# CHAPTER ONE

## 1.0 INTRODUCTION

Maize or Corn (*Zea mays*) is the most abundant cereal cultivated in Ghana (Asante, 2004). According to Asante (2004), production between 1995 and 2004 had hovered around a million metric tones per annum. This was accompanied by large quantities of maize agrowastes (husk, cobs and stove). The yields of various components of maize are: grains, 35%; husk and skins, 30%; corncobs, 30%; and skin trimmings, 5% (Rangkuti and Djajanegara, 1983).



Maize agrowastes do not pose as hazards to the environment but are underutilized resource. Currently the corncobs are burnt as fuel in the households of peasant rural farmers. The husks are used for wrapping kenkey and the stoves are usually burnt or left in the field. In large commercial farms where harvesting is mechanized, the agrowastes are left on the field and ploughed into the soil. This practice adds little to the soil nutrient status because maize agrowastes have high lignin, low nitrogen and readily available carbon content. Jigisha *et al.*, (2005) put nitrogen content per dry weight of corn cobs at 0.4%. It has been shown that high lignin, high carbon to nitrogen ratio and and low nitrogen contents of maize residues negatively affect their decomposition rate (Safari *et al.*, 2005). Maize agrowastes can be used to produce bioethanol.

Production of bio-ethanol from lignocellulose is relatively expensive. Currently, production cost is $2.65/gallon compared to $1.65/gallon for corn ethanol (www.nrel.gov, 2008; Collins, 2007). Cellulase is the costly part in the cellulosic ethanol

production, accounting for approximately 40% of the total cost (Howard *et al.*, 2003; Miyamoto, 1997). One way of reducing this cost is isolating a fungus with efficient cellulase system which is more likely to be found in a habitat where the lignocellulosic maize agrowastes are the predominant source of carbon and energy. At Ejura farms where harvesting is mechanized, the agrowastes are left on the field and ploughed into the soil making it a good habitat for cellulolytic fungi (Agana, 2007). Baig *et al.* (2003) isolated a cellulolytic fungus efficient in degrading banana agrowastes from a field where banana has been grown for 7 years.



### 1.1 Justification

Bio-ethanol was used as a fuel prior to the discovery of cheap petroleum and was resorted to when there was oil hikes in 1970s (Demirbas, 2005; Purwadi, 2006). Apart from being environmentally friendly and sustainable, bio-ethanol could insulate Ghana from high food and fuel prices because its source falls outside human food chain. It also adds value to the maize plant and improves the economic fortunes of maize farmers.

Maize agrowastes has been left to decompose in Ejura farms since its inception some 40 years ago. No work has been done on isolation of cellulolytic fungi from this farm. This project seeks to add value to the maize plant and improve the economic fortunes of the maize farmers.

### 1.2 Hypothesis

Soil from Ejura farms harbours cellulolytic fungi. Cellulases from cellulolytic fungi isolated from Ejura farms can efficiently hydrolyze maize agrowastes into glucose for bio-ethanol production.

### 1.3 Study Objective

The main objective of this thesis is to isolate fungi whose cellulases can efficiently hydrolyze maize agro-waste for bio-ethanol production.



#### 1.3.1. Specific objectives

1. Isolation of fungal species from soil sampled from Ejura farms.

2 Morphological identification of isolates.

3 Screening of isolates for cellulase activity.

4 Assaying of cellulolytic isolates for their cellulase activities.

5. Determination of optimum temperature, pH and substrate concentration for enzyme activity.

6. Simultaneous saccharification and fermentation of maize agro-waste to ethanol.

## CHAPTER TWO

## 2.0 LITERATURE REVIEW

## 2.1 Lignocellulose

Lignocellulosics are composed of heterogenous interwined polysaccharide chains of cellulose, hemicelluloses and pectins with varying degrees of crystallinity embedded in lignin. They also contain extractives such as unsaturated fatty acids and ashes (Taherzadeh and Karimi, 2007; Ohgren *et al*., 2006; Lynd *et al*., 2002). The compositions of these constituents may vary from one plant species to another. For example, hardwood has more cellulose constituent while wheat straw and leaves have more hemicellulose. The composition within a single plant varies with age, stage of growth and other conditions (Purwadi, 2006).

