STUDIES ON LIGNOCELLULOSE FROM FUNGI ISOLATED FROM FADAMA SOIL AT DUTSE LOCAL GOVERNMENT AREAS OF JIGAWA STATE

BY

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A THESIS SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, BAYERO UNIVERSITY KANO, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MASTERS OF SCIENCE (M.SC) DEGREE IN MICROBIOLOGY (INDUSTRIAL)

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DECLARATION

I SA'ADAT MUKHTAR ISMA'IL (SPS/13/MMB/00025) humbly declare that this project work entitled STUDIES ON LIGNOCELLULOSE FROM FUNGI ISOLATED FROM FADAMA SOIL AT DUTSE LOCAL GOVERNMENT AREAS OF JIGAWA STATE is as a result of my research effort carried out in the department of microbiology, Bayero University Kano, under the supervision of **Dr. Sani Yahaya**. I further wish to declare the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of other degree or diploma of any University or other institute of higher learning, except where due acknowledgement has been made in the text.

_____

Sa'adat Mukhtar Isma'il

Date: _____

CERTIFICATION

This is to certify that this dissertation entitled STUDIES ON LIGNOCELLULOSE FROM FUNGI ISOLATED FROM FADAMA SOIL AT DUTSE LOCAL GOVERNMENT AREAS OF JIGAWA STATE was carried out by Sa'adat Mukhtar Isma'il (SPS/13/MMB/00025) under my/our supervision.

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APPROVAL

The undersigned have certified that this work was carried out by Sa'adat Mukhtar Isma'il (SPS/13/MMB/00025) and it is adequate in scope and quality for the partial fulfillment of the requirements for the award of degree of M.Sc. in Microbiology (Industrial), Faculty of Science, Bayero Univeristy, Kano, Nigeria.

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DEDICATION

This project work is fully dedicated to God Almighty and to my beloved in-law, Alhaji Ibrahim Shettima (May his soul rest in peace).

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ABSTRACT

This research work was aimed at isolating and identifying fungal species with the potential to produce lignocellulose degrading enzymes and optimizing their culture production. Soil samples were obtained from Madaki, Dadin Duniya and Federal University Dutse from Dutse local government area of Jigawa State. The fungal species isolated from the soil using the pour plate technique were *Aspergillus flavus, Aspergillus fumigatus, Alteneria* spp. *Mucor* spp and *Rhizopus* spp. The five fungal species were screened for their ability to grow on wood containing agar medium and decolorization of synthetic dye, Remazol Brilliant Blue R (RBBR) and methyl green as an indicator for the production of lignocellulose degrading enzymes. *A. fumigatus* was found to have the highest lignocellulose degrading enzymes ability and hence was grown in sawdust and wheat bran liquid medium for optimization process.The amount of reducing sugar was estimated by the DNS method of Miller. The optimum temperature for cellulase activity using wheat bran as substrate was obtained at 45^{o}C with reducing sugar concentration of 0.47mg/ml with pH of 6 with concentration of 0.50mg/ml at 20 days of incubation with concentration of 0.51mg/ml, 1% substrate concentration of 0.53mg/ml with 10^{-3} spore suspension of inoculum size with reducing sugar concentration of 0.49mg/ml. Cellulase activity using sawdust was high at temperature of 45oC with concentration of 0.44mg/ml, pH of 4, at 15days of incubation with reducing sugar concentration of 0.50mg/ml with 4% substrate concentration and 10-4 inoculum size with reducing sugar concentration of 0.55mg/ml and 0.47mg/ml respectively. For xylanase, it was observed at temperature of 35^{o}C with reducing sugar concentration of 0.62mg/ml at pH of 6 with concentration of 0.57mg/ml at 20days of incubation using 1% substrate concentration (wheat bran) and 10^{-3} spore suspension with concentration of 0.58mg/ml and 0.56mg/ml respectively. Xylanase activity was optimum at temperature of 35^{o}C with concentration of 0.47mg/ml at pH of 5 with concentration of 0.51mg/ml at 10days of incubation with concentration of 0.52mg/ml using 5% substrate concentration (sawdust) with reducing sugar concentration of 0.6mg/ml and 10^{-4} inoculum size with concentration 0.50mg/ml. The isolate was found to produce high amount of cellulase and xylanase enzymes when they were grown in wheat brain liquid medium, although, lignin degrading enzymes production was negligible.

CHAPTER ONE

1.0 INTRODUCTION

In recent years the high cost of carbon sources for industrial production of microbial enzymes has necessitated a shift to other cheaper sources of carbon (Ajayi and Fagade, 2003). Plants are the most common source of renewable carbon and energy on earth with annually lignocellulose production estimate of about 4 x 10^9 tons (Coughlan and Mayer, 1990). Lignocellulose describes the three major constituents in plants, namely cellulose, hemicelluloses and lignin. The composition of lignocelluloses depends not only on the species but also on the growth conditions, the different parts of the plant and their age (Jørgensen, 2003). Lignocellulose is a network of lignin, cellulose and hemicellulose that is chemically bonded through non-covalent forces and covalent cross-linkages (Perez *et al.,* 2002), and it is the major structural component of woody plants. Cellulose and hemicellulose are carbohydrate polymers, while lignin is a complex aromatic polymer. In woods, lignin physically surrounds and protects the carbohydrate polymers from enzymatic hydrolysis and is also the most recalcitrant component of plant cell wall (Chandel *et al*., 2013).

Although, many microorganisms are capable of degrading and utilizing cellulose and hemicelluloses as carbon and energy sources, only a smaller group of filamentous fungi known as white-rot fungi possess the unique ability to efficiently degrade lignin (Sanchez, 2009). When wood-decomposing fungi attack woods, a range of degradative extracellular, enzymatic and non-enzymatic activities are carried out, and these alter the wood chemically and morphologically, resulting in three major types of rot: white, brown and soft rots. These enzymatic activities are performed by complex mixtures of cellulases, hemicellulases, and ligninases (Sanchez, 2009). The abundance of lignocellulosic materials make them potentially inexpensive and readily available natural resources for the manufacturing of high value compounds and for biofuels production in a sustainable environment. The use of these materials involves a separation of the polymeric compounds - cellulose and hemicelluloses. The spontaneous degradation of these compounds are extremely slow, but there are microorganisms in soil and in the rumen of ruminants, capable of degrading them to sugars which then can be utilized as energy and carbon source by various microorganisms for the production of different products. These organisms are capable of growing on lignocellulosic materials and thus produce a wide range of enzymes that could be of scientific or industrial importance.

Lignocellulose is a general term for an assemblage of lignin (18-30%), cellulose (28 – 50%), and hemicellulose (20– 30%) (Breznak and Brune 1994, Enari 1983). Lignin is a non-repetitive structure of various phenylpropanoid subunits (Breznak and Brune 1994). Composition of lignin varies; for example, hardwood lignin consists mainly of guaiacyl- and syringylpropane subunits, whereas softwood lignin consists mainly of guaiacylpropane subunits. Also, grass lignin mainly consists of guaiacyl-, syringyl-, and 4-hydroxyphenylpropane subunits with about 5 – 10 % esterified aromatic acids (Breznak and Brune 1994). Cellulose is an unbranched homopolymer of glucose molecules covalently joined by β-1,4-glycosidic linkages (Enari 1983, Martin 1987). Hemicellulose is a branched polymer of a variety of sugars, including L-arabinose, D-galactose, D-glucose, D-mannose, D-xylose, and 4-O-methylglucuronic acid (Breznak and Brune 1994, Enari 1983, Shallom and Shoham 2003). Most cellulose in plants is in highly ordered crystalline bundles called fibrils or microfibrils, which give a plant its rigidity. The cellulose fibrils are surrounded by a matrix of lignin and hemicellulose that are covalently bonded to each other at various intersections. The structural complexity of these polysaccharides increases the recalcitrance of each individual component. For example, cellulases cannot attack cellulose fibrils until the lignin-hemicellulose matrix is broken through (Breznak and Brune 1994, Enari 1983, Martin 1987). The combination of enzymes within the cellulosome act in synergy to degrade lignocellulose more effectively than any of the enzymes alone (Bayer *et al.,* 2004, Doi *et al.,* 2003). Many fungal species are saprophytic and efficient degraders of major polymers such as lignin and cellulose (Sivaramanan, 2014). The extracellular ligninolytic enzymes of white rot fungi have the ability to degrade a wide range of recalcitrant organo-pollutants such as chlorinated phenols and various types of synthetic dyes. Therefore, studying wood decay fungi and their abilities will not only help in the understanding of the economic threats they pose, but also in discovering their biotechnological abilities (Guillén *et al*., 2011). Natural pastures are one of Nigerian most important agricultural resources for it provides the cheapest source of animal feed. Natural grazing is threatened by expansion of the Karoo (semi-desert region) into grasslands, overgrazing, poisonous plants, poor grazing management practices, and bush densification.

Finally, expanding human activities (agriculture, housing, and industrialization) places a demand on already overburdened natural grazing resources. Therefore, the use and re-use of lignocellulosic wastes for animal feed will become a Nigerian reality in the near future. Much lignocellulose waste is often disposed of by biomass burning, which is not restricted to

underdeveloped countries alone, but is considered a global phenomenon. The problem of increasing the utility of lignocellulose wastes has been known for decades. In addition to the growing demand for traditional applications (paper manufacture, biomass fuels, composting, animal feed, etc.), novel markets for lignocellulosics have been identified in recent years. The intensity of research and the magnitude of capital investment in this field increased vastly once commercial viability seemed probable for many of these new applications. The most ambitious of these has been the conversion of lignocellulose to alternative energy carriers e.g. fuel ethanol, acetone and butanol, (Lee, 1997). The pulp and paper industry discovered lignocellulose biotechnology could improve process efficiency through savings in money and energy (Breen and Singleton 1999; Scott *et al.,* 1998). Others aimed at improving digestibility of nutritionally poor forages by exposing these lignocellulosics to white-rot fungi (Agosin and Odier 1985; Karunanandaa *et al.,* 1992).

Justification

Bacteria which has high growth rate as compared to fungi has good potential to be used in lignocellulase production. However, the application of Bacteria in producing lignocellulase is not widely used. Bacterial cellulase usually lacks one of the cellulase activities, which is FPase. However, fungal soil isolates capable of transforming lignocellulose derivatives could be interesting in soil biochemistry and applied biotechnology, particularly for soil bioremediation. This is needed especially in order to enhance the production.

Aims and objectives

Aims

To isolate and identify fungal species capable of degrading lignocellulose (cellulose, hemicellulose and lignin.) and evaluating their optimal culture condition for enzyme production.

Objectives

- To isolate and identify fungal species with lignocellulytic activity from fadama soil.
- To screen the isolate for their potential to degrade lignocellulose.
- To determine and evaluate optimal culture condition for enzyme production

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Lignocellulose

Cell walls of plants consist mainly of three organic compounds: cellulose, hemicellulose, and lignin. These compounds are also major components of natural lignocellulosic materials. Cellulose molecules arrange regularly, gather into bundles, and determine the framework of the cell wall. Fibers are filled with hemicellulose and lignin. The structure of the plant cell wall is compact. There is different bonding among cellulose, hemicellulose, and lignin. Cellulose and hemicellulose or lignin molecules are mainly coupled by a hydrogen bond. In addition to the hydrogen bond, there is the chemical bonding between hemicellulose and lignin, which results in the lignin, isolated from natural lignocelluloses, always contains a small amount of carbohydrates. The chemical bonds between the hemicellulose and lignin mainly refer to the chemical bonds between galactose residues, arabinose residues on the side chains of hemicellulose molecules and lignin, and carbohydrates. Cell walls mainly consist of cellulose, hemicellulose, and lignin in a 4:3:3 ratio. This ratio differs from sources such as hardwood, softwood, and herbs. Besides these three components, natural lignocellulosic materials contain a small amount of pectin, nitrogenous compounds, and the secret ash. For instance, the element content of wood is about 50% carbon, 6% hydrogen and 44% oxygen.

2.2 Cellulose

Enormous amounts of agricultural, industrial and municipal cellulosic wastes have been accumulating or used inefficiently due to the high cost of their utilization processes (Lee and Koo, 2007). Cellulose, a polymer of glucose residues connected by β-1, 4 linkages, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature. Therefore, it has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic wastes as inexpensive carbon sources. Cellulase is the enzyme that hydrolyzes the β-1, 4-glycosidic bonds in the polymer to release glucose units (Lednicka *et al*., 2002). This cellulose degrading enzyme can be used, for example, in the formulation of washing powders, extraction of fruit and vegetable juices and starch processing. Cellulases are produced by large number of microorganisms. They are either cell-bound or extracellular. Although a large number of microorganisms can degrade cellulose,

only a few of them produce significant quantities of free enzyme capable of completely hydrolyzing crystalline cellulose (Lynd *et al*., 2002).

Cellulose is a simple polymer, but it forms insoluble, crystalline microfibrils which are highly resistant to enzymatic hydrolysis (Beguin and Aubert, 1994). The ability to digest cellulose is restricted to microorganisms. Cellulolytic organisms are widely distributed among many genera in the domain bacteria and in the fungal groups within the domain Eukarya, although no cellulolytic members of the domain Archaea have yet been identified (Lynd *et al*., 2002). Until now the investigation of cellulolytic microorganisms in soil has been based on their cultivation (Lednicka *et al*., 2002; Waniick *et al.*, 2002).

Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulase is used extensively in the textile and food industries, bioconversion of lignocellulosic wastes to alcohol, animal feed industry as additive, isolation of plant protoplasts, in plant virus studies, metabolic investigations and genetic modification experiments (Evans and Bravo, 1983; Bhat, 2000).

Cellulase is one of the most useful enzymes in industry. Cellulase can be produced by fungi, bacteria or actinomycetes, but the most common producer is fungi. High cost of cellulase is mainly due to the substrates used in production, and also the slow growth rate of fungi.

Cellulose is the major component in wood, comprising nearly half of both softwoods and hardwoods. It is a high molecular weight homopolymer, comprised of β-D-glucopyranose units which are linked together by glycosidic bonds.It is these glycosidic bonds that allow cellulose to arrange in its crystalline form,which is also responsible for its low surface area. In nature, individual linear glucosyl chains undergo self-assembly at the site of biosynthesis (Brown and Saxena, 2000). An important feature of cellulose, unusual for a polysaccharide, is its crystalline structure. Also, the crystallinity of cellulose varies according to its source. For example, microcrystalline cellulose is nearly 60% crystalline, whereas the crystallinity of Scots pine and Norway spruce varied between 24%-31% and 23%-32%, respectively. The difference in crystallinity in pine and spruce samples is attributed to the location of the sample in the tree, with the higher crystallinity corresponding to older year rings (Andersson *et al*., 2004).

2.2.1 History of Cellulose

Cellulose was discovered in 1838 by the French Chemist Anselme Payen, who isolated it from plant matter and determined it's chemical formular (Crawford, 1981) cellulose was used to produce the first successful thermoplastic polymer, celluloid, by Hyatt manufacturing company in 1870. Hermann Staudinger determined the polymer structure of cellulose in 1920. Cellulose is the structural component of the primary cell wall of green plants, many forms of algae and the *oomycetes.* Some species of bacteria secret is to form biofilms (Andreas, 2005). Cellulose is the most common organic compound on earth. About 33% of all plant matter is cellulose (the cellulose content of cotton is 90% and that of wood is 40 – 50%).

2.2.2 Structure and Properties Cellulose

Cellulose has no taste, is odourless, hydrophilic with the contact angle of 20 – 30. It is insoluble in water and most organic solvents, is chiral and is biodegradable. It can be broken down chemically into its glucose units by treating it with concentrated acids at high temperature. Compared to starch, cellulose is also much more crystalline. Whereas starch undergoes a crystalline to amorphous transition when heated beyond 60 – 70^{o}C in water (as in cooking), cellulose requires a temperature of 320^{o}C and pressure of 25 MPa to become amorphous in water (Deguchi, 2006).

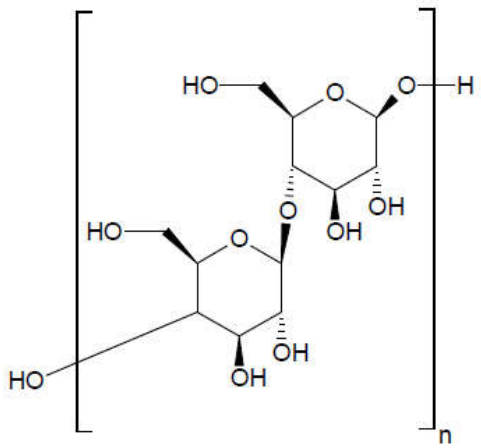
Several different crystalline structures of cellulose are known, corresponding to the location of hydrogen bonds between and within strands. Natural cellulose is cellulose I, with structures I and IB. cellulose produced by bacteria and algae is enriched in I while cellulose of higher plants consists mainly of IB. Cellulose in regenerated cellulose fibers is cellulose II. The conversion of cellulose I is metastable and cellulose II is stable. With various chemical treatments, it is possible to produce the structures cellulose III and cellulose IV. Native crystalline cellulose is insoluble and occurs as fibers of densely packed, hydrogen bonded, anhydroglucose chains of 15 to 10,000 glucose units. It's density and complexity make it very resistant to hydrolysis without preliminary chemical or mechanical degradation or swelling. In nature, cellulose is usually associated with other polysaccharides such as xylan or lignin. It is the skeletal basis of plant cell wall (Spano *et al.,* 1975). Cellulose is the most abundant organic source of food, fuel and chemicals. However, its usefulness is dependent upon it's hydrolysis to glucose. Acid and high temperature degradation are unsatisfactory, in

that, the resulting sugars are decomposed, also waste cellulose contains impurities that generate unwanted by-products under these harsh conditions (Spano *et al.,* 1975).

