Purification and Characterization of Cellulases and Xylanases from Bacterial Isolates for Hydrolysis of Pretreated Sugarcane Bagasse

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Dedication

This thesis is dedicated to my beloved Parents and my brothers. Thank you for all your endless love, sacrifices, prayers, and support.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Syed Yawar Saeed

Certificate

This thesis submitted by ***Syed Yawar Saeed*** is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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بسم الله الرحمن الرحيم

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LIST OF ABBREVIATIONS

˚C	Degree Celsius
%	Percentage
AEG	Applied Environmental Geomicrobiology
CMC	Carboxymethyl cellulose
BSA	Bovine Serum Albumin
LB	Lignocellulosic biomass
NARC	National Agricultural Research Council
CaCl2	Calcium chloride
DMSO	Dimethyl sulfoxide
$FeSO_4$	Iron Sulphate
Cu	Copper
$CuSO_4$	Copper sulfate
NMMO	N-methyl morpholine N-oxide
AFEX	Ammonia Fiber Explosion
CBH	Cellobiohydrolases
EC	Enzyme Commission
FTIR	Fourier transform infrared
XRD	X-Ray Diffraction
SEM	Scanning Electron Microscopy
SCB	Sugarcane bagasse
FAO	Food and Agriculture Organization
AGU	anhydroglucose unit
GHs	Glycoside hydrolases
BGL	β-glucosidases
hrs	Hours
i.e	That is
KCL	Potassium Chloride
kDa	Kilo Dalton
Km	Michaleous-Menton constant
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate

Vmax	Maximum velocity
$MgSO_4$	Magnesium Sulphate
$ZnSO_4$	Zinc Sulphate
$CuSO_4$	Copper Sulphate
Min	minutes
mM	milli Molar
Ml	Milli liter
Ml	microliter
DNS	3,5-Dinitrosalicylic acid
CFS	Cell free supernatant
SDS	Sodium dodecyl sulphate
nm	Nanometer
M	Molar
$MgSO_4$	Magnesium sulfate
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NiSO4	Nickel Sulfate
$(NH_4)_2SO_4$	Ammonium Sulphate
OD	Optical Density
pH	Paviour of hydrogen
RPM	Revolutions per minutes
UV	Ultra Voilet
ELISA	enzyme-linked immunosorbent assay
SA	Specific Activity

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Abstract

Enzymatic saccharification of organo-solvent pretreated sugarcane bagasse (SCB) by cellulases and hemicellulases (xylanase) is a widely used technique for the extraction of reducing sugar (cellulose and xylose). Sugarcane bagasse contains 25-30% hemicellulose and 35-50% cellulose which act as major substrates for these enzymes to obtain valuable products. In current study bacterial strains CC16 and CC4 have been screened to produce cellulase and xylanase enzyme respectively. Different culture parameters were optimized for CC16 and CC4 for maximum production of cellulase and xylanase. Cellulase and xylanase produced by CC16 and CC4 were further purified using column chromatography with Sephadex G-100 gel resin, and all proteins were eluted according to their molecular size. The maximum specific activity of purified cellulase from bacterial strain CC16 was 114.2 U/mg, 2.03 purification fold, and 55.6% yield and for purified xylanase from bacterial strain CC4 specific activity was 232.0U/mg, 1.74 purification fold, and 55.3% yield. Purified cellulase shows the highest activity and stability at pH 7.0 and temperature 45°C while purified xylanase revealed the highest activity and stability at pH 9.0 and 50°C temperature. The enzyme cellulase and xylanase were active in the presence of different metal ions like Cu^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+} whereas Pb strongly inhibited its activity. Surfactants such as Tween 20, Tween 80, CTAB, and Triton X 100 potentially enhanced activity of both enzymes while SDS significantly decreased their activity at both 1% and 10% concentrations in case of both enzymes. The enzyme cellulase from CC16 remained stable in the presence of 10% organic solvents such as Methanol, Acetone, Ethyl acetate, N-Hexane DMSO, and glycerol while xylanase from CC4 was found to be stable in the presence of Methanol, Acetonitrile, Acetone, DMSO and glycerol for 120 minutes. Enzymatic hydrolysis of pretreated sugarcane bagasse by cellulase and xylanase resulted in 33.2 g/L and 0.91 g/g of total reducing sugars (TRS). The significance of the current study is that all the selected parameters possess a substantial effect on the production of both cellulase and xylanase enzymes, which upon the optimization and purification process showed stability at a wide range of temperatures, pH, various concentrations of metal ions, organic solvents and surfactants leading to increased hydrolytic proficiency of cellulase and xylanase for saccharification of pre-treated sugarcane bagasse that can be used on industrial scale for the production of bioethanol and other valuable products.

Introduction

Lignocellulosic biomass (LB) is the most abundant and renewable plant-based resource on earth that primarily consists of polysaccharides cellulose (35–50%), hemicelluloses (25–30%), alongside aromatic polymer lignin (25–30%) (Hagen, 2015; Zoghlami & Paës, 2019). Renewable energy sources currently contribute approximately 14% of the global energy supply, in which LB contributes more than 70% of total renewable energy (Popp *et al*., 2021). The assimilation of lignocellulosic biomass wastes in substantial quantities poses environmental challenges. However, due to their chemical composition, these materials present an opportunity for recycling and production of various valuable products which include ethanol, food additives, organic acids, enzymes, and numerous others (Maitan-Alfenas *et al*., 2015). The utilization of LB for its applications relies on three fundamental steps which are: pretreatment, enzymatic hydrolysis, and microbial fermentation. Among these steps, enzymatic hydrolysis plays a pivotal role in the conversion of polysaccharides present in LB into fermentable monosaccharides, which can then be used for the generation of biofuels during the fermentation processes (Lima *et al*., 2014). Maize, wheat, rice, and sugarcane are the most important LB contributors in terms of both production volume and cultivated area. These four crops are responsible for generating the majority of lignocellulosic biomass within the agricultural sector (Cheng & Timilsina, 2011). The agricultural production of these crops generates a large amount of agricultural waste that often remains unused or burned openly. However, these agricultural wastes can be used as a potential alternative for the environmentally friendly production of biofuels, such as bioethanol. By utilizing these agricultural wastes, we can effectively convert this lignocellulosic biomass into valuable products like biofuels while mitigating environmental issues related to their disposal (Saini *et al*., 2015).

Sugarcane (Saccharum officinarum) is a highly cultivated crop in tropical countries, with larger production volumes. In 2017, the worldwide production of total sugarcane accounted to approximately 1.84 billion tons. The large scale production of sugarcane highlights the importance of sugarcane as a major lignocellulosic biomass commodity globally (Mahmud & Anannya, 2021). But this excessive production of sugarcane has also led to improper disposal of sugarcane waste which include the burning, one of the

method used for disposing of solid waste, which leads to environmental pollution (Mokhena *et al*., 2018). On other hand at industrial scale the sugarcane is utilized in sugar mills and alcohol mills but during their processing the entire sugarcane cannot be used and approximately 30% of the sugarcane is left as pulpy fibrous residue, known as bagasse (Pandey *et al*., 2000). Sugarcane bagasse typically consist of cellulose (32-34%), hemicellulose (19-24%), lignin (25-32%), extractives (6-12%), and ash (2-6%). This composition provides insight into the relative amounts of cellulose, hemicellulose, lignin, extractives, and ash present in sugarcane bagasse (Haghdan *et al*., 2016). The generation of very large quantities of sugarcane bagasse in nature has attracted the attention of the researchers, who have been exploring its potential for different applications in the energy sector and environmental sustainability. The effective utilization of bagasse for enzyme, biofuel production and other applications has been well studied which will not only resolve the future challenges posed by the fossil fuel crisis and air pollution resulting from fossil fuel but also offers a sustainable solution (Yadav *et al*., 2022). However, for effective utilization of sugarcane bagasse it should be proper pre-treated (Ajala *et al*., 2021). Pre-treatment is a method applied to lignocellulosic biomass before its processing. These pre-treatment methods are important to overcome the recalcitrant nature of sugarcane bagasse and enhance the efficiency of enzymatic hydrolysis process (Huang *et al*., 2018). Generally the aim of various pre-treatment methods is to change the physiochemical arrangement of LB to enhance the accessibility of cellulytic enzyme toward their substrate (Meng & Ragauskas, 2014).

Using lignocellulosic materials (sugarcane bagasse) in fermentation processes poses a major hurdle due to its complex polysaccharides that need to be converted into simple sugars that microorganisms can consume. To achieve this conversion, suitable pretreatments are applied to enhance the efficiency of either chemical or enzymatic hydrolysis (Karp *et al*., 2013). Different pretreatment methods, such as physical, chemical, and biological approaches, are being used to enhance the production efficiency (Guo *et al*., 2013). Physical or mechanical pretreatment methods involve the grinding, milling, and freezing, which decrease the size of the particle as well as the degree of polymerization while loosening the rigid structure of cellulose fibers in

lignocellulosic materials. Various techniques like ball milling, knife milling, hammer milling, two-roll milling, extruders, colloid milling, and attrition milling are utilized to reduce the size of particles during this pretreatment process (Croce *et al*., 2016). To disrupt the interactions between the macromolecules in lignocellulosic materials, different chemicals such as acids, alkalis, ionic liquids, organic solvents, ozone, and others are utilized in chemical pretreatment. Among these chemicals, H_2SO_4 (sulfuric acid) is extensively studied and widely used for biomass pretreatment. It is recommended because of its low cost, ease of use, and effectiveness in pretreating lignocellulosic biomass under relatively mild treatment parameters such as temperature, pressure, concentration, and treatment time (Gu *et al*., 2021; Qi *et al*., 2019). In chemical pretreatment, sodium hydroxide (NaOH) is frequently used to disrupt the ester and glycosidic linkages in lignocellulosic materials, producing structural alterations (Park & Kim, 2012). Organic solvents such as methanol, tetrahydrofuran, acetone, ethanol, and N-methyl morpholine N-oxide (NMMO) are used in organosolvent pre-treatment process. Ammonia Fibre Explosion (AFEX), CO2 explosion, and wet oxidation are examples of physicochemical pretreatments. These pretreatment methods aim to alter the structure and composition of LB to make it more amenable to subsequent processes and improve its overall conversion efficiency (Yadav *et al*., 2023). The ligninolytic enzyme system generated by various bacteria and fungi is used in biological pretreatment. These enzymes can degrade lignin in biomass while also hydrolyzing cellulose and hemicellulose (Sindhu *et al*., 2016). This biological approach is effective in breaking down the complex lignocellulosic structure, making it easier to access and utilize the cellulose and hemicellulose components for further processing or conversion (Nurika *et al*., 2022).

For hydrolysis of sugarcane bagasse, the important enzymes required are cellulases and hemicellulases. Novel approaches are being employed to lower enzyme expenses, and among them is the utilization of an enzyme cocktail comprising three essential enzymes necessary for cellulose hydrolysis: endoglucanases, exoglucanases, and β-glucosidases. These enzymes work together, with the first two alongside by cleaving cellulose chain to produce cellobiose and degenerate the crystalline structure, while the third enzyme transforms the cellobiose to single glucose monomers (Singh *et al*., 2019).

Endoglucanases (EG) (EC 3.2.1.4), cellobiohydrolases (CBHI and CBHII) (EC 3.2.1.91), and -glucosidases (BGL) (EC 3.2.1.21) are the three types of cellulases. During the hydrolysis process, each type of enzymes serves a distinctive role and has a distinct method of action (Bussamra *et al*., 2015). Cellulase enzymes work by breaking the β-1,4 bonds in the cellulose chain. These enzymes are classified on the basis of their function. Endocellulases hydrolyze the internal bonds in the cellulose chain, exocellulases (cellobiohydrolases) work on the ends of the cellulose chains (either reducing or non-reducing ends), and -glucosidases convert cellobiose into individual glucose monomers (Baldrian & Valášková, 2008). Bacteria such as *Bacillus* and *Clostridium* and *Paenibacillus* species have been identified as highly effective producing cellulases (Liang *et al*., 2014). Other bacterial genera such as *Clostridium, Cellulomonas, Streptomyces, Bacillus, Thermomonospora, Ruminococcus, Bacteriodes, Microbispora, Fibrobacter, Acetovibrio, Erwinia, Paenibacillus, and Cellulosimicrobium*. have also been observed to produce of cellulases in mild culture conditions (Jayasekara & Ratnayake, 2019). Various species of fungi, including *Penicillium funiculosum, Aspergillus niger, Sclerotium rolfsii, Penicillium pinophilum, Penicillium sp. CR-316, Penicillium sp., Trichoderma reesei, Fusarium oxysporum, Humicola sp., Gloeophyllum trabeum, Melanocarpus sp.,* and *Ascomycota,* have been reported as producers of cellulases (Tasia & Melliawati, 2017)

Hemicelluloses can be categorized into endo-β-1,4-xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), and accessory enzymes responsible for breaking downside chains. These enzymes target various polysaccharides that make up hemicellulose, which contain glucose, xylose, mannose, arabinose, galactose, fucose, as well as acetic, ferulic, glucuronic, and galacturonic acids. The diversity in hemicellulose composition requires enzymes that are specific to break down these different components (Juturu & Wu, 2013; Větrovský *et al*., 2014). The endo-1,4-β-xylanase and β-xylosidase target the backbone of xylan. Additionally, α-l-arabinofuranosidase (EC 3.2.1.55), acetyl esterase (EC 3.1.1.6), and α-d-glucuronidase (EC 3.2.1.1) are also essential as they act on the side chains of xylan, contributing to its complete hydrolysis (Pason *et al*., 2006). Among microbial sources bacteria and fungi have been reported as the best source of xylanases because they are easy to control and handle their products as well as high

multiplication rate (Walia *et al*., 2017). *Thermomyces lanuginosus, Thermoactinomyces thalophilus, Chaetomium thermophilum, Thermoascus aurantiacus,* and *Dictyoglomus, Arthrobacter Thermomonospora, Bacillus, Fusarium proliferatum, Melanocarpus albomyces, Nonomuraea flexuosa, Thermotogales, Clostridium abusonum, Streptomyces* are some of fungal and bacterial species that produce xylanases respectively (Sharma & Kumar, 2013).

Cellulases and xylanases have many important applications across different industries, including energy production, pulp and paper, textiles, and animal feed. Furthermore, their significance in the food sector, encompassing bakery, wine, and fruit and vegetable juice production (Kuhad *et al*., 2011). Because of the complex nature and structure of these enzyme systems and their high industrial importance, cellulases have now become a subject of extensive area of research for researchers (Bhardwaj *et al*., 2021). Currently, researchers are focusing on exploring the knowledge related to cellulases and xylanases production and addressing the challenges associated with improving the economic viability of various industries (Mmango-Kaseke *et al*., 2016). Both the cellulases and xylanases hold equal commercial importance due to their ability to facilitate the conversion of lignocellulosic materials and agricultural wastes into valuable products that can be used for a variety of application at industrial and commercial level (Beg *et al*., 2001; Benatti & Polizeli, 2023).

The improper treatment of sugarcane bagasse is a major problem in Pakistan and worldwide. By hydrolysis of sugarcane bagasse, it can be converted into valuable products like bioethanol. The current work focuses on the isolation as well as characterization of two enzymes, cellulase and xylanase from bacterial strains CC16 and CC4 to hydrolyze pre-treated sugarcane bagasse, respectively. Cellulase and xylanase report in this research was a stepwise optimization policy for maximum enzyme production, including elucidation of environmental constituents and medium that produce the enzyme in much higher quantity using statistical tools. The purified cellulase and xylanase were screened out for pre-treated sugarcane bagasse hydrolysis using analytical techniques i.e. FTIR spectroscopy and SEM. This research is an effort to reveal the efficacy of cellulase and xylanase, and approaches that can be used to

explore more effective and multipurpose cellulases and xylanases to achieve industrial requirements.

Aim and Objectives

Aim

The aim of this study is to purify and characterize cellulase and xylanase enzymes for hydrolysis of pre-treated sugarcane bagasse and trash.

Objectives

1. Screening of bacterial isolates for the production of cellulase and xylanase enzymes through qualitative and quantitative assay
2. Optimization of culture conditions for maximum cellulase and xylanase production
3. Purification of cellulase and xylanase by size exclusion column chromatography
4. Characterization of purified cellulase and xylanase enzyme using different parameters
5. Analysing the hydrolysis of sugarcane bagasse with purified cellulase and xylanase using analytical techniques

Literature Review

2.1 Sugarcane bagasse as Lignocellulosic Biomass

Every year, millions of tons of agricultural waste residue are generated globally. The prevailing practice of burning this waste directly contributes to elevated levels of CO2 and particulate matter in the atmosphere, leading to the critical issue of global warming, which is a major concern for all nations at the present time. (Giudicianni *et al*., 2021; Kumar *et al*., 2017). Sugarcane (Saccharum officinarum) bagasse, a biomass derived from the agricultural waste generated during sugarcane processing, is widely available worldwide. Its abundant availability in nature has led researchers to explore various applications of this biomass, particularly in the areas of energy production and environmental sustainability (Ajala *et al*., 2021).

Ensuring energy security and preserving the environment are expected to continue as prominent long-term challenges that humanity will face on a global scale (Sheikhdavoodi *et al*., 2015). In the context of the current energy crisis, lignocellulosic biomass, including sugarcane bagasse (SCB), corn stover, cereal straw, and various forest woody residues such as birch, spruce, and eucalyptus, possess significant energy potential. These biomass sources offer a promising solution to alleviate the growing energy crisis due to their high energy content (Ajala *et al*., 2020). Excessive production of sugarcane has led to the burning of bagasse as a method of disposing of solid waste, resulting in environmental pollution. Consequently, waste recycling has emerged as a key area of scientific research, primarily driven by environmental considerations (Mokhena *et al*., 2018). The utilization of sugar industry residue serves an important residue not only from an environmental perspective but also as a significant substrate for large-scale making of valued and innovative yields (Rabelo *et al*., 2015). Sugarcane bagasse is widely recognized as an environmentally friendly biomass option for generating second-generation biofuels such as ethanol. Sugarcane bagasse is widely recognized as an environmentally friendly biomass option for generating second-generation biofuels such as ethanol. Additionally, it serves as a valuable source for producing electricity, enzymes, sugars, and various other high-value products (Ázar *et al*., 2019).

Bagasse, the fibrous residue of sugarcane, possesses structural sugars akin to cellulose and hemicellulose, rendering it a promising source for generating biofuels, organic acids, enzymes, and other valuable derivatives within sugarcane mills. Nevertheless, the saccharification process of sugarcane bagasse is impeded by the presence of lignin, which obstructs the productive binding of enzymes, thus hindering efficient conversion (Florencio *et al*., 2019). The presence of lignin in bagasse is a significant obstacle to efficiently converting cellulose and hemicellulose into individual sugar molecules, which are crucial for the production of biofuels (Kim, 2018). According to the most current data published by the Food and Agricultural Organization (FAO) of the UN, India, China, Brazil, Pakistan, Colombia, Mexico, the Philippines, Indonesia, and the US were the top ten nations in the world for sugarcane production in 2018. The global production of sugarcane bagasse amounts to approximately annually 540 million metric tons (Zhao *et al*., 2015).

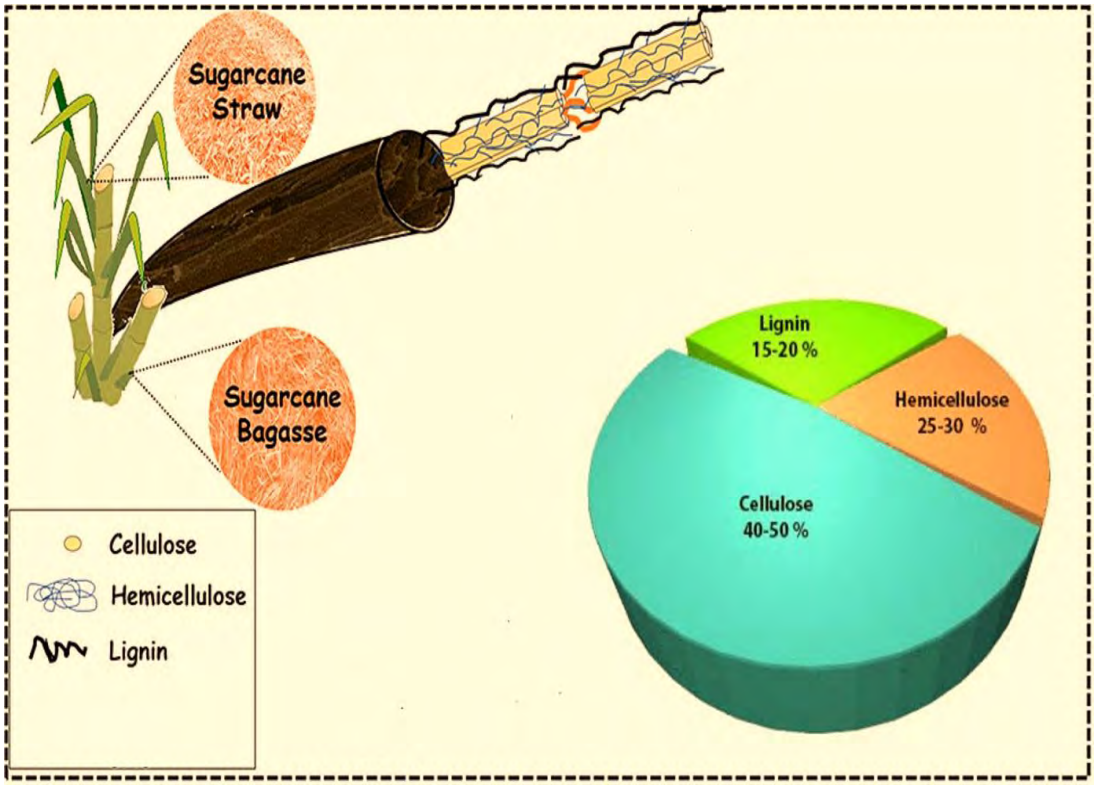

Figure 2.1: Composition of sugarcane lignocellulosic biomass (de Aguiar *et al*., 2020).

2.2 Chemical Composition of sugarcane bagasse

Cellulose stands as the most abundant polysaccharide polymer, a straight chain of the D- glucose molecules joined by β (1- 4) bond. This molecular arrangement generates

crystalline regions, enhancing its resistance to hydrolysis. Hemicellulose, the second most prevalent polysaccharide, differs from cellulose by being shorter and highly branched. It is composed of various sugars such as pentoses (arabinose and xylose) and hexoses (mannose, galactose, and glucose). The structure of hemicellulose differs depending on the source, and in the case of sugarcane bagasse, it mainly consists of heteroxylans with a higher abundance of xylose. Consequently, compared to cellulose, hemicellulose may be chemically degraded more quickly. The complex family of phenylpropanoid polymers known as lignin, on the other hand, is created when aromatic alcohols are polymerized. When combined with cellulose and hemicellulose, the cellulose hemicellulose lignin matrix imparts resistance to both chemical and enzymatic degradation processes (Ázar *et al*., 2019; Sabiha-Hanim & Abd Halim, 2018).

2.3 Hydrolysis of Sugarcane biomass

Sugarcane bagasse, a plant biomass residue obtained from the sugar-processing industry, serves as a renewable resource for the productions of biofuels and valuable macromolecules. It is utilized as a raw material in the production of biobased ethanol, contributing to sustainable energy solutions (Schmatz *et al*., 2020). Large-scale productions of ethanol from sugarcane juice transforms bagasse into an appealing raw material for the production of second-generation ethanol. The utilization of sugarcane lignocellulosic biomass for bioethanol production has gained significant attention due to the potential of cellulosic ethanol to replace gasoline, foster rural development, mitigate greenhouse gas emissions, and utilize non-edible materials. The hydrolysis process, which converts cellulose into glucose, can be catalyzed either by enzymes or acids. Acid-catalyzed hydrolysis effectively extracts sugars from highly intricate biomass; however, it typically require a high temperatures or concentrations of acid, rendering the process economically unviable (Carvalho *et al*., 2013; Dwivedi *et al*., 2009). The enzymatic saccharification process accounts for approximately 20% of the overall expenses in the production process, offering significant prospects for both cost reduction and innovation (Visser *et al*., 2015).

2.4 Enzyme-Catalyzed Hydrolysis

Enzymes are dynamic catalysts that effectively lower the activation energy, accelerating chemical reactions and reducing their overall duration, all without undergoing any permanent changes themselves. These catalysts are predominantly comprised of proteins, consisting of amino acid that are composed of many peptide bond, and possess a relatively high molecular weight. The term "enzyme" was initially coined by Wilhelm Kühne, a German physiologist, in 1877. Subsequently, James B. Sumner of Cornell University achieved a groundbreaking milestone in 1926 by successfully crystallizing and obtaining pure urease enzymes. The year 1959 witnessed Novozymes, a Danish company, becoming the first to commercialize the protease enzyme derived from *Bacillus*, while around 1969, a detergent manufacturing company embraced the application of *Bacillus* protease in detergent production. The food industry ventured into utilizing microbial enzymes in 1960, initially employing amylase in the starch processing sector as an alternative to acid hydrolysis. Remarkably, the starch processing industry now stands as the second-largest consumer of enzymes, trailing only the detergent industry in its utilization (Gurung *et al*., 2013). Cellulase and Xylanase enzymes offer significant advantages to the bio-based economy compared to chemical catalysts, as they facilitate various important transformations in an environmentally friendly manner. These enzymes possess key benefits such as low energy consumption, specificity and selectivity. Over the past decades, cellulases has become the third most widely used enzymes in diverse industrial process. Both cellulase and xylanase are extensively used enzymes, and numerous industrial applications necessitate their synergistic action. These application primarily include bioethanol production, deinking of wastepaper, animal feed processing, food processing, paper and pulp production, removal of fine fibers from textile materials (biostoning), and pharmaceuticals (Bajaj & Mahajan, 2019).

In 1955, the discovery of xylanases, initially referred to as pentosanes, marked a significant milestone. Later in 1981, the International Union of Biochemistry and Molecular Biology assigned the enzyme code EC 3.2.1.8 to xylanase. While various names have been used in scientific literature to describe xylanase, such as endo-xylanase, endo-1,4-β-D-xylanase, and beta xylanase, the official designation for this enzyme is endo-1,4-β-D-xylanase. Xylanases belong to the glycosidase family and

function as hydrolytic enzymes, specifically targeting xylan as their substrate. They catalyze the breakdown of xylan by cleaving the 1,4-β-linkage, resulting in the production of xylooligosaccharides. A wide range of organisms have been reported as producers of xylanases, including bacteria, gastropods, arthropods, protozoans, and fungi (Bhardwaj *et al*., 2019; Thapa *et al*., 2020).

"Cellulase" comprised of a broad range of cellulolytic enzymes, and the systems, having variety of the structures. This includes cellulases generated by microorganisms, whether they are cell-bound or extracellular. Moreover, cellulase can exhibit variations in their mechanisms of action (Korsa *et al*., 2022; Mattam *et al*., 2022). Cellulase is a general term encompassing a group that consists of three enzymes which are exoglucanases or cellobiohydrolase (EC 3.2.1.91), and the β-glucosidase and endo-1-4-β-glucanase (EC 3.2.1.4). These enzymes facilitate the breakdown of cellulose and related oligosaccharides. Specifically, endo-1-4-β-glucanase acts on carboxymethyl cellulose, producing the accidental cleavage of cellulose chain, resulting in the production of glucose and cello-oligosaccharides. On the other hand, exoglucanase breaks down micro crystals of cellulose (Avicel), releasing cellobiose as the main byproduct. Last but not least, -glucosidase releases glucose from cellobiose. Glucose is the result of the synergistic interaction between these enzymes. Numerous fungi and bacteria have been found to generate cellulase well to date (Bano *et al*., 2019).

Over the past few decades, biotechnology has placed significant emphasis on the bioconversion of lignocellulosic materials, which serve as a renewable energy source. Within these materials, xylan and cellulose play crucial roles as components of hemicelluloses, holding great potential for yielding valuable end products. Industries primarily rely on microbial xylanases and cellulases due to their numerous advantages. These enzymes demonstrate high specificity towards their respective substrates, resulting in minimal to no substrate loss. Moreover, they operate under mild catalytic conditions and generate low levels of byproducts. Consequently, xylanases and cellulases find extensive applications in diverse industries such as food, feed, and paper (Bhardwaj *et al*., 2021).

2.5 Cellulose overview

In 1838, Anselme Payen, a chemist from France, provided a detailed account of a fibrous element found in plant tissues. He discovered that this component could not be easily extracted using organic or aqueous solvents. Additionally, Payen determined the molecular formula of the substance to be $C_6O_5H_{10}$ and observed its resemblance to starch. In a subsequent report on his findings in 1839, he officially named this substance "cellulose" (McNamara *et al*., 2015). Cellulose stands as the predominant natural polymer in the biosphere, exhibiting an immense global production (and subsequent decomposition) of approximately 1.5 x 10^{12} tons annually. This quantity rivals the planetary reserves of crucial fossil fuels and mineral resources, highlighting its immense abundance in the natural world (Heinze, 2016).