Lignocellulose is the major structural component of woody plant and some non-woody plants such as grasses. Large amounts of lignocellulose are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries, some of which are shown on Table 2.1 and 2.2 (Howard *et al*., 2003; Purwadi, 2006).

**Table 2.1: Composition of some agricultural lignocellulose (% dry weight).**

| Lignocellulosic material | cellulose (%) | Hemicellulose (%) | Lignin (%) |
|---|---|---|---|
| Corncobs | 45 | 35 | 15 |
| Rice straw | 32.1 | 24 | 18 |
| Waste paper from pulps | 60-70 | 10-20 | 5-10 |

Source: Howard *et al*., (2003).

**Table 2.2: Carbohydrate compostion of some lignocellulose (% dry matter).**

| Raw material | glucan | mannan | galactan | xylan | arabinan | lignin |
|---|---|---|---|---|---|---|
| corn stover | 36.4 | 0.6 | 1.0 | 18.0 | 3.0 | 16.6 |
| rice straw | 34.2 | - | - | 24.5 | – | 11.9 |

- unknown

Source: Olofsson *et al.*, (2008).



### 2.1.1 Cellulose

Cellulose is an unbranched**β** -1,4-linked homopolymer of glucose. It is the most abundant biomass in nature found mainly in plant cell walls (Wyman *et al.*, 1993). It forms about 35 to 50% of dry weight of many plants except in few, notably cotton, where it forms more than 90% of its dry weight (Lynd *et al.*, 2002; Rayner and Boddy, 1988; Howard *et al.*, 2003). Synthesis of cellulose is estimated to be $4x10^{10}$ tons/year (Beguin, 1990; Sengbusch, 2008; Sjostrom, 1993). In most cases, cellulose is embedded in a matrix of biopolymers; primary hemicellulose and lignin.

Cellulose from different plants share many common characteristics. Unlike many polysaccharides, cellulose has quasi crystalline structure (Sengbusch, 2008; Lynd *et al.*, 2002). A cellulose molecule is a linear chain of glucose molecules linked by ß (1-4) glycosidic bonds (Fig 2.1). The successive glucose residues are rotated by 180˚ relative to each other. The repeating units of the cellulose molecule or chain are the cellobiose unit (Howard *et al.*, 2003, Sengbusch, 2008; Richard, 2008). The average degree of polymerization of plant cellulose varies between 2000 and 27000 glucose units, depending on the source (Sjostrom, 1993). The linear chains undergo self assembly at the site of synthesis under the influence of associated hemicellulose. About 30 individual

molecules ( linear chains) assemble together to form a lager molecule called elementary fibrils or protofibrils which in turn are packed together into larger molecules units called microfibrils which are also in turn assembled into familiar cellulose fibers (Brown and Saxena, 2000). A microfibril with a diameter of 20-30 nm contains about 2000 molecules (Sengbusch, 2008). The chains within a protofibril are strengthened by the interchain and intrachain hydrogen bonds. The hydroxyl functional groups in cellulose chain are able to interact with each other, with O-, N-, and S-containing functional groups, and water molecules to form hydrogen bonds. These hydroxyl groups make the surface of cellulose largely hydrophilic (Miettinen-Oinonen, 2004; Pizzi and Eaton, 1985).

There are various degrees of parallelism between the individual chains within an elementary fibril. The regions within the fibril where there are highly oriented chains are called crystalline regions whilst the regions where the chains are randomly oriented are referred to as amorphous region. These two regions are interspersed and run into each other. Amorphous regions offer greater surface exposure for enzyme penetration hence reaction proceeds faster than in crystalline regions. At the crystalline regions, atoms are fixed at discrete positions with respect to each other, impacting compact arrangement and prevent enzyme penetration thus hydrolysis rate is mainly dependent on the crystalline region. In addition to the crystalline and amorphous regions, cellulose fibers contain various types of irregularities, such as kinks or twists of microfibrils, or voids such as surface pores, large pits and capillaries thus making the total surface area of a cellulose fiber greater than surface area of an ideally smooth fiber of the same

dimension (Fan *et al.*, 1979; Lynd *et al.*, 2002; Miettinen-Oinonen, 2004; Sengbusch, 2008).