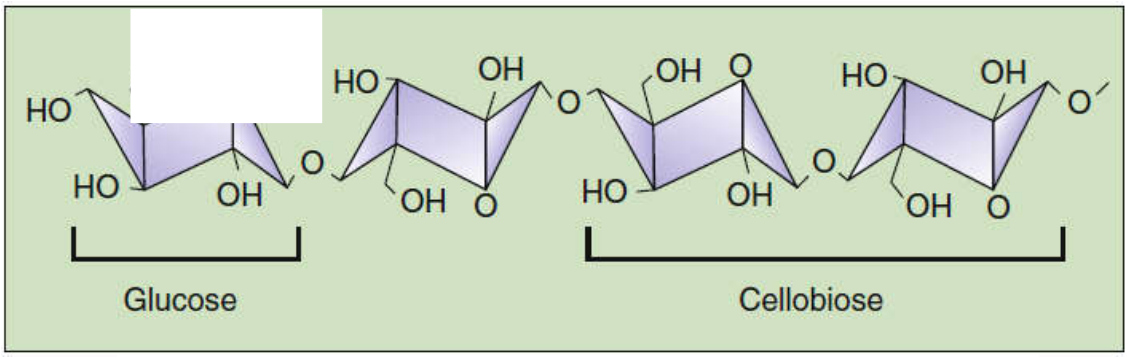
Many properties of cellulose depend on its chain length or degree of polymerization, the number of glucose units that make up one polymer molecule. Cellulose from wood pulp has typical chain lengths between 300 and 1700 units; cotton and other plant fibers as well as bacterial celluloses have chain lengths ranging from 800 to 10, 000 units, (Klemm, 2005). Molecules with very small chain length resulting from the breakdown of cellulose are known as cellodextrins; in contrast to long-chain cellulose, cellodextrins are typically soluble in water and organic solvents (Andreas, 2005).

2.2.3 Chemical Structure of Cellulose

Cellulose is a linear homopolymer composed of D-glucopyranose units linked by “-1,4-glycosidic bonds. It mainly contains carbon (44.44%), hydrogen (6.17%), and oxygen (49.39%). The chemical formula of cellulose is $(C_6H_{10}O_5)n$; n, called the degree of polymerization (DP), represents the number of glucose groups, ranging from hundreds to thousands or even tens of thousands. Cellulose consists of pure dehydrated repeating units of D-glucoses (as shown below), and the repeating unit of the cellulose is called cellobiose. Bundles of cellulose molecules aggregate together in the form of microfibrils with alternating highly ordered crystalline and disordered amorphous regions (Sjöström, 1981). The crystalline nature of cellulose dictates that the component molecules of each microfibril are packed tightly enough to prevent the penetration of other molecules even as small as water,thereby impeding hydrolysis and making the dissolution and subsequent treatment of cellulose difficult (Lynd *et al.,* 2002).



Chemical structure of Cellulose



Molecular chain structure of cellulose (Zugenmaier, 2001)

2.2.4 Physical Structure of Cellulose

The physical structure of cellulose refers to the spatial arrangement of different scale structural units, including the chain structure and aggregation structure of the polymer. The chain structure, also known as the primary structure, shows the geometric arrangement of the atoms or groups in the molecular chain. The short range structure is the first-level structure and refers to the chemical structure or stereochemical structure of one or several structural units in a single-molecule polymer. Remote structure is the second-level structure and refers to the size of a single-molecule polymer and a special structure. The aggregation structure, also called the secondary structure, refers to the inner structure of the whole polymer, including the crystal structure, noncrystal structure, orientational structure, and liquid crystal structure. The third-level structure term is used to describe how molecules in a polymeric aggregate accumulate each other, such as tangly clew structure and crystal structure formed with ordered folding chains. The chain structure of the polymer is the main structural hierarchy that reflects many characteristics of a polymer, such as melting point, density, solubility, viscosity, adhesion, and so on. The aggregation structure of a polymer is the major factor that determines the service performance of macromolecular compound products (Zhan, 2005).

2.2.5 Commercial Uses of Cellulose

Cellulose is used to make water–soluble adhesives and binders such as methyl cellulose and carboxymethyl cellulose which are used in wall paper paste. Microcrystalline cellulose and powdered cellulose are used as inactive fillers in tablets (Lois, 2002) and as thickeners and stabilizers in processed foods. Cellulose is used in the laboratory as the stationary phase for thin layer chromatography. Cellulose fibres are also used in liquid filtration, sometimes in

combination with diatomaceous earth or other filtration media to create a filter bed of inert material. Cellulose is further used to make hydrophilic and highly absorbent sponges. Cellulose insulation made from recycled paper is becoming popular as an environmental preferable material for building insulation. It can be treated with boric acid as a fire retardant.

Cellulose is mainly obtained from wood pulp and cotton. It is mainly used to produce paper board and paper; to a smaller extent it is converted into a wide variety of derivative products such as cellulosic ethanol which is under investigation as an alternative fuel source. Some animals particularly ruminants and termites can digest cellulose with the help of symbiotic microorganisms that live in their guts. Humans can digest cellulose to some extent, (Joshi, 1995). However, it is often referred to as "dietary fiber" or "roughage" (e.g outer shell of maize) and acts as a hydrophilic bulking agent for feces.

2.2.6 Types and Action of Cellulases

Five general types of cellulases based on the type of reaction catalyzed are: -

1. Endocellulase breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains.

2. Exocellulase cleaves two to four units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharides such as cellobiose. There are two main types of exocellulases (or cellobiohydrolases) one type works processively from the reducing end and one type works processively from the non reducing end of cellulose.

3. Cellobiase or beta – glucosidase hydrolyses the exocellulase product into individual monosaccharides.

4. Oxidative cellulases depolymerize cellulose by radical reactions, as for instance cellobiose dehydrogenase (acceptor).

5. Cellulose phosphorolases depolymerize cellulose using phosphates instead of water. (Sulzenbacher *et al*., 1997).

2.2.7 Application of cellulases

Celluloses were initially investigated several decades back for the bioconversion of biomass which gave way to research in the industrial applications of the enzyme in animals feed, food, textile and detergents and in the paper industry (Xia and Cen, 1999). With the shortage of

fossil fuels and the arising need to find alternative source of renewable energy and fuels, there is a renewable of interest in the bioconversion of lignocellulosic biomass using cellulases and other enzymes in the other fields, however, the technologies and products using cellulases have reaches the stay where these enzymes have become indispensable.

2.2.7.1 Textile Industry

Cellulases have become the largest groups of enzymes used in the industry (Xia and 1999). They are used in the biostoning of denim garments for producing softness and the faded look of denim garment replacing the use of pumice stones which were traditionally employed in the industry (Belghith *et al*., 2001). They act on the cellulose fibre to releases the indigo dye used for cooking the fabric, producing the faded look of denim. *H. insolens* cellulases is the most commonly employed in the biostoning, through use of acidic cellulases from *Trichoderma* along with proteases is found to be equally good. Cellulases are utilized for digesting of the small fibre ends protruding from the fabric resulting in a better finish (Galante *et al*., 1998). Cellulases have also been used in softening (Andersen, 2000), defibrillation and in processes for providing localized variation in the color density of fibers (Olson and Stanley, 1991).

2.2.7.2 Laundry and Detergents

Cellulases, in particular EG111 and CBH1 are commonly used in detergents for cleaning textile. Several reputs disclose that EG111 variants in particular from I. reasei, are suitable for the use in detergents. *T. viridue* and *T. harzianum* are also industrially utilized natural sources of celluases, as *A. niger*. Cellulases preparations mainly from species of *Humicola* (*H. insolens* and *H. grisea var. thermoses* that are active under mild alkaline conditions and at elevated temperatures, are commonly added in washing powders and in detergents (Uhlig, 1998).

2.2.7.3 Food and Animal Feed

In food industry, cellulases are used in extraction and clarification of fruit and vegetable juice, production of fruit nectars and purees, and in the extraction of olive oil (Galante *et al*., 1998). Glucanases are added to improve the malting of barley in beer manufacturing (Barbesgaard *et al*., 1984) and wine industry better maceration and color extraction is achieved by use of exogenous hemicelluloses and glucanases. Celluloses are also used in carotenoid extraction in the production of food coloring agents (Pajunen, 1986). Enzymes

preparations containing hemicelluloses and pectinase in addition to cellulases are used to improve the nutritive quality of forages. Improvements in feed digestibility and animal performance are reported with the use of cellulases in feed processing (Lewis *et al*., 1996).

2.2.7.4 Pulp and Paper Industry

In the pulp and paper industry, cellulases and hemicellulases have been employed for biomechanical pulping for modification of the coarse mechanical pulp and hand sheet strength properties (Bedford *et al*., 2003) de-inking of recycled fibrer and for improving drainage and renability of paper mills. Cellulasese are employed in the removing of inks, coating and toners from paper. Biocharacterization of pulp fibers is another application where microbial cellulases are employed. Cellulases are also used in preparation of easily biodegradable cardboard. The enzyme is employed in the manufacture of soft paper including paper towels and sanitary paper and preparations containing cellulases are used to remove adhered paper (Sharyo, 2002).

2.2.7.5 Biofuel

A potential application of cellulases is the conversion of cellulaosic materials to glucose and other fermentable sugars, which in turn can be used as microbial substrates for the production of single cell proteins or a variety of fermentation products like ethanol. Organisms with cellulases systems that are capable o converting biomass to alcohol directly are already reported (Sharyo, 2002). The strategy employed currently in bioethanol production from ligoncellulosic residues is a multi-step process involving pre-treatment of the residue to remove lignin and hemicellulases fraction, cellulases treatment at 50^0C to hydrolyze the cellulosic residue to generate fermentable sugars and finally use of a fermentative microorganism to produce alcohol from the hydrolyzed cellulosic material.

Apart from these common application cellulases are also employed in formulations for removal of industrial slime, in research for generation of protoplast (Wiatr, 1990), and for generation of antibacterial chit oligosaccharides, which could be used in food preservation, immuno- modulation (Tsai *et al*., 2000), and as a potent antitumor agent (Tsai, 2004). Currently, cellulase is commonly used in many industrial applications, especially in animal feed, textile, waste water, brewing and wine – making, (Beguin *et al*., 1985). With the shortage of petroleum,increase of green-house gases and air pollution due to the incomplete

combustion of fossil fuel,there has been increasing worldwide interest in the production of bioethanol from lignocellulosic biomass (Zaldivar and Nielson, 2001).

2.2.7.6 Bioethanol Industry. Enzymatic saccharification of lignocellulosic materials such as sugarcane bagasse, corncob, rice straw, Prosopis juliflora, Lantana camara, switch grass, saw dust, and forest residues by cellulases for biofuel production is perhaps the most popular application currently being investigated (Sukumaran *et al.,* 2005). Bioconversion of lignocellulosic materials into useful and higher value products normally requires multistep processes (Ghosh and Singh 1993). These processes include; pretreatment (mechanical, chemical, or biological), hydrolysis of the polymers to produce readily metabolizable molecules (e.g., hexose and pentose sugars), bioconversion of these smaller molecules to support microbial growth and/or produce chemical products, and the separation and purification of the desired products. The utility cost of enzymatic hydrolysis may be low compared with acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4–6 and temperature 45–50◦C) and does not have corrosion issues (Gupta *et al*., 2011). Technologies are currently available for all steps in the bioconversion of lignocellulosics to ethanol and other chemical products (Mosier *et al*., 2005). However, some of these technologies must be improved to produce renewable biofuel and other byproducts at prices, which can compete with more conventional production systems. Not only the recalcitrance of the substrate, but also several other factors that also limit cellulase efficiency during the hydrolysis process including end product inhibition, thermal deactivation of the native protein, nonspecific binding to lignin, and irreversible adsorption of the enzymes to the heterogeneous substrate (Taniguchi *et al.,* 2005).

2.2.7.7 Wine and Brewery Industry. Microbial glucanases and related polysaccharides play important roles in fermentation processes to produce alcoholic beverages including beers and wine (Bamforth, 2009). These enzymes can improve both quality and yields of the fermented products. Glucanases are added either during mashing or primary fermentation to hydrolyze glucan, reduce the viscosity of wort, and improve the filterability. In wine production, enzymes such as pectinases, glucanases, and hemicellulases play an important role by improving color extraction, skin maceration, must clarification, filtration, and finally the wine quality and stability. Glucosidases can improve the aroma of wines by modifying glycosylated precursors. Macerating enzymes also improve pressability, settling, and juice yields of grapes used for wine fermentation. A number of commercial enzyme preparations are now available to the wine industry. The main benefits of using these enzymes during wine

making include better maceration, improved color extraction, easyclarification, easy filtration, improved wine quality, and improved stability. Beer brewing is based on the action of enzymes activated during malting and fermentation. Malting of barley depends on seed germination, which initiates the biosynthesis and activation of α- and β-amylases, carboxypeptidase, and β- glucanase that hydrolyze the seed reserves (Bamforth, 2009).

2.2.7.8 Agricultural Industries. Various enzyme preparations consisting of different combinations of cellulases, hemicellulases, and pectinases have potential applications in agriculture for enhancing growth of crops and controlling plant diseases (Chet *et al.,*1998). Plant or fungal protoplasts produced using microbial hydrolases can be used to produce hybrid strains with desirable properties. Cellulases and related enzymes from certain fungi are capable of degrading the cell wall of plant pathogens in controlling the plant disease. Fungal β-glucanases are capable of controlling diseases by degrading cell walls of plant pathogens. Many cellulolytic fungi including *Trichoderma* sp*., Geocladium* sp*., Chaetomium* sp*., and Penicillium* sp. are known to play a key role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system and increased crop yields (Bailer and Lumsden, 1998). Although these fungi have both direct (probably through growth-promoting diffusible factor) and indirect (by controlling the plant disease and pathogens) effects on plants (Harman *et al*., 1998), it is not yet clear how these fungi facilitate the improved plant performance.

Cellulases have also been used for the improvement of the soil quality. Traditionally straw incorporation is considered an important strategy to improve soil quality and reduce dependence on mineral fertilizers (Tejada *et al*., 2008). Many studies have attempted to hasten straw decomposition via microbial routes. Cellulolytic fungi applications such as *Aspergillus, Chaetomium*, and *Trichoderma*, (Tiwari *et al.,* 1987), and *Actinomycetes* have shown promising results. Fontaine *et al.,* 2004 showed that exogenous cellulase supplementation accelerated decomposition of cellulose in soil. Therefore, using exogenous cellulase may be a potential means to accelerate straw decomposition and increase soil fertility.

2.2.7.9 Olive Oil Extraction. In recent years, extraction of olive oil has attracted the interest of international market because of its numerous health claims. Extraction of olive oil involves, crushing and grinding of olives in a stone or hammer mill; passing the minced olive paste through a series of malaxeurs and horizontal decanters; highspeed centrifugation to

recover the oil. To produce highquality olive oil, freshly picked, clean, and slightly immature fruits have been used under cold pressing conditions (Faveri *et al*., 2008). However, high yields have been obtained with fully ripened fruit, when processed at higher than ambient temperatures, but this resulted in oil with high acidity, rancidity, and poor aroma. Hence, an improved method for the extraction of high-quality olive oil was needed to meet the growing consumer demand. The commercial enzyme preparation, Olivex (a pectinase preparation with cellulase and hemicellulase from *Aspergillus aculeatus*), was the first enzyme mixture used to improve the extraction of olive oil (Faveri *et al.,* 1977). Furthermore, the use of macerating enzymes increased the antioxidants in extravirgin olive oil and reduced the induction of rancidity.

2.2.7.10 Carotenoid Extraction. Carotenoids are the main group of coloring substances in nature being responsible for many plant colors from red to yellow (Cinar, 2005). There is a continuously growing market for carotenoids as food colorants due to their desirable properties, such as their natural origin, null toxicity, and high versatility, providing both lipo- and hydrosoluble colorants with colors ranging from yellow to red. In addition, provitamin A activity, a role in lipid oxidation, and anti carcinogenic properties are very important biological functions of these pigments (Cinar, 2005). Usually a combination of cellulolytic and pectinolytic enzymes accelerates the rate of hydrolysis for achieving complete liquefaction. Cellulase randomly splits cellulose chains into glucose whereas commercial pectinase preparations from Aspergillus niger have pectinesterase (PE), polygalacturonase (PG), and pectin lyase (PL) activity. The use of pectinase and cellulase enzymes disrupts the cell wall of orange peel, sweet potato and carrot, and releases the carotenoids in the chloroplasts and in cell fluids. These pigments remain in their natural state still bound with proteins. This bonded structure prevents pigment oxidation and also affects color stability (Cinar, 2005), whereas solvent extraction dissociates the pigments from the proteins and causes water insolubility and ease of oxidation (Bassi *et al*., 1993).

2.2.7.11 Waste Management. The wastes generated from forests, agricultural fields, and agroindustries contain a large amount of unutilized or underutilized cellulose, causing environmental pollution (Milala *et al*., 2005). Nowadays, these so-called wastes are judiciously utilized to produce valuable products such as enzymes, sugars, biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds, and human nutrients (Kuhad *et al*., 1997).

2.3 Hemicellulose

Hemicellulose is the second most abundant renewable organic material, next to cellulose, on the Earth. In the conversion of lignocellulose to fuels and chemicals, utilization of hemicellulose as a byproduct is essential to make overall economics of processing wood into chemicals feasible. Hemicellulose is classified as a heterogeneous polysaccharide; it is an amorphous polymer that primarily contains five different sugar groups (Sjöström, 1981); more specifically:hexoses (glucose, mannose and galactose) and pentoses (xylose, arabinose). Hemicellulose chains are shorter than the chains in simpler cellulose, can be branched, and often have side groups, such as monosaccharides and acetyl groups (Mai *et al*., 2004). The general composition of hemicellulose differs between hardwoods and softwoods, with the hemicellulose in hardwoods primarily consisting of the pentoses, whereas the hemicellulose in softwoods includes hexoses (Phaiboonsilpa *et al*., 2010).

Hemicellulose functions as a supporting material in cell walls and is, when compared to cellulose, more readily hydrolyzed to its individual monomeric units. Typically, hemicellulose accounts for 20 to 30% of the mass of dried wood and it is important to note that the composition and structure of hemicellulose differs between not only hardwoods and softwoods, but also between the stem, branches, roots and bark of a given tree. This makes recovery of exact quantities of reducing sugars difficult (Sjöström, 1981).

2.3.1 Properties of Hemicellulose

Due to their branched structure, hemicelluloses are more soluble then cellulose and they can be isolated from wood by extraction. Hemicelluloses are easily hydrolysed by strong acid leaving cellulose and lignin intact, or by strong base (Fan *et al.,* 1982). In many cases diluted acid (0.5-1.0% H_2SO_4) pretreatment under elevated temperatures (140-190°C) will degrade most of the hemicellulose to soluble pentose and hexose sugars (Lloyd and Wyman, 2005). Even though this treatment is not particularly targeted towards solubilization of lignin, the lignin structure is disturbed and redistributed leading to much more favorable (pretreated) substrate for enzymatic hydrolysis (Yang and Wyman, 2004).

