KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

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DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

TITLE: Isolation and utilization of cellulolytic microorganisms for cellulase production through solid state fermentation of corncobs.

THIS DISSERTATION IS PRESENTED TO THE DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENT OF M.Sc. DEGREE IN BIOTECHNOLOGY.

BY

EDWARD AGYARKO

SEPTEMBER, 2011.

DECLARATION PAGE

I declare that I have wholly undertaken the study reported herein under the supervision of Dr. H. D. Zakpaa and that except portions where references have been duly cited, this dissertation is the outcome of my research.

Date: .....

27th September, 2011. EDWARD AGYARKO
(Student)

Date: .....

27th September, 2011. Dr. H. D. ZAKPAA
(Supervisor)

Date: .....

27th September, 2011. Mr. E. E. MAK-MENSAH
(Co-Supervisor)

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ABSTRACT

This research was conducted to investigate production of cellulase enzymes from a cellulolytic soil microorganism cultured on corncobs under solid state conditions. The cellulolytic fungi isolated from soil samples were *Aspergillus niger, Neurospora crassa, Penicillium sp., Rhizopus sp., Trichoderma sp.* The bacteria isolated from the same soil samples were *Streptomyces sp., Clostridium sp* and *Klebsiella pneumonia*. Out of these microorganisms, *A. niger* had the highest clearing zone to mycelium diameter ratio of 0.26 when cultured on carboxymethylcellulose agar and so was selected for the solid state fermentation experiments. The fermentation was carried out under different temperatures (27, 37, 47 and 57 °C) and initial spore concentrations of the fungi ($1.3x10^6$, $2.3x10^6$ and $4.5x10^6$) in order to investigate how these parameters affect the production of cellulase enzymes. Undiluted spore inoculum (containing about $4.5x10^6$ spores) gave the best fermentation results in terms of the amounts of glucose released (1.38 mg ml^{-1}) from the substrate and the concentration of enzymes produced by the fungi throughout the 15-day period. Although 47 °C was best for the lag phase of *A. niger* growth on corncobs, 37 °C is most suitable for exponential growth and enzyme production under solid state conditions. At 37 °C, enzyme activities of the fermentation filtrates were 0.35, 0.44 and 0.45 CMC units respectively for 5, 10 and 15 days of fermentation. These values were the highest obtained as compared with values obtained when the other temperatures were used. Thus the strain of *A. niger* used can be described as a highly cellulolytic mesophile.

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CHAPTER ONE

1.0 INTRODUCTION

Plant biomass such as agricultural and forestry residue is a major renewable natural resource of the world and represents a major source of renewable organic matter (Acharya *et al.*, 2008; Saha, 2004). Although they are mostly regarded as waste, they serve as low cost feedstock for production of fuel ethanol, reducing sugars, organic acids, proteins and amino acids, lipids, phenols, resins, improved animal feeds and enzymes (Tengerdy and Szakacs, 2003).

Lignocellulose is the major structural component of plant biomass (woody and non-woody plants). Lignocellulose consists of cellulose, hemicellulose and lignin (Mtui, 2009; Howard *et al*., 2003). Large amounts of lignocellulosic waste are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro industries. These materials pose an environmental pollution problem. The problem is often aggravated by disposing much of the lignocellulose waste by biomass burning (Howard *et al*., 2003).

The use of plant biomass for value added products requires prior conversion of the complex carbohydrates (cellulose and hemicellulose) and lignin to simple hydrolysates. This process can be achieved by direct microbial degradation or the use of enzymes harvested from the microorganisms. Since the microorganisms used in direct microbial degradation usually release extracellular enzymes onto the substrate, the enzymes alone can be harvested

and purified. Once the enzymes are harvested, they are easier and even tidier to use in industrial processes.

Although several microorganisms (mainly fungi and bacteria) have been isolated that can degrade and convert lignocellulose from these biomass feedstock into value added products, the recalcitrance of lignocellulose to microbial degradation has limited the microbial activities to some extent (Himmel *et al.*, 2007). It has therefore become necessary on biotechnologists to now focus on improving the strains that have been isolated as well as the processes of conversion of lignocellulose in order to improve the rate and volumes of products that are generated from the lignocellulosic biomass feedstock available. Hence one clear objective of the biofuel industry now is to make the production process cost-competitive in today's markets (Himmel *et al.*, 2007).

1.1 PROBLEM STATEMENT

Currently, the degradation of lignocellulosic biomass to fermentable sugars presents significant technical and economic challenges, and its success depends largely on the development of highly efficient and cost-effective enzymes for conversion of pretreated lignocellulosic substrates to fermentable sugars. Also, most industrial microorganisms are patented and may not be available (or must be purchased) for use outside their country of origin. This is of serious economic concern as it does not allow for rapid expansion of fermentation industries, hence the need to source for indigenous and suitable microorganisms from local substrates for sustainable ethanol production (Brooks, 2008).

After the feedstock and suitable microorganisms have been identified, it is also necessary to increase the product yield and rate of production of biomass hydrolysates to minimise production costs. The activities of cellulase enzymes for lignocellulose degradation can still be improved if production is carried out with microorganisms cultured on solid substrates.

1.2 JUSTIFICATION

This work seeks to use solid state fermentation as a simpler and less sophisticated method of hydrolysing lignocellulose to produced enzymes from microorganisms. Solid state fermentation, when used, results in higher enzyme yield and activity compared to submerged fermentation. Thus, the overall quantity of products per feedstock as well as the rate of production will be increased.

Determining the optimum conditions (ie. inoculum concentration, fermentation temperature and period) will lead to maximum production of enzymes per feedstock.

Finally, more value is added to maize plant production as the cobs are exploited in cellulase production.

1.3 HYPOTHESES

Soil microorganisms from Ejura farms are capable of utilizing cellulose as a carbon source due to the continual deposition of maize biomass. The use of these organisms in solid state fermentation of corncob will lead to the production of cellulases of high activity.

1.4 MAIN AIM

The main aim of this project was to produce cellulases of high activity through solid state fermentation of corncobs with isolated microorganisms.

1.5 SPECIFIC OBJECTIVES

- To isolate cellulolytic bacteria and fungi from soil and test the extent to which the isolated species can utilize cellulose material (carboxymethylcellulose).
- Use the best isolate in solid state fermentation of corncobs to produce cellulase enzymes.
- To study the effect of inoculum concentration, temperature and period of incubation on the activities of enzymes produced.

CHAPTER TWO

LITERATURE REVIEW

2.1 Lignocellulose

Lignocellulosic biomass represents the largest renewable reservoir of potentially fermentable carbohydrates on earth (Mtui and Nakamura, 2005). This resource is however mostly wasted in the form of pre-harvest and post-harvest agricultural losses and wastes of food processing industries. Due to their abundance and renewability, there has been a great deal of interest in utilizing lignocellulosic waste (LCW) for the production and recovery of many value-added products instead of starch crops (Pandey *et al*., 2000; Foyle *et al*., 2007). Among the main recovery products include enzymes, reducing sugars, furfural, ethanol, protein and amino acids, carbohydrates, lipids, organic acids, phenols, activated carbon, degradable plastic composites, cosmetics, biosorbent, resins, medicines, foods and feeds, methane, biopesticides, biopromoters, secondary metabolites, surfactants, fertilizer and other miscellaneous products (Saha, 2004; Mtui, 2007; Demirbas, 2008).

For lignocellulose to be amenable to fermentation, it needs to undergo treatments that release its monomeric sugars, which then can be converted by a suitable microorganism. Two main steps are involved in releasing the monomers: (1) a pretreatment (by physical or chemical procedures) that releases hexoses and pentoses from hemicellulose, and (2) an enzymatic treatment (or, alternatively, hydrolysis by chemical procedures) that generates glucose from cellulose.

Currently, the second generation bio-products such as bioethanol, biodiesel, biohydrogen and methane from lignocellulose biomass are increasingly being produced from wastes rather than from energy crops (jatropha, switchgrass, hybrid poplar and willow) because the latter competes for land and water with food crops that are already in high demand (www.novozymes.com). Thus utilization of a cheaper substrate such as lignocellulosic waste from plants could make bioethanol more competitive with fossil fuel. The processing and utilization of this substrate is complex, differing in many aspects from crop-based ethanol production. It has therefore become necessary to develop processes for effective treatment and utilization of lignocellulosic wastes as inexpensive carbon sources (Himmel *et al.*, 2007).

The barrier to the production and recovery of valuable materials from LCW is the structure of lignocellulose which has evolved to resist degradation due to crosslinking between the polysaccharides (cellulose and hemicellulose) and the lignin via ester and ether linkages (Yan and Shuya, 2006; Xiao *et al*., 2007). Cellulose, hemicelluloses and lignin form structures called microfibrils, which are organized into microfibrils that mediate structural stability in the plant cell (Rubin, 2008). The exact composition of lignocellulose may vary with different plant sources and maturity of the plant (Lynd *et al*., 1999). Wiselogel *et al*. (1996) reported the following general composition: cellulose (~45% of dry weight), hemicelluloses (~30% of dry weight), and lignin (~25% of dry weight). Table 1 below shows the composition of some agricultural lignocellulosic biomass (Saha, 2003).

Table 1 Composition of some agricultural lignocellulosic biomass

	Composition (%, dry basis)		
	Cellulose	Hemicellulose	Lignin
Corn fiber[a]	15	35	8
Corn cob	45	35	15
Corn stover	40	25	17
Rice straw	35	25	12
Wheat straw	30	50	20
Sugarcane bagasse	40	24	25
Switchgrass	45	30	12
Coastal bermuda grass	25	35	6

[a] Contains20% starch

Source: Saha, (2003).

2.1.1 Cellulose

Cellulose is composed of thousands of molecules of anhydroglucose linked by β (1, 4)-glycosidic bonds. The basic repeating unit is the disaccharide cellobiose (Delmer and Amor, 1995). Despite great differences in composition and in the anatomical structure of cell walls across plant taxa, they all possess high cellulose content (typically in the range of approximately 35 to 50% of plant dry weight) (Lynd *et al*., 1999). Once stripped of the protective effects of other plant biopolymers, cellulose in native plant material shares many characteristics across plant taxa, including its potential for complete hydrolysis and utilization under the proper microbial and environmental conditions (Lynd *et al*., 2002). Cellulose is synthesized in nature as individual molecules (linear chains of glucosyl residues) which undergo self-assembly at the site of biosynthesis (Brown and Saxena, 2002). Approximately 30 individual cellulose molecules are assembled into larger units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils, and

these are in turn assembled into the familiar cellulose fibers (Lynd *et al*., 2002; Somerville, 2006). The secondary and tertiary conformations of cellulose, as well as its close association with lignin, hemicellulose, starch, protein and mineral elements, make cellulose resistant to hydrolysis to some extent. Cellulose can however be hydrolyzed enzymatically by cellulases, or chemically by diluted or concentrated acid (Zaldivar *et al.*, 2001).

2.1.2 Hemicellulose

Hemicellulose is a highly branched heteropolymer containing sugar residues such as hexoses (D-galactose, L-galactose, D-mannose, L-rhamnose), pentoses (D-xylose, L-arabinose), and uronic acids (D-glucuronic acid) (Saha, 2003). It is the second most abundant polysaccharide in nature representing about 20-35% of lignocellulosic biomass (Saha, 2003). Unlike cellulose, hemicelluloses are not chemically homogeneous. Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans. Hemicellulose is more easily hydrolyzed than cellulose (Brigham *et al*., 1996). The composition of hemicellulose will depend on the source of the raw material (Wiselogel *et al*., 1996). Due to their relative abundance in lignocellulosic biomass, effective biodegradation of hemicellulose can lead to the efficient bioconversion of lignocellulosic materials to fuel ethanol and other value-added fermentation products (Saha, 2003).

2.1.3 Lignin

Lignin, the most abundant aromatic polymer in nature is the third most abundant constituent of plants (Vanholme *et al.*, 2010; Boerjan *et al.*, 2003). It serves as a matrix around the polysaccharide components of some plant cell walls, providing additional rigidity and compressive strength as well as rendering the walls hydrophobic and water impermeable (Whetten and Sederoff, 1995). Lignin is a macromolecule of phenolic character, being the dehydration product of three monomeric alcohols (monolignols), trans-p-coumaryl alcohol, trans-p-coniferyl alcohol, and trans-p-sinapyl alcohol, derived from p-cinnamic acid (Kirk *et al*., 1977). These monolignols are synthesised through the phenylpropanoid pathway (Vanholme *et al.*, 2010). The exact composition of these monomers differs from species to species therefore, lignin cannot be said to have a uniform structure (Boerjan *et al.*, 2003).