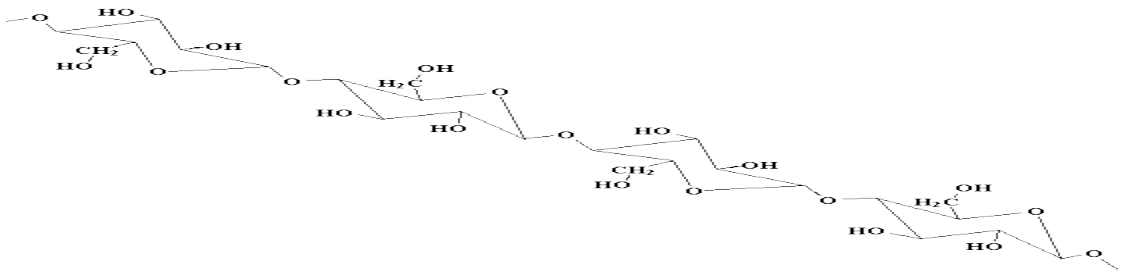
A model for cellulose structure is equivocal (Lynd *et al.*, 2002). Seven crystalline polymorphs have been identified for cellulose elementary fibrils, which are designated **Iα, Iβ , II, III** $_{I}$, $III_{II}$, $IV_{I}$, $IV_{II}$ (Miettinen-Oinonen, 2004). In nature, cellulose **Iα and Iβ** are the most abundant crystalline forms (Lynd *et al.*, 2002).



The interaction between the matrix and cellulose depends on type and maturity of plant cells (Lynd *et al.*, 2002). Utilization of cellulosic biomass is more complex due to both the complex matrices and diverse architecture of plant cells which limits accessibility, diffusion and transportation of cellulolytic agents to the site of attack (Lynd *et al.*, 2002).

Celluloses used in hydrolysis experiments are either pure or modified to enhance their hydrolysis. For instance microcrystalline celluloses such as Avicel and Sigmacell are nearly pure with their amorphous regions of the cellulose fibers removed. Solka floc is produced by delignification of wood and it contains substantial amounts of hemicelluloses and has low bulk density suggestive of swelling of cellulose fibers (Lynd *et al.*, 2002).

**Figure 2.1: Structure of cellulose molecule (linear chain)**



Source: http://pslc.ws/macrog/cell.htm., 2008



### 2.1.2 Hemicellulose

Hemicellulose is a collective term referring to those alkali soluble branched heteropolysaccharides associated with cellulose of plant cell wall (Obembe *et al.*, 2006; Ohgren *et al.*, 2006). The degree of polymerization of hemicelluloses is lower than 200 monomers and they include non-cellulose β -D-glucans, pectic substances (polygalacturonans), and several heteropolysaccharides such as arabinogalactans (galactose), galactogluco-and glucomannans (mannose) and arabinoglucurono- and glucuronoxylans (xylose) (Howard *et al.*, 2003). The principal sugar components of hemicellulose are hexoses: D-glucose, D-galactose, D-glucuronic acid, 4-O-methyl-D-glucuronic acid, D-galacturonic acid and mannose, and pentoses: xylose and L-arabinose, and to lesser extent, L-rhamnose, L-fucose and various O-methylated sugars (Howard *et al.*, 2003; Obembe *et al.*, 2006; Ohgren *et al.*, 2006; Richard, 2008). Fig 2.2 shows major monomers of hemicellulose. The amount of hemicellulose is usually between 11% and 37% of lignocellulosic dry weight (Sjostrom, 1993).

Hemicellulose from hardwood and agricultural residues are typically rich in xylans: primarily O-acetyl-4-O-methyl-glucuronoxylan in hardwood whilst grasses contain arabinoxylan. Softwood hemicellulose, on the other hand, contains more mannans: in

the form of galactoglucomannan and less xylan. In contrast to cellulose that is crystalline, strong, and resistant to hydrolysis, hemicellulose has a random, amorphous structure with little strength. It is easily hydrolyzed by dilute acid or base, and hemicellulase (Howard *et al.*, 2003).

