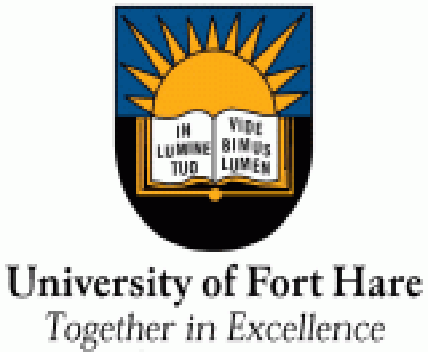
ISOLATION AND CHARACTERISATION OF LIGNOCELLULOSE DEGRADING BACTERIA FROM TYUME RIVER IN THE EASTERN CAPE PROVINCE, SOUTH AFRICA



Dissertation submitted in fulfilment of the requirements for award of Master of Science Degree in Microbiology

BY

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Dedication

I dedicate this dissertation to Abba Yahweh, my Almighty God and eternal Father who has been my source of everything, and for His word in Jeremiah 29:11. I also dedicate this dissertation to my parents (Mr & Mrs Papiyana), my siblings and aunts. Your support and prayers are priceless.

Acknowledgement

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Declaration

I, hereby, declare that this dissertation submitted to the University of Fort Hare for the Master's degree of Science in Microbiology in the Faculty of Science and Agriculture, and the work contained in this document is my original work with exemption to the citations and that this work has not been submitted at any other university in partial or entirely for the award of any other degree.

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ABSTRACT

This study focuses on the isolation and characterization of bacteria from lignocellulosic biomass obtained from the sediments of the Tyume River in Alice, Eastern Cape and to determine those bacterial isolates with good potential for modification and decomposition of lignocellulosic biomass for industrial application. Several bacterial isolates were recovered and screened for ability to degrade various lignocellulosic materials. Nine of the isolates were positive for lignocellulolytic activity. Four isolates were cellulase positive and six were xylanase positive. Moreover, one isolate (SB1) was positive for both xylanase and cellulase activities and showed the best hydrolysis zone on solid media. This isolate was then chosen as the best and identified molecularly. The 16S rDNA sequence analysis indicated that SB1 was a *Bacillus cereus* species. Factors affecting the cellulose and xylanase enzyme production by the organisms were studied. The organisms produced the enzymes maximally at earlier hours of incubation (12-30 hr) and optimally at acidic pH (3-5) and at moderate temperatures (35-45ºC). SB1 appears to hold promise in the decomposition of lignocellulosic wastes.

CHAPTER ONE

1.1 General Introduction

Lignocellulose is mainly an ubiquitous organic matter found in the biosphere and it characterizes many aerobic, facultative anaerobic and obligate anaerobic bacteria and fungi (Woo *et al.*, 2014). There are three types of polymers constituting lignocelluloses biomass, i.e. cellulose (35-50%), hemicellulose (25-30%) and lignin (25-30%). Cellulose and hemicelluloses are degradable into sugars, which are versatile materials for further conversion by fermentation, biocatalytic processes to value-added products, including biofuels, biopolymers and chemicals (Wongwilaiwalin *et al.*, 2010). Cellulose is a key structural component of plant cell walls, which is responsible for mechanical strength (Anwar *et al.*, 2014). This polymer has many properties which depend on its degree of polymerization (DP), defined as the number of monomeric glucose units that make up one polymer molecule (Malherb *et al.*, 2002).

In microbial ecology, cellulose, the most abundant naturally occurring biopolymer, is a vital component of the biospheric carbon cycle and its bioconversion to fuel and chemicals is of great interest (Lennox *et al.*, 2010). Cellulose is totally insoluble in water and has about 2000 – 10 000 glucose subunits with molecular weight determination value that ranges from 200 000 to about 2.4 million (Lennox *et al.*, 2010). Hemicellulose is the heteropolysaccharide made up of energy rich polymers of pentoses and hexoses (Zhang *et al.*, 2012). The different hexoses, pentose, and glucoronic acid of glucose, galactose and 4-O-methylglucose bound by (1-3)-,(1-6)-and (1-40)-glycosidic bonds make up the heteropolysaccharide called hemicelluloses (Hatakka *et al*., 2010). Unlike cellulose, hemicellulose is more soluble and is frequently branched with the degree of polymerization of 100 to 200. According to literature

hemicellulose is linked to lignin through cinnamate acid ester linkages, and to cellulose through inter chain hydrogen bonding, and to other hemicelluloses via covalent and hydrogen bonds (Hatakka *et al.*,2010).

Lignin is a natural polymer of aromatic compounds produced through biosynthetic processes and forms a protective seal around cellulose and hemicelluloses with hydrophobicity and highly irregular structure (Calvo-Flores & Dobado, 2010; Jiang, Nowakowski & Bridgwater, 2010; Menon & Rao, 2012; Anwar *et al.*, 2014). As a biopolymer, lignin is unusual due to its heterogeneity and lack of a defined primary structure (Zhang *et al.*, 2012). It consists of multiple cross-linked phenylpro-panoids, believed to be derived from three methoxylatedmonolignol monomers: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Mosier *et al.*, 2005).

Lignocellulose waste is accumulated in abundance annually, resulting to environmental problems. Nevertheless, they could be utilized for the production of some value added products, such as ethanol, food additives, organic acids, enzymes, and others, since its chemical composition is based on sugars and other compounds of interest (Bajaj & Manhas, 2012). Thus, besides the environmental problems caused by their accumulation in nature, the non-use of these materials constitutes a loss of potentially valuable resources (Mussatto & Teixeira, 2010).

1.2 STATEMENT OF THE PROBLEM

Resources are scarce, hence, the abundance of useful organic compounds trapped in lignocellulosic materials found ubiquitously in the biosphere makes it imperative to find efficient ways of recycling (Dixon & Linger, 2006). Several microbial species have been isolated from composts and these microbes have shown effectiveness in the degradation of lignocellulose as well as the conversion of intermediates into useful products like bio-ethanol,

animal feed, and methane gas (Tuomela *et al.*, 2000). Evidence is very scanty implicating freshwater environments as reservoirs of lignocellulose degrading microbes, yet these habitats have been well documented to habour microorganisms of diverse metabolic properties. Exploration for cost effective and efficient lignocellulose-degrading microbes with "high product yield" and "novel enzymes" capabilities has therefore become imperative.

1.3 HYPOTHESIS

The study hypothesized that Tyume River, a freshwater habitat in the Eastern Cape Province, is a potential reservoir of important lignocellulosic degrading bacteria.

1.4 AIM AND OBJECTIVES

The aim of the study was to isolate and characterize lignocellulose degrading bacteria from Tyume River.

1.5 SPECIFIC OBJECTIVES

1. To collect sediment samples from different depths and points locations of the Tyume River
2. To isolate and characterise cellulolytic and xylanolytic bacteria from the sediment samples
3. To select and identify one important cellulolytic and xalanolytic bacteria amongst the isolates and assess its ability to produce cellulases and xylanases.
4. To determine the optimum culture conditions for the production of these enzymes.

References

Anwar, Z., Gulfraz, M., & Irshad, M., 2014. Agro-industrial lignocellulosic biomass a key to unlock the future bioenergy: a brief review. *Journal of Radiation Research and Applied Sciences*, *7*(2), 163-173.

Bajaj, B. K., & Manhas, K., 2012. Production and characterization of xylanase from *Bacillus licheniformis* P11 (C) with potential for fruit juice and bakery industry. *Biocatalysis and Agricultural Biotechnology*, *1*(4), 330-337.

Calvo-Flores, F. G., & Dobado, J. A., **2010**. Lignin as renewable raw material.*ChemSusChem*, *3*(11), 1227-1235.

Dixon, N., & Langer, U., 2006. Development of a MSW classification system for the evaluation of mechanical properties.*Waste management*, *26*(3), 220-232.

Hatakka, A., &Hammel, K. E., **2011**.Fungal biodegradation of lignocelluloses.In *Industrial Applications.* pp. 319-340. Springer Berlin Heidelberg.

Jiang, G., Nowakowski, D. J., &Bridgwater, A. V., 2010. A systematic study of the kinetics of lignin pyrolysis.*ThermochimicaActa*, *498*(1), 61-66.

Lennox, J. A., Abriba, C., Alabi, B. N., &Akubuenyi, F. C., 2010. Comparative degradation of sawdust by microorganisms isolated from it. *Afr. J. Microbiol. Res*, *4*(13), 1352-1355.

Malherbe, S. & Cloete, T.E., 2002. Lignocellulose biodegradation: Fundamentals & applications. *Reviews in Environmental Science and Biotechnology*, *1*(2), 105-114.

McDonald, J. E., Rooks, D. J., & McCarthy, A. J., 2012. Methods for the isolation of cellulose-degrading microorganisms. *Methods Enzymol*, *510*, 349-374.

Menon, V., & Rao, M.., 2012. Trends in bioconversion of lignocellulose: biofuels, platform chemicals &biorefinery concept. *Progress in Energy and Combustion Science*, *38*(4), 522-550.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., &Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass.*Bioresource technology*, *96*(6), 673-686.

Mussatto, S. I., & Teixeira, J. A., 2010. Lignocellulose as raw material in fermentation processes. *Biotechnology advances*, *28*(6), 817-830.

Tuomela, M.,Vikman, M.,Hatakka A., Itavaara M.,2000. Biodegradation of lignin in a compost environment: a review. *Bioresource Technology 72*,169-183.

Wongwilaiwalin, S., Rattanachomsri, U., Laothanachareon, T., Eurwilaichitr, L., Igarashi, Y., & Champreda, V., 2010. Analysis of a thermophilic lignocellulose degrading microbialconsortium and multi-species lignocellulolytic enzyme system.*Enzyme and Microbial Technology*, *47*(6), 283-290.

Woo, H. L., Hazen, T. C., Simmons, B. A., & DeAngelis, K. M., 2014. Enzyme activities of aerobic lignocellulolytic bacteria isolated from wet tropical forest soils. *Systematic and Applied Microbiology*, *37*(1), 60-67.

Zhang, Z., Donaldson, A. A., & Ma, X., 2012. Advancements and future directions in enzyme technology for biomass conversion.*Biotechnology advances*, *30*(4), 913-919.

CHAPTER TWO

LITERATURE REVIEW

Human activity generates tons of wastes annually and the most abundant is of biological character, namely biomass. Biomass refers to all renewable organic matter, including all plantations, and associated residues, as well as animal, agricultural, industrial and municipal solid wastes. The biomass has a great potential that enables it to be used to meet the energy and nutritional demands of humans, used in the production of chemical feed stocks such as organic acids, solvents and enzymes for different industries (Claassen *et al.,* 1999; Laufenberg *et al.*, 2003; Wilke &Vorlop, 2004). Unfortunately, much of the lignocellulosic biomass is frequently disposed of by burning. Nevertheless, lignocellulosic biomasses have gained so much research interests and special importance due to their renewable nature (Anwar *et al.*, 2014).

Enormous amounts of agro industrial residues generated yearly from different economic activities signifies one of the most energy rich resources available on earth, and when not properly used, may result to an increase in environmental pollution (Heck *et al.*, 2005; Bajaj & Manhas 2012).

Recently, there has been an awareness and importance of preserving the natural resources such as lignocelluloses for the development of modern practical technologies and commercial scale manufacturing processes (Schneider, 1989; Maki *et al.,* 2012). As a result, the enormous amounts of lignocellulosic biomass can potentially be converted into diverse high value products including value added fine chemicals, and cheap energy sources for microbial fermentation and enzyme production (Isroi *et al.,* 2011; Iqbal *et al.*, 2013; Irshad *et al.*, 2013).

2.1 Historical background of lignocelluloses

Lignocellulose the main structural constituent of all plants and a renewable organic matter is made up of three major constituents: cellulose, hemicellulose and lignin **(**Sanchez & Cardona., 2008). Lignocellulosic compounds are the most abundant agricultural residues in the world and are frequently being replenished by photosynthesis. In nature, lignocellulose accounts for the major part of biomass and, thus its degradation is essential for the carbon cycle (Tuomela *et al.*, 2000).

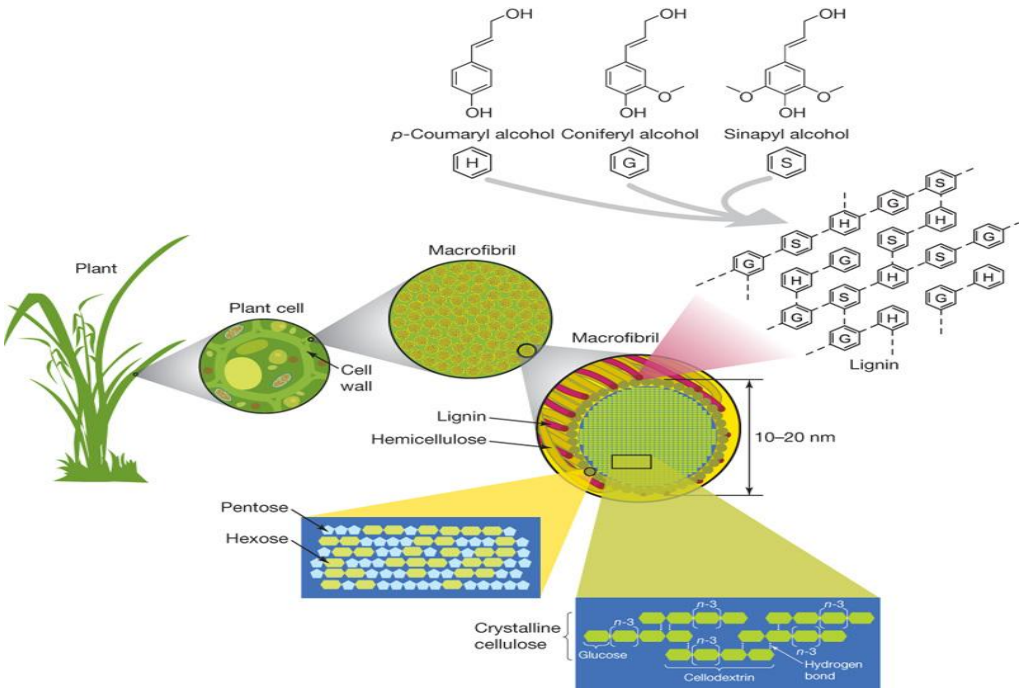


Figure 2. 1: Structure of lignocelluloses (Source: Rubin, 2008).

2.1.1. Internal structure and physical properties

Lignocellulosic biomass has a complex internal structure. It is made up of major components that also have complex structures. The description of each of the components are outlined in the following sections.