Cellulose, the primary component of the plant cell wall, is a linear homopolymer composed of D-glucose molecules connected by β-1,4 glycosidic bonds. It adopts a conformation known as the 4 C1-chair configurations, which is energetically favorable. These glucose units are linked together in a repeating pattern through β-1,4-glycosidic bonds, causing the cellulose chain axis to alternate its direction by 180 degrees. Hemicellulose, on the other hand, is a highly branched heteropolymer that also contributes to the structure of the plant cell wall (Barbosa *et al*., 2020). The presence of intermolecular hydrogen bonds among cellulose units gives rise to a structured arrangement of crystalline microfibrils, forming a matrix, alongside regions that lack a distinct order, known as amorphous regions (Álvarez *et al*., 2016). Cellulose is a vital polymer found in the structural framework of both gymnosperms and angiosperms, comprising approximately 40-50% of wood. It is accompanied by hemicellulose, which shares similarities with cellulose but has a lower molecular weight, and lignin, a non-saccharide polymer with aromatic properties. Together, these three components form the primary constituents of the plant cell wall. Cellulose, specifically, is a condensed, chiral, and biodegradable linear polymer composed of β-D-anhydroglucopyranose units. It possesses covalent C1-C4 β-glycosidic linkages, rendering it water-insoluble, tasteless, and odorless (Heinze, 2016) Cellulose is an extensively structured polymer composed primarily of D-anhydro glucopyranose units (AGU) and cellobiose units connected via β-1,4-D-glycosidic bonds. Within cellulose, the central ring of AGU possesses a secondary alcohol hydroxyl group at carbon

positions 2 and 3, while a primary alcohol hydroxyl group is present at carbon position 6 (Yurtsever *et al*., 2022). Cellulose, being a prominent complex polysaccharide, serves as an abundant and renewable biomass resource for fulfilling the material and energy requirements essential for the sustainable progress of human civilization. A promising approach towards addressing global energy challenges involves the conversion of cellulose into sugars, which can be further fermented by microorganisms to generate bioethanol (Ragauskas *et al*., 2006). Cellulases, which play a vital role in breaking down cellulose within deceased plant matter, are considered the most crucial enzymes involved in this process. These enzymes function by cleaving the β-1,4 bond present in the cellulose chain. In the conventional classification, cellulases are categorized based on their mechanisms of action. Endocellulases act by breaking internal bonds within the cellulose chain, exocellulases or cellobiohydrolases operate on either the reducing or non-reducing ends of cellulose chains, while β-glucosidases convert cellobiose into individual glucose molecules (Baldrian & Valášková, 2008).

Cellulose is abundantly present in various natural sources, such as agricultural and forestry residues, energy crops, municipal paper, detergent industries, and food waste. However, the process of breaking down cellulose into its components is extremely intricate. Unlike many other compounds, cellulose does not readily dissolve in water, which is why enzymatic conversion has emerged as a crucial technology for the biodegradation of lignocellulosic materials (Baldrian & Valášková, 2008). The intricate nature of cellulose necessitates the implementation of multiple pre-treatment techniques and a diverse range of cellulolytic enzymes working collaboratively to effectively break down cellulose into individual sugar molecules, which can be utilized in fermentation processes (Houfani *et al*., 2020). Cellulases are a class of glycoside hydrolases (GHs) that break down the β-1,4-D-glucan linkages present in cellulose, resulting in the production of glucose, cellobiose, and cellooligosaccharides as the main products of hydrolysis (Nordbring-Hertz *et al*., 2006). Cellulases are categorized based on their specific catalytic mechanisms. They work in coordination, simultaneously targeting distinct sites within amorphous cellulose, such as internal bonds (endoglucanase), chain ends (cellobiohydrolases), and cellobiose-to-glucose conversion (β-glucosidase) (Haldar *et al*., 2016). Endoglucanases, also known as

endo-1,4-β-D-glucan glucanohydrolases, perform random cleavage of cellulose chains within the amorphous regions. Their role is to initiate the enzymatic breakdown of cellulose, leading to a decrease in its polymerization level (Garg *et al*., 2016; Tomás-Pejó *et al*., 2010). Exoglucanases, also known as cellobiohydrolases (CBH), are enzymes that generate cellobiose at distinct ends of the cellulose molecule: CBH I at the reducing end and CBH II at the non-reducing end. β-glucosidases (BGL) or cellobiases, on the other hand, break down cellobiose and, in certain instances, cello-oligomers containing up to 6 glucose units. (Quiroz-Castañeda & Folch-Mallol, 2013). Cellulases find significant industrial applications across various sectors. In the textile industry, they are utilized for processes such as bio-polishing, bio-stoning, and bio-finishing. They also play a crucial role in starch processing, grain alcohol fermentation, and the brewing and malting of wine and beer in the respective industries. The food industry benefits from cellulases in the extraction and processing of fruit and vegetable juices. Additionally, cellulases are employed in the paper and pulp industries, in addition to in agriculture for controlling plant pathogens and diseases. Furthermore, they are incorporated into household laundry detergents to enhance fabric softness and brightness. These diverse applications of cellulases contribute to their wide-ranging industrial significance (Gulve & Deshmukh, 2011).

Numerous bacteria and fungi are known for producing cellulases, enzymes that break down cellulose. Among the extensively studied organisms with exceptional cellulolytic activity are various fungal species such as *Humicola, Trichoderma, Penicillium*, and *Aspergillus*. Additionally, certain bacterial species including *Pseudomonas, Bacilli*, *Actinomycetes, streptomycetes, Cellumonas, Streptomyces*, and *Actinomucor* exhibit cellulolytic capabilities. *Trichoderma reesai*, in particular, stands out as a highly researched fungus due to its ability to efficiently convert both native and desired cellulose into glucose (Imran *et al*., 2016).

2.5.1 Cellulolytic Complex

Cellulose, a major natural biopolymer present on Earth, serves as a primary component in the plant cell wall, specifically in its lignocellulosic form. Various microorganisms, including aerobic bacteria, fungi, yeast, and actinomycetes, possess the ability to produce cellulase enzymes. These enzymes facilitate the breakdown of cellulose by breaking down the β-1, 4-glycosidic linkages in the cellulose structure through

hydrolysis (Gulve & Deshmukh, 2011). The process of converting cellulose into glucose requires the coordinated activity of three enzyme groups: exo-cellobiohydrolases (EC 3.2.1.91), β-glucosidases (EC 3.2.1.21) enzymes and endo-β-1,4-glucanases (EC 3.2.1.4), (Benoliel *et al*., 2013)

2.5.1.1 Endo-β-1,4-glucanases

Endo-β-1,4-glucanase (EC 3.2.1.4) is synthesized by various microorganisms such as fungi, bacteria, and archaea. Its enzymatic activity involves the non-specific cleavage of the β-1,4-glycosidic bonds present between glucose units in cellulose molecules, leading to the production of cello-oligosaccharides with shorter chain lengths. (Gavande & Goyal, 2023). Based on the Carbohydrate Active Enzymes database (CAZy) available at http://www.cazy.org/, endoglucanase enzymes are classified under several glycoside hydrolase (GH) families, namely 5–10, 12, 44, 45, 51, 74, and 128 (Yennamalli *et al*., 2013). Endo-β-1,4-glucanase catalyzes the breakdown of β-1,4-glycosidic linkages present in β-1,4-glucans. These glucans are glucose polymers connected through β-1,4-glycosidic bonds. Cellulose, a β-1,4-glucan, exhibits a distinct composition characterized by both crystalline and amorphous regions. The crystalline regions display a well-organized structure, with parallel polymers being held together by hydrogen bonds. On the other hand, the amorphous regions lack this ordered arrangement. The action of endo-β-1,4-glucanase involves breaking internal β-1,4-O-glycosidic bonds within cellulose, resulting in the production of small oligomers consisting of 4–6 glucose units. These oligomers are subsequently subjected to hydrolysis, leading to the breakdown into smaller constituents (Linton, 2020; Ueda *et al*., 2014). Endoglucanases have a crucial function in enhancing the production of fruit juices, improving the filtration process in beer production and oil mining, in addition to enhancing the nutritional value of animal feed and bakery products. These enzymes are also becoming increasingly significant in the pulp and paper sector (Jagtap *et al*., 2014)

2.5.1.2 Exo-cellobiohydrolases

Exoglucanases, referred to as cellobiohydrolases (CBHs), are enzymes that operate at the closure of cellulose chain, discharging cellobiose and glucose in a sequential manner. CBHs are extensively studied and account for approximately 70 percent of

cellulases produced by cellulolytic fungi. These enzymes belong to glycoside hydrolase (GH) 6 and 7, as well as 48 other family (Annamalai *et al*., 2016; Takahashi *et al*., 2010). Various bacteria and fungi produce different types of cellobiohydrolases, which contain catalytic modules that are categorized into families 5, 6, 7, 9, 48, and 74 glycoside hydrolases. Aerobic fungus cellobiohydrolases are classified under families 6 and 7, while aerobic bacterial cellobiohydrolase are grouped into families 6 and 48. On the other hand, anaerobic fungal cellobiohydrolases are found in family 48, while anaerobic bacterial cellobiohydrolases can be found in families 9 and 48. It is noteworthy that family 7 cellobiohydrolases exclusively originate from fungi, whereas family 48 cellobiohydrolases primarily come from bacteria (Zhang & Zhang, 2013). Cellobiohydrolases or exoglucanases have the ability to efficiently operate on microcrystalline cellulose, potentially removing cellulose chains from its microcrystalline structure. Generally, CBHs exhibit activity on the crystalline sections of cellulose, while endoglucanases (1,4-β-glucanases) are commonly dynamic on the more solvable fluid area of the cellulose crystal (MUBAROK, 2018). Cellobiohydrolases or exoglucanases are enzymes that can be found in a diverse range of bacteria and fungi. These enzymes possess great potential for utilization in various industrial sectors, including feed production, food processing, and detergent manufacturing (Chaari & Chaabouni, 2019).

2.5.1.3 β-glucosidases

β-Glucosidases, also known as β-D-glucoside glucohydrolase (EC 3.2.1.21), have a widespread presence in bacteria, fungi, plants, and animal tissues. These enzymes play a crucial role in carbohydrate hydrolysis by effectively catalyzing the specific breaking of β-D-glucosidic bonds in oligosaccharide constituents (Mao *et al*., 2010). β-Glucosidases are a highly diverse group of enzymes that play a crucial role in biological systems. These enzymes, also known as β-D-glucopyrranoside glucohydrolase [E.C.3.2.1.21], are responsible for breaking down the glycosidic bond in carbohydrate molecules. This process results in the release of glycosyl residues, glycosides, and oligosaccharides (Singh *et al*., 2016). β-Glucosidases play a crucial role in the cellulase system, which consists of enzymes that break down cellulose. These enzymes are responsible for the final step in cellulose hydrolysis. Cellulase

enzymes break down cellulose into cellobiose and other smaller oligosaccharides, and these compounds are further broken down into glucose by β-glucosidase. This process is essential for cellulose metabolism (Teeri, 1997). β-Glucosidases play a significant role in multiple biotechnological applications, such as the extraction of biofuel and ethanol from cellulosic agricultural residues, as well as the creation of beneficial β-glucosides. These enzymes are essential in the enzymatic breakdown of glucosidic originators found in fermented and fruits products, releasing aromatic compounds (Krisch *et al*., 2010). β-Glucosidases are enzymes that break down β-glycosidic bond non-carbohydrate and carbohydrate component. The majority of characterized β-glucosidases belong to the GH1, GH3, and GH30 families and are classified as retaining enzymes. These enzymes carry out their catalytic process in two stages: glycosylation and deglycosylation (Davies & Henrissat, 1995). In the first step of glycosylation, the amino acid glutamate acts as a nucleophile and initiates a reaction by attacking the anomeric carbon. This reaction leads to the formation of an intermediate product consisting of glucose bound to the enzyme. In the second step, a water molecule is activated by an acid/base catalyst, which is a glutamate residue. The water molecule then acts as a nucleus loving to break the glycosidic linkage, resulting in the release of glucose.

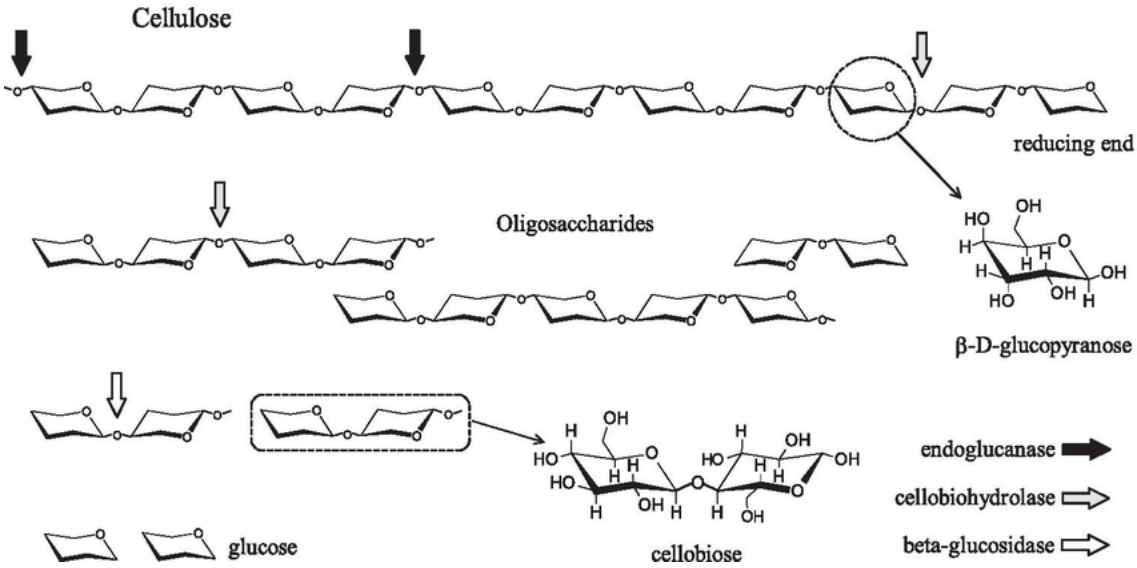

Figure 2.2: A schematic diagram illustrating the mechanism by which cellulolytic complex enzymes act on cellulosic material, leading to the liberation of oligosaccharides and monosaccharides (de Souza & Kawaguti, 2021).

2.5.2 Sources of cellulases

Cellulase enzymes are predominantly present in fungi and bacteria, and they hold significant significance in both industrial and natural settings. These enzymes play a crucial role in the global carbon cycle by breaking down insoluble cellulose into soluble sugars, thereby contributing to the degradation of cellulose in the environment (Abbasi Moud, 2022). Cellulose-degrading microorganisms can be discovered in decomposing grasses, foliage, and timber, as well as within cotton bundles, sewage residues, fermented silage, compost piles, muds, deteriorating plant material, and even in challenging surroundings such as hot and acidic volcanic regions, as well as alkaline springs. They are also prevalent in soil, swamps, marshes, aquatic environments, and sedimentary deposits in seawater (Siva *et al*., 2022). Various microorganisms of industrial importance, such as fungi like *Aspergillus fumigatus, Aspergillus tubingensis, Penicillium chrysogenum, Fusarium solani, Trichoderma reesei*, and *Sclerotium rolfsii*, are capable of producing cellulase enzyme. In addition to fungi, bacterial species such as *Pseudomonas fluorescens, Bacillus licheniformis, Ochrobactrum haematophilum, Micrococcus sp, Bacillus VITRKHB, Paenibacillus terrae ME27-1,* and *Enhydrobacter sp. ACCA2*, demonstrate the ability to produce cellulase using different substrates through submerged and solid-state fermentation *(Korsa et al., 2023; Sethi et al., 2013)*. Bacteria, known for their higher growth rates compared to fungi, exhibit promising potential in the production of cellulase *(Korsa et al., 2023).*

2.5.2.1Bacterial Sources of cellulases

The cellulosomal complex was initially observed in the early 1980s in the anaerobic thermophile *Clostridium thermocellum*. Subsequently, bacteria capable of producing cellulosomes have been discovered in various ecosystems, including both mesophilic and thermophilic environments (Liu *et al*., 2021). Bacteria that degrade cellulose are found widely across nature and have been isolated from diverse habitats. Bacterial cellulases possess several advantages, such as faster growth rates and genetic adaptability, which highlight their suitability and benefits compared to other sources of these enzymes (Menendez *et al*., 2015). Bacteria have been widely used in the production of cellulase due to their faster growth rate compared to fungi. In various culture conditions, bacteria have shown to be efficient in producing cellulase enzymes

(NAKAMURA & KITAMURA, 1982). Over the years, cellulase-producing bacteria have been isolated from diverse sources including soil, decaying wood, ruminant feces, and insect guts (Doi, 2008). These bacteria have the ability to synthesize complex protein structures that support cellulose hydrolysis enzymes, such as cellulosomes, xylosomes, and bifunctional or multifunctional enzymes. These advancements have attracted significant attention in the scientific community (Doi, 2008; Liu *et al*., 2021).

Numerous genera within the major classes *Bacilli* and *Clostridia* have been identified as highly effective in breaking down cellulose. For instance, *Bacillus* and *Paenibacillus* species have demonstrated remarkable cellulolytic abilities, which can be attributed to the presence of a diverse array of GH enzymes in their genomes (Liang *et al*., 2014). Additionally, bacteria belonging to the genera *Clostridium, Cellulomonas, Cellulosimicrobium, Thermomonospora, Bacillus* and many other genera have also been observed to produce various types of cellulase under both anaerobic and aerobic conditions (Jayasekara & Ratnayake, 2019). Cellulases have displayed immense potential for utilization in numerous industries, including food production, animal feed, brewing and winemaking, agriculture, biomass refining, pulp and paper, textile manufacturing, and laundry applications (Shaikh *et al*., 2013).

2.5.2.2 Fungal Sources of cellulases

Fungi are widely recognized for their ability to efficiently decompose cellulose, contributing to approximately 80% of cellulose decomposition on Earth. They play a crucial role as the main agents responsible for breaking down cellulose and lignin in forest ecosystems (Sajith *et al*., 2016). The industry primarily favors cellulases synthesized by aerobic fungi due to their high production potential, which is directly linked to fungal growth and their adaptability in natural environments (Park *et al*., 2010). Fungal cellulases have relatively simple structural features, characterized by distinct modular designs. Most of these enzymes possess a specific structural orientation, consisting of one cellulose binding and one catalytic site domain (Ahmed *et al*., 2017). To produce cellulases, two fermentation methods are commonly employed: submerged fermentation (SmF) and solid-state fermentation (SSF). In submerged fermentation, a nutrient broth or liquid medium is utilized to dissolve

essential nutrients, which are then utilized by the microbial source for enzyme production. The enzyme is generated within the fermentation medium, alongside the used medium. These can be separated through centrifugation. Submerged fermentation offers advantages such as easy sterilization, parameter monitoring, and a quick downstream process Various species of fungi, including *Penicillium funiculosum, Aspergillus niger, Sclerotium rolfsii, Penicillium pinophilum, Penicillium sp. CR-316, Penicillium sp., Trichoderma reesei, Fusarium oxysporum, Humicola sp., Gloeophyllum trabeum, Melanocarpus sp.,* and *Ascomycota,* have been reported as producers of cellulases (Tasia & Melliawati, 2017).

2.6 Xylan an overview:

Xylan constitutes the principal constituent of hemicelluloses, accounting for approximately 20-40% of the entire lignocellulosic biomass. It is considered the second most abundant naturally occurring polysaccharide after cellulose (Golestani, 2020; Zhu *et al*., 2022). Xylan biopolymers can be found in hardwood and perennial plants, including grasses, cereals, and herbs. Biopolymers of the xylan have a backbone composed of (1-4)-linked anhydroxylose, with some branching points containing arabinose, glucuronic acid, and acetic substituents at the 2 and 3 positions of xylose. Among the significant heteropolymers, xylan, mannan, galactan, and arabinan play crucial roles. The monomeric unit of xylan is formed by D-xylose and L-arabinose, while D-galactose units are present in galactan. Mannan contains D-mannose units, and arabinan consists of L-arabinose units (Broeker *et al*., 2018; Geng, 2019). Xylan polysaccharides are predominantly found in tropical plants, constituting approximately 20-35% of the plant's dry weight in hardwood. In contrast, xylan is less abundant in perennial plants, comprising around 8% of the total dry weight. It is primarily located in the secondary cell wall and forms covalent bonds with phenolic residues of lignin. Xylan works in conjunction with other polysaccharides such as pectin and glucan. The structural arrangement of xylan involves the linkage of its monomer, D-xylose, through β-1,4-glycosyl bonds (Qaseem & Wu, 2020).

Xylans are a prevalent component of hemicellulose, which constitutes a significant portion of plant biomass, typically accounting for 20-40% of the total biomass. Xylans serve as the primary constituent of raw materials and a vital food source for farm

animals, while also finding diverse industrial applications, including paper production and baking (Khaire *et al*., 2021). Xylan is a heteropolysaccharide characterized by its intricate structure, comprising diverse side chains such as 4-O-methyl-D-glucuronosyl and α-arabinofuranosyl residues, among others. Its intricate nature poses challenges for complete hydrolysis due to its heterogeneity. However, the use of xylanases can overcome this limitation by efficiently breaking down the heterogeneous β-1,4-glycoside linkage. Various enzymes, including α-L-arabinofuranosidases, α-D-glucuronidases, endo-β-D-xylanases, β-D-xylosidases, acetylxylan esterases, ferulic acid esterases, and p-coumaric acid esterases, play crucial roles in cleaving both the linear chain and side chains of xylan (Bhardwaj *et al*., 2019). Xylanases possess distinct enzymatic properties that enable them to effectively hydrolyze complex polysaccharides. These specific functions of xylanases contribute to their widespread utilization in various industries, including baking, food processing, animal feed production, and the pulp and paper industry (Tyagi & Sharma, 2021). A wide range of microorganisms, including bacteria, actinomycetes, and filamentous fungi, exhibit a substantial secretion of extracellular xylanase. However, among these microorganisms, filamentous fungi demonstrate a higher production of extracellular protein, specifically xylanase, in comparison to bacteria and actinomycetes (Ajeje *et al*., 2021).

2.6.1 Xylanolytic complex:

Xylan hydrolysis require the presence of xylanases for hydrolysis, which are enzymes secreted by microorganisms to degrade the polysaccharides found in plant cell walls. Various microorganisms, including bacteria, actinomycetes, protozoa, and algae, are capable of producing xylanase. However, filamentous fungi are widely recognized as the most efficient producers of this enzyme (Dhiman & Mukherjee, 2018).

Xylans, which constitute the primary component of plant hemicellulose, are complex polysaccharides characterized by a branched and heterogeneous structure. They possess a intricate backbone composed of xylopyranosyl units connected through β-1,4-glycosidic bonds. In addition to their branching nature, xylans also contain various side chain residues, including acetyl groups, arabinose, glucuronic acids, and other similar residues (Méndez-Líter *et al*., 2021). The breakdown of xylan, a complex

process, necessitates the involvement of multiple enzymes known as xylanolytic enzymes. The primary enzymes required for this degradation are endo-1,4 β-xylanase and xylosidase. In addition to these key enzymes, L-arabinofuranosidases, glucuronidases, ferulic/coumaric acid esterases and acetyl xylan esterases have been recognized as crucial enzymatic activities involved in the deconstruction of xylan (Nguyen-Thi, 2014; Rakotoarivonina *et al*., 2014).

2.6.3 Xylanolytic enzyme:

2.6.3.1 Endo-1, 4-β–xylanase

The activity of endo-1,4-β-xylanase leads to a decrease in polymerization by specifically breaking the glycosidic bonds of hetero-xylan. This cleavage process is not random and is influenced by various factors such as the molecular characteristics of the substrates, including the presence of substituents, the length of the chain, and the number of branches (Bhardwaj *et al*., 2019). Following hydrolysis, xylopyranosyl oligomers emerge initially, while smaller molecules such as mono-, di-, and trisaccharides of β-D-xylopyranosyl may be observed at a later stage. Xylanase enzymes can be classified into two categories based on their hydrolysis activity: debranching enzymes and non-debranching enzymes (Miao *et al*., 2015). Fungal species have the capability to produce two distinct types of xylanases: debranching xylanases and non-debranching xylanases. Debranching xylanases are responsible for breaking down arabinoxylan by cleaving it at the 1,3-α-L-arabinofuranosyl linkage, resulting in the production of arabinose. On the other hand, non-debranching enzymes like Endo-1, 4-β-xylanase are unable to act on the 1,3-α-L-arabinofuranosyl group of arabinoxylan, thus preventing the production of arabinose (Binod *et al*., 2019). Hence, molecules are categorized into two primary classes based on their molecular weight: basic proteins for those with low molecular weight below 30 kDa, and acidic proteins for those with high molecular weight exceeding 30 kDa (Ward, 2021).

2.6.3.2 β-D-Xylosidase

β-D-xylosidases are categorized based on their preference for xylose and high molecular weight xylooligosaccharides as substrates. Xylobiases and 1,4-β-xylanase are distinct enzymes; however**, t**hey are alternatively known as β-xylosidases due to

their shared ability in decomposing minor units of xylobiose and xylooligosaccharides, resulting in the creation of a β-D-xylopyranosyl residue from the terminal non-reducing end. The involvement of β-xylosidases is noteworthy as they come into play subsequent to a series of successive hydrolytic actions on xylan by xylanases (Li *et al*., 2018). The successive enzymatic breakdown processes lead to the buildup of shorter oligomers with β-D-xylopyranosyl units. These gathered oligomers could potentially impede the functioning of endoxylanase enzymes. However, the action of β-xylosidase on these oligomers serves to remove the inhibitors, thereby enhancing the efficiency of xylan hydrolysis (Gauterio *et al*., 2021).

2.6.3.3 Acetylxylan esterases

Removing O-acetyl group at the position number 2 and 3 of the β-D-xylopyranosyl residue leads to the modification of polysaccharides and oligosaccharides, resulting in the exposure of the primary xylan structure. This modification allows xylanases and β-xylosidase enzymes to effectively target and interact with the xylan substrate (Najjarzadeh *et al*., 2020). Acetyl xylan, a polysaccharide, undergoes expulsion of the O-acetyl branch at position number 2 and 3 on residue of the β-D-xylopyranosyle. This enzymatic process is facilitated by acetylxylan esterases, which act as catalysts. In the treatment of acetylated xylan, it is common to employ soluble bases that induce the elimination of acetyl groups from whole structure of xylan. Presence of acetyl side groups in xylan plays a crucial role in steric hindrance, preventing endoxylanases from accessing the polysaccharide backbone during xylan hydrolysis (Vermani *et al*., 2020).

2.6.3.4 Arabinase

Arabinase is responsible for the removal of L-arabinose residues that are substituted at either the 2 or 3 positions on the α-D-xylopyranosyle (Fujimoto *et al*., 2021). Arabinases can be classified into two categories based on their specific activity: exo-α-l-furanosidases and endo-1-5-α-l-arabinases. Exo-α-l-furanosidases are enzymes that can hydrolyze both p-nitrophenyl-α-l-furanoside and arabinose in a sequential manner, while endo-1-5-α-l-arabinases specifically degrade linear arabinose chains. Currently, the majority of purified and studied arabinases belong to the exo type I

(Valenzuela *et al*., 2016).

2.6.3.4 α-glucuronidase

Xylanolytic enzymes, like α-glucuronidase, possess the capability to break down α-1,2 bond which are located in the glucuronic acid residues, leading to the degradation of the β-D-xylopyranosyl structural backbone present in glucuronoxylan. The activity of microbial enzymes is influenced by the characteristics of the substrate. Enzyme specificity in relation to their substrates is determined by the enzyme's source (Obeng *et al*., 2017). There are certain types of glucuronidase enzymes that can break down entire polymer chains, while others specifically target smaller units known as oligomers of glucuronoxylan (de Aguiar *et al*., 2020).The effectiveness of α-glucuronidase, an enzyme involved in this process, is impaired by the presence of acetyl subunits located near glucuronosyl subunits .