2.2 Microbial utilization of lignocellulose

Microorganisms such as fungi and bacteria are known to play a major role in the degradation of cellulose and starch components (Coughlan, 1985). The breakdown of these components produces simple sugars that find many uses in the feed and fermentation industries. Cellulases are the enzymes that hydrolyze the β-1, 4-glycosidic bonds in the polymer to release glucose units (Ezekiel *et al*., 2010; Nishida, 2007). 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 enzymes capable of completely hydrolyzing crystalline cellulose (Koomnok, 2005).

Because of their relatively low cost and plentiful supply, reports show that plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity (Lynd *et al.*, 1999). Despite great differences in composition and in the anatomical structure of cell walls across plant taxa, high cellulose content is a unifying feature (Lynd *et al.*, 1999). In a few cases (notably cotton), cellulose is present in a nearly pure state. In most cases, however, the cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin. Although these matrix interactions vary with plant cell type and with maturity (Wilson, 1993), they are a dominant structural feature limiting the rate and extent of utilization of whole untreated biomass materials. Utilization of cellulosic biomass is more complex than that of pure cellulose, not only because of the former's complex composition (i.e., presence of hemicelluloses and lignin) but also because of the diverse architecture of plant cells themselves. Plant tissues differ tremendously with respect to size and organization. Some plant cell types (e.g., mesophyll) have thin, poorly lignified walls that are easily degraded by polysaccharide – hydrolyzing enzymes. Others, like sclerenchyma, have thick cell walls and a highly lignified middle lamella separating cells from one another (Wilson and Mertens, 1995). These cell walls must be attacked from the inside (luminal) surface out through the secondary wall (as opposed to particles of pure cellulose, which are degraded from the outside to inward). Thus, in addition to constraints imposed by the structure of cellulose itself, additional limitations are diffusion and transport of the cellulolytic agent to the

site of attack. These constraints may severely limit utilization in some habitats (Wilson and Mertens, 1995).

The central technological impediment to more widespread utilization of this important resource is the general absence of low-cost technology for overcoming the recalcitrance of lignocellulosic biomass. A promising strategy to overcome this impediment involves the development of native microorganisms that possess the required combination of substrate utilization and product formation properties (Lynd *et al.*, 1996). That is, microorganisms that already live and actively grow on LCW can be isolated and their ability to utilize these substrates improved to a greater extent. Also, the development of microorganism or consortium that can in addition to the production of cellulolytic enzymes and hydrolysis of biomass ferment the resulting sugars to desired products in a single process step seem promising. Such "consolidated bioprocessing" (CBP) offers very large cost reductions (Lynd *et al.*, 1996).

2.2.1 Cellulolytic Microorganisms

Current evolutionary studies based largely on measurements of sequence divergence among chronometric macromolecules, particularly small-subunit rRNAs (16S rRNA of prokaryotes and 18S rRNA of eukaryotes) reveal that the ability to digest cellulose is widely distributed among many genera in the bacteria and in the fungal groups (Woese, 2000; Olsen *et al*., 1994). Within the eubacteria there is considerable concentration of cellulolytic capabilities among the predominantly aerobic order *Actinomycetales* (phylum *Actinobacteria*) and the anaerobic order *Clostridiales* (phylum *Firmicutes*).

Fungal cellulose utilization is distributed across the entire kingdom, from the primitive, protist-like Chytridomycetes to the advanced Basidiomycetes. Fungi are well-known agents of decomposition of organic matter in general and of cellulosic substrates in particular (Carlile *et al*., 2001; Montegut *et al.*, 1991). A number of species of the most primitive group of fungi, the anaerobic Chytridomycetes, are well known for their ability to degrade cellulose in gastrointestinal tracts of ruminant animals. Although taxonomy of this group remains controversial (Carlile *et al*., 2001), members of the Order Neocallimastigales have been classified based on the morphology of their motile zoospores and vegetative thalli; they include the monocentric genera *Neocallimastix, Piromyces,* and *Caecomyces* and the polycentric genera *Orpimomyces* and *Anaeromyces* (Lee *et al.*, 1997). Cellulolytic capability is also well represented among the remaining subdivisions of aerobic fungi. Within the approximately 700 species of Zygomycetes, only certain members of the genus *Mucor* have been shown to possess significant cellulolytic activity, although members of this genus are better known for their ability to utilize soluble substrates. By contrast, the much more diverse subdivisions Ascomycetes, Basidiomycetes, and Deuteromycetes (each of which number over 15,000 species [Carlile *et al*., 2001]), contain large numbers of cellulolytic species. Members of genera that have received considerable study with respect to their cellulolytic enzymes and/or wood-degrading capability include *Aspergillus, Penicillium, Bulgaria, Chaetomium,* and *Helotium* (Ascomycetes); *Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula* (Basidiomycetes); and, *Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces* and *Trichoderma* (Deuteromycetes).

Among the bacteria, there is a distinct difference in cellulolytic strategy between the aerobic and anaerobic groups. With relatively few exceptions, anaerobes degrade cellulose primarily via complexed cellulase systems (Rainey *et al*., 1994 and Svetlichnyi *et al.*, 1990). It has been reported by Schwarz (2001) that cellulolytic enzymes in *Clostridium thermocellum* cultures are typically distributed both in the liquid phase and on the surface of the cells. However, several anaerobic species that utilize cellulose do not release measurable amounts of extracellular cellulase, and instead have localized their complexed cellulases directly on the surface of the cell or the cell-glycocalyx matrix (Schwarz, 2001). Most anaerobic cellulolytic species grow optimally on cellulose when attached to the substrate, and in at least a few species this adhesion appears to be obligate. Cellulolytic anaerobes resemble other fermentative anaerobes in that their cell yields are low, with the bulk of substrate being converted to various fermentation end products, including ethanol, organic acids, CO_2, and H_2.

The first step in developing a producer strain is the isolation of concerned microorganisms from their natural habitats (Apun *et al*., 2000). The microorganisms of industrial importance are, generally, bacteria, actinomycetes, fungi and algae. These organisms occur virtually everywhere, e.g., in air, water, soil, plant and animal surfaces and tissues. Often the ecological habitat from which a desired microorganism is more likely to be isolated will depend on the characteristics of the product desired from it, and of process development.

Both bacterial and fungal aerobic cellulose degraders utilize cellulose through the production of substantial amounts of extracellular cellulase enzymes that

are freely recoverable from culture supernatants (Rapp and Beerman, 1991; Schwarz, 2001), although enzymes are occasionally present in complexes at the cell surface (Bond and Stutzenberger, 1989; Wachinger *et al.*, 1989). The individual enzymes often display strong synergy in the hydrolysis of cellulose. While many aerobic bacteria adhere to cellulose, physical contact between cells and cellulose does not appear to be necessary for cellulose hydrolysis (Lynd *et al.*, 2002).

2.2.2 Mechanism of Cellulose Utilization by Microorganisms

Cellulolytic microbes are primarily carbohydrate degraders and generally are unable to use proteins or lipids (or their components) as energy sources for growth. Cellulolytic microbes native to soil (e.g., the bacteria *Cellulomonas* and *Cytophaga* and most fungi) can generally utilize a variety of other carbohydrates in addition to cellulose (Rajoka and Malik, 1997). Anaerobic cellulolytic species (e.g., those of the genera *Fibrobacter, Ruminococcus*, and *Clostridium*) are more limited in their carbohydrate range. They grow on cellulose and its hydrolytic products but often not on monosaccharides, oligosaccharides, and polysaccharides based on sugars other than glucose. The specialist nature of the anaerobic cellulolytic microbes probably results mainly from the specialized enzymatic machinery for cellulose hydrolysis, the significant metabolic effort devoted to its synthesis, and other features peculiar to cellulose utilization (Lynd *et al.*, 2002). These characteristics, along with the high caloric value and natural abundance of cellulose itself, apply a significant selective pressure on microbes for its utilization – particularly if the organism develops a strategy for positioning itself in such a way as to gain earliest access to the products of cellulose hydrolysis. A cellulolytic microbe,

well adapted to cellulose utilization, is unlikely to starve in any habitat (natural or man made) receiving a periodic input of plant biomass (Lynd *et al.*, 2002).

The nutrient requirements for growth of cellulolytic species include nitrogen, phosphorus, and sulphur, standard macro- and microminerals and various vitamins. Although additional nutrients present in complex media (e.g., peptones and yeast extract) are not usually required, they often stimulate the growth of individual strains.

Catabolism of cellulose involves both enzymatic depolymerisation of insoluble cellulose and cellular utilization of the hydrolytic products. There are two primary strategies for utilizing crystalline cellulose. One of the strategies, normally carried out by aerobic bacteria and fungi, is the production of noncomplexed cellulases and oxidization of hydrolytic products to CO_2 and water without adherence to cellulose (or weak adherence). Anaerobic bacteria and fungi on the other hand display a greater tendency to adhere to cellulose, produce primarily complexed cellulases (as in cellulosomes - complexes of cellulolytic enzymes created by some microorganisms), and produce a variety of fermentation end products (Lynd *et al.*, 2002). Thus for anaerobic species, adhesion of cells to cellulose appears to be a requirement for rapid and efficient cellulose hydrolysis. However, these two procedures are not completely mutually exclusive since various microbial species show different combinations of these characteristics. Hydrolysis of cellulose requires prior binding of enzymes to it, either as an enzyme-substrate binary complex or as a cellulose-enzyme-microbe (CEM) ternary complex (Lynd *et al*., 2002).

2.2.3 Cellulase Enzyme Systems

Cellulases are a group of hydrolytic enzymes that act in a coordinated synergistic manner to hydrolyze the β-1, 4-glycosidic bonds in cellulose to release glucose units (Ezekiel *et al*., 2010; Nishida *et al*., 2007; Lynd *et al*., 2002). For microorganisms to hydrolyze and metabolize insoluble cellulose, extracellular cellulases must be produced that are either free or cell associated. Although a large number of microorganisms can degrade cellulose, only a few of them produce significant quantities of free enzymes capable of completely hydrolyzing crystalline cellulose (Koomnok, 2005).

Based on their catalytic action, cellulases have been classified into three major groups as follows: (i) endoglucanases or 1, 4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), (ii) exoglucanases, including 1, 4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1, 4-β-Dglucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and (iii) β-glucosidases or β-glucoside glucohydrolases (EC 3.2.1.21) (Van-Elsas *et al*., 2007; Lynd *et al*., 2002). Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain, generating oligosaccharides of various lengths and consequently new chain ends. Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either glucose (glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (Lynd *et al*., 2002). Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze β-1, 4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β-1, 4-

glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base (Lynd *et al*., 2002).

2.3 Methods of lignocellulose conversion to useful products

Naturally, lignocellulosic biomass can undergo spontaneous fermentation by native microorganisms that inhabit them but the resulting products and rates of activities might be very low and inefficient. Harvesting products that result from such natural fermentation processes could be problematic. For maximum performance and product recovery however, these fermentations must be carried out under carefully controlled conditions with selected microorganisms while eliminating possible inhibitors of microbial activities. Two major methods used are the submerged and solid state fermentation methods (Vintilla *et al*., 2009; Jacob and Prema, 2006).

2.3.1 Submerged fermentation (SmF)

Submerged fermentation (SmF) systems can be defined as the cultivation of microorganisms in a liquid medium containing soluble carbon source and nutrients, maintained or not under agitation. This involves growing carefully selected microorganisms (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes and other products into solution. Submerged fermentation technology has long been used in the large-scale culture of microorganisms in fermentors, and the recovery of useful products contained within the microbial cells or released into the surrounding medium (Carlile *et al*., 2001). The commercial production of microbial metabolites with submerged fermentation

commonly involves fermentors that contain tens or even hundreds of thousands of litres of medium that is stirred with impellers and sparged with air. However laboratory research using this method is usually conducted with flasks that contain about 100 ml of medium and may be static or shaken. If the flasks are static most fungi grow as a mat on the surface, whereas with shaken liquid culture they occur as dispersed hyphae or pellets submerged in the medium (Carlile *et al*., 2001).

The development of microbial morphology in SmF is well investigated for a number of industrially important fermentations. In liquid environments, fungi grow as pellets or free mycelia, depending on the genotype of the strain and culture conditions (Papagianni, 1995). Each form has its own characteristics which greatly affect the process yields (Miles and Trinci, 1983). Bacteria on the other hand can move freely through a liquid medium either by diffusion or, in motile species, by active locomotion. Thus, as the cells grow and divide, the progeny are dispersed throughout the medium. Usually, as the concentration of cells increases, the medium becomes increasingly turbid. Certain bacteria are exceptional in that they tend to form a layer called pellicle on the surface of the medium. Below the pellicle, the medium may be almost free of cells. Some pellicles include bacterial products as well as the bacteria themselves.