Cellulose

Cellulose is the β-1,4-polyacetal of cellobiose (4-O-β-D-glucopyranosyl-D-glucose). It is a structural component of the primary cell wall of green plants and a linear polymer of glucose derived from D-glucose units (Yakubu *et al.,* 2011). The chemical formula of cellulose is $(C_6H_{10}O_5)n$ and **Figure 2.2** shows the structure of the polymer. It condenses through β (1- 4)-glycosidic bonds, usually set in microcrystalline structures, making its hydrolysis very difficult under natural conditions (Ghosal *et al.,* 2011). Cellulose accounts for about 50% of all the carbon utilized by plant biomass during photosynthesis yearly (Song *et al.,* 2013). Hence, cellulose degraders contribute to the cycling of about 90% of the overall plant production that enters soils and sediments as dead organic matter (Gessner *et al.,* 2010; Song *et al.,* 2013). In nature, cellulose is typically hydrolyzed by microorganisms, mainly fungi and bacteria. Fungi and bacteria produce either free cellulolytic enzymes under aerobic conditions or extracellular enzyme complexes known as cellulosomes under anaerobic conditions to hydrolyze cellulose (Demain *et al.,* 2005; Song *et al.,* 2013).

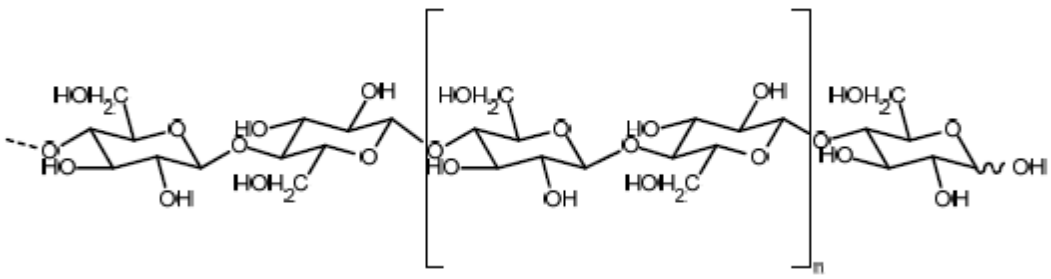


Figure 2. 2: Structure of cellulose (Source: Peterson *et al.,* 2008).

Hemicellulose

Hemicellulose is a heteropolysaccharide made up of different hexoses, pentoses, and glucoronic acid. The most regular types of polymers that belong to the hemicelluloses family of polysaccharides is xylan. Xylan (Figure 2.3A) is made of D-xylopyranosyl linked by β-1, 4-glycosidic bonds (Bajaj & Manhas, 2012). The xylan backbone can be customized with 4-O-methyl-D-glucuronic acid, acetic acid, uronic acids, L-arabinofuranose, and phenolic compounds (p-coumaric and ferulic acids) bound to xylose subunits. β-mannans are another type of hemicellulose can be comprised of repeating β-1,4-linked mannose or alternating mannose and glucose residues; galactomannan (Figure 2.3B) contains α-1,6-linked galactose side chains attached to the mannose backbone (Shallom & Shoham, 2003). Important aspects of the structure and composition of hemicelluloses are the lack of crystalline structure, mainly due to the highly branched structure and the presence of acetyl groups connected to the polymer chain (Kirk-Otmer, 2001).

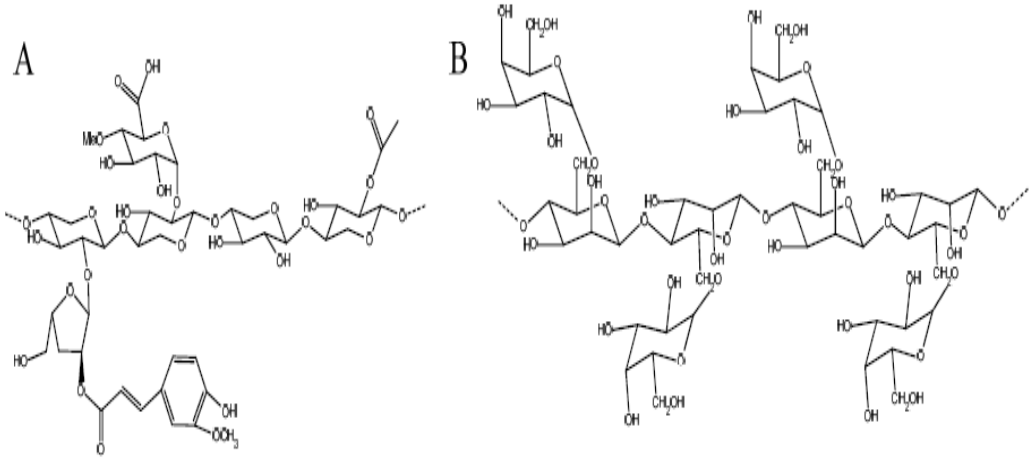


Figure 2. 3: Composite structures of hemicellulose: A) xylan and B) galactomannan (Source: Peterson *et al.*, 2008).

Hemicellulose extracted from plants has various characteristic sizes, shapes and masses (polydispersity, polydiversity and polymolecularity). Nevertheless, the degree of

polymerization does not go beyond the 200 units although the minimum limit can be approximately 150 monomers (Harmsen *et al.*, 2010). Hemicellulose is insoluble in water at low temperature. However, its hydrolysis starts at a temperature lower than that of cellulose, which renders it soluble at elevated temperatures (Harmsen *et al.*, 2010).

Lignin

Among all three lignocellulosic constituents, lignin is the most structurally complex noncarbohydrate, least abundant biopolymer that is covalently bound to hemicellulose within plant cell walls (Rahman *et al.*, 2013). It is the most recalcitrant of the biopolymers due to its complex structure related to heterogeneity, aromaticity, and carbon-carbon cross linking (Perez *et al.*, 2002). Lignin is composed of coniferyl alcohol, sinapyl alcohol, and p-coumaryl alcohol as its monomers and they form β- aryl ether, pheylcoumaran, resinol, biphenyl, and biphenyl ether motifs through crosslinking within the structure (Chang, 2007; Abd-Elsalam & El-Hanafy, 2009). Dividing higher plants into two categories, hardwood (angiosperm) and softwood (gymnosperm), it has been identified that lignin from softwood is made up of more than 90% of coniferyl alcohol with the remaining being mainly p-coumaryl alcohol units. Contrary to softwoods, lignin contained in hardwood is made up of varying ratios of coniferyl and sinapyl alcohol type of units (Kirk-Otmer, 2001).

The property of polydispersity, just as with hemicellulose, characterizes lignin as well. Different branching and bonding in other similar molecules are encountered (Lin & Lin, 2002). Lignin in wood behaves as an insoluble three-dimensional network. It plays an important role in the cell's endurance and development, as it affects the transportation of water, nutrients and metabolites in the plant cell (Brodeur *et al.*, 2011). Due to its high molecular weight and the presence of various biologically stable carbon-to- carbon and ether

linkages, it acts as a binder between cells creating a composite material that has a remarkable resistance to impact, compression and bending (Brodeur *et al.,* 2011).

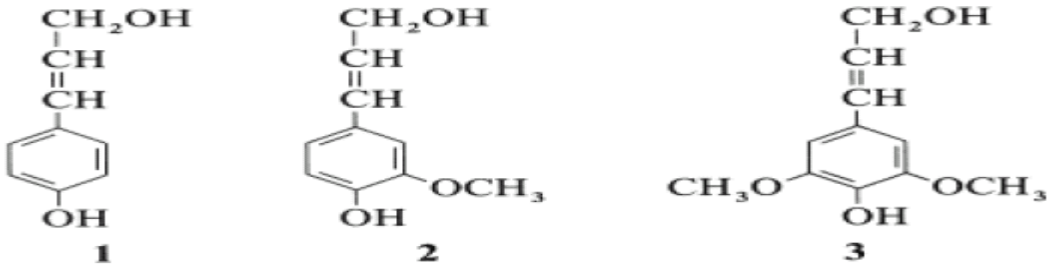


Figure 2. 4: P-coumaryl- , coniferyl- and sinapyl alcohol: dominant building blocks of the three dimensional polymer lignin (Source: Peterson *et al.,* 2008).

Low molecular solvents have been identified to significantly dissolve lignin i.e. dioxane, acetone, pyridine, and dimethyl sulfoxide. Moreover, it has been observed that at elevated temperatures, thermal softening of lignin takes place, allowing its depolymeristation reactions of acidic or alkaline nature to accelerate (O'Connor *et al.,* 2007).

2.1.2 Chemical interaction between components of lignocellulosic biomass

There are four main types of bonds identified in the lignocellulose complex. These include the ether type of bonds, ester bonds, carbon-to-carbon bonds and hydrogen bonds. These four bonds are the main types of bonds that provide linkages within the individual components of lignocellulose (intrapolymer linkages), and connect the different components to form the complex (interpolymer linkages). The positions and bonding functions of these linkages are summarized in **Table 2.1**.

Table 2. 1: Overview of linkages between the monomer units that form the individual polymers lignin, cellulose and hemicellulose, and between the polymers to form lignocellulose (Source: Huijgen*et al.,* 2010).

Bonds within different components (intrapolymer linkages)	
Ether bond	Lignin, (hemi)cellulose
Carbon to carbon	Lignin
Hydrogen bond	Cellulose
Ester bond	Hemicellulose
Bonds connecting different components (interpolymer linkages)	
Ether bond	Cellulose-Lignin Hemicellulose lignin
Ester bond	Hemicellulose-lignin
Hydrogen bond	Cellulose-hemicellulose Hemicellulose-Lignin Cellulose-Lignin

2.1.3 Intra-polymer linkages

The main types of bonds that connect the building molecules within the lignin polymer are ether bonds and carbon-to-carbon bonds (**Table 2.1**). Ether bonds may appear between allylic and aryl carbon atoms, or between aryl and aryl carbon atoms, or even between two allylic carbon atoms. The total fraction of ether type bonds in the lignin molecule is around 70% of the total bonds between the monomer units. The carbon-to-carbon linkages form the remaining 30% of the total bonds between the units. They can also appear between two aryl

carbon atoms or two allylic carbon atoms, or between one aryl and one allylic carbon atom (Kirk-Otmer, 2001).

The polymer of cellulose is formed on the basis of two main linkages:

1. The glucosidic linkage is the one that forms the initial polymer chain. More specifically, it is a 1-4 β D-glucosidic bond that connects the glucose units together. The glucosidic bond can also be considered as an ether bond, since it is in fact the connection of two carbon atoms with an elementary oxygen interfering.

2. The hydrogen bond is considered to be responsible for the crystalline fibrous structure of cellulose. The arrangement of the polymer in long straight parallel chains together with the fact that the hydroxyl groups are evenly distributed in both sides of the glucose monomer, allows the formation of hydrogen bond between two hydroxyl groups of different polymer chains (Huijgen*et al.*, 2010).

It has been identified that carboxyl groups are also present in cellulose in a fraction of 1 carboxyl per 100 or 1000 monomer units of glucose (Krassig & Schurz, 2002), although this does not appear obvious from the main structure of cellulose. As already mentioned, hemicellulose consists of polysaccharides other than cellulose. Its structure reveals that ether type of bonds, such as the fructosic and glucosidic bonds, is the main one that forms its molecule. The main difference with cellulose is that the hydrogen bonds are absent and that there is significant amount of carboxyl groups. The carboxyl groups can be present as carboxyl or as esters or even as salts in the molecule (Kirk-Otmer, 2001).

2.1.4 Inter-polymer linkages

In order to determine the linkages that connect the different polymers of the lignocellulose complex, lignocellulose is broken down and the individual components are separated.

However, their separation is commonly achieved by methods that result in alteration of their original structure. As a consequence, the conclusions on the connecting linkages between the polymers are not definite. It has been identified that there are hydrogen bonds connecting lignin with cellulose and with hemicellulose, respectively. Furthermore, the existence of covalent bonds between lignin and polysaccharides is identified. More specifically, it is certain that hemicellulose connects to lignin through ester bonds. It is also known that there are ether bonds between lignin and the polysaccharides. It is still not clear though whether the ether bonds are formed between lignin and cellulose, or hemicellulose. Hydrogen bonding between hemicellulose and cellulose is also identified. However, this linkage is not expected to be strong due to the fact that hemicellulose lacks a primary alcohol functional group that is external to the pyranoside ring (Faulon *et al.,* 1994).

2.2 Lignocellulose biodegradation

Lignocellulose is a complex substrate and its biodegradation is not dependent on environmental conditions alone, but also the degradation capacity of the microbial population. The enzymes responsible for the lignocellulosic biodegradation are: Cellulase, hemicellulase, and ligninase.

Cellulases

These are freely secreted enzymes or enzyme complexes that are attached to the surface of microorganisms and can be used for cellulose degradation (de Souza, 2013). Anaerobic organisms usually have cellulosomes while aerobic bacteria and fungi usually employ free enzymes. Cellulose degradation is achieved through the action of three types of enzymes, endo-glucanases, cellobiohydrolases (or exo-glucanases) and β-glucosidases (Figure 2.5). Endo- and exo-glucanases cut inside or at the end of the glucan chain, respectively, and are classified according to both their structural fold and catalytic mechanism (de Souza, 2013).

Cellulose hydrolysis by glucanases is catalyzed by two carboxyl groups in the active site and can either invert or retain configuration of the anomeric carbon.

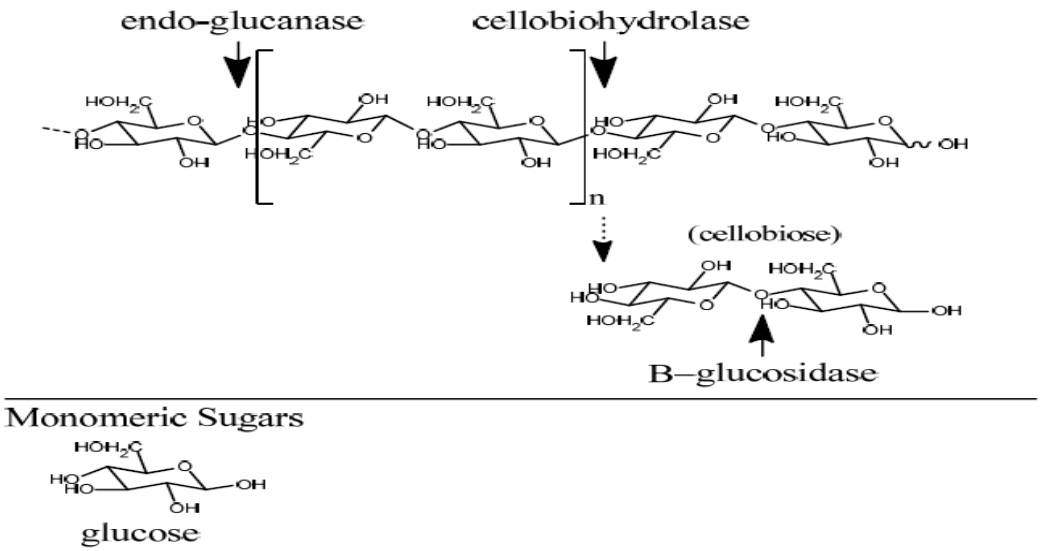


Figure 2. 5: Cellulases. Sites of enzymatic cleavage are indicated by arrows. Resultant monomeric sugars are listed below the structure (Source: Henriksen, 2008).

