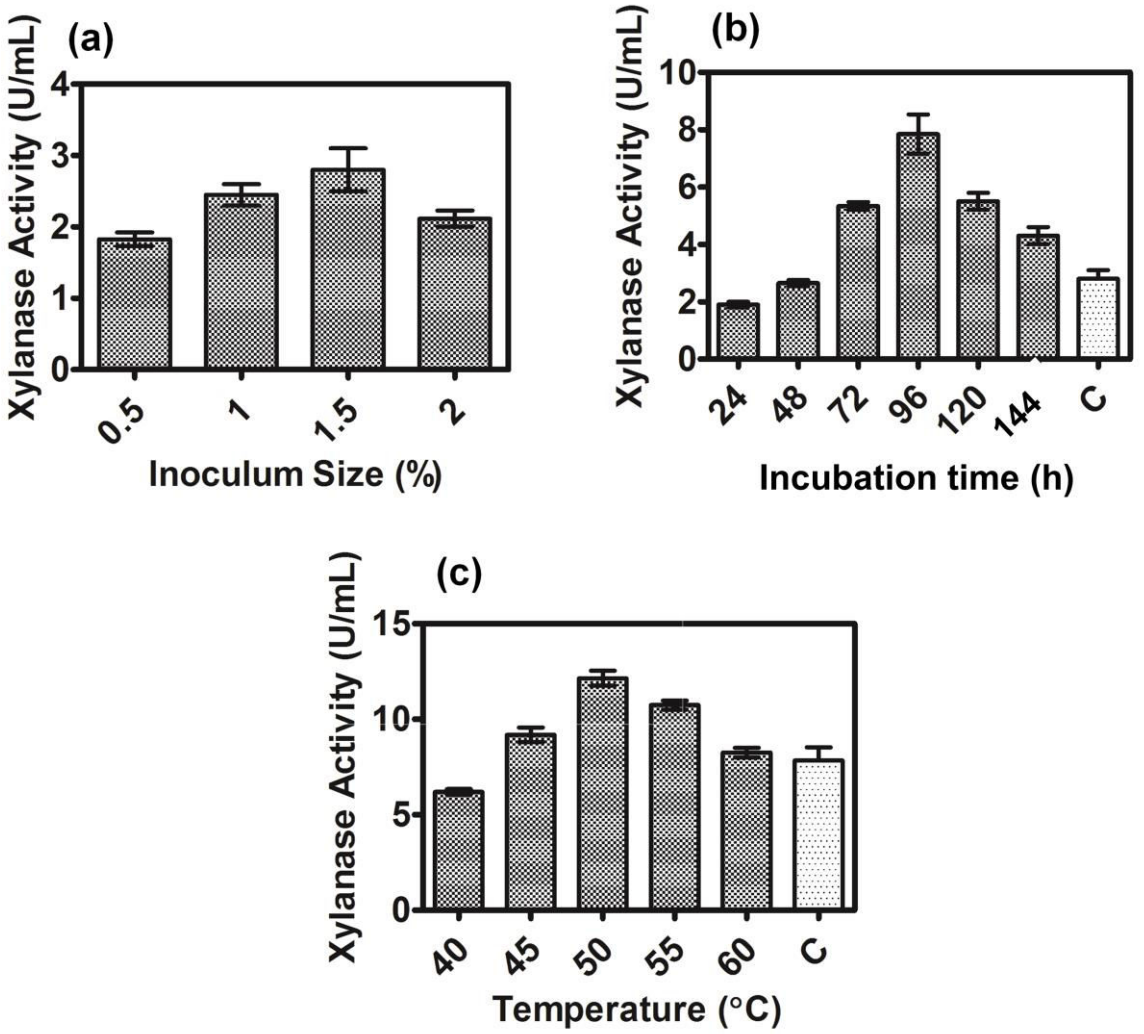


Figure 4.3: Influence of inoculums size, incubation time, temperature, pH and xylan concentration on xylanase production from *Geobacillus thermodenitrificans* X1: a) effect of inoculum size (0.5 to 2%) on xylanase production at 60°C, 150 rpm for 48 h. Minimal media with pH 7, 0.005 g/L yeast extract and 0.5% beechwood xylan was used. b) effect of incubation time (24 to 124 h) on xylanase production at 60°C and 150 rpm using 1.5% inoculum. Minimal media with pH 7, 0.005 g/L yeast extract and 0.5% beechwood xylan was used. c) effect of temperature (40 to 60°C) on xylanase production at 150 rpm for 96 h using 1.5% inoculum. Minimal media with pH 7, 0.005 g/L yeast extract and 0.5% beechwood xylan was used. All values are mean of two replicates ± SE. The control setup of preceding step has been denoted as C.

With increasing incubation time, increase in the xylanase activity was observed but beyond optimum value, no further increase was observed (Fig. 4.3b). Outflux of intracellular xylanase from the autolyzed aged cells, resulted in higher xylanase production during stationary phase

[39, 178]. While, beyond the optimum value; exhaustion of nutrients resulted in decreased activity. In literature, xylanases from *Geobacillus* sp. [39] and *Bacillus* SSP-34 [2] also rendered highest titer after 96 h.

4.3.2 Temperature and initial pH

Maximum xylanase of 12.15 U/mL was observed at 50°C (Fig. 4.3c). At higher temperature of 60°C 32% decline from maximum activity occured. At 40°C ~2.5-fold (6.2U/mL) decreased activity was observed compared to maximum. Since, GTX1 is a thermophilic bacterium; high temperature range is more favorable for its growth and metabolism. That is why, at 40°C comparatively more decline in the xylanase activity was observed with respect to higher temperature range. Study of pH profile revealed optimum pH range between pH 7-8 for xylanase production. Drift was towards alkaline region and acidic conditions (pH 4 and 5) were found unfavorable for xylanase production (Fig. 4.4a). At pH 7 (12.13 U/mL) and 8 (12.42 U/mL) high activities were observed.

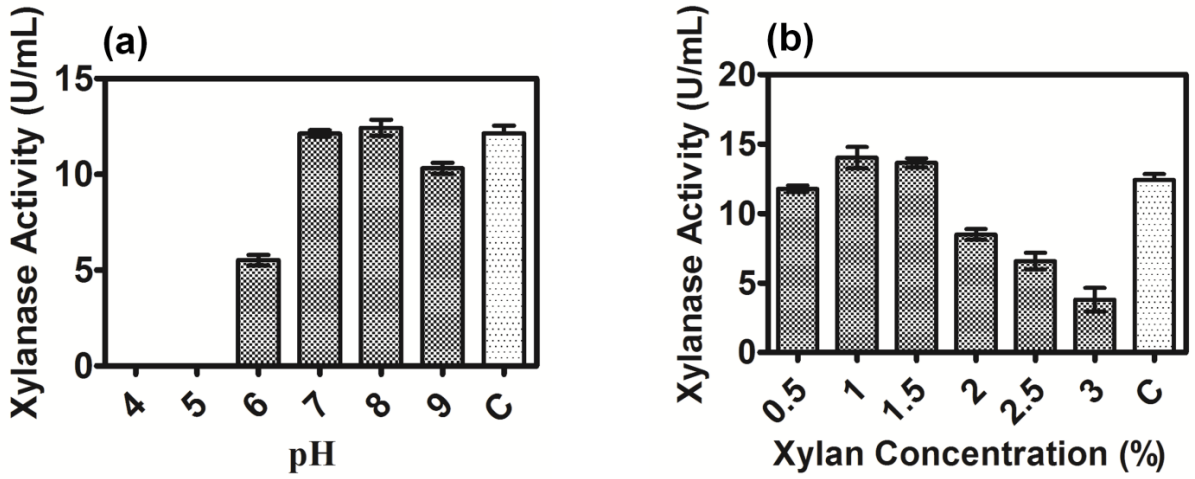


Figure 4.4: Influence of pH and xylan concentration on xylanase production from *Geobacillus thermodenitrificans* X1: a) effect of pH (4 to 9) on xylanase production at 50°C, 150 rpm for 96 h using 1.5% inoculum. Minimal media with pH 7, 0.005 g/L yeast extract, 0.5% beechwood xylan was used b) Effect of xylan concentration (0.5 to 3%) on xylanase production at 50°C, 150 rpm for 96 h using 1.5% inoculum. Minimal media with pH 8, 0.005 g/L yeast extract, 0.5% beechwood xylan was used. All values are mean of two replicates ± SE. The control setup of preceding step has been denoted as C.

Results clearly depicted pH as an influential factor. Specifically, secretion of an extracellular enzyme relies on transport machinery of cell membrane and H^+ ion concentration [4]. pH variations in the medium lead to limited nutrient access to microbial cells, disruption of their membrane and hinderence in their metabolic activities. [4]. In agreement with current work,

pH 8 gave highest production of xylanase from *Bacillus megaterium* [64] and *Bacillus mojavensis* AG137 [178].

4.3.3 Xylan concentration

Higher xylanase production (14.02 U/mL) was observed on using 1 to 1.5% (w/v) BW xylan as substrate. Using 0.5% xylan lead to 16% decline in activity (Fig. 4.4b). At higher xylan concentration (2% to 3%) xylanase production was decreased showing 8.5 U/mL and 3.8 U/mL xylanase activity, respectively. The increasing xylanase activity with increase in xylan concentration (1%, w/v) justified inducible nature of GT X1 xylanase. However, inducer (xylan) could enhance the production up to a limit. Beyond optimum concentration, no inducing effect was observed on xylanase production; likely due to product or feedback inhibition [64].

4.3.4 Nitrogen sources and concentration

Both peptone and yeast extract resulted in high activity of 14.3U/mL and 13.75 U/mL, respectively. On the other hand, urea and beef extract rendered comparatively lower activity (Fig. 4.5a). Peptone was used as nitrogen source for succeeding optimization. Among varying concentrations of peptone, range of 0.005-0.05 g/L was found optimum for xylanase activity (Fig. 4.5b). Literature reports depict that, choice of optimum nitrogen source varies among different bacteria. For instance, xylanase production from *Bacillus licheniformis* [179, 180] and *Bacillus* sp. [181, 182] was highest on using peptone. Whereas, using yeast extract and beef extract, lead to maximum xylanase production from *Streptomyces* sp. SU9 [55] and *Bacillus aerophilus* KGJ2 [71], respectively.

4.3.5 Agitation speed rate

Highest xylanase activity with 18.32 U/mL was attained at 200 rpm (Fig. 4.5c). Higher agitation results in uniform air and nutrient distribution which eventually results in enhanced enzyme production [60]. Observations in present study were similar to literature reports [62, 178]. At low agitation rate, limited availability of dissolved oxygen and improper mixing is detrimental for microbial cell growth [178]. Results obtained depicted significant influence of agitation rate during SmF [60].

4.3.6 Carbon sources

Wheat straw and BW xylan emerged as best carbon sources; with 18.5 U/mL and 18.2 U/mL activities, respectively (Fig. 4.5d).

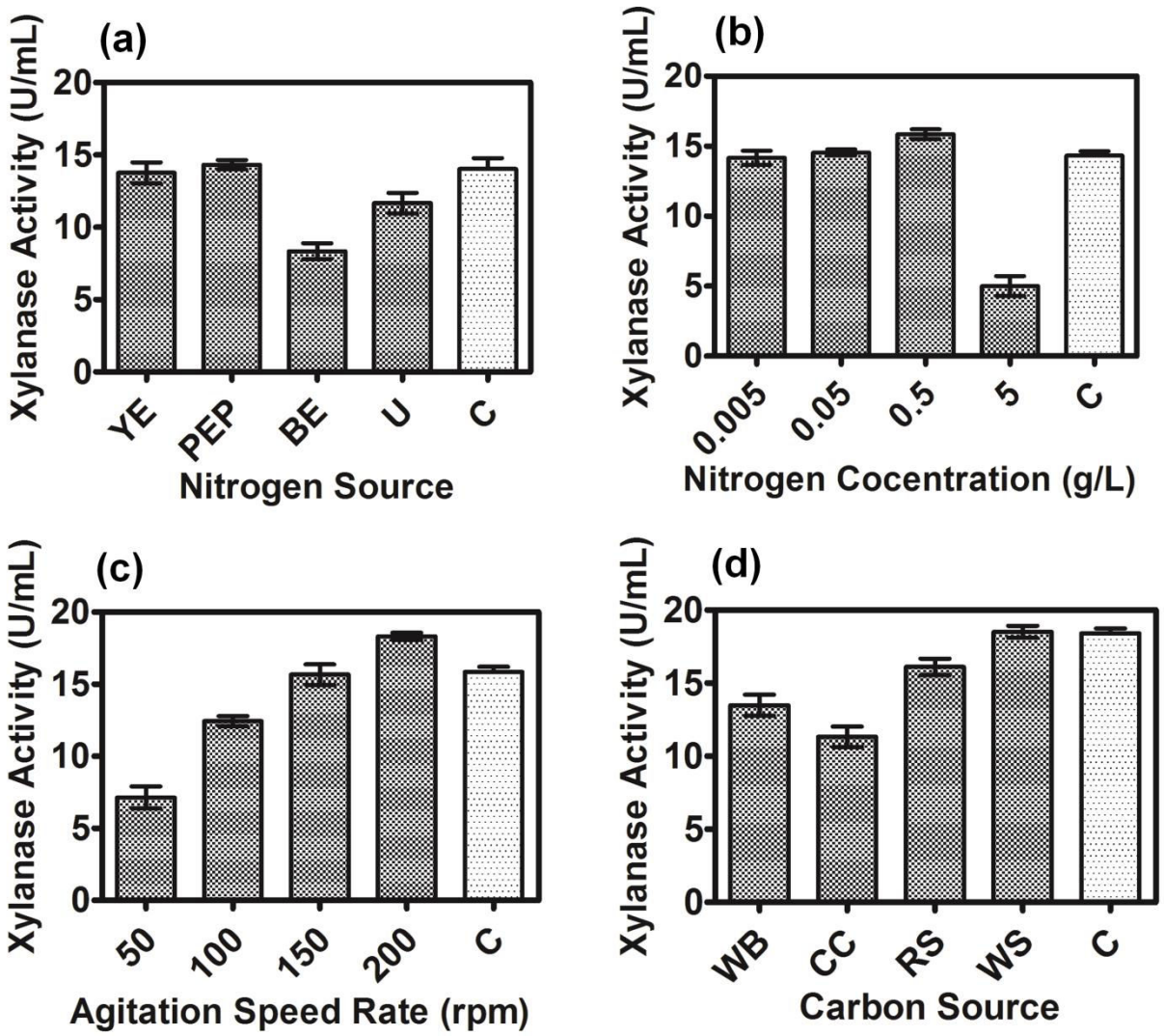


Figure 4.5: Influence of nitrogen sources, nitrogen concentrations, agitation speed rate and carbon sources on xylanase production from *Geobacillus thermodenitrificans* X1: (a) effect of nitrogen sources other than yeast extract (YE), such as peptone (PEP), beef extract (BE) and urea (U) at a concentration of 0.005 g/L on xylanase production at 50°C, 150 rpm for 96 h using 1.5% inoculum. Minimal media with pH 8, 1.5% beechwood xylan was used. (b) effect of nitrogen concentration (peptone at concentrations 0.005–5 g/L) on xylanase production at 50 °C, 150 rpm for 96 h using 1.5% inoculum. Minimal media with pH 8, 1.5 % beechwood xylan was used. (c) effect of agitation speed rate (50–200 rpm) on xylanase production at 50 °C for 48 h using 1.5 % inoculum. Minimal media with pH 8, 0.5 g/L peptone, 1.5 % beechwood xylan was used. (d) effect of carbon source (XY= beechwood xylan, WS =wheat straw, RS= rice straw, CC= corn cob and WB= wheat bran) at 1.5% concentration on xylanase production at 50 °C, 200 rpm for 96 h using 1.5% inoculum. All values are mean of two replicates ± SE. The control setup of preceding step has been denoted as C.

Rice straw (16.1 U/mL), wheat bran (13.5 U/mL) and corn cob (11.3 U/mL) rendered lower xylanase production, comparatively. In 2015, Marcolongo L et al, observed higher production

of xylanase in case of *Geobacillus thermodenitrificans* A333 on replacing commercial xylan with lignocellulosic substrate. Although in present study, xylanase production was almost equal with wheat straw and BW xylan. However, from economical point of view, wheat straw would be optimum substrate for xylanase production from GT X1. Wheat straw is commercially viable raw material; being wordwide abundantly avaialable [183, 184, 185].

4.4 Statistical optimization through response surface methodology

Three significant variables namely temperature, incubation time and agitation were selected from OFAT optimization. CCD design of these three variables alongwith 20 experimental trials and the response (xylanase activity) were shown in Table 4.1.

Table 4.1: Factors and levels in the response surface central composite design arrangement along with the experimental and predicted results.

Run	A	B	C	Response (Xylanase activity)	
				Experimental (U/mL)	Predicted (U/mL)
1	55	80	150	22.9	22.5
2	55	80	150	21.7	22.5
3	70	40	200	2.5	4.8
4	55	80	150	22	22.5
5	55	12.8	150	0.7	0
6	70	40	100	1.02	1.6
7	55	80	65.9	16.5	14.3
8	40	40	200	12.2	11.8
9	70	120	200	0.9	1.8
10	55	80	150	22.5	22.5
11	55	80	150	23.5	22.5
12	40	40	100	0.9	2.7
13	55	80	234.1	21.8	20.2
14	29.8	80	150	12	11.1
15	40	120	100	13	13.4
16	80.2	80	150	0	0
17	40	120	200	15.2	17.3
18	70	120	100	0.7	3.8
19	50	80	150	21.8	22.5
20	55	147.3	150	8.5	5.9

A: Temperature (°C), B: Incubation time (hour) and C: Agitation speed (rpm)

Run 11 rendered maximum response (23.5 U/mL), while no xylanase production was shown by run 16. A second-order polynomial equation was deduced from multiple regression analysis. The final coded equation was presented as: Xylanase activity (U/mL) = +22.51 - 4.13A + 1.93B + 1.76C - 2.13AB - 6.51A^2 - 7B^2 - 1.86C^2. Where A symbolized temperature (degree Celsius), B-incubation time (hour) and C-agitation speed rate (rpm). Through ANOVA data regarding statistical significance of the factors and model was attained (Table 4.2).

Table 4.2: ANOVA for response surface quadratic model (xylanase activity).

Source	Sum of Squares	df	Mean Square	F Value	*p*-value	Prob>F
Model	1596.9	9	177.4	34.8	<0.0001	*
A-Temperature	232.6	1	232.6	45.6	<0.0001	
B-Incubation time	50.6	1	50.6	9.93	0.0103	
C-Agitation	42.5	1	42.5	8.3	0.0162	
AB	36.2	1	36.2	7.1	0.0237	
AC	17.5	1	17.5	3.43	0.0939	
BC	13.47	1	13.4	72.64	0.1351	
A^2	610.1	1	610.1	119.7	<0.0001	
B^2	706.48	1	706.4	8138.6	<0.0001	
C^2	49.72	1	49.7	29.75	0.0108	
Residual	50.97	10	5.1			
Pure Error	2.5	5	0.5			
Cor Total	1647.9	19				

*significant value

The model F-value was 34.81 and value of ‘‘Prob>F’’ was < 0.05; implying model to be significant. Variables A, B, C, AB, A^2, B^2 and C^2 were concluded as significant model terms according to ANOVA data. The data obtained depicted that variable A and B were significant for xylanase production at linear, quadratic and interactive level. The significant interaction between A and B factors has been presented in Fig.. 4.6 through contour plots. Elliptical contours depict significant interaction [186]. Value for “determination coefficient” R^2 was

0.9691 (close to unity); showing good correlation between experimental and predicted values. This implies that about 97% of the variability could be explained by the model.