Submerged fermentation can be carried out in batch, continuous or fed-batch culture systems. A batch culture system is one in which nothing is added to or removed from an environment after a medium of appropriate composition is inoculated with living cells. A batch system can support cell multiplication for only a limited time and with progressive changes in the original medium. With continuous culture, a fresh supply of medium is added continuously at the

same rate that culture is withdrawn. This technique theoretically allows continuous exponential growth of the culture. Fed-batch culture is a modified form of batch culture in which transition from exponential growth of the microorganism to the stationary phase is prevented by adding fresh medium from time to time. In fed-batch, the volume of the liquid medium in the fermenter increases during fermentation. This technique allows substantial improvements in cell mass or product formation over the ordinary batch system (Carlile *et al*., 2001; Miles and Trinci, 1983).

2.3.2 Solid state fermentation (SSF)

Solid-state fermentation (SSF) processes can be defined as "the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water (Pandey *et al*., 2004; Archana; Satyanarayana, 1997; Moo-Young *et al.,* 1983). SSF holds tremendous potential for the production of value added products from lignocellulosic waste, with the main focus being, hydrolytic enzymes, organic acids, gibberelins, flavours and biopesticides (Raimbault, 1998). It has been also widely used for bioremediation of hazardous compounds, biological detoxification of agroindustrial residues, nutritional enrichment, biopulping, biopharmaceutical products etc. (Pérez-Guerra *et al.*, 2003).

The use of SSF for enzyme production has recently gained ground for a range of enzymes and isolates. It is of special interest in those processes where the crude fermented products may be used directly as enzyme sources. The methods often give higher enzyme productivity as compared to submerged fermentation (SmF) techniques (Arora and Bhatnagar, 2003). Viniegar-

Gonzalez *et al.* (2002) have hypothesized that this was due to differences in the local micro-gradients of oxygen and substrate concentration within the fungal aggregates formed on the surface of solid support or within the liquid of fungal suspensions.

A large number of microorganisms, including bacteria, yeast and fungi can grow on solid substrates, and find application in SSF processes. The selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate, environmental conditions and the products of interest (Pandey *et al*., 1999). The microorganisms used are aerobes, and depend on oxygen in air-filled spaces between substrate particles. The water content of the substrate has hence to be kept low so that the air-filled spaces persist. The resulting low moisture levels mean that the organisms have to tolerate low water potentials (Carlile *et al*., 2001). Bacteria are mainly involved in composting, ensiling and some food processes (Doelle *et al*., 1992). Yeasts can be used for ethanol and food or feed production (Raimbault, 1998). But filamentous fungi are the most important group of microorganisms used in SSF process owing to their physiological, enzymological and biochemical properties. The hyphal mode of fungal growth and their good tolerance to low water activity (a_w) and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates (Pandey *et al*., 1999; Carlile *et al*., 2001).

Generally, hydrolytic enzymes, e.g. cellulases, xylanases, pectinases, etc. are produced by fungal cultures, since such enzymes are used in nature by fungi for their growth. *Trichoderma* spp. and *Aspergillus* spp. have most widely been used as sources for these enzymes (Pandey *et al*., 1999; Arora and

Bhatnagar, 2003). Amylolytic enzymes too are commonly produced by filamentous fungi and the preferred strains belong to the species of *Aspergillus* and *Rhizopus* (Pandey *et al*., 1999).

Agro-industrial residues are generally considered the best substrates for the SSF processes. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes and other secondary metabolites. Some of the substrates that have been used include sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull, sago hampas, grapevine trimmings dust, saw dust, corncobs (Pandey *et al.*, 1999; Jacob and Prema, 2006). The selection of a substrate for production of any microbial metabolite (primary or secondary) in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. Thus ideally, the substrate that provides all the needed nutrients to the microorganisms growing in it should be preferred. However, some of the nutrients may be available in sub-optimal concentrations, or even absent in the substrates. In such cases, it would become necessary to supplement them externally with those nutrients. The substrates usually receive pretreatment to facilitate microbial attack (Carlile *et al*., 2001). Examples are grinding or milling to increase surface area, soaking to soften, and treatment with acids and/or bases.

Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size and moisture level/water

activity are the most critical (Pandey *et al*., 1999). Generally, smaller substrate particles provide larger surface area for microbial attack and, thus, are a desirable factor. However, too small substrate particles may result in clumping of the substrate, which may interfere with microbial respiration /aeration, and therefore result in poor growth. In contrast, larger particles provide better respiration/aeration efficiency (due to increased inter-particle space), but provide limited surface for microbial attack. This necessitates a compromised particle size for a particular process (Pandey *et al.*, 1999).

Solid substrate fermentations have many merits. The raw materials are commonly cheap, sterilization is not usually needed, energy requirements are low and the wastes needing disposal are compact and semisolid (Pérez-Guerra *et al.*, 2003). Comparative studies between submerged fermentation (SmF) and SSF claim higher yields for products made by SSF compared to those obtained in the corresponding submerged cultures (Cannel and Moo-Young, 1980). On the other hand, the fermentation process is often slow and the range of organisms able to tolerate the low water potentials involved is limited (Carlile *et al*., 2001). There are also drawbacks in terms of the scale up of the process, largely due to heat transfer and culture homogeneity problems (Mitchell *et al*., 2000).

2.4 Value – added products from lignocellulose

During growth of microorganisms, they generate certain intermediates and products of metabolism for various purposes of growth and development. These products (metabolites) can be harvested at various stages of the fungal growth as products of industrial importance. Depending on the role of these

metabolites they can be classified as primary or secondary. The kind of metabolite/value added products released into the media of growth also largely depends on the microorganism cultured and the composition of media on which it is grown (Howard *et al*., 2003).

Primary metabolites are those that have to be produced for growth to occur. Examples are nucleic acids, proteins, carbohydrates and lipids (Carlile *et al*., 2001). Vigorous primary metabolism is inevitably associated with the phase of rapid growth. In batch culture the maximum accumulation of primary metabolites tends to be towards the end of the growth phase, since in the stationary phase they may be further metabolized (Carlile *et al*., 2001). Continuous culture, in which the organism is maintained in the phase of exponential growth, is ideal for the production of primary metabolites, but requires greater investment and maintenance than batch culture. Most of the pathways of primary metabolism are widespread, so an economically interesting primary metabolite may occur in a wide range of microorganisms. Hence, for the production of a specific metabolite, the biotechnologist may be able to select from a wide range of bacteria and fungi (Carlile *et al*., 2001).

Secondary metabolites are those that are not essential for vegetative growth in pure culture. Examples include antibiotics, ergot alkaloids, naphtalenes, nucleosides, peptides, phenazines, quinolines, terpenoids and some complex growth factors. Secondary metabolism occurs as growth rate declines and during the stationary phase, and often is associated with differentiation and sporulation (Carlile *et al*., 2001; Doull and Vining, 1990). Thus batch rather than continuous culture is usually favoured for secondary metabolite production.

2.4.1 Simple Sugars

Simple sugars (eg. glucose, xylose, xylitol, arabinose, pentose and galactose) are usually the first products to be expected when microorganisms are grown on lignocellulose (Singh *et al*., 2008). Lignocellulose has only complex carbohydrates and must first be converted to monosaccharides (as sources of carbon) that can be utilized easily by the microorganism for growth. Most of the simple sugars, because they are fermentable, can then be further processed (by fermentation) to other industrially important products such as biofuels.

Mtui (2009) reported that yield of hydrolysable sugars up to 83.3% has been achieved at the reaction temperatures of 37 - 50°C for 6 – 179 h at pH 5 - 6. Among several factors that affect the physiology of microorganisms grown on lignocellulose, concentration of substrate has a great impact on the amount of saccharification products (Baig *et al*., 2004). Some transgenic plant residues have been reported to yield nearly twice as much sugar from cell walls compared to wild-types (Chen and Dixon, 2007). Glucose seems to be the major monosaccharide product from LCW (Mtui, 2009). The challenge facing depolymerization of hemicellulose into fermentable sugars is the requirement for a consortium of enzymes to complete the hydrolysis of hemicellulose, leading to high enzyme costs. Efforts to overcome the problem include process improvement and the use of modified microorganisms that produce the required hemicellulose enzymes (Lu and Mosier, 2007).

2.4.2 Enzymes

Lignocellulosic enzymes, mainly from fungi and bacteria, are important commercial products of LCW bioprocessing used in many industrial

applications including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Howard *et al*., 2003; Oberoi *et al*., 2008). Many microorganisms produce extracellular enzymes as secondary metabolites (secreted from the cells onto the medium of growth) that enable them to break down polysaccharides and proteins into sugars and amino acids that can be assimilated easily (Carlile *et al.*, 2001). These enzymes are of considerable industrial importance. They have, for example, largely replaced acid hydrolysis as a method for converting starches into sugars; enzymes, unlike acid, do not cause undesirable side reactions or corrode reactor vessels. Some of the most popularly studied extracellular enzymes include hydrolytic enzymes such as cellulases; hemicellulases and pectinases; degradative enzymes like amylases, proteases; and ligninolytic enzymes like laccases, peroxidases and oxidases (Mtui, 2009).

Cellulases have already found important roles in several industries but intensive studies are still being conducted on them in connection with the utilization of lignocellulose, which are the cheapest and most abundant of organic carbon sources, but one of the most intractable (Carlile *et al.*, 2001; Oberoi *et al*., 2008; Mtui, 2009). Hemicellulolytic enzymes, mainly xylanases, are produced from a wide range of LCW biomass (Pandey and Pandey, 2002). Pectinases such as endopolygalacturonase (endo-PG), exo-polygalacturonase (exo-PG) and pectin lyase are mainly produced from solid state fermentation processes utilizing agricultural residues (Jacob and Prema, 2006), while proteases have been produced by *Penicillium janthinellum* in submerged cultures (Oliveira *et al.*, 2006). Among the ligninases produced from LCW, laccases are the mostly studied (Couto and Sanromána, 2005; 2006), followed

by manganese peroxidase and lignin peroxidase (Couto and Sanromána, 2005). Very high enzyme activities (31,786 U/L) have been reported when the experiments were carried out under optimal conditions (pH 5.5 - 6: temperature 30 - 45°C) (Rosales *et al*., 2007).

Mostly, for industrial purposes, these enzymes are used as crude enzymes from the fermentation filtrates. However, for food and medical purposes, recovery of the pure enzymes may be achieved through 50 - 80% $(NH_4)_2SO_4$ saturation followed by chromatographical purification techniques (Mtui and Nakamura, 2008). Several efforts have been made to increase the production of enzymes through strain improvement by mutagenesis and recombinant DNA technology. Cloning and sequencing of the various genes of interest could economize the enzymes production processes (Kumar *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

The microorganisms were isolated from soil at Ejura farms, where corn agrowaste has been left to decompose into the soil since the beginning of corn cultivation about 40 years ago. Corncobs were also collected from the CSIR – Crop Research at Fumesua in Kumasi. All isolation and fermentation experiments were carried out at the Microbiology Laboratory of the Department of Theoretical and Applied Biology, KNUST, Kumasi. Analyses of fermentation filtrates were carried out at the Analytical Lab of the Department of Biochemistry and Biotechnology.

3.1 Soil Sampling

Five g of soil samples were taken from five separate plots at the Ejura farms. For each plot, samples were taken from three different depths (surface, 10 and 30 cm deep). In total, 75 samples of soil were collected for isolation of microorganisms.

3.2 Isolation of Microorganisms

The soil samples (5 g each) were weighed into 250 ml conical flasks containing 100 ml of sterile distilled water. The suspension was mixed vigorously to obtain a uniform mixture. The suspension was then serially diluted up to 10^{-10} and pour plated on nutrient agar (NA) and potato dextrose agar (PDA) supplemented with chloramphenicol for selective isolation of bacteria and fungi respectively. Inoculated media were incubated at 25°C for 7 days (for fungi isolation) and 37°C for 24 – 48 hrs (for bacteria isolation).

Individual colonies observed (based on morphological appearance) were subcultured until pure cultures were obtained (Prescott *et al.*, 1999). Isolated pure cultures were stored at -4°C for identification and subsequent studies. All isolation experiments were carried out in a laminar flow cabinet to ensure that results were obtained under sterile conditions.

3.3 Identification of Microorganisms

Isolated fungi were identified with assistance from the Department of Plant Pathology of the Faculty of Agriculture, KNUST. Identifications were based on mycelia properties and spore structure observed under the light microscope. Bacterial colonies were identified based on morphological and biochemical properties. Morphological characteristics such as colony form, colour and margin were observed and recorded. Biochemical features such as Gram reaction, catalase, urease, indole, citrate and oxidase activity tests were performed at the Komfo Anokye Teaching Hospital. The results of these reactions were then looked up in the Bergey's Manual of Determinative Bacteriology (Volume 2) in order to identify the bacteria (Garrity *et al.*, 2004; Prescott *et al.*, 1999).