Enzymes that retain chirality use a double-displacement mechanism with a covalent enzyme-substrate intermediate while enzymes that invert chirality function by a single-step concerted mechanism (Henriksen, 2008). β-glucosidases are essential for overall cellulose degradation and cleave cellobiose to monomeric glucose. Glucanases activity can be inhibited by accumulation of cellobiose and glucose (Henriksen, 2008).

Hemicellulases

Hemicellulases are either glycoside hydrolases or carbohydrate esterases and are classified into families based on their activity and homology of primary sequence. The hydrolysis of glycosidic bonds is carried out by glycoside hydrolases enzymes, whereas carbohydrate esterases enzymes cleave the ester linked acetate and ferulic acids side chains. As the structure of hemicellulose is known to be heterogeneous, a wide range of enzymes is essential for its hydrolysis (Shallom & Shoham, 2003). Furthermore, many hemicellulases have

carbohydrate-binding modules in addition to catalytic domains; as much of the hemicellulose structure can be insoluble, the carbohydrate-binding modules are vital for targeting of the enzymes to the polymers (Henriksen, 2008).

Xylan is one major type of hemicellulose (Figure 2.6). The β-1,4glycosidic bonds of the xylose backbone found in the xylan are cleaved by xylanases. On the other hand, xylosidases hydrolyze resultant oligomers to monomeric xylose. The ester bonds of ferulic acid and acetate side chains are cleaved by ferulic acid esterases and acetyl-xylanesterases respectively. Arabinofuranosidases hydrolyze arabinofuranosyl side chains from the xylose backbone and can have an altering specificity as to the location of the arabinofuranosyl group. Ultimately, glucuronic acid side chainare cleaved by glucuronidases (Shallom & Shoham, 2003).

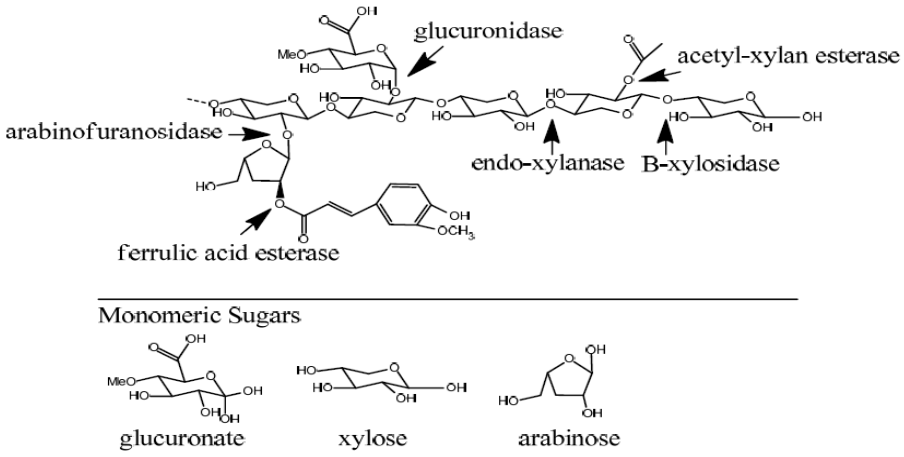


Figure 2. 6: Xylanases. Sites of enzymatic cleavage are indicated by arrows. Resultant monomeric sugars are listed below the structure (Source: Henriksen, 2008).

2.3 Lignocellulose biomass recalcitrance

Lignocellulose is the primary building block of plant cell walls. The complex hierarchy structure of lignocellulosic biomass is the main obstacle for key components fractionation, where cellulose, hemicellulose, and lignin are hindered by many physicochemical, structural,

and compositional factors. Generally, cellulose fibrils are coated with hemicellulose to form an open network, whose empty spaces are gradually filled up with lignin (Cosgrove, 2005).

In order to utilise the cellulose component of lignocelluloses, they must first be separated from lignin, which binds cellulose sheets in an enzymatically inaccessible matrix. Aside from lignin removal being a barrier, cellulose itself is a relatively intractable polymer with strong inter glucose hydrogen bonding making it resistant to enzymatic degradation, as well as hydrophobic sheet formations imparting resistance to acid hydrolysis via the presence of a dense water layer near the hydrated cellulose surface (Matthews, *et al.,* 2006).

Different types of biomass, such as woody plants, herbaceous plants, grasses, aquatic plants, agricultural crops and residues, municipal solid waste and manures, contain different amounts of cellulose, hemicelluloses, lignin and extractives (Chandra *et al.,* 2007; Agbor *et al.,* 2011). Generally plant biomass contains 40-50% cellulose, 20-40 % hemicelluloses, 20-30% lignin by weight (Mackendry, 2002; Chandra *et al.,* 2007; Agbor *et al.,* 2011). Biomass recalcitrance to bioprocessing is directly related to the inherent properties of the biomass source. Properties such as lignin content, cellulose accessibility to cellulase (CAC), and cellulose crystallinity (CC) determine the overall digestibility of the biomass (Agbor *et al.,* 2011). The complexity of a given biomass type is reflected in the relationship between its structural and carbohydrate components. The factors that contribute to biomass recalcitrance include: Crystallinity and degree of polymerization of cellulose, accessible surface area, protection of cellulose by lignin, cellulose sheathing by hemicelluloses and fibre strength (Mosier *et al.,* 2005; Agbor *et al.,* 2011). It is this variability that accounts for differences in the digestibility or hydrolysis of a given biomass feedstock. Removal of lignin enhances the biomass digestibility up to the point where the effect of lignin present is no longer sufficient

to limit enzymatic hydrolysis or microbial digestibility. It has also been shown that highly crystalline cellulose (Chang & Holtizapple, 2000) and the cellulose accessibility to cellulase is one of the most important factors in enzymatic hydrolysis when the effect of lignin is minimized (Jeoh *et al.,* 2007; Agbor *et al.,* 2011).

2.4. Lignocellulose degrading microorganisms

In nature, lignocellulosic biomass is degraded with the cooperation of many microorganisms, mainly including diverse fungal and bacterial genera producing a variety of cellulolytic and hemicellulolytic enzymes under aerobic and anaerobic conditions (Wongwilaiwalin *et al.,* 2010). The composition of wastes is diverse amongst which is the most abundant lignocellulosic matter, responsible for limiting degradation (Dixon & Linger, 2006). Hence, the ability of microorganisms to decompose lignocelluloses is a key factor in recycling of wastes and mainly, in composting. Lignocellulose degrading microorganisms are naturally present in wastes and take part in the composting process (Tuomela *et al.,* 2000).

There has been a known challenge in the isolation and cultivation of individual lignocellulose degrading microbial species from complex environments, but an important improvement has been made in recovering cellulolytic taxa from a range of ecological niches including the human, herbivore, termite gut, terrestrial, and aquatic environment (McDonald *et al*., 2012) .
The lignocellulosic biomass can be degraded by both fungi and bacteria. Although lignocellulolytic fungi such as *Aspergillus, Penicillium, Schizophyllum*, *Trichoderma*, *Phanerochaete* and *Sclerotium* can secrete industrial quantities of extracellular enzymes,bacterial enzyme production can be more cost effective (Woo *et al.,* 2014). This is due to the fact that they grow more rapidly, produce multi enzyme complexes with increased functionality and higher specificity, and can stand larger and more various environmental stress (Maki *et al.,* 2009).

Lignocellulolytic bacteria could also potentially allow better separation of lignin from cellulose and thereby increasing the value of lignin, which is currently a waste product, and cellulose. The few bacterial species currently known to breakdown cellulose and lignin are within *Pseudomonas* (order *Pseudomonadales*), *Cellulomonas* (order *Actinomycetales*), *Streptomyces* (order *Actinomycetales*), and other genera within the order *Actinomycetales* (Lynd *et al.,* 2002). Some of the most important microorganisms that breakdown the lignocellulosic biomass to produce lignocellulolytic enzymes include *Aspergillus, Trichoderma, Streptomyces, Phanerochaetes, Chytridiomycetes, Ruminococcus, Fibrobacteres, Clostridium sp.* and *Bacillus* (Motta *et al.*, 2013).

Microorganisms developed cellular mechanisms to take energy from plant biomass which includes the production and secretion of carbohydrate-active enzymes. These enzymes breakdown the plant cell wall to release sugar monomers such as glucose that can be used as substrates for the metabolism of the microorganism. The microbial use of plant biomass is responsible for large portions of carbon flux in the biosphere (Woo *et al.,* 2014).

2.4.1. Bacillus genus

The *Bacillus* genus is ubiquitously found both above and below the surface of the earth. Six closely related species have so far been described in this taxonomic group: *Bacillus anthracis, Bacillus thuringiensis, Bacillus mycoides, Bacillus pseudomycoides, Bacillus weihenstephanensis* and *B. cereus (sensustricto).* According to some literature these species are not strictly based on genomic variance (Helgason *et al*., 2004; Priest *et al*., 2004), but rather on subjective consideration of practical usefulness such as virulence (*B. anthracis, B. thuringiensis*), physiology (*B. weihenstephanensis*), morphology (*B. mycoides*and *B.*

pseudomycoides) and ill-defined features (*B. cereus sensustricto*) (Pirttijarvi *et al*., 2000). These species vary in practical importance. *Bacillus anthracis* is known to be the causal agent of anthrax. *Bacillus cereus* (*sensustricto*) includes pathogenic strains that may cause food poisoning, eye infections and periodontal disease in humans (Helgason *et al*., 2000). By contrast, certain *B. cereus* strains are used as probiotics. Spore and crystal toxin preparations from *B. thuringiensis*are used as commercial biopesticides. *Bacillus weihenstephanensis, B. mycoides* and *B. pseudomycoides* have not been described as food poisoning agents, but their toxigenic potential remains uncertain (Helgason *et al*., 2000). *Bacillus cereus* is a Gram positive, aerobic to facultative, spore forming rod found ubiquitously in the environment both above and below the Earth's surface. The *Bacillus cereus* bears a close phenotypic and genetic (16S rRNA) relationships to reveal other *Bacillus* species (Bottone, 2010). *Bacillus cereus* may be the most common aerobic spore bearer in any types of soil and in sediments, dust, and plants (Woo *et al.,* 2014). Microbiologically, members of the *B. cereus* group exclusive of *B. anthracis* display a range of morphological forms depending upon the milieu in which they are observed. The studies have shown that it is also frequently present in food production environments due to the adhesive nature of its endospores. This characteristic enables the bacterium to spread to all kinds of food.

2.5 Potential uses of lignocellulosic waste

Biomass is the mass of organic material from any biological material or any large mass of biological matter. A broad diversity of biomass resources is available on our planet for conversion into bioproducts (Table 2.2). These may include whole plants, processing byproducts, materials of marine origin and animal byproducts, municipal and industrial wastes (Howard *et al*., 2003).

Table 2. 2: Types of Lignocellulosic waste materials and their current uses (Source: Howard *et al*., 2003).

Lignocellulosic material	Residues	Competing use
Grain harvesting Wheat, rice, oats barley and corn	Straw, cobs, stalks, husks	Animal feed, burnt as fuel, compost, soil conditioner
Processed grains Corn, wheat, rice, soybean	Waste water, bran	Animal feed
Fruit and vegetable harvesting	Seeds, peels, husks,stones, rejected whole fruit and juice	Animal and fish feed, some seeds for oil extraction
Fruit and vegetable processing	Seeds, peels, husks, shells, stones, rejected whole fruit and juice	Animal and fish feed, some seeds for oil extraction
Sugar cane, other sugar products	Bagasse	Burnt as fuel
Oils and oil seed plants Nuts, cotton seeds, olives, soy bean etc.	Shells, husks, lint, fibre, sludge, presscake, waste water	Animal feed, fertilizer, burnt fuel
Animal waste	Manure, other waste	Soil conditioners
Forestry-paper and pilp Harvesting of logs	Wood residuals, barks, leaves etc.	Soil conditioners, burnt
Saw- and plywood waste	Woodchips, wood shavings, saw dust	Pulp and paper industries, chip and fibre board
Pulp and paper mills	Fiber waste, sulphite liquor	Reused in pulp and board industry as fuel
Lignocelluloses waste from communities	Old newspapers, paper, cardboard, old boards, disused furniture	Small percentage recycles, others burnt
Grass	Unutilised grass	Burnt

2.5.1 Bio-fuel

Ethanol demand has the most important market, where ethanol is either used as a chemical feedstock, petrol additive and as an octane enhancer. Global crude oil production is predicted to decline from 25 billion barrels to approximately 5 billion barrels in 2050 (Balat & Balat, 2009). Ethanol production from sugars or starch has a negative impact on economic processes; hence ethanol is more expensive compared to fossil fuels. Thus technology development focus for ethanol production has shifted towards the use of residual lignocellulosic materials to lower production costs (Balat & Balat, 2009).

2.5.2 Chemicals

Bioconversion of lignocellulosic wastes could make an important input to the production of organic chemicals. Five primary base-chemicals: ethylene, propylene, benzene, toluene and xylene produce over 75% of organic chemicals used to synthesize other organic compounds that are then used to produce various chemical products including polymers and resins (Howard *et al.,* 2003). Lignin produces aromatic compounds while the low molecular mass aliphatic compounds can be derived from ethanol produced by fermentation of sugar generated from the cellulose and hemicellulose. Table 2.3 shows estimations of total demands for chemicals which could be made by fermentation.

Table 2. 3: Annual production of chemicals which could potentially be made from fermentation (Howard *et al.,* 2003).