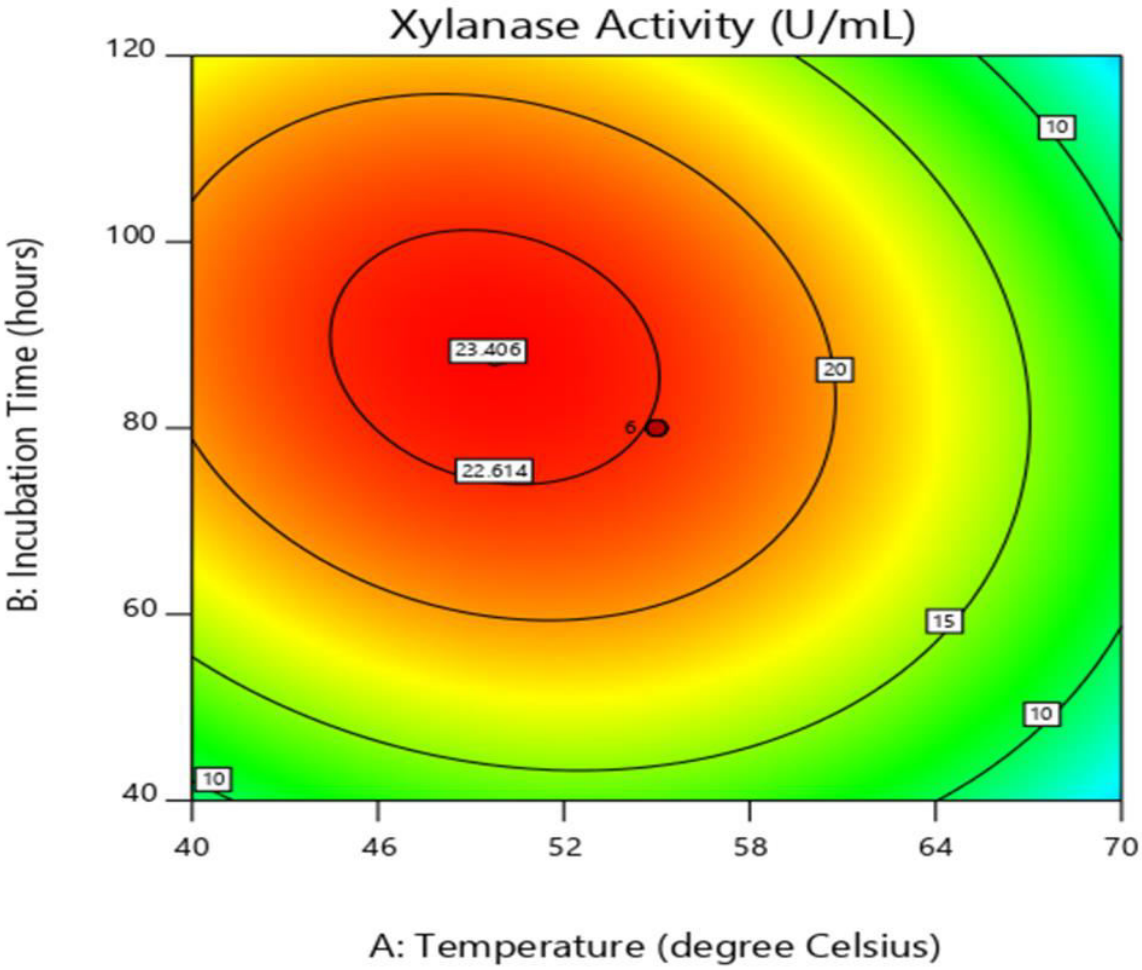


Figure 4.6: Contour plot showing interaction between temperature A and incubation time.

The correlation between experimental and predicted values was also presented in the form of parity plot (Fig.. 4.7). Data points located in the vicinity of diagonal of parity plot, represented high accuracy of the model. In present study, the "Predicted R^2" of 0.7681 was in reasonable agreement with "Adjusted R^2" of 0.9412. The signal to noise ratio is measured by adequate precision value. The ratio of our model was 15.876, indicating that this model can be used to navigate the design space. The low value (18.79 %) for "coefficient of variation" indicated good precision and reliability of the experiments conducted [187].

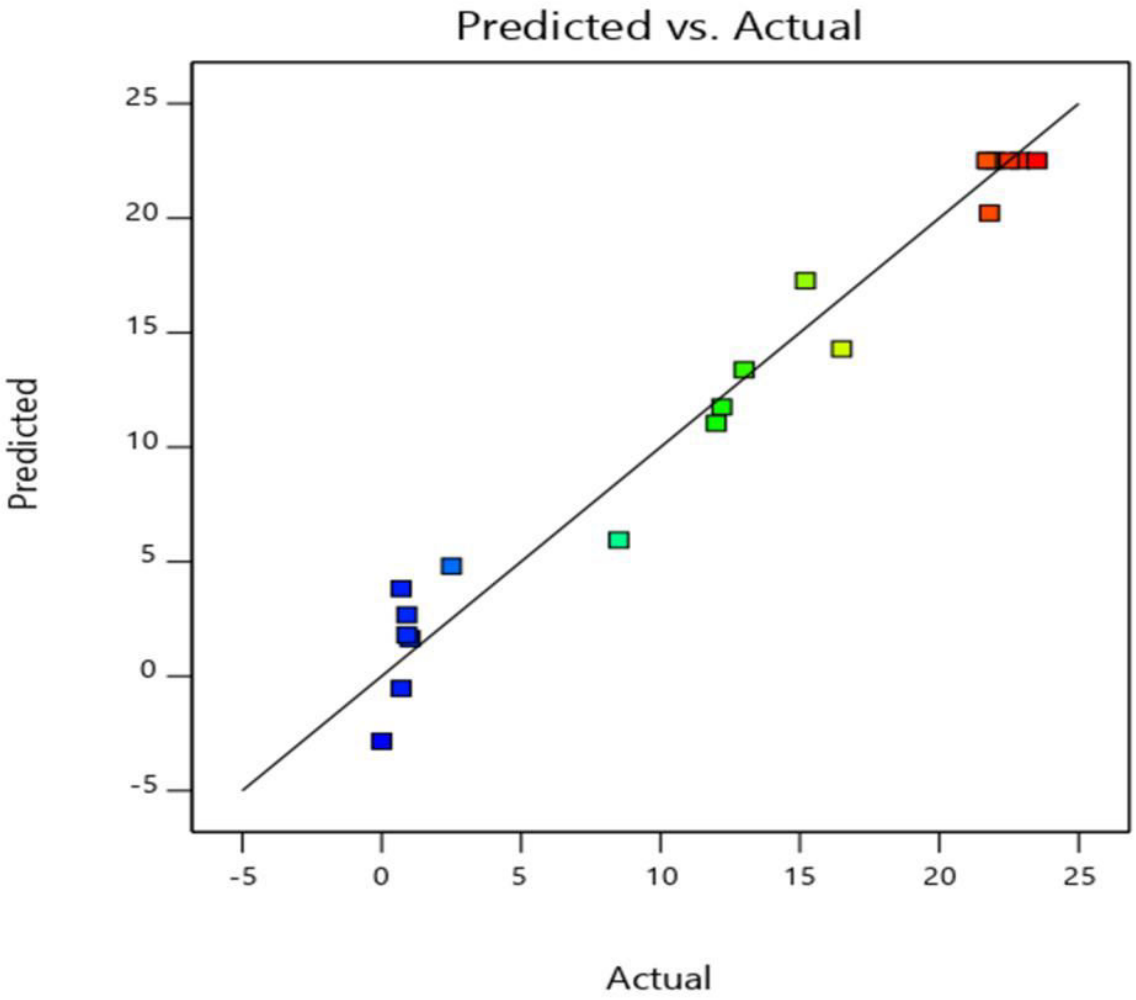


Figure 4.7: Parity plot showing correlation between the experimental and predicted values.

4.5 Model validation

The optimum conditions predicted by the model for xylanase production comprised of 50°C, 87 h and 177 rpm with predicted response of 23.9 U/mL. For validation, xylanase production was carried out on predicted conditions and response value of 24 ± 2 U/mL was attained. The close proximity between the predicted and experimental value of response justifies the validity of model. Comparative study of GT X1 xylanase with respect to other bacterial species revealed that activity of GTX1 xylanase was highest among all other *Geobacillus* sp. reported till date (Table 4.3). Comparison with *Bacillus* sp. showed that the two species (*tequilensis* & *pumilis*) [88, 168] renderd comparatively lower activity whereas higher activity was shown by the other two (*Bacillus subtilis* & *Geobacillus sp.* TC-W7) [189, 190]. However, *Bacillus* sp. with higher activity had used wheat bran and corn cob as substrates

whereas in our study wheat straw was used; which is comparatively more abundant raw material in the region.

Table 4.3: Comparison of xylanase yield of *Geobacillus thermodenitrificans* X1 with other bacterial species.

Organism	Carbon source	Xylanase activity (U/mL)	Reference
Geobacillus thermodenitrificans X1	Wheat straw	24 ± 2	Present study
Geobacillus thermodenitrificans A333	Wheat straw	0.35	[38]
Geobacillus sp. WSUCF1	Corn stover	20.9	[39]
Bacillus sp.	Wheat bran	3.5	[168]
Bacillus pumilus	Corn husk	2.5	[88]
Bacillus tequilensis SH0	Wheat bran	41.3	[189]
Bacillus subtilis	Corn cob	295	[190]
Geobacillus thermodenitrificans TSAA1	Wheat bran	~2.75	[57]

4.6 Assay of cellulases and accessory hemicellulases

Assay of GT X1 xylanase for FPase showed an activity of 70±20 mU/mL. This implies that GT X1 is associated with negligible traces of cellulase (Table 4.4). Being cellulase free, GT X1 crude xylanase can be suitably exploited for biobleaching of pulp in paper industry. Similarly, very low fraction of other cellulolytic enzymes and accessory hemicellulases were found associated with crude GT X1 xylanase (Table 4.4).

Table 4.4: Cellulases and hemicellulases associated with crude xylanase.

S.No.	Enzyme	Substrate	Activity (mU/mL)
1.	FPase	Filter paper	70 ± 20
2.	Endo-1,4-β-D-glucanase	CMC	36 ± 12
3.	Exo-1,4-β-D-glucanase	pNPC	1 ± 1
4.	β-D-glucosidase	pNPG	5 ± 2
5.	β-D-xylopyranosidase	pNPX	9 ± 3
6.	a-L-arabinofuranosidase	pNPA	8 ±1
7.	Feruloyl esterase	pNP ferrulate	1 ± 1
8.	Acetyl xylan esterase	pNP acetate	20 ± 10

CMC (Carboxymethyl cellulose), pNPC (4-nitrophenyl-β-D-cellobioside), pNPG (4-nitrophenyl-β-D-glucopyranoside), pNPX (4-nitrophenyl-β-D-xylopyranoside), pNPA (4-nitrophenyl-α-l- arabinofuranoside)

4.7 Characterization of GTX1 xylanase

Temperature optima for activity of GTX1 xylanase was 70°C (Fig.. 4.8a) as reported by Marcolongo et al. [38] and Anand et al. [57]. The optimum pH was found to be 8 (Fig.. 4.8b), similar to that reported in case of other isolates of *Geobacillus thermodenitrificans* [190, 191]. The enzyme showed appreciable stability at 60°C and 50°Cwith 90.8% and 99.9% residual activity, respectively after 4h (Fig.. 4.8c) and was stable in the broad range of pH; showing higher stability in alkaline range (Fig.. 4.9a). Among all the metal ions, Ca^{+2} ions showed maximum stimulation and Mn^{+2} ions showed maximum inhibition on xylanase activity (Fig.. 4.9b).

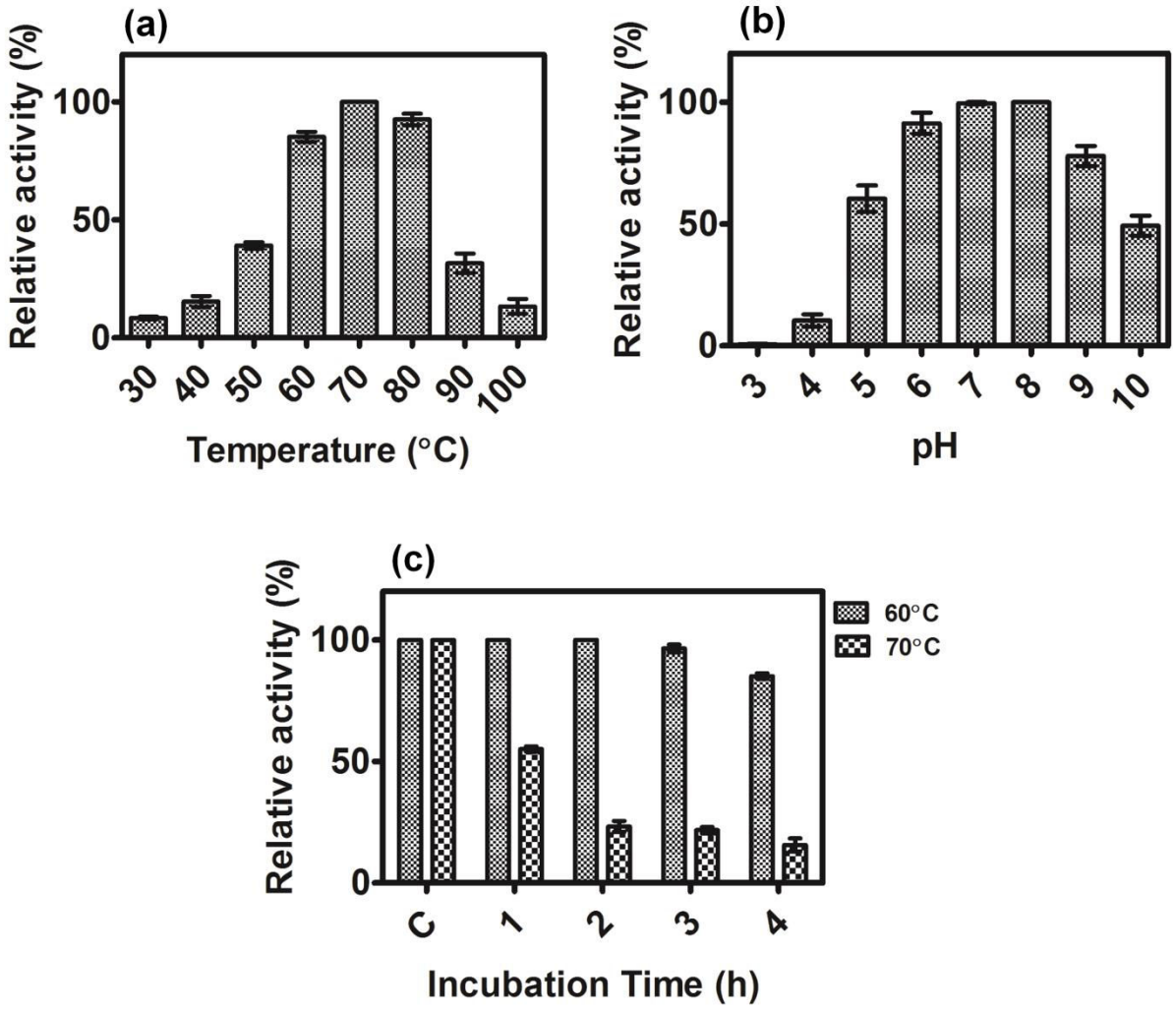


Figure 4.8: Characterization of GTX1 xylanase (a) effect of temperature (30˚C-90˚C) on xylanase activity at pH 7. Maximum activity (100%) was 24.8 U/mL (b) effect of pH (pH 3-10): on xylanase activity at 70˚C. Maximum activity (100%) was 25.3 U/mL (c) thermostability of xylanase at 50˚C, 60˚C and 70˚C at pH 8, with reference to the control (C) as 100%. All values are mean of three replicates ± SE.

GTX1 xylanase efficiently hydrolyzed BW xylan at alkaline pH 8, producing xylose (0.22 ± 0.01 g/L), xylobiose (1.46 ± 0.02 g/L), xylotriose (1.64 ± 0.02 g/L) and xylotetrose (0.65 ± 0.01 g/L). This potential of producing XOs makes xylanase a reliable applicant in aquaculture and poultry industry. The sequencing of PCR product (amplified xylanase gene) revealed gene length of 1,224 bp which has been submitted to NCBI under the accession no. MG874777. Zymogram analysis of crude xylanase showed a light yellow band near 45 kDa, against the red background of Congo red dye (Fig.. 4.9c). The molecular weight of GTX1 xylanase estimated through zymogram lies in the same range as reported for xylanase from other *Geobacillus thermodenitrificans* isolates [57, 192].

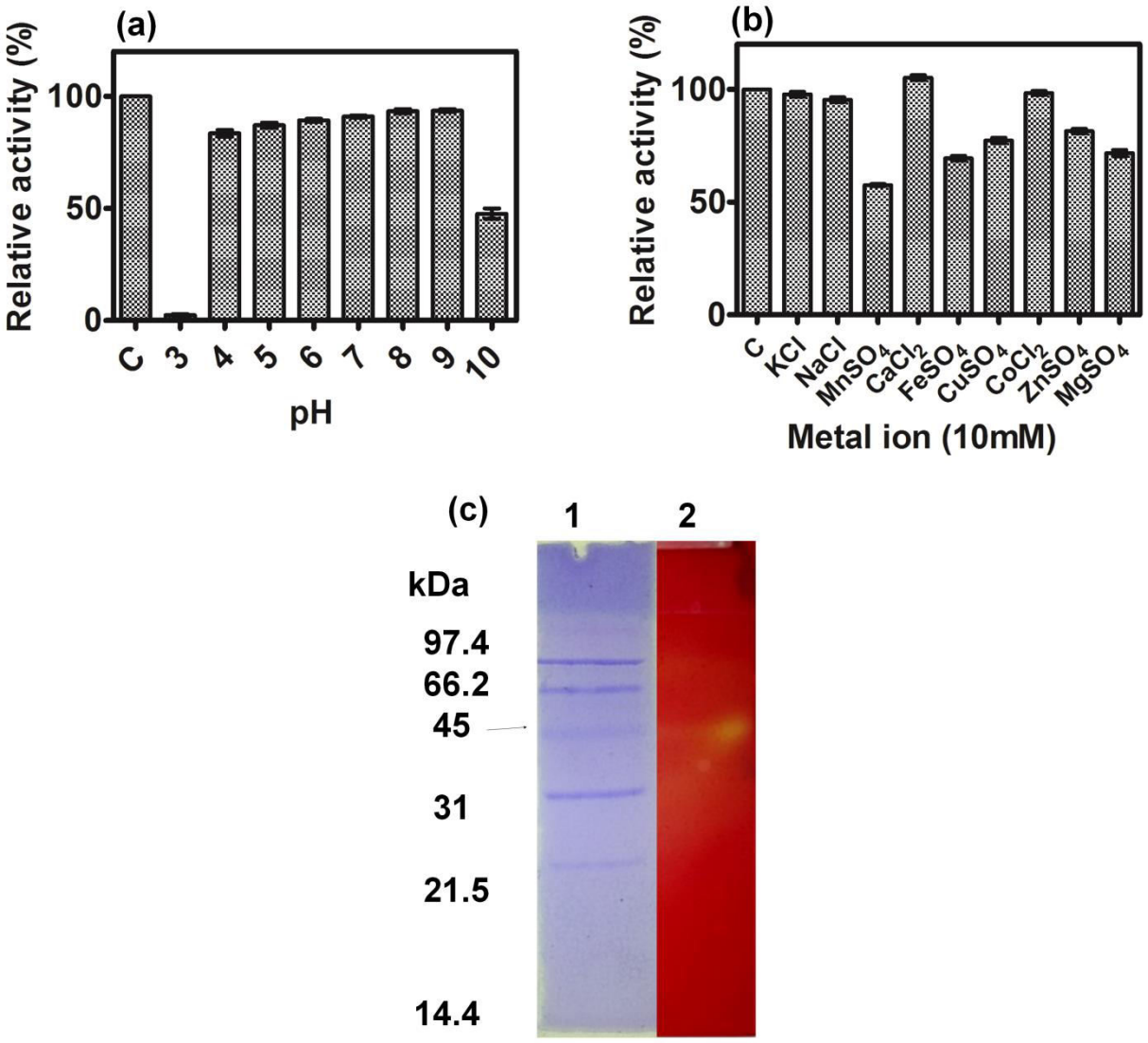


Figure 4.9: Characterization of GTX1 xylanase (a) pH stability of xylanase at pH 3-10 and temperature 60°C with reference to the control (C) as 100% (b) effect of metal ions (10mM) at pH 8 and 60°C on xylanase activity; with reference to the control (C) as 100%. All values are mean of three replicates ± SE. (c) Zymogram of xylanase; 1= ladder, 2= zymogram band of concentrated crude xylanase.