3.3.1 Gram Staining

This was used to classify the bacteria on the basis of their Gram reactions (ie. whether they are positive or negative) and cellular morphologies. The isolates were smeared and fixed on a clean slide by air drying and heat fixing it. The fixed specimens were first stained with crystal violet for 30 s and next treated with iodine also for 30 s to promote dye retention. The smears were then

treated with ethanol to decolorize them. Lastly the samples were counterstained with safranin for 30 s (Isenberg, 2007).

3.3.2 Catalase Test

A drop of hydrogen peroxide was placed on a cleaned microscopic slide. Using sterile glass rod small part of isolate colony was taken and emulsified in the hydrogen peroxide drop. The production gas bubbles were considered a positive reaction (Isenberg, 2007).

3.3.3 Urease Test

Urea test medium of Christensen was inoculated with the bacteria isolates, incubated at 37 °C, and examined for up to seven days. Urea hydrolysis was indicated by the change of medium to pink colour (Isenberg, 2007).

3.3.4 Indole Test

This test was done to determine if bacteria can breakdown the amino acid tryptophan into indole. Tryptic soy broth (TSB) was inoculated using an inoculation needle. After incubating the bacteria for 48 hours, Kovac's reagent was added to the media to detect if indole has been produced by the bacteria. The development of a red/pink layer on top of the media indicated a positive result (the bacteria can breakdown tryptophan to form indole). The absence of a red layer is a negative result (indole was not formed from tryptophan) (Isenberg, 2007).

3.3.5 Citrate Utilization Test

A single streak was done over the surface of slant of Simmon's citrate medium, incubated at 37°C, and examined daily up to seven days for growth. A change in colour from dark green to blue that indicated citrate utilization (Isenberg, 2007).

3.3.6 Oxidase Test

This test is useful in the initial characterization of gram-negative bacteria. It tests the ability of a bacterium to oxidize Kovacs' reagent to form a deep purple compound, indol phenol. A few drops of the Kovacs' reagent were added to the bacteria isolates (cultured on a Petri dish). The plate was tilted to allow air to reach the colonies and observations for a purple colour change were made between 10 and 60 s. A colour change to purple observed within this period indicates that the isolate is positive. No colour change is a negative result (Isenberg, 2007).

3.4 Test for Microorganisms' ability to utilize cellulose

Samples of the colonies of bacterial and mycelia of fungal isolates were inoculated onto CMC agar media under a laminar flow cabinet. In order to obtain separate colonies, the samples were picked with an inoculating needle and mixed in 10 ml distilled water. This suspension was then serially diluted before spreading onto the solid CMC agar. The Petri dishes were then incubated at 25 °C for a week. To visualize the hydrolysis zones, the plates were flooded with an aqueous solution of Congo-red dye (1 mg/ml) for 15 min and washed with 1 M NaCl (Apun *et al.*, 2000).

Colony diameter and clearing zone diameter of the isolated colonies were measured on the third and fifth days after incubation. The colony diameter gives an indication of the extent to which the isolates are able to utilize carboxymethylcellulose, a soluble form of cellulose, for growth. The clearing zone to colony/mycelia diameter ratios was also calculated to determine the ability of the isolates to exude extracellular enzymes into the media (Zakpaa *et*

al., 2009). The ratios were calculated by dividing the clearing zone diameter by the sum of the colony diameter and the clearing zone diameter. The best isolate, based on the clearing zone to colony diameter ratios was selected and stored for solid state fermentation experiments.

3.5 Solid state fermentation (SSF) experiments

The SSF method used by Vintila *et al.*, (2009) was adopted in this research. In this method, the biomass substrate was washed to remove glucose and also periodically flushed to remove reducing sugars, harvest enzymes, and add nutrients. The washing was done by rinsing the substrate with 100 ml of nutrient solution and filtering the mixture through a sieve (pore-size less than 0.20mm). Fifteen g of the corncob powder was weighed into 300 ml conical flasks and autoclaved at 121°C for 30 minutes. This treatment served the functions of sterilizing the substrate and pretreating it with steam pressure. The substrates were washed twice with 100 ml distilled water to remove any glucose molecules produced during pretreatment. After the second wash, 100 ml of nutrient solution (containing the following: $(NH_4)_2SO_4$ 1.4 g/l; KH_2PO_4 2.04 g/l; $CaCl_2$ 0.3 g/l; $MgSO_4.7H_2O$ 0.3 g/l; Urea 2.1g/l; Citric acid 0.25 g/l; Tween 80 2 ml/l; Peptone1 g/l; and Trace metal stock solution, 1 ml/l) was used to wash the substrate twice in order to saturate the substrate with additional nutrients prior to inoculation. The concentration of glucose in each filtrate after washing was determined by the DNS method (Appendix B).

Spore inoculums were prepared by washing 4 – day old cultures of *Aspergillus niger* with 10 ml of sterile nutrient solution with the aid of a camel hair brush. The number of spores in the inoculum was estimated by counting the spores

with a haemacytometer. To determine the spore concentrations for optimum saccharification and enzyme activities, the initial spore concentrations in the inoculums were varied by diluting the original inoculum. The initially washed inoculum contained 4.5 x 10^6 spores (representing 100 %). This number was diluted to 50 and 25 % by pipetting 5 and 2.5 ml respectively from the initial inoculum. The volumes were then topped up with sterile nutrient solution to 10 ml each. Thus the 3 inoculums used contained the following spore concentrations per 10 ml: 4.5 x10^6 (undiluted inoculum/100%); 2.3 x 10^6 (inoculum diluted to 50 %) and 1.3 x 10^6 (inoculum diluted to 25 %). The codes 1.0, 0.5 and 0.25 were used to represent the spore concentrations, 100, 50 and 25 % respectively in the inoculums.

Each flask (containing 15 g of the substrate) was inoculated with 10 ml of inoculum prepared above in duplicate. The flasks were incubated at 27, 37, 47 and 57 °C for 15 days. After every 5 days, each flask was washed with 100 ml of nutrient solution to harvest any glucose and extracellular enzymes secreted into the medium and also to replenish the substrate with fresh nutrients. After every flushing, total reducing sugars, filtrate proteins and cellulase activities were determined in the fermentation filtrate.

3.6 Experimental design and analyses

Each fermentation test was carried out in duplicate although analyses for total glucose, crude proteins and enzyme activities in fermentation filtrates were done in triplicate. Two-way ANOVA with Bonferroni's post test was performed using GraphPad Prism version 5.02 for Windows. Graphs were also plotted using the same software.

CHAPTER FOUR

RESULTS

4.1 Isolated fungi from soil

4.1.1 *Aspergillus niger*

A. niger mycelia formed black colonies on PDA as shown in Plate 1. Conidiophores were perpendicular to the substrate hyphae and terminated in dark-brown to black vesicles. Conidiophores were smooth-walled and hyaline turning dark towards the vesicle. The vesicles had short hyphae (phialides) which actually bore the conidia. Each conidium was globose, rough-walled and dark brown to black in colour. The conidia were produced in chains on the phialides.

Plate 1: *Aspergillus niger* culture on PDA slants.

4.1.2 *Neurospora crassa*.

Neurospora crassa grew readily on PDA (especially in pure culture) and had large hyphae which spread rapidly. Its aerial mycelium rising above the

medium consisted largely of branched chains of orange macroconidia. The macroconidia were roughly ellipsoidal, containing several nuclei.

Plate 2: *Neurospora crassa* culture on PDA slant.

4.1.3 *Penicillium* sp.

Penicillium sp. mycelia formed flat, green patch with rough surfaces and white margins on PDA. Conidiophores arose from mycelium singly and branched near the apex, into penicillates ending in phialides. Conidia were hyaline, globose and dry. They were arranged in chains on the phialides.

Plate 3: *Penicillium sp.* culture on PDA

4.1.4 *Rhizopus* sp.

Rhizopus sp. had white fluffy mycelia at the beginning of growth on PDA but at maturity, black spores were produced at the tips of the hyphae, giving it the black upper surface as seen in plate 4. Hyphae of *Rhizopus sp*. were multinucleate without septa and grew very rapidly (reached maturity within 4 days). They had long and unbranched sporangiophores which terminated in a dark, round sporangium that contained several oval, colourless spores.

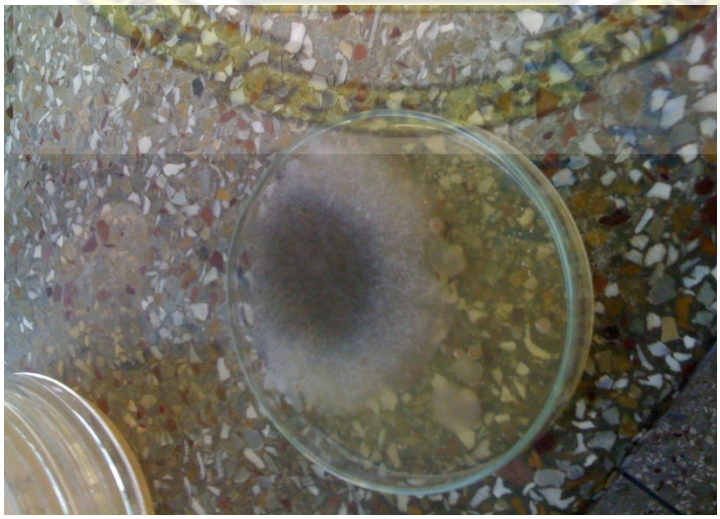

Plate 4: *Rhizopus sp*. culture on PDA

4.1.5 *Trichoderma* sp.

Trichoderma sp. was white at early stage and later developed into deep green compact tufts in concentric ring-like zones on the PDA. Conidiophores were hyaline, much branched, but not verticillate and bearing single or group of phialides. Conidia were green, single-celled, and ovoid with smooth or rough walls usually borne in small terminal clusters.

4.2 Isolated Bacteria from soil

4.2.1 *Streptomyces sp.*

These bacteria produced filamentous colonies on nutrient agar. The cells were Gram positive and appeared rod-shaped (bacilli) with extensive branching under the microscope.

4.2.2 *Clostridium sp.*

These bacteria formed large irregularly circular colonies on nutrient agar. The cells were Gram positive rods which were arranged in pairs. The colonies showed negative results for catalase, citrate, indole and oxidase tests.

4.2.3 *Klebsiella pneumoniae*

Klebsiella pneumoniae showed white, rounded colonies on nutrient agar and had smooth margins. They were negative for Gram staining and cells had rod shapes when viewed under the microscope. They tested positive for catalase and citrate tests but negative for both indole and oxidase tests.

4.3 Cellulose utilizability tests

It was observed that after 3 days of inoculating CMC agar with the isolated bacteria and fungi, no significant clearing zones were formed albeit growth

was seen in some of the fungi. Clearing zones were therefore measured only after 5 days of incubation. Bacteria isolates did not show any significant clearing zones even after the fifth day, although growth was seen as early as 24 hours after incubation. Table 1 below shows the clearing zone to mycelium diameter ratios of fungi after 5 days of incubation. *Aspergillus niger* showed the highest clearing zone to colony diameter ratio followed by *Trichoderma* sp.

Table 1: **Clearing zone to mycelium diameter ratios of fungi isolates.**

Name of Isolate	Mean Mycelium Diameter (cm)	Mean Clearing Zone (cm)	Clearing Zone to Mycelium diameter Ratio
Aspergillus niger	2.3	0.8	0.26
Neurospora crassa	*	*	*
Penicillium sp.	3.4	0.2	0.06
Rhizopus sp.	1.9	0.1	0.05
Trichoderma sp.	3.2	0.8	0.20

* Measurement of clearing zone was not possible because mycelia spread over the entire plate.

4.4 Amounts of glucose released from corncobs after fermentation by *A. niger*

The saccharification ability of *A. niger* on corncobs under solid state conditions was tested by measuring the concentration of glucose in the fermentation filtrate harvested every 5th day for 15 days. Figures 1 – 3 show the amounts of glucose released from the flasks inoculated with different spore concentrations and incubated at temperatures ranging from 27 to 57 °C. From the figures, undiluted spore inoculums (containing 4.5 x10^{6} spores) resulted in the highest glucose concentrations from the 5th day of fermentation to the 15th

day. Analyses of the variance observed shows that the spore concentration significantly affected the observed saccharification results at all temperatures (Appendix D).