2.3.2 Sources and structure of Hemicellolose

Hemicelluloses are the second most abundant polysaccharides in nature after cellulose. They occur in close association with cellulose and lignin and contribute to the rigidity of plant cell walls in lignified tissues. Hemicelluloses constitute about 20–30% of the total mass of annual and perennial plants and have a heterogeneous composition of various sugar units, depending on the type of plant and extraction process, being classified as xylans (1,4-linked D-xylose units), mannans (1,4-linked D-mannose units), arabinans (1,5-linked L-arabinose units), and galactans (1,3-linked D-galactose units) (Belgacem and Gandini, 2008). Xylans are the main hemicelluloses in hardwood and they also predominate in annual plants and cereals making up to 30% of the cell wall material and one of the major constituents (25–35%) of lignocellulosic materials. The most potential sources of xylans include many agricultural crops such as straw, sorghum, sugar cane, corn stalks and cobs, and hulls and husks from starch production, as well as forest and pulping waste products from hardwoods and softwoods.

The structural diversity and complexity of xylans are shown to depend on the botanic source. Various suitable extraction procedures for the isolation of xylans from different plant sources are described and compared in the literature. It is suggested that certain structural types of xylans, such as glucuronoxylan, arabinoglucuronoxylan, and arabinoxylan, can be prepared from certain plant sources with similar chemical and physical properties. Its general structure has a linear backbone consisting of 1,4-linked D-xylopyranose residues, a reducing sugar with five carbon atoms. These may be substituted with branches containing acetyl, arabinosyl, and glucuronosyl residues, depending on the botanic source and method of extraction (Habibi and Vignon, 2005).

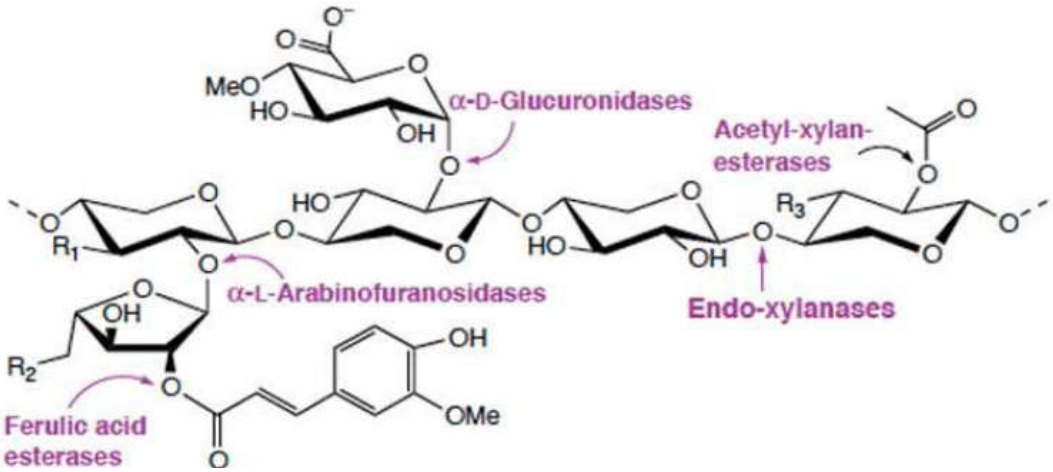
2.3.3 Chemical Structure of Hemicellulose

The content and structure of hemicellulose in various plants are different. The research on the chemical structure is mainly about the composition of the main chain and branched chains of glucans in hemicellulose. The main chain may consist of one or more types of glycosyls, and the connections between glycosyl are also different. Raw materials from different producing areas and different parts have different glycan compositions. Therefore, to illustrate the chemical structure, glycans must be classified first. It is generally believed that hemicellulose is the glucan in the matrix of the cell, and the main components are xylan, xyloglucan, glucomannan, mannan, galactomannan, callose,e.t.c (Yin and Fan, 1999).

2.3.3.1 Chemical Structure of Xylan Hemicelluloses

Almost all plants contain xylan. D-Xylosyls are linked with each other to form homopolymer linear molecules as the main chain. Xylan hemicellulose is the glucan with a backbone of 1,4-D-xylopyranose and with branch chains of 4-oxymethylglucuronic acid. The hemicellulose of hardwoods and gramineous forbs is mainly composed of this kind of polysaccharide. The hemicellulose of Gramineae also contains Larabinofuranose linking to the main chain as branch chains. The number of branch chains depends on different kinds of plants. The typical molecular structure of hemicellulose of Gramineae is chiefly composed of D-xylopyranosyl, which is linked by “-1,4-glucosidic bonds. Branch chains consist of L-arabinofuranosyl and D-glucuronopyranosyl, respectively, on C3 and C2 of the main chain; there are also branch chains composed of xylosyl and acetyl (xylosyl acetate). The Depolimerization Point of hemicellulose in Gramineae is less than 100. Xylan hemicelluloses in timber are composed of linear xylans linked by “-1,4-glucosidic bonds, with some different short-branch chains linked to the main chain, similar to the Gramineae.

However, average polymerization is higher than 100. In addition, hemicellulose from softwoods and hardwoods also has the distinction. Hemicellulose of hardwood is chiefly acidic xylans that have been partly acetylized; for example, the content of this hemicellulose in birch is about 35%, while this content in Euonymus bungenus is only 13% (Xu *et al.,* 2003). Xylan hemicellulose in softwoods is 4-O-methyl-glucuronic acid arabinose-xylan with almost no acetyl, while O-acety-L-4-O-methyl-glucuronic acid xylan is the most important hemicellulose in hardwoods (Yang, 2008).



Chemical structure of Xylan (Shallon and Shoham, 2003)

2.3.4 Chemical Properties of Hemicellulose

Because of the low depolymerization point DP and few crystalline structures, hemicellulose is more easily degraded in acidic medium than cellulose. But, the category of glycosyl in hemicellulose varies, including the pyran type, furan ,glycoside bond-linked type, L-configuration type, D- configuration type, e.t.c. The ways of linkage between glycosyls are various, such as 1-2, 1-3, 1-4 and 1-6 links (Yang, 2008). Most studies showed that hydrolysis of methyl-rabopyranoseis the fastest; the others are arranged in decreasing speed as follows: methyl- D-galactopyranoside, methyl-D-xylopyranoside, methyl-D-mannopyranoside, and methyl-D-galactopyranoside, which is the most stable. The “-D type of glycoside is easier to hydrolyze than the ’-D type. Generally, the hydrolysis rate of the furan type is faster than that of the pyran type. The hydrolysis rate of glucuronide is 40,000; perhaps the carboxyl has positive control in the glucoside bond. Hemicellulose is an inhomogeneous glycan composed of a variety of glycosyls, so the reducing ends have many kinds of glycosyls and some branch chains.

Similar to cellulose, hemicellulose can have a peeling reaction under mild alkaline conditions. At high temperature, it would have alkaline hydrolysis. Research showed that the speed of alkaline hydrolysis of furan glycosides was many times faster than that of pyran glycosides. Hemicellulose can dissolve in both alkali solution (5% Na_2CO_3 solution) and acid solution (2% HCl solution). It has a relative affinity to water, which can make it form a viscous state or become a gelling agent. In rheologic studies of the viscosity of hemicellulose, this phenomenon can be well observed. For example, when the concentration of hemicelluloses in water reaches 0.5%, the aqueous solution of hemicellulose has a certain consistency. That is the same as in human saliva; when the concentration is 2%, the solution cannot flow because of the viscosity generated. When the concentration reaches 4%, the solution is to be regarded as a gel.

The affinity of hemicellulose is closely related to its pentose; for example, arabinose and xylose are responsible for fixing water masses on to different structures of the hemicellulose. The greatest benefit brought by this characteristic is to apply pentose in food technology. This feature also illustrates that if the percentage of the pentose in the hemicellulose is too low, the spatial organization keeps pentose away from water, resulting in low affinity of hemicellulose to water (Chen *et al*., 1996).

2.3.5 Applications of Hemicellulose

The potential applications of xylanases also include the bioconversion of lignocellulosic material and agro-wastes into fermentative products, the clarification of juices, the improvement of the consistency of beer and the digestibility of animal feedstocks (Wong *et al*., 1988). One of the most important biotechnological applications of xylanase is its use in pulp bleaching (Viikari *et al.,* 1994). Xylanases may also be applicable to the production of rayon, cellophane and several chemicals such as cellulose esters (acetates, nitrates, propionates and butyrates) and cellulose ethers (carboxymethylcellulose and methyl and ethyl cellulose), which are all produced by dissolving pulp and purifying fibers from other carbohydrates (Subramaniyan and Prema, 2002).

2.3.5.1 The paper and pulp industries

During the past several years, the use of enzymes in paper and pulp bleaching has caught the attention of researchers and industries all over the world. Xylanase enzymes have proven to be a cost-effective means for mills to take advantage of a variety of bleaching benefits (Bajpai, 2012). Xylanases and other side-cleaving enzymes have been used in pulp bleaching primarily to reduce lignin and increase the brightness of the pulp (Sunna *et al*., 1997). The importance of xylanase in the pulp and paper industries is related to the hydrolysis of xylan, which facilitates the release of lignin from paper pulp and, consequently, reduces the usage of chlorine as the bleaching agent .

Bleaching is the process of lignin removal from chemical pulps to produce bright or completely white finished pulp (Beg *et al*., 2001). Thus, the bleaching of pulp using enzymes or ligninolytic microorganisms is called biobleaching (Perez *et al.,* 2002). This process is necessary due to the presence of residual lignin and its derivatives in the pulping process, which causes the resultant pulp to gain a characteristic brown color. The intensity of this pulp color is related to the amount and chemical state of the remaining lignin (Subramaniyan and Prema, 2002). The bleaching of pulp involves the destruction, alteration or solubilization of the lignin, colored organic matter and other undesirable residues on the fibers . Bleaching of kraft pulp usually requires large amounts of chlorine-based chemicals and sodium hydrosulfite, which cause several effluent-based problems in the pulp and paper industries. The use of these chemicals generates chlorinated organic substances, some of which are toxic, mutagenic, persistent, and highly resistant to biodegradation, in addition to causing

numerous harmful disturbances in biological systems and forming one of the major sources of environmental pollution (Beg, 2001).

As hemicellulose is easier to depolymerize than lignin, biobleaching of pulp appears to be more effective with the use of xylanases than with lignin-degrading enzymes. This is due to the fact of the removal of even a small portion of the hemicellulose could be sufficient to open up the polymer, which facilitates removal of the residual lignin by mild oxidants (Juturu *et al*., 2011). The use of xylanase in bleaching pulp requires the use of enzymes with special characteristics. A key requirement is to be cellulose-free, to avoid damaging the pulp fibers (Haltrich *et al.*,1996), as cellulose is the primary product in the paper industry. Other desirable characteristics are stability at high temperatures (Chidi *et al.,* 2008) and an alkaline optimal pH (Perez *et al*., 2002)

2.3.5.2. Bioconversion of lignocellulose in biofuels

Currently, second-generation biofuels are the primary products of the bioconversion of lignocellulosic materials. According to Taherzadech and Karimi (2008), ethanol is the most important renewable fuel in terms of volume and market value, and following the fossil fuel crisis, it has been identified as an alternative fuel. Despite the primarily first-generation production of ethanol, from sugar and starch, the second-generation production of ethanol has only begun to be tested in pilot plants (Taherzadech and Karimi, 2008) . And, unlike first-generation biofuels, secondgeneration biofuels do not compete with food production and can provide environmental,economic, and strategic benefits for the production of fuels (Viikari *et al.,* 2012).

Xylanase, together with other hydrolytic enzymes, can be used for the generation of biological fuels, such as ethanol, from lignocellulosic biomass (Olsson and Hahn-Hagerdal, 1996). However, enzymatic hydrolysis is still a major cost factor in the conversion of lignocellulosic raw materials to ethanol . In bioethanol fuel production, the first step is the delignification of lignocellulose, to liberate cellulose and hemicellulose from their complex with lignin. The second step is a depolymerisation of the carbohydrate polymers to produce free sugars, followed by the fermentation of mixed pentose and hexose sugars to produce ethanol (Lee, 1997). Simultaneous saccharification and fermentation is an alternative process, in which both hydrolytic enzymes and fermentative microorganism are present in the reaction (Chandrakant and Bisaria, 2008).

2.3.5.3 The pharmaceutical, food and feed industries.

Xylanase, together with pectinase, carboxymethylcellulase and amylase, can be used for the clarification of juices because the turbidity observed is due to both pectic materials and other materials suspended in a stable colloidal system (Parajo *et al*., 1998). Xylanase may also improve the extraction of coffee, plant oils, and starch. The xylose resulting from xylan depolymerization may also be converted to xylitol, a valuable sweetener that has applications in both the pharmaceuticaland food industries.

In the bakery industry, xylanase may improve the quality of bread, by increasing the bread's specific volume. According to Collins *et al.,* 2006 psychrophilic enzymes may be suitable for use in the baking industry as they are generally optimally active at the temperatures most frequently used for dough preparation (at or below 35°C). These enzymes could also be used as more efficient baking additives than the currently used commercial mesophilic enzymes, which are optimally active at higher temperatures.

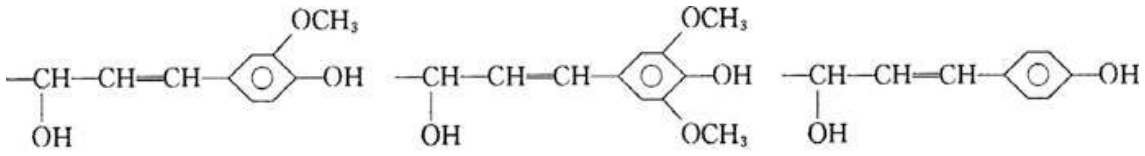
Xylanase may also improve the nutritional properties of agricultural silage and grain feed. The use of this enzyme in poultry diets showed that the decrease in weight gain and feed conversion efficiency in rye-fed broiler chicks has been associated with intestinal viscosity (Paridon *et al.*, 1992). The incorporation of xylanase from *Trichoderma longibrachiatum* into the rye-based diet of broiler chickens reduced intestinal viscosity, thus, improving both the weight gain of the chicks and their feed conversion efficiency (Bedford and Classen, 1992).

Xylanases can also be used in cereals as a pretreatment for arabinoxylan-containing substrates, as arabinoxylans are partly water soluble and result in a highly viscous aqueous solution. This high viscosity of cereal grain water extract may lead to brewing problems, by decreasing the rate of filtration or haze formation in beer. Additionally, it is unfavorable in the cereal grains used in animal feeding (Dervilly and Saulnier, 2001).

2.4 Lignin

Lignin is one of the most abundant organic polymers in plants, just behind cellulose. It is the exclusive chemical composition of gymnosperm and angiosperm. The content of lignin in wood and Gramineae is 20–40% and 15–20%, respectively. Lignin is the name of a group of substances; their inhomogeneity is manifested in different species of plants, length of growing season, and different parts of the plants. Even in the different morphologies of cells of the same xylem or different cell wall layers, the structures of lignin are not the same (Jiang

et al., 2001). Lignin is a polymer made up of phenylpropane monomers, which are polymerized by peroxidases or phenoloxidases during biosynthesis and are derived from coniferyl, coumaryl and sinapyl alcohols (Mai *et al.,* 2004; Ragauskas *et al*., 2006). The structure of lignin, like hemicellulose, depends both on the wood species and on where the lignin is located in the plant. In general, softwoods have higher lignin content than hardwoods (Pandey, 1999). Additionally ,softwood lignin is largely based on a coniferyl alcohol unit, whereas hardwood lignin is comprised of different base units, including coniferyl alcohol, in varying proportions (Pandey, 1999). A schematic of a representative section of coniferyl alcohol unit in soft wood lignin is shown below (Pandey, 1999).



Structure of coniferyl alcohol unit

Lignin is a complex composed of complicated phenylpropane units nonlinearly and randomly linked; three main monomers are coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Because of the different monomers, lignin can be divided into three types;

1 Syringyl lignin polymerized by syringyl propane,

2 Guaiacyl lignin polymerized by guaiacyl propane,

3 Hydroxy-phenyl lignin polymerized by hydroxy-phenyl propane.

Usually, gymnosperm mainly contains guaiacyl (G) lignin; the dicotyledon mainly contains guaiacyl-syringyl (GS) lignin; the monocotyledon mainly contains guaiacyl-syringyl-hydroxy-phenyl (GSH) lignin (Wei and Song, 2001).

Lignin is a class of complex organic polymers. Lignins are one of the main classes of structural materials in the support tissues of vascular plants and some algae. Lignins are particularly important in the formation of cell walls, especially in wood and bark, because they lend rigidity and do not rot easily. Chemically lignins are cross-linked phenol polymers (Schrick *et al*., 2004).

2.4.1 History of Lignin

Lignin was first mentioned in 1813 by the Swiss botanist A. P. de Candolle, who described it as a fibrous, tasteless material, insoluble in water and alcohol but soluble in weak alkaline solutions, and which can be precipitated from solution using acid.(Candolle *et al*., 1813) He named the substance "lignine", which is derived from the Latin word lignum meaning wood, (Sjostrom, 1993) . It is one of the most abundant organic polymers on Earth, exceeded only by cellulose. Lignin constitutes 30% of non-fossil organic carbon, (Ralph *et al*., 2003) and a quarter to a third of the dry mass of wood.

2.4.2 Structure of Lignin

Lignin is a cross-linked racemic macromolecule with molecular masses in excess of 10,000 unit. It is relatively hydrophobic and aromatic in nature. The degree of polymerisation in nature is difficult to measure, since it is fragmented during extraction and the molecule consists of various types of substructures that appear to repeat in a haphazard manner. Different types of lignin have been described depending on the means of isolation, (Samuel *et al*.,2002).

There are three monolignol monomers, methoxylated to various degrees: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol These lignols are incorporated into lignin in the form of the phenylpropanoids p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), respectively.Gymnosperms have a lignin that consists almost entirely of G with small quantities of H. That of dicotyledonous angiosperms is more often than not a mixture of G and S (with very little H), and monocotyledonous lignin is a mixture of all three,(Ralph *et al*., 2003). Many grasses have mostly G, while some palms have mainly S.(Kuroda *et al.,* 2001) All lignins contain small amounts of incomplete or modified monolignols, and other monomers are prominent in non-woody plants, (Lange *et al.,* 1995).

2.4.4 Composition of Lignin

The composition of lignin varies from species to species. An example of composition from an aspen sample is 63.4% carbon, 5.9% hydrogen, 0.7% ash, and 30% oxygen (by difference), (Peter *et al*., 1983) corresponding approximately to the formula $(C_{31}H_{34}O_{11})n$. As a biopolymer, lignin is unusual because of its heterogeneity and lack of a defined primary structure. Its most commonly noted function is the support through strengthening of wood (xylem cells) in trees, (Chabannes *et al*., 2001).