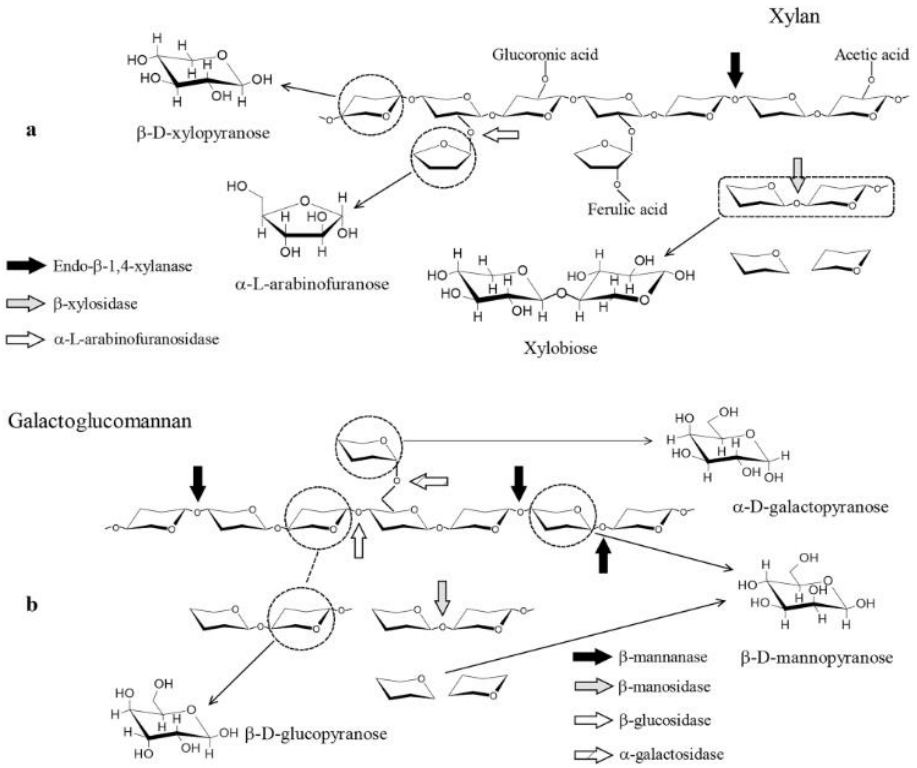

Figure 2.3: Shows Mechanism of action of specific Xylanolytic enzyme, resulting in the release of oligosaccharides and monosaccharides (de Souza & Kawaguti, 2021).

2.6.4 Sources of xylanase

Xylanases are enzymes that belong to the class of xylosidic hydrolases. They are produced by a diverse range of organisms, encompassing both prokaryotes and eukaryotes. These organisms include insects, crustaceans, snails, seeds, protozoans, termites' intestinal bacteria, actinomycetes bacteria, and fungi. Xylanases function by breaking down the glycoside bond found in xylan, leading to the formation of hemi acetyls and glycans (Basu *et al*., 2018; Motta *et al*., 2013) whereas, bacteria (Sarrouh *et al*., 2012) and fungi (Kumar *et al*., 2016) had been characterized the best source of valuable enzymes because easy to control and handle their products as well as high multiplication rate. The primary origins of microbes responsible for the production of industrial enzymes are found in natural environments. However, the extreme conditions encountered during industrial processes, such as high temperatures, acidity, alkalinity, and the presence of various solvents, can pose challenges at different stages. Therefore, it is necessary to develop enzymes that can withstand these harsh conditions and exhibit optimal catalytic activity (Galbe & Wallberg, 2019). In recent decades, a wide range of bacterial and fungal species have been identified as potential sources of enzymes for industrial catalysts. These include *Thermomyces lanuginosus, Thermoactinomyces thalophilus, Chaetomium thermophilum, Thermoascus aurantiacus, Dictyoglomus, Arthrobacter Thermomonospora, Bacillus, Fusarium proliferatum, Melanocarpus albomyces, Nonomuraea flexuosa, Thermotogales, Clostridium abusonum,* and *Streptomyces,* among others. These organisms have been found to produce xylanases, which are enzymes with significant applications in various industrial processes (Sharma & Kumar, 2013). Filamentous fungi exhibit a greater capacity to secrete xylanase compared to bacteria. Among these fungi, the species *T. lanuginosus* stands out as the most proficient and predominant producer of xylanase (Knob *et al*., 2014).

2.6.4.1 Bacteria

Xylanases are naturally derived from various biological sources, and several bacteria, such as *Caldocellum saccharolyticum*, Bacillus *thermantarcticus*, *Bacillus aerophilus*, and *Streptomyces althioticus*, have been documented for their ability to produce xylanolytic enzymes (Basu *et al*., 2018; Goswami *et al*., 2014). Xylanase extracted

from various bacterial species has undergone purification and characterization based on biochemical principles (Irfan *et al*., 2014). Bacterial enzymes possess remarkable resilience in the face of challenging environmental conditions such as high temperatures and extreme pH levels. This unique adaptability allows these enzymes to effectively enhance lignocellulose bioconversion, offering expanded functionality and synergistic effects (Irfan *et al*., 2014). A strain of *Paenibacillus* bacteria, previously classified within group 3 of the *Bacillus* genus, has been identified for its remarkable capacity to break down various carbohydrates. Moreover, this bacterium exhibits the remarkable ability to produce specific extracellular enzymes that play a crucial role in industrial applications (Ghio *et al*., 2018).

Xylanolytic bacteria have been observed in a wide range of bacterial groups, indicating their widespread presence. Various bacteria, including extremophiles and those specifically known for producing xylanase, such as *Bacillus subtilis, Bacillus pumilus, Bacillus megatorium, Bacillus amyloliquefaciens, Bacillus circulans, Bacillus cereus, Bacillus stearothermophilus, Bacillus licheniformis, Streptomyces roseiscleroticus, Streptomyces sp., Streptomyces actuosus, Streptomyces cuspidosporus, Clostridium absonum, Pseudonomas sp.,* and *Thermoactinomyces thalophilus*, have been found to possess xylanases that exhibit characteristics like thermostability, cold adaptivity, or alkalo-stability. These xylanases have been isolated and purified from these bacteria (Burlacu *et al*., 2016; Mandal, 2015). Research findings have shown that Bacillus sp. bacteria exhibit increased xylanase activity under conditions of elevated temperatures and alkaline pH (Çiçekler, 2022). Xylanases are enzymes synthesized by bacterial species found in various fields such as forestry, agriculture, and industries. These bacteria can thrive in a wide temperature range, including thermophiles, mesophiles, and psychrophiles. Notably, xylanases produced by thermophilic eubacteria and archaea exhibit significantly longer half-lives (T1/2) at temperatures of 80°C or higher compared to those derived from thermophilic fungi. While bacterial xylanase activities are generally low, they possess essential characteristics like specificity, thermostability, and extreme pH tolerance, which are crucial for numerous industrial and environmental applications (Kapilan & Arasaratnam, 2017).

2.6.4.2 Fungi

Fungi are commonly employed as significant producers of xylanase and other enzymes involved in xylan degradation, surpassing yeast and bacteria due to their remarkable ability to secrete these enzymes at substantially higher levels within the growth medium (Ho, 2017). Certain fungal genera, including *Trichoderma, Pichia, Aspergillus*, and *Fusarium,* have been identified as significant producers of xylanases. Recently, a novel fungus called *Cladosporium oxysporum* has been discovered, which exhibits the ability to produce specific extracellular xylanases. These xylanases hold potential applications in various industries such as paper production, feed manufacturing, and textile processes (Del-Cid *et al*., 2014; Singh *et al*., 2019). *Cuninghamella subvermispora* demonstrated an increased production of xylanases when exposed to polysaccharides found in the cell walls of plants as well as wood chips (Abena, 2021). Thermophilic fungi are recognized as the prevailing producers of xylanase, surpassing their mesophilic fungal counterparts in terms of enzymatic production (Patel & Rawat, 2021). In recent research, significant interest has been garnered by thermophilic fungal isolates like *Thermoascus aurantiacus, Melanocarpus, Humicola insolens, Malbranchea,* and *Chaetomium thermophilum* due to their ability to produce xylanase. These isolates have attracted attention in the scientific community for their biotechnological applications, as they generate thermos-alkali stable xylanases and contribute to enhanced pulp bio-bleaching (Dahiya *et al*., 2019). In the context of bioenergy, food production, and animal feed, there is a strong demand for materials that exhibit stability even in conditions of high temperature and acidic pH levels (Srivastava *et al*., 2018). Further enhancements are required as it has been documented that the majority of xylanases fail to meet the standards necessary for industrial utilization (Uday *et al*., 2016). Researchers are currently exploring unexplored extreme environments and utilizing various techniques such as protein engineering and genetic engineering to enhance industrial enzymes, with a particular focus on improving xylanases. To provide an overview of commercially available fungal xylanases, 15 different companies have been identified. These companies utilize fungal strains such *as T. reesei, T. longibrachiatum, T. viride*, and *Humicola insolens* for the production of xylanases, which are primarily recommended for the food and feed industries. However, it should be noted that none

of these enzymes have optimal pH and temperature above 6 and 60˚C, respectively. In contrast, *Thermomyces lanuginosus* has been found to produce more promising xylanases. These enzymes have an optimum temperature of 70˚C and exhibit stability across a wide pH range (Li *et al*., 2012; Madhavan *et al*., 2017; Singh *et al*., 2003).

2.7 Applications of Cellulases and xylanases

Cellulases and xylanases exhibit great potential for utilization in various industries, including energy production, pulp and paper, textile, and animal feed. Additionally, their importance in food industries, such as bakery, wine, and fruit and vegetable juice production, cannot be overlooked (Kuhad *et al*., 2011). Given the complexity of these enzyme systems and their immense industrial value, cellulases have become a subject of extensive research for both academic and industrial groups. Currently, there is a significant focus on advancing the facts of cellulase generation and addressing the complications associated with improving the economic viability of various industries. Similarly, xylanases hold equal commercial importance due to their ability to facilitate the conversion of lignocellulosic materials and agricultural wastes into valuable products (Beg *et al*., 2001; Benatti & Polizeli, 2023).

2.7.1 Cellulase and Xylanases used in Pulp and paper

The paper and packaging industry holds great importance in the overall global economy which contribute a crucial role globally. Cellulase, an intricate enzyme produced by various microorganisms, has immense potential in the pulp and paper sector. Cellulase accounts for 10% of the global demand for industrial enzymes, and there is substantial room for its biotechnology application to grow commercially, opening up new possibilities in pulp and paper manufacturing. Key enzymes like cellulase, xylanase, laccase, and lipase are highly significant for various processes in the pulp and paper industry. In paper production, the initial stage involves the elimination of lignin from lignocellulose materials. The utilization of cellulase and xylanase in this process reduces the need for chemical usage, resulting in a significant reduction in the kappa number, while simultaneously enhancing brightness and viscosity (Henrissat *et al*., 1998; Roncero *et al*., 2005).

Cellulases and xylanases find numerous applications in the paper and pulp industries. These enzymes are used for biopulping, enzymatic deinking, dewatering, biocharacterization of pulp fibers, biobleaching of kraft pulps, reducing drainage problems, and improving the handsheet strength of fibers. Additionally, they play a crucial role in the production of biodegradable cardboard, soft paper like towels and sanitary paper, as well as in the removal of adhered paper (Beg *et al*., 2000; Buchert *et al*., 1994).

Xylanases primarily target the xylan that has been relocated and reprecipitated on the outer layer of pulp fibers. The enzymatic breakdown of this particular type of xylan results in the increased permeability of the fiber structure, facilitating the more efficient removal of leftover lignin from the fibers (Kuhad *et al*., 2016). Xylanases possess remarkable dynamism, renewable properties, and exhibit high specificity in their ability to improve the quality of paper. These enzymes work on xylan, while cellulases act on cellulose fibers, consequently enhancing the effectiveness of paper bleaching (Kuhad *et al*., 2016). Cellulases and hemicellulases are extensively employed in industries for the de-inking of waste pulp. These enzymes, particularly cellulases and xylanases, play a crucial role in bio pulping, facilitating the production of recyclable paper and other paper products. Additionally, cellulases are utilized as co-additives in biobleaching processes to enhance drainage, reduce chlorine consumption during bleaching, improve fiber brightness, and aid in the bio de-inking of pulp (Lee *et al*., 2006; Sims & Bates, 1994).

2.7.2 Cellulases and Xylanase in fruit juice enrichment

Raw juice is often cloudy and thick, causing it to separate during storage. The cloudiness and thickness of the juice are primarily caused by polysaccharides like hemicelluloses, starch, pectin, cellulosic, and bound lignin (Nagar *et al*., 2012). Adding enzymes that break down cell walls can enhance the clarification process for fruits, wine, and oil. Currently, a combination of macerating enzymes, such as xylanase, cellulose, and pectinase, is used to extract and clarify juice from fruits like apricots, peaches, bananas, and papayas. The use of cellulases and xylanases enzymes has been shown to improve the yield and clarity of fruit juices while reducing viscosity in bananas, kiwifruits, citrus fruits, apples, and pineapples (Bhat, 2000) (Mojsov,

2014). The utilization of cellulases and xylanases enzymes can enhance both production output and operational efficiency without requiring additional financial investment. Typically, cellulases and xylanases are employed in two stages: firstly, after the fruit pulp has been crushed, these enzymes macerate the pulp, leading to either partial or complete liquefaction. This procedure offers several advantages, such as augmenting juice output, shortening processing duration, and enhancing the retrieval of precious constituents from fruits. Following juice extraction, another step involves the application of pectinases to facilitate clarification. This action contributes to the reduction of fruit juice viscosity, aiding in improved filtration speed and the stability of the ultimate product. Consequently, the incorporation of cellulases, xylanases, and pectinases in food biotechnology assumes a pivotal role. Anticipated to witness rising demand, these enzymes find utility across a wide spectrum of fruits and vegetables for juice extraction (Belghith *et al*., 2001).

2.7.3 Cellulases and Xylanase in Textile industry

The process of extracting textile fibers typically requires significant amounts of energy, water, and chemicals, leading to the production of harmful wastewater. This wastewater contains various contaminants and causes significant environmental issues. To address these concerns and promote sustainability in the textile industry, environmentally friendly technologies are being utilized. These technologies involve the use of biological catalysts such as living organisms (microbes) or their active components (enzymes), as well as molecular techniques. By employing these methods in fiber processing, the industry aims to achieve industrial sustainability while reducing the negative impact on the environment (Fu *et al*., 2012). The use of cellulases in textile processing began in the late 1980s, specifically in denim finishing (Miettinen-Oinonen *et al*., 2004). Cellulases from microbial origin are now globally employed in different industries like textile industry for various purposes such as biostoning jeans, biopolishing textile fibers, improving fabric quality, enhancing absorbance properties of fibers, softening garments, increasing stability of cellulosic fabrics, removing excess dye from fabrics, and restoring color brightness (Fu *et al*., 2012). Cellulases find prominent roles in the textile industry, particularly in techniques like bio-stoning and biopolishing. In the bio-stoning process, these enzymes work to

diminish the strength of surface fibers. Subsequently, a mechanical action takes place, leading to the elimination of weakened fibers that carry indigo dye. Since the indigo dye predominantly resides on the denim fabric's surface, the application of cellulases induces a faded appearance, unveiling the preferred white yarn effect sought after by numerous customers (Benedetti *et al*., 2019).

Xylanases play a crucial role in the textile industry as they can effectively process complex plant fibers such as flax, hemp, cotton, and jute. It is important, however, that xylanases used for processing plant fibers do not contain cellulase components. Enzyme-based pretreatment of textile fibers has gained considerable attention as it is a fundamental requirement in the textile industry (Patel *et al*., 2019). Bacterial-derived xylanases have been successfully employed in fabric bioprocessing. Particularly, alkaline pH and high-temperature active xylanases are utilized for the bio scouring of jute fabric. One highly effective technique involves incubating grass stems or dried ramie with xylanase enzymes to facilitate the release of cellulose fiber (Nwamba *et al*., 2021).

2.7.4 Cellulases and Xylanases in Biofuel Production

The utilization of cellulases in the conversion of LB for the generation of biofuel is considered as a modern area of research. Lignocellulosic biomass, which is readily available and cost-effective, serves as a suitable substrate for the development of second-generation biofuels and various valuable chemicals through biorefinery platforms (Mohsin *et al*., 2019). The extraction of bioethanol from lignocellulosic biomass holds significant importance as a renewable source of transportation fuel. It offers numerous benefits such as the reduction of greenhouse gas emissions, decreased reliance on fossil fuels, enhanced energy security, and lowered food costs, all while maintaining food production levels (Sun & Cheng, 2002). There are numerous methods available for the degradation of lignocellulosic biomass, including chemical, physical, and enzymatic processes. Enzymatic approaches have several advantages over chemical and physical pretreatments. They are environmentally friendly and do not require the use of harmful chemicals like acids or bases. Among all the known processes, the enzyme-based hydrolysis of lignocellulose feedstock using cellulase and xylanase is considered the best. This is because it offers enhanced specificity,

avoids the production of toxic substances, and prevents any loss of substrate. Cellulase and xylanase are the primary enzymes needed for the efficient hydrolysis of lignocellulosic biomass (Gupta & Verma, 2015). Cellulases are enzymes that specifically focus on cellulose, which is the primary component of plant cell walls. They catalyze the process of hydrolysis, breaking down cellulose into glucose. These enzymes are crucial in the saccharification stage of biofuel production, as they convert cellulose into glucose, which can then be fermented into biofuels like ethanol. In contrast, xylanases target hemicellulose, the intricate carbohydrate matrix that surrounds cellulose (Sun & Cheng, 2002). Xylanases play a crucial role in improving the overall effectiveness of biomass conversion by breaking down hemicellulose into sugars such as xylose. These enzymes are especially valuable in the bioconversion of lignocellulosic feedstocks, which are widely available and recognized as environmentally sustainable sources of biofuel (Gupta & Verma, 2015). In order to increase the efficiency of producing biofuels, xylanases can increase the accessibility of cellulose to cellulases and contribute to increased sugar yields during the saccharification process (Chen *et al*., 2019).

Materials and Methods

The current research study was conducted in the Applied Environmental and Geomicrobiology Lab (AEG), Department of Microbiology, Quaid-i-Azam University, Islamabad. The whole research was in accordance with standard microbiological procedures.

3.1 Isolation of Bacteria from Compost Sample:

The bacterial strains CC16 and CC12 were selected, previously isolated by my research fellow. The selection was made based on the production of cellulase and xylanase enzymes by CC16 and CC4 respectively.

3.2 Qualitative Screening of bacterial isolates for Cellulase and Xylanase Enzymes Production:

3.2.1 Qualitative Screening of bacterial isolates for Cellulase Production:

Screening of cellulase producing bacterial isolates was performed by inoculating a 24-hour fresh culture on carboxymethyl cellulose (CMC) containing medium (Castrillo *et al.,* 2020) having the composition given in (Table 3.1). The plates were then placed in incubator for 48 hours at 37˚C. After 24 hours incubation, the plates were then stained using 1% Congo red dye, subsequently de-staining with 1M NaCl for 30 mins (Irfan *et al.,* 2017). The basic concept behind this xylan plate assay is the utilization of Congo red dye, which permanently binds to the polysaccharides and temporarily binds to reducing sugars. After the de-staining with a NaCl solution, free sugars are removed, revealing clear zones of hydrolysis on the plate. These clear zones indicate the hydrolysis of xylan and serve as confirmation of cellulase production. Bacteria that exhibited a clear zone of hydrolysis for cellulase were subjected to a quantitative cellulase assay to calculate their specific activity.

3.2.2 Qualitative Screening of Bacterial Isolates for Xylanase Production:

Qualitative enzyme assay was performed for bacterial isolates that were analyzed for the xylanase enzyme by inoculating the bacterial isolates on nutrient agar plates containing 0.5% beechwood xylan and incubated for 48 hrs at 37˚C. After 24 hrs incubation, the plates were afterward the stained with 1% Congo red stain, followed by

de-staining with 1M NaCl for 30 minutes, and plates were then checked for zone of hydrolysis. Following this, bacterial isolates that show a clear zone of hydrolysis for xylanase were subsequently subjected to a quantitative xylanase assay to calculate their specific activity (Kumar and Shukla, 2018).

Table 3.1: Composition of CMC media g/L

Ingredients	g/L
CMC	5
KCl	1
$NaNO_3$	1
$MgSO_4$	0.5
K_2HPO_4	1
yeast extract	0.5
agar	20

3.3 Quantitative Screening of Bacterial Isolates for Maximum Cellulase and Xylanase Production:

The bacterial isolates that produce cellulase and xylanase in qualitative plate assay were then used for quantitative enzyme assay and protein estimation to screen for the highest cellulase-specific and xylanase-specific activity.

3.3.1 Cellulase Production Medium for Cellulase Quantitative Assay:

The inoculum preparation for the cellulase production media was performed by inoculating the 24 hours fresh cultures of the isolates into the nutrient broth, followed by incubation at 35˚C for 24 hours at 150rpm in a shaking incubator. After 24 hours incubation, about 1% inoculum was added to the cellulase production medium using micropipette. The production medium used for the cellulase was composed of; CMC (5g/L), KCl (1g/L), $NaNO_3$ (1g/L), $MgSO_4$ (0.5g/L), K_2HPO_4 (1g/L), and yeast extract (0.5g/L). About 2ml sample from the cellulase production medium was taken at 24 hours, 48 hours, 72hours and 96 hours intervals, followed by centrifugation at 4˚C for 10 minutes at 10,000rpm. The enzyme assay and protein estimation were then performed for the cell free supernatant.

3.3.2 Xylanase Production Medium for Xylanase Quantitative Assay:

The inoculum for xylanase production media was performed by inoculation of 24-hour fresh culture into nutrient broth incubation at 35˚C for 24 hrs at 150rpm in a shaking incubator. About 1% inoculum was transferred to the xylanase production medium after 24 hours of incubation using a micropipette. The composition of the production medium used for the xylanase is as follows; 0.5% xylan and nutrient broth (13g/L). About 2ml sample from the xylanase production medium was taken at 24 hrs, 48 hrs, 72 hrs, and 96 hrs intervals, then centrifuged at 4˚C for 10 minutes at 10,000rpm. The enzyme assay and protein estimation were then performed from the cell-free supernatant.

3.4 Preparation for Cellulase Enzyme Assay:

3.4.1 Preparation of Sodium Citrate Buffer and 1% CMC stock solution:

Sodium phosphate buffer was prepared by taking 100 ml of distilled water in a flask. Then 1.28g of sodium citrate and 0.6g of citric acid to 100ml were added to the flask. Subsequently, about 1.5g of CMC was added to a reagent bottle containing 50ml of distilled water. Then CMC solution (50ml) was added to the buffer (100ml), to achieve 1% CMC concentration in 0.05M sodium citrate buffer, followed by adjustment of the pH to 5 using 4M HCl and 4M NaOH and storage at 4˚C in a refrigerator.

3.5 Preparation for Xylanase Enzyme Assay:

3.5.1 Preparation of Sodium Phosphate Buffer:

The sodium phosphate buffer was prepared in a 100mM concentration, by mixing 1.549g of Na_2HPO_4 and 0.583g of NaH_2PO_4 in 100 ml of distilled water and adjusting the final pH to 8 using 1M HCl and 1M NaOH.

3.5.2 Beechwood Xylan Stock Solution Preparation:

Preparation of 5% xylan stock solution for the xylanase assay was performed in 50 ml of distilled water. Initially 2.5g of beechwood xylan was gradually added to 40ml of distilled water in a flask. The mixture was stirred using a magnetic stirrer on a hot plate to aid in complete dissolution. Once dissolved, the solution was cooled by placing the flask on an ice pack while continuing to stir with the magnetic stirrer for approximately

30 minutes. After the 30-minute, an additional 10ml of distilled water was added to reach a final volume of 50ml. To remove any suspended particles, the resulting stock solution was then centrifuged at 8000rpm for about 10 minutes. After centrifugation, the stock solution was then filtered through a syringe filter and stored in a refrigerator at 4˚C for further use in the xylanase assay.

3.6 Preparation of DNS Reagent:

DNS reagent was prepared for the estimation of reducing sugars, according to the following protocol.

3.6.1 Solution A (200ml):

For solution A preparation, firstly about 105 g of Potassium sodium tartarate was gradually dissolved in 100ml of distilled water using magnetic stirrer. Secondly, 4g of Na_2SO^3 was dissolved in 100ml of distilled water and then both solutions were mixed together and then 0.5 ml of phenol was added to it. Phenol was handled in dark due to its light sensitivity.

3.6.2 Solution B (300ml):

For preparation of solution B, firstly 8g of NaOH was added to 100 ml of distilled water. Secondly 8g of DNS (handled in dark due to light sensitivity) was dissolved in 200ml of distilled water with constant stirring using magnetic stirrer. Then both solutions were mixed together.

Solution A was added to solution B gradually using micropipette and constant stirring, followed by filtration through filter paper. The DNS reagent was then stored in a glass bottle covered with aluminum foil to avoid light exposure, at 4˚C.

3.7 Determination of Total Proteins:

The estimation of the total extracellular proteins present in a crude supernatant of the enzyme was analyzed by the method previously described by Lowry *et al.* (1951).

Reagents for Protein Estimation:

The details of the protein estimation reagents used for total protein assay are as follows.

Solution A = 2% Na_2CO_3 in 0.1N NaOH

Solution B = 1% potassium sodium tartarate

Solution C = 0.5% $CuSO_4$. $5H_2O$

Solution D = Solution A+ Solution B+ Solution C = 48:1:1

Solution E = Folin Phenol Reagent + Distilled Water = 1:1

Solution A preparation:

About 4 g of NaOH was dissolved in 900ml of distilled water, with the addition of 20g of Na_2CO_3 , to attain the final volume of 1000ml.

Solution B preparation:

Solution B was prepared by adding 1g of potassium sodium tartarate in 100 ml of distilled water.

Solution C preparation:

About 0.5g of $CuSO_4$. $5H_2O$ was dissolved in 100 ml of distilled water to prepare solution C.

Solution D preparation:

Solution A, B, and C were mixed in a ratio of 48ml: 1ml: 1ml to get solution D.

Solution E preparation:

Solution E was prepared by mixing the Folin phenol reagent with distilled water in a ratio of 1:1 in the dark. Solution E was prepared freshly for each protein estimation value because of its high sensitivity to light.

3.8 Standard Curve for Glucose, Xylose and BSA:

3.8.1 Standard Curve for Glucose:

The standard curve for glucose was prepared by making a 0.5% glucose stock solution in distilled water. It was then further diluted to attain a concentration of 100µg/ml in first test tube and 1000µg/ml in last test tube (Table 3.2). About 500µl of each dilution was taken in an Eppendorf tube and then 500µl of DNS reagent was added to each tube. The blank was prepared using 500µl distilled water instead of glucose dilution. All Eppendorf tubes were incubated at room temperature for 10 minutes, followed by heating at 98℃ in water bath for 15 minutes. The OD was then measured at 540nm against blank. A graph was plotted between glucose concentration and absorbance at 540nm using Microsoft Excel software. To determine the unknown values for the reducing sugars released, equivalent to the concentration of glucose, a trend line was added to the graph (Figure 3.1).

Table 3.2 Standard Curve Development for Glucose

Test tube #	Stock solution of Glucose (µl)	Distilled water (ml)	Final glucose concentration (µg/ml)	Absorbance at 540nm
1	60	2.94	100	0.403
2	120	2.88	200	1
3	180	2.82	300	1.76
4	240	2.76	400	2.4
5	300	2.70	500	2.84
6	360	2.64	600	3.4
7	420	2.58	700	4.2
8	480	2.52	800	4.9
9	540	2.46	900	5.6
10	600	2.40	1000	6.12

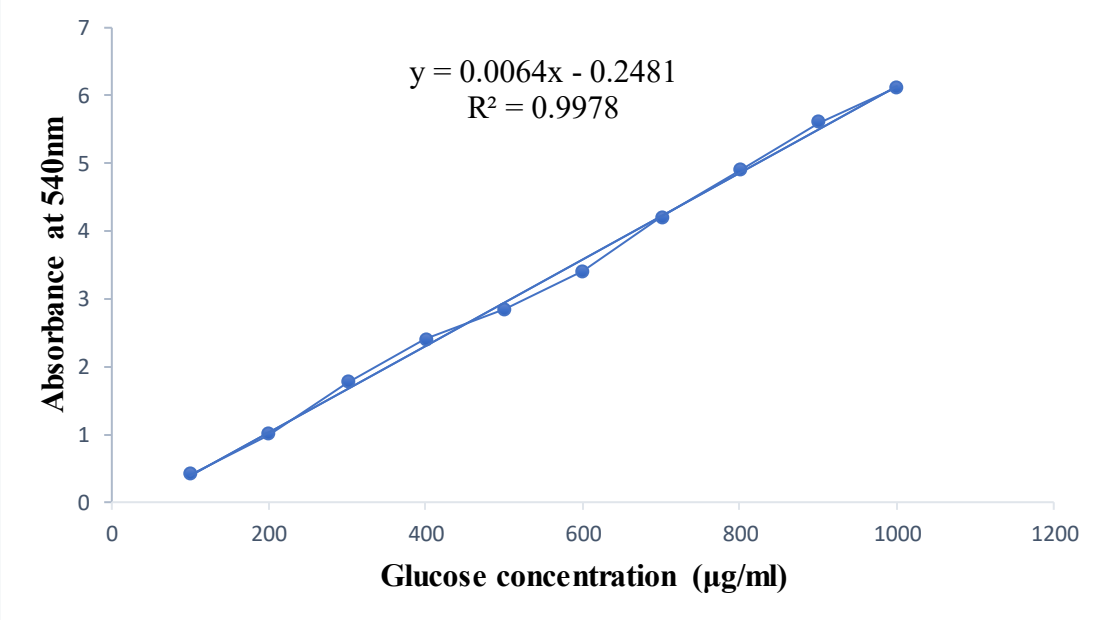

Figure 3.1 Formula y = 0.0.0064x - 0.1248 was derived from the graph. While Y represent amount of glucose in unknown sample and X represent optical density of unknown sample

3.8.2 Standard Curve for Xylose:

The standard curve for xylose was prepared by making 0.5% xylose stock solution in distilled water. It was then further diluted to attain a concentration of 100µg/ml in first test tube and 1000µg/ml in last test tube (Table 3.3). About 500µl of each dilution was taken in an Eppendorf tube and subsequently, a volume of 500µl of DNS reagent was added into each respective tube. The blank was prepared using 500µl distilled water instead of xylose dilution. Each Eppendorf tube was subjected to incubation at room temperature for a duration of 10 minutes, succeeded by heating at 98˚C in a water bath for a period of 15 minutes. The OD was then measured at 540nm against blank. A graph was then plotted among xylose concentration and absorbance at 540nm using Microsoft Excel software. To determine the unknown values for the reducing sugars released, equivalent to the concentration of xylose, a trend line was added to the graph (Figure 3.2).