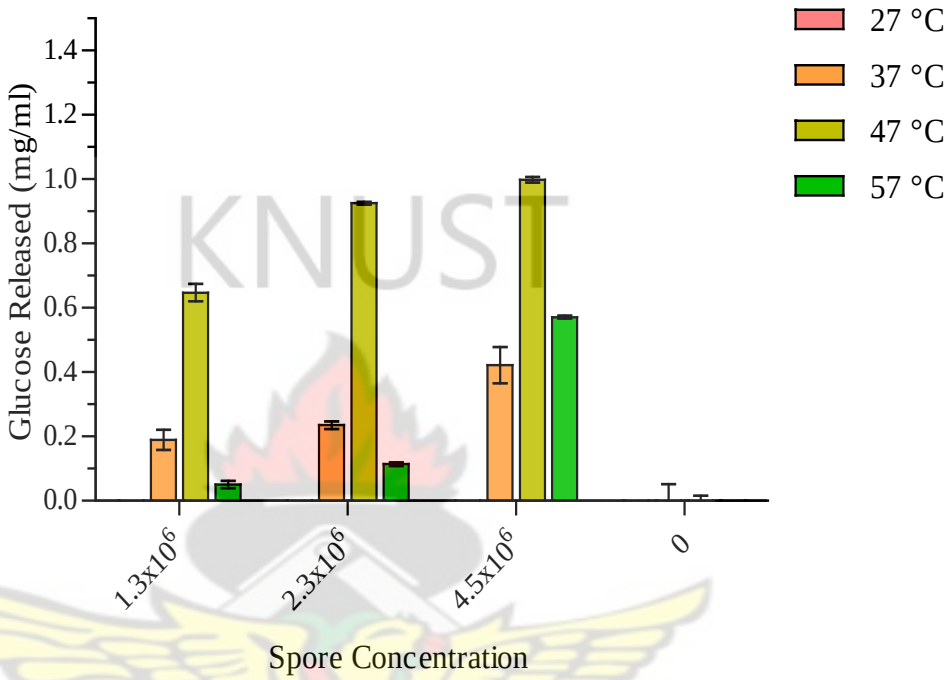

Fig. 1: Influence of inoculum size of *A. niger* on release of glucose from fermented corncobs at varying temperatures after 5 days

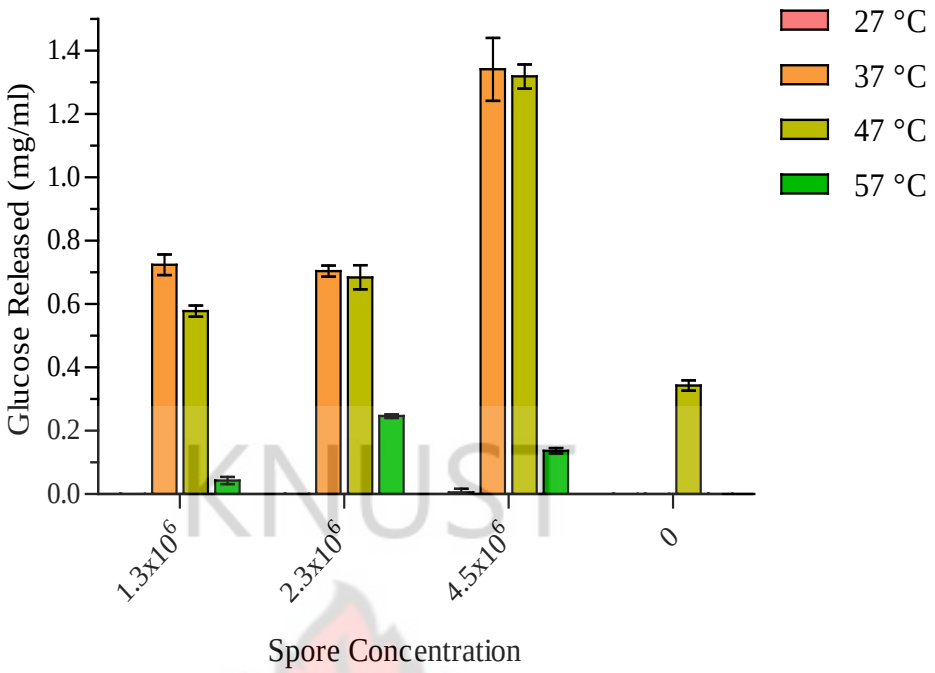

Fig. 2: Influence of inoculum size of *A. niger* on release of glucose from fermented corncobs at varying temperatures after 10 Days

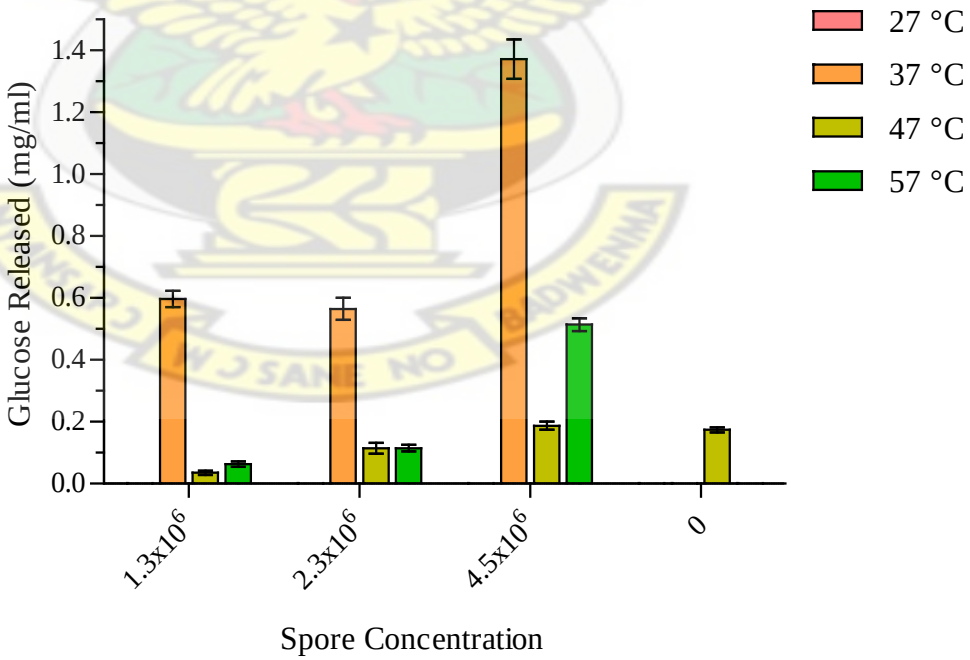

Fig. 3: Influence of inoculum size of *A. niger* on release of glucose from fermented corncobs at varying temperatures after 15 days

4.5 Effect of fermentation temperature on saccharification of corncobs by *A. niger*

Temperature also contributed significantly to the difference in saccharification abilities observed. Figures 1, 2 and 3 above indicate that from days 5 to 15, fermentation temperatures of 37 and 47 °C resulted in high saccharification. Figure 3, shows that the highest saccharification activity (1.38 mg of glucose per ml of filtrate) was achieved when fermentation was carried out at 37 °C for 15 days and this value was significantly different ($p<0.001$) from all the other values obtained during the fermentation period.

At 47 °C, the saccharification was high within the first 5 days and increased slightly through day 10 and declined sharply thereafter. At 37 °C however, the saccharification ability increased throughout the fermentation period. The increase in saccharification from the 5^{th} day to the 10^{th} day was sharp while that from day 10 to 15 was marginal but statistically significant ($p< 0.01$). At 27 °C, the saccharification values were very low or negligible throughout the fermentation period.

4.6 Amount of crude proteins released in fermentation filtrate

Figures 4 – 6 show the amounts of crude proteins harvested from the fermentation flasks from day 5 to day 15 respectively. At all the temperatures of incubation, undiluted spore concentrations gave the highest crude proteins production in fermentation filtrates. Also during the first 5 days of fermentation, flasks incubated at 47 °C produced the highest protein values (Fig. 4). The protein concentrations in those flasks however decreased gradually down to the 15^{th} day of fermentation (Fig. 5 and 6). Fermentation in

flasks incubated at 37 °C was the direct opposite of those incubated at 47 °C in that the protein concentrations started low and increased gradually from day 5 to 15.

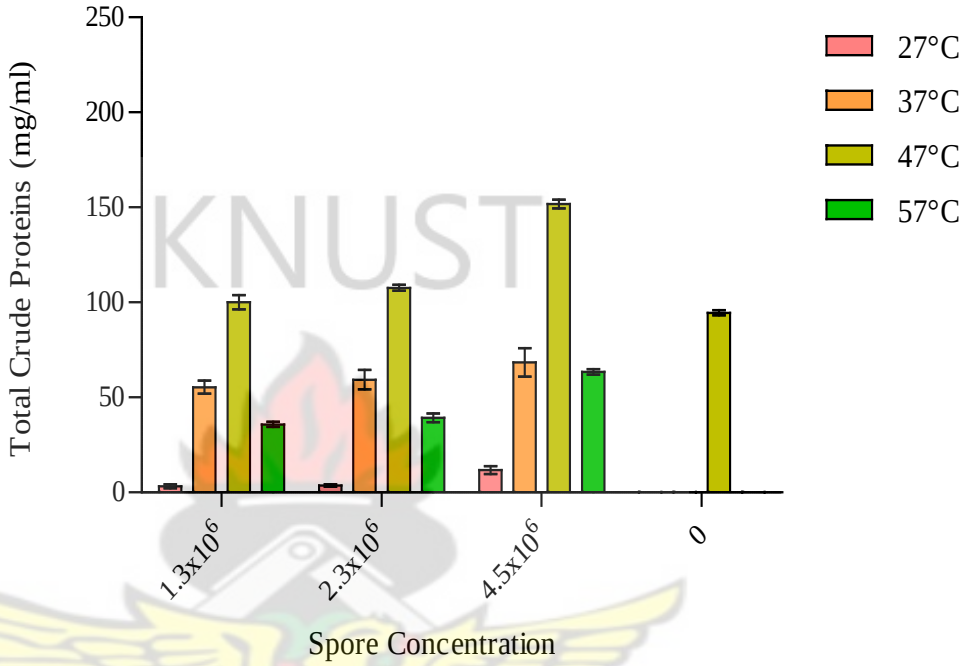

Fig 4: Influence of spore concentration of *A. niger* on crude proteins production from fermented corncobs after 5 days

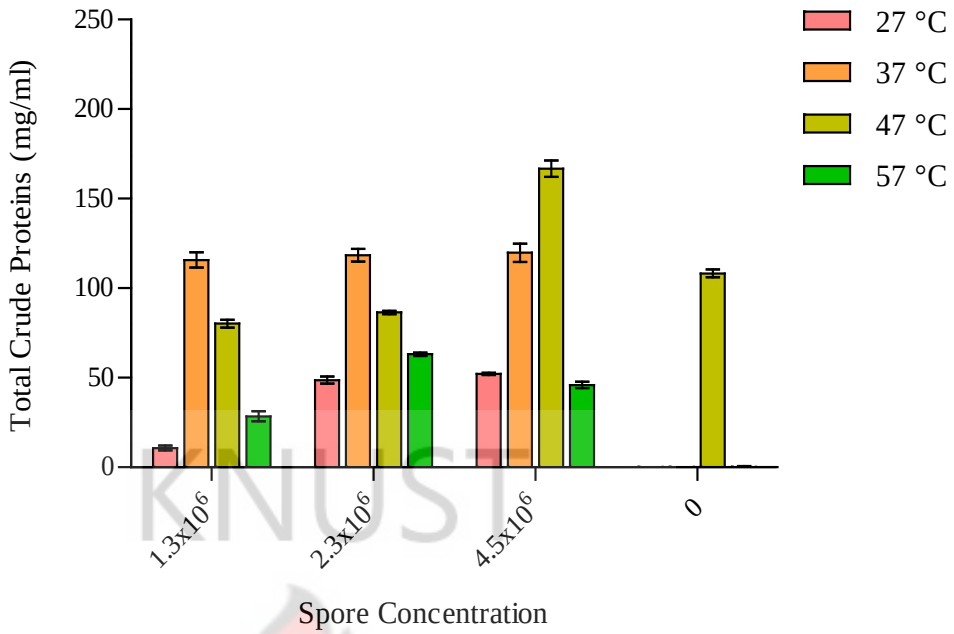

Fig 5: Influence of spore concentration of *A. niger* on crude proteins production from fermented corncobs after 10 days