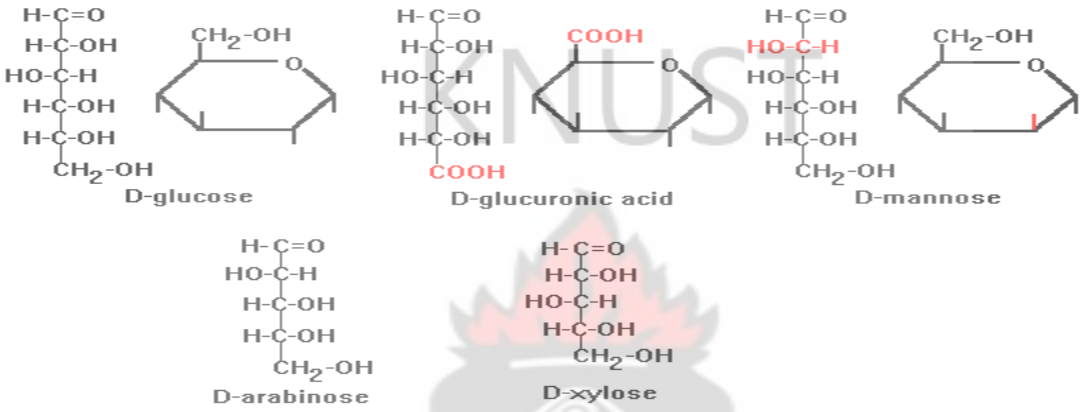


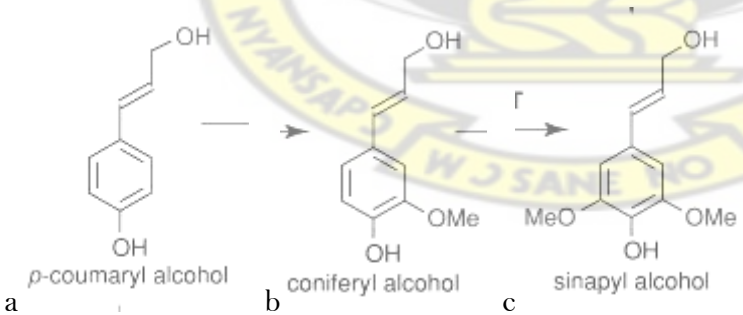

**Figure 2.2: Structures of major hemicellulose monomers**

### 2.1.3 Lignin

Lignin accounts for approximately 30% of the organic carbon in the biosphere and is the second most abundant biopolymer (Boerjan *et al.*, 2003; Dam *et al.*, 2008; Jennings and Lysek, 1996). It is a complex, amorphous, three-dimensional racemic heteropolymer of phenylpropane units, which are cross-linked to each other by a variety of chemical bonds (McCrady, 2008; Richard, 2008). The three hydroxycinnamyl alcohol monomers (monolignols) *p*-coumary, coniferyl, and sinapyl alcohols which differ in the degree of methoxylation produce *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) phenylpropanoid units respectively. The phenylpropanoid units are then incorporated into the lignin polymer (Lewis and Yamonoto, 1990; Howard *et al.*, 2003). Fig 2.3 shows the monomers and portion of lignin polymer. These monolignols are synthesised by