Table 3.3 Standard Curve Development for Xylose

Test tube #	Stock solution of Xylose (µl)	Distilled water (ml)	Final glucose concentration (µg/ml)	Absorbance at 540nm
1	60	2.94	100	0.029
2	120	2.88	200	0.387
3	180	2.82	300	0.707
4	240	2.76	400	1.02
5	300	2.70	500	1.313
6	360	2.64	600	1.697
7	420	2.58	700	1.891
8	480	2.52	800	2.341
9	540	2.46	900	2.57
10	600	2.40	1000	2.854

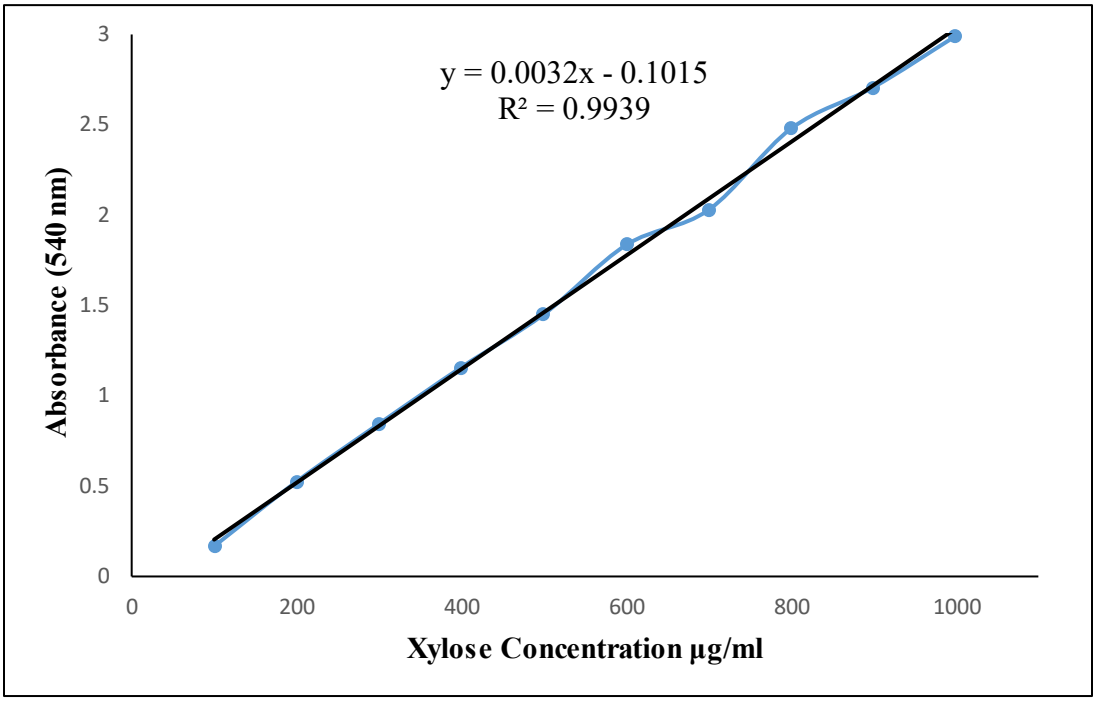

Figure 3.2 Formula y = 0.0.0032x - 0.1015 was derived from the graph. While Y represent amount of xylose in unknown sample and X represent optical density of unknown sample

3.8.3 Bovine Serum Albumin (BSA) Standard Curve Development:

A stock solution of BSA was prepared with a 1mg/ml concentration. It was then diluted to attain different concentrations ranging 0.1-1mg/ml of BSA (Table 3.4), followed by protein quantification at 650nm. The absorbance (OD) was then plotted into graph against BSA concentration (µg/ml), using Microsoft Excel software. The trend line was added to the graph, followed by the derivation of the equation that was then utilized for the estimation of the unknown concentration of the reducing sugars (Figure 3.3).

Table 3.4 Standard curve development for bovine serum albumin (BSA)

Test tube #	Distilled water (ml)	BSA stock solution (ml)	Protein concentration (mg/ml)	Absorbance at 650nm
1	1	0.00	0.00	0
2	0.9	0.1	0.1	0.112
3	0.8	0.2	0.2	0.296
4	0.7	0.3	0.3	0.307
5	0.6	0.4	0.4	0.423
6	0.5	0.5	0.5	0.584
7	0.4	0.6	0.6	0.632
8	0.3	0.7	0.7	0.746
9	0.2	0.8	0.8	0.836
10	0.1	0.9	0.9	0.901
11	0.00	1.0	1.0	0.977

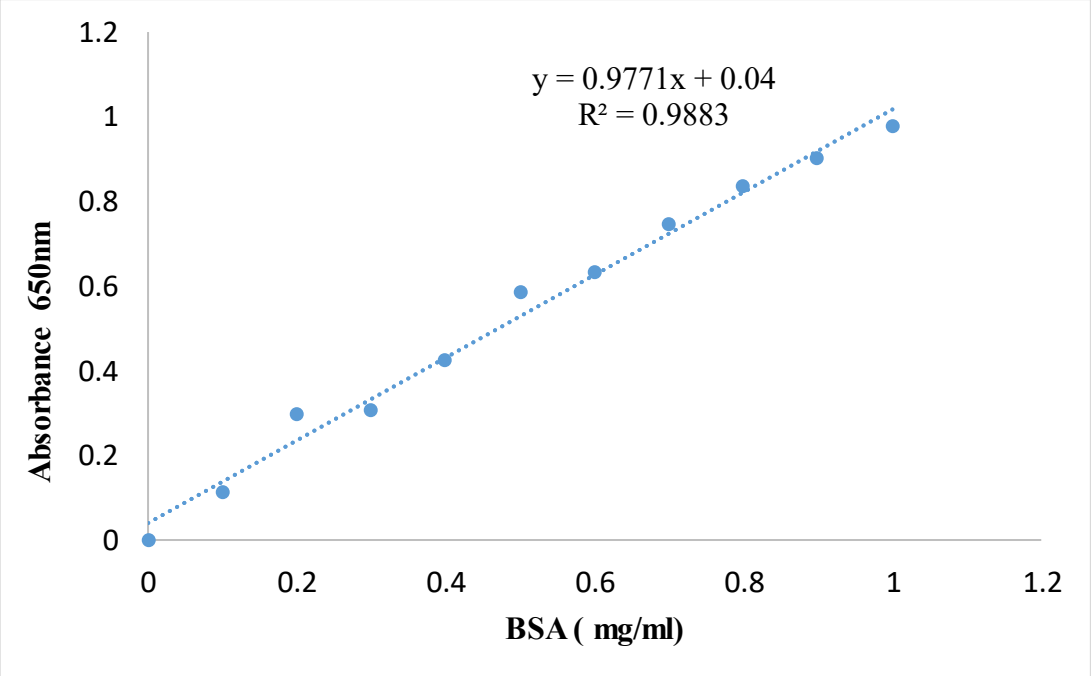

Figure 3.3 Standard Curve Development for BSA

3.9 Cellulase Quantitative Enzyme Assay and Protein Estimation:

In order to perform the quantitative enzyme assay for cellulase, a 500µl from 1% stock solution of CMC in a 0.05M sodium citrate buffer and 500µl of crude enzyme supernatant were added in a test tube. For control, 500µl of enzyme production media without inoculation was added instead of crude enzyme extract. The reaction mixture was incubated at 45˚C for 30 minutes and then 500µl of DNS reagent was added to the reaction tubes and then they were subjected to heating at 98˚C in water bath for 10 minutes. About 100µl of sample from the enzyme assay mixture was filled in the 96-well microtiter plate using micropipette. Then OD was measured in triplicates, at 540nm using ELISA microplate reader.

Enzyme Unit:

One unit of cellulase is defined as the amount of enzyme that will release 1µmol/ml of glucose per minute.

Specific Activity (U/mg):

Cellulase specific activity was determined using formula;

$$Specific\ Activity = \frac{Unit\ per\ ml\ of\ enzyme\ activity}{mg\ per\ ml\ of\ total\ protein}$$

For the protein estimation, 500µl of the crude enzyme extract and 500µl of the solution D were added to a test tube and incubated at room temperature for 10 minutes, followed by addition of 50µl of solution E and incubating it for 30 minutes in dark at room temperature. The OD was measured at 650nm using ELISA microplate reader.

The isolates showing the highest specific activity for cellulase were then further optimized to attain the maximum cellulase production.

3.10 Xylanase Quantitative Enzyme Assay and Protein Estimation:

Quantitative enzyme assay for xylanase was performed by adding 175µl of distilled water, 125µl of sodium phosphate buffer, 100µl of 5% xylan stock solution and 100µl of crude enzyme supernatant in a test tube. For control, 100µl of enzyme production media without inoculation was added instead of crude enzyme extract. The test tubes

were incubated at 45˚C for 30 minutes and then 500µl of DNS reagent was added to the reaction tubes and then they heated at 98˚C in a water bath for 10 minutes (Kumar and Shukla, 2018). About 100µl of sample from the enzyme assay mixture was filled in the 96-well microtiter plate using micropipette. Then OD was measured in triplicates, at 540nm using ELISA microplate reader.

Enzyme Unit:

One unit of xylanase is defined as the amount of enzyme that will release 1µmol/ml of xylose per minute.

Specific Activity (U/mg):

Xylanase specific activity was determined using formula;

$$Specific\ Activity = \frac{Unit\ per\ ml\ of\ enzyme\ activity}{mg\ per\ ml\ of\ total\ protein}$$

For protein estimation, 500µl of the crude enzyme extract and 500µl of solution D were mixed in a test tube and left to incubate at room temperature for 10 minutes. Subsequently, 50µl of solution E was added, and the mixture was incubated in darkness at room temperature for 30 minutes. The OD was measured at 650nm using ELISA microplate reader. The isolates exhibiting the highest specific activity for xylanase were used in additional optimization, with the aim of achieving the utmost xylanase production.

3.11 Identification of the Cellulase and Xylanase Producing Bacterial Strains:

Both strains; CC16 (cellulase producer) strain and strain CC4 (xylanase producer), were identified biochemically and by observation of morphological characteristics.

3.11.1 Morphological Examination:

The strain CC16 and CC4 were examined for their morphological characteristics by growing them on nutrient agar plates and observing their colonies after 24hrs incubation at 37˚C.

3.11.1.1 Gram Staining for Microscopic Examination:

Gram staining was performed for bacterial strains CC16 and CC4. For Gram Stainig, a droplet of normal saline was applied onto a sterile glass slide. The bacterial colony was then placed onto the droplet, and a thin layer was produced by distributing the colony across the slide's surface. The bacterial strains CC16 and CC4 were heat-fixed by passing the slide through the flame 3-4 times, then allowing it to cool to the touch before

staining. The smear was stained for 1 minute with crystal violet and then carefully washed for 2 seconds with ionized water. After that, the slide was saturated with Gram's iodine for 1 minute and then gradually cleaned with tap water. Drop by drop ethanol was added to the slide to decolorize for 10- 15 seconds until it ran clear. Next, the slide was immersed in safranin counterstain for a duration of 1 minute and subsequently rinsed with tap water until the effluent displayed no coloration. The slide was dried with absorbent paper.

3.11.2 Biochemical Identification:

3.11.2.1 Triple Sugar Iron (TSI) Test:

The TSI test aimed to investigate the ability to ferment glucose, sucrose, dextrose, or lactose, as well as the production of hydrogen, hydrogen sulfide, or carbon dioxide. To conduct the test, a 24-hour fresh culture was obtained using a sterile inoculation needle to stab the butt of the TSI agar slant and then streak the culture on the slant. TSI agar test tubes were subjected to incubation at a temperature of 37˚C for a duration of 24 hours in order to analyze the results. The interpretation of results was as follows: a yellow butt and slant indicated dextrose, sucrose, or lactose fermentation; a yellow butt with a red slant indicated dextrose fermentation; a red slant and butt indicated the absence of sugar fermentation; the development of black coloration indicated hydrogen sulfide gas production, and the formation of cracks in the TSI agar indicated carbon dioxide or hydrogen gas production.

3.11.2.2 Catalase Test:

Catalase test was performed to check the ability of bacterial isolates for detection of catalase enzyme. For this purpose, a sterile inoculation loop was used to prepare a smear of the 24 hours fresh culture of all bacterial strains on sterile glass slides. Then a drop of hydrogen peroxidase (catalase reagent) was applied to the smear. The formation of the bubbles was interpreted as the presence of catalase enzyme activity.

3.11.2.3 Citrate Utilization Test:

The purpose of the Citrate test was to investigate the utilization of sodium citrate as a carbon source and ammonium hydrogen phosphate as a nitrogen source by bacterial

isolates. To conduct this test, a 24-hour fresh culture was streaked on the surface of the Simmon citrate agar and incubated for 24 hours at 37˚C. The culture was then observed for the transition of color from green to blue, which would indicate a positive result.

3.11.2.4 Oxidase Test

This test was conducted for the detection of the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. For this purpose, a small piece of the filter paper was soaked in Kovac's reagent and loop was used to pick a colony from 24 hours fresh culture of all bacterial strains, followed rubbing onto treated filter paper. The formation of the blue color was interpreted as the presence of oxidase enzyme activity.

3.12 Bulk Production of Cellulase and Xylanase under Optimal Conditions:

The cellulase and xylanase enzymes were produced in bulk at their optimized conditions. This step was performed to facilitate the process of enzymatic hydrolysis of the alkali-organosolvent pretreated sugarcane bagasse (SCB) and sugarcane trash (SCT).

3.12.1 Bulk Production of Cellulase under Optimal Conditions:

About 500ml of cellulase production media was prepared in 1000 ml Erlenmeyer flask for strain CC16 with composition (g/L) as follows; Sucrose, 1; yeast extract, 6; peptone, 1; K_2HPO_4, 0.90; CMC, 5; KH_2PO_4, 1.60; $FeSO_4.7H_2O$, 0.1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.22; $CaCl_2$, 0.01. About 1% (5ml) inoculum of strain CC16 was added to the cellulase production medium, with a pH adjusted to 5 and incubated at 40˚C for 48 hours. Then the medium was centrifuged in 50ml falcon tubes at 8000rpm for 20 minutes and the supernatant was collected and analyzed for its specific activity was calculated.

3.12.2 Bulk Production of Xylanase under Optimal Conditions:

About 500ml of xylanase production media was prepared in 1000ml Erlenmeyer flask with composition (g/L) as follows; Yeast extract, 6.2; peptone, 8.3; K2HPO4, 0.25; xylan, 3.8; KH_2PO_4, 0.50; $FeSO_4.7H_2O$, 0.1; $MgSO_4.7H_2O$, 0.5; NaCl, 1.2; $CaCl_2$, 0.02. About 1% (5ml) inoculum of strain CC4 was added to the xylanase production medium, with a pH adjusted to 8 and incubated at 30˚C for 48 hours. Then the medium was

centrifuged in 50ml falcon tubes at 8000rpm for 20 minutes and supernatant was collected, and analyzed for its specific activity was calculated.

3.13 Protein Purification and precipitation from bacterial strains CC16 and CC4

Purification of cellulase and xylanase enzyme from bacterial strain was done using ammonium sulfate precipitation protocol and gel permeation chromatography (Sephadex G-100) method.

3.13.1 Ammonium Sulfate Precipitation of Crude Enzyme in Supernatant

A volume of 100 ml of cell-free supernatant (CFS) was extracted from bacterial strains CC16 and CC4. The required amount of ammonium sulfate was first calculated which was 10% in first instance via online calculator of EnCor Biotechnology Inc. For the precipitation, ammonium sulphate was added slowly into cell free supernatant flask. CFS is stirred with the aid of a magnetic stirrer at 4˚C. After the ammonium sulphate is dissolved completely, the solution can stand for 24 hours at 4˚C. Following this, the solution was centrifuged at 8,000rpm for a duration of 15 minutes. The resultant pellet and supernatant were then collected separately. Subsequently, the pellet of CC16 was dispersed within a sodium citrate buffer with a pH of 5.0, while the pellet of CC4 was dispersed within a sodium phosphate buffer with a pH of 8.0. Afterward, an enzyme assay and protein estimation were conducted on the resulting pellet and supernatant using DNS and Lowry's methods, respectively. These methods were employed for the purpose of calculating and analyzing their respective specific activities. The supernatant collected earlier was then further tested at 20%, 30%, 40%, 50%, 60%, 70% and 80% ammonium sulfate treatment using the same protocol described earlier. The fractions resulted from these variations were stored at 4˚C. Precipitation of bulk amount was carried out at that specific percentage of ammonium sulfate in which high yield of cellulase and xylanase activity for CC16 and CC4 respectively was figured out.

3.13.2 Dialysis of crude enzyme

Following the precipitation step, the enzyme solution was subjected to dialysis to eliminate the presence of ammonium sulfate. The crude enzyme cellulase was dissolved in sodium citrate buffer (pH 5.0) and xylanase was dissolved in phosphate buffer (pH

8.0). After precipitation enzymes were transferred to a dialysis tube and then placed in their respective buffer at 4˚C for 24 hours.

3.13.3 Protein purification (Sephadex G-100) column Chromatography

The fundamental principle of gel permeation chromatography resides in the separation of molecules based on their respective sizes (Determann, 2012)

Procedure

For this process Sephadex gel was prepared by appropriately mixing 2.3g of Sephadex G-100, 0.03g sodium-azide and 0.03g nastatin in 130 ml of sodium phosphate buffer and incubated at 45˚C for 24 hours. The gel perfectly swelled after incubation time, sodium-azide inhibits bacterial growth while nastatin inhibits fungal growth. The glass column was carefully washed with distilled water and inspected for any leakage. After that, the gel and buffer solution were exposed to 30 minutes of sonication to remove any trapped gas. Packing of the column with gel was performed carefully, ensuring that no bubbles were formed. The column was packed up to a length of 27 cm, with 21 cm consisting of gel and the remaining filled with phosphate buffer. The gel was allowed to settle undisturbed in the column for 24 hours at room temperature, resulting in a firmly packed gel bed. The flow rate of the column was carefully maintained at 0.33 ml per minute. Following this, 3 ml of a previously partially purified enzyme was introduced into the Sephadex column. To ensure a continuous flow of the sample within the column, sodium citrate buffer for cellulase and sodium phosphate buffer for xylanase were continually supplied. This technique produced 30 fractions, each of which was collected separately. Following that, the absorbance of each fraction was measured using UV light at 280nm to determine the protein amount in each. Following that, enzyme assays and protein estimations were performed for each fraction. Fractions exhibiting high cellulase and xylanase activity were mixed separately and kept at 4 C for subsequent analysis.

3.13 Characterization of Purified Cellulase and Xylanase:

Characterization of purified cellulase xylanase was carried out using the following method described by (Coral *et al.,* 2002). The relative activity of the cellulase and xylanase was calculated by:

Residual Activity (%) = (Activity (U/mg) of crude enzyme / Activity (U/mg) of the purified enzyme) × 100

3.13.1 Effect of Temperature on the activity and stability

To find the optimal temperature, the activity of the purified enzymes was assessed across a range of temperatures (30°C to 80°C), over a duration of 30 minutes. The impact of these temperatures was quantified in terms of relative activity (%). The stability of the purified enzyme was investigated by subjecting it to incubation within a temperature range of 30°C to 80°C, in the absence of substrate, for a maximum of 150 minutes. The enzyme's activity was monitored at intervals of 30 minutes during this incubation. Following the incubation period, the substrate was added, and the mixture was incubated for 30 minutes at the identified optimum temperature. After this incubation, DNS reagent was added, and the solution was incubated again for 10 minutes at 98˚C.

3.13.2 Effect of pH on the activity and stability

The activity of the purified enzyme was evaluated over a variety of pH values ranging from 5.0 to 10.0 to find the optimum pH. Different buffer systems were employed to achieve specific pH levels: 0.1M sodium acetate (pH 3.0-5.0), sodium phosphate (pH 6.0-8.0), and 0.1M NaOH Glycine buffer (pH 9.0-11.0). The enzyme assay was conducted at the identified optimum temperature for a duration of 30 minutes. Cellulase and xylanase were incubated at various pH values (ranging from 5.0 to 10.0) to determine their pH stability in the absence of substrate for a total duration of 180 minutes. The enzyme's activity was monitored at intervals of 30 minutes during this incubation period. After the incubation time, the substrate was introduced, and the mixture was incubated for 30 minutes at the previously determined optimum temperature. Following this, DNS reagent was added, and the solution was incubated again for 10 minutes at 98°C.

3.13.3 Effect of Metal Ions on the activity of enzyme:

The effect of metal ions on purified enzyme was determined by incubating enzyme solution with metal ions and then cellulase and xylanase activity was measured. Enzyme activity was checked against different metal ions i.e. zinc sulfate, ferrous

sulfate, copper sulfate, calcium chloride, sodium chloride, nickel sulfate, manganese sulfate, lead nitrate and magnesium sulfate. Metals were used in two concentrations, 5mM and 10 mM. The relative activity of metal ions was used to determine their effects.

3.13.4 Effect of Surfactants on the activity of enzyme

To assess the impact of surfactants on xylanase activity, the purified enzyme was analyzed for various surfactants. Surfactants were used at a concentration of 0.5% and 1.0 %. These include; cetyl trimethyl ammonium bromide (CTAB), polyethylene glycol (PEG), Triton X-100, SDS, EDTA, Tween 20, Tween 40, Tween 60 and Tween 80. The effects of surfactants at different concentration was determined in terms of relative activity.

3.13.5 Effect of organic solvents on the activity of enzyme

The effect of organic solvents on purified enzyme activity was analyzed by measuring activity in the presence of different organic solvents. Organic solvents include Glycerol, butanol, propanol, acetone, methanol, ethanol, acetonitrile, ethyl acetate, and DMSO. The effects of organic solvents at different concentrations were determined in terms of relative activity.

3.13.6 Determination of kinetic parameters

Cellulase from bacterial strain CC16 was analyzed at 45˚C and pH 7.0 in a reaction mixture where cellulase was present in various concentrations (0.5–35 mg/ml). Similarly, xylanase from bacterial strain CC4 was analyzed at 50˚C and pH 9.0 in a reaction mixture where xylanase was present in various concentrations (0.5–35 mg/ml). The data for both enzymes were plotted to the Lineweaver-Burk software in order to determine the Vmax and Km of kinetic constant.

3.14 Analysis of Hydrolysate from Enzymatically Digested Pretreated Bagasse for Fermentable Sugars

3.14.1 Pretreatment of Sugarcane bagasse (SCB) and Sugarcane trash (SCT)

SCB was grinded into fine powder form by mechanical grinder. 15 grams of the powdered bagasse was taken in a flask. Methanol was added by 1:15 total solid to liquid ratio. NAOH was added 4% to the total volume. Flask was shaken for proper mixing and incubated at 50˚C for 2 hours. Following incubation, the solution was then centrifuged for a period of 10 minutes at 8000 rpm. supernatant was collected in separate flask. The syrup was then washed with distilled water three times at 8000 rpm for 10 minutes. The pellets were collected and dried overnight in oven at 60˚C.

3.15 Enzymatic Hydrolysis of Alkali-Organosolvent Pretreated SCB and SCT using Bacterial purified Enzymatic Extracts

The potential of purified enzymatic extracts of bacterial cellulase, and xylanase to produce high fermentable sugars such as glucose and xylose sugars was studied.

3.15.1 Hydrolysis Analysis

To ensure the best utilization of the enzymatic activities of cellulase and xylanase for the enzymolysis of the 4% (w/v) pretreated bagasse, a two-step strategy was applied. The pretreated bagasse was incubated with xylanase at the optimum condition of 30℃ for 48 hours, using sodium phosphate buffer (100mM) with pH adjusted to 9. The reaction mixture was then centrifuged at 8000 rpm for 10 minutes. The hydrolysate obtained was then quantified for xylose concentration using DNS method. The xylanase (34U/g of bagasse) treated bagasse was then subjected to the cellulase catalyzed hydrolysis by incubating it with cellulase (61U/g of bagasse) at 40℃ for 24 hours, in the presence of the sodium citrate buffer (0.05M) with pH adjusted to 7. The control group for the enzymatic hydrolysis was the direct incubation of cellulase enzyme with NaOH-catalyzed methanol pretreated bagasse without prior treatment with xylanase. In addition, 0.03% of sodium azide (antibacterial) and 100µl of nystatin (antifungal) were added to avoid microbial contamination in the enzymatic hydrolysis process (Wei et al., 2019). After 24 hrs. incubation, 1ml of the sample was taken from the enzymatic hydrolysis reaction mixture, followed by centrifugation at 8000rpm for 5 minutes. The hydrolysate obtained was then further analyzed, using DNS method, for the calculation of the fermentable glucose yield.

3.15.2 DNS method:

For DNS method, 500µl of the enzymatically treated hydrolysate was mixed with 1ml of DNS was added to the mixture in test tube, followed by heating at 98˚C in water bath for 5 minutes, then transferring the test tubes to the cold water and then measuring absorbance at 540nm.

3.15.3 Calculation of the Total Reducing Sugars Yield:

The Total Reducing Sugars (TRS) yield were calculated using following formulas (Yuan et al., 2018);

$$Total\ Reducing\ Sugars\ (TRS)\ yield\ (\frac{g}{g}) = \frac{TRS\ released\ \left(\frac{g}{L}\right) \times Volume of\ the\ reaction\ mixture\ (L)}{Amount\ of\ raw\ sugarcane\ bagasse\ (g)}$$

3.15.4 FTIR Analysis

To identify the alteration in the functional groups in the chemical structure of non-treated, pre-treated enzymatically hydrolyzed SCB and SCT was analyzed by FTIR spectrophotometer. Non pretreated, pretreated, and enzymatically hydrolyzed bagasse were placed on sample plate of FTIR. For each sample, a single spectrum of 500-4000 wave numbers cm^{-1} was used.

Results

4.1 Isolation of bacterial strains

The previously isolated bacterial strains CC16 and CC4 were selected on basis of production cellulase and xylanase enzymes respectively, which will be used for the enzymatic hydrolysis of SCB.

4.2 Qualitative screening of bacterial strain for Cellulase and Xylanase production

On CMC agar plate CC16 formed a clear zone of hydrolysis by utilizing CMC as substrate and CC4 revealed a clear zone of hydrolysis on nutrient agar plate supplemented with 0.5% beechwood xylan. Extracellular enzymes diffuse through the medium and degrade the substrate, resulting in a clear zone around the colony on the agar plate. Fig 4.1 shows zone of hydrolysis for CC16 (Fig 4.1A) and CC4 (Fig 4.1B).