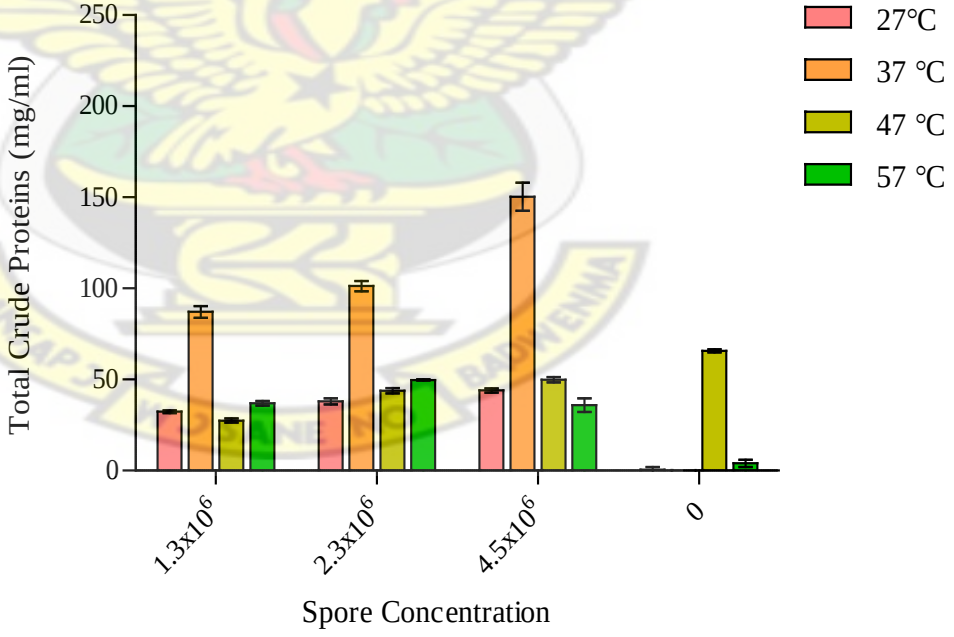

Fig 6: Influence of spore concentration of *A. niger* on crude proteins production from fermented corncobs after 15 days

4.7 Enzyme activities of fermentation filtrate

Enzyme activities of fermentation filtrates in flasks inoculated with varied spore concentrations of *A. niger* gave an indication of the effect of spore concentration on the activities of cellulases in the medium. The enzyme assay used measured mainly the endoglucanases activity of the filtrate. Undiluted spore inoculums ($4.5x10^6$ spores per 10ml) yielded highest activities throughout the period of fermentation. The highest activities of 0.35, 0.44 and 0.45 CMC units were recorded for flasks incubated at 37 °C through day 5, 10 and 15 respectively (Figs. 7 – 9). According to the Bonferroni post tests carried out on the results, each of these values were significantly different (at $p < 0.01$) from all other enzymes harvested at the respective days. Thus, undiluted spore inoculums gave the highest enzyme activities at a fermentation temperature of 37 °C. The highest activity of 0.45 CMC units was recorded after 15 days of fermentation at 37 °C.

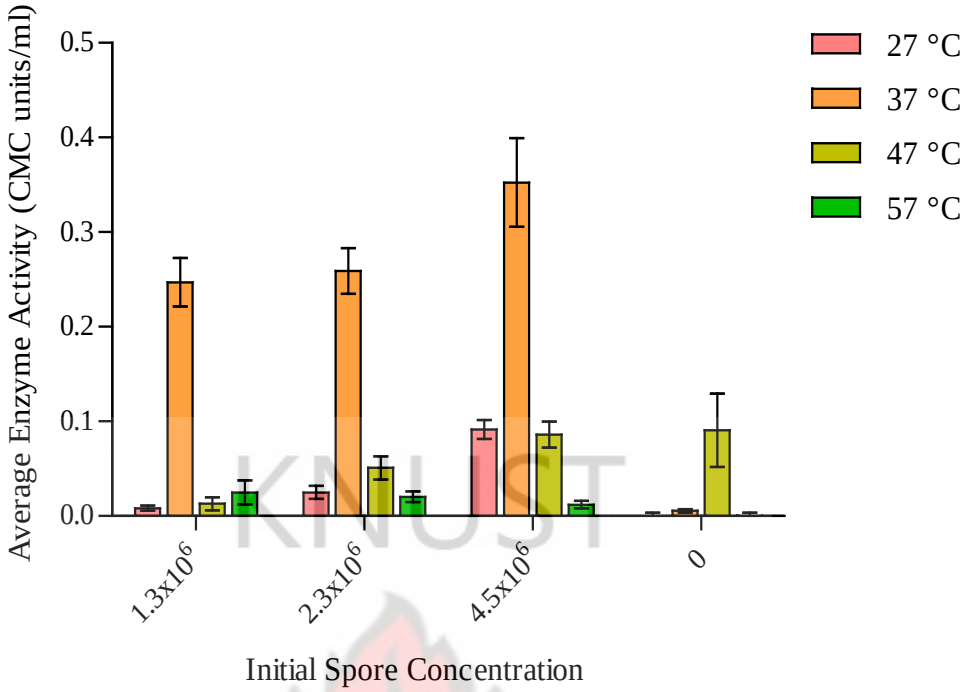

Fig 7: Activities of enzymes produced after 5 days of fermentation of corncobs at different incubation temperatures

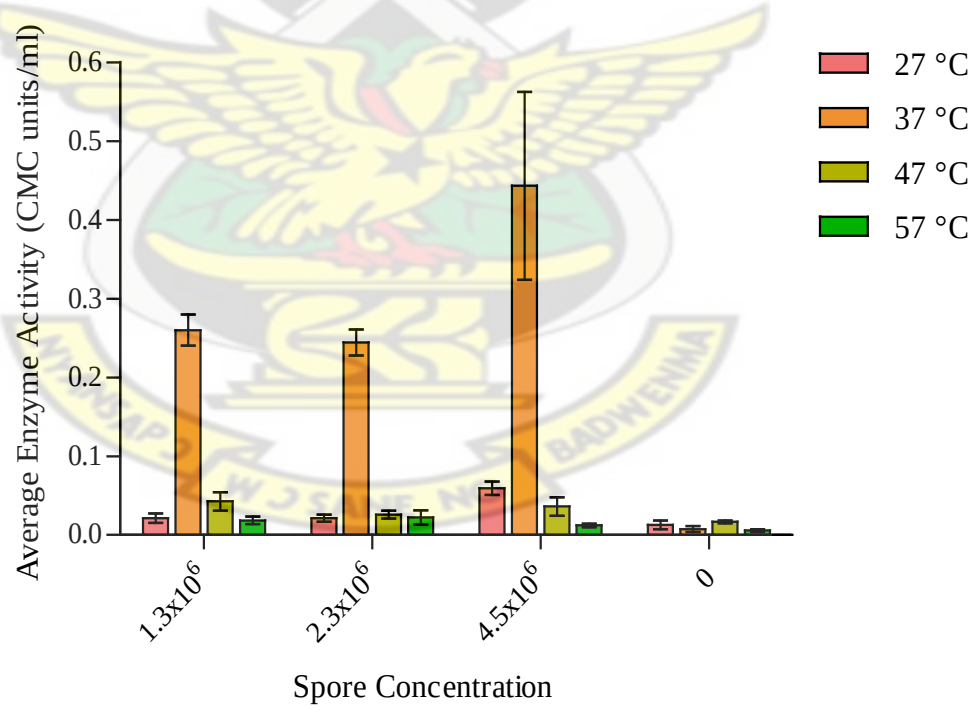

Fig 8: Activities of enzymes produced after 10 days of fermentation of corncobs at different incubation temperatures

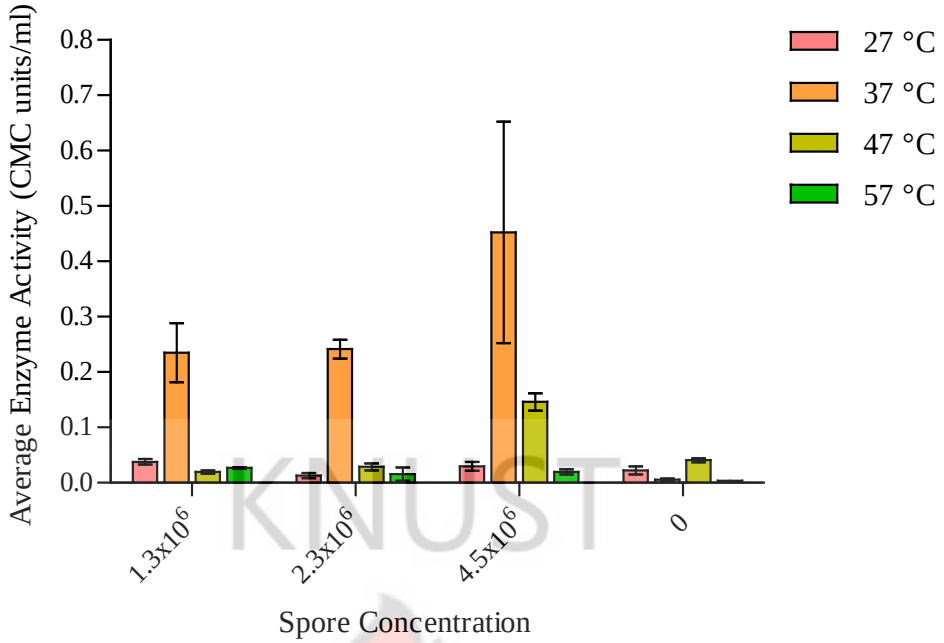

Fig 9: Activities of enzymes produced after 15 days of fermentation of corncobs at different incubation temperatures

4.8 Effect of temperature on enzyme activities of *A. niger* on corncobs

Figures 7 – 9 show that flasks incubated at 37 °C had the highest enzyme activities which increased gradually from 5 to 15 days. This effect of increasing activities from 5 to 15 days was however not seen at temperatures 27, 47 and 57 °C.

Two-way analyses of variance carried out on temperature and spore concentration for fermentation results after 5, 10 and 15 days of fermentation indicate that temperature significantly affects the enzyme activities (at each concentration of spores inoculated) between 5 and 10 days of fermentation. Beyond 10 days of fermentation, temperature no longer has a significant effect on activities of enzymes produced with respect to the concentration of spores inoculated onto the medium (Appendix D).

CHAPTER FIVE

DISCUSSION

The isolated fungi and bacteria represent the culturable microorganisms from soil of an agricultural land where maize biomass has been left to degrade for many years. Their presence in soil and other biomass waste has been reported for many years by much research on lignocellulose biodegradation and utilization (Mojsov, 2010; Zakpaa *et al*., 2009; Baig *et al*., 2004). These reports of their ability to utilize cellulose as carbon source were confirmed in this project by the ability of the isolates to exhibit growth on carboxymethyl cellulose (a crystalline form of cellulose) (Carlile *et al*., 2001; Hankin and Anagnostakis, 1977).

For any organism to successfully utilize cellulose in a medium as a carbon source for growth, it must first hydrolyze the cellulose to glucose before utilizing the glucose. This cellulolytic ability is possible if the organism can produce cellulases and exude them from its cells onto the cellulose medium (Apun *et al*., 2000). Thus cellulose hydrolysis is extracellular. This cellulolytic ability was quantified in this work by calculating clearing zones to colony diameter ratios of the isolates. *A. niger* produced the highest ratio amongst the other isolates after 5 days of incubation on CMC agar and therefore was considered to have the highest cellulolytic ability. This result was also obtained by Zakpaa *et al.*, (2009) in which similar fungi were isolated and *A. niger* was found to have the highest cellulolytic ability on Mandel's agar medium based on the clearing zone to colony diameter ratios.

The concentration of spores used as inoculum for the fermentation experiments proved to have a significant effect on the saccharification of the corncobs ($p < 0.01$). Figures 1 – 3 show that the optimum concentration of spores that yields the highest saccharification (measured by the concentration of glucose in the fermentation filtrates) is $4.5x10^6$ spores per 10 ml of inoculum, representing the undiluted spore suspension prepared from the slants of 4 day old cultures. This same concentration of spores led to high total crude proteins in the filtrate (Figs. 4, 5 and 6) as well as high enzyme activities in fermentation filtrates (Figs. 7, 8 and 9). Mosjov (2010) also reported that $6.0x10^6$ spores of *A. niger* in an inoculum yielded the highest concentration of pectinolytic enzymes (higher than $4.0x10^6$, $8.0x10^6$ and $1.0x10^7$ spores per ml) in a submerged fermentation of pectin.