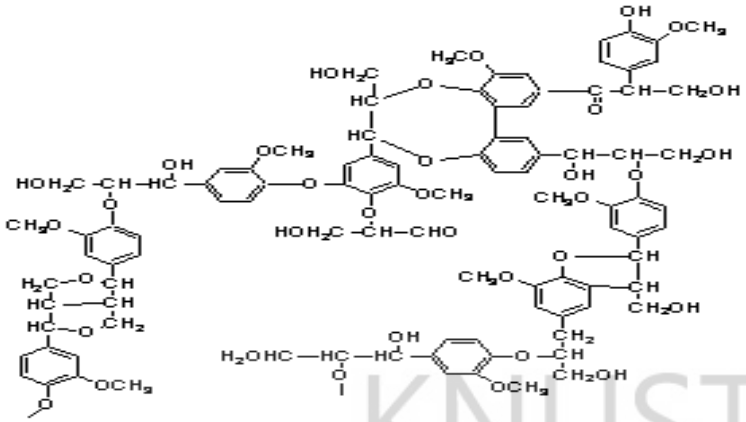
removal of water from sugars through phenylpropanoid pathway (Hudson, 1987). Polymerisation of the monolignols to make the lignin polymer is thought to take place through peroxidase/laccase assisted oxidative activation of the precursors. The formed radicals are polymerised in a random fashion and the most important factor guiding the process is the rate of monolignol feeding to the polymerisation site. The template polymerisation model, suggests that the pre-existing lignin polymer dictates the linkages that are formed between the polymer and the monolignol radicals. The polymerising enzymes have broad substrate specificities (Lewis andYamonoto, 1990).

The amount and composition of lignin depends on taxa, cell types, individual cell wall layers and developmental and environmental factors (Howard *et al.*, 2003; Lewis and Yamonoto, 1990). Although exceptions exist, lgnins of dicotyledonous angiosperm (hardwood) consist principally of G and S units and traces of H units, whereas those of gymnosperm (softwood) are composed mostly of G units with low levels of H units. Lignins from grasses (monocots) incorporate G and S units at comparable levels, and more H units than dicots (Lewis and Yamonoto, 1990; Howard *et al.*, 2003).




Source: Boerjan *et al.*, (2003)

**Figure 2.3: Monomers of lignin**





Source: www.lignin.org-Dialogue/Newsletters.htm

**Figure 2.4: Portion of lignin polymer**

## 2.2 Cellulolytic Fungi

Large numbers of microorganisms are capable of degrading cellulose (Miyamoto, 1997; Jennings and Lysek, 1996). Members of genera that have received considerable study under aerobic conditions are *Chaetomium*, and *Helotium* (Ascomycetes); *Coriolus*, *Phanerochaete*, *Poria*, *Schizophyllum* and *Serpula* (Basidiomycetes); *Aspergillus*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Myrothecium*, *Paecilomyces*, *Penicillium*, and *Trichoderma* (Deuteromycetes) and *Mucor* (Zygomycetes). The genera with prominent cellulolytic activity within anaerobic fungal division Chytridiomycetes are *Neocallimastix*, *Piromyces*, *Caecomyces*, *Orpimomyces and Anaeromyces* (Lynd *et al.*, 2002; Miyamoto, 1997; Boer *et al.*, 2005). However, only a few of these microorganisms produce significant quantities of cell-free enzymes capable of completely hydrolysing crystalline cellulose *in vitro*. Fungi of the genera *Trichoderma* and *Aspergillus* are thought to be prominent cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use (Bon and Ferrara, 2007;

Rajesh *et al.*, 2008; Haq *et al.*, 2005). Other relevant fungi include strains of *Humicola*, *Talaromyces*, *Acrophialophora*, *Thermoascus* and *Penicillium* species (Bon and Ferrara, 2007). Species of fungi in the genus *Trichoderma* produce relatively large quantities of endo-ß-glucanase and exo-ß-glucanase, but low levels of ß-glucosidase, while those of the genus *Aspergillus* produce relatively large quantities of endo-ß-glucanase and ß-glucosidase with low levels of exo-ß- glucanase production (Miyamoto,1997).