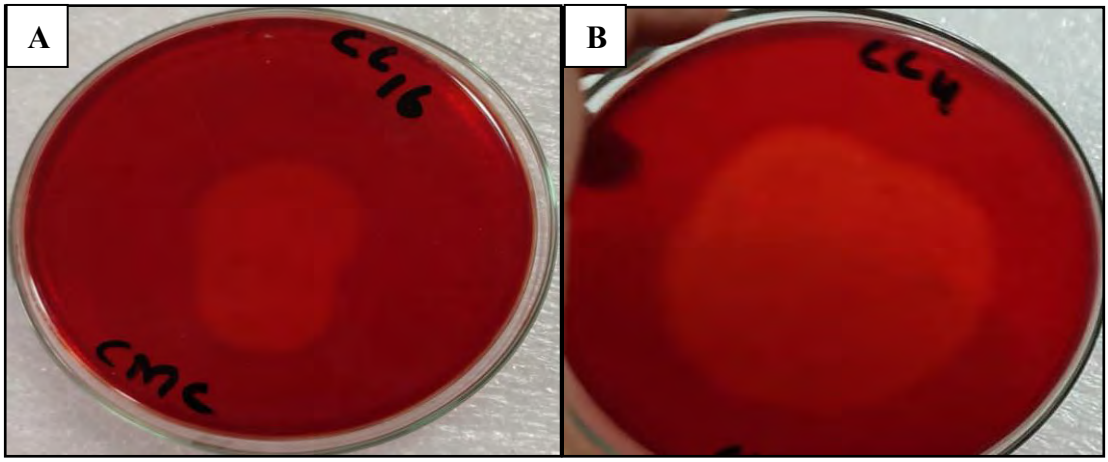

Fig 4.1: Clear zone of hydrolysis around bacterial strains stained with Congo red (A) Cellulase on CMC agar plate around strain CC16 (B) Xylanase on xylan agar plate around strain CC4.

4.3 Morphological and Biochemical Characterization of CC16 and CC4

4.3.1 Growth of Bacterial Strains

Bacterial strains CC16 and CC4 were grown on nutrient agar plates. Rapid growth of strain CC16 and CC4 was achieved at 37°C within 24 hours.

4.3.2 Morphological Examination of CC16 and CC4

The colonial characteristics were used to examine the cellular morphology of strains CC16 and CC4. On nutrient agar plate, colony morphology of strain CC16 revealed characteristics such as small, flat colonies with irregular margins, mucoid, and white in color (Fig 4.2A) and CC4 revealed large, opaque, flat colonies with white to yellowish color (Fig 4.3A). CC16 and CC4 strains were found gram positive and rod-shaped, according to microscopic observations (Fig 4.2A and Fig 4.3B respectively).

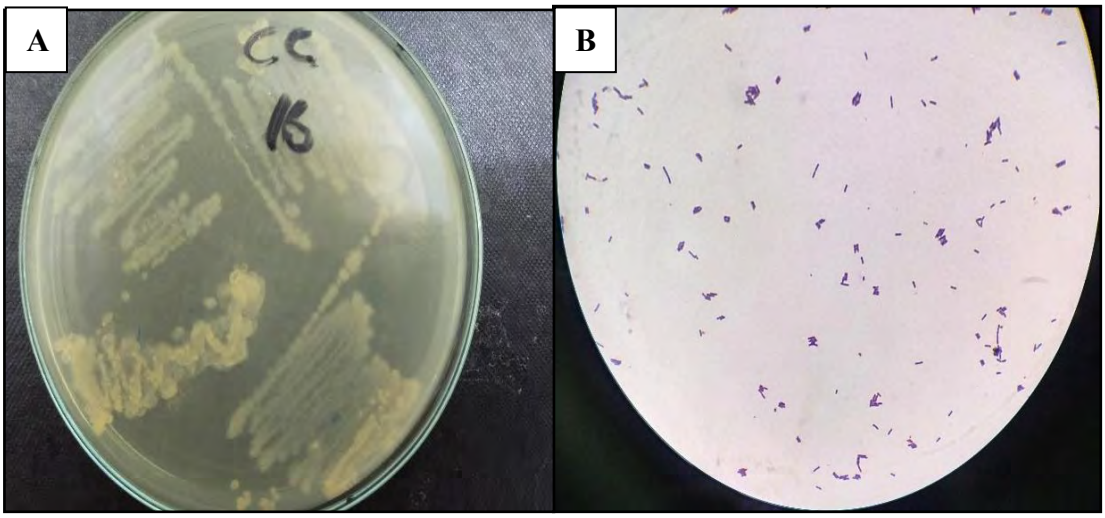

Fig 4.2: Morphological and microscopic observation of CC16 strain (A) Colony morphology (B) Gram identification showed Gram positive rod shaped.

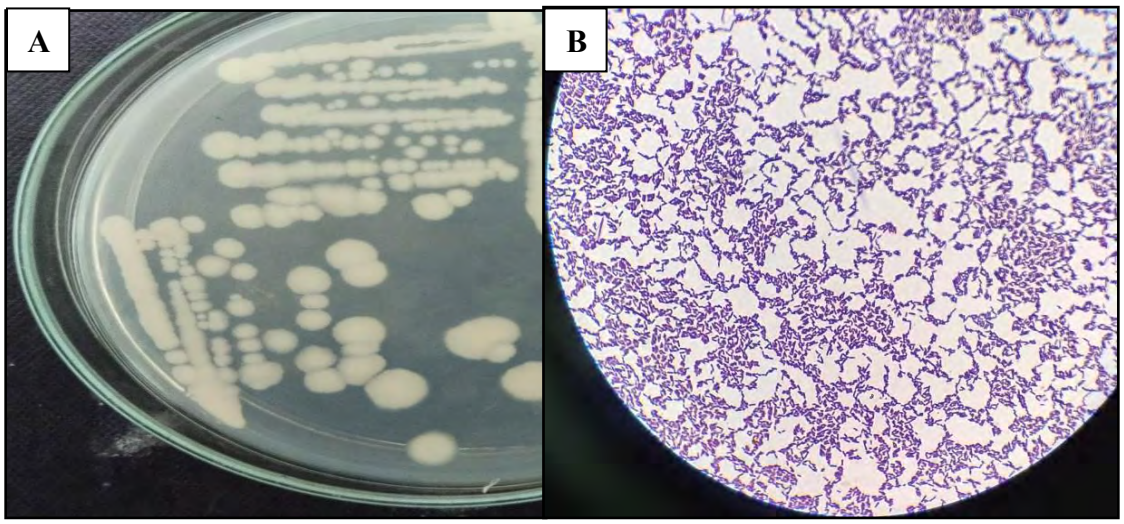

Fig 4.3: Morphological and microscopic observation of CC4 strain (A) Colony morphology (B) Gram identification showed Gram positive rod shaped.

4.3.3 Biochemical Characteristics of CC16 and CC4

The biochemical and morphological properties of the bacterial strains CC16 and CC4 are given in Table 4.1. Strain CC16 was positive for oxidase, catalase, and citrate while negative for amylase. TSI results revealed that CC16 is a glucose fermenter. Biochemical analysis of CC4 showed positive results for citrate and negative results for oxidase, and catalase while TSI show that CC4 is not a glucose fermenter neither lactose nor sucrose. The biochemical and morphological properties of both strains are presented in Table 4.1.

Table 4.1: Biochemical characteristics of CC16 and CC4

Test	CC16	CC4
Oxidase activity	+	-
Catalase activity	+	-
Citrate	+	+
Amylase	-	-
Triple sugar iron	+ (Glucose fermenter)	- (Lactose, Glucose, and Sucrose are not being fermented)

4.4 Optimized Culture conditions for production of Cellulase and Xylanase

4.4.1 Effect of Temperature

Production of cellulase from bacterial strain CC16 and xylanase from bacterial strain CC4 was observed by incubating the reaction mixture at different range of temperatures; 30˚C to 50˚C. Maximum specific activity of CC16 for cellulase was recorded 4.22 U/mg at 40˚C (Fig 4.4) and for xylanase the maximum specific activity 12.74 U/mg was achieved at 30˚C (Fig 4.5).

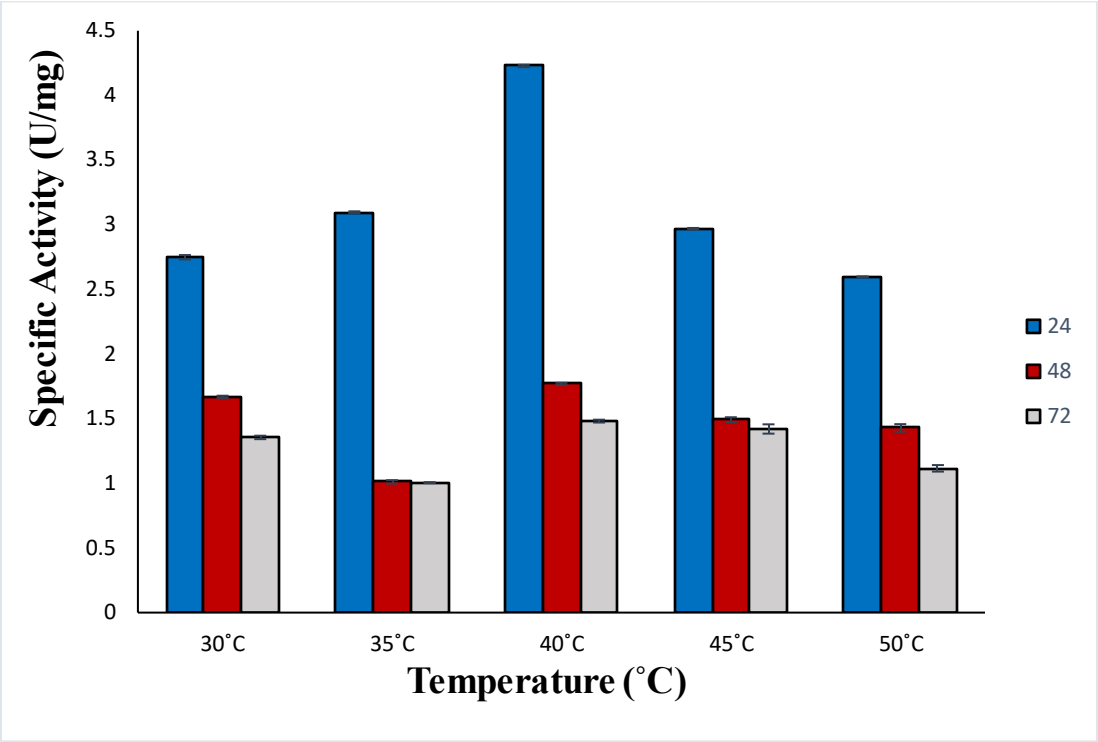

Figure 4.4: Effect of temperature on production of cellulase from strain CC16 within 24-72 hours of incubation.

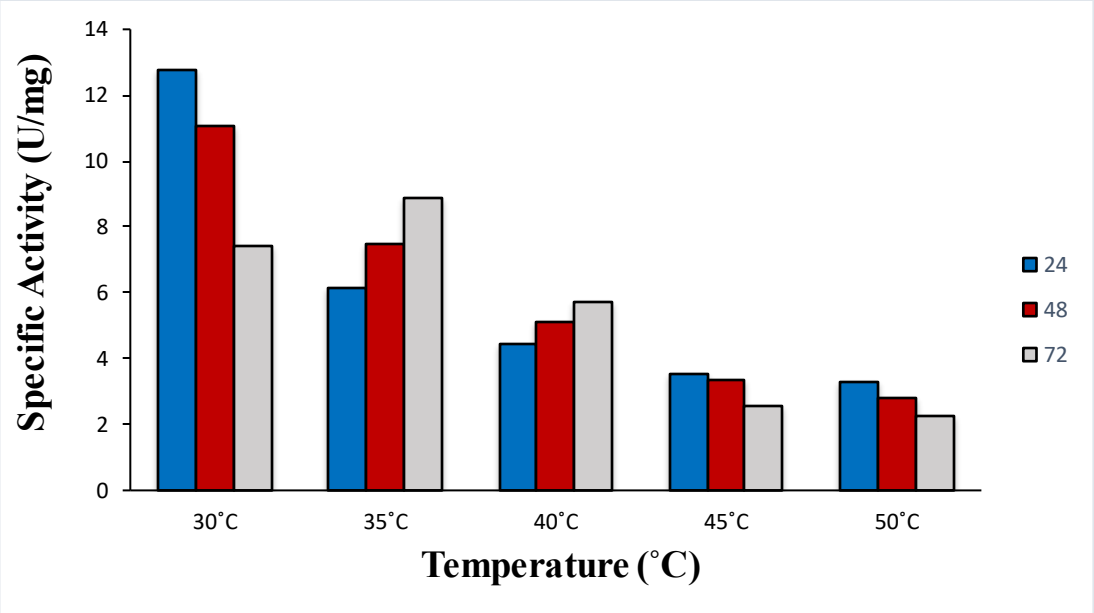

Figure 4.5: Effect of temperature on production of xylanase from strain CC4 within 24-72 hours of incubation.

4.4.2 Effect of pH

The impact of pH on the production of cellulase from bacterial strain CC16 and xylanase from CC4 was studied within a broad pH range of 3.0 to 11.0. Production and. The optimum pH range was analyzed by incubating the reaction mixture and determining the specific activity of both strains at different ph. The maximum specific activity of CC16 for cellulase of 8.195 U/mg was achieved at pH 5.0 after 48 hours (Fig 4.6) and for xylanase, the maximum specific activity of 16.52 U/mg was achieved at pH 8.0 after 48 hours, above and below pH 8.0 specific activity decreased (Fig 4.7).

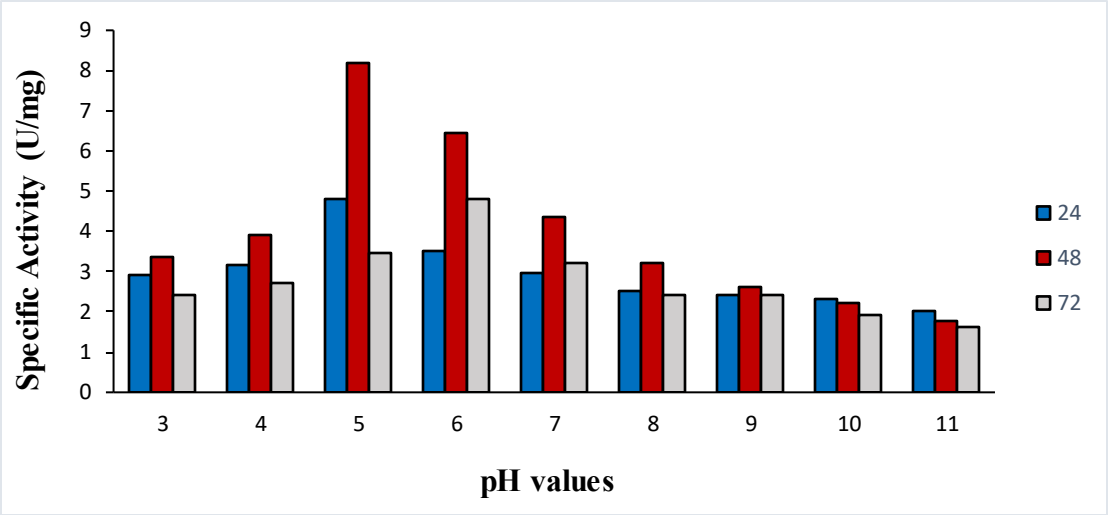

Figure 4.6: Effect of different pH level on production of cellulase from CC16 within 24 – 72 hours of incubation.

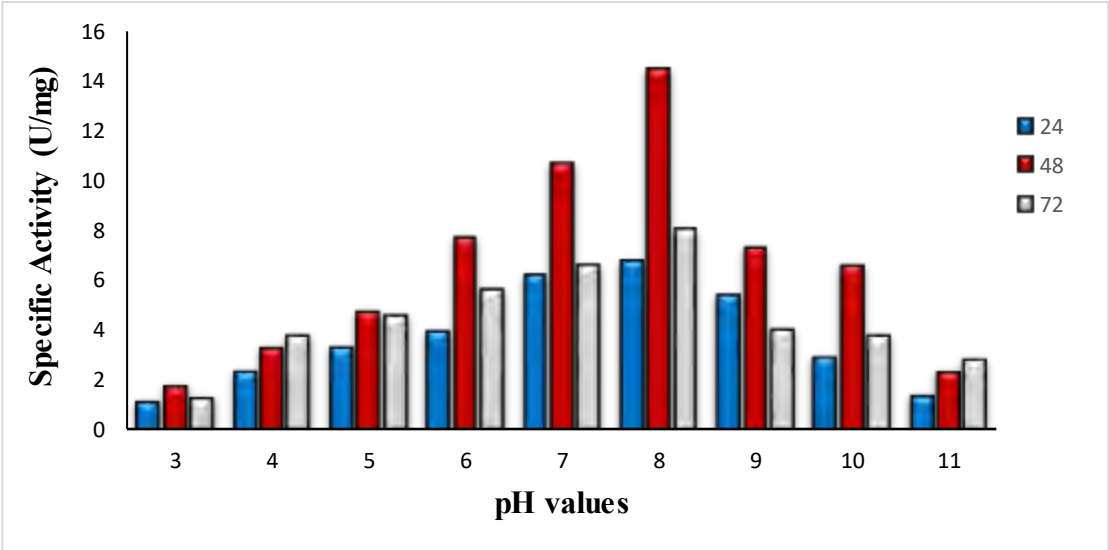

Figure 4.7: Effect of different pH level on production of xylanase from CC4 within 24 – 72 hours of incubation

4.4.3 Effect of Incubation Time

The effects of incubation time on production of cellulase and xylanase from CC16 and CC4 strains were analyzed after every 24 hours for a maximum period of 96 hours respectively. Maximum specific activity of 10.703 U/mg for cellulase from CC16 was achieved after 48 hours of incubation along with other optimum conditions such as temperature and pH (Fig 4.8). Moreover, following 48 hours of incubation under other optimized conditions, xylanase had a maximum specific activity of 16.52 U/mg (Fig 4.9).

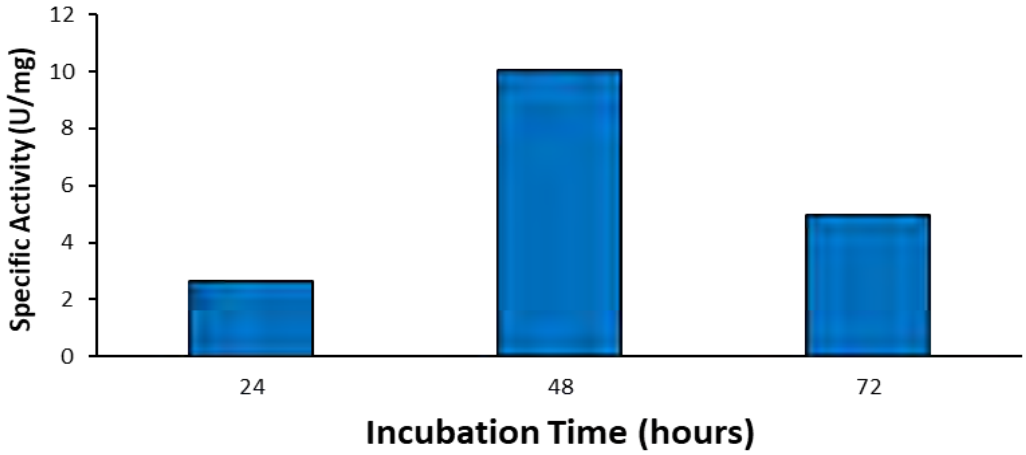

Figure 4.8: Effect of incubation time on production of cellulase from CC16 from 24 – 72 hours of incubation

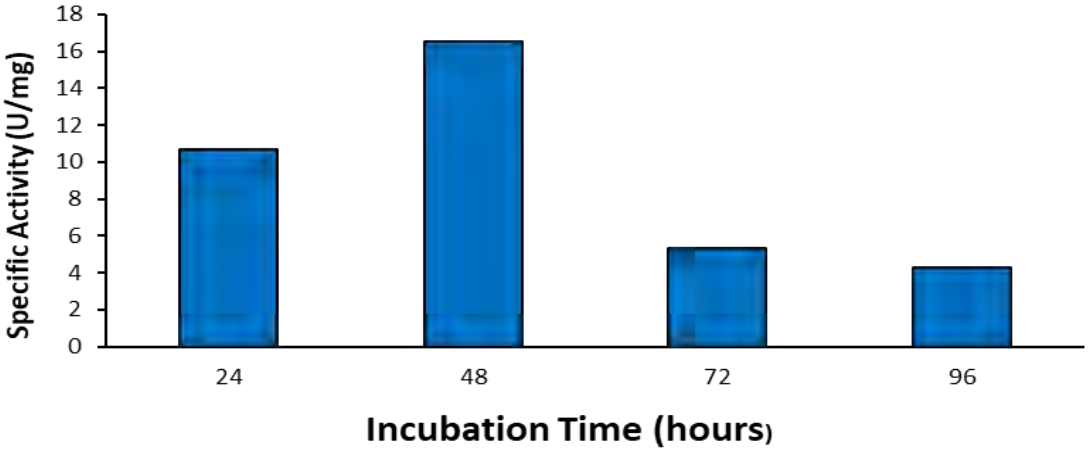

Figure 4.9: Effect of incubation time on production of xylanase from CC4 from 24 – 96 hours of incubation

4.5 Purification of Cellulase and Xylanase Enzyme from Strain CC16 and CC4

4.5. Precipitation with ammonium sulfate

Ammonium sulfate was used in various concentrations dissolved in cell free supernatant for maximum precipitation of proteins obtained from CC16 (cellulase) and CC4 (xylanase). Maximum cellulase and xylanase activity was obtained in precipitates obtained at 70% and 60% concentration respectively. Whereas further addition of ammonium sulfate resulted into a drop-in both cellulase xylanase activity, while low cellulase and xylanase concentration/activity was obtained at below 70% ammonium sulfate concentration for cellulase and 60% ammonium sulfate concentration for xylanase. For the extraction of cellulase at optimum 70% ammonium sulfate concentration the specific activity was 135.6 U/mg (Fig. 4.10) and for extraction of targeted xylanase, 60% was optimum and specific activity was 232.0 U/mg (Fig. 4.11).

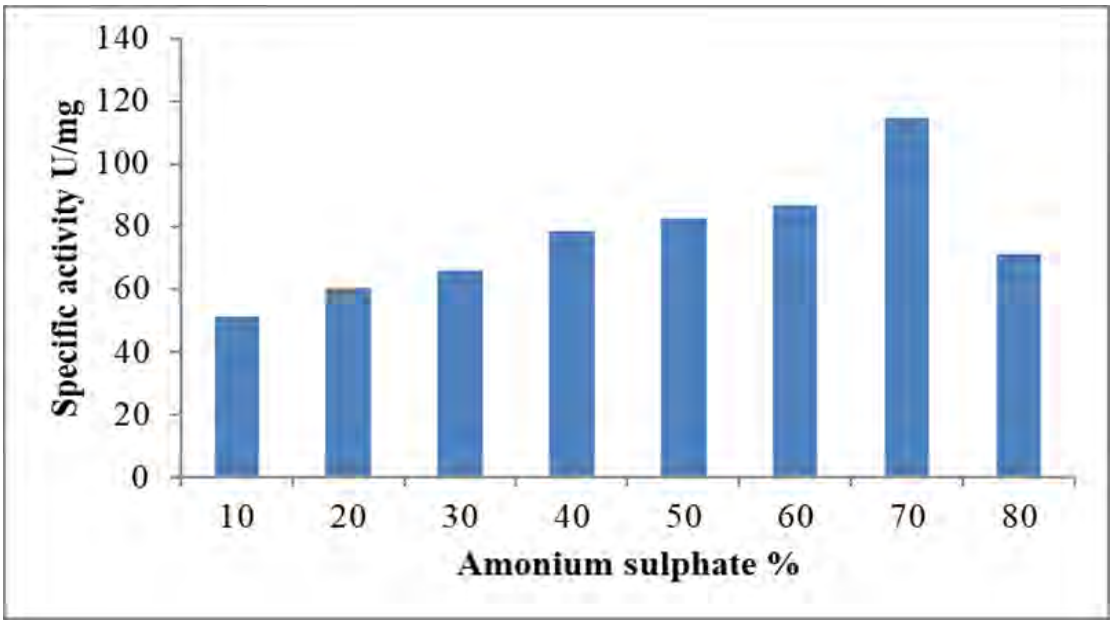

Figure 4.10: Ammonium sulfate precipitation of crude cellulase from CC16 and evaluation of specific activities of pellets at various percentages of ammonium sulfate precipitation

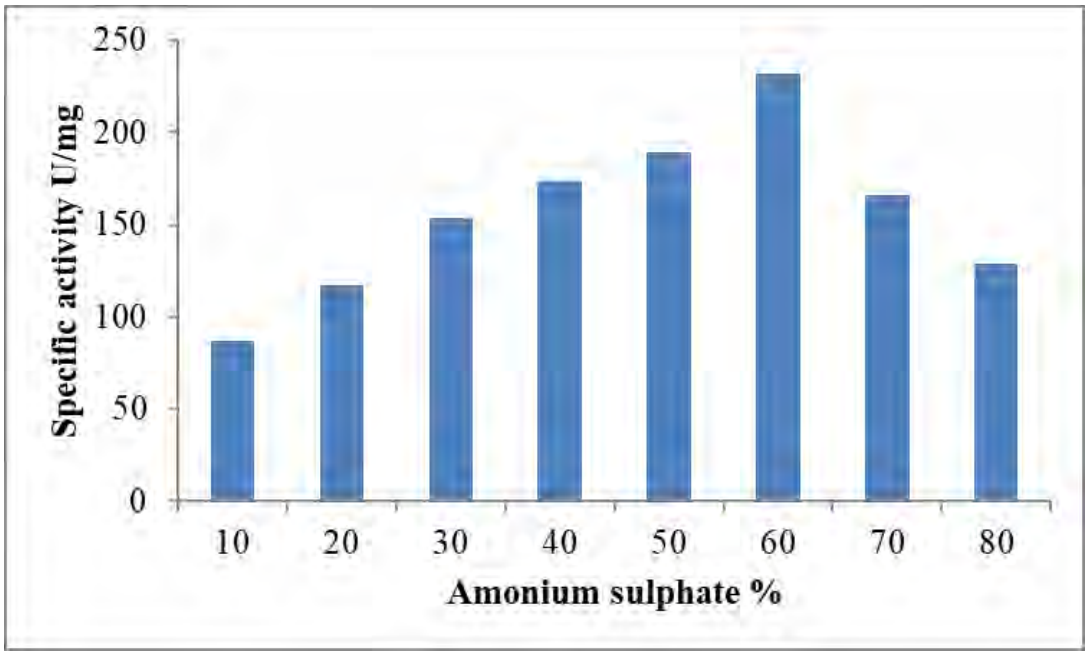

Figure 4.11: Ammonium sulfate precipitation of crude xylanase from CC4 and evaluation of specific activities of pellets at various percentages of ammonium sulfate precipitation.

4.5.3 Protein purification (Sephadex G-100) column Chromatography

Following ammonium sulfate dialysis, the partially purified enzymes (cellulase and xylanase) were further processed using column chromatography with Sephadex G-100 gel resin. The separation was based on molecular size. The peak activity was observed in fractions 7 to 18, prompting the pooling of these fractions to enhance cellulase concentration for optimal activity (Figure 4.12). Similarly, the fractions from the partially purified xylanase were subjected to analysis and highest activity was observed in fractions 8 to 15, thus these fractions were combined to concentrate xylanase for maximal activity (Fig. 4.13).

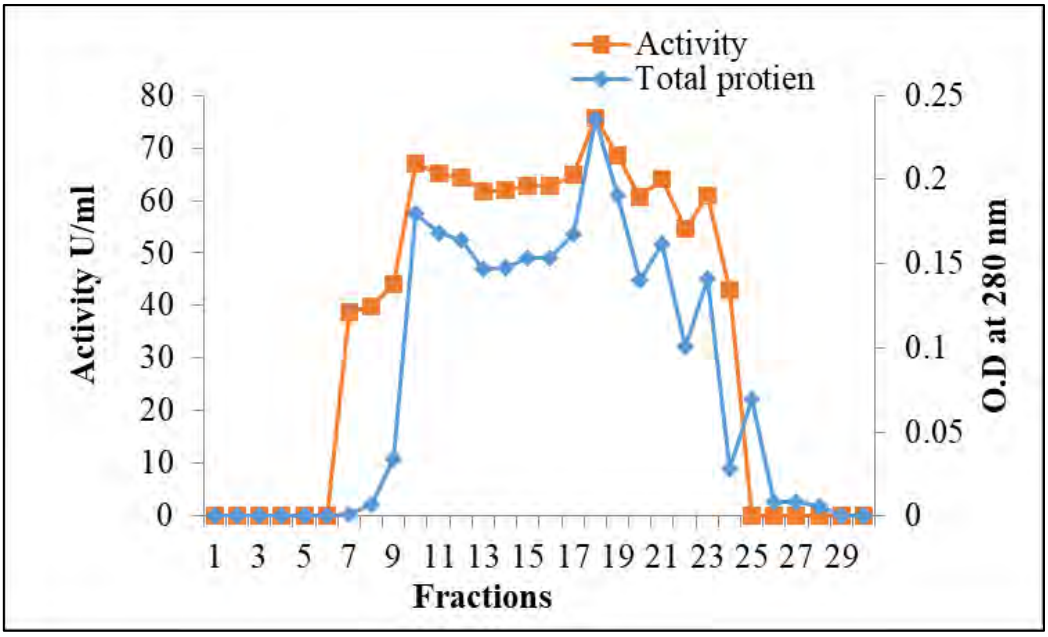

Figure 4.12: Total protein and specific activity profile of ammonium sulfate precipitated. Purification steps of cellulase from strain CC16 through Sephadex G-100 gel column.