2.4.5 Biological function of Lignin

Lignin fills the spaces in the cell wall between cellulose, hemicellulose, and pectin components, especially in xylem tracheids, vessel elements and sclereid cells. It is covalently linked to hemicellulose and, therefore, crosslinks different plant polysaccharides, conferring mechanical strength to the cell wall and by extension the plant as a whole, (Lebo *et al.,*2001). It is particularly abundant in compression wood but scarce in tension wood, which are types of reaction wood. Lignin plays a crucial part in conducting water in plant stems. The polysaccharide components of plant cell walls are highly hydrophilic and thus permeable to water, whereas lignin is more hydrophobic. The crosslinking of polysaccharides by lignin is an obstacle for water absorption to the cell wall. Thus, lignin makes it possible for the plant's vascular tissue to conduct water efficiently (Sarkanen *et al.,*1971).

Lignin is present in all vascular plants, but not in bryophytes, supporting the idea that the original function of lignin was restricted to water transport. However, it is present in red algae, which seems to suggest that the common ancestor of plants and red algae also synthesised lignin. This would suggest that its original function was structural; it plays this role in the red algae Calliarthron, where it supports joints between calcified segments (Martone *et al.,* 2009). Another possibility is that the lignin in red algae and in plants are result of convergent evolution, and not of a common origin.

2.4.6 Ecological function of Lignin

Lignin plays a significant role in the carbon cycle, sequestering atmospheric carbon into the living tissues of woody perennial vegetation. Lignin is one of the most slowly decomposing components of dead vegetation, contributing a major fraction of the material that becomes humus as it decomposes (Sjostrom *et al.,* 1993). The resulting soil humus, in general, increases the photosynthetic productivity of plant communities growing on a site as the site transitions from disturbed mineral soil through the stages of ecological succession, by providing increased cation exchange capacity in the soil and expanding the capacity of moisture retention between flood and drought conditions (Kuroda *et al.,* 2001)

2.4.7 Chemical Properties of Lignin

The chemical properties of lignin include halogenation, nitration, and oxidation reactions on the phenyl ring; reactions on the benzyl alcohol, the aryl ether bond, and an alkyl ether bond in the side chain; lignin-modified chromogenic reaction; and so on. The chemical reactions of

the lignin structural unit are divided into two major categories: nucleophilic reactions and electrophilic reactions.

Chemical reactions of lignin structural unit on the side chain Reactions on the lignin side chains are associated with pulping and lignin modification; the reaction is a nucleophilic reaction. The following reagents can conduct nucleophilic reactions with lignin:

In alkaline medium, the effect of HO-, HS-, and S2-nucleophilic reagents leads to the cleavage of the main ether bond (e.g., ’-aryl ether bond, a phenol-type ’- alkoxy ether bond, and phenol-type “-aryl ether bond) and fragmentation and partial dissolution of macromolecule lignin. In alkaline medium, the phenoltype structural unit is separated into phenolate anions, and an oxygen atom that affects the benzene ring by induction and a conjugative effect, which activates their ortho- and para- positions and thereby affects the stability of the CO bond and breaks theether bond, then generating a methylene quinone intermediate and resulting in the aromatization of methylene quinone to generate a 1,2-diphenylethene structure(Tao and Guan, 2003).

2.4.8 Physical Properties of Lignin

Molecular weight and polydispersity any type of separation method may cause some partial degradation and changes in lignin. Accordingly, the molecular weight of the original lignin is unsure. The molecular weight of the separated lignin varies with the separation method and conditions. Its molecular weight distribution can range from several hundred to several million. Under the effect of mechanical action, enzymes, or chemical reagents, the three-dimensional net structure is degraded into different size lignin fragments, which leads to the molecular weight polydispersity of lignin (Zhang *et al.*, 2001).

2.4.8.1 Solubility

Hydroxyls and many polar groups exist in the lignin structure, resulting in strong intramolecular and intermolecular hydrogen bonds, and making the intrinsic lignin insoluble in any solvent. Condensation or degradation make the separated lignin able to be divided into soluble lignin and insoluble lignin; the former has an amorphous structure, and the latter is the morphological structure of the raw material fibers. The presence of phenolic hydroxyl and carboxyl makes the lignin able to be dissolved in alkaline solution. Separated Brauns lignin and organosolv lignin can be dissolved in dioxane, Dimethyl sulfoxide (DMSO), methanol, ethanol, acetone, methyl cellosolve, and pyridine. Alkali lignin and lignosulfonate usually

can be dissolved in a dilute alkali, water, and salt solution. Brauns lignin, phenol lignin, and many organosolv lignins can be completely dissolved in dioxane. Acid lignin is not soluble in any solvents. The best solvents for most separated lignin are acetyl bromide and hexafluoroisopropanol in acetic acid.

Thermal properties of Lignin is an amorphous thermoplastic polymer. It has slight friability under high temperature and cannot form film in solution. It also has glassy transfer properties. Under the glassy transfer temperature, lignin is in the solid glass phase; it begins to move when it is above the glassy transfer temperature. The lignin is softened to become sticky and has adhesive force. The glassy transfer temperature of separated lignin varies with the raw materials, separation method, molecular weight, and water content. The softening temperature of absolutely dried lignin ranges from 127^{O}C to 129^{O}C, which remarkably decreased with increased water content, indicating that water acts as a plasticizer in lignin. The higher the lignin molecular weight,the higher the softening point. For example, for lignins with a MW of 85,000 and 4,300, the softening points, respectively, are 176 and 127^{O}C (Jiang, 2001).

2.4.8.2 Relative density

The relative density of lignin is roughly between 1.35 and 1.50. Values vary with the liquid for measurement if measured by water. The relative density of sulfuric acid lignin isolated from pine is 1.451 and is 1.436 if measured by benzene. The relative density of dioxane lignin is 1.33 when measured by water at 20^{O}C and is 1.391 by dioxane. Lignin prepared by different methods has different relative densities, such as the relative density of pine glycol lignin is 1.362, but it is 1.348 for pine hydrochloride lignin (Jiang, 2001).

2.4.8.4 Color

Intrinsic lignin is a white or nearly colorless substance; the color of lignin we can see is the result of the separation and preparation process. For example, the color of lignin isolated by Brauns and named after him is light cream, and the colors of acid lignin, copper ammonia lignin, and periodate lignin vary from fawn to deep tan (Yu *et al*., 2003).

2.4.9 Economic significance

Highly lignified wood is durable and therefore a good raw material for many applications. It is also an excellent fuel, since lignin yields more energy when burned than cellulose.

Mechanical, or high-yield pulp used to make newsprint contains most of the lignin originally present in the wood. This lignin is responsible for newsprint's yellowing with age, (Wittkowski *et al.*, 1992). Lignin must be removed from the pulp before high-quality bleached paper can be manufactured.

In sulfite pulping, lignin is removed from wood pulp as sulfonates. These lignosulfonates have several uses; Dispersants in high performance cement applications, water treatment formulations and textile dyes Additives in specialty oil field applications and agricultural chemicals Raw materials for several chemicals, such as vanillin, Dimethyl sulfoxide (DMSO), ethanol, xylitol sugar, and humic acid. Environmentally sustainable dust suppression agent for roads the first investigations into commercial use of lignin were reported by Marathon Corporation, a paper company based in Rothschild Wisconsin, starting in 1927. The first class of products that showed promise were leather tanning agents.

Lignin removed via the kraft process (sulfate pulping) is usually burned for its fuel value, providing energy to run the mill and its associated processes. Higher quality lignin presents the potential to become the main renewable aromatic resource for the chemical industry in the future, with an addressable market of more than $130bn. In 1998, a German company, Tecnaro, developed a process for turning lignin into a substance, called Arboform, which behaves identically to plastic for injection molding. Therefore, it can be used in place of plastic for several applications. When the item is discarded, it can be burned just like wood, (Tao and Guan, 2003).

In 2007, lignin extracted from shrubby willow was successfully used to produce expanded polyurethane foam, (Qiu and Chen, 2006). In 2012, it was shown carbon fiber can be produced from lignin instead of from fossil oil. In 2013, the Flemish Institute for Biotechnology was supervising a trial of 448 poplar trees genetically engineered to produce less lignin so that they would be more suitable for conversion into bio-fuels.(Qiu and Chen, 2006) Biodegradation of lignin by white rot fungi leads to destruction of wood on the forest floor and man-made structures such as fences and wooden buildings. However biodegradation of lignin is a necessary prerequisite for processing biofuel from plant raw materials. Current processing setups show some problematic residuals after processing the digestible or degradable contents. The improving of lignin degradation would drive the output from biofuel processing to better gain or better efficiency factor. Lignin is indigestible by animals, which lack the enzymes that can degrade this complex polymer. Some fungi

(such as the Dryad's saddle) and bacteria do however biodegrade lignin using so-called ligninases (also named lignases). The mechanism of the biodegradation is speculated to involve free radical pathways. Yu *et al.,* (2003) Well understood ligninolytic enzymes are manganese peroxidase and lignin peroxidase. Because it is cross-linked with the other cell wall components and has a high molecular weight, lignin minimizes the accessibility of cellulose and hemicellulose to microbial enzymes such as cellobiose dehydrogenase.

Hence, in general lignin is associated with reduced digestibility of the overall plant biomass, which helps defend against pathogens and pests. Syringyl (S) lignol is more susceptible to degradation by fungal decay as it has fewer aryl-aryl bonds and a lower redox potential than guaiacyl units. This means that organic matter that is enriched with G lignol (like the bark of woody vascular plants) is more resistant to microbial attack. Lignin is degraded by micro-organisms including fungi and bacteria. Lignin peroxidase (also "ligninase", EC number 1.14.99) is a hemoprotein firstly isolated from the white-rot fungus *Phanerochaete chrysosporium* with a variety of lignin-degrading reactions, all utilizing hydrogen peroxide as an oxygen source (Tien, 1983). Other microbial enzymes may be involved in lignin biodegradation, such as manganese peroxidase and the copper-based laccase, (Wittkowski *et al.,* 1992).

2.5 Pretreatment of lignocellulose

In economically feasible industrial processes for conversion of lignocellulosic materials into energy carriers such as ethanol and butanol, or various other products, such as organic acids, acetone or glycerol, both cellulose and hemicellulose needs to be hydrolyzed to sugars that in proceeding steps can be further converted (Wyman, 2002). The ideal pretreatment method, thus, needs to integrate several processes; e.g. hydrolysis of hemicellulose to predominantly pentoses (5-carbon sugars), reduction, modification and/or redistribution of lignin, and reduction in crystallinity and increase of surface area of cellulose. Physical, physico-chemical, chemical and biological processes have been used for pretreatment of lignocellulosic material (Fan, *et al*., 1982; Sun and Cheng, 2002). The drawback of the pretreatment processes, besides the obvious economical impact, is the generation of inhibitory compounds that can negatively influence the action of enzymes and microorganisms. Inhibitors are produced as a consequence of extreme pH and high temperature treatment of lignocellulosic materials (Olsson *et al*., 2004).

Each pretreatment process should, therefore, be carefully chosen and critically justified. Physical treatments can be classified into two general categories, mechanical and non-mechanical pretreatments (irradiation, high pressure steaming and pyrolysis), A common purpose of both categories is to subdivide lignocellulosic materials into fine particles which are substantially susceptible to acid or enzymatic hydrolysis. The smaller particles have a large surface-to-volume ratio thus making cellulose more accessible to hydrolysis (Fan *et al*., 1982). Chemical pretreatment methods have been traditionally used in paper and pulp industry for delignification of cellulose and for destroying its crystalline structure. The main advantages of these methods are high effectiveness (high glucose yield) and Cellulosic material minimal formation of inhibitors (in particular with the concentrated acid treatment). On the other side, the need of specialized corrosion resistant equipment, and necessity of subsequent extensive washing, together with the disposal of chemical waste should be stated as the main disadvantages of these processes. Biological pretreatment utilizes wood attacking microorganisms that can degrade lignin White rot fungi have been identified as the most promising group for the lignocellulose pretreatment (Ander and Eriksson, 1979).

2.6 Degradation of Lignocellulose

Lignin, cellulose, and hemicellulose together form a complex structure (Breznak and Brune 1994, Enari 1983, Martin 1987). Most cellulose in plants is in highly ordered crystalline bundles called fibrils or microfibrils, which give a plant its rigidity. The cellulose fibrils are surrounded by a matrix of lignin and hemicellulose that are covalently bonded to each other at various intersections. The structural complexity of these polysaccharides increases the recalcitrance of each individual component. For example, cellulases cannot attack cellulose fibrils until the lignin-hemicellulose matrix is broken through (Breznak and Brune 1994, Enari 1983, Martin 1987b).

Hemicellulose is more readily degraded than cellulose and lignin (Breznak and Brune 1994, Martin 1987a). Fungi, bacteria and protozoa produce a variety of enzymes to degrade hemicellulose since it is composed of a variety of sugars (Martin 1987, Shallom and Shoham 2003). For example, hemicellulose can be degraded by glycoside hydrolases such as arabinase, galactanase, glucanase, mannanase, and xylanase, or carbohydrate esterases, such as ferulic acid esterase, depending on the specific hemicellulose composition (Martin 1987, Shallom and Shoham, 2003). Presence of active hemicellulases can be determined by analyzing the degradation of a variety of p-nitrophenyl- or methylumbelliferyl- conjugated

sugars, such as pnitrophenyl-α-L-arabinofuranoside and 4-methylumbelliferyl-β-D-mannopyranoside.

Cellulose is less readily degraded than hemicellulose, but more readily degraded than lignin (Breznak and Brune 1994). Cellulase enzymes of the white rot fungi have been extensively studied (Enari 1983 and Martin 1987). White rot fungi produce three major types of cell-free cellulolytic enzymes that all hydrolyze the same β-1,4-glycosidic linkage, but differ by specifically attacking cellulose with different amounts of polymerization or attacking different areas of the macromolecular structure (Martin 1987). The three enzyme types are endo-β-1,4-glucanases (Cx-cellulases), exo-β-1,4-glucanases (C1-cellulases), and β-1,4-glucosidases. Endo-β-1,4-glucanases randomly hydrolyze β-1,4-glycosidic linkages, but do not hydrolyze cellobiose.Exo-β-1,4-glucanases attack the non-reducing end of the sugar polymer releasing cellobiose or glucose molecules. Cellobiose and cello-oligosaccharides are hydrolyzed to glucose molecules by β-1,4-glucosidases. Together, these three cellulases can hydrolyze crystalline cellulose and are together referred to as a complete cellulase complex (Martin, 1987b).

In contrast to fungal cellulases, bacterial cellulases are usually cell-bound; therefore, the bacterial cells require close contact to the substrate in order for degradation to occur (Enari 1983 and Martin 1987b). Examples of bacteria that produce cellulases are the *Actinomycetes, myxobacteria*, *pseudomonads*, and members of the genera *Bacillus, Bacteroides, Cellulomonas, Clostridium, and Ruminococcus* (Enari, 1983). Many anaerobic cellulolytic bacteria produce cellulosomes (Bayer *et al.*, 2004). These multienzyme complexes are composed of a variety of cellulases and hemicellulases, and may include pectate lyases and chitinases, that are docked on a scaffold attached to the cell surface (Doi, 2003). The combination of enzymes within the cellulosome act in synergy to degrade lignocellulose more effectively than any of the enzymes alone (Bayer *et al.*, 2004).

Presence of active cellulases can be determined by testing the degradation of model substrates (Enari 1983 and Martin 1987). Filter paper, Avicel, cotton, and microcrystalline cellulose are model substrates representing various forms of crystalline cellulose. Carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC) are common substrates used to demonstrate endo-β-1,4-glucanase activity. Analysis for the production of reducing sugars demonstrates exo-β-1,4-glucanase hydrolysis of the non-reducing ends of Avicel and amorphous cellulose. Supplying the substrates cellobiose, p-nitrophenyl-β-D-glucoside or 4-

methylumbelliferyl-β-D-glucoside and analyzing for the production of glucose, p-nitrophenol or methylumbelliferone, respectively, can demonstrate β-1,4-glucosidase activity (Enari 1983 and Martin 1987).

As detritus is processed and labile compounds decomposed, its lignin composition increases, since lignin is less readily degraded than both hemicellulose and cellulose (Cummins & Klug 1979). The recalcitrance of lignin is attributed to its chemically stable linkages, complex structure, and large size (Breznak and Brune, 1994). Various fungi and bacteria, mainly actinomycetes, are capable of hydrolyzing lignin. The white rot fungi and some *Streptomyces* spp. produce extracellular peroxidases to randomly hydrolyze lignin (Breznak and Brune 1994). Also, carboxylesterases may contribute to the degradation of lignin by releasing aromatic esters from the polymeric structure (Breznak and Brune 1994).

2.7 Biotechnological application of lignocellulose and its biodegradation

The primary objective of lignocellulose pretreatment by the various industries is to access the potential of the cellulose and hemicellulose encrusted by lignin within the lignocellulose matrix. The combination of solid-state fermentation (SSF) technology with the ability of white-rot fungi to selectively degrade lignin has made possible industrial-scale implementation of lignocellulose-based biotechnologies. The advantages and disadvantages of SSF have received attention by Mudgett (1986). SSF offers the advantages of a robust technology and outperforms conventional fermentation technologies with respect to simplicity, cost effectiveness, and maintenance requirements. These advantages make SSF an attractive technology for environmental problems where money and expertise are limited. Problems commonly associated with SSF are heat build-up, bacterial contamination, scale-up, biomass growth estimation, and control of substrate moisture content (Lonsane *et al*., 1985). At this stage, engineering aspects come into play and the success of scale-up will depend on bioreactor design and operation (Lonsane *et al*., 1992).

2.7.1 Lignocellulose-based technologies using unsterile substrates

Silage manufacture is a good example of a working technology based on unsterile lignocellulose substrates. Silage is the material produced by the controlled fermentation of moist plant material (McDonald, 1981). Water authorities consider silage effluent a serious threat to natural water supplies (Haigh, 1994). From the farmer’s perspective, silage effluent constitutes a loss of valuable nutrients (McDonald, 1981).

Arnold *et al.,* (2000) used *Candida utilis* and *Galactomyces geotrichum* to reduce the polluting potential of silage effluent with an initial chemical oxygen demand (COD) of 80 000 mg/l. COD, phosphate and ammonia concentrations were reduced by 74–95%, 82–99% and 16–64% respectively. A similar effluent is produced during oyster mushroom cultivation (personal observation). These effluents represent sources of carbon and nutrients and might even in future serve as cheap sources of fermentation adjuvants.