**2.3 Cellulase systems**

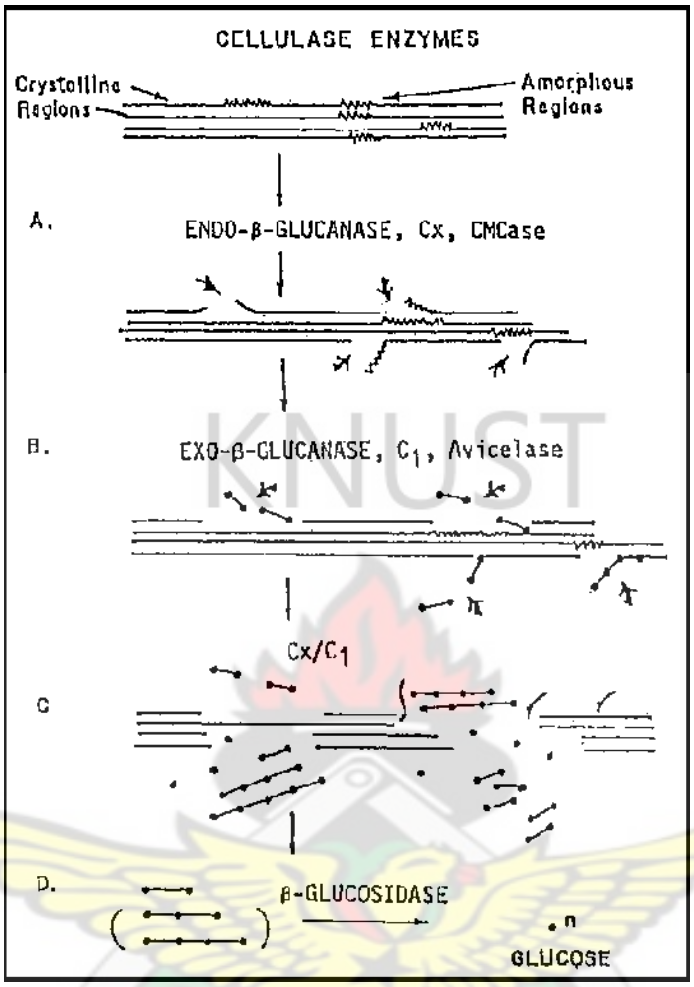
Cellulase is a term used to describe a mixture of cellulolytic enzymes whose synergistic action is required for effective breakdown of cellulose to its monomeric units. An enzyme system which hydrolyses cellulose is also active on hemicellulose (Howard *et al.*, 2003; Lynd *et al.*, 2002). Cellulase system has three major types of enzymatic components: (i) endoglucanases also known as 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), CMCase or Cx (ii) exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4- β –D glucan cellobiohydrolases (also known as cellobiohydrolases, CBH or Avicelase), (EC 3.2.1.91), and (iii) β-glucosidases or β-glucoside glucohydrolases (EC3.2.1.21) (Lynd *et al.*, 2002; Miettinen-Oinonen, 2004; Howard *et al.*, 2003; Miyamoto,1997; Maheshwari *et al.*, 2000, Haq *et al.*, 2005; Woodward *et al.*, 1988).

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 non-reducing ends of cellulose polysaccharide chains, liberating either glucose

(glucanohydrolases) or cellobiose (cellobiohydrolase) as major products. Exoglucanases can also act on microcrystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure. β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (Lynd *et al.*, 2002; Howard *et al.*, 2003; Miyamoto, 1997). The activities of cellulase components are illustrated in Fig. 2.5.



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. The hydrolysis products can either result in the inversion or retention (double replacement mechanism) of the anomeric configuration of carbon-1 at the reducing end (Lynd *et al.*, 2002; Miettinen-Oinonen, 2004).




Source: Miyamoto, 1997

**Figure 2.5: Illustration of cellulase components on different regions of cellulose**

The insoluble, recalcitrant nature of cellulose represents a challenge to cellulase systems. Most cellulases have modular structure consisting of catalytic and carbohydrate-binding modules (CBMs). The CBMs bind to the insoluble cellulose and it is believed that these facilitates hydrolysis of cellulose by bringing the catalytic domain very close to the cellulose and initiate its process by exoglucanase. CBMs also slough off cellulose fragments from cellulosic surfaces to enhance cellulose hydrolysis (Lynd *et al.*, 2002; Miettinen-Oinonen, 2004; Howard *et al.*, 2003).