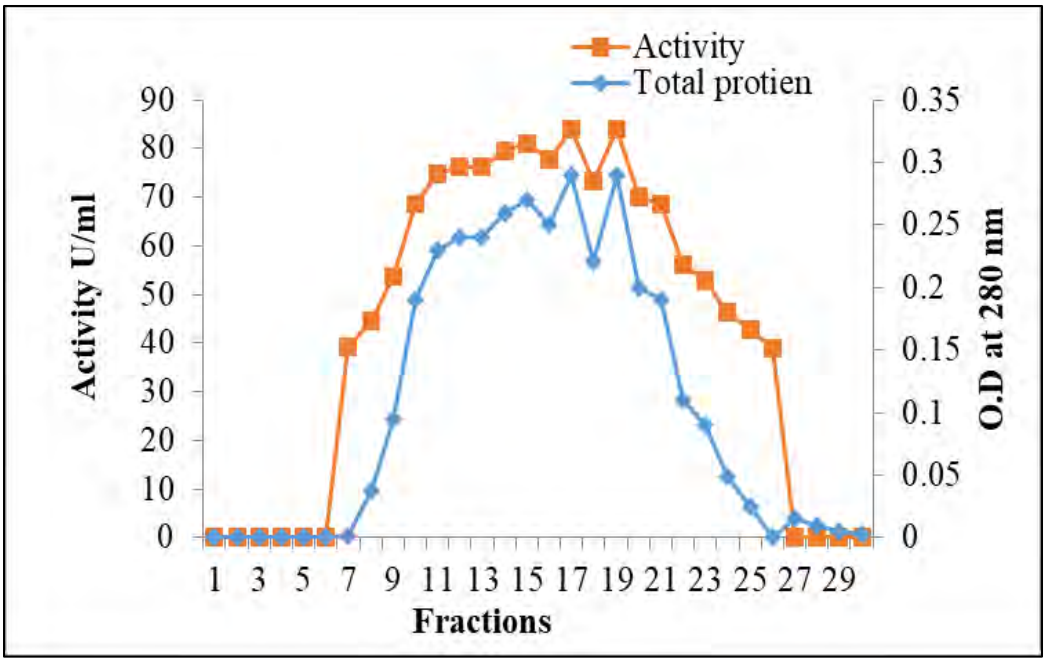

Figure 4.13: Total protein and specific activity profile of ammonium sulfate precipitated. Purification steps of cellulase from strain CC4 through Sephadex G-100 gel column.

4.5.4 Total yield of Purified Cellulase from strain CC16 and Xylanase from CC4

The determination of total yield and purification fold was performed for both precipitated and gel-filtered cellulase extracted from strain CC16. The cellulase CC16 exhibited a total yield of 34.55% and a purification fold of 6.53. These values are presented in (Table 4.2). Similarly, the xylanase extracted from CC4 demonstrated a total yield of 44.2% and a purification fold of 4.19 (Table 4.3).

Table 4.2: Purification steps of cellulase from bacterial strain CC16

Purification steps	Total Activity (U/ml)	Protein (mg/ml)	SA (U/mg)	Yield (%)	Purification Fold
Crude Extract	24382.66	1.4	54.8	100	1
$(NH4)_2SO_4$	13564.06	1.1	114.2	55.6	2.03
Sephadex (G 100)	8425.62	0.5	345.8	34.5	6.5

Table 4.3: Purification steps of xylanase from bacterial strain CC4

Purification steps	Total Activity (U/ml)	Protein (mg/ml)	SA (U/mg)	Yield (%)	Purification Fold
Crude Extract	98917.19	2.5	133.2	100	1
$(NH4)_2SO_4$	54734.38	2.3	232.0	55.3	1.74
Sephadex (G 100)	43768.75	1.9	558.5	44.2	4.19

4.6 Characterization of Purified Cellulase and Xylanase:

4.6.1 Effect of temperature on activity of cellulase from bacterial strain CC16 and Xylanase from bacterial strain CC4

The effect of temperature on the action of purified cellulase from bacterial strain CC16 and purified xylanase from bacterial strain CC4 was examined by incubating it with CMC and xylan substrate respectively at 30°C, 40°C, 45°C, 50°C, 60°C, 70°C and 80°C for 30 min. At 45°C, cellulase preserved its peak residual activity (100%), while at 50°C, it retained more than 80% of its activity. At temperatures ranging from 60°C to 80°C, enzyme activity was at its lowest (Fig 4.14). The xylanase enzyme showed peak residual activity at 50°C, while at 30°C, and 40°C, it retained more than 80% of its activity. At temperatures ranging from 70°C to 80°C, enzyme activity was at its lowest (Fig 4.15).

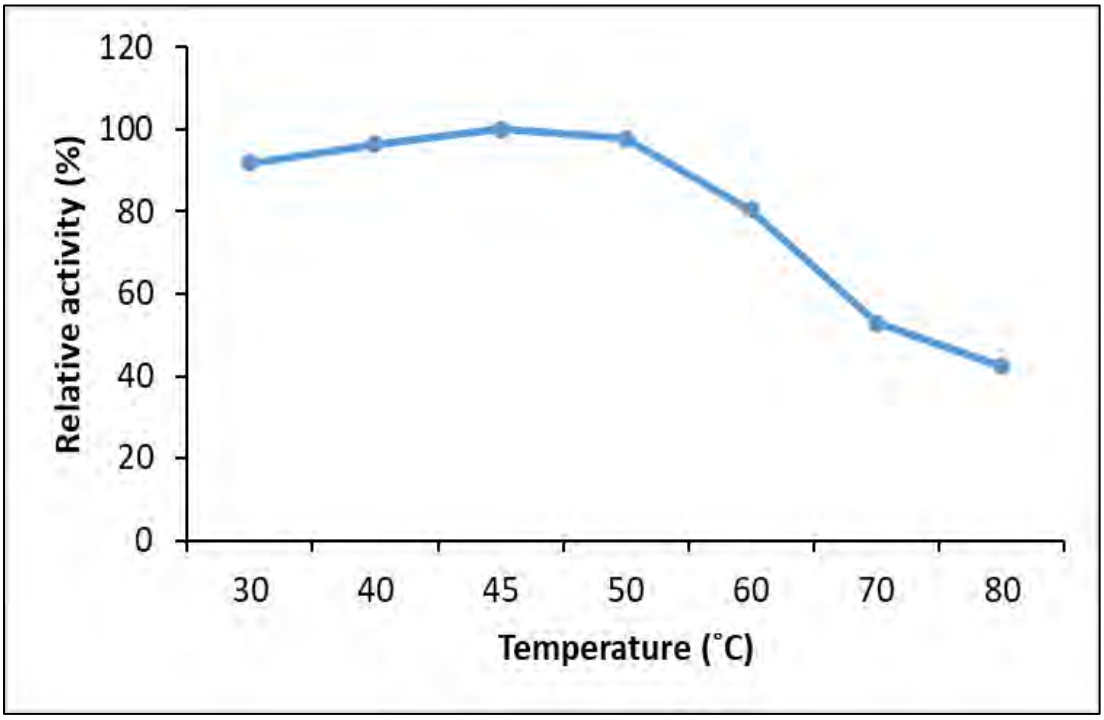

Figure 4.14: Effect of temperature on specific activity of purified cellulase from bacterial strain CC16.

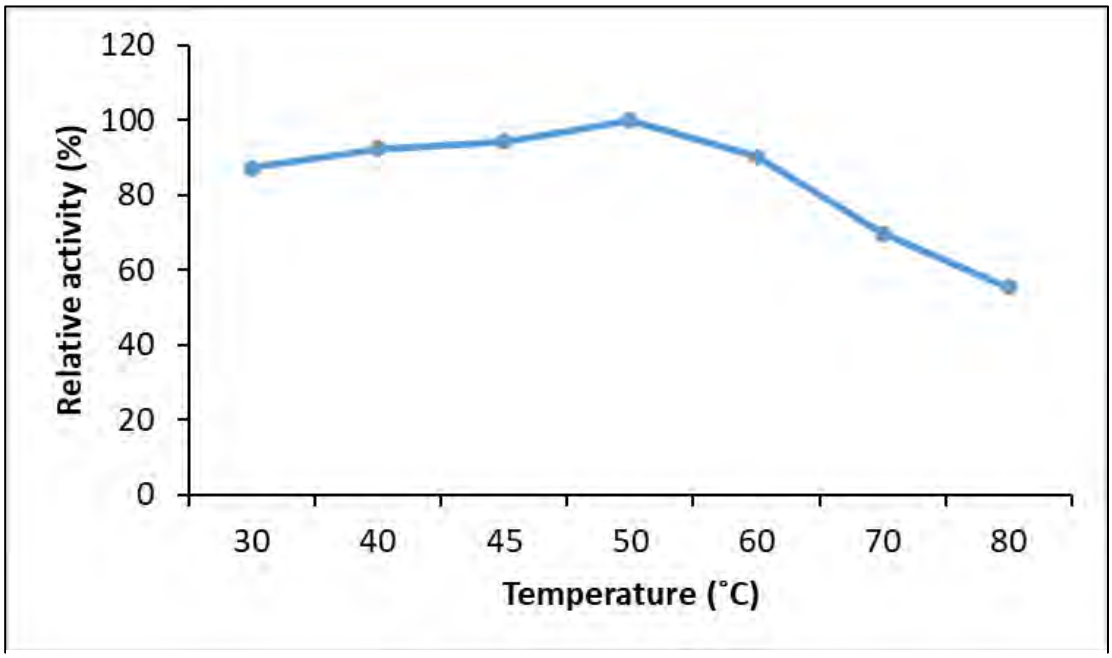

Figure 4.15: Effect of temperature on specific activity of purified xylanase from bacterial strain CC4.

4.6.2 Temperature stability profile cellulase of cellulase from bacterial strain CC16 and Xylanase from bacterial strain CC4

The stability of temperature on the action of purified cellulase from bacterial strain CC16 and purified xylanase from bacterial strain CC4 was studied. For cellulase, the100% stability was achieved at 45°C for 150 min. At 40°C for 150 minutes, the enzyme maintained more than 95% of its stability. At 30°C for 150 minutes, the enzyme maintained more than 70% of its stability. Temperatures between 50 and 80 °C for 150 minutes, cellulase stability was reduced by more than half (Fig 4.16).

In the case of xylanase from CC4, 100% stability was achieved at 50°C for 150 min. At 45°C for 150 minutes, the enzyme maintained more than 90% of its stability. At 30°C for 150 minutes, the enzyme maintained more than 70% of its stability. At 70°C and 80°C for 150 minutes, xylanase stability was reduced by more than half (Fig 4.17).

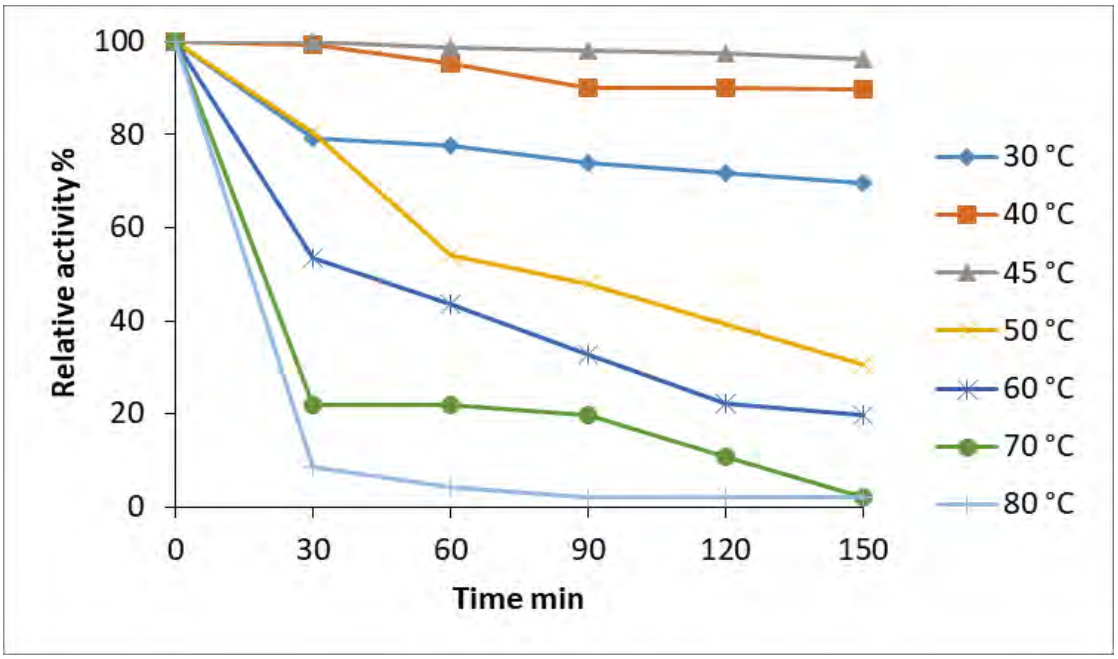

Figure 4.16: Effect of temperature on stability of purified cellulase from bacterial strain CC16

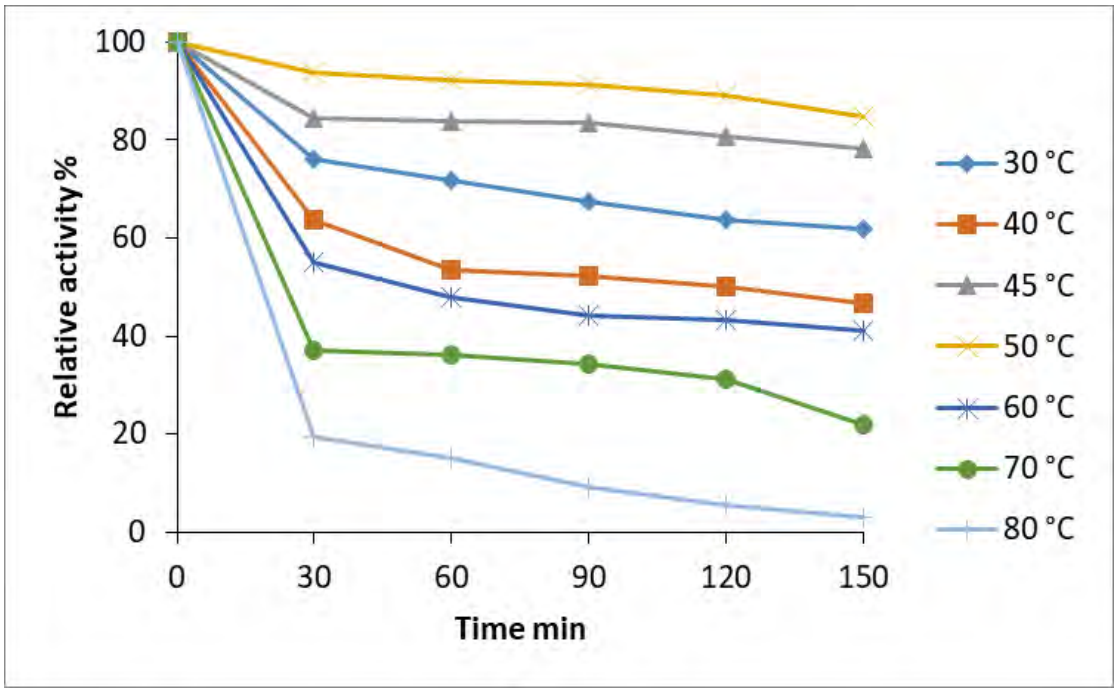

Figure 4.17: Effect of temperature on stability of purified xylanase from bacterial strain CC4.

4.6.3 Effect of pH on the activity of cellulase from bacterial strain CC16 and Xylanase from bacterial strain CC4

The effect of different pH on specific activity of purified cellulase isolated from bacterial strain CC16 and purified xylanase from bacterial strain CC4 was examined. The cellulase residual activity peaked at pH 7.0, cellulase activity was decreased at pH levels above and below 7.0 (Fig 4.18) while that of xylanase at pH 9.0 and it9 activity was decreased at pH levels above and below 7.0 (Fig 4.19).

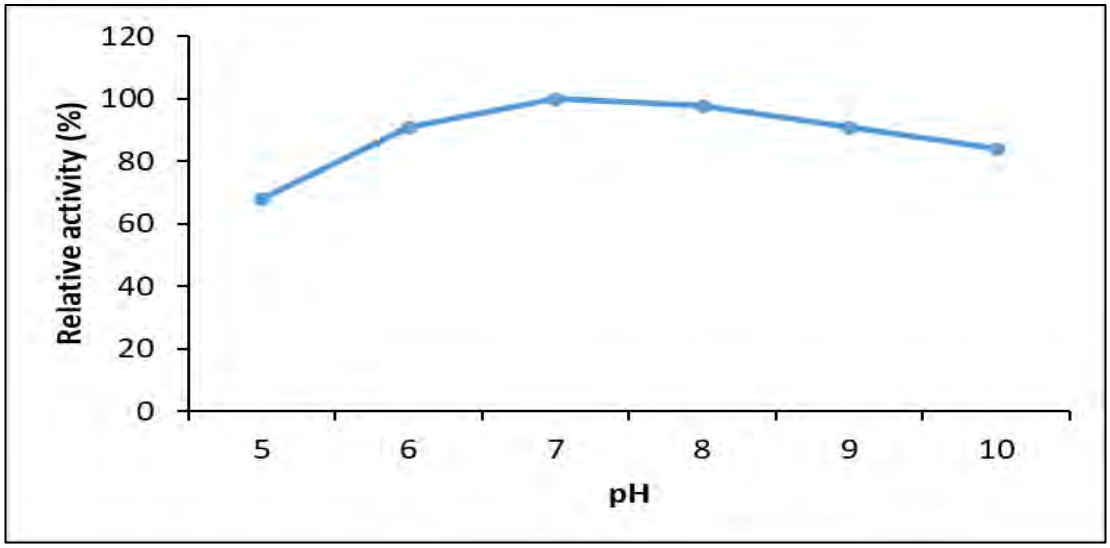

Figure 4.18: Effect of pH on specific activity of purified cellulase from bacterial strain CC16

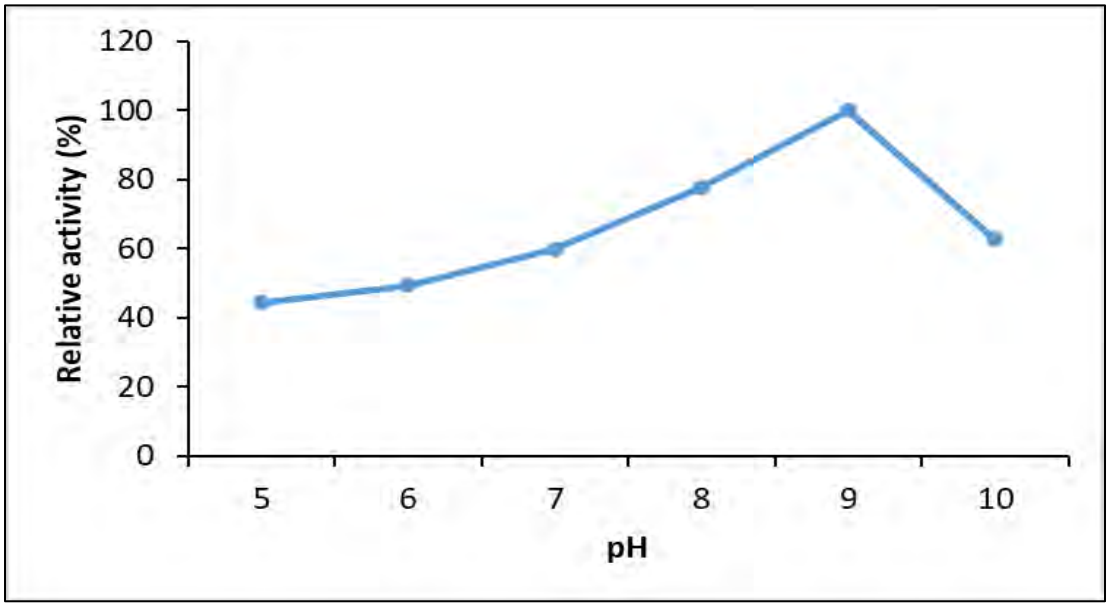

Figure 4.19: Effect of pH on specific activity of purified xylanase from bacterial strain CC4

4.6.4 pH stability profile of cellulase from bacterial strain CC16 and Xylanase from bacterial strain CC4

The stability of pH on the action of purified cellulase from bacterial strain CC16 and purified xylanase from bacterial strain CC4 was examined. Cellulase enzyme was stable at pH 7.0, enzyme stability was maintained 100% for 150 min. At a pH of 6.0, and pH 8.0 more than 80% of the stability was maintained. After being exposed to pH 9.0 and above, cellulase stability declined (Fig 4.20).

Xylanase enzyme was stable at pH 9.0. At pH 9.0 enzyme stability was maintained 100% for 150 min. At a pH of 10.0 more than 90% of the stability was maintained. After being exposed to pH 8.0 and below, xylanase stability declined (Fig 4.21).

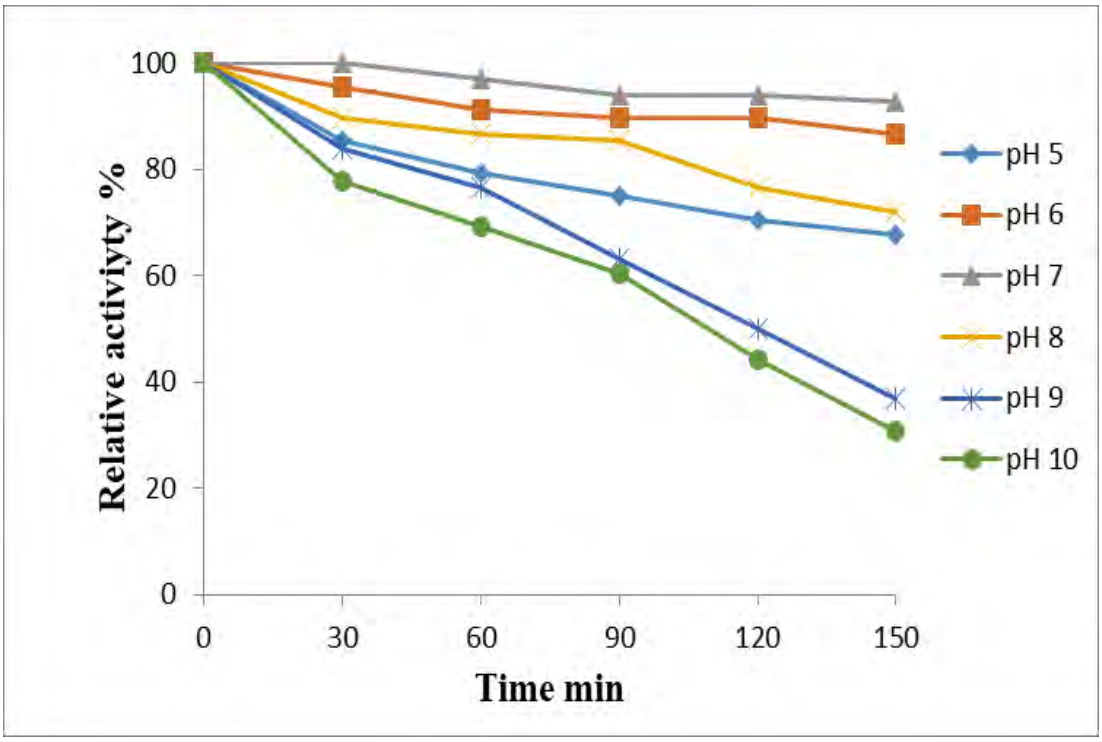

Figure 4.20: Effect of pH on stability of purified cellulase from bacterial strain CC16

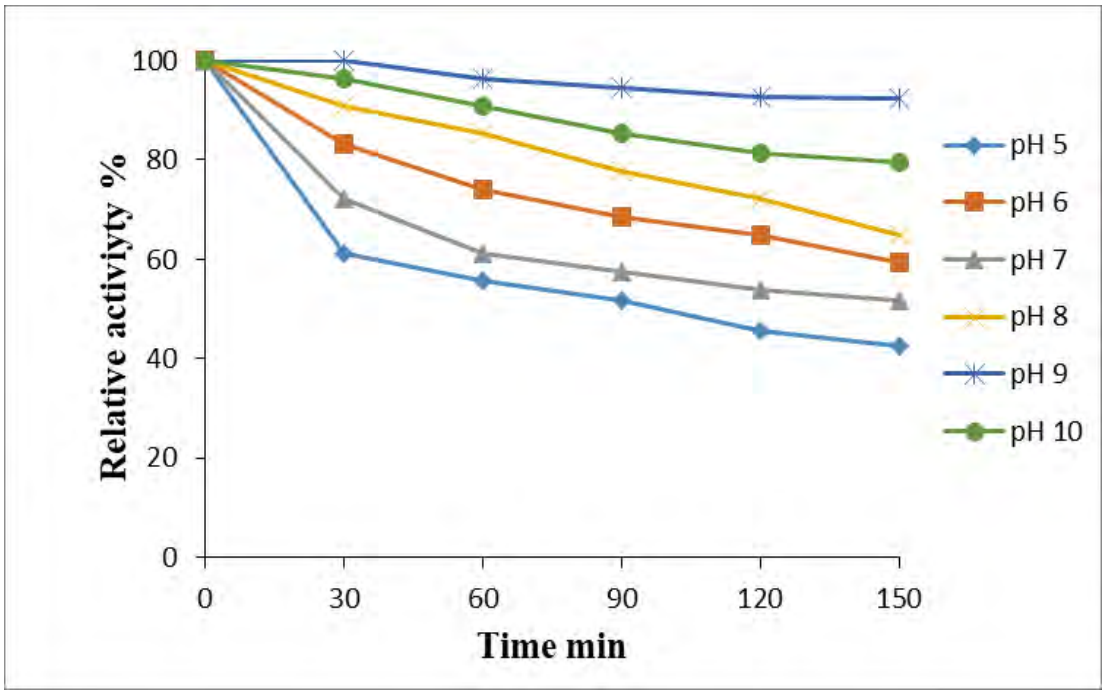

Figure 4.21: Effect of pH on stability of purified xylanase from bacterial strain CC4.

4.6.5 Effect of metals on activity of cellulase from bacterial strain CC16 and Xylanase from bacterial strain CC4

At concentrations of 5mM and 10mM, the effect of various metals on purified cellulase from bacterial strain CC16 and purified xylanase from bacterial strain CC4 was examined. $FeSO_4$, enhanced cellulase activity by 127% at both concentrations. Cellulase activity was also enhanced by more than 100% in presence of $CaCl_2$, $CuSO_4$, and $MnSO_4$ at both concentrations (2mM and 10mM) while $Pb(NO_3)_2$ decreased the activity of cellulase 25% at 2mM concentration and 32% at 10mM concentration (Fig 4.22).

Generally, the xylanase activity was enhanced by most of the metals with increase in metals concentration. $CuSO_4$ increased 119% of xylanase activity at 2mM concentration and 152% at 10mM concentration. $CaCl_2$, $NiS0_4$, NaCl, $MgSO_4$, and $MnSO_4$ increased the xylanase activity while $ZnSO_4$ decreased the enzyme activity at both concentrations. $Pb(NO_3)_2$ decreased xylanase activity by 44% at 2mM concentration and 49% at 10mM concentration (Fig 4.23).