2.7.2 Biopulping

Lignin becomes problematic to cellulose-based wood processing, because it must be separated from cellulose at enormous energy, chemical and environmental expense. Biopulping is therefore a solid-state fermentation process during which wood chips are treated with white-rot fungi to improve the delignification process. Biological pulping has the potential to reduce energy costs and environmental impact relative to traditional pulping operations (Breen and Singleton 1999). The benefits of biopulping was demonstrated by Scott *et al.,* (1998) using 40-ton scale experiments: tensile, tear and burst indexes of the resulting paper were improved (indicative of higher degree of cellulose conservation during pulping process); brightness of the pulp was increased (indicating improved lignin removal); and improved energy savings of 30–38%. Problems endemic to SSF still plague this concept. Inoculation, aeration and heat removal are key parameters that influence fungal activity. Also, poor colonization of wood chips by white-rot fungi has been attributed to competition with naturally occurring microorganisms or to inhibition by wood chemical components (Wolfard *et al.,* 1999). Substrate sterilization is usually a major expense, and secondary contamination by airborne microorganisms must be prevented at additional costs as well. Breen and Singleton (1999) summarized decades of dissatisfaction associated with high capital costs to make SSF viable for the pulp and paper industry: “Overcoming these challenges will determine, in a large part, if biopulping becomes a reality.”

2.7.3 Animal feed

Cellulose is the most important source of carbon and energy in a ruminant’s diet, although the animal itself does not produce cellulose-hydrolyzing enzymes (Czerkowski, 1986). Rumen microorganisms utilize cellulose and other plant carbohydrates as their source of carbon and energy. Thus, the microorganisms convert the carbohydrates in large amounts of acetic, propionic and butyric acids, which the higher animal can use as its energy and carbon sources (Colberg, 1988).

The concept of preferential delignification of lignocellulose materials by white-rot fungi has been applied to increase the nutritional value of forages (Agosin and Odier,1985; Akin *et al*., 1995; Zadrazil and Isikhuemhen 1997). This increased digestibility provides organic carbon that can be fermented to organic acids in an anaerobic environment, such as the rumen. However, upgrading of animal feed by white-rot fungi failed to reach industrial proportions. A possible explanation can be that the animals' instincts prevent them from ingesting mushrooms, for they can contain toxicants or they can be toxic to their rumen microflora and, hence, toxic to the animal also.

2.7.4 Applications of genetic engineering

The scope of lignocellulose-based applications is expanding rapidly towards applications of genetic engineering. Recently, repression of lignin biosynthesis was achieved in Populus tremuloides resulting in cellulose accumulation and healthy growth of such transgenic trees (Hu *et al*., 1999). Cellulose and lignocellulose fibers can be chemically modified to render it useful to miscellaneous applications in the textile industry (Ghosh and Gangopadhyay 2000). Lignocellulose degrading Actinomycetes have been used as biocontrol agents to preserve golf green turf-grass (Chamberlain and Crawford 2000). Currently, metabolic engineering is being applied to facilitate simultaneous fermentation of hexoses and pentoses to ethanol (Aristidou and Penttilä 2000). The future might see the application of genetically engineered microorganisms (containing lignocellulases) to biotechnological applications where lignocellulosic wastes serve as the on-site carbon and nutrient source. Commercial byproducts of lignocellulose conversion to fuel ethanol has found application as absorbents of organic pollutants and as enterosorbents (Dizhbite *et al.,* 1999). Therefore, commercializing lignin waste production can offset process costs. Lignocellulose is also of potential medical value. Apart from being essential in the human diet as fiber, lignocellulose can be a source of compounds with biological activity. Such compounds have potential as stimulators of the human immune system and as antiviral agents (Kiyohara *et al.,* 2000; Sakagami *et al.,* 1999).

2.7.5 Potential of lignocellulose in space exploration

Advances in lignocellulose research will enable scientists to contribute to space science exploration. Space travel will benefit from this research, in the near future as the transport of lignocellulose to space can result in substantial cost savings. Lignocelluloses can be a feedstock to provide for all basic needs: fuel, energy, feedstock chemicals, food, and water. Recycling of inedible plant material by white-rot fungi (*Pleurotus ostreatus*) has been

investigated in a Closed Ecological Life Support System (CELSS) (Sarikaya and Ladish, 1997). Incineration technology have been proposed as another way of recycling the elemental resources found in spent lignocelluloses to support agriculture in a CELSS (Wignarajah *et al*., 2000). Lignocellulose can therefore be the "super fuel" of the future – being a compact natural polymer containing enough potential energy to sustain man and machine in space.

2.8 Ecology of lignocellulose biodegradation

Lignocellulose degradation is essentially a race between cellulose and lignin degradation (Reid, 1989). This contest is even more extensive and complex in nature (Rayner and Boddy 1988). Decomposition curves for complex substrates incubated in soil, such as plant residues, usually yield a multislope decomposition curve (Paul and Clark 1989).

Fungi with restricted metabolic capabilities (e.g. soft rots like *Mucor* spp.) develop mutualistic relationships with and thrive alongside fungi degrading cellulose and lignin. Microorganisms unable to overcome the lignin or physical barrier can obtain energy from the low molecular weight intermediates released from lignocellulose by the true white-rot fungi. Such complex associations have been observed under natural conditions (Blanchette *et al*., 1978).

2.8.1 Actinomycetes and bacteria

Lignocellulose biodegradation by prokaryotes is essentially a slow process characterized by the lack of powerful lignocellulose degrading enzymes, especially lignin peroxidases. Grasses are more susceptible to Actinomycete attack than wood (Antai and Crawford 1981; McCarthy, 1987). Together with bacteria, Actinomycetes play asignificant role in the humification processes associated with soils and composts (Trigo and Ball 1994). The enzymatic ability to cleave alkyl-aryl ether bonds enable bacteria to degrade oligomeric and monomeric aromatic compounds released during fungal lignin degradation (Vicuna *et al*., 1993; Vicuna 2000; White *et al*., 1996). Therefore, lignocellulose biodegradation by prokaryotes is of ecological significance, but lignin biodegradation by fungi, especially white-rot fungi, is of commercial importance.

2.8.2 Fungi

Most fungi are capable cellulose degraders. However, their ability to facilitate rapid lignocellulose degradation attracted attention from scientists and entrepreneurs alike. White-

rot fungi comprise powerful lignin degrading enzymes that enable them in nature to bridge the lignin barrier and, hence, overcome the rate-limiting step in the carbon cycle (Elder and Kelly 1994). Of these, *Phanerochaete chrysosporium* is the best studied. New information regarding the identities of the cellulose, hemicellulose or lignin degrading enzymes, their unique catalytic capabilities, the physiological conditions required for optimum secretion or activity etc. is constantly being added to an already impressive volume of work and varies between fungi and bacterial genera, species and even strains. Anaerobic fungi (*Piromyces* spp*., Neocalli-mastix* spp*. and Orpinomyces* spp*.)* form part of the rumen microflora. These fungi produce active polymer degrading enzymes, including cellulases and xylanases (Hodrova *et al*., 1998). Their cellulases are among the most active reported to date and able to solubilise both amorphous and crystalline cellulose (Wubah *et al*., 1993). These fungi can be used in situations where process principles and design necessitate anaerobic conditions. In such a scenario, ruminant manure will serve as inoculum and this waste product will meet a crucial requirement in biotechnology – cost effectivity versus optimum utility.

2.9 Physical and chemical characteristics of lignocellulosic biomass

The term "lignocellulosic biomass" is used when referring to higher plants, softwood or hardwood. The main components of the lignocellulosic materials are cellulose, hemicellulose and lignin. Cellulose is a major structural component of cell walls, and it provides mechanical strength and chemical stability to plants. Solar energy is absorbed through the process of photosynthesis and stored in the form of cellulose. (Raven *et al*., 1992) Hemicellulose is a copolymer of different C5 and C6 sugars that also exist in the plant cell wall. Lignin is polymer of aromatic compounds produced through a biosynthetic process and forms a protective layer for the plant walls. In nature, the above substances grow and decay during the year. It has been estimated that around $7.5x10^{10}$ tonnes of cellulose are consumed and regenerated every year (Kirk-Otmer, 2001). It is thereby the most abundant organic compound in the world. Apart from the three basic chemical compounds that lignocellulose consists of, water is also present in the complex. Furthermore, minor amounts of proteins, minerals and other components can be found in the lignocellulose composition as well. The composition of lignocellulose highly depends on its source. There is a significant variation of the lignin and (hemi)cellulose content of lignocellulose depending on whether it is derived from hard-wood, softwood, or grasses.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample collection

Soil samples were collected from different locations i.e Madaki, Dadin Duniya and Federal University Dutse of Jigawa State from a depth of 1-5 inches from the top and sieved through a 2mm sieve constituted the soil sample. The sample were dispensed into clean polythene bag and immediately transported to Microbiology Research laboratory of Bayero University Kano. Two hundred grams (200g) of wheat bran and saw dust ware purchased from Yankura market in Kano Metropolis, Nigeria which was used as substrate for enzyme production.

3.2 Soil Sample processing

One gram (1g) of soil sample was weighed and dissolved in 9 millilitres of sterile distilled water. An aliquot of 1 ml was taken and serially diluted to six fold dilution, which was used for inoculation.

3.3 Media preparation. (Potatoe Dextrose Agar)

Thirty nine grams (39g) of potatoe dextrose agar was weighed and dispensed in one litre capacity cornical flask and one litre of distilled water was added gradually and gently shaked.The mixture was heated on the hot plate to achieve total dissolution of the agar. It was then corked with cotton wool and aluminium foil and was sterilized in an autoclave at $121^{o}C$ for 15 minutes.The media was allowed to cool naturally at room temperature and 15 -20mls aliquot was dispensed into sterile petridishes and allowed to solidify.

3.4 Microbiological Analysis

3.4.1. Isolation of fungal species.

An aliquot of 0.1millilitres from test tube 10^4 and 10^5 was transferred using sterile pipette onto sterile potatoe dextrose agar plate. It was spread using a sterile bent glass rod and incubated at 30^0C for 5 days, (Lekk *et al*., 2014)

3.4.2. Maintenance of Isolates

The colonies that developed after 5 days of incubation were continually subcultured until a pure culture was obtained. The pure cultures were then subculture on potatoe dextrose agar

slants and incubated at 30^{o}C for 5 days and then refrigerated at -18^{o}C. The isolates were maintained on the slant until when required.

3.5 Identification

The identification of fungal species was done according to the method of Refa'i, (1979) using colonial appearance and microscopic examination.

3.6 Screening of potential degraders

Screening test for the ability of organisms to grow on wood-containing agar plates was performed in wood-containing agar medium. The medium contained 2% w/v wood powder, 5% v/v stock salt solution, 0.02% v/v trace elements, 0.05% w/v glucose and 1.5 % w/v agar. The stock salt solution contained the following (per liter): $NaNO_3$ 6g, KCl 0.52g, $MgSO_4.7H_2O$ 0.52g and KH_2PO_4 0.82g. The trace elements solution contained the following (per liter) $ZnSO_4.7H_2O$ 2.2g ,H_3BO_3 1.1g , $MnCl_2.4H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.5g, $CoCl_2.6H_2O$ 0.16g, $CuSO_4.6H_2O$ 0.16g, $(NH4)_6Mo7O_{24}.4H_2O$ 0.11g and Na_2EDTA 5g.

3.6.1 Dye decolorization test.

In dye decolorization tests, PDA medium containing 0.04% w/v Remazol Brilliant Blue R, (RBBR) and 0.04% w/v methyl green) was used. RBBR and methyl green were added as a sterilized filtered solution. Each fungal strain was inoculated onto the media plates and incubated at 30^{o}C and the growth was observed for a period of 2 weeks, Kyi, 2011).

3.7 Assessment for lignocellulose degrading enzyme production.

Lignocellulose degrading enzymes production was assessed in two different liquid media

- Wood liquid medium containing 2% (w/v) wood powder, 0.05% (w/v) glucose, 5% (v/v) stock salt solution and 0.02% (v/v) trace elements.
- Wheat bran liquid media containing 2% (w/v) wheat bran, 0.05% (w/v) glucose, 5% (v/v) stock salt solution and 0.02% (v/v) trace element.

3.8 Enzyme extraction for Cellulase and Hemicellulase Assay.

A cornical flasks filled with 150 ml of liquid media were autoclaved at 121^{o}C for 20 min. Mycelia from an agar plate were used for inoculation. The flasks were incubated at 30^{o}C while agitating at 180rpm and samples were collected after 5 days. The collected samples were centrifuged at 5100 rpm for 15 minutes and the supernatant was stored for further analyses.

3.8.1 Enzyme extraction for Laccase Assay

The enzyme extract was prepared by homogenizing 0.5ml of the liquid media in 2.0ml of an extraction medium containing tris HCl, sorbitol and NaCl. The homogenate was centrifuged at 5100rpm for 15 minutes and the supernatant was used for the assay.

3.9 Enzyme Assay

3.9.1. Cellulase Assay

Cellulase was assayed by adding one millilitre of enzyme (fermented broth supernatant) in test tube containing 1ml of 0.1M citrate buffer pH 5 followed by the addition of 1ml of 1% carboxymethyl cellulose solution. The test tubes were incubated for 30 minutes at 50^{o} C. After incubation 3ml Dinitrosalicylic acid (DNS) reagent was added and then boiled for 15 minutes in boiling water bath followed by the addition of 1ml sodium potassium tartarate. After cooling to room temperature absorbance was measured at 540nm.

Cellulase was defined as the amount of enzyme which released 1 unit of reducing sugar measured as glucose per minute under the assay condition.

3.9.2. Hemicellulase Assay

Hemicellulase was assayed by adding 1 millilitre of enzyme (fermented broth supernatant) in test tube, 1ml of citrate buffer pH 5 was added and finally 1ml xylan solution was added the mixture was incubated at 50^{o} C for 30 minutes on water bath. After incubation 3ml of DNS reagent was added and placed in boiling water bath for 15 minute followed by the addition of sodium potassium tartarate. After cooling absorbance was measured at 540nm.

Xylanase activity was defined as the amount of enzyme which released 1 unit of reducing sugar measured as xylan per minute under the assay condition.

3.9.3 Lignin Assay

Phosphate buffer (2.5ml) and 0.3ml of catechol solution was added in the cuvette and the spectrophotometer was set at 495nm.The enzyme extract (0.2ml) was added and change in absorbance was recorded for every 60 seconds up to 5 minutes in a spectrophotometer.

One unit of laccase is defined as the amount of enzyme that transform 1µ mole of quinone per minute

3.10 Optimization of enzyme production

3.10.1. Effect of fermentation time.

In order to determine the effect of fermentation time on lignocellulose activity, the selected fungal isolate was grown in sawdust and wheat bran seperately and then incubated for 5, 10, 15, 20 and 25 days. Culture broths were then centrifuged at 140 rpm for 20 minutes for collection of the supernatant.

3.10.2. Effect of pH.

The effect of media pH on lignocellulose activity was conducted by adjusting the pH to 3, 4, 5, 6, and 7 with NaOH and HCl before fungal inoculation. After 5days of incubation at 30ºC, culture broths were then centrifuged at 140 rpm for 20 minutes for collection of the supernatant.

3.10.3. Effect of Temperature.

To evaluate the effect of temperature on lignocellulose activity, the selected fungal isolate was grown at 30ºC in sawdust and wheat bran. After incubation at 25, 30, 35, 40 and 45^{o}C, culture broths were centrifuged at 140 rpm for 20 minutes for collection of the supernatant.

3.10.4. Effect of substrate concentration.

To evaluate the effect of substrate concentration on lignocellulose activity, the selected fungal isolate was grown at 30ºC in 1, 2, 3, 4 and 5% of saw dust and wheat bran and incubated for 5 days, culture broths were centrifuged at 140 rpm for 20 minutes for collection of the supernatant.

3.10.5. Effect of inoculum size.

To evaluate the effect of inoculum size on lignocellulose activity, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of the selected fungal isolate was grown at 30ºC in 2% of saw dust and wheat bran and

then incubated for 5days, culture broths were centrifuged at 140 rpm for 20 minutes for collection of the supernatant.

CHAPTER FOUR

4.0 RESULT

Study was conducted to isolate fungal species from fadama soil with potential to produce lignocellulose degrading enzymes. Result from identification of the fungal isolate revealed the presence of *Mucor* spp, *Alteneria* spp, *Rhizopus* spp, *A. flavus* and *A. fumigatus*. (Table 4.1).

All the strains were screened for their ability to grow on wood with or without addition of glucose, as an inducer for growth at the beginning of cultivation. Most of the species grew well on wood supplemented with glucose. However, all the organisms, except *Rhizopus* spp were able to grow adequately even in the absence of supplemented glucose, indicating the capability to produce enzyme necessary for the degradation of wood, as represented in Table 4.2.

The ability of the species to decolorize the different indicators such as Remazol brilliant blue R (RBBR) and methyl green were examined on PDA plates with 0.04% w/v of the respective indicators. Positive reaction with the indicators are observed as a colorless halo around the fungal growth. Four (4) out of the five species examined were positive on RBBR indicator plate expect for *Rhizopus* spp. which was negative and only two of the species were positive on methyl green indicator plate. The positive result indicated the production of lignocellulose degrading enzymes which decolorize the dyes. Due to it's capability to grow on wood and the ability to decolorize the dye, *A. fumigatus* was selected to further investigate their lignocellulose degrading enzyme ability, (Table 4.3).

Table 4.1: Identification of the fungal species.

Colonial appearance	Morphological characteristics	Organism identified
White to yellow	Septate hypae	*Aspergillus flavus*
Grey to green	Conidiophore bornelatterally the hypae	*Aspergillus fumigatus*
Wooly brown and olive green colonies	It has both transverse and longitudinal septate hypae, conidia borne on in chain at the top of conidophore.	*Alternaria* spp.
The colony was initially white and later grey with black dots, smooth in appearance	Hypae-thick and non septate. Reproductive structure-columella is round and bear the sporangia.	*Mucor* spp
Large colony initially white and later turned brown and black	Non-septate hypae and cottony cottony mycelium produce cluster of root-like structure-rhizoid and stolon	*Rhizopus* spp.

Table 4.2: Screening of potential degraders

The growth of Fungi on wood agar medium with and without addition of glucose.