Products	**World demand (thousand of tonnes)**
Fumaric acid	60
Citric acid	300
Acetic acid	2539
Glycerol	414
Butanol	1400
Acetone	1659
Ethanol	16 000

2.6 Industrial applications of lignocellulases

2.6.1 In detergent and textile industries

Alkaline cellulases found in the composition of the detergent can easily pass through the inter fibril spaces and help in effective removal of stains from cloths. Furthermore, cellulases process the cellulose fibrils. They are also normally used in textile processing and finishing of the cellulose based textiles. Cellulases are useful in removing furriness of the cellulose threads which are used in textile production with insignificant weight loss, developing

smooth and glossy appearance of the cloth and giving color brightness (Sukumaran *et al.,* 2005).

2.6.2 In animal feed industries

Cellulases along with hemicellulases are used in the production of animal and poultry feeds. Advantages of adding enzymes during feed processing are partial hydrolysis of cellulase and hemicellulase components present in the silage, dehulling of cereal grains and better emulsification of feeds (Cinar, 2005).

2.6.3 In food processing industries

Cellulases have broader applications in food processing industry. Cellulases together with xylanases and pectinases are known as macerating enzymes. They have been used in extraction and clarification of fruit and vegetable juices to increase their yields. Macerating enzymes reduce viscosity; improve cloud stability and aromatic properties of the fruit juices and their pulps during processing (Sukumaran *et al.,* 2005).

2.7 Application of xylanases

Potential application of xylanases in biotechnology include biobleaching of wood pulp, treating animal feed to increase digestibility, processing food to increase clarification and converting lignocellulosic substances to feedstock and fuels (Motta *et al.,* 2013).The most common of which is in paper and pulp industries.

2.7.1 The paper and pulp industries

Xylanases have been used in pulp bleaching mainly to decrease lignin and increase the brightness of the pulp (Bajpai, 2011). The significance of xylanase in the pulp and paper industries is associated with xylan hydrolysis, which speeds up the release of lignin from paper pulp and, as a result, reducing the use of chlorine as the bleaching agent (Subramaniyan and Prema, 2002). Bleaching is referred to as the process of removing lignin from chemical

pulps producing bright and completely white finished pulp (Beg *et al.,* 2001). Hence, biobleaching is the bleaching of pulp using enzymes or lignocellulolytic microorganisms (Pe´rez *et al.,* 2002). The presence of residual lignin and its derivatives in the pulping process makes the resultant pulp to gain a brown color, thus, biobleaching is necessary. The bleaching of pulp includes destruction, alteration or solubilization of the lignin, colored organic matter and other unwanted residues on the fibers (Subramaniyan & Prema, 2002)

The use of xylanase in bleaching pulp requires the use of enzymes with special characteristic which include being cellulose free and avoiding damage of the pulp fibers (Motta *et al.,* 2013), since cellulose is the primary product in the paper industry (Subramaniyan & Prema, 2002). Other desirable characteristics are stability at high temperatures (Chidi *et al.,* 2008) and an alkaline optimal pH (Perez *et al.,* 2002)

References

Abd-Elsalam, **H. A., & El-Hanafy, A. A., 2009**. Lignin biodegradation with ligninolytic bacterial strain and comparison of *Bacillus subtilis* and *Bacillus sp*. isolated from Egyptian soil. *American-Eurasian Journal of Agricultural & Environmental Sciences, 5*, 39-44.

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., & Levin, D. B., 2011. Biomass pretreatment: fundamentals toward application. *Biotechnology advances*, *29*(6), 675-685.

Anwar, Z., Gulfraz, M., & Irshad, M., 2014. Agro-industrial lignocellulosic biomass a key to unlock the future bioenergy: a brief review. *Journal of Radiation Research and Applied Sciences*, *7*(2), 163-173.

Bacovsky, D**., 2009**. "2nd generation biofuels-state of the art".IEA Bioenergy. Task 39, from http://biofuels.abc-energy.at,http://www.a3pa.at

Bajaj, B. K., & Manhas, K., 2012. Production and characterization of xylanase from *Bacillus licheniformis* P11 (C) with potential for fruit juice and bakery industry. *Biocatalysis and Agricultural Biotechnology*, *1*(4), 330-337.

Bajpai, P., 2011. *Biotechnology for pulp and paper processing*. Springer.

Balat, M., &Balat, H., 2009. Recent trends in global production and utilization of bio-ethanol fuel. *Applied Energy*, *86*(11), 2273-2282.

Beg, Q., Kapoor, M., Mahajan, L., & Hoondal, G. S., 2001. Microbial xylanases and their industrial applications: a review. *Applied Microbiology and Biotechnology*, *56*(3-4), 326-338.

Bottone, E. J., 2010. *Bacillus cereus,* a volatile human pathogen. *Clinical Microbiology Reviews*, *23*(2), 382-398.

Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K. B., & Ramakrishnan, S., 2011. Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. *Enzyme Research*, *2011*.

Chandra, R, Raj, A, Purohit ., H.J, Kapley A., 2007. Characterisation and optimisation of three potential aerobic bacterial strains for kraft lignin degradation from pulp paper waste. *Chemosphere; 67*: 839–846.

Chang, V.S., Holtzapple, M.T., 2000. Fundamental factors affecting biomass enzymatic reactivity. *Applied Biochemistry and Biotechnology, 84,* 5–37.

Chidi, S. B., Godana, B., Ncube, I., Van Rensburg, E. J., Cronshaw, A., &Abotsi, E. K., 2008. Production, purification and characterization of celullase-free xylanase from *Aspergillusterreus UL 4209.African Journal of Biotechnology*, *7*(21).

Çinar, I., 2005. Effects of cellulase and pectinase concentrations on the colour yield of enzyme extracted plant carotenoids. *Process Biochemistry*, *40*(2), 945-949.

Classen, P.A.M., van Lier, J.B., Lopes Contreras, A.M., van Niel, E.W.J., Sijtsma, L., Stams, A.J.M., Weushuis, R.A., 1999. Utilisation of biomass for the supply of energy carriers.*Applied Microbiology and Biotechnology 52,* 741-755.

Cosgrove, D. J., 2005.Growth of the plant cell wall. *Nature reviews molecular cell biology*, *6*(11), 850-861.

de Souza, W. R. 2013. Microbial Degradation of Lignocellulosic Biomass.

Demain, A.L., Newcomb, M., & Wu, J.D., 2005. Cellulase, clostridia, and ethanol. *Microbiology and molecular biology reviews* 69, *1*(2005)*:* 124-154.

Dixon, N., & Langer, U., 2006. Development of a MSW classification system for the evaluation of mechanical properties.*Waste management*, *26*(3), 220-232.

Faulon, J., G.A. Carlson, et al., 1994. A three-dimensional model for lignocellulose from gymno-spermous wood*. Organic Geochemistry 21*: 1169-1179.

Fujii, K., Kuwahara, A., Nakamura, K., & Yamashita, Y., 2011. Development of a simple cultivation method for isolating uncultured cellulose-producing microbes.*Applied Microbiology and Biotechnology .91*, 1183-1192.

Gessner , M.O., Swan , C.M., Dang, C.K., McKie , B.G., Bardgett, R.D., Wall , D.H., Ha¨ttenschwiler, S., 2010. Diversity meets decomposition.*Trends of Ecological Evolution 25:*372–380

Ghosal, K., **Chakrabarty, S., & Nanda, A., 2011**. Hydroxypropyl methylcellulose in drug delivery. *Der Pharmacia Sinica*, 2(2).

Gilbert, H.J., 2007. Cellulosomes: Microbial nanomachines that display plasticity in quaternary structure. *Molecular Microbiology*, 63,1568-1576.

Haichar, F.E.Z., Achouak, W., Christen, R., Heulin, T., Marol, C., Marais, M.F., Mougel, C., Ranjard L., Balesdent J. & Berge O., 2007. Identification of cellulolytic bacteria in soil by stable isotope probing.*Environmental Microbiology*.9, 625-634.

Heck, J. X., Flôres, S. H., Hertz, P. F., & Ayub, M. A. Z. 2005. Optimization of cellulase-free xylanase activity produced by *Bacillus coagulans* BL69 in solid-state cultivation. *Process Biochemistry*, 4*0*(1), 107-112.

Helgason, E., Tourasse, N. J., Meisal, R., Caugant, D. A., & Kolstø, A. B., 2004. Multilocus sequence typing scheme for bacteria of the *Bacillus cereus* group. *Applied and Environmental Microbiology*, *70*(1), 191-201.

Henriksen, **E. D. 2008**. *The application of pectinolytic enzymes in the conversion of lignocellulosic biomass to fuel ethanol* (Doctoral dissertation, University of Georgia).

Howard, R.L**.; Abotsi, E.; Jansen van Rensburg E.L. and Howard, S., 2003**. Lignocellulose biotechnology: issues of bioconversion and enzyme production. *African Journal of Biotechnology*, *2* (12), 602-619.

Huijgen, W., Bermudez, L., & Bakker, R., 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass (pp. 978-9).*Wageningen UR, Food & Biobased Research.*

Iqbal, H. M. N., Kyazze, G., & Keshavarz, T., 2013. Advances in valorization of lignocellulosic materials by bio-technology: an overview. *Biological Resources, 8*(2), 3157e3176.

Irshad, M., Anwar, Z., But, H. I., Afroz, A., Ikram, N., & Rashid, U., 2013. The industrial applicability of purified cellulase complex indigenously produced by Trichoderma viride through solid-state bio-processing of agro-industrial and municipal paper wastes. *Biological Resources, 8*(1), 145e157.

Isroi, M. R., Syamsiah, S., Niklasson, C., Cahyanto, M. N., Lundquist, K., & Taherzadeh, M. J., 2011. Biological pretreatment of lignocelluloses with white-rot fungi and its applications: a review. *Biological Resources, 6*(4), 5224e 5259.

Jeoh, T., Ishizawa, C. I., Davis, M. F., Himmel, M. E., Adney, W. S., & Johnson, D. K., 2007. Cellulase digestibility of pretreated biomass is limited by cellulose accessibility. *Biotechnology and bioengineering, 98*(1), 112-122.

Kirk-Otmer 4th edition 2001, vol 20.

Krassig, **H.& J. Schurz., 2002. Ullmann's Encyclopedia of Industrial Chemistry,** Sixth edition, Weinheim, Germany, Wiley-VCH.

Laufenberg, G., Kunz, B., Nystroem, M., 2003. Transformation of vegetable waste into value added products:(A) the upgrading concept; (B) practical implementations. *Bioresource Technology 87,* 167–198.

Lin, S.Y.&, I.S. Lin ., 2002. Ullmann's Encyclopedia of Industrial Chemistry, Sixth edition**, Wein-heim, Germany,** Wiley-VCH.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., & Pretorius, I. S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and molecular biology reviews*, *66*(3), 506-577.

Maijala, P., Kleen, M., Westin, C., Poppius-Levlin, K., Herranen, K., Lehto, J. H., & Hatakka, A., 2008. Biomechanical pulping of softwood with enzymes and white-rot fungus< i> Physisporinus rivulosus</i>. *Enzyme and Microbial technology*, *43*(2), 169-177.

Maki, M., Leung, K. T., & Qin, W. 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass.*International Journal of Biological Sciences*, *5*(5), 500.

Maki, M.L., Indrees, A., Leung, K.T., & Qin, W., 2012. Newly Isolated and characterized bacteria with great application potential for decomposition of lignocellulosic. *Journal of Molecular Microbiology and Biotechnology, 22* (3), 156-166.

Malherbe, S. & Cloete, T.E., 2002. Lignocellulose biodegradation: *Fundamentals & applications.*

Matthews, J. F., Skopec, C. E., Mason, P. E., Zuccato, P., Torget, R. W., & Sugiyama, J., 2006. Computer simulation studies of microcrystalline cellulose I beta. *Carbohydrate Research, 341*(1), 138–152.

McCarthy, A.J., 1987. Lignocelluloses degrading actinomycetes. *FEMS Microbiology letters, 46*(2), 145-163.

McDonald, J. E., Rooks, D. J., & McCarthy, A. J., 2012. Methods for the isolation of cellulose-degrading microorganisms. *Methods Enzymol*, *510*, 349-374.

McKendry, P., 2002. Energy production from biomass (part 1): Overview of biomass. *Bioresource Technology, 83*(1), 37e46.

Menon, V., & Rao, M.., 2012. Trends in bioconversion of lignocellulose: biofuels, platform chemicals &biorefinery concept. *Progress in Energy and Combustion Science*, *38*(4), 522-550.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y.,Holtzapple, M., &Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, 96(6), 673-686.

Motta, F. L., Andrade, C. C. P., & Santana, M. H. A., 2013. "A Review of Xylanase production by fermentation of xylan : Classification, characterization and application", In : *Sustainable degradation of Lignocellulosic Biomass Techniques Application and Commercialization, A.K. Chandel and S.S. da Silva (eds), InTech, Shangai,* p.p. 251-275.

O'Connor, R.P., R. Woodley, J.J. Kolstad, R. Kean, D.A. Glassner, B. Mastel, J.M. Ritzenthaler, H. John, J. Warwick, J.R. Hettenhaus & R.K. Brooks., 2007. Process for fraction-atingligno-cellulosic biomass into liquid and solid products. assignee U. S. A. Nature-works LLC, patent number WO 2007120210.

Pérez, J., Munoz-Dorado, J., de la Rubia, T. D. L. R., & Martinez, J., 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *International Microbiology*, *5*(2), 53-63.

Peterson, **A. A., Vogel, F., Lachance, R. P., Fröling, M., Antal Jr, M. J., & Tester, J. W., 2008**. Thermochemical biofuel production in hydrothermal media: a review of sub-and supercritical water technologies. *Energy & Environmental Science*, *1*(1), 32-65.

Pirttijärvi, T. S. M., Andersson, M. A., & Salkinoja-Salonen, M. S., 2000. Properties of *Bacillus cereus* and other bacilli contaminating biomaterial-based industrial processes.*International journal of food microbiology*, *60*(2), 231-239.

Priest, F. G., Barker, M., Baillie, L. W., Holmes, E. C., & Maiden, M. C., 2004.Population structure and evolution of the *Bacillus cereus* group.*Journal of*

Bacteriology, *186*(23), 7959-7970.Production by the Fermentation of Xylan: Classification, Characterization and Applications.

Rahman, N. H. A., Aziz, S. A., & Hassan, M. A., 2013. Production of Ligninolytic Enzymes by Newly Isolated Bacteria from Palm Oil Plantation Soils.*BioResources*, *8*(4), 6136-6150.

Rubin, E. M., 2008. Genomics of cellulosic biofuels. *Nature*, *454*(7206), 841-845.

Sanchez, **O. J., & Cardona, C. A. 2008**.Trends in biotechnological production of fuel ethanol from different feedstocks.*Bioresource technology*, *99*(13), 5270-5295.