4.8 Immobilization of xylanase by CLEA preparation

4.8.1 Optimization of parameters affecting preparation of Xy-CLEAs

4.8.1.1 Protein concentration and type of precipitant

Among varied range (0.8-3 mg/mL) of protein concentration, 1.7 mg/mL was optimum for CLEA preparation (Fig.. 4.10a). At higher concentrations (2.5 and 3.3 mg/mL), relative activity decreased. This decrease comes from negative influence of extreme protein loading on CLEA surface. Enzyme crowding leads to crashing among protein molecules of enzyme,

thus reducing the enzyme mobility [151]. Afterwards, evaluation of various organic solvents (30% v/v) as precipitating agents was conducted for synthesis of Xy-CLEAs. The relative activity of the precipitated protein was comparesd with control (without organic solvent). Acetone was observed to be the best precipitant as 100% xylanase activity was retained in its presence (Fig.. 4.10b). By optimizing the precipitant, one can foresee its impact on enzyme activity, prior to cross-linking [130, 155]. Organic solvents have been reported as efficient precipitants in earlier studies as well. Easa et al. [154] used 65% acetone for *Zophobas morio* amylase precipitation. Whereas, Dalal et al. [158] reported 100% activity in case of pectinase, xylanase and cellulase on precipitation with n-propanol. Bilal et al. [149] on preparation of Manganese peroxidase CLEAs mentioned acetone as best precipitant. However some studies report organic solvents to be less efficient compared to salts like ammonium sulfate [13, 155]. Talekar et al. [155] reported variations in precipitant choice with respect to the variety of proteins under study. Such variations occur due to differences among proteins from varying enzymes in terms of biochemical and structural properties and even among same enzymes from different sources.

4.8.1.2 Protein: precipitant ratio

Crude xylanase (1.7 mg/mL protein) was precipitated with varying ratio of protein:acetone (v/v). Ratio of 1:10 (v/v) showed maximum Xy-CLEA recovery (Fig.. 4.10c). Organic solvents are known to facilitate protein aggregation via electrostatic interactions. They cause aggregation of proteins by displacing surrounding water molecules and reducing dielectric constant of the solution [155, 176]. Recent studies reported high retention of CLEA activity at high precipitant concentration; as a consequence of competition between aggregating and denaturing proteins. At higher concentrations, immediate adequate precipitation of protein occurs; eliminating chances of partial inactivation or denaturation of protein [130].

4.8.1.3 Precipitation time

Activity of Xy-CLEAs was observed maximum after carrying out precipitation for duration of 120 min (Fig.. 4.10d). The time requirement for precipitation resulting in maximum CLEA recovery varies among various proteins [193]. Talekar et al. [194] reported 30 min duration as an optimum precipitation time for p-CLEAS of *Saccharomyces cerevisiae* invertase. Whereas, Easa et al. [154] observed maximum recovery of amylase CLEAs after a long duration of 16 h.

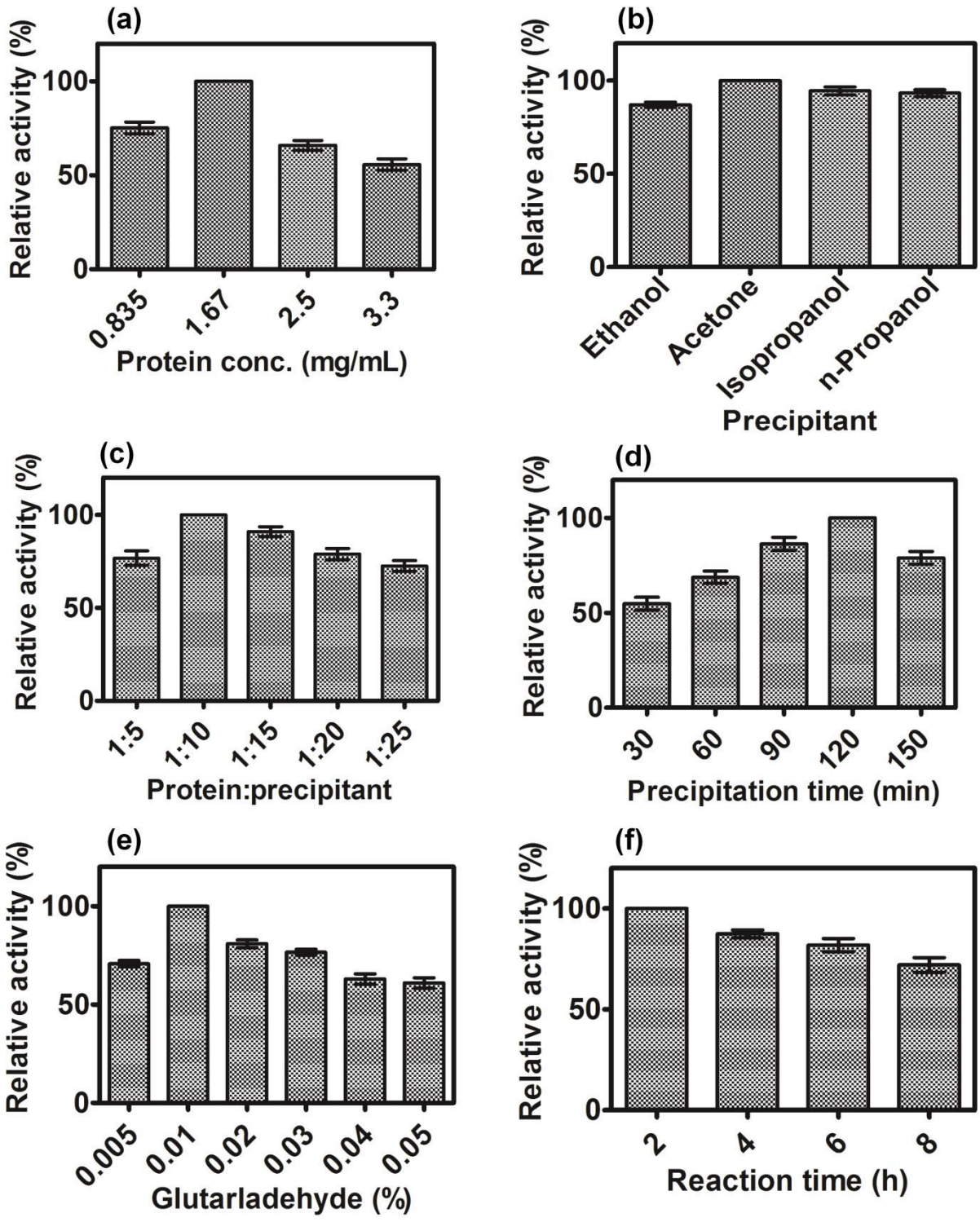


Figure 4.10: Optimization of various factors for Xy-CLEAs preparation (a) effect of protein conc. (0.835-3.3 mg/mL) (b) effect of various precipitants (c) effect of protein:precipitant ratio (1:5-1:25) (d) effect of precipitation time (30-150 min) (e) effect of glutaraldehyde conc. (0.005-0.05%) (f) effect of reaction time (2-8 hrs). All values are mean of three replicates ± SE.

4.8.1.4 Cross-linker concentration and reaction time

Low cost and easy availability of GA makes it most reliable cross-linker on commercial platform [194]. However, some studies also suggest use of dextran polyaldehyde as a cross-linker to overcome activity loss by CLEAs [137]. In the present study, 0.01% v/v GA concentration (Fig.. 4.10e) and duration of 2 h for cross-linking (Fig.. 4.10f) resulted in maximum recovery of CLEA activity. Similar to earlier reports, decreased CLEA activity was observed at higher GA concentrations [14, 195]. Also, increasing cross-linking time lead to decline in activity of Xy-CLEAs. Reason for the observed trends in both cases is the decreased structural flexibility of enzyme molecules [194]. Also there is production of large sized and highly rigid CLEAs with decreased activity and limited mass transfer [196]. On the contrary, very low cross-linker concentrations and short cross-linking time lead to insufficient cross-linking and production of highly flexible CLEAs with low activity [13, 155].

4.8.2 Characterization of Xy-CLEAs

4.8.2.1 Morphological analysis

Analysis of shape and size of Xy-CLEAs through SEM (Fig.. 4.11 a&b) and DLS (Fig.. 4.11c) respectively, revealed spherical structures of 202 nm diameter with rough and porous surface texture. The porous surface of Xy-CLEAs enhances mass diffusion by increasing the accessibility of CLEAs to substrate molecules, which leads to increase in reaction rate 150. Xy-CLEAs were categorized under type I aggregates of CLEAs, as they appeared clusters of ball like structures [130]. Type I category of CLEAs have been reported to have high surface area which makes the catalytic sites more accessible towards the substrate molecules [14, 195]. Size of CLEAs also has significance because it influences the filterability as well as mass transfer. By optimizing the cross linking time and ratio of enzyme with respect to cross linker; the size of CLEAs can be modified [125]. The observations from the morphological analysis of GTX1 Xy-CLEAs were similar to Xy-CLEAs of *Cohnella* sp. AR92, reported in the recent study [14].

4.8.2.2 Chemical characterization

Through FTIR analysis variations in the absorption spectra of immobilized xylanase were observed with respect to the free xylanase (Fig.. 4.11d). Peaks of free xylanase were within the range of 3650-3590 cm^{-1}, corresponding to symmetric stretching vibration of O-H bond.

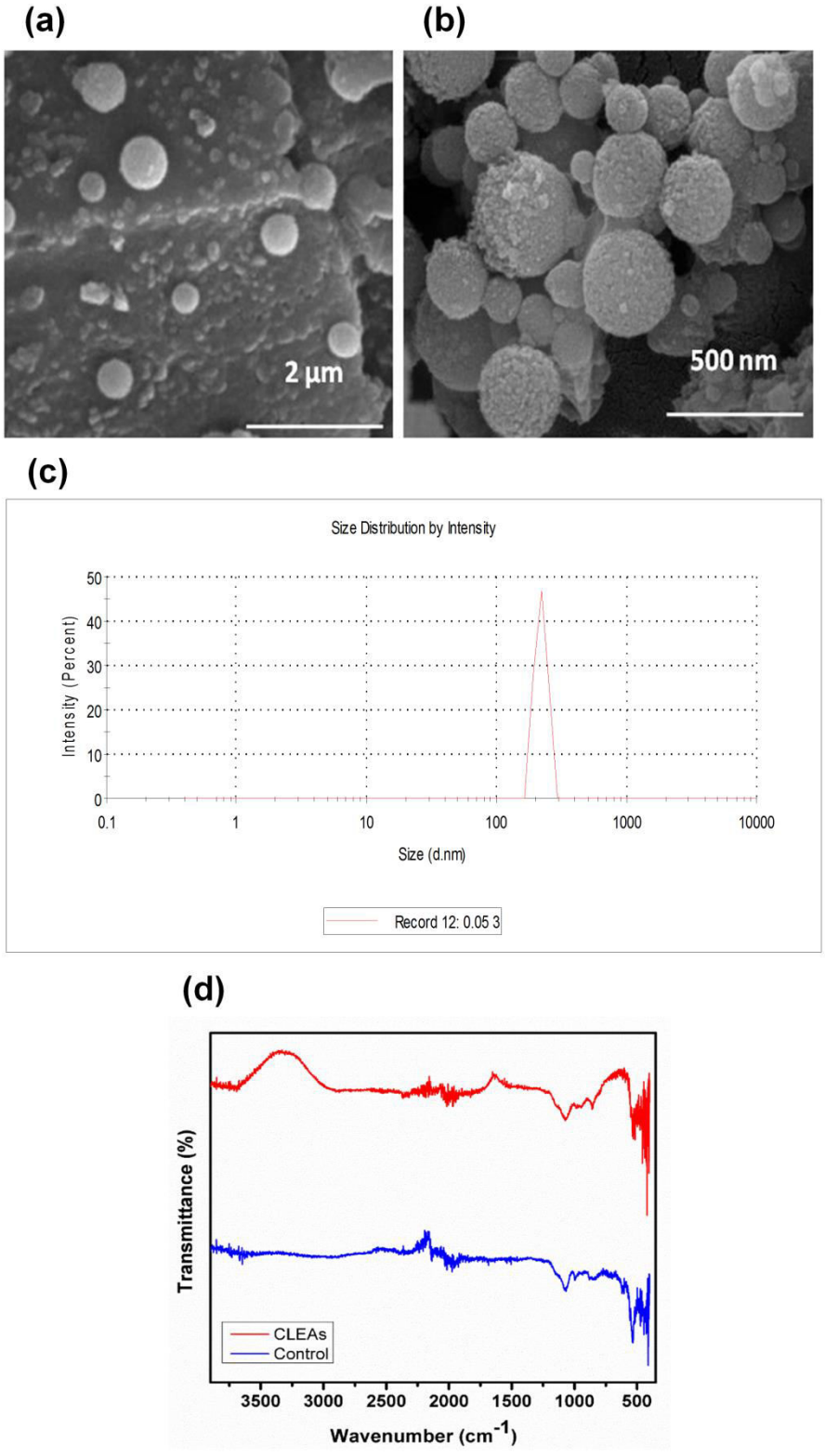


Figure 4.11: Morphological and structural characterization of Xy-CLEAs (a) SEM image of Xy-CLEAs at magnification 40,000 x (b) SEM image of Xy-CLEAs at magnification 100, 000 x (c) DLS analysis of Xy-CLEAs (d) FTIR spectra of free xylanase (control) and Xy-CLEAs.

Xy-CLEAs rendered peak at 1548 cm^{-1} corresponding to amide II band (1550-1520 cm^{-1}) and a sharp peak at 859 cm^{-1} again corresponding to N-H bond. Whereas, in free xylanase spectra, the two peaks (1548 cm^{-1} and 859cm^{-1}) were found shifted to 1538 cm^{-1} and 856 cm^{-1}, respectively [14]. This shifting of peaks corresponds to the conformational sensitivity of amide bonds as a result of cross-linking. Variations in the position of peaks confirmed cross-linking which lead to conformational alterations of amide band. The shifting in peaks of N-H bond is attributed to varaitions in hydrogen bonds and sulphur bridges within protein structure; as reported in earlier reports [197].

4.8.2.3 Biochemical characterization

4.8.2.3.1 Optimum pH and temperature

The optimum temperature for both, free xylanase and Xy-CLEAs was 70°C, as shown in Fig. 4.12a.

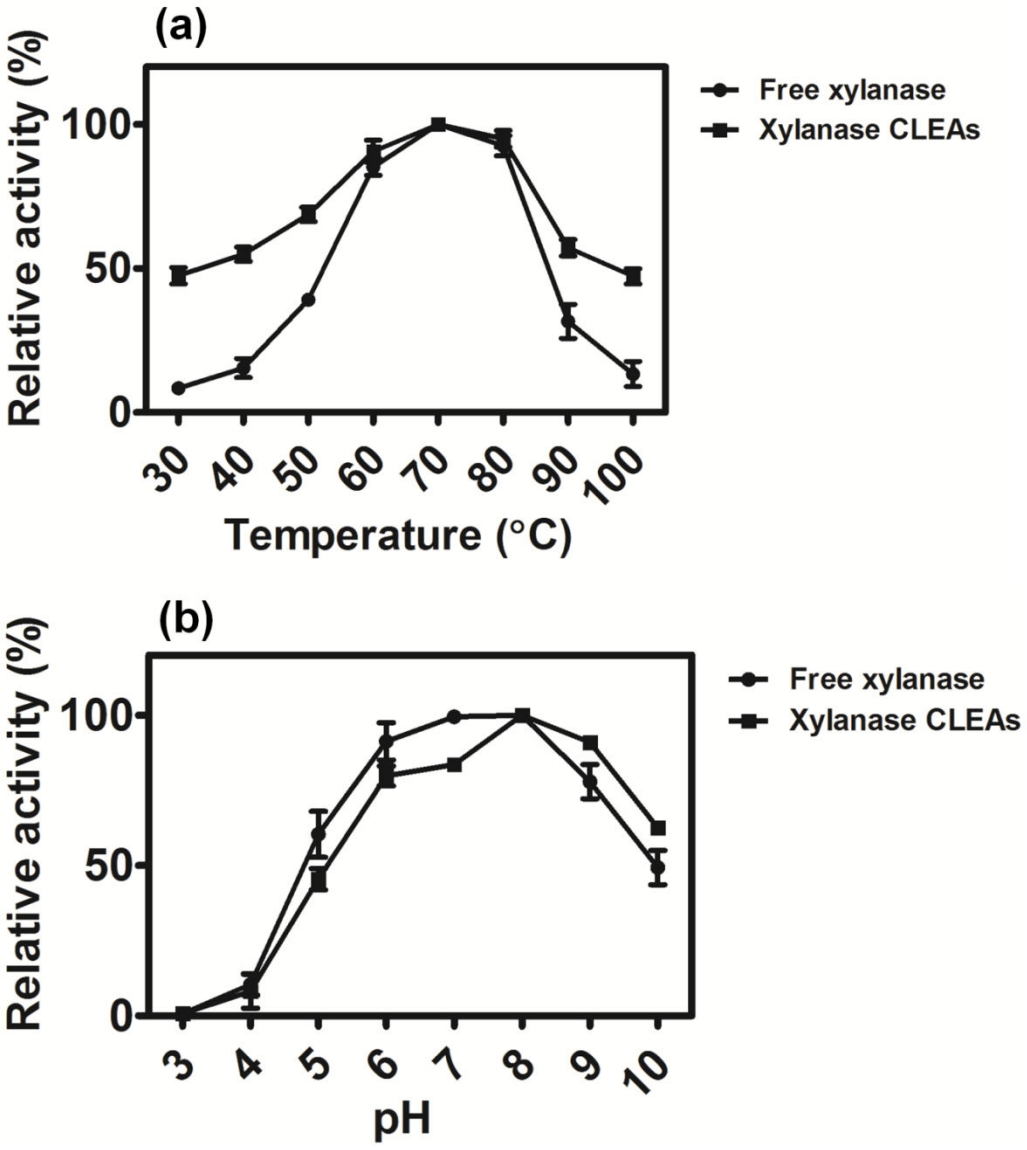


Figure 4.12: Tempearture and pH optima of Xy-CLEAs (a) effect of varying temperature (30-100°C) on free and immobilized xylanase from *Geobacillus thermodenitrificans* X1 (b) effect of varying on free and immobilized xylanase from *Geobacillus thermodenitrificans* X1. All values are mean of three replicates ± SE.