Fungal strains	**Wood + glucose**	**Wood**
A.flavus	++	+
A.fumigatus	++	+
Alteneria spp.	++	+
Mucor spp.	++	+
Rhizopus spp.	+	+

Key:

+ = Total growth of less than 2cm in size

++ = Total growth of more than 2cm in size

Table 4.3: Dye decolorization effect during growth of different Fungal species on RBBR and Methyl green indicators.

Fungal isolates	RBBR	Methyl green
A. flavus	+	-
A. fumigatus	+	+
Alteneria spp.	+	-
Mucor spp.	+	-
Rhizopus spp.	-	+

Key:

+ = Positive for decolorization of dye

-= negative for decolorization of dye

4.1 Effect of Temperature on Wheat bran

The effect of temperature on the enzyme activity using wheat bran as substrate is presented in figure 4.1.The result shows that the cellulase and xylanase yield was maximum at 45^{o}C and 35^{o}C respectively with an enzyme activity of 0.47mg/ml and 0.62mg/ml respectively and showed reduction in the activity as the temperature decreases.

4.2 Effect of PH on Wheat bran

The effect of PH on the cellulase and xylanase activity of *A.fumigatus* was examined at various pH ranging from 3 to 7 as shown in figure 4.2. The maximum activity was displayed at pH of 6 with reducing sugar concentration of 0.50mg/ml for cellulase and 0.57mg/ml for xylanase and least activity was displayed at pH of 3 with an activity of 0.30mg/ml and 0.33mg/ml of the respective enzymes.

4.3 Effect of incubation period on Wheat bran

Figure 4.3 shows a gradual increase in enzyme activity through 5,10,15 and maximum at 20days with reducing sugar concentration of 0.51mg/ml for cellulase and 0.60mg/ml for xylanase. The enzyme activity showed a gradual decrease on further extension of incubation period beyond 25days.

4.4 Effect of substrate concentration on Wheat bran

The effect of substrate concentration (wheat bran) is shown in figure 4.4. From the figure, substrate concentration of 1% gave the highest activity of both enzymes with reducing sugar concentration of 0.53mg/ml for cellulase and 0.58mg/ml xylanase while the substrate concentration of 5% gave the lowest activity of 0.32mg/ml and 0.35mg/ml respectively from *A.fumigatus.*

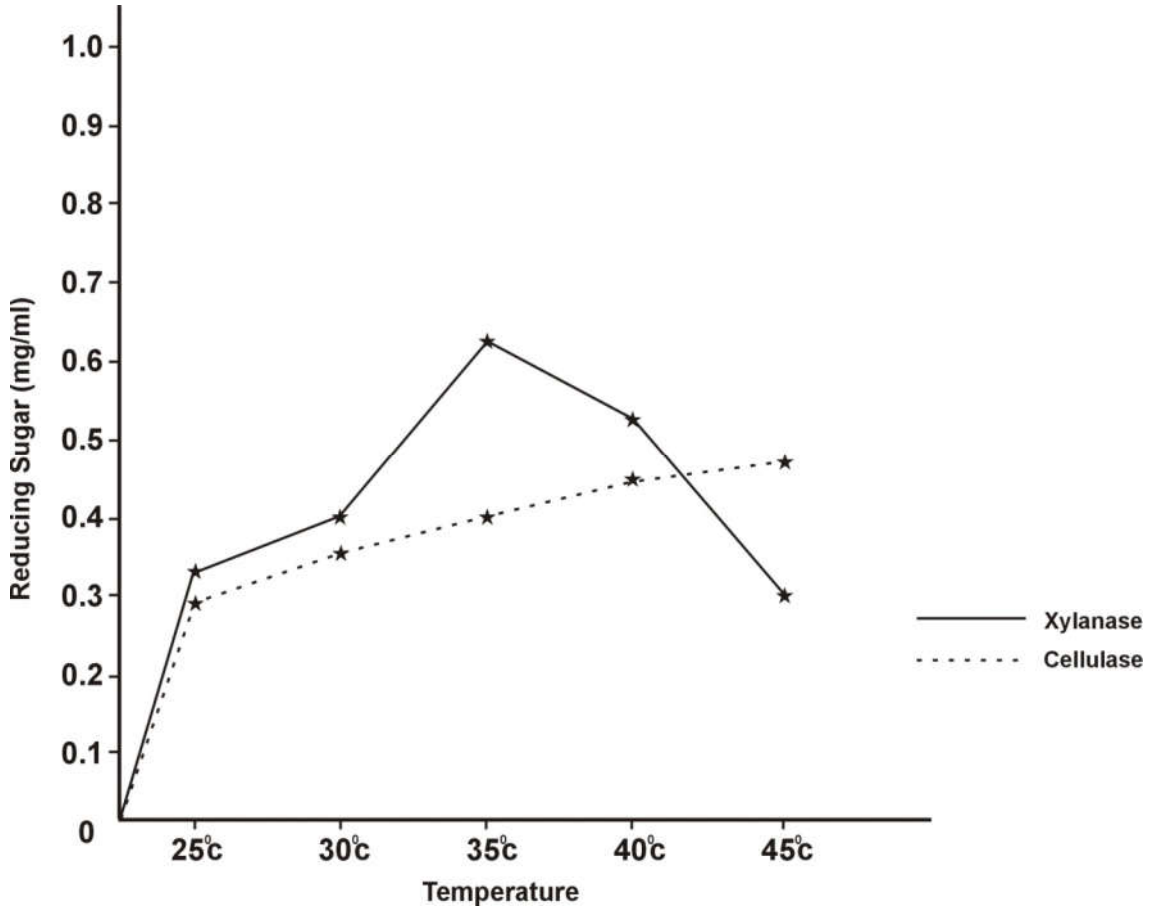


Fig 4.1: Effect of Temperature on cellulase and xylanase produced by *A. fumigatus* on wheat bran.

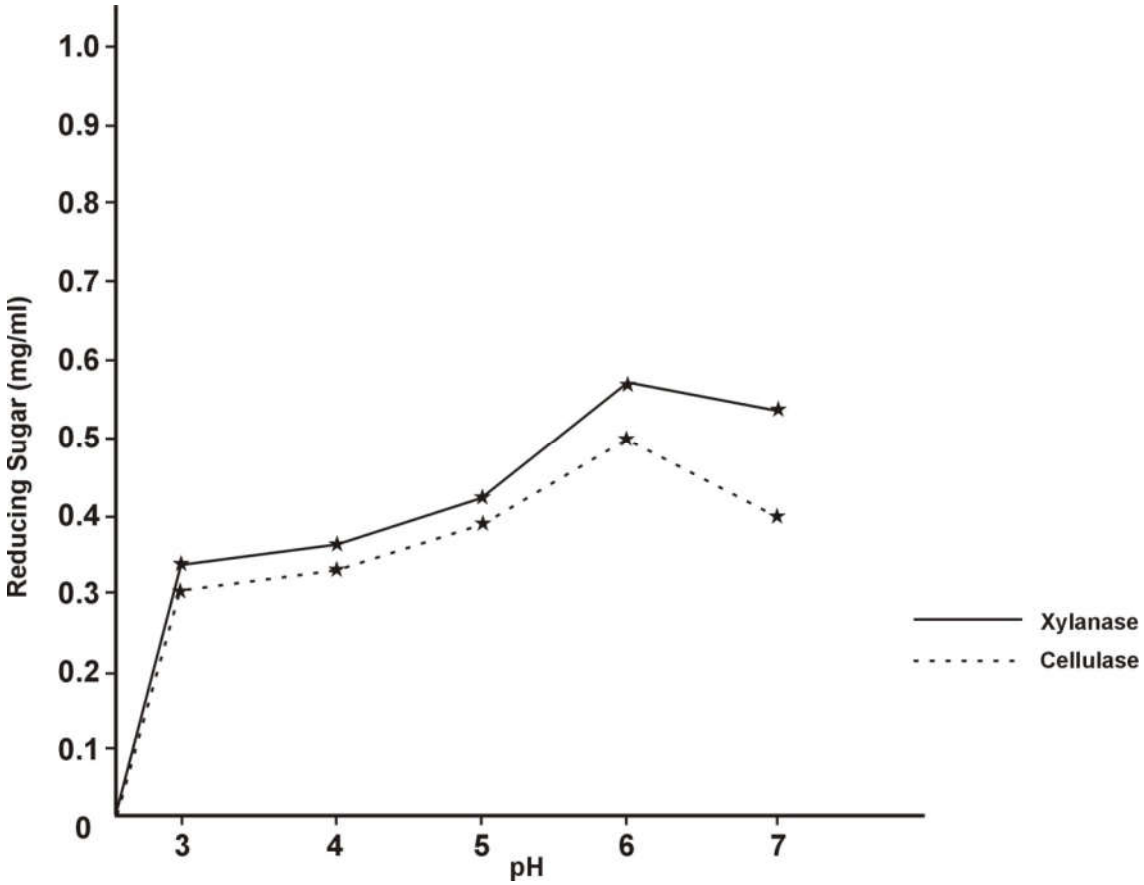


Fig 4.2: Effect of pH on cellulase and xylanase produced by *A. fumigatus* on wheat bran.

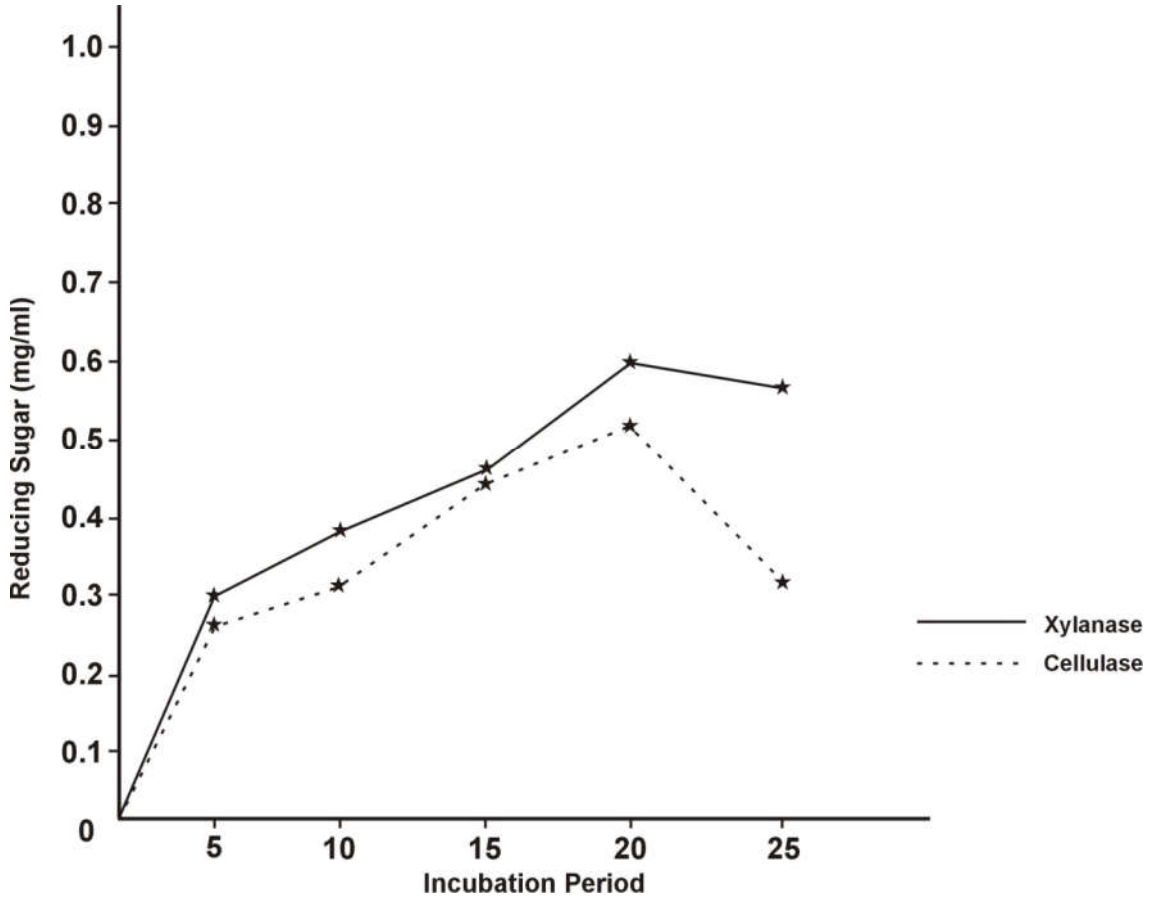


Fig 4.3: Effect of incubation Period (Days) on cellulase and xylanase produced by *A. fumigatus* on wheat bran.

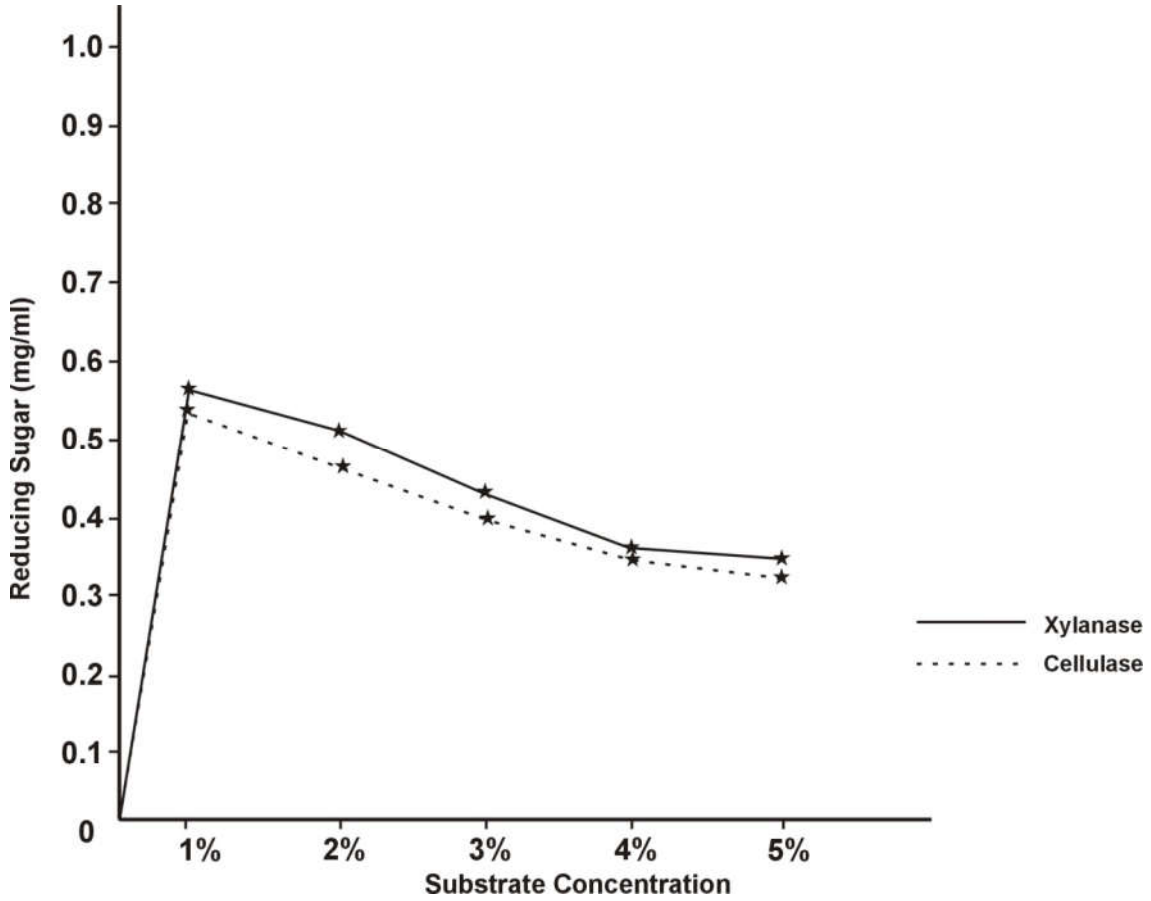


Fig 4.4: Effect of Substrate concentraton on cellulase and xylanase produced by *A. fumigatus* on wheat bran.

4.5 Effect of inoculum size on Wheat bran

There was no increase in enzyme activity when the size of the inoculums was further increased (figure 4.5).The maximum activity for cellulase and xylanase was 0.49mg/ml and 0.56mg/ml respectively when 10^{-3} of inoculums were used.

4.6 Effect of Temperature on Sawdust

The temperature stability result of cellulase and xylanase obtained from *A. fumigatus* is shown in (Figure 4.6). The figure revealed that the enzymes remained stable at 45^{o}C and 35^{o}C for the respective enzymes with reducing sugar concentration of 0.44mg/ml and 0.47mg/ml. The enzyme stability decline as the temperature decreases with least activity of 0.23mg/ml and 031mg/ml.

4.7 Effect of pH on Sawdust

The effect of varying pH values of 3, 4, 5, 6, and 7 of media containing sawdust was presented in figure 4.7. At pH of 4, maximum activity of cellulase was 0.44mg/ml and 0.51mg/ml for xylanase at pH of 5.

4.8 Effect of Incubation period on Sawdust

Figure 4.8 shows the enzymes activity of *A. fumigatus*. The peak activity was observed on the 15th day of cultivation with reducing sugar concentration of 0.50mg/ml for cellulase and on the 10th day for xylanase at 0.52 mg/ml.

4.9 Effect of Substrate concentration on Sawdust

The effect of substrate concentration on the enzymes activities was observed from 1% to 5% with highest cellulase activity of 0.55 mg/ml at 4% while xylanase was 0.61mg/ml at 5% as presented in figure 4.9.