Schneider, S.H., 1989. The greenhouse effect: science and policy. *Science; 243*:771-781.

Shallom, **D., & Shoham, Y. 2003**. Microbial hemicellulases. *Current opinion in microbiology*, *6*(3), 219-228.

Slepecky, R. A., & Hemphill, H. E., 2006. The genus Bacillus - nonmedical. In *The prokaryotes*. pp. 530-562.

Song, N., Cai, H. Y., Yan, Z. S., & Jiang, H. L., 2013. Cellulose degradation by one mesophilic strain< i> Caulobacter</i> sp. FMC1 under both aerobic and anaerobic conditions. *Bioresource technology*, *131*, 281-287.

Subramaniyan, S., & Prema, P., 2002. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Critical Reviews in Biotechnology*, *22*(1), 33-64.

Sukumaran, R. K., Singhania, R. R., & Pandey, A., 2005. Microbial cellulases-production, applications and challenges. *Journal of Scientific and Industrial Research*, *64*(11), 832.

Tuomela, M.,Vikman, M., Hatakka, A., Itavaara, M., 2000. Biodegradation of lignin in a compost environment: a review. *Bioresource Technology 72*,169-183.

Wahyudi, A., Cahyanto, M. N., Soejono, M., & Bachruddin, Z., 2010. potency of lignocell lose degrading bacteria isolated from buffalo and horse gastrointestinal tract and

elephant dung for feed fiber degradation. *Journal of the Indonesian Tropical Animal Agriculture*, *35*(1), 34-41.

Wilke, T., Vorlop, K.D., 2004. Industrial bioconversion of renewable resources as an alternative to conventional chemistry. *Applied Microbiology and Biotechnology 66*, 131–142.

Wongwilaiwalin, S., Rattanachomsri, U., Laothanachareon, T., Eurwilaichitr, L., Igarashi, Y., & Champreda, V., 2010. Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system. *Enzyme and Microbial Technology*, *47*(6), 283-290.

Woo, H. L., Hazen, T. C., Simmons, B. A., & DeAngelis, K. M., 2014. Enzyme activities of aerobic lignocellulolytic bacteria isolated from wet tropical forest soils. *Systematic and Applied Microbiology*, *37*(1), 60-67.

Yakubu, A**., Umar, T., & Mohammed, S., 2011**. Chemical Modification of microcrystalline cellulose : Improvement of Barrier Surface Properties to enhance Surface Interactions with some synthetic polymers for biodegradable packaging material processing and application in textile, food and.. *Advances in Applied Science Research*, *2* (6), 233-237

CHAPTER THREE

Abstract

Lignocellulose is the main structural component of plant cell walls and can be degraded into simple sugars with the help of hydrolytic enzymes. Lignocellulose is composed of three polymers: cellulose, hemicelluloses and lignin. Certain bacteria have evolved the ability to degrade lignocelluloses, most of which are gram positive and spore formers. A lignocelluloses degrading bacteria isolated from the fresh water sediments of the Tyume River in the Eastern Cape province of South Africa was assessed for its potential to produce lignocellulolytic enzymes i.e. cellulase and xylanase under submerged fermentation. Based on 16S r DNA sequence analysis, the isolate was identified as *Bacillus cereus sp.* The bacteria produced the lignocellulolytic enzymes optimally at acidic pH (3-5) and at moderate temperatures (35 and 45º C). The time course for the enzyme production was optimal at 12-30 hr of incubation.

Keywords: *Bacillus cereus sp.*, Lignocellulose, Sediments

3.1 Introduction

Lignocellulose is a renewable, plentiful and cheap natural resource which could be converted to biofuels and other bioproducts (Maki *et al.,* 2011). It is the main structural component of plant cell wall and can be degraded into simple sugars with the help of hydrolytic enzymes (Maki *et al.,* 2011). It is comprised of mainly cellulose which is a homologous polymer of glucose molecules connected by β-1, 4 linkages (the most abundant organic polymer in the world). Lignocellulose also contains some heterologous polymer including the 5- and 6-carbon sugars (hemicelluloses) and a less complex aromatic polymer (lignin). The various sources of lignocellulosic biomass include municipal waste, agricultural residues, forestry or pulp and paper excesses, and, switch grass (Greene, 2004).

Several microbes have evolved the ability to degrade lignocelluloses and to this effect cell-bound enzymes and multi-protein complexes expressing cellulase and hemicellulase activity have effectively degraded lignocellulosic materials (Woo *et al.*, 2014). Cellulose is degraded to glucose by the synergistic action of three distinct classes of enzymes: endoglucanases, exoglucanases and β-glucosidases. However, due to the variability in the structure and organisation of hemicellulose, its degradation requires various enzymes with diverse modes of action. The enzyme batteries involved in hemicellulose degradation includes endoxylanases, endomannanases, β-xylosidases, β-mannosidases, β-galactosidases, α-glucuronidases, α-arabinofurnosidases, α-galactosidases, acetyl xylan esterases, feruloyl esterases and glucuronyl esterases. Hemicellulose has been anticipated as a physical obstruction for cellulose hydrolysis in the utilization of lignocellulosic materials for value added products (Yoshida *et al.,* 2008). Nonetheless, the hemicellulose-degrading enzyme activities of most commercial cellulase enzymes are insufficient as the achievement of complete conversion of hemicelluloses is not usually the situation (Hu *et al.,* 2011; Qing &

Wyman, 2011). Xylanases, on the other hand, are fast becoming a major group of industrial enzymes finding significant application in paper and pulp industry (Shoham *et al.,* 1993). They are of importance to the pulp and paper industries, the hydrolysis of xylan facilitates the release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent (Shoham *et al.,* 1993). The importance of xylanases is not bound to the paper and pulp industry alone as there are other industries where xylanases are of equal importance and applicability. The potential applications of xylanases also include the bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, improvement in consistency of beer and the digestibility of animal feed stock (Agustini *et al.,*. 2012)

The need for enhanced lignocellulose bioconversion to value added product has motivated the continual exploration of different ecological milieu for microbial species with enzyme batteries sufficient degrade the hemicellulose and release the abundant glucose held up as cellulose. Consequently, this work aimed at exploring the bacterial diversity of the sediments of Tyume River of the Eastern Cape Province of South Africa.

3.2 Materials and Methods

3.2.1 Description of the study area

The Tyume River is located in Alice town in the Eastern Cape province of South Africa, with geographical co-ordinates of 32º 46’ 44.6’’S, and 26º 51’ 21.5’’E. The River flows next to the University of Fort Hare, under Nkonkobe District Municipality. Currently, the river serves as source of water in use for grazing animal flows from the upper part of the Amathole Mountains in Hogsback, passing through the lower coastal escarpment down to Alice, through several rural settlements, and finally joins the Keiskamma River at Manqulweni community.

3.2.2. Sample collection

The murky water samples (disturbed water and sediments) were collected from two different points of the Tyume River, Eastern Cape using 1.7 L sterile bottles and transported in ice packs to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, University of Fort Hare for analysis.

3.2.3 Media for Bacterial Isolation, Cellulose Utilization

The M1 medium amended with Nystastin (50mg/l) to prevent the fungal growth was used for the cultivation of bacteria from the samples in accordance to the method described by Jensen et al. (1991). The media was composed of the following (g/l) starch (10) l, yeast extract (4), peptone (2) and agar (18) (Mincer *et al.,* 2002).

Basal salt medium supplemented with xylan and CMC were respectively used for the screening of cellulase and xylanase activity. The media was composed as follows; substrate carboxymethyl cellulose or beechwood xylan (5g), $NaNO_3$ (1g), K_2HPO_4 (1g), KCl (1g), $MgSO_4$ (0.5g), Yeast extract (0.5g) and agar (15g) in 1 L of sterile deionised water.

3.2.4. Bacterial isolation

With slight modifications, bacterial species were isolated on M1 agar (composition is as seen in section 3.2.3) in accordance with the methods of Mincer (2002). The experiment was carried out by shaking and heating 100ml of murky (sediment and water) sample at 55 ^oC for 15 minutes in a conical flask. A series of dilutions of the sample suspension from 10^{-0} to 10^{-8} was carried out using phosphate buffer saline (PBS). Approximately 100 µl of the suspension was pipetted and spread onto M1 medium amended with Nystatin (50mg/L) and nalidixic acid (20mg/L). The culture experimentation was in triplicate. All the plates were then incubated at 28^o C for 3 weeks. The morphology, size and color of bacterial cultures were documented and discrete colonies of distinct isolates were then selected and purified. After

purification, distinct culture colonies were stocked in 30% glycerol solution at 4°C until further use.

3.2.5 Inoculum standardization for cellulase and xylanase activity screening

About 10 ml of sterile basal salt media was inoculated with a loop full of purified isolate and incubated at 28 ^{o}C for 48 hr at an agitation speed of 200 rpm. After incubation, 1ml of the culture was taken from the test tubes and transferred to sterile 2.5 ml capacity eppendorf tubes, centrifuged at 10000 g for 5-10 min to sediment the cells. The supernatant was carefully decanted. Approximately, 1ml of sterile distilled water was added to the tube and vortexed to re-suspend the cells. The suspension was aseptically transferred to sterile McCartney bottle containing 9 ml of sterile distilled water and vortexed afterwards. The suspension was read spectrophotometrically at an optical density (OD) of 600 nm and adjusted to an OD of about 0.1.

3.2.6 Screening for cellulase activity

The method previously described by Maki *et al.,* (2012) was used to screen isolates for CMCase activity. Isolates were grown in 10 ml of sterile basal medium broth amended with the appropriate substrates for 72 hours, at 28°C, with shaking at 12000 rpm. After incubation, the resulting broth cultures were screened for cellulase activity using the Gram' iodine method (Kasana *et al.,* 2008). The inoculum standardization was done as described earlier. Approximately, 5 µ l of each culture suspension was singly dropped onto a plastic Petri dish containing CMC agar: 5g of a substrate (CMC, beechwood xylan, 1g $NaNO_3$, 1g K_2HPO_4 , 1g KCl, 0.5g $MgSO_4$,0.5g Yeast extract ,15 agar in 1L sterile deionised water) and incubated for 120 hours at 28 °C. After incubation, the plates were taken out to be flooded with Gram's iodine solution (2.0 g KI and 1.0 g I, per 300 ml ddH 2 O) for 5 min to visualize and snap those that have the halo zone. The agar containing CMC stained brown and areas without CMC (degraded) were clear. The clear areas are described as halo zones, as seen in Figure 3

and 4. Halo diameters were measured using a ruler for a semi-qualitative comparison of cellulose activity among the isolates after 120 h of incubation. The presence of a halo measurement is used to confirm cellulase activity of the bacteria.

3.2.7. Screening for xylanase activity

The xylanase activity of all of the isolates was evaluated using the same method described above for the screening of cellulase activity. However, for the xylanase activity, 0.5 g of beechwood xylan (Sigma Aldrich) was used instead of CMC. Similarly, the presence of halos after staining with Gram's iodine was positive for xylanase activity.

3.2.8. Identification of the bacterial isolate by 16s rRNA gene sequencing

The genomic DNA (Deoxyribo nucleic acid) used for the PCR was prepared from pure culture of the bacterial isolate grown on nutrient agar plates. The total genomic DNA from the positive strain was extracted employing the DNA purification Kit according to the manufacturer's instruction. The 16S rRNA gene fragment was amplified using the universal primers. The PCR conditions were standardized with initial denaturation at 94 ^{o}C for 3 minutes followed by 30 cycles amplification (Denaturation at 94 ^{o}C for 60 sec, annealing temperature was 55 ^{o}C for 60 seconds and extension at 72 ^{o}C for 60 sec) and an addition of 5 minutes at 72 ^{o}C as final extension. The amplification reactions were carried with the total volume of 50 µL consisting of 25 µL master mix, 0.5 µL each of forward and reverse primers, 10 µL of template DNA and 14 µL nuclease free water in a gradient PCR. PCR product was analysed.

3.2.9. Enzyme production

The production of crude enzyme was carried out in the same medium used for screening without the addition of agar. A loop full of culture from agar plates was inoculated into glass tubes containing 5 ml of production medium, and incubated at 120 rpm and 30°C. This

culture was then inoculated (9×10^4 CFU/ml) into a 250-ml capacity Erlenmeyer flask containing 100 ml of the same medium, 2 ml aliquots was drawn out and centrifuged at 10,000 g for 10 min. The enzyme activity was measured in cell-free supernatant was then used as the source of crude enzymes (El-Sersy *et al.,* 2010).

3.2.10 Cellulase and Xylanase activity

Cellulase activity was assayed using a modified method described by Lo *et al.,* (2010). About 0.2 ml of culture filtrate was added to 1.8 ml of 1% CMC prepared in 0.05M phosphate buffer (pH 7) in a test tube and incubated at 40 °C for 30 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in a water bath at 100°C for 15 min. 1 ml of Rochelle salt solution (40 g Rochelle salt in distilled water to make the volume 100 ml) was then added to stabilize the colour. Using spectrophotometer, the absorbance was recorded at 575 nm against the blank (0.05 M sodium citrate buffer). One unit of CMCase activity was expressed as 1.0 μM of glucose liberated per ml enzyme per min (Ariffin *et al.,* 2006). By using a calibration curve for glucose, one unit of enzyme activity was defined as the amount of enzyme that released 1 μM of glucose per min (Ahmed *et al.*, 2009; El-Sersy *et al.*, 2010).

Xylanase activity was determined using the same method as cellulose; 0.2ml of enzyme, solution appropriately diluted in phosphate buffer (pH7) with 1.8ml of aqueous suspension containing 1% xylan. The reaction was carried out at 37^o C for 30 min. The amount of reducing sugar released was estimated with DNS method using xylose as standard. The DNS method for the protein estimation was performed according to the method of Miller 1959 as follows: The standard was pipette out solution into the dry, clean test tubes in the range of 0 to 2ml, the final volume in all tubes was made up to 2ml with distilled water concentrations ranging from 0-750mg. 1ml of DNS reagent was then added to all the test tubes, mixed well and capped to avoid the loss of liquid due to evaporation, the test tubes with the reaction

mixture were then put into the boiling water bath for ten minutes. The tubes were taken out and cooled to room temperature and the extinction was read at 540nm against the blank.

3.2.11 Optimum conditions determination for the production of cellulase and xylanase enzymes.

In order to obtain the maximum cellulase and xylanase production, the effects of pH, temperature and incubation time were evaluated. The Initial pH of the fermentation medium was varied from 3 to 9. The pH was adjusted by using 1M-HCl or NaOH prior to autoclaving. The temperatures ranged from 25 to 50º C and the incubation time was varied from 6 to 54 h. The crude enzyme was extracted and assayed at regular intervals of 6 h (Adhyaru *et al.*, 2014).