At higher temperatures (90°C and 100°C), immobilized xylanase comparatively showed higher activity (57.2% and 47.3% respectively) due to the strong covalent cross-linking within CLEA structure that prevents enzyme denaturation by heat [198]. Similar observations were made by Vrsanska et al. [193], who reported similar temperature optima for both free and immobilized laccase from *Trametes versicolor* and *Fomes fomentarius*. Unlike temperature, the pH profile was different for free and immobilized xylanase from GTX1. Range of pH 7-8 was optimum for free xylananse, whereas only pH 8 was optimum for maximum activity of Xy-CLEAs (Fig. 4.12b). After immobilization, alterations in the pH profile were due to conformational modifications in the enzyme, resulting from interactions between cross-linker and free basic residues on enzyme surface [155]. Studies by Xu et al. [199] and Vrsanska et al. [193] reported similar pH optima for both; CLEAs and free form of tyrosinase and laccase respectively.

4.8.2.3.2 Thermostability and pH stability

Enhancement of stability at high temperature and diverse pH is one of the objectives of immobilizing enzymes. However, the structural integrity and stability attained by immobilized enzyme also depends on the type of immobilization strategy opted [200, 201]. In the present study, both CLEAs and free xylanase showed similar thermostability at 60°C after 2 h incubation (Fig. 4.13a). However, after 3 and 4 h incubation at 60°C, Xy-CLEAs were comparatively more stable. At 70°C, tremendous increase in the thermostability was observed for Xy-CLEAs with 53% retained xylanase activity after 4 h incubation. On the contrary, free xylanase showed comparatively lower thermostability by retaining only 15% of initial xylanase activity. Strong covalent bonds within the structure of CLEAs make them highly stable at high temperatures [145, 155, 199]. Menfaatli & Zihnioglu reported in their studies that trypsin CLEAs showed higher thermostability by retaining 15% xylanase activity in comparison to free trypsin with 5% xylanase relative activity [195]. Further, kinetic analysis revealed the deactivation constant (K_d) of free xylanase as 0.04 (at 60°C) and 0.465 (at 70°C), whereas for immobilized xylanase it was 0.013 (at 60°C) and 0.161 (at 70°C). On the other hand, $t_{1/2}$ of free xylanase was 17 h (at 60°C) and 1.5 h (at 70°C) whereas after immobilization it increased to 53 h (at 60°C) and 4 h (at 70°C).

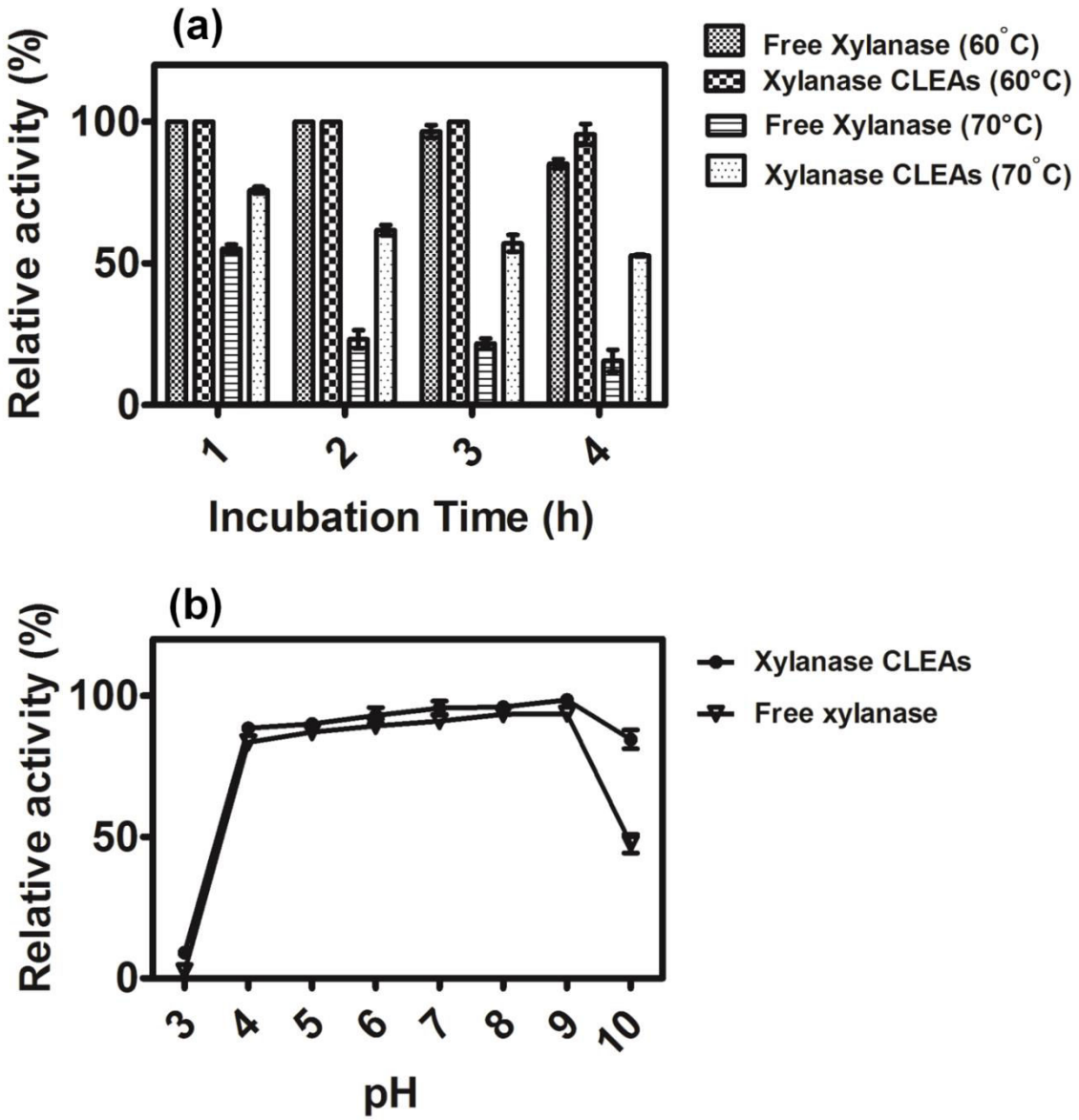


Figure 4.13: Thermostability and pH stability of Xy-CLEAs (a) thermostability of free and immobilized xylanase at 60°C and 70°C (b) stability of free and immobilized xylanase at different pH (3-10). All values are mean of three replicates ± SE.

Also, enhancement in the pH stability of xylanase was observed after its immobilization in the form of CLEAs (Fig. 4.13b). Specifically, in the alkaline range (9-10), Xy-CLEAs rendered comapratievly higher activity. Earlier studies had similar observation with respect to trypsin CLEAs with higher stability in range of pH 9-11 [195]. Similarly, studies by Vrsanska et al. [193] reported enhanced pH stability of laccase after immobilization. The enhanced pH stability of CLEAs is attributed to the cross-linked network of inter and intramolecular bonds within CLEA structure. Cross-linked network protects the reactive groups on the surface of

enzyme from the influence of pH alterations [202]. Also, cross-linking stabilizes the structural integrity of enzyme under varying pH.

4.8.2.3.3 Reusability and storage stability

Xy-CLEAs exhibited potential reusability till six consecutive cycles with 53.5% of retained xylanase activity after completion of sixth cycle (Fig. 4.14a).

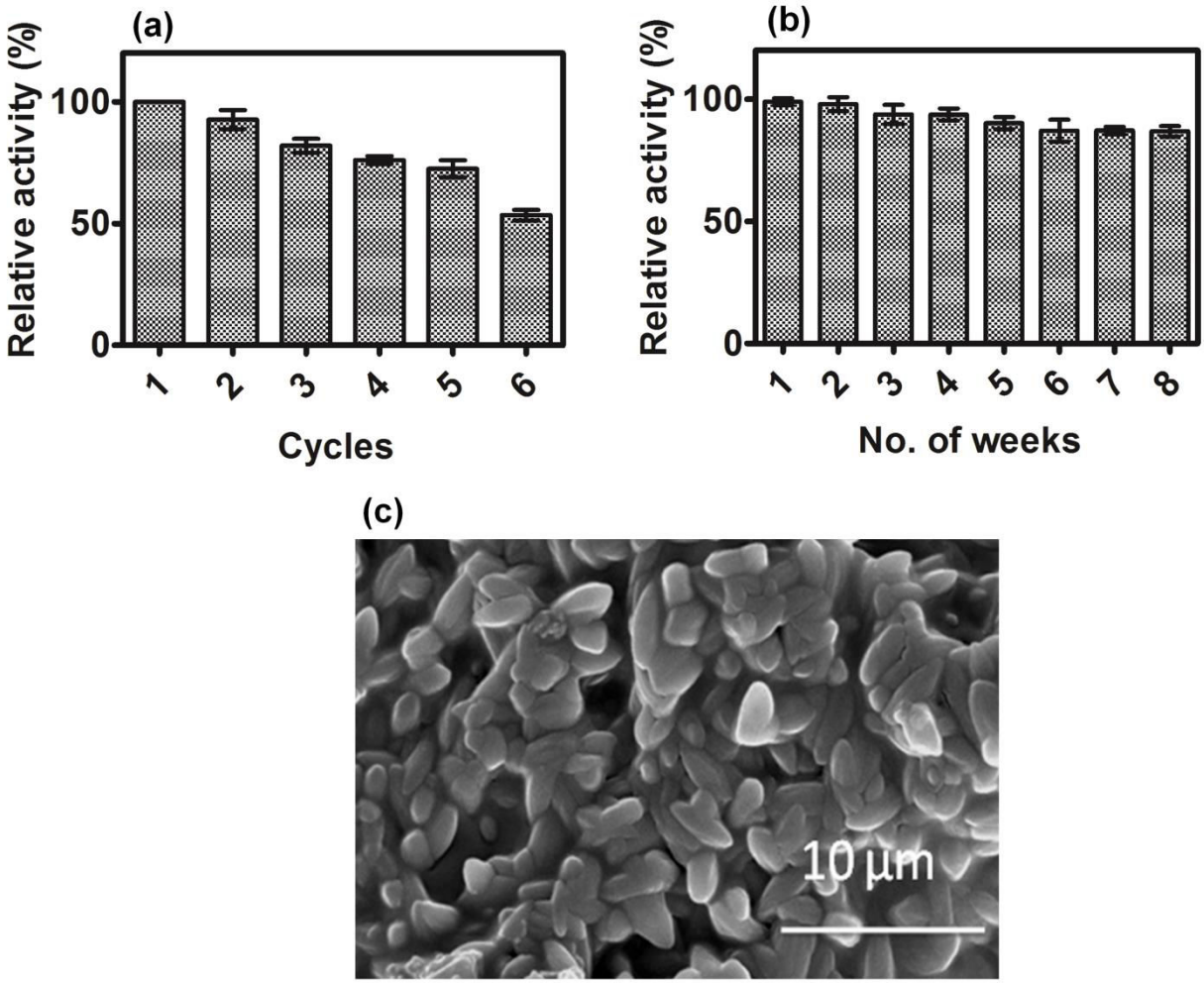


Figure 4.14: Operational stability and storage stability of Xy-CLEAs (a) reusability of Xy-CLEAs (b) storage stability of Xy-CLEAs. All values are mean of three replicates ± SE. (c) SEM images of Xy-CLEAs after six cycles of reusability; 12,000 X.

Decrease in the activity was observed with increasing cycles. The decrease in activity of Xy-CLEAs is because of structural alterations and mechanical shearing of CLEAs during centrifugation in each cycle. Clumping of CLEAs after centrifugation and loss of CLEAs due to washing with buffer after each cycle are additional reasons for decreased activity of Xy-CLEAs [158, 199]. The distortion of structure of Xy-CLEAs after being reused for six cycles

was observed under SEM imaging (Fig. 4.14c). Clumping and distortion of CLEAs also promotes decrease in activity due to mass transfer limitations [200, 203]. In earlier studies also, CLEAs have been reported to show tremendous reusability. Xylanase CLEAs from *Conhella sp.* AR92 retained 50% relative activity after being reused for repetitive four cycles [14]. 50% relative laccase activity was retained by laccase CLEAs from *Fomes fomantarius* after six repetitive cycles [193]. In addition to reusability, CLEAs are beneficial in terms of showing better storage stability than free enzymes. GTX1 Xy-CLEAs retained 86% xylanase activity after 8 weeks storage at 4°C (Fig. 4.14b). Higher storage stability of CLEAs is because of cross-linked matrix, which limits conformational variations during the storage duration. Comparatively, free enzyme is more prone to such variations, which further results in decreased activity [204]. Literature studies evident variations in storage stability of CLEAs. Commercial pancreatic trypsin CLEAs on 4 weeks storage at 4°C; retained 69% of initial activity [195]. Whereas, CLEAs of peroxidase from *Brassica rapa* showed no activity loss on storage at 4°C for more than three months [150].

4.9 Application of crude xylanase for bio-bleaching of paper pulp

4.9.1 Pretreatment of pulp with crude xylanase

The agro-pulp prepared obtained after sodium cooking had an initial pH of 8.6. Considering alkaline stability potential of GTX1 xylanase, it was directly used for pretreating the alkaline pulp.

Table 4.5: Pretreatment of agropulp with crude xylanase.

DECKER STAGE (XYLANASE PRETREATMENT)				
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample**
1.	pH	-	8.6	8.6
2.	Kappa no.	-	13.4 ± 0.1	13.4 ± 0.1
3.	Brightness	ISO	38.7 ± 0.3	38.7 ± 0.3
4.	Temperature	°C	70.0	70.0
5.	Enzyme	U/mL/g of pulp	-	50.0
6.	Retention time	Minutes	90	90

This is an added advantage as there was no need for neutralization of the pulp before pretreating with xylanase. After xylanase pretreatment, values of kappa no. and brightness were same for both control and test samples (Table 4.5). Similar observations were made during pretreatment of wheat straw pulp with commercial endoxylanase Cat X2753 [205]. No immediate variations in kappa no. and brightness were observed after xylanase pretreatment because, xylanase was not directly involved in lignin removal. Its action, rather partially hydrolyze the lignin molecules and also elevates the porosity of pulp, which further enhances the accessibility of pulp towards other bleaching agents [8]. Consequently, variations in kappa no. and brightness are observed in the succeeding bleaching stages.

4.9.2 Chlorination Stage

Chlorine selectively solubilizes lignin, a part of which is removed after washing and the residual lignin becomes more accessible for further bleaching. After chlorination, increased brightness was observed in all the samples (Table 4.6) with T1 showing maximum brightness (46.4 ± 0.6 ISO), followed by T2 (44.6 ± 0.7 ISO) and control sample (42.8 ± 0.6 ISO).

Table 4.6: First stage of bleaching, using chlorine as a chemical bleaching agent.

CHLORINATION STAGE					
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample (T1)**	**Test sample (T2)**
1.	Chlorine dosing	%	4.0	4.0	3.2
2.	Consistency	-	3.0	3.0	3.0
3.	Initial pH	-	2.6	2.6	2.8
4.	Temperature	°C	30	30	30
5.	Retention time	Minutes	45	45	45
6.	pH after washing		7.0	7.2	7.3
7.	Brightness	ISO	42.8 ± 0.6	46.4 ± 0.6	44.6 ± 0.7

Comparatively, higher brightness of both the test samples (T1 and T2) with respect to the control sample highlights the positive influence of xylanase pretreatment. Moreover, better brightness was shown by test sample in comparison to control, despite reducing the chorine dosage by 20%. Earlier studies also report reduction in chlorine consumption after pretreatment of pulp with xylanase. Chlorine reduction by 18% was observed after pretreating bagasse pulp with xylanase from *Thermomyces lanuginosus* SSBP [206]. Chlorine consumption by 12.5% was reduced by application of xylanase from *Cellulosimicrobium cellulans* CKMX1 for pretreating wheat straw pulp [8].Similarly, chlorine reduction by 17% and 24% was observed on carrying out pulp biobleaching with xylanase from *Streptomyces griseorubens* [191] and recombinant *Bacillus subtilis* [57], respectively.

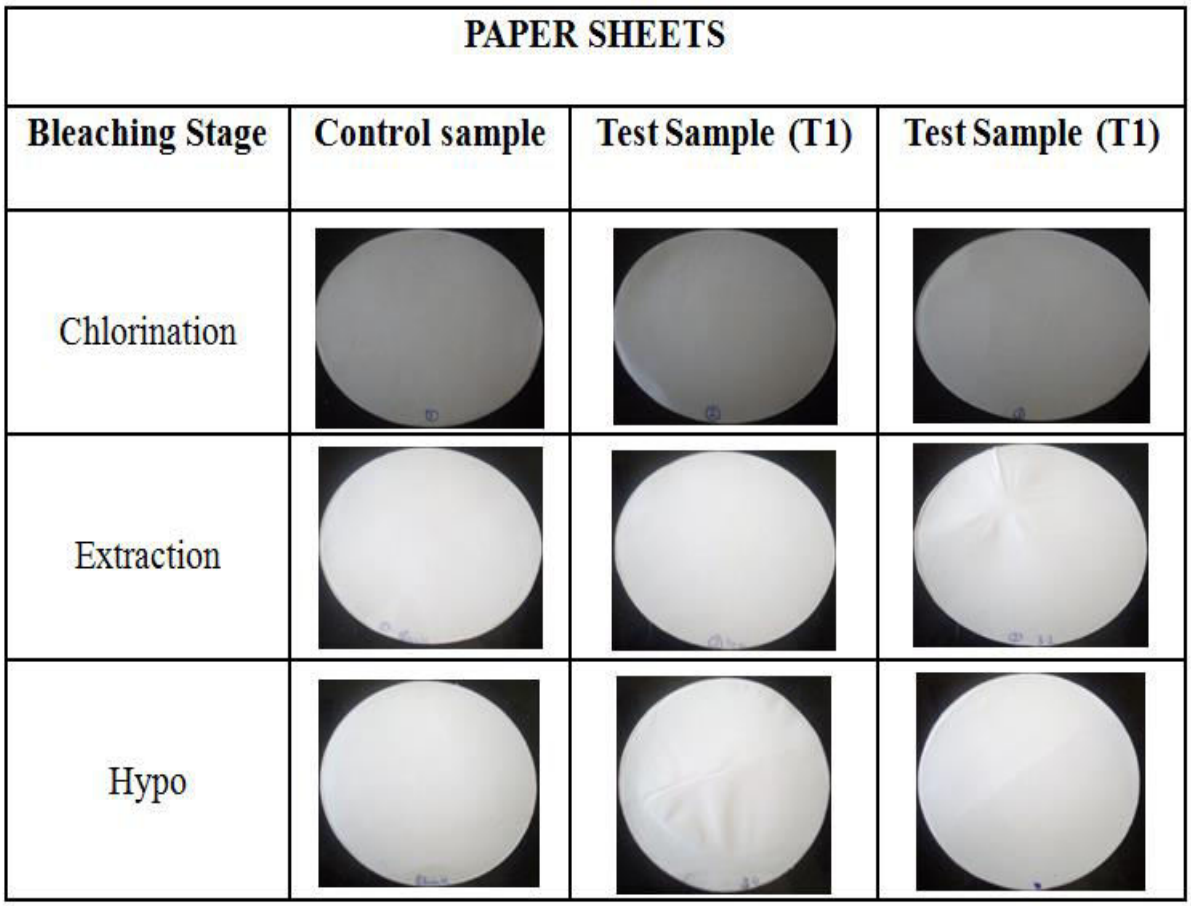


Figure 4.15: Paper sheets generated from the pulp samples after each bleaching stage.