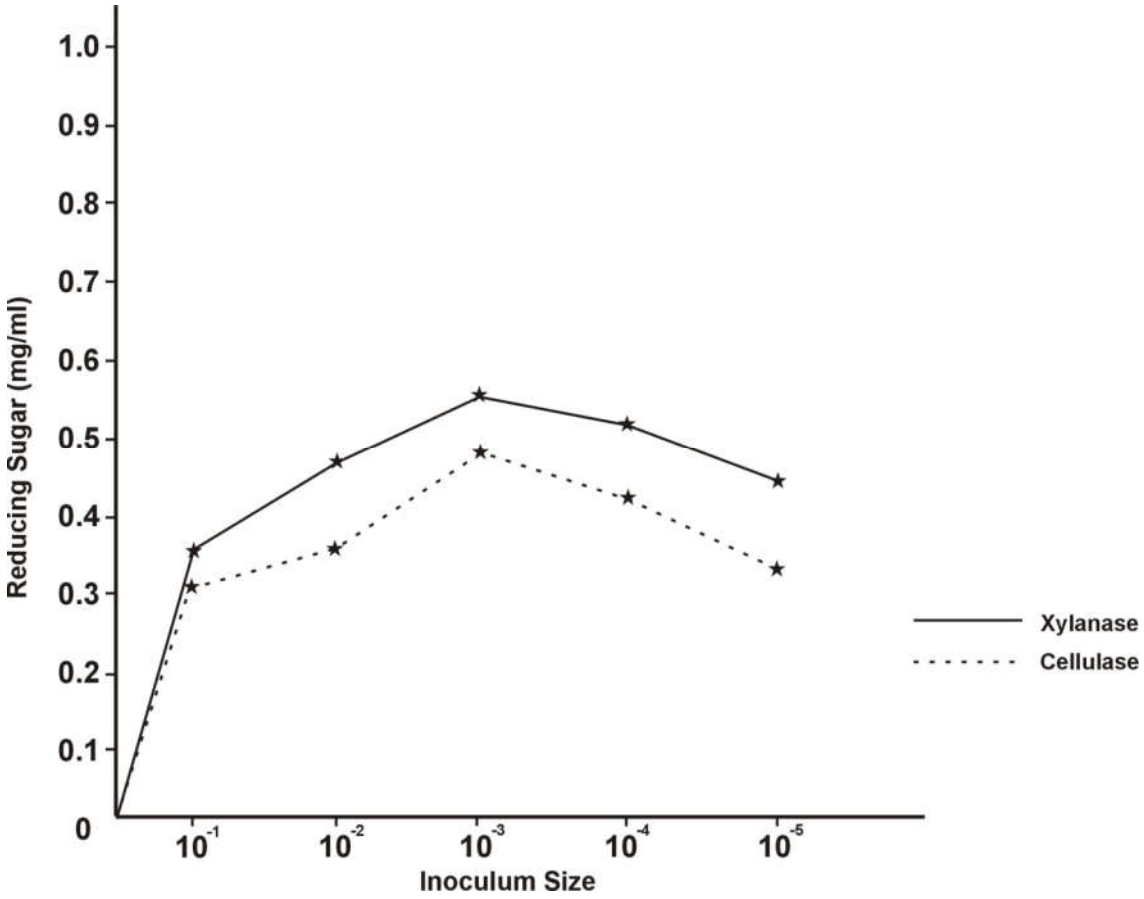


Fig 4.5: Effect of Inoculum size on cellulase and xylanase produced by *A. fumigatus* on wheat bran.

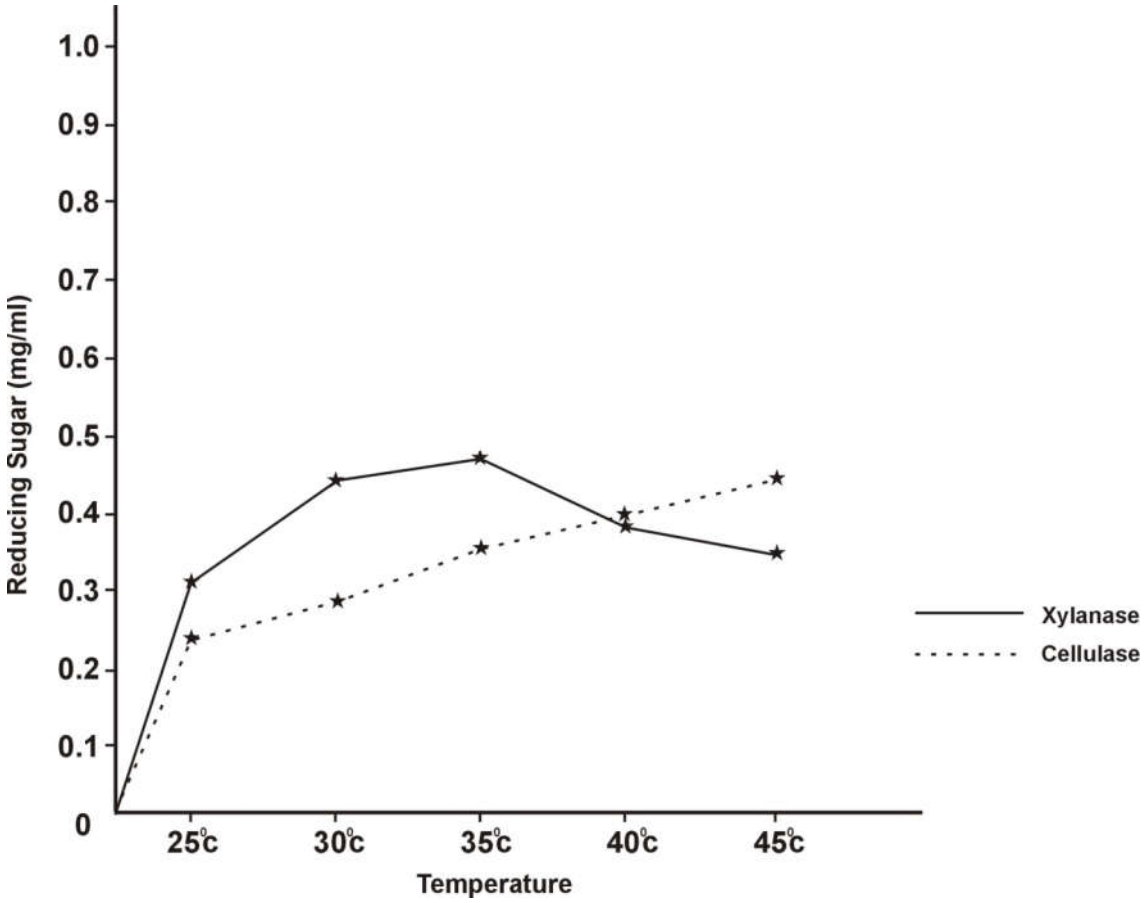


Fig 4.6: Effect of Temperature on cellulase and xylanase produced by *A. fumigatus* on sawdust.

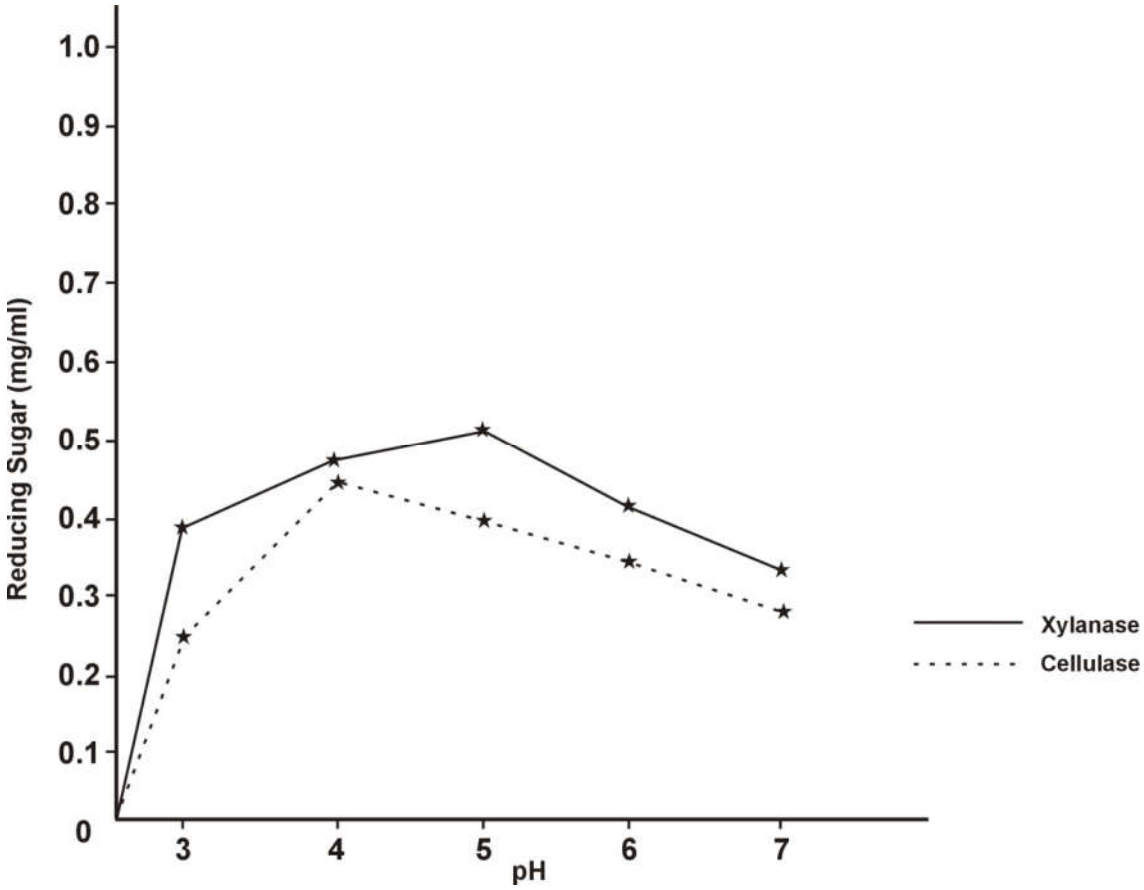


Fig 4.7: Effect of pH on cellulase and xylanase produced by *A. fumigatus* on sawdust.

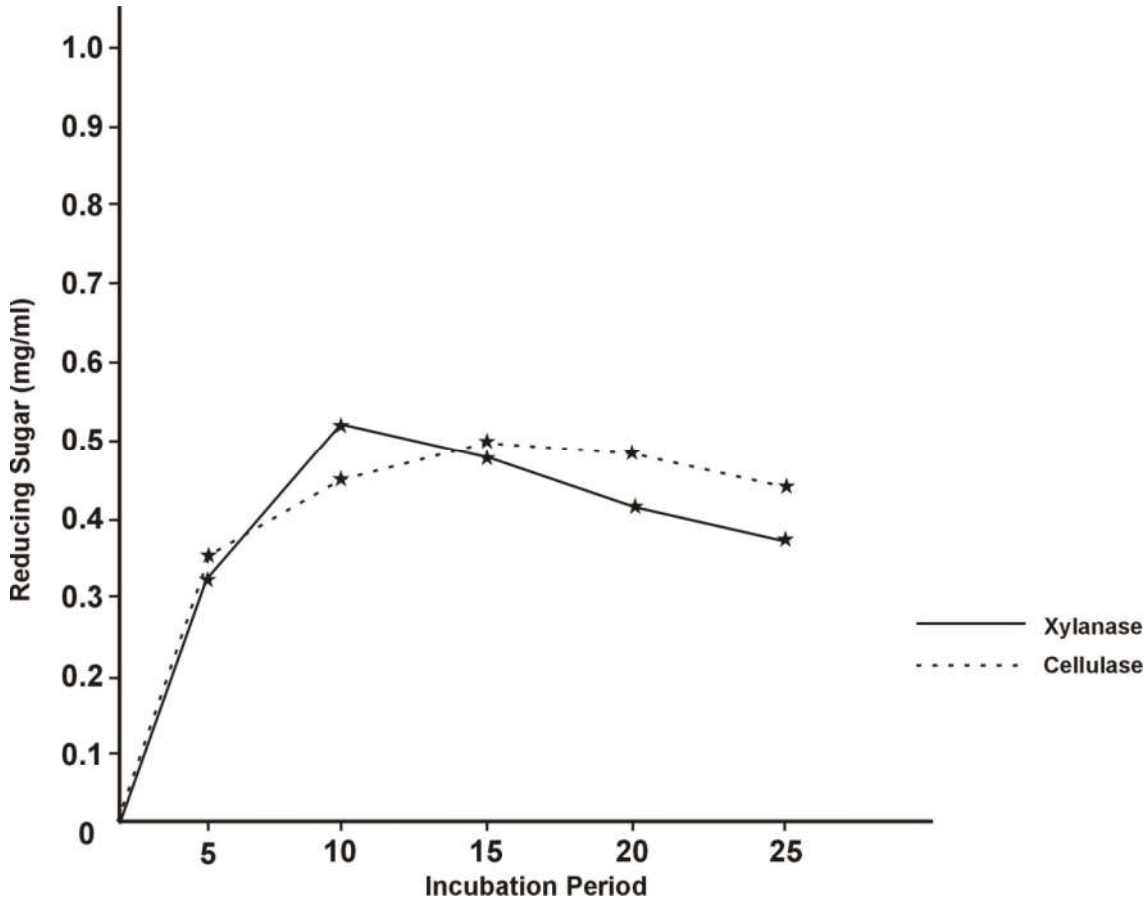


Fig 4.8: Effect of Incubation period on cellulase and xylanase produced by *A. fumigatus* on sawdust.

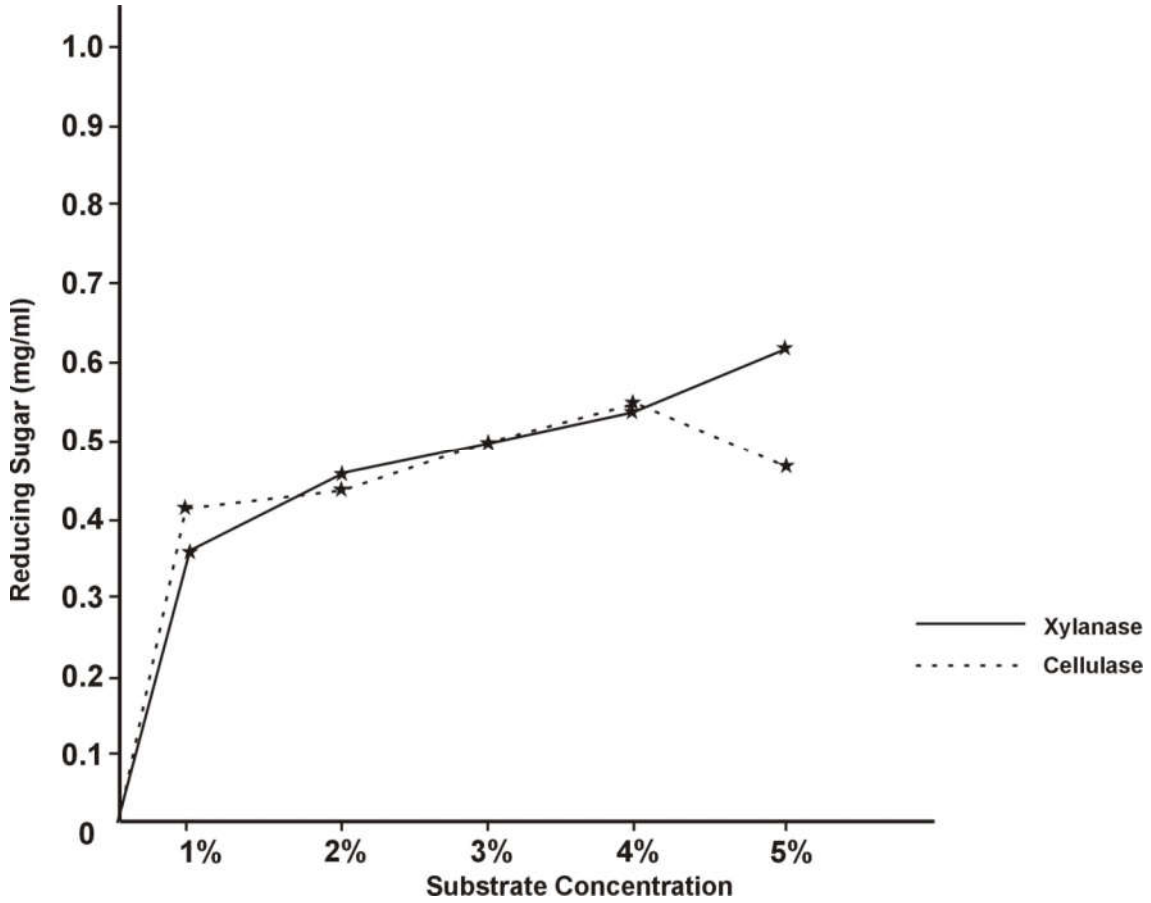


Fig 4.9: Effect of Substrate concentration on cellulase and xylanase produced by *A. fumigatus* on sawdust.

4.10 Effect of Inoculum size on Sawdust

Figure 4.10 shows the cellulase and xylanase activity of *A. fumigatus* was observed at 10^{-3} of the spore suspension with reducing sugar concentration of 0.47mg/ml for cellulase and 0.50 mg/ml for xylanase at 10^{-4} of the spore suspension.