3.3 Results and Discussion

3.3.1. Isolation of lignocellulase producing bacteria

After isolation, the bacteria were then selected according to their morphology, size and colour and then picked and streaked onto fresh M1 medium plates for purification and were incubated at 28 ^{o}C for a week. After purification, the cultures were observed and those with similar color, morphology and size were selected and coded for identification purposes. The codes were termed based on the colony characteristics and the sample from which it was obtained. For instance, SB1 was the code for the organism isolated from the sediment sample with a beige-creamish colour and was from a 10^{-1} plate.

The photograph of some of the isolates that were taken after isolation (**Figure 3.1.)**

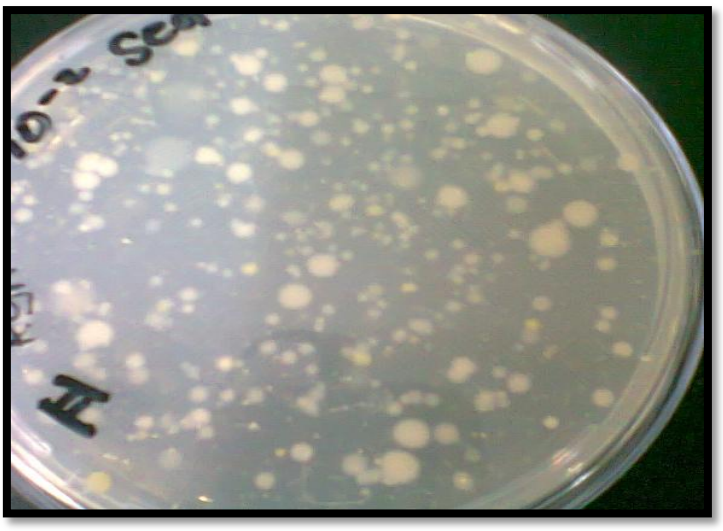


Figure 3. 1: Colonies on M1 culture plates obtained through spread plate technique

3.3.2. Screening for Carboxymethyl cellulose activity

A total of 23 isolates were obtained from the isolation stage. From the 23 isolates 14 were removed as they had similar cultural characteristics. The remaining 9 isolates were then screened for the hydrolysis of cellulose and xylan on agar plates containing the substrates (CMC and xylan). Four of these were positive for cellulose hydrolysis, and 6 for xylan hydrolysis respectively. One (SB1) isolate out of the 9 showed the degradation for both xylan and CMC as it is shown in **Figure 3.2,** and exhibited the greatest enzyme activity shown by the halo (**Figure 3.2 and Figure 3.3)** zone diameter and in **Table 3.1.** The morphological characteristics of the bacterium revealed the colony to be creamish beige, with a rod shape, and smooth surface. The 16S rDNA revealed the bacteria to have 98% similarity to *Bacillus cereus species.*The genus *Bacillus* are Gram positive, aerobic to facultative, spore forming rod found ubiquitously in the environment both above and below the earth's surface *Bacillus cereus* may be the most common aerobic spore bearer in any types of soil and in sediments, dust, and plants (Woo *et al.,* 2014). Microbiologically, members of the *B. cereus* group exclusive of *B. anthracis* display a range of morphological forms depending upon the milieu in which they are observed. The studies have shown that it is also frequently present in food production environments due to the adhesive nature of its endospores. This characteristic

enables the bacteria to spread to all kinds of food. By contrast, certain *B. cereus* strains are used as probiotics (Carlandrelli *et al.*, 2008). Although a number of enzymes have been reported to be produced by some of the species belonging to the *Bacillus* genus, such as *Bacillus subtilis*, to the best of our knowledge ours is the first report implicating the *Bacillus cereus* in lignocellulolytic enzyme production.

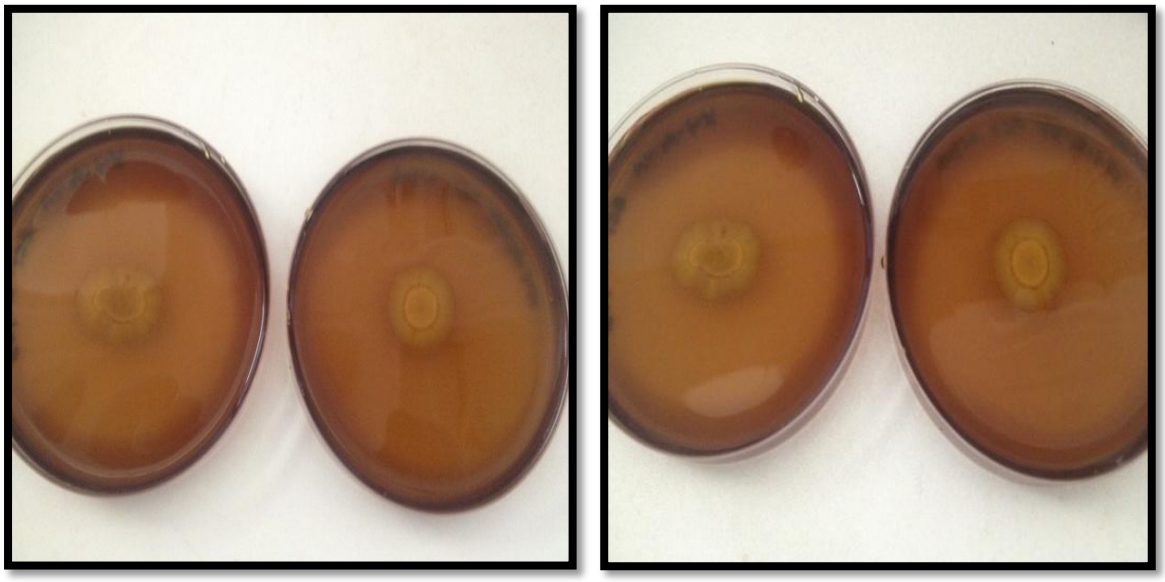


Figure 3. 2 : Xylanase activity shown by the clearance, the halo zone around the colony.

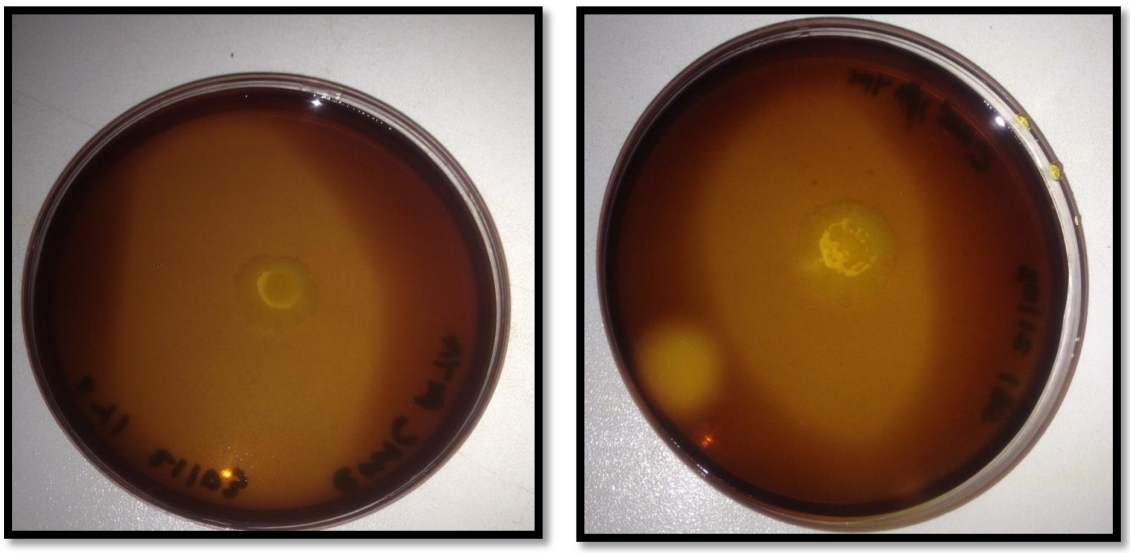


Figure 3. 3: Cellulase activity shown by the clearance of the halo zone around the colony.

Table 3. 1: Summary of Isolates based on their activity to degrade xylan and cellulose showed by the average halo zone diameter and standard deviation (SD).

Isolate code	Halo zone diameter (mm) (±SD)	
	Xylan	Cellulose,
SB1	**71.6 (±2.9)**	**61.5 (±2.1)**
WY0	**72.5 (±3.5)**	**-**
SC1	**64.5 (±4.5)**	**-**
WC2	**47.5(±3.5**	**-**
WC1	**65**	**-**
SC2	**56 (±7.8)**	**-**
SY1	**-**	**66.3 (±14.2)**
SY2	**-**	**46 (±5.7)**
SY0	**-**	**42 (±5.7)**

3.3.3. Enzyme production

Both the cellulase and xylanase enzymes were produced by SB1 under submerged fermentation conditions. The enzymes were produced at the temperature of 30 ºC at 1200-1500 rpm and pH 7 after 72 hours. Culture conditions (pH, temperature and incubation time) for the production of the enzymes, they were optimized by shaken flask fermentation experiments. The optimal pH of the enzyme came out to be ranging between 4 and 5 (Figure 3.4) with the enzyme activities: 16.29 U/ml and 19.91 U/ml. The enzyme activity dropped at pH 6 and then slowly increased to pH 8 and it dropped rapidly to pH. This therefore suggests

that SB1 is able to produce the enzyme at slightly alkaline to alkaline pH, in corroboration of previous report (Verma *et al.,* 2012).

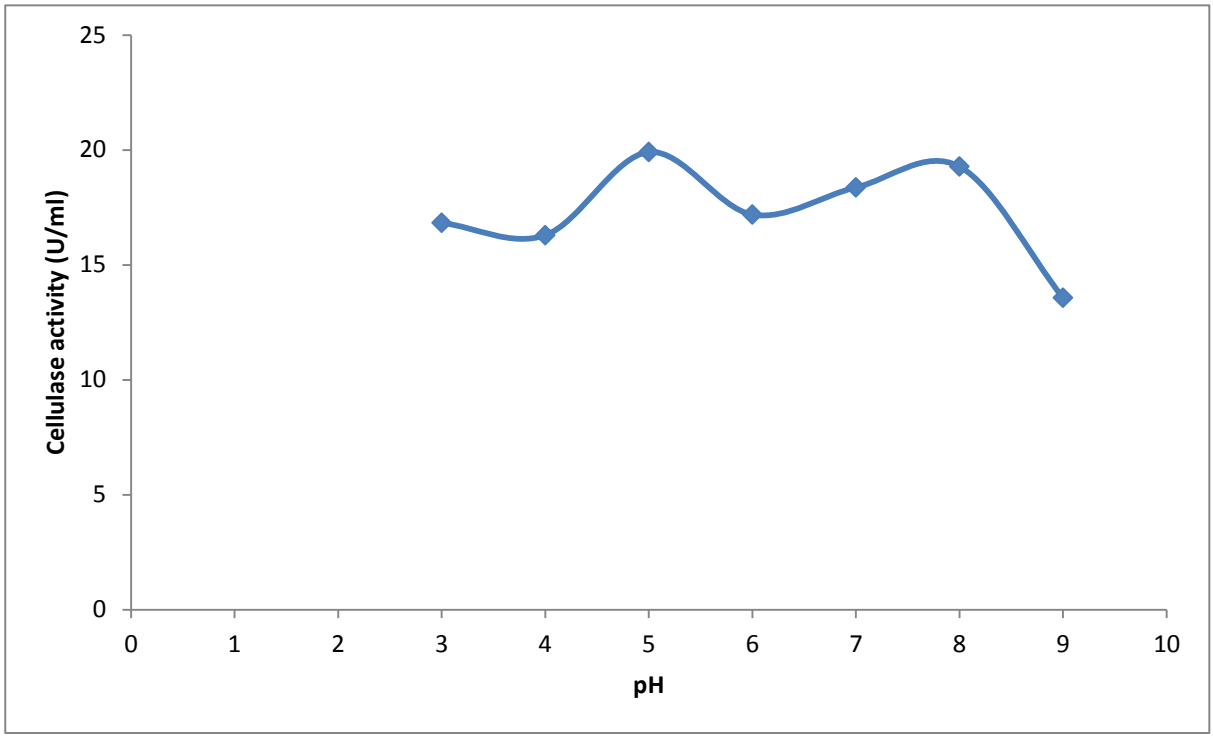


Figure 3. 4: Effect of pH on cellulase production by SB1.

Also, with regards to effect of pH on xylanase production by SB1, the maximum xylanase production occurred at the medium pH 3-4 (Figure 3.4) with enzyme activity calculated to range between 289.32 U/ml-271.83 U/ml, and mostly at acidic or slightly alkaline pH in support of the reports of Sanghi *et al*., (2010) and Bajaj & Manhas, (2012).

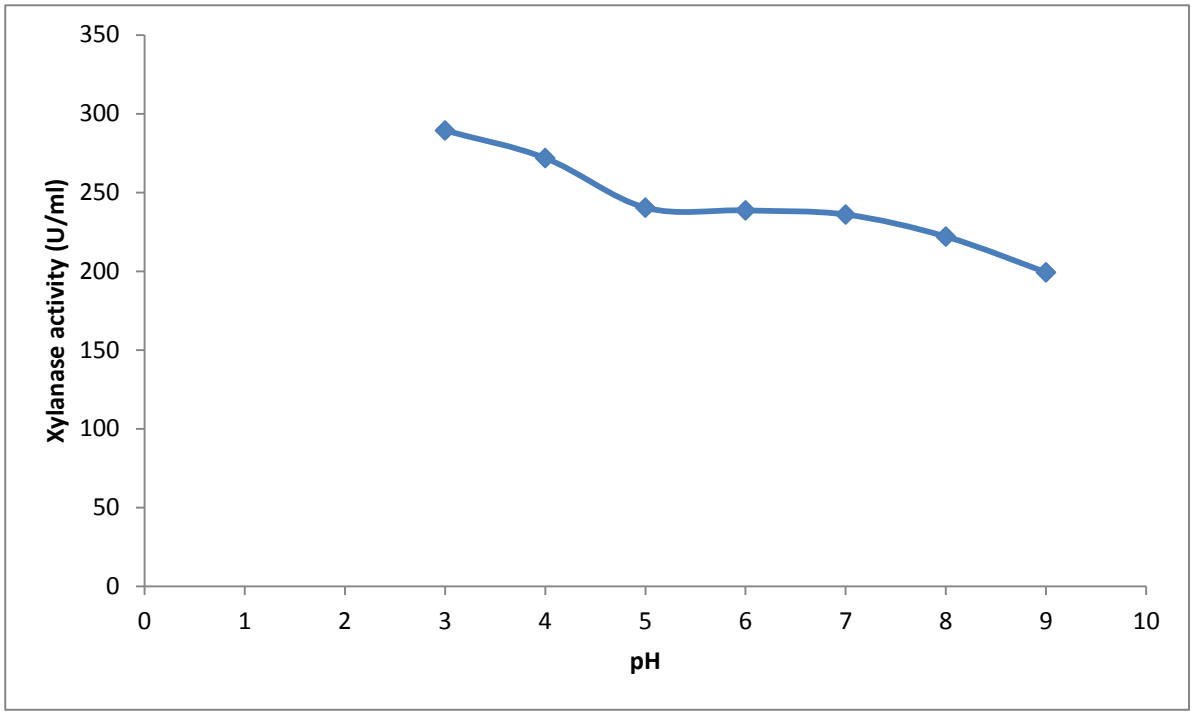


Figure 3. 5: Effect of pH on xylanase production by *Bacillus cereus*.