In the present study, paper sheets generated from the chlorinated pulp appeared pale-yellowish in color as shown in Fig. 4.15, due to the presence of residual lignin molecules which require further bleaching for complete removal.

4.9.3 Extraction stage

Lignin gets easily solubilized under alkaline conditions; which makes its removal by bleaching agents easier under alkaline pH. That is why in the extraction stage, wheat-straw bagasse pulp was treated with 2% caustic soda to create alkaline conditions. For boosting up the efficiency of caustic soda, hydrogen peroxide was also added. Again brightness of the test samples T1 (78.0 ± 0.7 ISO) and T2 (77.0 ± 0.6 ISO) was greater than the control (75.2 ± 0.7 ISO) sample, shown in Table 4.7.

Table 4.7: Hydrogen-peroxide reinforced extraction of lignin in alkaline environment.

EXTRACTION STAGE					
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample (T1)**	**Test sample (T2)**
1.	Caustic dosage	%	4	4	3.2
2.	H_2O_2 dosage	Kg/ton	12	12	12
3.	Consistency	%	10.1	10.1	10.1
4.	Initial pH	-	11.6	11.3	11.1
5.	Temperature	°C	70	70	70
6.	Retention time	Minutes	120	120	120
7.	Final pH	-	10.5	11.2	11.0
8.	Kappa no.	-	1.5 ± 0.2	1.1 ± 0.2	1.3 ± 0.1
9.	Brightness	ISO	75.2 ± 0.7	78.0 ± 0.7	77.0 ± 0.6

Also, the kappa no. values for both the test samples was lower than control which shows better lignin removal in case of test samples. These observations mark the potential of GTX1 xylanase to enhance the efficiency of lignin removal by other bleaching agents. Recent studies report also report comparatively greater brightness and lower kappa no. after extraction stage in case of xylanase pretreated pulp, with respect to the untreated one [207, 208]. Paper sheets generated from the pulp after alkali extraction were brighter and off white in color (Fig. 4.15).

4.9.4 Hypo stage

In this stage, hypochlorite decolorizes the chromophoric groups of pulp fibers and imparts brightness to pulp. Hypochlorite treatment needs to be carried out carefully under optimal pH and temperature conditions because hypochlorite also reacts with the cellulose fraction of pulp. However, pH plays the most significant role during this bleaching stage. When the process is carried out under alkaline conditions above pH 8, hypochlorous anion (OCl^-) is formed.

Table 4.8: Final stage of bleaching, using sodium hypochlorite as bleaching agent and sulphamic acid as an enhancer of this bleaching reaction.

HYPO STAGE					
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample (T1)**	**Test sample (T2)**
1.	Hypochlorite dosing	%	2.5	2.5	2.5
2.	Sulphamic acid	%	0.1	0.1	0.1
3.	Consistency	%	10.5	10.5	10.5
4.	Initial pH	-	9.2	9.2	9.3
5.	Temperature	°C	45	45	45
6.	Retention time	Minutes	120	120	120
7.	Final pH	-	6.7	6.8	6.8
8.	Brightness	ISO	82.8 ± 0.7	85 ± 0.7	84 ± 0.8

On the other hand, when pH is below 8, there occurs formation of chorine (Cl_2) and hypochlorus acid (HOCl), which has deteriorating effect, on cellulose. In this context, initial pH of hypochlorite reaction in the current study was maintained as 9.2 for all the pulp samples. After hypo stage, paper sheets generated appeared brighter than in the previous stage (Fig.. 4.11c). Test samples T1 and T2 rendered brightness of 85 ± 0.7 ISO and 84 ± 0.8 ISO, respectively (Table 4.8). Whereas, comparatively lower brightness was observed for control sample; showing 82.8 ± 0.7 ISO brightness. Thus, after the final bleaching stage it was

concluded that xylanase pretreated samples showed increase in brightness by 2.2 (T1 sample) and 1.2 (T2 sample) points in comparison to untreated sample (control). Especially, >80 ISO brightness shown by test sample (T2) despite 20% reduction in chlorine dosage is appreciable at commercial level and also an environment friendly approach. Other studies have also reported increased final brightness of paper generated from xylanase pretreated pulp. Application of crude xylanase from *Cellulosimicrobium cellulans* CKMX1 for pulp biobleaching lead to increase in final brightnes of paper by 0.8 points [8]. Many other literature studies had observations in agreement with the current study [209, 210, 211].

4.9.5 Properties of paper

Alongwith brightness, physical and strength paper properties have significant contribution in deciding the quality of paper. Thus evaluation of properties of paper is an indispensable step after completion of the bleaching process. Also, the assessment of paper properties reflects the efficiency and aftereffects of bleaching steps on the paper quality. In the present study, properties of the paper generated from respective pulp samples were compared to each other (Table 4.9). It was observed that, the properties like basis weight, bulk weight, burst factor and viscosity were comparable among the control and test samples, whereas freeness, breaking length and tear factor were increased in case of xylanase treated pulp samples. Freeness was increased by 1 point and a decrease of 1 point was observed for basis wt. Tremendous increase was observed for breaking length and tear factor for test samples T1 (4100 ± 4.7) and T2 (4065 ± 4.8), with respect to the control (3865 ± 7.2). From the results it is concluded that xylanase pretreatment had positive impact on the properties of paper and significantly reduced the consumption of chlorine dosage as well. In agreement with the current results, literature reports evident about modifications in the properties of paper after xylanase pretreatment. Some properties may get increased [61], while some bear the negative impetus [205] of enzyme pretreatment. Walia et al. [8] reported decrease in burst factor and tear factor for xylanase pretreated paper.Whereas, increase in breaking length and bulk weight for the same sample. Buzala et al (2018) observed positive impact on paper properties after paper pulp pretreatment with xylanase form *N. patriciarum* xylanase [212].

Table 4.8: Final stage of bleaching, using sodium hypochlorite as bleaching agent and sulphamic acid as an enhancer of this bleaching reaction.

HYPO STAGE					
S.No.	**Particulars**	**Unit**	**Control sample**	**Test sample (T1)**	**Test sample (T2)**
1.	Hypochlorite dosing	%	2.5	2.5	2.5
2.	Sulphamic acid	%	0.1	0.1	0.1
3.	Consistency	%	10.5	10.5	10.5
4.	Initial pH	-	9.2	9.2	9.3
5.	Temperature	°C	45	45	45
6.	Retention time	Minutes	120	120	120
7.	Final pH	-	6.7	6.8	6.8
8.	Brightness	ISO	82.8 ± 0.7	85 ± 0.7	84 ± 0.8

Increase of 13% was observed in tensile index whereas tear index remained unaffected. They also concluded that pretreatment of paper pulp with the cellulase free xylanase or the one with low cellulolytic activity is beneficial in terms of improving the paper strength properties. However, sometimes the paper properties are not significantly modified after xylanase pretreatment. Chen et al. [213], reported that the tear, tensile and burst indices of paper remained unchanged after xylanase pretreatment of paper pulp. The positive impetus of xylanase pretreatment of pulp in the present study, unveils the efficiency of GTX1 xylanase in bio-bleaching. The dosage of GTX1 xylanase as a pre-bleaching agent, lead to decrease in chlorine dosage consumption and also enhanced the strength properties of paper. Moreover, GTX1 xylanase rendered the required thermostability and alkaline stability in paper pulp bleaching process. Thus, GTX1 xylanase holds promising potential to be exploited in paper industry.

SUMMARY & CONCLUSION

The current study elucidates the potential of *Geobacillus thermodenitrificans* X1 (GTX1) for cost-effective xylanase production through valorization of lignocellulosic residues. GTX1 xylanase thus produced was observed as efficient biobleaching agent to be used in paper industry. The major findings have been summarized as follows:

Thermophilic bacteria were isolated from Tattapni hot spring soil sample. All the isolates were screened for xylanase activity through Congo red assay and estimation of xylanase activity in liquid production medium through dinitrosalicylic acid method. The isolate with maximum xylanase activity was identified as *Geobacillus thermodenitrificans* X1, through phylogenetic analysis. Xylanase from GTX1 was produced under submerged fermentation conditions. Various influential physico-chemical and nutritional parameters were optimized one by one for xylanase production, using OFAT method. The optimized conditions attained from OFAT were 1.5% inoculums size, 96 h incubation time, 50°C, pH 8, 1.5% xylan concentration, 0.05g/L of peptone and 200 rpm agitation speed rate. For cost-effective xylanase production, commercial xylan was replaced by cheaper lignocellulosic residues such as wheat straw, rice straw, corn cob and wheat bran. Maximum xylanase production was observed on using wheat straw as substrate. Further, statistical optimization was practiced through RSM to study mutual interactions among the significant parameters which were temperature, agitation speed rate and incubation time. The central composite design of RSM generated 20 different combinations of the selected parameters. Xylanase production was carried out under all the combinations and xylanase activity observed was recorded as the response value for each combination. From ANOVA it was concluded that only one interaction was significant, i.e. between temperature and incubation time. Eventually, the optimized conditions predicted by the statistical software of RSM (Design Expert 10) were 50°C, 87 h and 177 rpm with predicted activity of 23.9 U/mL. Xylanase production was thus carried out under these predicted conditions and 24±2 U/mL activity was observed. The statistical model was thus validated as the experimental value of xylanase activity was close to the predicted one. The crude xylanase produced under optimized conditions was also assayed for accessory cellulases and hemicellulases. Negligible traces of accessory hemicellulases and cellulases were observed; showing endoxylanase is the major enzyme in the crude preparation and is cellulase free. Further, characteristic features of GTX1 xylanase were studied. The temperature and pH optima was 70°C and 8 respectively and the enzyme

showed appreciable thermostability with 90.8% retained activity at 60°C, after 4 h. Also, GTX1 xylanase showed higher stability in alkaline range of pH 8-10. In the presence of Ca^{+2} ions, GTX1 xylanase activity was highly stimulated while it was strongly inhibited by the effect of Mn^{+2} ions. Hydrolytic efficiency of GTX1 was assessed by quantifying the hydrolysis products through HPLC. Variety of xylooligosaccharides were generated; 0.22 ± 0.01 g/L xylose, 1.46 ± 0.02 g/L xylobiose, 1.64 ± 0.02 g/L xylotriose and 0.65 ± 0.01 g/L xylotetrose. Molecular characterization and zymogram of GTX1 xylanase revealed gene lenghth of 1,224 bp (accession no. MG874777) and 45 kDa molecular weight of GTX1 xylanase. Furhter, to enhance the industrial applicability of GTX1 xylanase, it was immobilized through carrier free immobilization to form crude xylanase CLEAs. Conditions for preparation of Xy-CLEAs were optimized and were observed as 1.7 mg/mL of protein concentration, 120 min precipitation time, 0.01% v/v glutaraldehyde as cross-linker and 2 h reaction time for cross-linking. GTX1 Xy-CLEAs prepared were found spherical in shape with 202 nm diameter. Variations in the FTIR spectra of free and immobilized xylanase confirmed structural and functional variations after the preparation of Xy-CLEAs. The optimum pH and temperature of free xylanase and Xy-ClEAs was similar (pH 8 & 70°C), but deviation in the overall pH and temperature profile was observed after immobilization due to conformational alterations. Thermostability of Xy-CLEAs was higher than the free xylanase at both 60°C and 70°C. Tremendous increase in thermostability was observed at 70°C, where retained xylanase activity increased from 15% (free xylanase) to 53% (Xy-CLEAs). Kinetic studies revealed that $t^{1/2}$ value was increased by 69.7% at 60°C and 62.5% at 70°C, after the formation of Xy-CLEAs. Similarly, pH stability was also increased after immobilization; specifically the percent increase was more prominent in the alkaline range. GTX1 xylanase CLEAs also rendered 53.5% reusability after six cycles and retained 86% activity after 8 weeks storage at 4°C. Eventually, the main objective of our study was to estimate the potential of GTX1 xylanase in paper pulp bleaching. Application study was carried out in R&D lab of Kauntum Papers Ltd. paper industry, Hoshiarpur, Punjab. Application of GTX1 xylanase increased the brightness of the paper by 2.2 ISO. Moreover, despite 20% reduction in chlorine dosage, brightness was increased by 1.2 ISO. Paper properties like breaking lenghth and tear factor were tremendously increase after xylanase application; whereas other properties like basis weight, bulk weight, burst factor, viscosity and freeness were comparable with the control.

In nutshell, it could be concluded that thermophilic bacteria *Geobacillus thermodenitrificans* X1 can substantially produce xylanase by utilizing cheaper lignocellulosic substrates. The characteristics of GTX1 xylanase are suitable for its application in the robust conditions of paper industry. Application studies conducted in the present study reinforce the potential of GTX1 xylanase as an efficient biobleaching agent for increasing the brightness and properties of paper. Reduction in the chlorine consumption by application of GTX1 xylanase is an added advantage for curbing the environmental impact.

REFERENCES

1. T. Collins, C. Gerday, and G. Feller, "Xylanases, xylanase families and extremophilic xylanases," *FEMS Microbiol. Rev.*, vol. 29, pp. 3-23, 2005.
2. S. Subramaniyan and P. Prema, "Biotechnology of Microbial Xylanases: Enzymology, Molecular Biology, and Application," *Crit. Rev. Biotechnol.*, vol. 22, pp. 33-64, 2002.
3. B. S. Chadha, B. Kaur, N. Basotra, A. Tsang, and A. Pandey, "Thermostable xylanases from thermophilic fungi and bacteria: Current perspective," *Bioresour. Technol.*, vol. 277, pp. 195-203, 2019.
4. Z. Bibi, A. Ansari, R. R. Zohra, A. Aman, and S. A. Ul Qader, "Production of xylan degrading endo-1, 4-β-xylanase from thermophilic *Geobacillus stearothermophilus* KIBGE-IB29," *J. Radiat. Res. Appl. Sci.*, vol. 7, pp. 478-485, 2014.
5. A. C. Mongra, "Distribution pattern of Cyanobacteria in hot water springs of Tattapani, Himachal Pradesh, India," *J. Acad. Ind. Res.*, vol. 1, 2012.
6. D. Kumar *et al.*, "Xylanases and their industrial applications: A review," *Biochem. Cell. Arch.*, vol. 17, pp. 353-360, 2017.
7. S. Singh, A. M. Madlala, and B. A. Prior, "*Thermomyces lanuginosus*: properties of strains and their hemicellulases," *FEMS Microbiol. Rev.*, vol. 27, pp. 3-16, 2003.
8. A. Walia, P. Mehta, S. Guleria, and C. K. Shirkot, "Modification in the properties of paper by using cellulase-free xylanase produced from alkalophilic *Cellulosimicrobium cellulans* CKMX1 in biobleaching of wheat straw pulp," *Can. J. Microbiol.*, vol. 61, pp. 671-681, 2015.
9. S. K. S. Patel *et al.*, "Fe_2O_3 yolk-shell particle-based laccase biosensor for efficient detection of 2,6-dimethoxyphenol," *Biochem. Eng. J.*, vol. 132, pp. 1-8, 2018.
10. R. C. Rodrigues *et al.*, "Immobilization of proteins in poly-styrene-divinylbenzene matrices: functional properties and applications," *Curr. Org. Chem.*, vol. 19, pp. 1707-1718, 2015.
11. H. Yamaguchi, Y. Kiyota, and M. Miyazaki, "techniques for preparation of cross-linked enzyme aggregates and their applications in bioconversions," *Catalysts*, vol. 8, p. 174, 2018.
12. S. S. Nadar, A. B. Muley, M. R. Ladole, and P. U. Joshi, "Macromolecular cross-linked enzyme aggregates (M-CLEAs) of α-amylase," *Int. J. Biol. Macromol.*, vol. 84, pp. 69-78, 2016.

13. S. Talekar, V. Shah, S. Patil, and M. Nimbalkar, “Porous cross linked enzyme aggregates (p-CLEAs) of *Saccharomyces cerevisiae* invertase,” *Catal. Sci. Technol.*, vol. 2, pp. 1575-1579, 2012.
14. J. S. Hero, C. M. Romero, J. H. Pisa, N. I. Perotti, C. Olivaro, and M. A. Martinez, “Designing cross-linked xylanase aggregates for bioconversion of agroindustrial waste biomass towards potential production of nutraceuticals,” *Int. J. Biol. Macromol.*, vol. 111, pp. 229-236, 2018.
15. G. Malhotra and S. S. Chapadgaonkar, “Production and applications of xylanases – an overview,” *BioTechnologia*, vol. 99, pp. 59-72, 2018.
16. J. Hu, V. Arantes, and J. N. Saddler, “The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect?,” *Biotechnol. Biofuels*, vol. 4, p. 36, 2011.
17. P. Khonzue *et al.*, “Optimization of xylanase production from *Aspergillus niger* for biobleaching of eucalyptus pulp,” *Biosci. Biotechnol. Biochem.*, vol. 75, pp. 1129-1134, 2011.
18. S. Bhuvaneshwari, H. Hettiarachchi, and J. N. Meegoda, “Crop residue burning in india: policy challenges and potential solutions,” *Int. J. Environ. Res. Public. Health*, vol. 16, p. 832, 2019.
19. R. L. Whistler and E. Masak, “Enzymatic hydrolysis of xylan1,” *J. Am. Chem. Soc.*, vol. 77, pp. 1241-1243, 1955.
20. R. A. Prade, “Xylanases: from biology to biotechnology,” *Biotechnol. Genet. Eng. Rev.*, vol. 13, pp. 101-132, 1996.
21. E. A. Rennie and H. V. Scheller, “Xylan biosynthesis,” *Curr. Opin. Biotechnol.*, vol. 26, pp. 100-107, 2014.
22. S. K. Chanda, E. L. Hirst, J. K. N. Jones, and E.G.V.Percival, “262. The constitution of xylan from esparto grass (*Stipa tenacissima*, L.),” *J. Chem. Soc.* (Resumed), pp.1289-1297, 1950.
23. S. Eda, A. Ohnishi, and K. Kato, “Xylan isolated from the stalk of *Nicotiana tabacum*,” *Agric. Biol. Chem.*, vol. 40, pp. 359-364, 1976.
24. V. C. Barry and T. Dillon, “Occurrence of xylans in marine algae,” *Nature*, vol. 146, p. 620, 1940.
25. A. Sunna and G. Antranikian, “Xylanolytic enzymes from fungi and bacteria,” *Crit. Rev. Biotechnol.*, vol. 17, pp. 39-67, 1997.