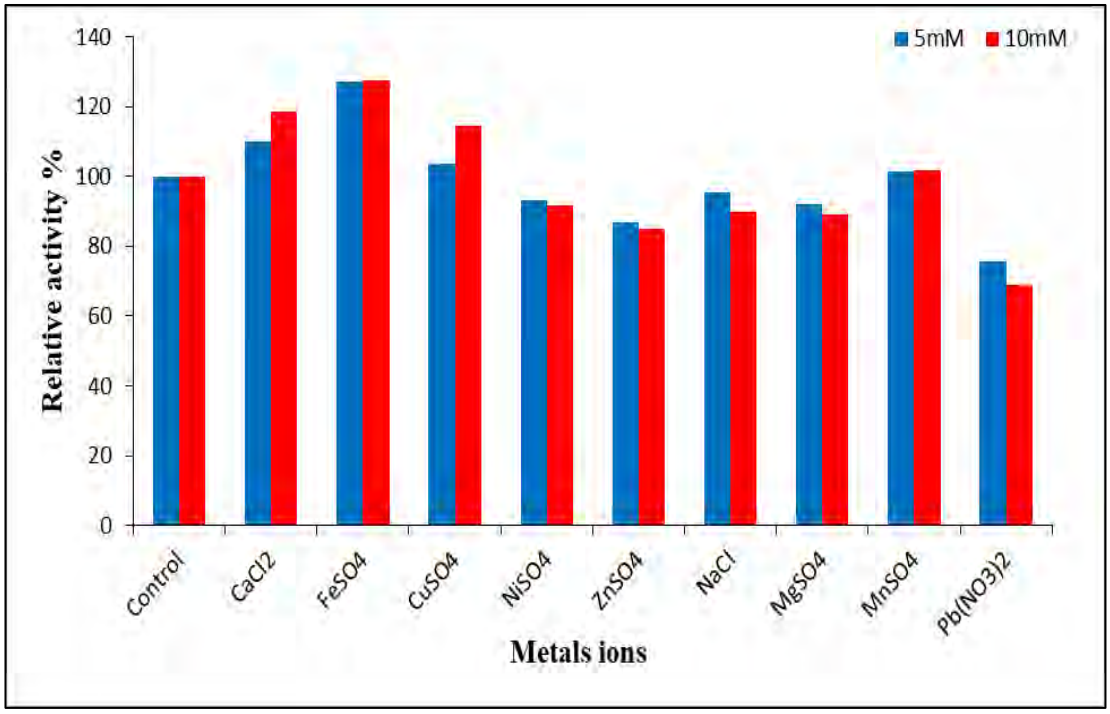

Figure 4.22: Effect of different metal ions on stability of purified cellulase from bacterial strain CC16.

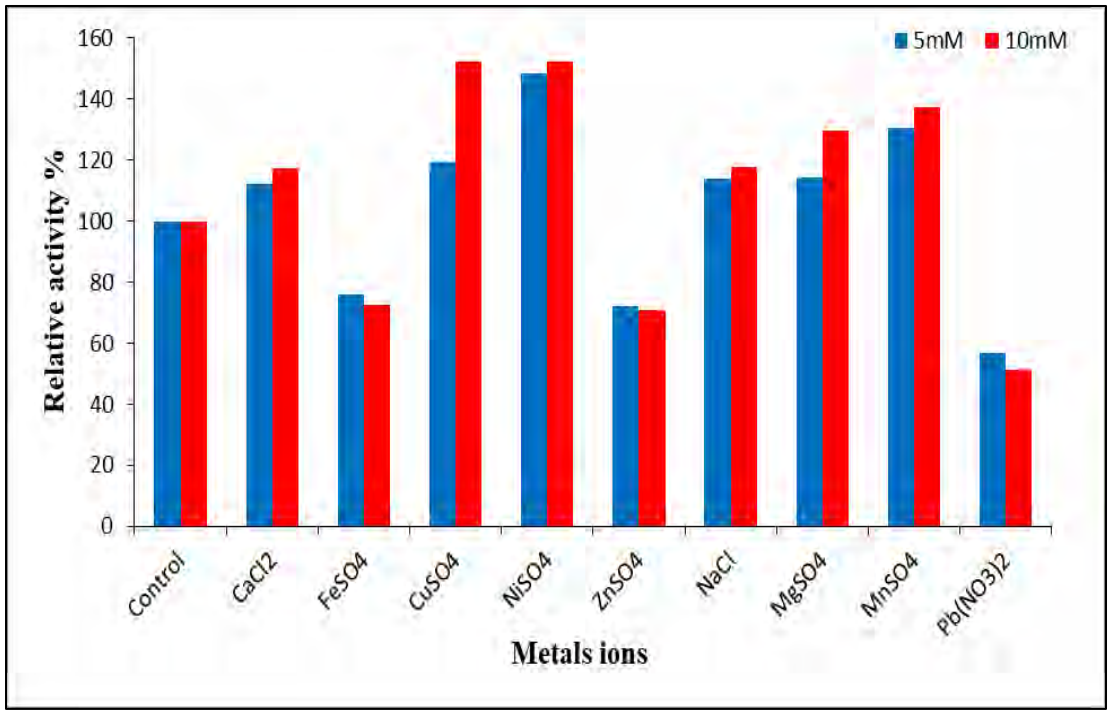

Figure 4.23: Effect of different metal ions on stability of purified xylanase from bacterial strain CC4.

4.6.6 Effect of Surfactants on the activity of of cellulase from bacterial strain CC16 and Xylanase from bacterial strain CC4

Different surfactants at 1.0% and 10.0% concentration were used in order to determine their effect on the activity of purified enzyme. In general, SDS inhibited 88% of the enzyme activity at high concentration (10.0%) while 73% at low concentration (1.0%). Comparatively, Tween 20, Tween 80, CTAB and Triton X 100 potentially enhanced cellulase activity at both concentrations (1.0% and 10.0%) (Fig. 4.24).

For xylanase SDS inhibited 83% of the enzyme activity at high concentration (10.0%) while 68% at low concentration (1.0%). Comparatively, Tween 20, CTAB and Triton X 100 potentially enhanced cellulase activity at both concentrations (1.0% and 10.0%). Tween 80 enhanced xylanase activity by 71% of the enzyme activity at high concentration (10.0%) (Fig. 4.25).

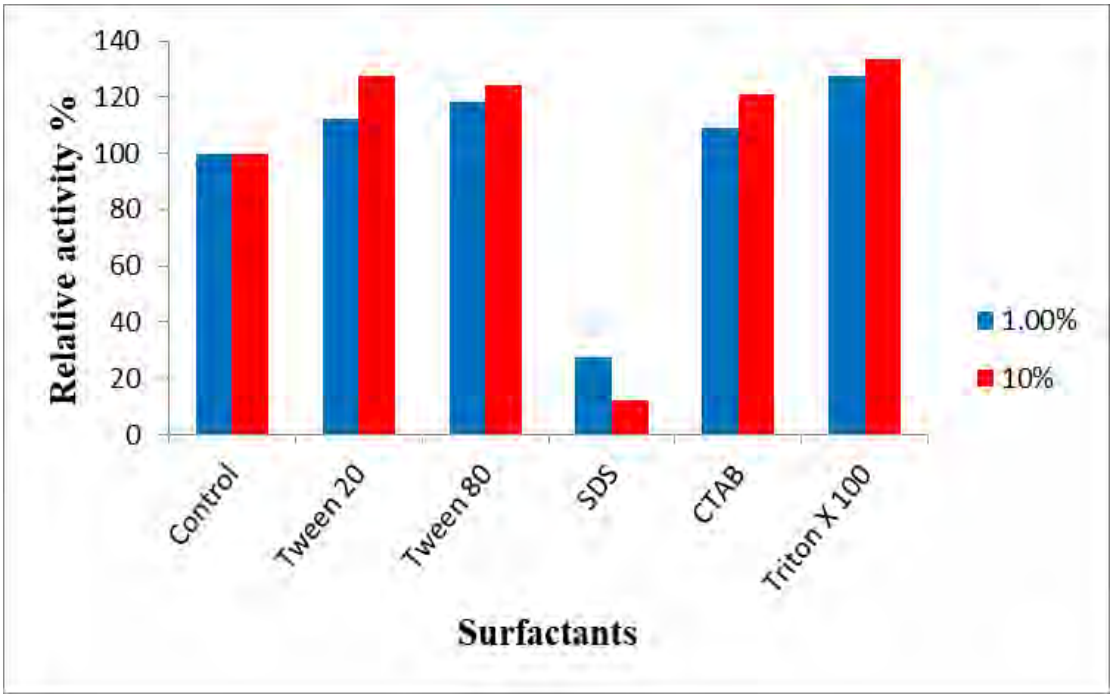

Figure 4.24: Effect of different surfactants on stability of purified cellulase from bacterial strain CC16.

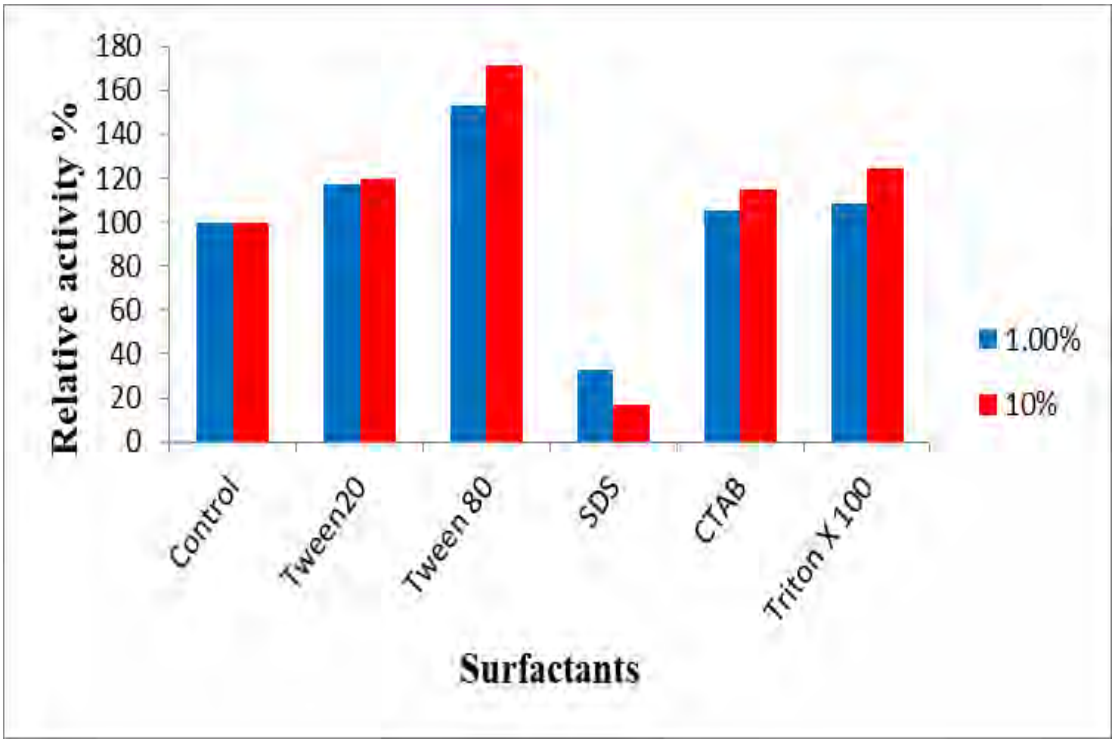

Figure 4.25: Effect of different surfactants on stability of purified xylanase from bacterial strain CC4.

4.6.7 Effect of organic solvents on the activity of cellulase from bacterial strain CC16 and Xylanase from bacterial strain CC4

Different organic solvents (10%) final concentration was used in order to determine the effect on the activity of purified enzyme for 120 minutes. In general, Acetonitrile, Ethyle acetate, N-Hexane, Butanol, and DMSO enhanced cellulase activity for 120 minutes while, enzyme maintained 100% activity within the presence of organic solvent methanol for 120 minutes. Glycerol enhanced 10% of cellulase activity for 120 minutes. Acetonitrile and propanol decreased the activity of purified enzyme with increase in time duration (Fig. 4.26).

In case of xylanase the enzyme activity was enhanced in presence of ethanol, methanol, acetonitrile, acetone with time for 120 minutes. Glycerol enhanced 26% of cellulase activity in 120 minutes. Ethyle acetate, N-Hexane, propanol, butanol and DMSO decreased the xylanase activity with time for 150 minutes (Fig. 4.27).

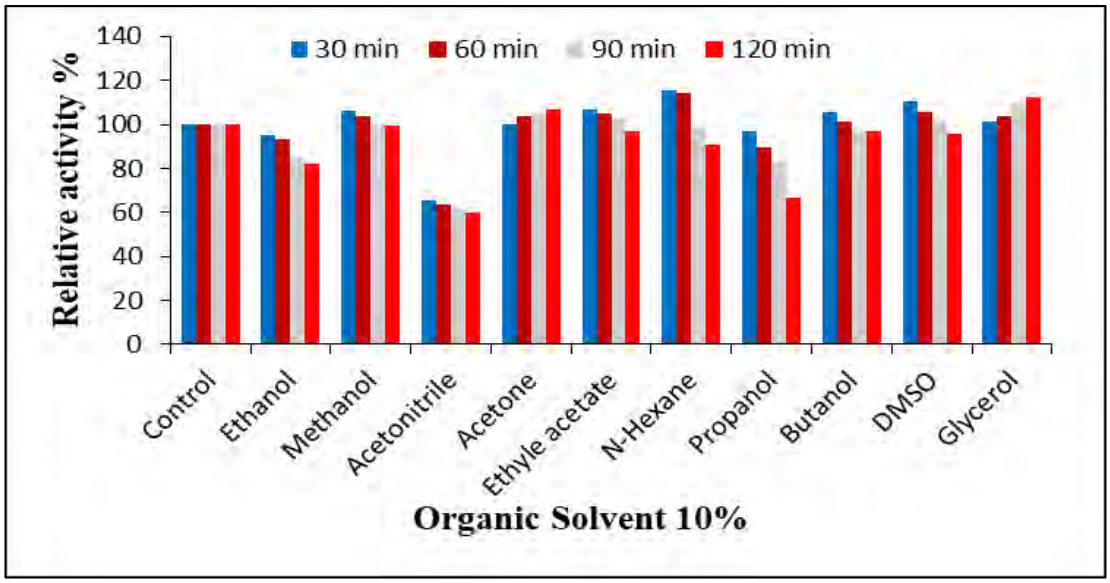

Figure 4.26: Effect of different organic solvents on purified cellulase from bacterial strain CC16.

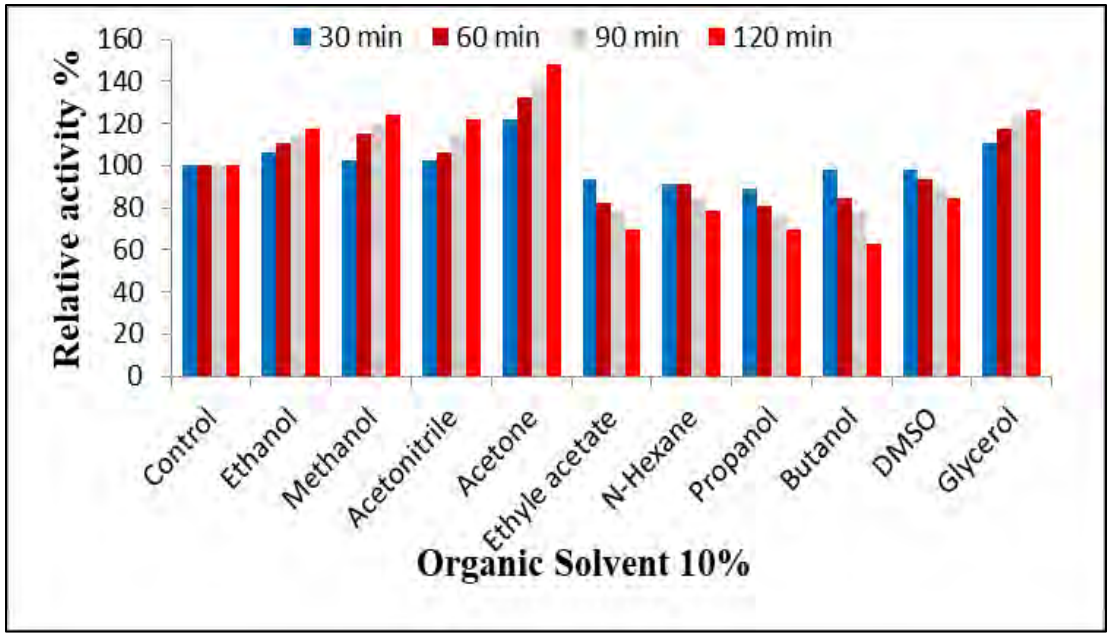

Figure 4.27: Effect of different organic solvents on purified xylanase from bacterial strain CC4.

4.6.8 Determination of kinetic parameters

The kinetic parameters of pure cellulase from bacterial strain CC16 and purified xylanase from bacterial strain CC4 were determined, Lineweaver and Burk (1934) plot was used to calculate the Vmax and Km constant. The Km value of bacterial strain CC16 cellulase (CMC) was calculated as 1.72 mg/ml. The Vmax of CC16 cellulase was 129.8 µmolmg $^{-1}$min^{-1} for CMC (Fig. 4.28). Similarly, the Km value for strain CC4 xylanase (for beechwood xylan) was 3.7 mg/ml. Respectively, the Vmax of CC4 xylanase was 1428.5 µmolmg -1min-1 for beechwood xylan (Fig. 4.29).

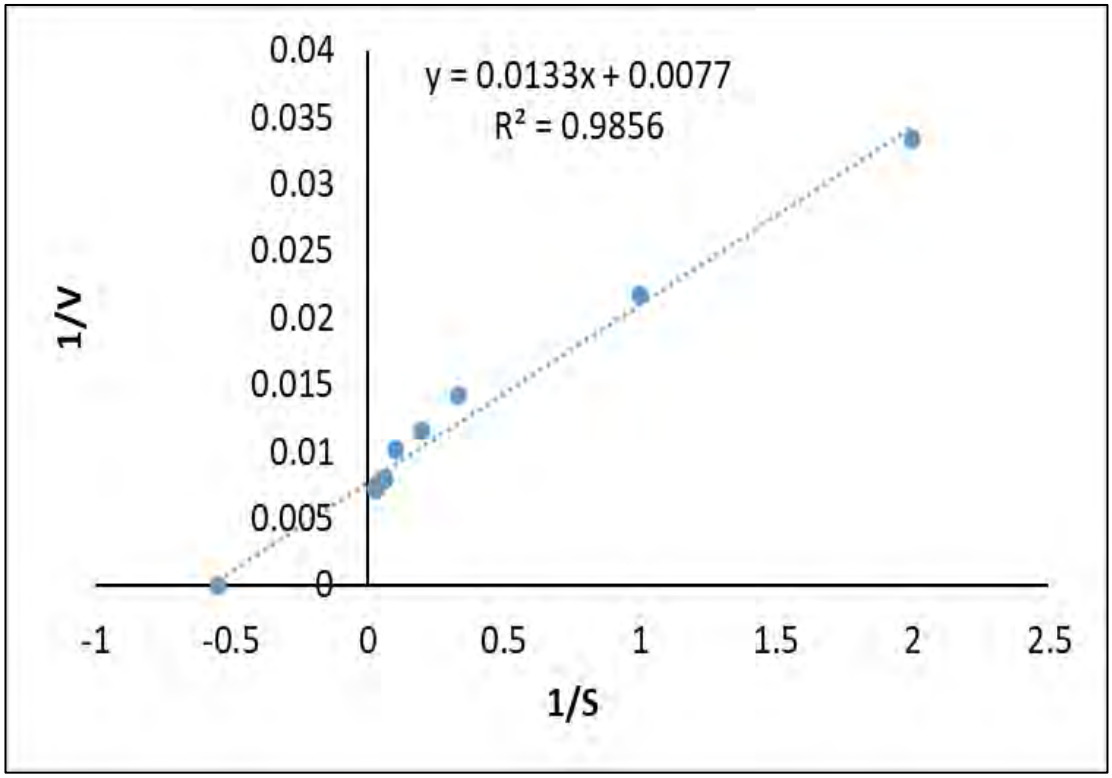

Figure 4.28: Kinetics analysis of cellulase from bacterial strain CC16 (Km and Vmax) value were observed using Lineweaver-Burk plot.

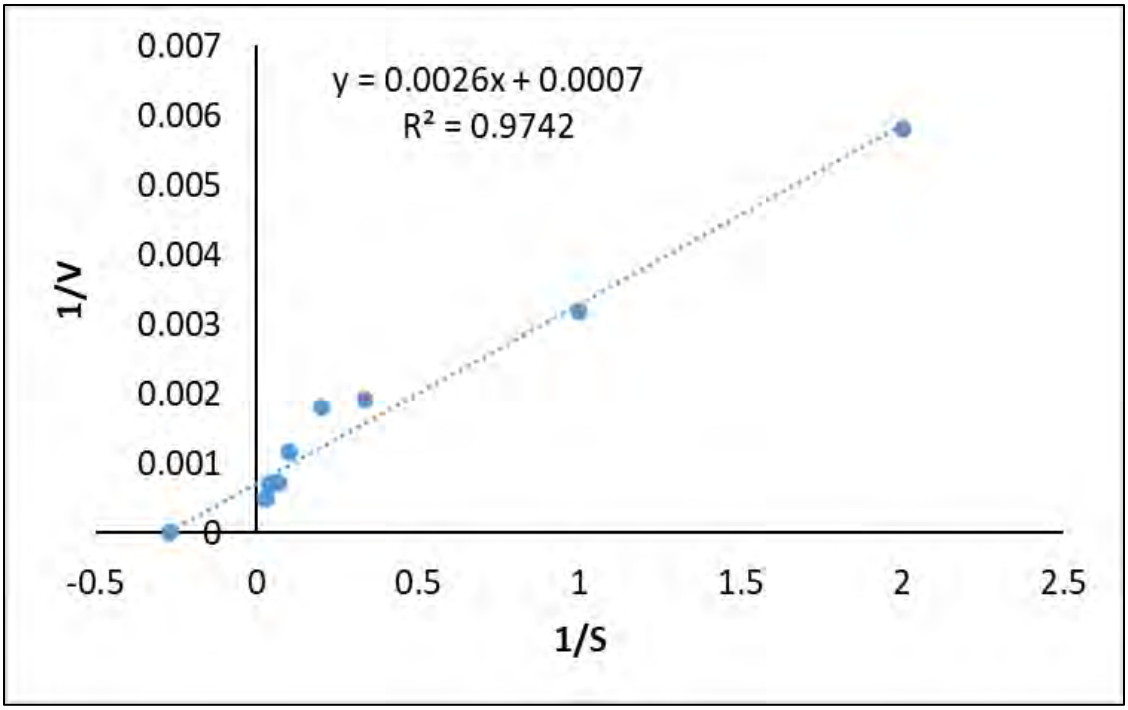

Figure 4.29: Kinetics analysis of xylanase from bacterial strain CC4 (Km and Vmax) value were observed using Lineweaver-Burk plot.

4.7 Two-Step Enzymatic Hydrolysis of the Pretreated Bagasse:

4.7.1 TRS (g/L) and TRS yield (g/g):

In first step of enzymolysis of the NaOH-catalyzed methanol pretreated bagasse, the treatment with xylanase resulted in 9.8 g/L of the TRS while the post-cellulase hydrolysis resulted in a hydrolysate with 33.2 g/L of the TRS. The TRS yield calculated was 0.27 g/g for the hydrolysate obtained from xylanase treatment of pretreated bagasse while it was 0.91 g/g for the hydrolysate obtained from cellulase treatment as shown in Figure 4.30 and Table 4.19.

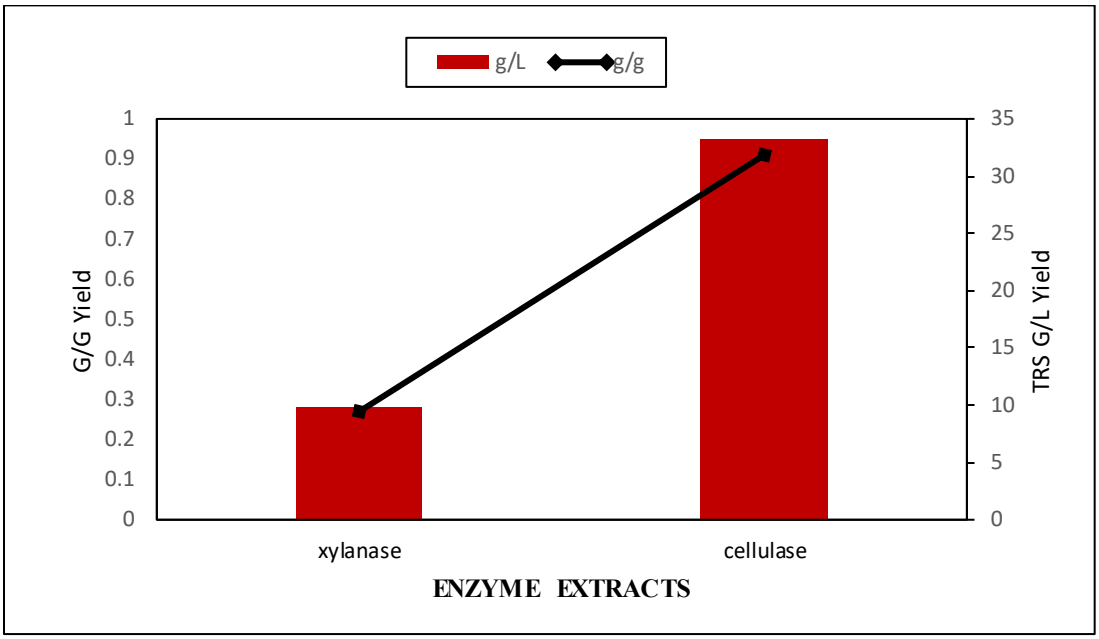

Figure 4.30: The TRS (g/g) for the hydrolysate from the xylanase treatment followed by cellulase treatment of pretreated bagasse represented on the primary axis while the TRS yield (g/L) represented on the secondary axis.

Table 4.4: The TRS (g/L) and TRS yield (g/g) of the hydrolysate obtained from subjecting the bagasse initially to the xylanase hydrolysis (48 hrs.) and then to the cellulase hydrolysis (24 hrs.).

Purified Enzyme Extracts

Crude Enzymatic Extracts	TRS (g/L)	TRS yield (g/g)
Xylanase	9.8	0.27
Cellulase	33.2	0.91

4.8 FTIR (Fourier-transform infrared spectroscopy) analysis of Untreated, Pretreated and Saccharified Bagasse

Samples of untreated (Fig 4.31), pretreated(Fig 4.32), and saccharified sugarcane bagasse (Fig 4.33) were analyzed by FTIR to examine the changes in specific functional groups that are responsible for lignin, cellulose, and hemicellulose. The band at 3321

cm^{-1} in the untreated sugarcane bagasse represents OH stretching, the band at 2919 cm^{-1} was responsible for C–H bond whereas the band at 1618 cm^{-1} represents C=C stretching that is present in the aromatic structure of lignin. The aromatic structure peak at 1514 cm^{-1} confirmed the presence of lignin in the untreated substrate. The presence of the lignin's aryl group was shown by a signal for C–O at 1239 cm^{-1}. Additionally, the occurrence of lignin in untreated samples was confirmed by considering the region between 1300 and 1500 cm^{-1} where high molecular coupling has been observed. The enzymatic hydrolysis of hemicellulose and cellulose in the saccharified sugarcane bagasse was indicated by changes in the area between 1030 and 1583 cm^{-1}, which confirms the hydrolysis of sugarcane bagasse by cellulases and xylanases. The Peak at 832 cm^{-1} confirms the C-H deformations of the aromatic ring of lignin, which is absent in spectra of pretreated and saccharified sugarcane bagasse thus confirming the removal of lignin and the increase in line width and asymmetry of the peaks between 3000 and 3800 cm^{-1} in saccharified sugarcane bagasse was due to disturbed H-bonding.