Figure 3.5 shows that the SB1 was able to produce the cellulase enzyme maximally at 45 ºC with the enzymatic activity of 39.82 U/ml. Also, results revealed that the enzyme remained stable after 50 ºC and the enzyme stability declined at temperatures above 50 ºC in support of another report (Verma *et al.*, 2012).

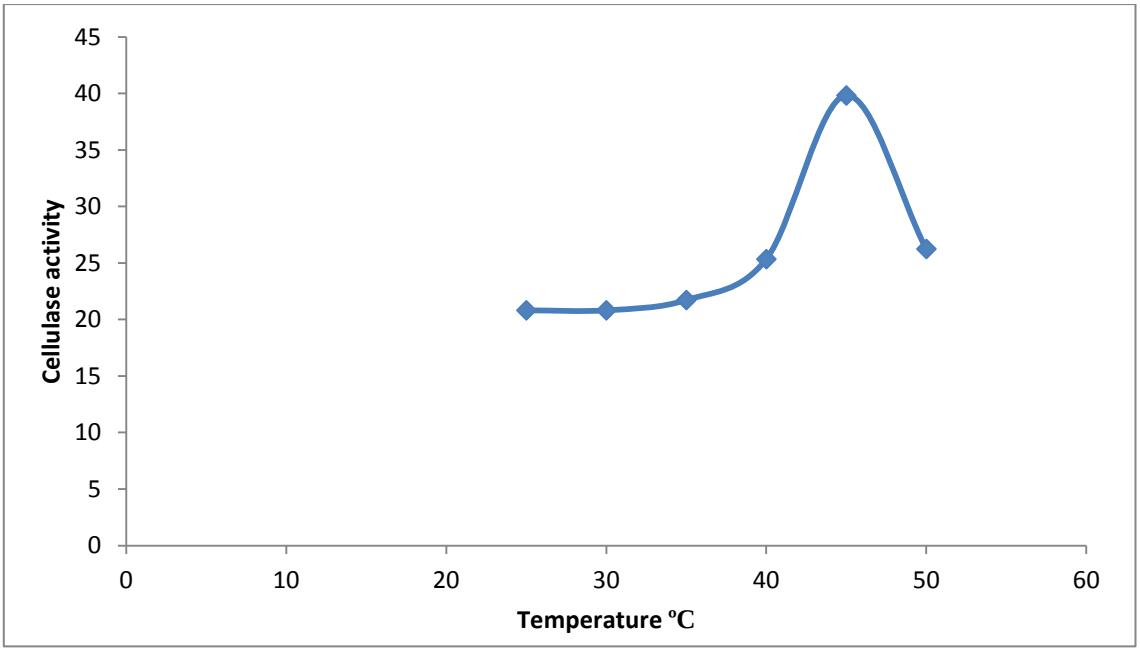


Figure 3. 6 : Effect of temperature on cellulase production by SB1.

With respect to effect of temperature on xylanase production, results showed that xylanes was produced over a temperature range of 25ºC-50 ºC (Figure 3.6). The maximum xylanase activity as observed at 35 ºC (273.58 U/ml). However, substantially high activity was observed at 25º C and even at 40º C. Bajaj *et al., (*2012) reported a similar observation for *Bacillus pumilus* SS1, and previous reports suggest that most of the bacterial xylanases show optimum activity at 50 ºC-60 ºC (Bajaj & Manhas, 2012).

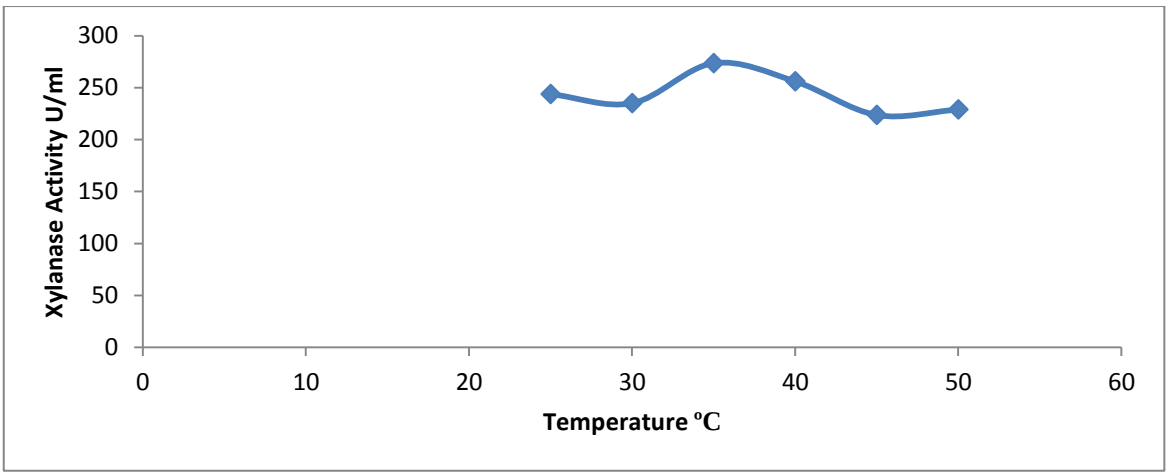


Figure 3. 7: Effect of temperature on xylanase production by SB1.

Time course studies of cellulase and xylanase enzymes productions by SB1 were carried out and the results revealed that with respect to cellulose production, the enzyme production increased with time and attained a peak after 30 hr of incubation with the enzyme activity of 27.15 U/ml (Figure 3.8) after which production declined. However, xylanase production on the other hand was short-lived reaching a peak in about 12 hours and thereafter significantly decreased.

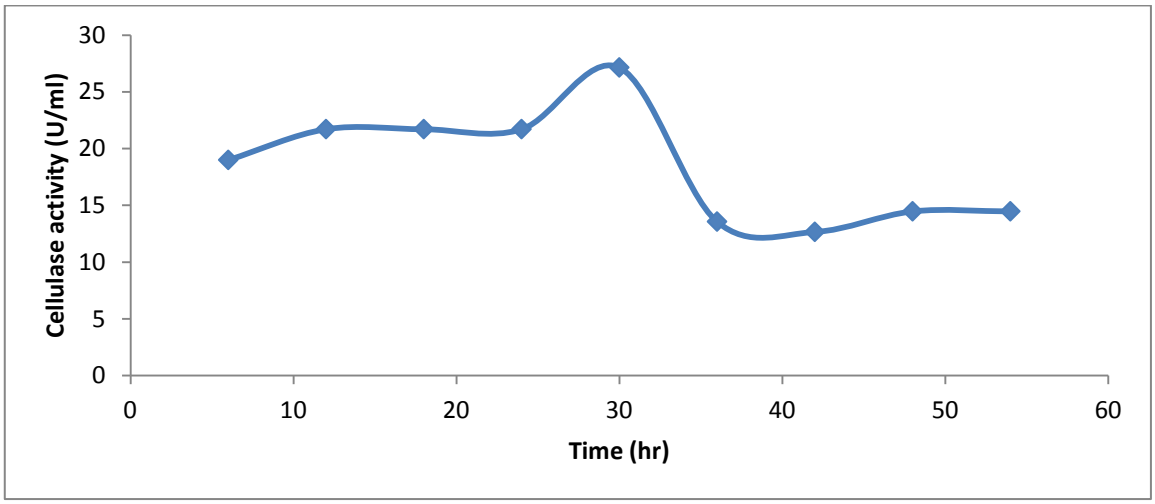


Figure 3. 8: Effect of incubation time on cellulase production by SB1.

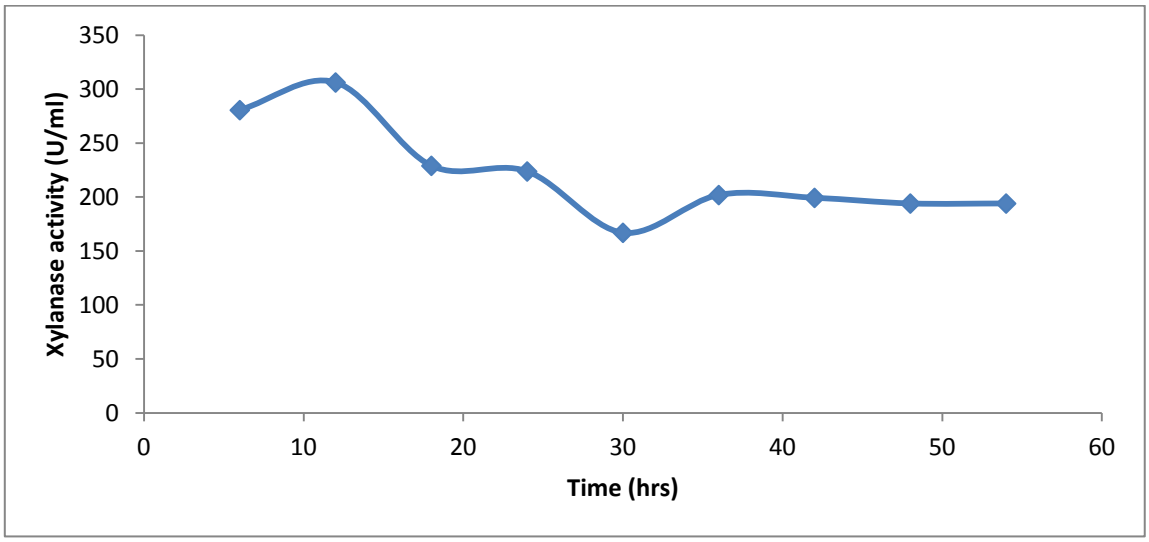


Figure 3. 9: Effect of incubation time on Xylanase activity production by *Bacillus cereus.*

Conclusion

This study revealed that Tyume River is an important source of cellulase and xylanase producing bacteria. The *Bacillus cereus* strain investigated (SB1) showed good promise as a cellulose and xylanase producer that could be beneficial in the bioconversion of lignocellulosic wastes to simple sugars for bioethanol production which is the future prospect of this research.

References

Adhyaru, D.N., Bhatt, N.S., Modi, H.A., 2014. Enhanced production of cellulase-free, thermo-alkali-solvent-stable xylanase from *Bacillus altitudinis* DHN8, its characterization and application in sorghum straw saccharification. Biocatalysis and *Agricultural Biotechnology*. *3*(2), 182-190.

Agustini, L., Efiyanti, L., Faulina, S. A., & Santoso, E., 2012. Isolation and Characterization of Cellulase-and Xylanase-Producing Microbes Isolated from Tropical Forests in Java and Sumatra. *International Journal of Environmental Biengineering*, *3*(3), 154-167.

Ahmed, S., Bashir, A., Saleem, H., Saadia, M., & Jamil, A., 2009. Production and purification of cellulose degrading enzymes from a filamentous fungus *Trichoderma harzianum.Pakistan Journal of Botany, 41(*3), 14111-1419.

Ariffin , H., Abdullah, N., Umi Kalsom, M. S., Shirai, Y., Hassan, M. A., 2006. Production and characterization of cellulase by *Bacillus pumilus* EB3. *International Journal of Engineering and Technology, 3*(1), 47-53.

Bajaj, B. K., **& Manhas, K., 2012**. Production and characterization of xylanase from *Bacillus licheniformis* P11 (C) with potential for fruit juice and bakery industry. *Biocatalysis and Agricultural Biotechnology*, *1*(4), 330-337

Bajaj, **B. K., Khajuria, Y. P., & Singh, V. P., 2012**. Agricultural residues as potential substrates for production of xylanase from alkali-thermotolerant bacterial isolate. *Biocatalysis and Agricultural Biotechnology, 1*(4), 314-320.

De Angelis, K.M., Allgaier, M., Chavarria, Y., Fortney, J.L., Hugenholtz, P., Simmons, B.A., Sublette, K., Silver, W.L., Hazen, T.C. (2011). Characterization of trapped lignin-degrading microbes in tropical forest soil. *Plos One, 6*(4): 1- 9 (e19306).

De Angelis, K.M., Gladden, J.M., Allgaier, M., D'haeseleer, P., Fortney, J.L., Reddy, A., Hugenholtz, P., Singer, S.W., Gheynst, J.S.V., Silver, W.L., Simmons, B.A., Hazen, T.C. 2010. Strategies for enhancing the effectiveness of metagenomic-based enzyme discovery in lignocellulolytic microbial communities. *Bioenergy Research, 3*: 146-158.

El-Sersy, N.A., Abd-Elnaby, H., Abou-Elela, G. M., Ibrahim, H.A., & El-Toukhy, N.M., 2013. Optimization, economization and characterization of cellulasenproduced by marine *Streptomyces ruber*. African *Journal of Biotechnology, 9*(38), 6355-6364.

Greene, N., 2004. Growing energy. How biofuels can help end America's oil dependence. *Growing energy: how biofuels can help end America's oil dependence*.

Hu, J., Arantes, V., Saddler, J.N., 2011. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect?. *Biotechnology for Biofuel, 4:*36.

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., & Gulati, A., 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current Microbiology, 57*(5), 503-507.

Maki, M. L., Broere, M., Leung, K. T., & Qin, W., 2011. Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers. *International journal of biochemistry and molecular biology*, *2*(2), 146.

Maki, M.L., Indrees, A., Leung, K.T., & Qin, W., 2012. Newly Isolated and characterized bacteria with great application potential for decomposition of lignocellulosic. *Journal of Molecular Microbiology and Biotechnology, 22* (3), 156-166.