26. P. Biely, J. Puls, and H. Schneider, “Acetyl xylan esterases in fungal cellulolytic systems,” *Febs Letters*, vol. 186, pp.80-84, 1985.
27. S. Shulami, O. Gat, A. L. Sonenshein, and Y. Shoham, “The glucuronic acid utilization gene cluster from *Bacillus stearothermophilus* T-6,” *J. Bacteriol.*, vol. 181, p. 10, 1999.
28. A. Teplitsky, S. Shulami, S. Moryles, Y. Shoham, and G. Shoham, “Crystallization and preliminary X-ray analysis of an intracellular xylanase from *Bacillus stearothermophilus* T-6,” *Acta Crystallogr. D Biol. Crystallogr.*, vol. 56, pp. 181-4, 2000.
29. F. L. Motta, C. C. P. Andrade and M. H. A. Santana, “A review of xylanase production by the fermentation of xylan: classification, characterization and applications,” in *Sustainable degradation of lignocellulosic biomass-techniques, applications and commercialization*, vol. 1, 2013.
30. T. Collins, M.-A. Meuwis, I. Stals, M. Claeyssens, G. Feller, and C. Gerday, “A Novel family 8 xylanase, functional and physicochemical characterization,” *J. Biol. Chem.*, vol. 277, pp. 35133-35139, 2002.
31. B. L. Cantarel, P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, and B. Henrissat, “The Carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics,” *Nucleic Acids Res.*, vol. 37, pp. D233-D238, 2009.
32. B. Henrissat and A. Bairoch, “New families in the classification of glycosyl hydrolases based on amino acid sequence similarities.,” *Biochem. J.*, vol. 293, pp. 781-788, 1993.
33. G. Paes, J. G. Berrin, and J. Beaugrand, “GH11 xylanases: structure/function/properties relationships and applications,” *Biotechnol. Adv.*, vol. 30, pp. 564-592, 2012.
34. A. Torronen and J. Rouvinen, “Structural and functional properties of low molecular weight endo-1,4-β -xylanases,” *J. Biotechnol.*, vol. 57, pp. 137-149, 1997.
35. R. Gaur, S. Tiwari, P. Rai and V. Srivastava, “Isolation, production, and characterization of thermotolerant xylanase from solvent tolerant *Bacillus vallismortis* RSPP-15.” *Int. J. Polym. Sci.*, 2015.
36. A. Khasin, I. Alchanati, and Y. Shoham, “Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6,” *Appl. Env. Microbiol*, vol. 59, pp. 1725-1730, 1993.

37. T. Satyanarayana, A. Sharma, D. Mehta, A. K. Puri, V. Kumar, M. Nisha and S. Joshi, "Biotechnological applications of biocatalysts from the *Firmicutes Bacillus* and *Geobacillus* Species," in *Microorganisms in Sustainable Agriculture and Biotechnology*, T. Satyanarayana and B. N. Johri, Eds. Dordrecht: Springer Netherlands, 2012, pp. 343-379.
38. L. Marcolongo, F. La Cara, A. Morana, A. Di Salle, G. Del Monaco, S. M. Paixao, L. Alves, and E. Ionata, "Properties of an alkali-thermo stable xylanase from *Geobacillus thermodenitrificans* A333 and applicability in xylooligosaccharides generation," *World J. Microbiol. Biotechnol.*, vol. 31, pp. 633-648, 2015.
39. A. Bhalla, K. M. Bischoff, and R. K. Sani, "Highly thermostable xylanase production from a thermophilic *Geobacillus* sp. strain WSUCF1 utilizing lignocellulosic biomass," *Front. Bioeng. Biotechnol.*, vol. 3, 2015.
40. S. Aikawa *et al.*, "Characterization and high-quality draft genome sequence of *Herbivorax saccincola* A7, an anaerobic, alkaliphilic, thermophilic, cellulolytic, and xylanolytic bacterium," *Syst. Appl. Microbiol.*, vol. 41, pp. 261-269, 2018.
41. M. Mechelke *et al.*, "Characterization of the arabinoxylan-degrading machinery of the thermophilic bacterium *Herbinix hemicellulosilytica*—Six new xylanases, three arabinofuranosidases and one xylosidase," *J. Biotechnol.*, vol. 257, pp. 122-130, 2017.
42. N. Boucherba *et al.*, "Biochemical properties of a new thermo- and solvent-stable xylanase recovered using three phase partitioning from the extract of *Bacillus oceanisediminis* strain SJ3," *Bioresour. Bioprocess.*, vol. 4, p. 29, 2017.
43. S. Mathew, A. Aronsson, E. N. Karlsson, and P. Adlercreutz, "Xylo- and arabinoxylooligosaccharides from wheat bran by endoxylanases, utilisation by probiotic bacteria, and structural studies of the enzymes," *Appl. Microbiol. Biotechnol.*, vol. 102, pp. 3105-3120, 2018.
44. M. Bibra, V. R. Kunreddy, and R. K. Sani, "Thermostable xylanase production by *Geobacillus* sp. strain DUSELR13, and its application in ethanol production with lignocellulosic biomass," *Microorganisms*, vol. 6, p. 93, 2018.
45. F. J. St John, C. Crooks, D. Dietrich, and J. Hurlbert, "Xylanase 30 A from *Clostridium thermocellum* functions as a glucuronoxylan xylanohydrolase," *J. Mol. Catal. B Enzym.*, vol. 133, pp. S445-S451, 2016.

46. R. Biswas, V. Sahai, S. Mishra, and V. S. Bisaria, "Development of mutants of *Melanocarpus albomyces* for hyperproduction of xylanase," *Biotechnol. Bioprocess Eng.*, vol. 15, pp. 800-809, 2010.
47. H. Xiong *et al.*, "Characterization of the xylanase produced by submerged cultivation of *Thermomyces lanuginosus* DSM 10635," *Enzyme Microb. Technol.*, vol. 35, pp. 93-99, 2004.
48. Y. Du *et al.*, "Characterization of three novel thermophilic xylanases from *Humicola insolens* Y1 with application potentials in the brewing industry," *Bioresour. Technol.*, vol. 130, pp. 161-167, 2013.
49. K. McPhillips, D. M. Waters, C. Parlet, D. J. Walsh, E. K. Arendt, and P. G. Murray, "Purification and characterisation of a β-1,4-xylanase from *Remersonia thermophila* CBS 540.69 and its application in bread making," *Appl. Biochem. Biotechnol.*, vol. 172, pp. 1747-1762, 2014.
50. A. M. Winger, J. L. Heazlewood, L. J. G. Chan, C. J. Petzold, K. Permaul, and S. Singh, "Secretome analysis of the thermophilic xylanase hyper-producer *Thermomyces lanuginosus* SSBP cultivated on corn cobs," *J. Ind. Microbiol. Biotechnol.*, vol. 41, pp. 1687-1696, 2014.
51. J. T. Ellis and T. S. Magnuson, "Thermostable and alkalistable xylanases produced by the thermophilic bacterium *Anoxybacillus flavithermus* TWXYL3," *ISRN Microbiol.*, vol. 2012, 2012.
52. A. Burlacu, C. P. Cornea, and F. Israel-Roming, "Microbial xylanase : a review," *Scientific Bulletin. Series F. Biotechnologies*, pp.335-342, 2016.
53. S. Guleria, A. Walia, A. Chauhan, and C. K. Shirkot, "Optimization of cultural conditions for cellulase-free xylanase production by mutant strain of alkalophilic *Cellulosimicrobium* sp. CKMX1 in submerged fermentation.," *Appl. Biol. Res.*, vol. 15, pp. 137-144, 2013.
54. S. Subramaniyan, "Isolation, purification and characterisation of low molecular weight xylanase from *Bacillus pumilus* SSP-34," *Appl. Biochem. Biotechnol.*, vol. 166, pp. 1831-1842, 2012.
55. B. K. Bajaj and N. P. Singh, "Production of xylanase from an alkalitolerant *Streptomyces* sp. 7b under solid-state fermentation, its purification, and characterization," *Appl. Biochem. Biotechnol.*, vol. 162, pp. 1804-1818, 2010.

56. P. Yadav *et al.*, "Production, purification, and characterization of thermostable alkaline xylanase from *Anoxybacillus kamchatkensis* NASTPD13," *Front. Bioeng. Biotechnol.*, vol. 6, 2018.
57. A. Anand, V. Kumar, and T. Satyanarayana, "Characteristics of thermostable endoxylanase and β-xylosidase of the extremely thermophilic bacterium *Geobacillus thermodenitrificans* TSAA1 and its applicability in generating xylooligosaccharides and xylose from agro-residues," *Extremophiles*, vol. 17, pp. 357-366, May 2013.
58. L. Cunha *et al.*, "Optimization of xylanase production from *Aspergillus foetidus* in soybean residue," *Enzyme Research*, 2018.
59. G. Ramanjaneyulu and B. Rajasekhar Reddy, "Optimization of xylanase production through response surface methodology by *Fusarium* sp. BVKT R2 isolated from forest soil and its application in saccharification," *Front. Microbiol.*, vol. 7, 2016.
60. S. Nagar, A. Mittal, D. Kumar, and V. K. Gupta, "Production of alkali tolerant cellulase free xylanase in high levels by *Bacillus pumilus* SV-205," *Int. J. Biol. Macromol.*, vol. 50, pp. 414-420, 2012.
61. A. Sanghi, N. Garg, K. Kuhar, R. C. Kuhad, and V. K. Gupta, "Enhanced production of cellulase-free xylanase by alkalophilic *Bacillus subtilis* ash and its application in biobleaching of kraft pulp," *Bioresources*, vol. 4, pp. 1109-1129, 2009.
62. L. Kumar *et al.*, "Modulation of xylanase production from alkaliphilic *Bacillus pumilus* VLK-1 through process optimization and temperature shift operation," *3 Biotech*, vol. 4, pp. 345-356, 2014.
63. M. Irfan, U. Asghar, M. Nadeem, R. Nelofer, and Q. Syed, "Optimization of process parameters for xylanase production by *Bacillus* sp. in submerged fermentation," *J. Radiat. Res. Appl. Sci.*, vol. 9, pp. 139-147, 2016.
64. A. Karim, M. A. Nawaz, A. Aman, and S. A. Ul Qader, "Hyper production of cellulose degrading endo (1,4) β-d-glucanase from *Bacillus licheniformis* KIBGE-IB2," *J. Radiat. Res. Appl. Sci.*, vol. 8, pp. 160-165, 2015.
65. V. Juturu and J. C. Wu, "Microbial xylanases: engineering, production and industrial applications," *Biotechnol. Adv.*, vol. 30, pp. 1219-1227, 2012.
66. A. K. Ray, A. Bairagi, K. S. Ghosh, and S. K. Sen, "Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut," *Acta Ichthyol. Piscat.*, vol. 1, pp. 47-53, 2007.

67. B. M. Forster and H. Marquis, “Protein transport across the cell wall of monoderm Gram-positive bacteria,” *Mol. Microbiol.*, vol. 84, pp. 405-413, 2012.
68. S. Kumar, I. Haq, J. Prakash, S. K. Singh, S. Mishra, and A. Raj, “Purification, characterization and thermostability improvement of xylanase from *Bacillus amyloliquefaciens* and its application in pre-bleaching of kraft pulp,” *3 Biotech*, vol. 7, p. 20, 2017.
69. A. Nkohla, K. Okaiyeto, A. Olaniran, U. Nwodo, L. Mabinya, and A. Okoh, “Optimization of growth parameters for cellulase and xylanase production by *Bacillus* species isolated from decaying biomass,” *J. Biotech Res.*, vol. 8, pp. 33-47, 2017.
70. S. Subramaniyan, G. S. Sandhia, and P. Prema, “Control of xylanase production without protease activity in *Bacillus* sp. by selection of nitrogen source,” *Biotechnol. Lett.*, vol. 23, pp. 369-371, 2001.
71. D. Gowdhaman, G. Jeyalakshmi, K. Sugumaran, N. S. Subramanian, R. S. Santhosh and V. Ponnusami, “Optimization of the xylanase production with the newly isolated *Bacillus aerophilus* KGJ2,” *Turk. J. Biochem.*, vol. 39, 2014.
72. D. N. Adhyaru, N. S. Bhatt, and H. A. Modi, “Enhanced production of cellulase-free, thermo-alkali-solvent-stable xylanase from *Bacillus altitudinis* DHN8, its characterization and application in sorghum straw saccharification,” *Biocatal. Agric. Biotechnol.*, vol. 3, pp. 182-190, 2014.
73. S. Garg, R. Ali, and A. Kumar, “Production of alkaline xylanase by an alkalothermophilic bacteria, *Bacillus halodurans*, MTCC 9512 isolated from dung,” *Curr. Trends Biotechnol. Pharm.*, vol. 3, pp. 90-96, 2009.
74. A. N. de Sales, A. C. de Souza, R. de O. Moutta, V. S. Ferreira-Leitao, R. F. Schwan, and D. R. Dias, “Use of lignocellulose biomass for endoxylanase production by *Streptomyces termitum*,” *Prep. Biochem. Biotechnol.*, vol. 47, pp. 505-512, 2017.
75. A. Knob, D. Fortkamp, T. Prolo, S. C. Izidoro, and J. M. Almeida, “Agro-residues as alternative for xylanase production by filamentous fungi,” *Bioresources*, vol. 9, pp. 5738-5773, 2014.
76. R. Bandikari, V. Poondla, and V. S. R. Obulam, “Enhanced production of xylanase by solid state fermentation using *Trichoderma koeningi* isolate: effect of pretreated agro-residues,” *3 Biotech*, vol. 4, pp. 655-664, 2014.

77. M. Camassola and A. J. P. Dillon, “Production of cellulases and hemicellulases by *Penicillium echinulatum* grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation,” *J. Appl. Microbiol.*, vol. 103, pp. 2196-2204, 2007.
78. A. Kumar and T. Satyanarayana, “Production of endoxylanase with enhanced thermostability by a novel polyextremophilic *Bacillus halodurans* TSEV1 and its applicability in waste paper deinking,” *Process Biochem.*, vol. 49, pp. 386-394, 2014.
79. M. Bibra, S. Kumar, J. Wang, A. Bhalla, D. R. Salem, and R. K. Sani, “Single pot bioconversion of prairie cordgrass into biohydrogen by thermophiles,” *Bioresour. Technol.*, vol. 266, pp. 232-241, 2018.
80. N. Kulkarni, A. Shendye, and M. Rao, “Molecular and biotechnological aspects of xylanases,” *FEMS Microbiol. Rev.*, vol. 23, pp. 411-456, 1999.
81. H. U. Rehman, S. A. U. Qader, and A. Aman, “Polygalacturonase: Production of pectin depolymerising enzyme from *Bacillus licheniformis* KIBGE IB-21,” *Carbohydr. Polym.*, vol. 90, pp. 387-391, 2012.
82. A. Sharma, S. Adhikari, and T. Satyanarayana, “Alkali-thermostable and cellulase-free xylanase production by an extreme thermophile *Geobacillus thermoleovorans*,” *World J. Microbiol. Biotechnol.*, vol. 23, pp. 483-490, 2007.
83. I. Seyis and N. Aksoz, “Effect of carbon and nitrogen sources on xylanase production by *Trichoderma harzianum* 1073 D3,” *Int. Biodeterior. Biodegrad.*, vol. 55, pp. 115-119, 2005.
84. G. S. Lakshmi, P. L. Bhargavi, and R. S. Prakasham, “Sustainable bioprocess evaluation for xylanase production by isolated *Aspergillus terreus* and *Aspergillus fumigatus* under solid - state fermentation using oil palm empty fruit bunch fiber,” vol. 5, p. 12, 2011.
85. H.-D. Jang and K.-S. Chang, “Thermostable cellulases from *Streptomyces* sp.: scale-up production in a 50-l fermenter,” *Biotechnol. Lett.*, vol. 27, pp. 239-242, 2005.
86. X. Xia, S. Lin, X.-X. Xia, F.-S. Cong, and J.-J. Zhong, “Significance of agitation-induced shear stress on mycelium morphology and lavendamycin production by engineered *Streptomyces flocculus*,” *Appl. Microbiol. Biotechnol.*, vol. 98, pp. 4399-4407, 2014.
87. A. Kumar, R. Gupta, B. Shrivastava, Y. P. Khasa, and R. C. Kuhad, “Xylanase production from an alkalophilic actinomycete isolate *Streptomyces* sp. RCK-2010, its

characterization and application in saccharification of second generation biomass," *J. Mol. Catal. B Enzym.*, vol. 74, pp. 170-177, 2012.

88. A. Khusro, B. K. Kaliyan, N. A. Al-Dhabi, M. V. Arasu, and P. Agastian, "Statistical optimization of thermo-alkali stable xylanase production from *Bacillus tequilensis* strain ARMATI," *Electron. J. Biotechnol.*, vol. 22, pp. 16-25, 2016.
89. Y. Cao, D. J. Meng, J. Lu and J. Long, Statistical optimization of xylanase production by *Aspergillus niger* AN-13 under submerged fermentation using response surface methodology. *Afr. J. Biotechnol.,* vol. 7, pp. 631-638, 2008.
90. A. Walia, P. Mehta, A. Chauhan, S. Kulshrestha, and C. K. Shirkot, "Purification and characterization of cellulase-free low molecular weight endo β-1,4 xylanase from an alkalophilic *Cellulosimicrobium cellulans* CKMX1 isolated from mushroom compost," *World J. Microbiol. Biotechnol.*, vol. 30, pp. 2597-2608, 2014.
91. H. Chakdar *et al.*, "Bacterial xylanases: biology to biotechnology," *3 Biotech*, vol. 6, p. 150, 2016.
92. G. Goswami and R. R. Pathak, "Microbial xylanases and their biomedical applications: a review," *Int. J. Basic Clin. Pharmacol.*, vol. 2, pp. 237-246, 2013.
93. M. T. H. Emam, K. A. Mohamed, F. M. I. Badawy, and S. A. Ibrahim, "Cloning, sequencing and expression of the xylanase gene from *Bacillus pumilus* GH in *Escherichia coli*," *Int. J. Chemtech. Res.*, vol. 9, pp. 114-124, 2016.
94. X. Yi *et al.*, "Hyperexpression of two Aspergillus niger xylanase genes in *Escherichia coli* and characterization of the gene products," *Braz. J. Microbiol.*, vol. 41, pp. 778-786, 2010.
95. U. Comlekcioglu *et al.*, "Coexpression of rumen fungal xylanase and bifunctional cellulase genes in *Escherichia coli*," *Braz. Arch. Biol. Technol.*, vol. 60, 2017.
96. S. Chang, Y. Guo, B. Wu, and B. He, "Extracellular expression of alkali tolerant xylanase from *Bacillus subtilis* Lucky9 in *E. coli* and application for xylooligosaccharides production from agro-industrial waste," *Int. J. Biol. Macromol.*, vol. 96, pp. 249-256, 2017.
97. D. Verma and T. Satyanarayana, "Production of cellulase-free xylanase by the recombinant *Bacillus subtilis* and its applicability in paper pulp bleaching," *Biotechnol. Prog.*, vol. 29, pp. 1441-1447, 2013.