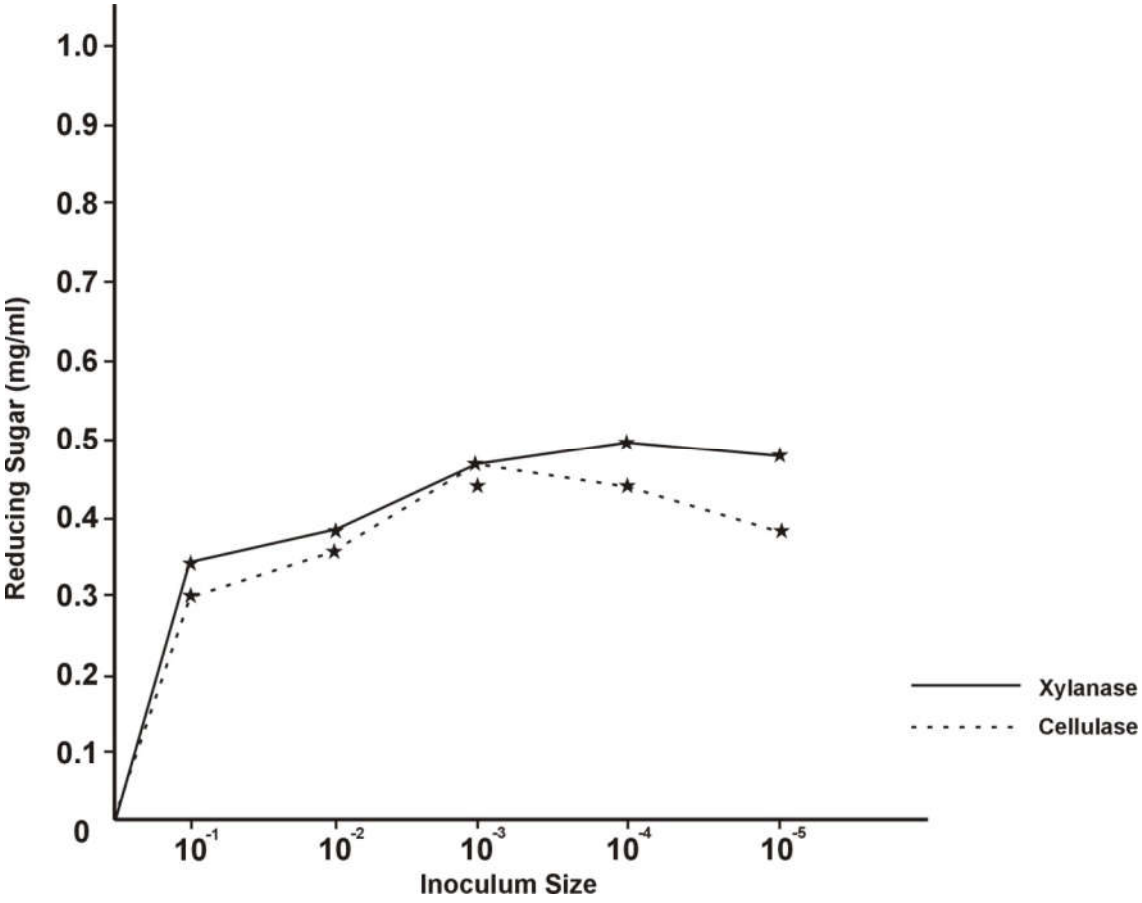


Fig 4.10: Effect of Inoculum size on cellulase and xylanase produced by *A. fumigatus* on sawdust.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.0 DISCUSSION

Five different fungal species were isolated identified using the method of Rufa'i (1979) as presented in table 4.1. All the strains were screened for their ability to grow on wood media with or without addition of glucose, as an inducer for growth at the beginning of cultivation. Most of the species grew well on wood supplemented with glucose. However, all the organisms, except *Rhizopus* spp were able to grow adequately even in the absence of supplemented glucose, indicating the capability to produce enzyme necessary for the degradation of wood, as represented in Table 4.2. Table 4.3 shows the screening of the fungal isolates on PDA media containing coloured indicator compounds that enable the visual detection of lignocellulose enzymes production. The use of coloured indicators is generally simpler because there is no requirement for sample handling and measurement (Kalmis *et al*., 2008). Several different compounds have been used as indicators for ligninolytic enzymes production. RBBR and guaiacol are used frequently and the result correlate well to each other (Kiiskinen *et al*., 2004). Methyl green was also used in this project for lignocellulose enzymes screening. The lack of detection of growth for *A .flavus, Alteneria* spp. and *Mucor* spp on methyl green containing media could be attributed to the high concentration of the indicator, which might have an inhibitory effect (Eichlerova *et al*., 2006). *A. fumigatus* showing positive reactions in the screening in wood solid medium and dye decolorization assay, were grown in different lignocellulosic materials such as sawdust and wheat bran to induce the production of lignocellulose degrading emzymes. Wheat bran has been used as substrate in most of studies for the production of different enzymes such as cellulase, xylanase and ligninolytic enzymes e.t.c. (Chandra *et al.,* 2007 and Souza *et al.,* 2006).

Aspergillus fumigatus in this study showed maximum activity within the temperature range of 45^{o}C and 35^{o}C for cellulase and xylanase respectively using wheat bran as substrate, (Figure 4.1). The reported cellulases from *Bacillus species* present an optimum activity from 40^{o}C to 70^{o}C (Hakamada *et al.,* 1997; Christakopoulos *et al.,* 1999; Mawadza *et al.,* 2000; Kim *et al.,* 2005. This is in coincidence with the result obtained by Padmavathi and Venkatesh, 2009, as *A. niger* produces maximum of cellulase at 45^{o}C.

Enzymes have pH range within which they function best with their activity maximum at optimum pH and at higher or lower pH values, their activity decreases (Lehinger *et al*., 1993). The cellulase and xylanase production using wheat bran as substrate showed that optimum pH was found to be near neutral (PH of 6) as shown in Figure 4.2. Endoxylanse I and II from *A. awamori* shows an optimum pH at 5.5 and 6 respectively (Kormelink *et al*., 1992).

Figure 4.3 shows the effect of incubation period on the enzymes production increased progressively and attained the peak activity at the 20th day of incubation and then declined on the 25th day. The current result differs from that of (Kyi, 2011) who reported peak activity at the 17th day of incubation. The decline in enzymes activity on the 25th day may be explained by the fact that at this stage the isolates have entered their late stationary phase. Further increase in wheat bran beyond one percent (1%) did not result in proportionate increase in enzymes yield.

Mandels and Reese (1959) also reported that maximum yield of cellulase were obtained on 1% substrate (Figure 4.4). This may be due to the fact that certain sugars are inhibitors of enzyme production while others stimulate enzyme production. Ibrahim et al, 2011 reported that Adansonia digitata fruit pulp inhibited the production of Amylase by *B. licherniformis*.

At temperature of 45^{o}C and 30^{o}C saw dust were found to support highest cellulase and xylanase activity respectively, as presented in table 4.6. This is in line with the findings of Ogundero, (1982) who reported that culture cellulase of *A. niger* were found to be more active at temperature 45-50^{o}C. *Fusarium oxysporioum* in shake flask culture also produces maximum xylanases yield at incubation temperature of 30^{o}C (Kuhad *et al.,* 1998). So also Kheng and Omar, 2005, produces maximum xylanases at 28^{o}C using *A. niger* USMA1. This observation was in agreement with those reported by Biswas *et al.,* 1990 who showed that the highest xylanase activities of *Fusarium solani* was at room temperature when the temperature increased or decreased from the optimum, the growth of the fungus was inhibited (Yaun and Rugyu, 1999). The enzymes activity gradually decreased probably due to enzyme denaturation, conformation change, as enzymes are proteins. Therefore, pre-treatment of lignocellulosic material enhances enzyme activity and maximum saccharification was achieved within the range 30-45^{o}C coinciding with the characteristics of mesophiles (Baig and Ysmeen, 2004). There is no statistical significant difference between wheat bran and sawdust in terms of enzyme production based on temperature variation, (p = 0.358).

At pH of 4, cellulase was found to have its highest activities while at pH value of 5 xylanase have its highest activity when saw dust was used as substrate, figure 4.7. Bakri and Arabi (2000) produced xylanase from newly isolated *Cochilobolus sativus* in submerged fermentation and reported that initial medium pH of 4-5 was optimum for the enzyme production. Different investigation on xylanase production reported that initial medium pH of 4.5 (Fadel, 2001) 6.0 (Qinnghe *et al*., 2004) and 6.5 (Carmona, 2005) were best for the enzymes production by different fungi in fermentation process. The reports indicates that most of the fungus exhibit acidic environment for their growth. This in agreement with the work of Ali *et al.,* (1991) in which pH of 3 and 4 were reported as favouring high yield of cellulase enzymes. Effect of pH on glucose production by the organism supports the findings

of lee *et al.,* (2002) who reported that CMCase, Avicelase and FPase activities exhibit a PH optimum of approximately 4. The instability of the enzymes at very low or very high pH values is due to the fact that they are proteins which are generally denatured at extreme pH values. Optimum pH not only provides suitable condition for growth and enzymes production but will also determine the enzymatic action on the substrate and enhance the enzyme stability (Padmavathi *et al.,* 2011). This implies that both enzymes holds commercial value for industries that carry out their operation at neutral pH values. There is no statistical significant difference between wheat bran and sawdust in terms of enzyme production based on pH variation ($p = 0.661$).

Figure 4.8 shows the maximum cellulase and xylanase enzyme production by *A. fumigatus* using saw dust as substrate were obtained on the 15th day and 10th day of cultivation respectively. This is in similar with the findings of Ray *et al.,* (1993) who reported maximum production of cellulase on the 15th day of cultivation on wheat bran by *A. niger.* Abdel- satera and El- Said (2001) obtained maximum production of xylanase from *Trichoderma harzanum* after 8th days of cultivation period. Goyal and Sareen (2008) achieved maximum enzyme production for 14-17 days of fermentation period using strain of *T. viride.* Increased fermentation time and decreased enzymes synthesis might be due to the depletion of macro and micro nutrients in the fermentation medium with the passage of time, which altered the fungal physiology resulting in the inactivation of secretary machinery of the enzymes, (Nochure and Roberts, 1993). There is no statistical significant difference between wheat bran and sawdust in terms of enzyme production based on incubation period ($p = 0.162$).

The increase in enzyme activity in saw dust as substrate from 1% - 5% suggests the ability of the saw dust to produce more of the enzymes at optimal substrate concentration of 5%, figure 4.9. Abubakar *et al.,* (2013) reported an optimum cellulase production at a substrate concentration of 6%. Reis *et al*., (2003) obtained maximum xylanase activity with 5%

sugarcane bagasse as a carbon source using *A. nidulans.* Since the substrate contain different minerals apart from carbon which may serve as nutrient supplement, increase in substrate concentration leads to increase in the nutrients which may adversely affect the cell concentration. The increase in glucose production until the optimum that was obtained was due to the availability of cellulose in the medium while a decrease in production beyond optimum concentration is explained to be as a result of an inhibitory effect of accumulated cellobiose and cellodextrins of low degree of polymerization of the growth medium. It might also be due to the specific binding of the enzymes with the substrate (Wang *et al.,* 2006). Brien and Craig, (1996) also reported that decrease in enzymes production beyond the optimum may be reduced due to sugar depletion from the substrate into the medium. There is no statistical significant difference between wheat bran and sawdust in terms of enzyme production by varying substrate concentrations ($p = 0.235$).

Inoculum size of 10^{-3} dilution resulted in a higher cellulase and xylanase production compared to other inoculum sizes, as presented in figure 4.10. Higher enzyme production of higher inoculum is related to the rapid growth of the fungus, which resulted higher degradation of the substrates and increase availability of the nutrients. Inoculum size beyond this level declined the enzymes production, inoculum size controls and shortens the lag phase, smaller inoculum size increased the moisture content which ultimately decreases the growth and enzymes production (Sharma *et al*., 1996).

Decrease in enzymes activity with further increase in inoculum might be due to the clumping of cells which could have reduced sugar and oxygen uptake rate and also enzyme release and may probably be due to limiting nutrients at higher inoculum size (Dhanya *et al*., 2006). There is no statistical significant difference between wheat bran and sawdust in terms of enzyme production based on inoculum size ($p = 0.827$).

The enzyme activity is species dependent and the type of substrate used and cultivation method also effect the production (Elisashvili *et al.,* 2008). The growth or enzyme production detected in samples used in lignin production using the substrates was negligible due to low accessibility of nutrient for fungal growth.

P. pinophilum TERIDBI was shown to be able to produce lignin degrading enzymes on corn cobs molasses and wheat straw (Pant and Adholega, 2007). These lignin degrading enzymes were not detected in the samples of the present study, possibly because it was a different strain than the one reported and the substrate used were different. These is similar with the findings of (Kyi, 2011) who reported lignocellulose degrading enzymes production such as cellulase and xylanase using *P. pinophilum* IBTI0872, although no lignin degrading enzymes were detected in liquid fermentation. It has also been reported in the literature that solid state fermentation SSC has been found to be an efficient inducer for the production of ligninolytic enzymes as it mimics the natural habitat of the fungus and holds great potential for the production of the enzymes.

5.1 Conclusion

In conclusion, the fungi identified were *A. flavus*, *A. fumigatus*, *Mucor* spp, *Alteneria* spp. and *Rhizopus* spp. Screening test was done on wood media and dye decolorization test using Remazol brilliant blue R and methyl green (RBBR) which was observed as a colourless halo around the microbial growth. The production of the enzymes by *A. fumigatus* on wheat bran was maximum at pH of 6, 20days of incubation using 1% substrate concentration with 10^{-3} of the inoculum while the optimum temperature for cellulase production on sawdust was achieved at 45^{o}C, pH of 4, 15days of incubation using 10^{-3} of the inoculum, and xylanase activity was maximum at 30^{o}C, pH of 5, at 10days of incubation using 5% substrate concentration. The results highlight the potential of the substrates as possible raw materials

for the enzymes production using *A. fumigatus*, with wheat bran being slightly better even though there is no statistical significant difference between them.

5.2 Recommendation

1. Wheat bran should be used with this organisms to produce cellulase and xylanase in bioreactors (Pilot scale).
2. Large scale industrial production of cellulase and xylanase should be carried out in other to harness the substrate potential.
3. Further research should be carried out to characterize the cellulase and xylanase produced by the *A. fumigatus* in this work.

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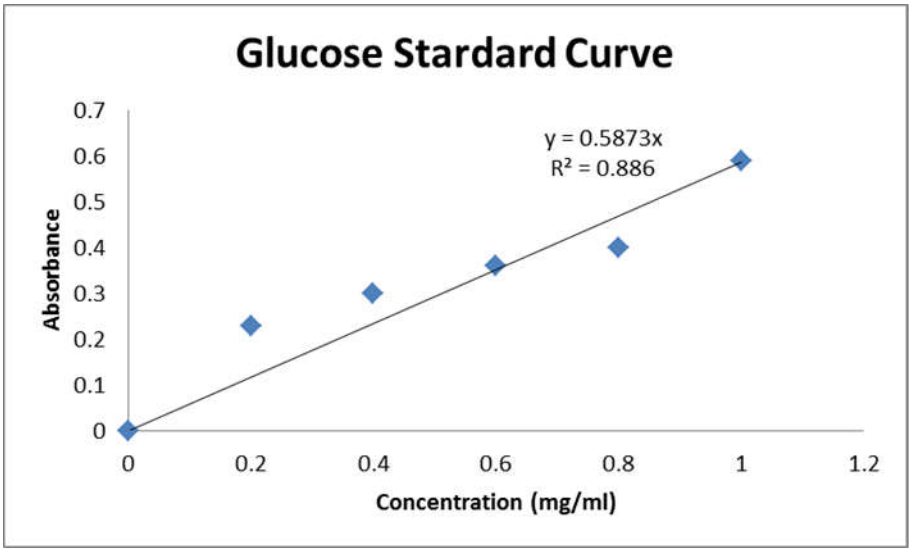
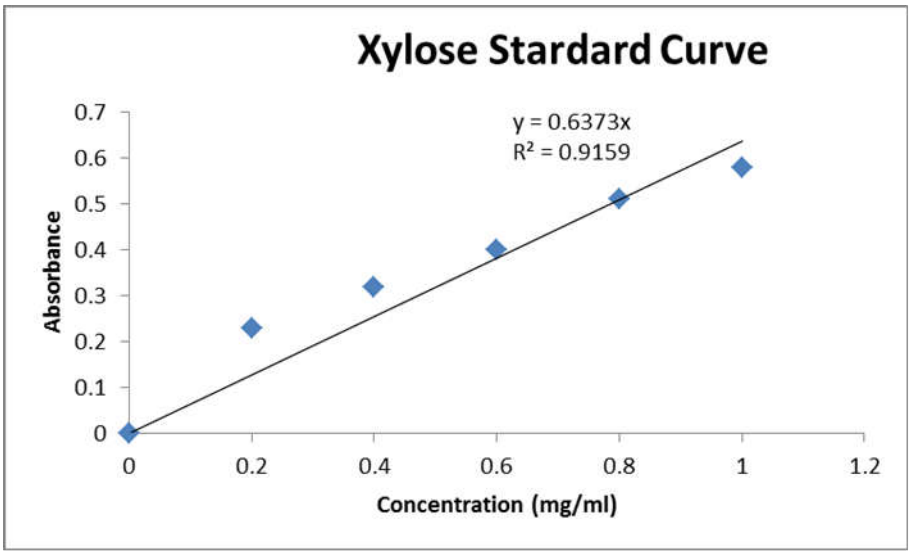
APPENDICES

Appendix I: Glucose Standard Curve



Appendix II: Xylose Standard Curve



APPENDIX III

t-test

Data source: Data Temperature

Normality Test: Passed (P = 0.565)

Equal Variance Test: Passed (P = 0.570)

Group Name	N	Missing	Mean	Std Dev	SEM
WB-Temp	5	0	0.392	0.0705	0.0315
SD- Temp	5	0	0.344	0.0844	0.0378

Difference 0.0480

t = 0.976 with 8 degrees of freedom. (P = 0.358)

95 percent confidence interval for difference of means: -0.0654 to 0.161

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.358).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

t-test

Data source: Data of pH

Normality Test: Passed (P = 0.697)

Equal Variance Test: Failed (P < 0.050)

Group Name	N	Missing	Mean	Std Dev	SEM
WB-pH	5	0	0.382	0.0789	0.0353
SD-pH	5	0	0.362	0.0585	0.0262

Difference 0.0200

t = 0.455 with 8 degrees of freedom. (P = 0.661)

95 percent confidence interval for difference of means: -0.0813 to 0.121

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.661).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

t-test

Data source: Data Incubation period

Normality Test: Passed (P = 0.686)

Equal Variance Test: Passed (P = 0.141)

Group Name	N	Missing	Mean	Std Dev	SEM
WB-Inc	5	0	0.368	0.102	0.0457
SD-Inc	5	0	0.448	0.0554	0.0248

Difference -0.0800

t = -1.540 with 8 degrees of freedom. (P = 0.162)

95 percent confidence interval for difference of means: -0.200 to 0.0398

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.162).

Power of performed test with alpha = 0.050: 0.171

The power of the performed test (0.171) is below the desired power of 0.800.
Less than desired power indickates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.

t-test

Data source: Data Substrate

Normality Test: Passed (P = 0.791)

Equal Variance Test: Passed (P = 0.140)

Group Name	N	Missing	Mean	Std Dev	SEM
WB-Subs	5	0	0.412	0.0881	0.0394
SD-Subs	5	0	0.472	0.0559	0.0250

Difference -0.0600

t = -1.286 with 8 degrees of freedom. (P = 0.235)

95 percent confidence interval for difference of means: -0.168 to 0.0476

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.235).

Power of performed test with alpha = 0.050: 0.106

The power of the performed test (0.106) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.
t-test

Data source: Data Inoculum size

Normality Test: Passed (P = 0.844)

Equal Variance Test: Passed (P = 0.852)

Group Name	N	Missing	Mean	Std Dev	SEM
WB-innoc	5	0	0.382	0.0733	0.0328
SD-Innoc	5	0	0.392	0.0669	0.0299

Difference -0.01000

t = -0.225 with 8 degrees of freedom. (P = 0.827)

95 percent confidence interval for difference of means: -0.112 to 0.0923

The difference in the mean values of the two groups is not great enough to reject the possibility that the difference is due to random sampling variability. There is not a statistically significant difference between the input groups (P = 0.827).

Power of performed test with alpha = 0.050: 0.050

The power of the performed test (0.050) is below the desired power of 0.800.
Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.