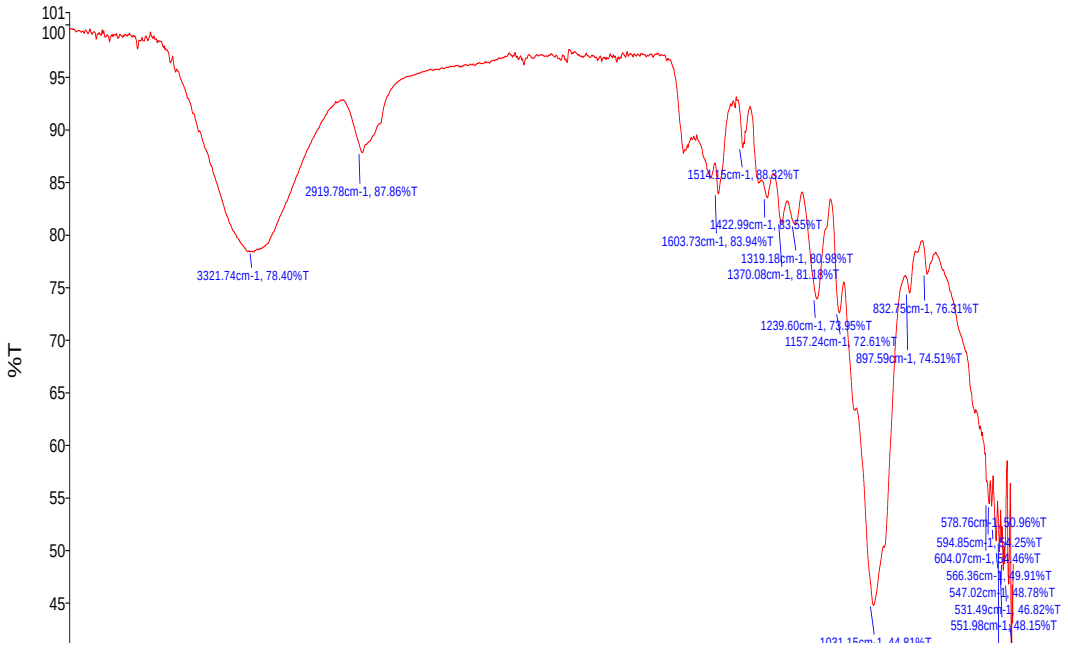

Figure 4.31 The Fourier Transform Infra-Red (FT-IR) absorption pattern of Untreated Sugarcane Bagasse

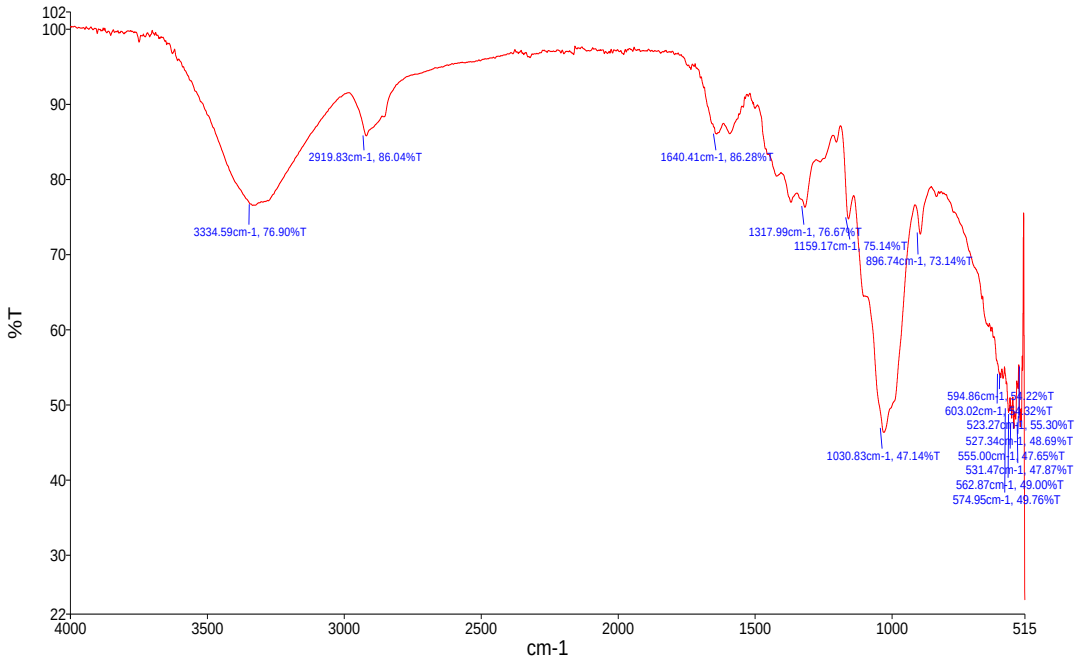

Figure 4.32 The Fourier Transform Infra-Red (FT-IR) absorption pattern of Alkali Organo-solvent Pretreated Sugarcane Bagasse

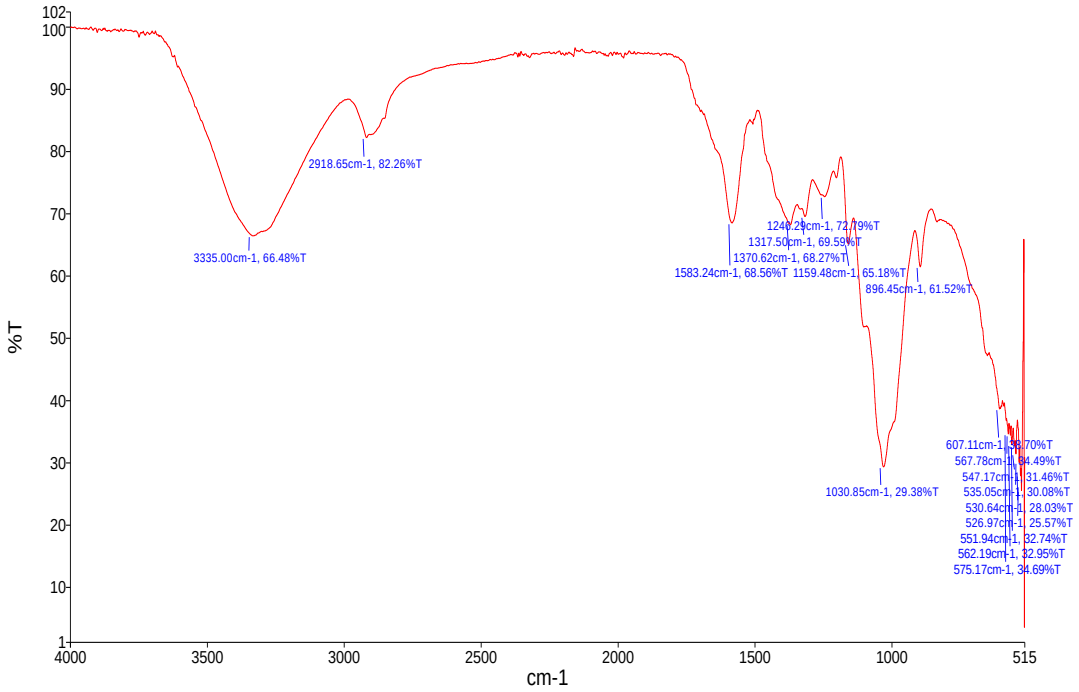

Figure 4.33 The Fourier Transform Infra-Red (FT-IR) absorption pattern of Enzymatically Saccharified Sugarcane Bagasse

Discussion

With enzyme discovery, the industrial world experienced a revolution as enzymatic processes replaced chemical-based procedures. In industrial process utilization of chemicals has serious environmental consequences, but the environmental regulation agencies proposed rules and regulation to move the industries to a safe direction (Abdelraheem *et al*., 2019). Attractive characteristics of the enzymes include they are renewable, and considered safe based on biodegradation, treatment and byproduct formation. As the industrial processes occur in harsh conditions and it is thought that bacteria are considered as potential candidates as these enzymes can bear the hostile industrial conditions and remain active in industrial processes. Application of enzymes on industrial scale is becoming the dominant technology, and remarkable research has been done on the implementation of enzymes in pulp and paper industry, biofuel production, textile industry, baking industry, food production and pharmaceuticals processes (Liu & Kokare, 2023) (Tatta *et al*., 2022). Sugarcane (Saccharum officinarum) bagasse, a biomass derived from the agricultural waste generated during sugarcane processing, which is widely available worldwide. Excessive production of sugarcane has led to the burning of bagasse as a method of disposing of solid waste, resulting in environmental pollution (Ázar *et al*., 2019). In last two decades among different enzymes, a lot of attention has been given to cellulases and xylanases. Currently, cellulases and xylanases are commonly used for bioethanol production, deinking of waste paper, processing of animal feed, in processing of food, paper, and pulp production (Motta *et al*., 2013).

The high production of cellulase and xylanase provides an opportunity to utilize lignocellulosic biomass and convert it into a valuable product like biofuels (Saini *et al*., 2015). Lignocellulosic biomass is found abundant in nature but due to lack of proper handling, it is an environmental burden. This biomass instead of burning should be utilized sustainably in different industries (Safian *et al*., 2022). The synergistic use of cellulase and xylanase plays an important role in converting the lignocellulosic biomass into bioethanol and its derivatives (Thanigaivel *et al*., 2022). Sugarcane bagasse is among the highly generated LB waste. The high cellulosic content of sugarcane bagasse is about 50% and hemicellulosic content about 25% is an ideal substrate for cellulase

and xylanase but it requires pretreatment to overcome the recalcitrant nature of lignocellulose (Li *et al*., 2014).

Although diverse organisms are reported for cellulase and xylanase production, the microorganisms are considered as predominant ones. Short doubling time, simple and easy to grow and handle, utilization of low grade substrate for growth, and ability to tolerate the environmental shifts are some advantages due to which microbial enzymes are preferred. Researchers have paid a lot of attention on fungi and bacteria for xylanase production. Cellulase and xylanase produced from bacteria have an advantage over fungi that fungal strains face difficulties during the submerged fermentation because of their mycelial growth patterns. Other culture parameters including the transfer of oxygen can also vary for different fungal strains due to which bacterial strains possess more feasibility to work with and produce enzymes (Iram *et al*., 2021).

Purification and characterization of xylanases from bacteria isolated from the compost sample were the goals of the current study. The sample was obtained from the National Agricultural Research Council (NARC), Islamabad, and was made from fruit and vegetable waste using a lab-scale composter. A cellulase producing bacterial strain CC16 and xylanase producing bacterial strain CC4 was previously isolated from compost soil. After 48 hours of incubation, the zone of hydrolysis of the strain CC16 on the CMC agar plate and the strain CC4 on the nutritional agar enriched with xylan as a substrate was measured. Larger zones indicate that the isolated strain CC16 and CC4 produced cellulase and xylanase in large amount respectively. Cellulase producing bacteria have also been reported to be isolated from different region including paper industry waste, municipal waste, sugarcane farm and wood furnishing region (Shaikh *et al*., 2013). Among bacteria Pseudomonas fluorescens, *Bacillus subtilus, E. coli,* and *Serratia marscens* have been reported to produce cellulase at 40 to 60°C (Sethi *et al*., 2013). The Bacillus strain has been reported to produce significant amounts of extracellular xylanases at 60 °C in recently published literature. Additionally reported xylanase producers include *Bacillus subtilis BE-91*, *B. pumilus*, and *Streptomyces* species from composted soil (Rodrigues *et al*., 2019).

Multiple enzymatic reactions and the movement of various components through the cytoplasmic membrane were found to simultaneously affect the pH of the medium. pH

is the integral parameter of growth for microorganisms in growth medium which effect the production of metabolites (Ray *et al*., 2007). Most microbial cellulases and xylanases function optimally over a wide pH range (5.5–9.5) (Kumar & Satyanarayana, 2014; Ladeira *et al*., 2015). The incubation temperature and time also affect the production of enzymes by microorganisms. Cellulase produced from CC16 showed maximum activity at pH 5. The maximum specific activity of CC16 for cellulase of 8.195 U/mg was achieved at pH 5.0 after 24 hours. In another study cellulase production was reported at pH 6.5-7.0 and incubation of 120 hour at 30 °C temperature (Jampala *et al*., 2017). This might be due to differences in strain used for production of cellulase or incubation time and inoculum size. In the present study the maximum specific activity for xylanase was observed 16.52 U/mg at pH 8.0 after 48 hours. Similar results are reported by Irfan *et al*., (2016) in which xylanase produced by B. *subtilis BS04* and *B. megaterium BM07 i* exhibited maximum xylanase production of 51.60 U/ml at 37 °C and pH 8.0 in 48 hours (Irfan *et al*., 2016).

For a high-grade pure enzyme, analytical techniques are required, however purity is not necessary for industries like detergent, pulp and paper, and food processing due to financial limitations. But it's crucial to get rid of some other undesirable proteins. Cellulase from the bacterial strain CC16 precipitated at a concentration of 70% ammonium sulphate, while xylanase from CC4 precipitated at a concentration of 60% ammonium sulphate. Other methods have been reported for cellulase and xylanase precipitation such as, ethanol, acetone (Bhattacharya & Pletschke, 2015; Gad *et al*., 2022). However, ammonium sulfate $(NH4)_2SO_4$) method is commonly observed in the literature for xylanases precipitation because of non-toxic high purity and economical (Dzuvor *et al*., 2018). Precipitation serves as an important technique for fractionating specific proteins from a solution containing multiple proteins. It can also be employed as a highly precise approach to isolate an individual protein of interest following a purification procedure (Matulis, 2016).

Purification of cellulase from CC16 by column chromatography (Sephadex G-100) increased specific activity from 114.2 to 345.8 Umg-1 (34.5% yield) with 6.5 purification fold and the specific activity of xylanase from CC4 increased from 232 to 558.5 Umg-1 (44.2% yield) with 4.1 purification fold. Dehghanikhah *et al*., (2020)

conducted a study in which with purification of cellulase enzyme, specific activity was increased from 4.29 to 10.86 U/mg and purification fold was 2.72 (Dehghanikhah *et al*., 2020). In a another research study, xylanase was isolated from bacteria and purified, with a purification fold of 2.8, recovery of 2.08%, and specific activity of 250.02 U/mg of protein (Kallel *et al*., 2015).

The activity and stability of purified cellulase from CC16 and xyalanse enzyme from CC4 was tested at different temperatures ranging from 30-90˚C. The optimum temperature for cellulase was found as 45˚C and for xylanase 50˚C was observed as optimum temperature. Cellulase purified from *Bacillus amyoliquefaciens* DL-3 showed optimum cellulase production in range of 45 to 50˚C (Lee *et al*., 2008). Yin *et al*., (2010) reported xylanase from *Bacilus* that also show the optimum acivty at 50˚C (Yin *et al*., 2010). In current study cellulase from CC16 maintained more than 95% of its stability at 40°C for 150 minutes. At 30°C for 150 minutes, cellulase maintained more than 70% of its stability and xylanase from CC4 achieved maximum activity at 50°C for 150 min. At 45°C for 150 minutes, xylanase maintained more than 90% of its stability. At 30°C for 150 minutes, xylanase maintained more than 70% of its stability. At 70°C and 80°C for 150 minutes, xylanase stability was reduced by more than half. Several researchers have reported cellulase and xylanase with variable stability at different temperature ranges (Gaur & Tiwari, 2015; MARCO *et al*., 2017; Yadav *et al*., 2023; Yadav *et al*., 2018). This alteration may be due to the isolation of strain from difference in area of origin.

Cellulase and xylanase from CC16 and CC4 respectively, showed stability at wide range of pH. The cellulase enzyme was stable at pH 7.0 exhibit 100% activity, its stability was maintained 100% for 150 min. At a pH of 6.0, and pH 8.0 more than 80% of the stability was maintained. In recently reported literature similar results were observed and purified cellulase from *Achromobacter spanius* showed maximum stability at pH 7.0 (Mousa *et al*., 2019). In this study xylanase from CC4 was stable at pH 9.0. At pH 9.0 enzyme stability was maintained 100% for 150 min. At a pH of 10.0 more than 90% of the stability was maintained. After being exposed to pH 8.0 and below, xylanase stability declined. Whereas, in another study effect of pH on xylanase activity was investigated within the pH range of 5.5 to 8.5. The optimum pH range for

the activity was determined between 6.5 and 7.0. Specifically, enzyme activity percentages at pH 6.0, 7.0, and 8.5 were 87%, 97%, and 64% relative to that observed at pH 6.5 (Cordeiro *et al*., 2002).

The various agro-industrial wastes contain metal ions that may significantly decrease the activity of enzymes. (Marcolongo *et al*., 2019). In current study, it was observed that $FeSO_4$ significantly improved cellulase activity by 127% across both concentrations. Similarly, the presence of Ca^{2+}, Cu^{2+}, and Mn^{2+} also led to an increase of over 100% in cellulase activity at both 2mM and 10mM concentrations but of Pb^{2+} resulted in a reduction of cellulase activity by 25% at 2mM concentration and 32% at 10mM concentration. In particular, the enzyme was resistant to the heavy metals Ca^{2+}, Mn^{2+}, and Cu^{2+}. The reaction mechanism and structure of the enzymes recovered from strain CC16 could be one factor (Liu & Smith, 2021). Gaur *et al*., (2015) conducted a study in which he reported similar results showing Ca^{2+}, Mg^{2+}, and Cu^{2+} ions strongly elevated cellulase activity of cellulase (Gaur & Tiwari, 2015) Furthermore, xylanase activity from CC4 was generally positively influenced by most metals, with higher metal concentrations leading to increased activity. Cu^{2+}, exhibited a significant enhancement of 119% at 2mM concentration and 152% at 10mM concentration. Other metals like Ca^{2+}, Ni^{2+}, Na^{2+}, Mg^{2+}, and Mn^{2+} also contributed to elevated xylanase activity. However, $ZnSO_4$ and $Pb(NO_3)_2$ led to a substantial reduction in xylanase activity. Pb^{2+} decrease the xylanase activity by 44% at 2mM concentration and 49% at 10mM concentration. In a current study on xylanase from bacterial isolates, Ca^{2+}, Zn^{2+}, and Mg^{2+} demonstrated limited enhancing effects, while Cu^{2+} significantly enhanced xylanase activity. However, the enzyme activity experienced partial inhibition due to Co^{2+}, Ba^{2+}, Fe^{2+}, Mn^{2+}, and Fe^{2+}. Furthermore, Pb^{2+} exhibited strong inhibition, potentially through its interaction with sulfhydryl groups. This suggests the presence of a significant cysteine residue either within or proximate to the enzyme's active site (Luo *et al*., 2016).

In this study different surfactants at 1.0% and 10.0% concentration were used in order to determine their effect on the activity of purified enzyme. In general, SDS inhibited 88% of the enzyme activity at high concentration (10.0%) while 73% at low concentration (1.0%). Comparatively, Tween 20, Tween 80, CTAB and Triton X 100

potentially enhanced cellulase activity at both concentrations (1.0% and 10.0%). The cellulase enzyme exhibited activation in the presence of both anionic and non-ionic surfactants. Cellulase activity reported by other researchers showed an increase with SDS (129.73%), Triton X-100 (113.51%), and Tween-80 (105.41%) concentrations. A similar enhancement in cellulase activity through these surfactants has been noted in prior studies by Seki *et al*. (2015) and Asha *et al*. (2012). This phenomenon might be attributed to the surfactants capacity to alter surface properties, thereby potentially reducing the irreversible deactivation of cellulase. Consequently, these surfactants hold the potential to effectively stimulate cellulase activity within the cellular context (Potprommanee *et al*., 2017) (Asha *et al*., 2012; Seki *et al*., 2015).

The xylanase activity was inhibited by SDS by 83% at high concentration (10.0%) while 68% at low concentration (1.0%). Comparatively, Tween 20, CTAB and Triton X 100 potentially enhanced cellulase activity at both concentrations (1.0% and 10.0%). Tween 80 enhanced xylanase activity by 71% of the enzyme activity at high concentration (10.0%). Comparatively, according to recently published literature, the xylanase enzyme maintained 72% of its activity in the presence of SDS, but it completely inhibited itself when exposed to numerous detergents like Tween-20, Triton 100, and Tween-80. The amylases obtained from the *Bacillus methylotrophicus* strain and *Anoxybacillus sp. IB-A 37* strain, in comparison, were much more stable. They retained 110% and 98% of their activity in the presence of SDS, respectively, and exhibited considerable activity even in the presence of the other detergents (Shukla & Singh, 2015).

To assess the impact on purified enzyme activity over a 120-minute duration, a variety of organic solvents at a final concentration of 10% were employed. Generally, Acetonitrile, Ethyl acetate, N-Hexane, Butanol, and DMSO exhibited an enhancing effect on cellulase activity throughout the 120-minute period. Cellulase enzyme from CC16 activity remained at 100% in the presence of methanol over the same time frame. Glycerol led to a 10% enhancement in cellulase activity for 120 minutes. However, both Acetonitrile and propanol resulted in a decline in enzyme activity as time progressed. Similarly, xylanase from CC4, enzyme activity experienced improvement in the presence of ethanol, methanol, acetonitrile, and acetone over the 120-minute duration.

Glycerol contributed to a notable 26% increase in xylanase activity within the same time frame. On the contrary, Ethyl acetate, N-Hexane, propanol, butanol, and DMSO caused a decrease in xylanase activity over a 150-minute period. Similar results have been observed by other researchers, one of study revealed that how certain organic solvents. The results indicate that xylanase from strain C9 is suitable for various applications such as in production of the alcoholic beverages and bioethanol production. In a study by Li & Yu (2012), it was discovered that after being incubated with acetone, N,N-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO), more than 90% of the cellulase activity was still present. DMF and acetone, interestingly, even increased the enzyme activity to 118.4 and 127.3%, respectively (Li & Yu, 2012). In another study, the impact of different organic solvents (at a concentration of 30%, v/v) on cellulase activity and stability was investigate. Optimal test conditions were used to determine the enzyme's activity. The cellulase activity increased after being exposed to n-dodecane, n-decane, hexane, iso-octane, methanol, toluene, and n-butanol, achieving 141% (48 h), 132% (48 h), 111% (48 h), 129% (24 h), 117% (48 h), 113% (24 h), and 119% (48 h),. However, the cellulase activity was slightly decreased when exposed to benzene, xylene, propanol, and ethanol, leaving behind residual activities of 81%, 85%, 79%, and 82%, respectively (Irfan *et al*., 2017). Recently reported literature on xylanase show similar results which revealed that the enzyme was stable in the presence of different organic solvents such as Ethyl acetate, N-Hexane, propanol, butanol, and DMSO except formaldehyde and glycerol that inhibited enzyme activity (Ullah *et al*., 2019). The stability and increase in activity is because in presence of organic solvents can impact enzyme stability and activity by interacting with the water molecules present around the enzyme. Enzymes typically function effectively in water-based environments, where water molecules contribute significantly to maintaining the enzyme's structure and function. The introduction of organic solvents can disrupt the arrangement of water molecules surrounding the enzyme. Interestingly, there are situations where organic solvents can be harmful. They can create a protective layer around the enzyme's active site, shielding it from harmful effects that could cause the enzyme to lose its function and decrease in activity occurs. This protective effect prevents unfavorable interactions that might lead to the deactivation of the enzyme.

Moreover, certain organic solvents can increase the enzyme's adaptability, potentially improving its ability to bind to substrates and carry out catalytic reactions.

Cellulase from bacterial strain CC16 and purified xylanase from bacterial strain CC4, Lineweaver and Burk (1934) plot was used to calculate the Vmax and Km constant. Cellulase from strain C116 had high specific activity (345.8) against CMC. The Km of strain CC16 cellulase (for CMC) was 1.72 mg/ml. The Vmax of CC16 cellulase was 129.8 µmolmg -1min-1 for CMC. CC16 has higher specific activity, Km and Vmax value that *B. amyloliquefaciens AK9* having specific activity of 44.12 (U/ml), reported in literature (Irfan *et al*., 2017). In a different investigation, cellulase was shown to have specific activity of 62.9 units (U) mg1 with Vmax and Km values of 37.87 mol min1 mg1 and 3.02 mg ml^{-1}, respectively. These values are lower than CC16 (Bano *et al*., 2019). Similarly, the Km value for strain CC4 xylanase (for beechwood xylan) was 3.7 mg/ml. Respectively, the Vmax of CC4 xylanase was 1428.5 $\mu molmg^{-1}min^{-1}$ for beechwood xylan. CC4 has a very high Km and Vmax as compared to xylanase produced by *Anoxybacillus kamchatkensis* which have Km 0.7 mg/ml and Vmax 66.64 $\mu molmg^{-1}min^{-1}$ (Yadav *et al*., 2018). Chang *et al*., (2017) reported xylanase from *Bacillus subtilis CCMI 996* he Km values for xylanase from *Bacillus subtilis CCMI 996* Km value of 4.5 mg/ml higher than CC4 toward beech-wood xylan but Vmax (333.0 µmol/mg min^{-1}) was lower that that of CC4 (Chang *et al*., 2017).

In this study the NaOH-catalyzed methanol pretreated bagasse was first subjected to xylanases which resulted in 9.8 g/L of the TRS and then treated with the cellulases that resulted in a hydrolysate with 33.2 g/L of the TRS. The TRS yield calculated was 0.27 g/g for the hydrolysate obtained from xylanase treatment of pretreated bagasse while it was 0.91 g/g. The lower TRS yield from xylanase treatment may suggest a more specific breakdown of hemicellulose, resulting in a selective release of sugars. In another study the TRS yield for cellulase and xylanase from hydrolysis of sugarcane bagasse was recorded 32.82 and 13.6 g/L respectively which are almost similar to our findings (Ascencio *et al.,* 2020). But in this case xylanases yielded a substantially higher TRS content, indicating a more extensive breakdown of hemi cellulose which could be due to factors like efficiency of enzymatic action, and substrate accessibility.

In current study FTIR analysis of untreated, pretreated and saccharified sugarcane bagasse revealed that lignin was efficiently removed by pretreatment with an alkali organo-solvent, as the pretreated substrate did not exhibit the lignin aromatic ring absorption bands located at 1239, 1370, 1422, 1514, and 1603 cm^{-1}. The enzymatic hydrolysis of hemicellulose and cellulose in the saccharified sugarcane bagasse was indicated by changes in the area between 1030 and 1583 cm^{-1}, which confirms the hydrolysis of sugarcane bagasse by cellulases and xylanases. The Peak at 832 cm^{-1} confirms the C-H deformations of the aromatic ring of lignin, which is absent in spectra of pretreated and saccharified sugarcane bagasse thus confirming the removal of lignin. In a previous study similar results were observed in which Primary and secondary hydroxyl (OH) groups were observed at 1051 cm^{-1} and 1163 cm^{-1}, respectively. The absorption peak at 2912 cm^{-1} is associated with the aliphatic axial deformation of C-H bonds in the CH2 and CH3 groups found in cellulose, lignin, and hemicellulose (Moutta *et al.,* 2013). The peaks observed at 1604 cm−1 and 1735 cm−1 are designated as acetyl groups within the hemicellulose structure in sugarcane bagasse. Additionally, the peak at 875 cm−1 is associated with the β-1-4-glycosidic bonds connecting monosaccharide units in saccharified sugarcane bagasse as reported in the literature (Laluce *et al.,* 2019).

The cellulase from CC16 and xylanase from CC4 exhibited stability towards high temperature, broad pH range, as well as showing resistance to organic solvent, metals and detergent. They also possess high total reducing sugars yield. Thus, enzyme from these strains could be excellent candidate for various biotechnological applications such as bio-bleaching of paper pulp, clarification and reduction of turbidity in juices, deinking of paper, animals feed, textile industry, and bioethanol production.

Conclusion

In this study, CC16 and CC4 bacterial strains isolated from fruits and vegetable compost collected from NARC Islamabad were screened for cellulase and xylanase enzyme production. Qualitative and quantitative assays confirmed that CC16 was cellulase producing bacteria and CC4 was able to produce xylanase. The strain CC16 (cellulase producing) and CC4 (xylanase producing) ability to produce maximum units of enzyme was achieved by optimizing their temperature, pH, and incubation time. The bulk production of cellulase and xylanase was achieved under optimal conditions, followed by partial purification of cellulase from CC16 using 70% ammonium sulphate saturation. Similarly, xylanase from CC4 was partially purified using 60% ammonium sulphate saturation. The purified cellulase enzyme from column chromatography yielded specific activity of 114.2 U/mg, 2.03-fold purification, and a 55.6% yield and purified xylanase showed specific activity of 232.0 U/mg, 1.74-fold purification, and a 55.3% yield. Characterization studies revealed that enzyme cellulase and xylanase were active in the presence of different metal ions like Cu^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+} whereas Pb^{2+} strongly inhibited its activity of both strains purified cellulase and xylanase. Surfactants such as Tween 20, Tween 80, CTAB, and Triton X 100 potentially enhanced cellulase and xylanase activity while SDS significantly decreased their activity at both 1% and 10% concentrations. The enzyme cellulase from CC16 remained stable in the presence of 10% organic solvents such as Methanol, Acetone, Ethyl acetate, N-Hexane DMSO, and glycerol while xylanase from CC4 was found to be stable in the presence of Methanol, Acetonitrile, Acetone, DMSO and glycerol for 120 minutes. The kinetics of cellulase and xylanase resulted in Km and Vmax values of 1.72 mg/ml and 129.8 mol mg^{-1} min^{-1} for cellulase, and 3.7 mg/ml and 1428.5 mol mg^{-1} min^{-1} for xylanase. Sugarcane bagasse was pretreated with alkali-organosolvent (NaOH-methanol). NaOH-catalyzed methanol pretreated bagasse was then subjected to xylanase which resulted in 9.8 g/L of the TRS while the post-cellulase hydrolysis resulted in a hydrolysate with 33.2 g/L of the TRS. The TRS yield calculated was 0.27 g/g for the hydrolysate obtained from xylanase treatment of pretreated bagasse while it was 0.91 g/g for the hydrolysate obtained from cellulase treatment This study provides insight into the enzymatic abilities of CC16 and CC4 bacteria, revealing their suitability for a wide range of different applications.

Future prospects

- Enzyme Immobilization of cellulase and xylanase on various supports to improve their stability and reusability during multiple hydrolysis cycles.
- It is also important to study the synergistic effects of combining cellulase and xylanase enzymes with other enzymes or additives, aiming to boost hydrolysis efficiency and reduce enzyme loading.
- Exploring the potential for utilizing the waste/byproducts generated during pretreatment and enzymatic hydrolysis for other value-added products, like bio-based chemicals or materials.
- Experiments could be conducted to validate the feasibility at larger scales, considering factors like reactor design, heat and mass transfer, and economics.

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