98. M. Ahmad, M. Hirz, H. Pichler, and H. Schwab, "Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production," *Appl. Microbiol. Biotechnol.*, vol. 98, pp. 5301-5317, 2014.

99. C. Zhou, Y. Wang, M. Wu, W. Wang, and D. Li, "Heterologous expression of xylanase ii from *Aspergillus usamii* in *Pichia pastoris*," *Food Technol. Biotechnol.*, vol. 47, pp. 90-95, 2009.

100. D. Driss, F. Bhiri, R. ghorbel, and S. E. Chaabouni, "Cloning and constitutive expression of His-tagged xylanase GH 11 from *Penicillium occitanis* Pol6 in *Pichia pastoris* X33: purification and characterization," *Protein Expr. Purif.*, vol. 83, pp. 8-14, 2012.

101. C. Vieille and G. J. Zeikus, "Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability," *Microbiol. Mol. Biol. Rev.*, vol. 65, pp. 1-43, 2001.

102. M. Irfan, H. I. Guler, A. A. Shah, F.A. Sal, K. Inan, and A. O. Belduz, "Cloning, purification and characterization of halotolerant xylanase from *Geobacillus thermodenitrificans* C5," *J. Microbiol. Biotechnol. Food Sci.*, vol. 5, pp. 523-529, 2016.

103. M. Sharma, B. S. Chadha, and H. S. Saini, "Purification and characterization of two thermostable xylanases from *Malbranchea flava* active under alkaline conditions," *Bioresour. Technol.*, vol. 101, pp. 8834-8842, 2010.

104. R. A. Deshmukh, S. Jagtap, M. K. Mandal, and S. K. Mandal, "Purification, biochemical characterization and structural modelling of alkali-stable β-1,4-xylan xylanohydrolase from *Aspergillus fumigatus* R1 isolated from soil," *BMC Biotechnol.*, vol. 16, p. 11, 2016.

105. G. Sanghvi, M. Jivrajani, N. Patel, H. Jivrajani, G. B. Bhaskara, and S. Patel, "Purification and characterization of haloalkaline, organic solvent stable xylanase from newly isolated halophilic bacterium-OKH," *International Scholarly Research Notices*, 2014.

106. C. C. Terrone, C. de Freitas, C. R. F. Terrasan, A. F. de Almeida, and E. C. Carmona, "Agroindustrial biomass for xylanase production by *Penicillium chrysogenum*: purification, biochemical properties and hydrolysis of hemicelluloses," *Electron. J. Biotechnol.*, vol. 33, pp. 39-45, 2018.

107. R. A. Sheldon, “Enzyme immobilization: the quest for optimum performance,” *Adv. Synth. Catal.*, vol. 349, pp. 1289-1307, 2007.

108. A. Pal, L. Ray, and P. Chattopadhyay, “Purification and immobilization of an *Aspergillus terreus* xylanase: use of continuous fluidized bed column reactor,” *Indian J. Biotechnol.* vol 5, 2006.

109. K. L. Chang *et al.*, “Improving the remaining activity of lignocellulolytic enzymes by membrane entrapment,” *Bioresour. Technol.*, vol. 102, pp. 519-523, 2011.

110. M. Sardar, I. Roy, and M. N. Gupta, “Simultaneous purification and immobilization of *Aspergillus niger* xylanase on the reversibly soluble polymer EudragitTM L-100,” *Enzyme Microb. Technol.*, vol. 27, pp. 672-679, 2000.

111. R. Gaur, Lata, and S. K. Khare, “Immobilization of xylan-degrading enzymes from *Scytalidium thermophilum* on Eudragit L-100,” *World J. Microbiol. Biotechnol.*, vol. 21, pp. 1123-1128, 2005.

112. Z. Ai, Z. Jiang, L. Li, W. Deng, I. Kusakabe, and H. Li, “Immobilization of *Streptomyces olivaceoviridis* E-86 xylanase on Eudragit S-100 for xylo-oligosaccharide production,” *Process Biochem.*, vol. 40, pp. 2707-2714, 2005.

113. Z. S. Akdemir, S. Demir, M. V. Kahraman, and N. Kayaman Apohan, “Preparation and characterization of UV-curable polymeric support for covalent immobilization of xylanase enzyme,” *J. Mol. Catal. B Enzym.*, vol. 68, pp. 104-108, 2011.

114. M. Kapoor and R. C. Kuhad, “Immobilization of xylanase from *Bacillus pumilus* strain MK001 and its application in production of xylo-oligosaccharides,” *Appl. Biochem. Biotechnol.*, vol. 142, pp. 125-138, 2007.

115. N. Rueda, J. C. S. dos Santos, R. Torres, O. Barbosa, C. Ortiz, and R. Fernandez-Lafuente, “Reactivation of lipases by the unfolding and refolding of covalently immobilized biocatalysts,” *RSC Adv.*, vol. 5, pp. 55588-55594, 2015.

116. S. Velasco-Lozano, F. Lopez-Gallego, J. C. Mateos-Diaz, and E. Favela-Torres, “Cross-linked enzyme aggregates (CLEA) in enzyme improvement – a review,” *Biocatalysis*, vol. 1, pp. 166-177, 2016.

117. A. Bhattacharya and B. I. Pletschke, “Magnetic cross-linked enzyme aggregates (CLEAs): A novel concept towards carrier free immobilization of lignocellulolytic enzymes,” *Enzyme Microb. Technol.*, vol. 61–62, pp. 17-27, 2014.

118. A. Bhattacharya and B. I. Pletschke, “Strategic optimization of xylanase–mannanase combi-CLEAs for synergistic and efficient hydrolysis of complex lignocellulosic substrates,” *J. Mol. Catal. B Enzym.*, vol. 115, pp. 140-150, 2015.

119. S. Talekar, S. Nadar, A. Joshi, and G. Joshi, “Pectin cross-linked enzyme aggregates (pectin-CLEAs) of glucoamylase,” *RSC Adv.*, vol. 4, pp. 59444-59453, 2014.

120. L. Cao, L. van Langen, and R. A. Sheldon, “Immobilised enzymes: carrier-bound or carrier-free?,” *Curr. Opin. Biotechnol.*, vol. 14, pp. 387-394, 2003.

121. S. Shah, A. Sharma, and M. N. Gupta, “Preparation of cross-linked enzyme aggregates by using bovine serum albumin as a proteic feeder,” *Anal. Biochem.*, vol. 351, pp. 207-213, 2006.

122. F. Lopez-Gallego, L. Betancor, A. Hidalgo, N. Alonso, R. Fernandez-Lafuente, and J. M. Guisán, “Co-aggregation of enzymes and polyethyleneimine: a simple method to prepare stable and immobilized derivatives of glutaryl acylase,” *Biomacromolecules*, vol. 6, pp. 1839-1842, 2005.

123. G. Fernández-Lorente *et al.*, “Glutaraldehyde cross-linking of lipases adsorbed on aminated supports in the presence of detergents leads to improved performance,” *Biomacromolecules*, vol. 7, pp. 2610-2615, 2006.

124. H. Torabizadeh, M. Tavakoli, and M. Safari, “Immobilization of thermostable α-amylase from *Bacillus licheniformis* by cross-linked enzyme aggregates method using calcium and sodium ions as additives,” *J. Mol. Catal. B Enzym.*, vol. 108, pp. 13-20, 2014.

125. R. A. Sheldon, “Characteristic features and biotechnological applications of cross-linked enzyme aggregates (CLEAs),” *Appl. Microbiol. Biotechnol.*, vol. 92, pp. 467-477, 2011.

126. K. J. Khorshidi, H. Lenjannezhadian, M. Jamalan, and M. Zeinali, “Preparation and characterization of nanomagnetic cross-linked cellulase aggregates for cellulose bioconversion,” *J. Chem. Technol. Biotechnol.*, vol. 91, pp. 539-546, 2016.

127. E. Lanfranchi *et al.*, “Production of hydroxynitrile lyase from *Davallia tyermannii* (DtHNL) in *Komagataella phaffii* and its immobilization as a CLEA to generate a robust biocatalyst,” *Chem. Bio. Chem.*, vol. 19, pp. 312-316, 2018.

128. R. A. Sheldon and S. van Pelt, “Enzyme immobilisation in biocatalysis: why, what and how,” *Chem. Soc. Rev.*, vol. 42, pp. 6223-6235, 2013.

129. M.-Q. Xu, S.-S. Wang, L.-N. Li, J. Gao, and Y.-W. Zhang, "Combined cross-linked enzyme aggregates as biocatalysts," *Catalysts*, vol. 8, p. 460, 2018.

130. R. Schoevaart *et al.*, "Preparation, optimization, and structures of cross-linked enzyme aggregates (CLEAs)," *Biotechnol. Bioeng.*, vol. 87, pp. 754-762, 2004.

131. H. W. Yu, H. Chen, X. Wang, Y. Y. Yang, and C. B. Ching, "Cross-linked enzyme aggregates (CLEAs) with controlled particles: application to *Candida rugosa* lipase," *J. Mol. Catal. B Enzym.*, vol. 43, pp. 124-127, 2006.

132. S. Talekar, V. Ghodake, A. Kate, N. Samant, C. Kumar, and S. Gadagkar, "Preparation and characterization of cross-linked enzyme aggregates of *Saccharomyces cerevisiae* invertase," p. 7, 2010.

133. S. Montoro-Garcia, F. Gil-Ortiz, J. Navarro-Fernandez, V. Rubio, F. Garcia-Carmona, and A. Sanchez-Ferrer, "Improved cross-linked enzyme aggregates for the production of desacetyl β-lactam antibiotics intermediates," *Bioresour. Technol.*, vol. 101, pp. 331-336, 2010.

134. L. Zhao, L. Zheng, G. Gao, F. Jia, and S. Cao, "Resolution of N-(2-ethyl-6-methylphenyl) alanine via cross-linked aggregates of *Pseudomonas* sp. Lipase," *J. Mol. Catal. B Enzym.*, vol. 54, pp. 7-12, 2008.

135. X. Yang, P. Zheng, Y. Ni, and Z. Sun, "Highly efficient biosynthesis of sucrose-6-acetate with cross-linked aggregates of Lipozyme TL 100 L," *J. Biotechnol.*, vol. 161, pp. 27-33, 2012.

136. Y. Wine, N. Cohen-Hadar, A. Freeman, and F. Frolow, "Elucidation of the mechanism and end products of glutaraldehyde crosslinking reaction by X-ray structure analysis," *Biotechnol. Bioeng.*, vol. 98, pp. 711-718, 2007.

137. C. Mateo, J. M. Palomo, L. M. van Langen, F. van Rantwijk, and R. A. Sheldon, "A new, mild cross-linking methodology to prepare cross-linked enzyme aggregates," *Biotechnol. Bioeng.*, vol. 86, pp. 273-276, 2004.

138. Q. Zhen, M. Wang, W. Qi, R. Su, and Z. He, "Preparation of β-mannanase CLEAs using macromolecular cross-linkers," *Catal. Sci. Technol.*, vol. 3, pp. 1937-1941, 2013.

139. A. Wang *et al.*, "A facile technique to prepare cross-linked enzyme aggregates using p-benzoquinone as cross-linking agent," *Korean J. Chem. Eng.*, vol. 28, pp. 1090-1095, 2011.

140. S. Velasco-Lozano, F. Lopez-Gallego, R. Vazquez-Duhalt, J. C. Mateos-Diaz, J. M. Guisan, and E. Favela-Torres, "Carrier-free immobilization of lipase from *Candida*

rugosa with polyethyleneimines by carboxyl-activated cross-linking," *Biomacromolecules*, vol. 15, pp. 1896-1903, 2014.

141. H. Ayhan, F. Ayhan, and A. Gulsu, "Highly biocompatible enzyme aggregates crosslinked by L-lysine," *Turk. J. Biochem.*, vol. 37, pp. 14-20, 2012.
142. O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R. Torres, R. C. Rodrigues, and R. Fernandez-Lafuente, "Glutaraldehyde in bio-catalysts design: a useful crosslinker and a versatile tool in enzyme immobilization," *RSC Adv.*, vol. 4, pp. 1583-1600, 2014.
143. R. R. and S. S., "Improved biochemical characteristics of crosslinked β-glucosidase on nanoporous silica foams," *J. Mol. Catal. B Enzym.*, vol. 85–86, pp. 111-118, 2013.
144. F. Sulek, D. P. Fernández, Z. Knez, M. Habulin, and R. A. Sheldon, "Immobilization of horseradish peroxidase as crosslinked enzyme aggregates (CLEAs)," *Process Biochem.*, vol. 46, pp. 765-769, 2011.
145. I. Migneault, C. Dartiguenave, M. J. Bertrand, and K. C. Waldron, "Glutaraldehyde: behavior in aqueous solution, reaction with proteins, and application to enzyme crosslinking," *BioTechniques*, vol. 37, pp. 790-802, 2004.
146. S. Dalal, M. Kapoor, and M. N. Gupta, "Preparation and characterization of combi-CLEAs catalyzing multiple non-cascade reactions," *J. Mol. Catal. B Enzym.*, vol. 44, pp. 128-132, 2007.
147. R. Taboada-Puig *et al.*, "Combined cross-linked enzyme aggregates from versatile peroxidase and glucose oxidase: Production, partial characterization and application for the elimination of endocrine disruptors," *Bioresour. Technol.*, vol. 102, pp. 6593-6599, 2011.
148. S. S. Mahmod, F. Yusof, M. S. Jami, S. Khanahmadi, and H. Shah, "Development of an immobilized biocatalyst with lipase and protease activities as a multipurpose cross-linked enzyme aggregate (multi-CLEA)," *Process Biochem.*, vol. 50, pp. 2144-2157, 2015.
149. M. Bilal, M. Asgher, H. M. N. Iqbal, H. Hu, and X. Zhang, "Bio-based degradation of emerging endocrine-disrupting and dye-based pollutants using cross-linked enzyme aggregates," *Environ. Sci. Pollut. Res.*, vol. 24, pp. 7035-7041, 2017.
150. N. Tandjaoui, A. Tassist, M. Abouseoud, A. Couvert, and A. Amrane, "Preparation and characterization of cross-linked enzyme aggregates (CLEAs) of *Brassica rapa* peroxidase," *Biocatal. Agric. Biotechnol.*, vol. 4, pp. 208-213, 2015.

151. L. Fernandez-Lopez, S. G. Pedrero, N. Lopez-Carrobles, B. C. Gorines, J. J. Virgen-Ortíz, and R. Fernandez-Lafuente, "Effect of protein load on stability of immobilized enzymes," *Enzyme Microb. Technol.*, vol. 98, pp. 18-25, 2017.

152. H. Cabana, J. P. Jones, and S. N. Agathos, "Preparation and characterization of cross-linked laccase aggregates and their application to the elimination of endocrine disrupting chemicals," *J. Biotechnol.*, vol. 132, pp. 23-31, 2007.

153. T. Dong, L. Zhao, Y. Huang, and X. Tan, "Preparation of cross-linked aggregates of aminoacylase from *Aspergillus melleus* by using bovine serum albumin as an inert additive," *Bioresour. Technol.*, vol. 101, pp. 6569-6571, 2010.

154. N. Easa, and F. Yusof, "Optimizing the preparation of cross-linked enzyme aggregates (CLEA)-amylase from super mealworm (*Zophobas morio*)," *ARPN J. Eng. Appl. Sci.*, vol. 10, pp. 9710-9716, 2015.

155. S. Talekar, A. Joshi, G. Joshi, P. Kamat, R. Haripurkar, and S. Kambale, "Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs)," *RSC Adv.*, vol. 3, pp. 12485-12511, 2013.

156. H. Chen, J. Ullah, and J. Jia, "Progress in *Bacillus subtilis* spore surface display technology towards environment, Vaccine Development, and Biocatalysis," *J. Mol. Microbiol. Biotechnol.*, vol. 27, pp. 159-167, 2017.

157. S. A. Chaudhari and R. S. Singhal, "A strategic approach for direct recovery and stabilization of *Fusarium* sp. ICT SAC1 cutinase from solid state fermented broth by carrier free cross-linked enzyme aggregates," *Int. J. Biol. Macromol.*, vol. 98, pp. 610-621, 2017.

158. S. Dalal, A. Sharma, and M. N. Gupta, "A multipurpose immobilized biocatalyst with pectinase, xylanase and cellulase activities," *Chem. Cent. J.*, vol. 1, p. 16, 2007.

159. A. D. H. and C. Ramalingam, "Xylanases and its application in food industry: a review," *J. Exp. Sci.*, 2010.

160. A. G. J. Voragen, "Technological aspects of functional food-related carbohydrates," *Trends Food Sci. Technol.*, vol. 9, pp. 328-335, 1998.

161. G. Dervilly, C. Leclercq, D. Zimmermann, C. Roue, J.-F. Thibault, and L. Saulnier, "Isolation and characterization of high molar mass water-soluble arabinoxylans from barley and barley malt," *Carbohydr. Polym.*, vol. 47, pp. 143-149, 2002.

162. P. Biely, "Diversity of microbial endo-β-1,4-xylanases," in *Applications of enzymes to lignocellulosics*, vol. 855, American Chemical Society, 2003, pp. 361-380.

163. G. K. Goswami, M. Krishnamohan, V. Nain, C. Aggarwal, and B. Ramesh, "Cloning and heterologous expression of cellulose free thermostable xylanase from *Bacillus brevis*," *Springer Plus*, vol. 3, p. 20, 2014.

164. T. O. O. Babalola, D. F. Apata, and J. O. Atteh, "Effect of β-xylanase supplementation of boiled castor seed meal-based diets on the performance, nutrient absorbability and some blood constituents of pullet chicks," *Trop. Sci.*, vol. 46, pp. 216-223, 2006.

165. K. Shimoda, H. Hamada, and H. Hamada, "Synthesis of xylooligosaccharides of daidzein and their anti-oxidant and anti-allergic activities," *Int. J. Mol. Sci.*, vol. 12, pp. 5616-5625, 2011.

166. P. K. Gupta, P. Agrawal, P. Hegde, N. Shankarnarayan, S. Vidyashree, S. A. Singh and S. Ahuja, "Xylooligosaccharide-a valuable material from waste to taste: a review," *J. Environ. Res. Develop.*, vol. 10, p.555, 2016.

167. S. Garg, "Xylanase: applications in biofuel production," *Curr. Metabolomics*, vol. 4, pp. 23-37, 2016.

168. C. Azeri, U. A. Tamer, and M. Oskay, "Thermoactive cellulase-free xylanase production from alkaliphilic *Bacillus* strains using various agro-residues and their potential in biobleaching of kraft pulp," *Afr. J. Biotechnol.*, vol. 9, 2010.

169. B. R. Goluguri *et al.*, "Potential of thermo and alkali stable xylanases from *Thielaviopsis basicola* (MTCC-1467) in biobleaching of wood kraft pulp," *Appl. Biochem. Biotechnol.*, vol. 167, pp. 2369-2380, 2012.

170. R. M. Teather and P. J. Wood, "Use of congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen.," *Appl. Environ. Microbiol.*, vol. 43, pp. 777-780, 1982.

171. A. Archana and T. Satyanarayana, "Purification and characterization of a cellulase-free xylanase of a moderate thermophile *Bacillus licheniformis* A99," *World J. Microbiol. Biotechnol.*, vol. 19, pp. 53-57, 2003.