Although temperature had a significant effect on the saccharification of corncob by *A. niger*, the effect was not regular throughout the 15 days of fermentation for all the initial spore concentrations used (Figs. 1 – 3). This observation can be attributed to the fact that temperature affects the different growth parameters of lag time, specific growth rate and total yield differently (Carlile *et al*., 2001). Since spores were used in the inoculation, it is assumed that the lag phase (including germination) falls within the first 5 days of incubation. Thus the most suitable temperature for the lag phase should enable the organism to move from the lag phase faster and begin utilizing cellulose by breaking it down to glucose for vegetative growth. Figure 1 shows that, 27 °C was too low for germination and any significant saccharification to occur under the solid state conditions and so the amount of glucose released was below 0.01 mg/ml. Flasks incubated at 47 °C had the highest glucose values

within 5 days of fermentation. At this temperature, spore germination took place fast enough to enable vegetative growth, and hence, cellulose utilization to get underway. Temperature 37 °C gave the next highest sets of glucose concentrations indicating the next favourable temperature for the lag phase. Although at 57 °C, there was slight saccharification activity, this value quickly declined by the 10^{th} day of fermentation before rising again to almost the same value as in day 5 (Figs. 1-3). This behaviour can be explained by the principle of microcycle sporulation (ie. when spore germination is soon followed by sporulation) (Carlile *et al.*, 2001). This phenomenon occurs if spore germination is initiated but then conditions (such as temperature) prove unsuitable for growth. A comparison of saccharification patterns at temperatures 37 and 47 °C (Figs. 1-3) shows that, although 47 °C was the best temperature for germination/lag phase, it was not very suitable for saccharification of cellulose into glucose for vegetative growth. This is why the glucose concentration declined sharply after the tenth day of fermentation. Temperature 37 °C was the best temperature for growth on corncob as substrate under solid state fermentation (Carlile *et al.*, 2001). On the basis of the high vegetative growth and enzyme activity on corncobs at 30 °C, *A. niger* can be described as a mesophile (microorganisms with optimum growth within 20 – 45 °C) (Prescott, 1999).

The carboxymethyl cellulase assay was used to confirm the presence and to quantify the activities of cellulases in the fermentation filtrate harvested at every fifth day of the fermentation period. The highest enzyme activities were recorded in flasks inoculated with undiluted spores. These flasks (with undiluted spores) also produced the highest total filtrate proteins. Thus most of

the crude proteins detected from the filtrates were extracellular enzymes secreted onto the medium. The high enzyme activities of filtrates obtained from flasks incubated at 37 °C at the 15th day of fermentation (Fig. 9) also corresponds to the time when the highest amount of glucose was released from the flasks (Fig. 3). This observation augments the fact that the glucose released into the filtrate was as a result of extracellular enzyme activity on the cellulose components of the substrate. Also it can be said that the solid state method used in this research (where substrate was washed periodically to remove fermentation products) did not encourage catabolite repression since the glucose generated was periodically removed (Vintila *et al*., 2009).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

The use of fungi in solid state fermentation of corncobs to produce cellulases has been studied. Amongst the five species of fungi isolated in this research, *A. niger* exhibited the best cellulolytic ability. Maximum growth of the fungi in solid state fermentation will be achieved when concentrated spore inoculum (containing about $4.5x10^6$ spores/10ml) is used.

The temperature for fermentation must also be varied depending on the stage of growth of the fungi used. Thus 47 °C was the best for the lag phase/spore germination, while 37 °C is optimum for growth and enzyme production. These conclusions are evident from figures 1-3 where flasks incubated at 47 °C had highest concentration of glucose after 5 days of incubation (lag phase/spore germination) but those at 37 °C increased through 10 to 15 days (period of growth and enzyme production) to give very high glucose concentrations per filtrate.

6.2 RECOMMENDATIONS

It is recommended that these enzymes are used in simultaneous saccharification and fermentation experiment with yeasts in order to study their performance in ethanol production. This will test the performance of the enzymes in their application of cellulose hydrolysis and production of value added products from lignocellulose.

It is also recommended that the other isolated fungi, *Penicillium sp*., *Trichoderma sp*., *Neurospora crassa* and *Rhizopus sp*. be used to ferment

corncobs in order to compare the saccharification abilities and activities of enzymes produced by each of them.

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APPENDICES

APPENDIX A: Preparation of Media

All media used in this research were prepared according to standard reported protocols as described below:

a) Nutrient agar (Oxoid)

Nutrient agar is composed of the following ingredients (g/litre): beef extract 3.0; peptone 5.0; agar 15.0. Twenty three g of the mixture was suspended in 1 L of distilled water and mixed thoroughly. The mixture was heated to the boil for 1 minute to enable total dissolution. The medium was then autoclaved at 121°C for 15 minutes.

b) Potato Dextrose Agar (PDA) (Oxoid)

PDA is composed of the following ingredients (g/litre): potato extract 4.0 (equivalent to 200 g of infusion from potatoes); dextrose 20.0; agar 15.0. Thirty nine g of the powder was suspended in 1 L of distilled water and dissolved completely by bringing to the boil on a hot plate. The medium was then autoclaved at 121°C for 15 minutes.

c) Carboxymethylcellulose (CMC) agar medium (BDH)

CMC agar medium is a minimal medium adapted from Apun *et al*. (2000). It consists of the following ingredients (g/litre): $NH_4H_2PO_4$ 1.0; KCl 0.2; $MgSO_4.7H_2O$ 1.0; yeast extract 1.0; carboxymethylcellulose (BDH, low viscosity) 26.0; agar 15.0. The above ingredients were dissolved in 1 L distilled water on a hot plate magnetic stirrer. After the ingredients had dissolved completely, it was autoclaved at 121°C for 15 minutes.

APPENDIX B: Analysis of Fermentation Filtrate for total glucose, proteins and enzyme activity.

a) Determination of total glucose concentration in fermentation effluent

Total glucose released was measured by adapting the dinitrosalicyclic acid assay described by Wang (1997). The reagents used were a 1% dinitrosalicyclic acid (DNS) solution and 40 % potassium sodium tartrate solution. DNS reagent (1 %) was prepared by dissolving the following in 1 litre of distilled water: Dinitrosalicylic acid 10 g; Sodium sulfite 0.5 g; Sodium hydroxide 10 g. Phenol was omitted in order to prevent the coloured product from being too intense. 40 % potassium sodium tartrate solution was prepared by dissolving 40 g of potassium sodium tartrate solution in 100 ml of distilled water.

Three millilitres of the DNS reagent was added to 1 ml each of the fermentation filtrates (in addition to glucose solutions of known concentrations) and incubated in boiling water for 5 minutes. After that 1 ml of the 40 % potassium sodium tartrate solution was added. The mixture was then cooled immediately and the absorbance was read with a spectrophotometer (Nicolet Evolution 300) at 540 nm. A standard glucose curve was drawn by plotting the absorbance of the known glucose solutions against their concentrations. The concentration of glucose (unknowns) in fermentation filtrates were then extrapolated from the glucose standard curve (Appendix C).

b) Determination of filtrate protein concentration

The total proteins from the fermentation filtrates were determined by the Biuret method (Zakpaa *et al.*, 2009). The Biuret reagent was prepared with the following ingredients per litre: $CuSO_4.5H_2O$ 1.5 g; $NaKC_4O_6.4H_2O$ 6.0 g; and NaOH 30 g. The first two components ($CuSO_4.5H_2O$ and $NaKC_4O_6.4H_2O$) were dissolved in 500 ml of distilled water. Thirty grams of the NaOH was then dissolved in 300 ml of distilled water to make a 10 % (w/v) NaOH solution. This was then added to the mixture and made up to 1 L.

The Biuret assay consists of (i) egg albumin standards (with known protein concentrations), (ii) reagent blank and (iii) test samples (with unknown protein concentrations). Egg albumin stock solution (10mg ml^{-1}) was prepared by dissolving 1 g of egg albumin powder in 100 ml of distilled water. Dilutions were prepared from the stock by pipetting 0.2, 0.4, 0.6, 0.8, and 1 ml of the stock solution into 5 test tubes. The first 4 volumes were topped up to 1 ml to make 2 mg ml^{-1}; 4mg ml^{-1}; 6mg ml^{-1} and 8 mg ml^{-1} of egg albumin standards respectively. The reagent blank consisted of 1 ml nutrient solution used in preparing the fermentation samples. One milliliter each of the filtrate samples were measured into test tubes. To each of these test tubes (standards, reagent blank and test samples), 4 ml of Biuret reagent was added and mixed. All the test tubes were incubated at 37 °C for 20 minutes. After the incubation period, absorbance of each of the tubes was read with a spectrophotometer (Nicolet Evolution 300) at 540 nm.

The spectrophotometer was zeroed with the reagent blank before all the readings were carried out. A standard graph was drawn by plotting the

absorbance at 540 nm against the egg albumin concentrations. The total protein present in a milliliter of each filtrate sample was extrapolated from the graph using their absorbance (Appendix C).

c) Cellulase activity assays

The carboxymethylcellulose assay described by Jeffries (1987) (http://calvin.biotech.wisc.edu/jeffries/cellulases/) was used for testing cellulase activities in the isolates.

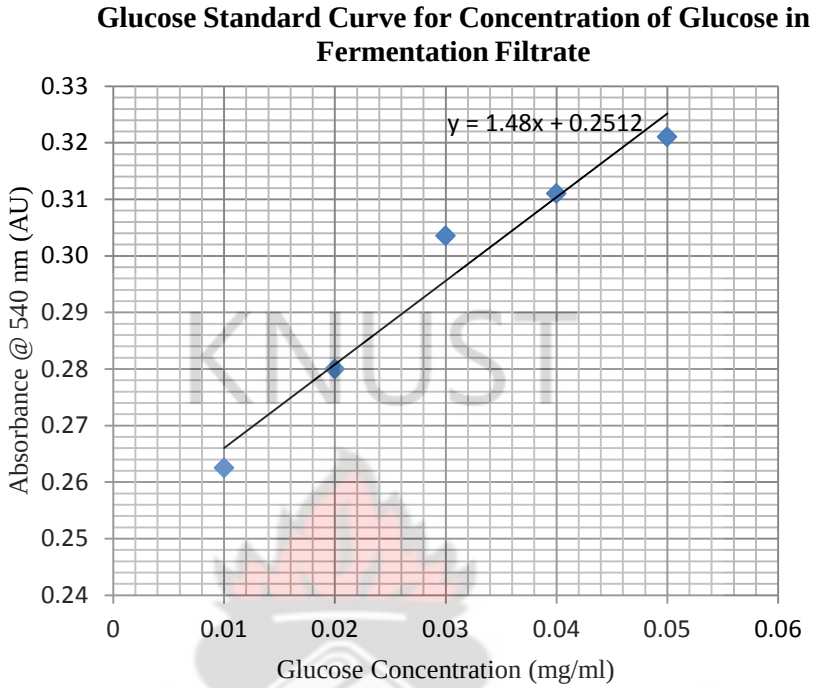
1% carboxymethylcellulose solution was prepared by dissolving 10 g carboxymethylcellulose powder in 800 mL hot water by adding the dry powder slowly with continuous stirring**.** 100 ml of 0.05 M citrate buffer pH 4.8 was added to the mixture. The solution was topped up to 1 litre with distilled water and refrigerated.

The assay included glucose standards, enzyme samples from fermentation and blanks of enzyme without substrate (control) and substrate without enzymes. Glucose standards were prepared as follows: A stock glucose solution was prepared by diluting 0.2 g of glucose in 100 ml of distilled water to make 2 mg ml^{-1}. The stock solution (2 mg ml^{-1}) was diluted in 0.05 M citrate buffer to the following concentrations: 0.25 mg ml^{-1}, 0.50 mg ml^{-1}, 0.75 mg ml^{-1} and 1 mg ml^{-1}. Tubes containing substrate without enzymes were prepared by pipetting 0.5 ml of 1% carboxymethylcellulose into the test tube. Half a millilitre of nutrient solution was also added and incubated for 30 minutes at 50 °C. After the incubation period, 3 ml of DNS was added and the amount of glucose present was measured with the DNS method. Assay mixtures consisted of 0.5 ml of fermentation filtrate in test tubes with 0.5 ml of 1%

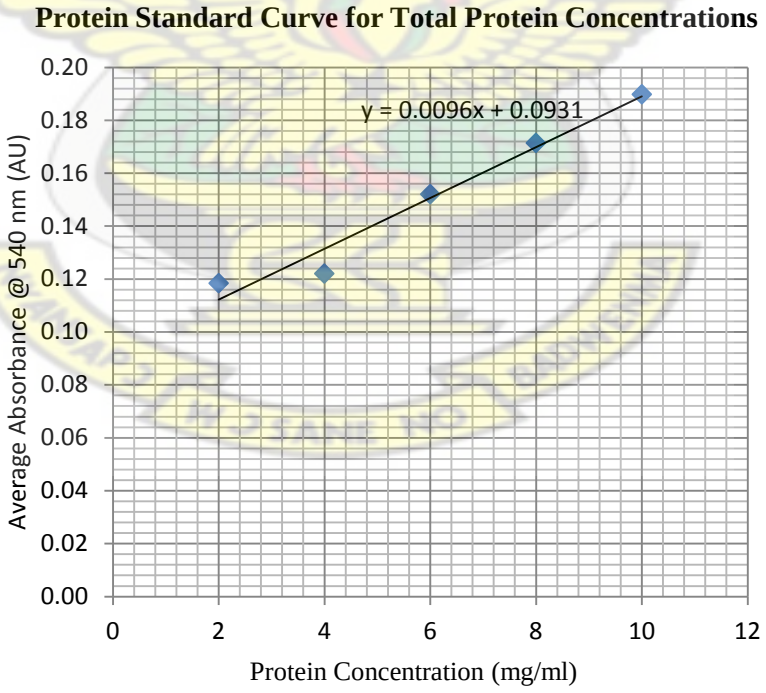
carboxymethylcellulose solution added and incubated at 50 °C for 30 minutes. Three ml of DNS reagent was added to the mixture after 30 minutes to stop the reaction. Test tubes were placed in boiling water for 5 minutes and the reducing sugars were determined as glucose released by reading the absorbance at 540 nm with a spectrophotometer. The control tubes contained 0.5 ml of enzyme sample plus 0.5 ml of citrate buffer. These tubes were also taken through the same procedure as the assay mixtures. The amount of glucose released from these tubes was deducted from that obtained from the fermentation filtrates before conversion to actual cellulase activities.