Miller, G.L, 1959. Use of dinitrosalicylic acid for the determination of reducing sugar. *Annalytical Chemisrty.31,* 426-428.

Mincer, T. J., Jensen, P. R., Kauffman, C. A., & Fenical, W., 2002. Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments. *Applied and environmental microbiology*, *68*(10), 5005-5011.

Parton, W., Silver, W.L., Burke, I.C., Grassens, L., Harmon, M.E., Currie, W.S., King, J.Y., Adair, E.C., Brandt, L.A., Hart, S.C., Fasth, B., 2007. Global-scale similarities in Nitrogen release pattern during long-term decomposition. *Science, 315*: 361-364.

Qing, Q., Wyman, C.E., 2011. Supplementation with xylanase and β-xylosidase to reduce xylooligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover. *Biotechnology for Biofuels, 4*: 18.

Shoham, Y., Schwartz, Z., Khasin, A., Gat, O., Zosim, Z., & Rosenberg, E. (1993). Delignification of wood pulp by a thermostable xylanase from Bacillus stearothermophilus strain T-6. In *Microorganisms to Combat Pollution* (pp. 83-94). Springer Netherlands.

Verma, V., Verma, A., & Kushwaha, A., 2012. Isolation and production of cellulase enzyme from bacteria isolated from agricultural fields in district Hrdoi, Uttar Pradesh, India. *Advances in Applied Science Research, 3*(1), 171-174.

Wanderley, **K.J., Torres, F.A., Moraes, L.M., & Ulhoa, C.J., 2004**. Biochemical characterization α-amylase from the yeast Cryptococcus flavus. *FEMS Microbiology Letters, 231* (2), 165-169.

Yoshida, M., Lin, Y., Uchida, S., Kawarada, K., Ukagami, Y., Ichinose, H., Kaneko, S., Fukuda, K. (2008). Effects of cellulose crystallinity, hemicellulose and lignin on the enzymatic hydrolysis of Miscanthus sinensis to monosaccharides. *Bioscience, Biotechnology, and Biochemistry., 72*: 805-810.

CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSION

In the past years, culturable, cellulase and xylanase producing bacteria have been isolated from a wide variety of environments including agricultural wastes, composting heaps, decaying plant material, the feces of ruminant animals such as cows, soil and organic matter, and extreme environments like hot springs, to name a few (Doi, 2008). On this work, a number of isolates were obtained from the isolation stage mostly from the sediment samples of the fresh water milieu compared to disturbed water samples. The sediment samples gave a variety of colonies as compared to the disturbed water sample. The colonies differed in size, colour and morphological characteristics and amongst these were the ones that were removed due to similar characteristics.

Screening for cellulase and xylanase production can be done by enrichment growth on microcrystalline cellulose and or beechwood xylan as a sole source of carbon, followed by the extraction of 16S rDNA/RNA to determine the molecular community structure of the environment and analyze whether families containing cellulase or xylanase producing species are present. Strains with lignocellulase potential can be isolated by sub-culturing from the enrichment culture on the agar containing substrates as sole carbon sources. This method was used to identify cellulase-producing bacteria in the deep subsurface of the Homstake gold mine, Lead, South Dakota, USA (Rastogi *et al.,* 2009**)**. For this study the screening for bacterial xylanase and cellulase activity in microbial isolates was performed on carboxymethylcellulose (CMC) containing plates and for xylanase activity beechwood xylan containg plates were used. This method can be timely and zones of hydrolysis are not easily evident but we managed to get good results (Maki *et al.,* 2009).

Kasana and colleagues have recently found that Gram's iodine for plate flooding gave more rapid and highly discernible results compared to hexadecyltrimethyl ammonium bromide or Congo red (Kasana *et al.,* 2008). Similar to our study, the Gram's iodine solution was used as well for flooding the plates during screening and the results were good.

The characterization of cellulase and xylanase producing bacteria has gained more attention due to the readily available abundance of cellulosic and hemicellulosic carbon sources in the world, which can be degraded into reducing sugars and optionally fermented to valuable by-products such as bioethanol as an alternative source of renewable energy (Ragauskas *et al.,* 2006; Maki *et al.,* 2012).

Of all the isolates screened for cellulase and xylanase production, only one isolate **SB1** was able to hydrolyse both cellulose and xylan. Other isolates were only capable of hydrolyzing one substrate each respectively. Five isolates (WY0, SC1, WC2, WC1, SC2) hydrolysed only xylan and the three isolates (SY1, SY2, SY0) hydrolysed carboxymethyl cellulose. However, they all showed a potential in lignocelluloses degradation. SB1 shows to be the best candidate for greater lignocellulolytic activities including degradation of crystalline cellulose, xylanase activities and the ability to degrade lignin. Generally, all of the isolates showed a good industrial potential for degradation of lignocellulosic biomass. However, SB1, which degraded both CMC and xylan, with the greatest halo zone displayed the greatest potential.

Screening requires knowledge or rather an objective for the isolation of specific enzymes with specific activities whether it be with activity on microcrystalline cellulose or endoglucanases with activity on soluble cellulose such as carboxymethyl cellulose (Duan *et al.,* 2009). For this study the screening was targeted on endoglucanases and xylanases since

we screened for carboxymethyl cellulose and beechwood xylan. The isolation and identification of lignocellulases has been limited in the past to culturable microorganisms. However, recent advances in molecular techniques, such as the creation of metagenomic libraries will extend the pool of lignocellulolytic enzymes available for future research.

Bacteria present attractive potencies for exploitation of cellulase and hemicellulase due to

their rapid growth rate and enzyme complexity (Maki *et al.,* 2009). Xylan, the major hemicelluloses component, like carboxymethyl cellulose, requires the synergistic action of several hemicellulase enzymes for its complete hydrolysis to monomer sugars. The major enzyme in this process is endo-1, 4-β-xylanase, which cleaves the glycosidic bonds between xylosides, generating short xylooligosaccharide. Xylanases that comply with bad industrial process conditions are enviable for many biotechnological applications and such enzymes can be specially made by protein engineering or immense microbial diversity could be exploited to get the organisms which produce process suitable xylanases (Bajaj *et al.*, 2012).

Maximum xylanase production was observed after 12 h of fermentation, and thereafter xylanase activity decreased (Fig. 3.9). This may be due to the interaction of xylanase with some other medium/cell-secreted components or due to the inhibition of the enzyme by the end products (Sudan & Bajaj, 2007). The maximum cellulase production was observed after 24 h. The enzyme was also found to be produced maximally at pH 3 and at 45 ºC. Nevertheless, the optimum time for maximum enzyme production by different *Bacillus spp.* has been reported to be as low as 18 h or up 2–4 days (Yang *et al.*, 1995; Sa´. Pereria *et al.*, 2002). Thus, our study has revealed the same for our *Bacillus cereus.*

In conclusion, as the world's present economy is highly dependent on various fossil energy sources such as oil, coal, natural gas, to name a few. These are being used for the production

of fuel, electricity and other goods. Excessive consumption of fossil fuels, particularly, in urban areas, has resulted in generation of high levels of pollution during the few decades. The level of greenhouse gases in the earth's atmosphere has drastically increased**.** In this regard, lignocellulosic biomass holds considerable potential to meet the current energy demand of the modern world. This is also crucial to overcome the excessive dependence on petroleum for liquid fuels. Further advanced biotechnologies are crucial for discovery, characterization of new enzymes, and production in homologous or heterologous systems and ultimately lead to low-cost conversion of lignocellulosic biomasses into bio-fuels and bio-chemicals. In the current situation, future trends are being directed to lignocellulose biotechnology and genetic engineering for improved processes and products. To overcome the current energy problems it is advised that lignocellulosic biomass in addition of green biotechnology will be the main focus of the future research.

References

Bajaj, **B. K., Khajuria, Y. P., & Singh, V. P., 2012**. Agricultural residues as potential substrates for production of xylanase from alkali-thermotolerant bacterial isolate. *Biocatalysis and Agricultural Biotechnology*, *1*(4), 314-320.

Doi, R. 2008. Cellulases of mesophhilic microorganisms. *Annals of the New York Academy of Sciences 1125*, 1(2008): 267.

Duan, C.J., Xian, L., Zhao, G.C., Feng, Y., Pang, H., Bai, X.L., Tang, J.L., Ma, Q.S., Feng, J.X., 2009. Isolation and partial characterization of novel genes encoding acidic cellulases from metagenomes of buffalo rumens. Journal of *Applied Microbiology, 107*(1), 245-256.

Kasana, R. C., Salwan, R., Dhar, H., Dutt, S., &Gulati, A., 2008.A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. *Current microbiology*, *57*(5), 503-507.

Maki, M., Leung, K. T., & Qin, W., 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. *International Journal of Biological Sciences*, *5*(5), 500.

Maki, M.L., Indrees, A., Leung, K.T., & Qin, W., 2012. Newly Isolated and characterized bacteria with great application potential for decomposition of lignocellulosic. *Journal of Molecular Microbiology and Biotechnology, 22* (3), 156-166.

Ragauskas , **AJ, Williams CK, Davison BH, Britovsek G, Cairney J, Eckert CA, Frederick WJ Jr, Hallett JP, Leak DJ, Liotta CL, Mielenz JR, Murphy R, Templer R, Tschaplinski T:, 2006**. The path forward for biofuels and biomaterials. *Science; 311*: 484–489.

Rastogi, G., Muppidi, G.L., Gurram, R.N., Adhikari, A., Bischoff, K.M., Hughes, S.R., Sani, R.K., 2009. Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. *Journal of Industrial Microbiology & Biotechnology*, *36* (4), 585-598.

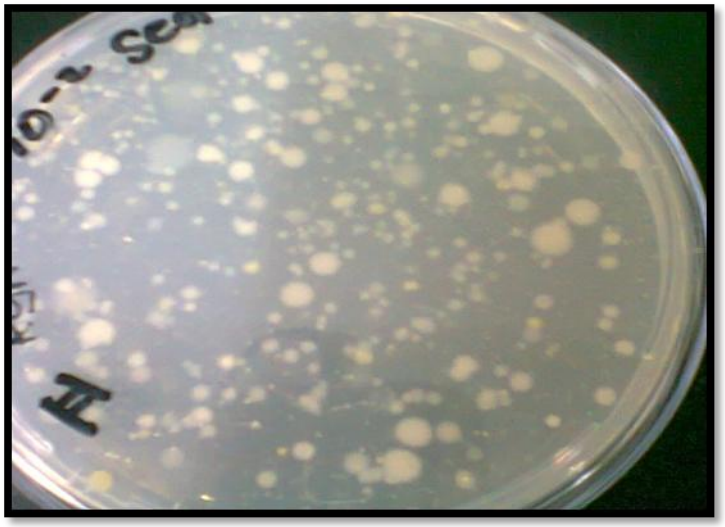
Sa Pereria, P., Mesquita, A., Duarte, J.C., Barroe, M.R.A., Costa-Ferreira, M., 2002. Rapid production of thermostable cellulase –free xylanase by a strain of Bacillus subtilis and its properties. *Enzyme Microbiol. Technol. 30,*924-933.

Sudan, R., Bajaj, B.K., 2007. Production and biochemical characterization of xylanase from an alkalitolerant novel species *Aspergillus niveus* RS2. *World journal of Microbiology and Biotechnology.23*, 491-500.

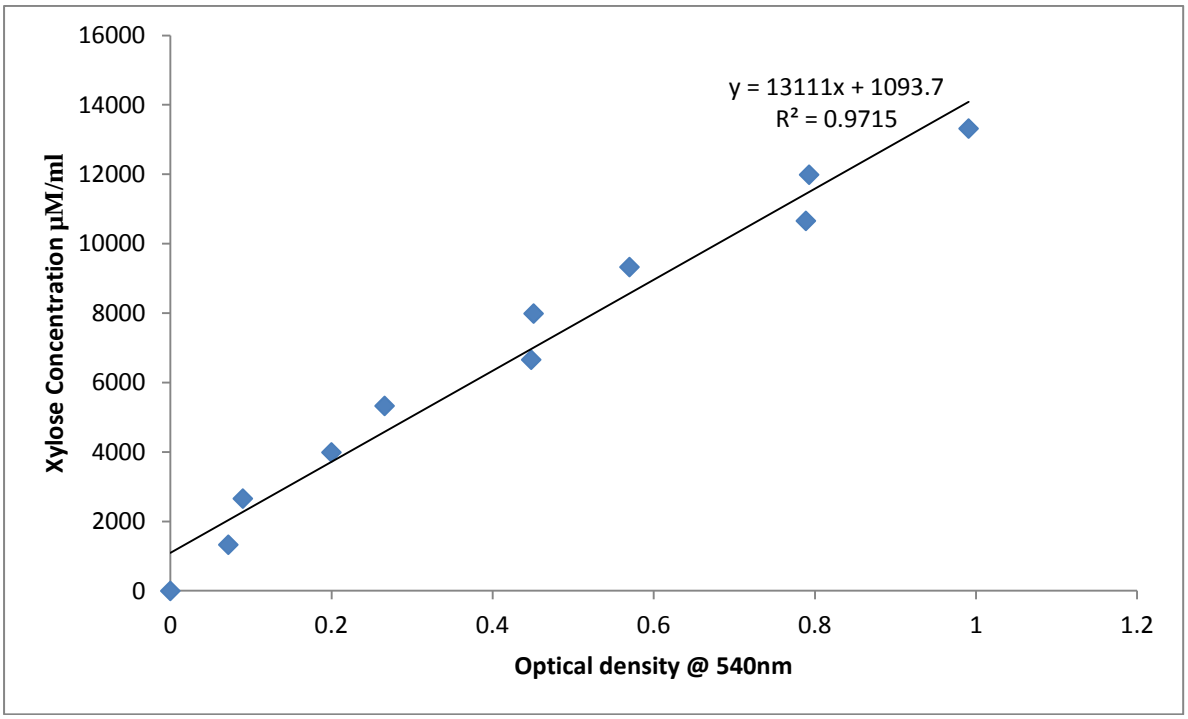
Yang, V.W., Zhuang, Z, Elegir, G., Jeffries T.W., 1995. Alkaline active xylanase produced by an alkalophilic *Bacillus sp.* Isolated from kraft pulp. *Journal of Indian microbiology. 15,* 434-441.

APPENDIX A: Sample collection by student

APPENDIX B: Colonies obtained through spread plate technique on M1 agar plate



APPENDIX C: Xylose standard curve



APPENDIX D: Glucose Standard curve