172. A. Kumar, H. S. Saini, and S. Kumar, "Bioleaching of gold and silver from waste printed circuit boards by *Pseudomonas balearica* SAE1 isolated from an e-waste recycling facility," *Curr. Microbiol.*, vol. 75, pp. 194-201, 2018.

173. K. Tamura, J. Dudley, M. Nei, and S. Kumar, "MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0," *Mol. Biol. Evol.*, vol. 24, pp. 1596-1599, 2007.

174. T. K. Ghose, “Measurement of cellulase activities,” *Pure Appl. Chem.*, vol. 59, pp. 257-268, 1987.

175. N. Basotra, B. Kaur, M. Di Falco, A. Tsang, and B. S. Chadha, “*Mycothermus thermophilus* (Syn. *Scytalidium thermophilum*): repertoire of a diverse array of efficient cellulases and hemicellulases in the secretome revealed,” *Bioresour. Technol.*, vol. 222, pp. 413-421, 2016.

176. S. Rehman, H. N. Bhatti, M. Bilal, and M. Asgher, “Cross-linked enzyme aggregates (CLEAs) of *Pencilluim notatum* lipase enzyme with improved activity, stability and reusability characteristics,” *Int. J. Biol. Macromol*., vol. 91, pp.1161-1169, 2016.

177. M. Irfan, M. Nadeem, and Q. Syed, “One-factor-at-a-time (OFAT) optimization of xylanase production from *Trichoderma viride*-IR05 in solid-state fermentation,” *J. Radiat. Res. Appl. Sci.*, vol. 7, pp. 317-326, 2014.

178. A. A. Sepahy, S. Ghazi, and M. A. Sepahy, “Cost-effective production and optimization of alkaline xylanase by indigenous *Bacillus mojavensis* AG137 fermented on agricultural waste,” *Enzyme research*, 2011.

179. M. I. Abou-Dobara, A. K. El-Sayed and R. A. El Fayoumy, “Optimization and physiochemical properties of xylanase from *Bacillus coagulans* and *Bacillus licheniformis*,” *J. Am. Sci.*, vol. 7, pp.661-670, 2011.

180. S. Chaturvedi, R. Singh, and S. P. Khurana, “Isolation and identification of xylanolytic enzyme from an effective strain *Bacillus licheniformis* isolated from the decaying wood,” 2013.

181. S. C. D. Sharma, M. S. Shovon, A. K. M. Asaduzzaman, M. S. Jahan, T. Yeasmin, and N. Roy, “Optimization of alkali-thermostable and cellulase-free eylanase production from *Bacillus Sp*.,” *J. Bio-Sci*., vol. 19, pp. 7-14, 2011.

182. S. Dholpuria, B. K. Bajaj, S. Karri, A. Bhasin, K. Razdan, N. Gupta, N. Sethi, “Production and purification of alkali stable xylanase from *Bacillus* sp.,” *Int. J. Curr. Microbiol. Appl. Sci.*, vol. 3, pp. 365-377, 2014.

183. A. A. Dias et al., “Enzymatic saccharification of biologically pre-treated wheat straw with white-rot fungi,” *Bioresour. Technol.*, vol. 101, pp. 6045-6050, 2010.

184. J. Cilerdzic, M. Galic, J. Vukojevic, I. Brceski, and M. Stajic, “Potential of selected fungal species to degrade wheat straw, the most abundant plant raw material in Europe,” *BMC Plant Biol*., vol. 17, p. 249, 2017.

185. F. Talebnia, D. Karakashev, and I. Angelidaki, "Production of bioethanol from wheat straw: An overview on pretreatment, hydrolysis and fermentation," *Bioresour. Technol*., vol. 101, pp. 4744-4753, 2010.

186. S. Narang, V. Sahai, and V. S. Bisaria, "Optimization of xylanase production by *Melanocarpus albomyces* IIS68 in solid state fermentation using response surface methodology," *J. Biosci. Bioeng*., vol. 91, pp. 425-427, 2001.

187. V. Kumar, V. Kumar, R. S. Chauhan, H. Sood, and C. Tandon, "Cost effective quantification of picrosides in *Picrorhiza kurroa* by employing response surface methodology using HPLC-UV," *J. Plant Biochem. Biotechnol*., vol. 24, pp. 376-384, 2015.

188. S. Kumar, N. Sharma, S. Pathania, "Purification, characterization and gene encoding of xylanase produced from *Bacillus tequilensis* SH0 isolated from compost using low cost wheat bran as substrate," *Global J. Biol. Agric. Health Sci.*, vol. 3, pp. 1-15, 2014.

189. S. Naz, M. Irfan and M. U. Farooq, "Xylanase production from *Bacillus subtilis* in submerged fermentation using box-behnken design", *Pak. J. Biotechnol*., vol. 14, pp. 151-156, 2017.

190. L. Bin, N. N. Zhang, C. Zhao, B. X. Lin, L. H. Xie, and Y. F. Huang, "Characterization of a recombinant thermostable xylanase from hot spring thermophilic *Geobacillus* sp. TC-W7," *J. Microbiol. Biotechnol.*, vol. 22, pp. 1388-1394, 2012.

191. S. Canakciv, K. Inan, M. Kacagan, A. O. Belduz, "Evaluation of arabinofuranosidase and xylanase activities of *Geobacillus* sp. isolated from some hot springs in Turkey", *World. J. Microbiol. Biotechnol.* vol.17, p. 1262, 2007.

192. J. Gerasimova and N. Kuisiene, "Characterization of the novel xylanase from the thermophilic *Geobacillus thermodenitrificans* JK1," *Microbiology*, vol. 81, pp. 418-424, 2012.

193. M. Vrsanska, S. Voberkova, A. M. Jimenez Jimenez, V. Strmiska, and V. Adam, "Preparation and optimisation of cross-linked enzyme aggregates using native isolate white rot fungi *Trametes versicolor* and *Fomes fomentarius* for the decolourisation of synthetic dyes," *Int. J. Environ. Res. Public. Health*, vol. 15, p. 23, 2018.

194. S. Talekar, S. Waingade, V. Gaikwad, S. Patil, and N. Nagavekar, “Preparation and characterization of cross linked enzyme aggregates (CLEAs) of *Bacillus amyloliquefaciens* alpha amylase,” p. 6, 2011.

195. E. Menfaatli and F. Zihnioglu, “Carrier free immobilization and characterization of trypsin,” *Artif. Cells Nanomedicine Biotechnol.*, vol. 43, pp. 140-144, 2015.

196. F. Kartal, M. H. A. Janssen, F. Hollmann, R. A. Sheldon, and A. Kılınc, “Improved esterification activity of *Candida rugosa* lipase in organic solvent by immobilization as CROSS-linked enzyme aggregates (CLEAs),” *J. Mol. Catal. B Enzym.*, vol. 71, pp. 85-89, 2011.

197. A. Kumar, G. Wu, and Z. Liu, “Synthesis and characterization of cross linked enzyme aggregates of serine hydroxyl methyltransferase from *Idiomerina leihiensis*,” *Int. J. Biol. Macromol.*, vol. 117, pp. 683-690, 2018.

198. B. S. Aytar and U. Bakir, “Preparation of cross-linked tyrosinase aggregates,” *Process Biochem.*, vol. 43, pp. 125-131, 2008.

199. D.-Y. Xu, Y. Yang, and Z. Yang, “Activity and stability of cross-linked tyrosinase aggregates in aqueous and nonaqueous media,” *J. Biotechnol.*, vol. 152, pp. 30-36, 2011.

200. E. A. Manoel, J. C. S. dos Santos, D. M. G. Freire, N. Rueda, and R. Fernandez-Lafuente, “Immobilization of lipases on hydrophobic supports involves the open form of the enzyme,” *Enzyme Microb. Technol.*, vol. 71, pp. 53-57, 2015.

201. G. H. Peters, O. H. Olsen, A. Svendsen, and R. C. Wade, “Theoretical investigation of the dynamics of the active site lid in *Rhizomucor miehei* lipase,” *Biophys. J.*, vol. 71, pp. 119-129, 1996.

202. M. A. Rahman, U. Culsum, A. Kumar, H. Gao, and N. Hu, “Immobilization of a novel cold active esterase onto Fe_3O_4∼cellulose nano-composite enhances catalytic properties,” *Int. J. Biol. Macromol.*, vol. 87, pp. 488-497, 2016.

203. F. A. P. Lage, J. J. Bassi, M. C. C. Corradini, L. M. Todero, J. H. H. Luiz, and A. A. Mendes, “Preparation of a biocatalyst via physical adsorption of lipase from *Thermomyces lanuginosus* on hydrophobic support to catalyze biolubricant synthesis by esterification reaction in a solvent-free system,” *Enzyme Microb. Technol.*, vol. 84, pp. 56-67, 2016.

204. R. Xu, R. Tang, Q. Zhou, F. Li, and B. Zhang, “Enhancement of catalytic activity of immobilized laccase for diclofenac biodegradation by carbon nanotubes,” *Chem. Eng. J.*, vol. 262, pp. 88-95, 2015.

205. S. Ates, Y. Ni, and C. Atik, “Effects of the endoxylanase treatment on fiber characteristics, brightness stability and strength properties of bleached wheat straw pulp,” *Cell. Chem. Technol*. vol. 43, p. 17, 2009.

206. S. Bissoon, L. Christov, and S. Singh, “Bleach boosting effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp,” *Process Biochem.*, vol. 37, pp. 567-572, 2002.

207. L. Zheng, Y. Du, and J. Zhang, “Biobleaching effect of xylanase preparation from an alkalophilic *Bacillus* sp. on ramie fibers,” *Biotechnol. Lett*., vol. 22, pp. 1363-1367, 2000.

208. J. Angayarkanni, M. Palaniswamy, B. V. Pradeep, and K. Swaminathan, “Biochemical substitution of fungal xylanases for prebleaching of hardwood kraft pulp,” *Afr. J. Biotechnol*., vol. 5, 2006.

209. A. Das et al., “Production of cellulolytic enzymes by *Aspergillus fumigatus* ABK9 in wheat bran-rice straw mixed substrate and use of cocktail enzymes for deinking of waste office paper pulp,” *Bioresour. Technol*., vol. 128, pp. 290-296, 2013.

210. A. Sridevi, G. Ramanjaneyulu, and P. Suvarnalatha Devi, “Biobleaching of paper pulp with xylanase produced by *Trichoderma asperellum*,” *3 Biotech*, vol. 7, p. 266, 2017.

211. A. Sridevi, A. Sandhya, G. Ramanjaneyulu, G. Narasimha, and P. S. Devi, “Biocatalytic activity of *Aspergillus niger* xylanase in paper pulp biobleaching,” *3 Biotech*, vol. 6, p. 165, 2016.

212. K. Przybysz Buzała, H. Kalinowska, J. Borkowski, and P. Przybysz, “Effect of xylanases on refining process and kraft pulp properties,” *Cellulose*, vol. 25, pp. 1319-1328, 2018.

213. J. C. Chen, G. H. Yang, F. G. Kong, L. A. Lucia, and Y. Liu, “Influence of xylanase pretreatment on refining energy and brightness of P-RC APMP pulp of Italian black poplar branches”, *Cell Chem. Technol.*, vol. 46, p.283, 2012.

APPENDIX

A.1 *Geobacillus thermodenitrificans* X1 16S ribosomal RNA gene, partial (KT899095)

GCGTGAAAGTGGCGAGCGGCTCTCTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGGTCAC
ACCCTTTAGTGCTGCAGCTAACGCGATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCCCTGACAACCCAAGAGATTGGGCGTTCCCCCTTCGGGGGG
ACAGGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGCCTCTAGTTGCCAGCATTCAGTTGGGCACTCTAGAGGGACTGCCGGCT
AAAAGTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGGCGGTACAAAGGGCTGCGAACCCGCGAGGGGGAGCGAATCCCAAAAAGCCGCTC
TCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGCTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGCTTGCAA
CACCCGAAGTCGGTGAGGTAACCCTTACGGGAGCCACCCGCCGAAGGTGGGGCAAGTGATTGGGG
TGAGTCTAAAAAAAAGGTATAAA

A.2 *Geobacillus thermodenitrificans* X1 xylanase gene, complete cds (MG874777)

ATGTTGAAAAGATCGCGAAAAGCGATAATAGTTGGATTCTCATTTATGCTGTTGCTCCCTTTAGG
GATGACGAATGCATTGGCAAAAACTGAACAATCATACGCTAAAAAGCCTCAAATCAGCGCATTGC
ATGCCCCACAATTGGACCAGCGCTACAAAGATTCATTCACTATTGGTGCGGCTGTTGAACCTTAT
CAGCTACTAAACGAGAAAGACGCACAAATGCTAAAACGCCATTTTAACAGCATTGTCGCTGAGAA
CGTTATGAAGCCGATTAATATTCAACCGGAAGAAGGAAAATTCAATTTTGCTGAGGCGGATCAAA
TCGTTCGGTTTGCTAAAAAACACCATATGGATATCCGTTTCCACACGCTCGTTTGGCATAGCCAA
GTACCTCAATGGTTCTTTCTTGACAAGGAAGGCCAACCAATGGTCAATGAAACGGACCCTGTGAA
ACGCGAACAAAATAAACGGCTGTTATTAAAACGGATCGAAACCCATATTAAAACGATTGTCGAGC
GGTATAAAGATGACATCAAATATTGGGACGTTGTAAATGAGGTAGTCGGGGATGATGGAGAATTG
CGCGATTCCCCATGGTATCAAATCGCTGGCATCGATTATATTAAGGTAGCGTTCCAAACAGCGAG
AAAATATGGCGGCAACAAGATTAAACTTTACATTAATGATTACAATACGGAAGTTGAACCAAAGC
GAAGCGCTCTTTATAACTTGGTGAAACAATTAAAAGAAGAGGGCATTCCCATTGATGGTATTGGC
CATCAGTCCCACATCCAAATTGACTGGCCTTCTGAAGAGGAAATCGAAAAAACGATTATCATGTT
TGCCGATCTAGGGTTAGACAACCAAATTACTGATCTGGATGTGAGCATGTACGGCTGGCCGCCTC
GTCCTTACCTGTCTTATGACGCCATTCCGGAGCAAAAGTTTTTGGACCAAGCGGATCGCTATGAT
CGATTGTTTAAGCAGTATGAAAAACTCAGCGATAAAATCAGTAACGTCACCTTCTGGGGCATCGC
CGACAACCATACGTGGCTCGACAGTCGAGCGGATGTTTACTATGATGCTGATGGGAATGTGATTG
TAGACCCGAAAGCCCCATATACGAGAGTGGAAAAAGGGAATGGAAAAGATGCGCCATTTGTGTTC
GACCCCGAATACAACGTAAAACCTGCGTATTGGGCTATTATCGATCATAAGTGAGG

LIST OF PUBLICATIONS

Journal Publications

1. **R. Verma**, A. Bhalla and S. Kumar. "Valorization of Lignocellulosic Residues for Cost-Effective Production of Thermo-Alkali-Stable Xylanase by *Geobacillus thermodenitrificans* X1 of Indian Himalayan Hot Spring", *Waste Biomass Valorization*, pp.1-11, July 2018. **IF: 2.3 [Scopus, SCIE]**

2. **R. Verma**, A. Kumar and S. Kumar. "Synthesis and characterization of cross-linked enzyme aggregates (CLEAs) of thermostable xylanase from *Geobacillus thermodenitrificans* X1", *Process Biochem.*, vol. 80, pp. 72-79, Jan. 2019. **IF: 2.8 [Scopus, SCI]**

3. **R. Verma**, S. Chechi and S. Kumar. "Biobleaching of wheat straw-bagasse pulp using thermo-alkali-stable xylanase from *Geobacillus thermodenitrificans* X1". (Due for submission)
4. **R. Verma**, A. K. Tripathi, A. Yadav and S. Kumar. "Comparative analysis of various pretreatment methods for sugar recovery from waste foliage of *Pinus roxburghii* and *Pinus wallichiana*". (Due for submission)

Conference paper

R. Verma and A. Kumar, "Biogas Plants for Community Service", in *National Biogas convention for Sustainable Energy Access in Rural Areas: Proc. of 2nd National Conf. on Current and Emerging Trends in Indian Biogas and Bio-Fertilizer Development, CETIBBD-2015, September 15-17, 2015, Indian Institute of Technology Delhi, Hauz Khas, New Delhi-110016*, V. K. Vijay, R. Chandra, A. Trivedi, B. Jha, V. Vijay, Eds. IIT Delhi, 2015. pp. 113-122.

Book Chapter

R. Verma, A. K. Tripathi and S. Kumar, "Conversion of Lignocellulosic Feedstocks into Biogas" in *Extremophilic Microbial Processing of Lignocellulosic Feedstocks to Biofuels, Value-Added Products, and Usable Power*, 1st ed. R. Sani and R. N. Krishnaraj, Ed. Switzerland:Springer Nature, 2018, pp. 111-143.

Correspondence article

R. Verma, A. Kumar and S. Kumar, "CO_2 levels and Coral Reefs", *Curr. Sci.*, vol. 111 (8), pp. 1288, Oct. 2016 **[Scopus]**

NCBI GenBank submissions

Verma R and Kumar S (2015): KT899095

Verma R and Kumar S (2018): MG874777, AYF56152

Conference Abstracts

1. W. Sharma, **R. Verma** and S. Kumar. "Process optimization for xylanase production using thermophilic bacteria isolated from biogas slurry". 2nd Himachal Pradesh science Congress (HPSC) 2017-18 organized by Himachal Pradesh Council for Science, Technology & Environment (HIMCOSTE), held at Hotel Peterhoff, Shimla, Nov. 20-21st , 2017.

2. P. Sethi, **R. Verma** and S. Kumar. "Bioprospecting for xylanse producing bacteria with potent application in lignocellulosic conversion". 2nd International Conference: Innovative Research in Engineering, Science and Technology (IREST-2017) held at Eternal University during April 7-8, 2017.

3. **R. Verma**, A. Bhalla and S. Kumar. "Isolation of thermophilic xylanase from hot spring soil sample". Second International Conference on 'Recent Advances in Bio-energy Research', held at National Institute of Bioenergy, Kapurthala, Jalandhar, Feb. 25-27, 2016.

4. **R. Verma**, A. Bhalla and S. Kumar. "Extremophilic xylanases for efficient conversion of lignocellulosic biomass". Second International Conference on 'Recent Advances in Bio-energy Research', held at National Institute of Bioenergy, Kapurthala, Jalandhar, Feb. 25-27, 2016.

5. **R. Verma**, Anil Kumar, Abhilash Kumar Tripathi, Ashish Kumar and Sudhir Kumar. "Biogas for community services". National biogas convention 2015 on Current and emerging

trends in Indian biogas and bio-fertilizer development, organized by biogas development and training center, center for rural development and technology, IIT Delhi, Sept. 15-16, 2015.

Trainings and Workshops

1. Participated in one day workshop on "Patent Drafting" held on 13th October 2017 at Jaypee Uiversity of Information Technology, Wakanghat, Solan, Himachal Pradesh.

2. Participated in one day workshop on "Innovation and Intellectual Property Rights" held on 9th December 2016 at Jaypee Uiversity of Information Technology, Wakanghat, Solan, Himachal Pradesh.

3. Participated in one week training program on Hands on Analytical and Molecular Tehniques 'Biomass based Biorefineries: An Emerging Incentive for Advanced Biofuels and Value-added Products' during February 23-27, 2015 held at National Institute of Renewable Energy Jalandhar, Punjab.