APPENDIX C: Standard Graphs for Glucose, Protein and Carboxymethyl cellulase assays.

a)

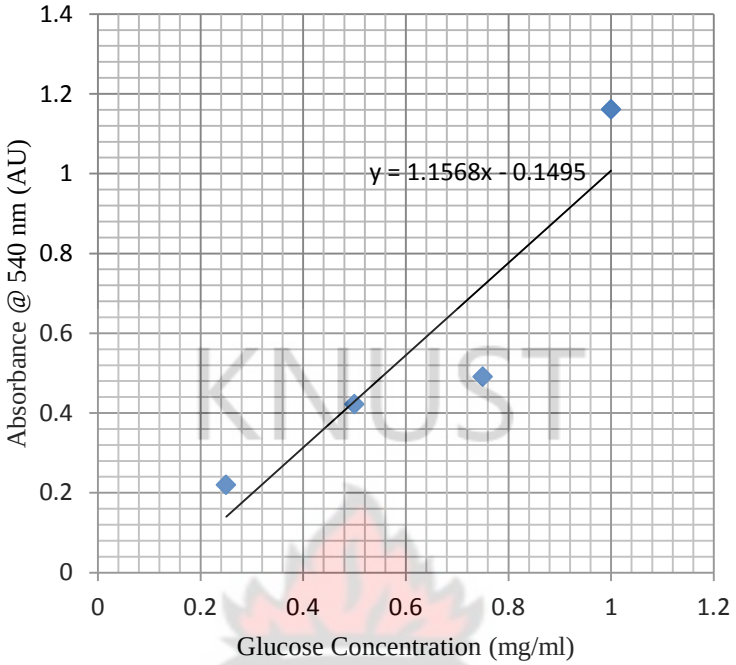


b)



c)

Glucose Standard Curve for Carboxymethyl Cellulase assay



APPENDIX D: DATA ANALYSIS

i) Data analyzed: Amount of Glucose Released from Corncobs After 5 days

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	5.49	1.83
Spore Concentration	3.0	2.72	0.908
Interaction	9.0	1.38	0.153
Residual (error)	68.0	0.260	0.00383
Total	83.0		

Does Temperature have the same effect at all values of Spore Concentration?

Interaction accounts for approximately 11.63% of the total variance.
F = 40.07. DFn=9 DFd=68
The P value is < 0.0001

If there is no interaction overall, there is a less than 0.01% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.
Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?

Temperature accounts for approximately 46.31% of the total variance.

F = 478.59. DFn=3 DFd=68

The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Spore Concentration affect the result?

Spore Concentration accounts for approximately 22.96% of the total variance.

F = 237.30. DFn=3 DFd=68

The P value is < 0.0001

If Spore Concentration has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

ii) Data analyzed: Amount of Glucose Released from Corncobs After 10 Days

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	8.99	3.00
Spore Concentration	3.0	4.33	1.44
Interaction	9.0	3.19	0.355
Residual (error)	68.0	0.455	0.00670
Total	83.0		

Does Temperature have the same effect at all values of Spore Concentration?

Interaction accounts for approximately 16.11% of the total variance.
F = 53.00. DFn=9 DFd=68
The P value is < 0.0001

If there is no interaction overall, there is a less than 0.01% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?

Temperature accounts for approximately 45.34% of the total variance.
F = 447.37. DFn=3 DFd=68
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Spore Concentration affect the result?

Spore Concentration accounts for approximately 21.85% of the total variance.
F = 215.58. DFn=3 DFd=68
The P value is < 0.0001

If Spore Concentration has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

iii) Data analyzed: Amount of Glucose Released from Corncobs after 15 days

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	4.81	1.60
Spore Concentration	3.0	3.06	1.02
Interaction	9.0	2.95	0.328
Residual (error)	68.0	0.247	0.00363
Total	83.0		

Does Temperature have the same effect at all values of Spore Concentration?
Interaction accounts for approximately 22.23% of the total variance.
F = 90.25. DFn=9 DFd=68
The P value is < 0.0001

If there is no interaction overall, there is a less than 0.01% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?
Temperature accounts for approximately 36.21% of the total variance.
F = 441.10. DFn=3 DFd=68
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Spore Concentration affect the result?
Spore Concentration accounts for approximately 23.07% of the total variance.
F = 281.03. DFn=3 DFd=68
The P value is < 0.0001

If Spore Concentration has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

iv) Data analyzed: Effect of Temperature on Saccharification ability of A. niger on Corncobs

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	12.01	4.002
Fermentation Time (Days)	2.0	0.6483	0.3241
Interaction	6.0	7.606	1.268
Residual (error)	60.0	0.5942	0.009903
Total	71.0	20.85	

Does Temperature have the same effect at all values of Fermentation Time (Days)?
Interaction accounts for 36.47% of the total variance.
F = 128.01. DFn=6 DFd=60
The P value is < 0.0001

If there is no interaction overall, there is a less than 0.01% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?
Temperature accounts for 57.57% of the total variance.
F = 404.15. DFn=3 DFd=60
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Fermentation Time (Days) affect the result?
Fermentation Time (Days) accounts for 3.11% of the total variance.
F = 32.73. DFn=2 DFd=60
The P value is < 0.0001

If Fermentation Time (Days) has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

v) Data analyzed: Concentration of Crude Proteins in Filtrate After 5 Days

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	125343	41781
Spore Concentration	3.0	24750	8250
Interaction	9.0	9059	1007
Residual (error)	68.0	3955	58.2
Total	83.0		

Does Temperature have the same effect at all values of Spore Concentration?
Interaction accounts for approximately 5.17% of the total variance.
F = 17.31. DFn=9 DFd=68
The P value is < 0.0001

If there is no interaction overall, there is a less than 0.01% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?
Temperature accounts for approximately 71.48% of the total variance.
F = 718.33. DFn=3 DFd=68
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Spore Concentration affect the result?
Spore Concentration accounts for approximately 14.11% of the total variance.
F = 141.84. DFn=3 DFd=68
The P value is < 0.0001
If Spore Concentration has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

vi) Data analyzed: Concentration of Crude Proteins after 10 Days

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	94103	31368
Spore Concentration	3.0	46539	15513
Interaction	9.0	41819	4647
Residual (error)	68.0	3137	46.1
Total	83.0		

Does Temperature have the same effect at all values of Spore Concentration?
Interaction accounts for approximately 21.30% of the total variance.
F = 100.71. DFn=9 DFd=68
The P value is < 0.0001

If there is no interaction overall, there is a less than 0.01% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?
Temperature accounts for approximately 47.94% of the total variance.

F = 679.85. DFn=3 DFd=68
The P value is < 0.0001
If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Spore Concentration affect the result?
Spore Concentration accounts for approximately 23.71% of the total variance.
F = 336.22. DFn=3 DFd=68
The P value is < 0.0001

If Spore Concentration has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

vii) Data analyzed: Concentration of Crude Proteins after 15 Days

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	35475	11825
Spore Concentration	3.0	25510	8503
Interaction	9.0	36655	4073
Residual (error)	68.0	3158	46.4
Total	83.0		

Does Temperature have the same effect at all values of Spore Concentration?
Interaction accounts for approximately 30.50% of the total variance.
F = 87.69. DFn=9 DFd=68
The P value is < 0.0001

If there is no interaction overall, there is a less than 0.01% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?
Temperature accounts for approximately 29.52% of the total variance.
F = 254.62. DFn=3 DFd=68
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.
Does Spore Concentration affect the result?
Spore Concentration accounts for approximately 21.23% of the total variance.

F = 183.09. DFn=3 DFd=68
The P value is < 0.0001

If Spore Concentration has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

viii) Data analyzed: Activities of Enzymes Produced After 5 Days of Fermentation

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	0.331	0.110
Spore Concentration	3.0	0.0747	0.0249
Interaction	9.0	0.121	0.0134
Residual (error)	40.0	0.0536	0.00134
Total	55.0		

Does Temperature have the same effect at all values of Spore Concentration?
Interaction accounts for approximately 16.62% of the total variance.
F = 10.00. DFn=9 DFd=40
The P value is < 0.0001

If there is no interaction overall, there is a less than 0.01% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?
Temperature accounts for approximately 45.64% of the total variance.
F = 82.41. DFn=3 DFd=40
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Spore Concentration affect the result?
Spore Concentration accounts for approximately 10.29% of the total variance.
F = 18.58. DFn=3 DFd=40
The P value is < 0.0001

If Spore Concentration has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

ix) Data analyzed: Activities of Enzymes Produced after 10 Days of Fermentation

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	0.442	0.147
Spore Concentration	3.0	0.0888	0.0296
Interaction	9.0	0.178	0.0198
Residual (error)	40.0	0.185	0.00464
Total	55.0		

Does Temperature have the same effect at all values of Spore Concentration?
Interaction accounts for approximately 16.39% of the total variance.
F = 4.27. DFn=9 DFd=40
The P value = 0.0006

If there is no interaction overall, there is a 0.062% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered extremely significant.

Since the interaction is statistically significant, the P values that follow for the row and column effects are difficult to interpret.

Does Temperature affect the result?
Temperature accounts for approximately 40.68% of the total variance.
F = 31.79. DFn=3 DFd=40
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Spore Concentration affect the result?
Spore Concentration accounts for approximately 8.17% of the total variance.
F = 6.38. DFn=3 DFd=40
The P value = 0.0012

If Spore Concentration has no effect overall, there is a 0.12% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered very significant.

x) Data analyzed: Activities of Enzymes Produced After 15 Days of Fermentation

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	0.396	0.132
Spore Concentration	3.0	0.129	0.0430
Interaction	9.0	0.195	0.0216
Residual (error)	40.0	0.525	0.0131
Total	55.0		

Does Temperature have the same effect at all values of Spore Concentration?
Interaction accounts for approximately 13.68% of the total variance.
F = 1.65. DFn=9 DFd=40
The P value = 0.1347

If there is no interaction overall, there is a 13% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered not significant.

Does Temperature affect the result?
Temperature accounts for approximately 27.85% of the total variance.
F = 10.06. DFn=3 DFd=40
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Spore Concentration affect the result?
Spore Concentration accounts for approximately 9.06% of the total variance.
F = 3.27. DFn=3 DFd=40
The P value = 0.0308

If Spore Concentration has no effect overall, there is a 3.1% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered significant.

xi) Data analyzed: Effect of Fermentation Time on Enzyme Activity

Source of Variation	Degrees of Freedom	Sum of Squares	Mean square
Temperature	3.0	1.21	0.403
Fermentation Time (Days)	2.0	0.00687	0.00344
Interaction	6.0	0.0496	0.00827
Residual (error)	36.0	0.688	0.0191
Total	47.0	1.95	

Does Temperature have the same effect at all values of Fermentation Time (Days)?
Interaction accounts for 2.54% of the total variance.
F = 0.43. DFn=6 DFd=36
The P value = 0.8521

If there is no interaction overall, there is an 85% chance of randomly observing so much interaction in an experiment of this size. The interaction is considered not significant.

Does Temperature affect the result?
Temperature accounts for 61.88% of the total variance.
F = 21.08. DFn=3 DFd=36
The P value is < 0.0001

If Temperature has no effect overall, there is a less than 0.01% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered extremely significant.

Does Fermentation Time (Days) affect the result?
Fermentation Time (Days) accounts for 0.35% of the total variance.
F = 0.18. DFn=2 DFd=36
The P value = 0.8362

If Fermentation Time (Days) has no effect overall, there is a 84% chance of randomly observing an effect this big (or bigger) in an experiment of this size. The effect is considered not significant.