Cellulase systems are not merely an agglomeration of enzymes representing the three enzyme groups (endoglucanases, exoglucanases, and β -glucosidases, with or without CBMs), rather the different groups act in a coordinated manner to efficiently hydrolyze cellulose. Cellulase systems exhibit synergism. Four forms of synergism have been reported: (i) endo-exo synergy between endoglucanases and exoglucanases, (ii) exo-exo synergy between exoglucanases processing from the reducing and non-reducing ends of cellulose chains, (iii) synergy between exoglucanases and β -glucosidases that remove cellobiose (and cellodextrins) as end products of the first two enzymes, and (iv) intramolecular synergy between catalytic domains and CBMs (Lynd *et al.*, 2002; Miettinen-Oinonen, 2004; Howard *et al.*, 2003; Olofsson *et al.*, 2008).



There are 2 types of cellulase systems namely non-complex and complex cellulase systems or Cellulosomes. Cellulolytic filamentous fungi have the ability to penetrate cellulosic substrates through hyphal extensions and present their cellulase systems in confined cavities within cellulosic particles. The free cellulases, with or without CBMs present is referred to as non-complex system. The enzymes in this system do not form stable high-molecular weight complexes. Cellulosomes are protuberances produced on the cell wall of cellulolytic bacteria when growing on cellulosic materials. These protuberances are stable high-molecular weight enzyme complexes that are firmly bound to the bacteria cell wall but flexible enough to also bind tightly to microcrystalline cellulose (Lynd *et al.*, 2002).

### 2.4.1 Organization of cellulase genes

The genes encoding cellulases are chromosomal and are usually randomly distributed over the genome with each gene having its own transcription regulatory elements. A comparison of the promoter regions of the cellulose genes *cbh1*, *cbh2*, *eg1*, and *eg2* of *Trichodeerma reesei* revealed the presence Carbon catabolite repressor element of (CRE1) -binding sites through which catabolite repression is exerted. Transcriptional factors, Activator of cellulase expression I and II (ACE I and ACE II) activate transcription by binding to at least the *cbh1* promoter region (Lynd *et al*., 2002).



**2.4.2 Regulation of cellulase production**

Cellulase is an inducible enzyme and is induced only in the presence of cellulose or products of it (Miyamoto, 1997; Lynd *et al*., 2002). Its production is suppressed when easily utilizable sugars, such as glucose, are available due to economics of its production. Cellulose is insoluble and cannot enter the cell to act as inducer. Sophorose (β -1,2-glucobiose) has been identified as a strong inducer of cellulases in *T. reesei* (Stenberg and Mandels, 1979, Lynd *et al*., 2002) Cellobiose, β -cellobiose-1,5-lactone, and other oxidized products of cellulose hydrolysis, or even xylo-biose have not been ruled out as natural inducer(s). The possibility of cellobiose functioning as an inducer is more complex because at high levels it inhibits cellulase production (Lynd *et al*., 2002).

Production of cellulases by *T. reesei* is regulated at the transcriptional level. Expression of the cellulase genes (*cbh1*, *cbh2*, *egl1*, *egl2*, and *egl5*) of *T. reesei* QM9414 is coordinated through transcription factors ACEI and ACEII. ACEII is homologous to XlnR, a transcriptional activator in *Aspergillus niger*. ACEII also stimulates the expression of

cellulase and xylanase genes. The general carbon catabolite repressor protein CRE1 represses the transcription of cellulase genes in *T. reesei* (Lynd *et al*., 2002).

### 2.4.3 Classification of cellulase

Cellulases belong to the O-glycioside hydrolases (EC 3.2.1-) which consist of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety (Miettinen-Oinonen, 2004; Shuler and Kargi, 1997; Moses, 2008).



## 2.5 Nutrition of cellulolytic fungi

The nutrient requirements for growth of cellulolytic fungal species are available carbon, nitrogen, phosphorus, and sulfur, plus standard macro- and microminerals and various vitamins. A few cellulolytic microbes have additional requirements (e.g., four and five-carbon). 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, sometimes dramatically (Lynd *et al*., 2002; Atlas, 1995).

Carbon is the basic source of energy and building block of their cells. Most cellulolytic fungi are capable of utilizing other carbohydrates. The most widely used nitrogen sources are ammonia and ammonium salts (ammonium chloride, ammonium sulphate and ammonium nitrate), proteins, peptides, and amino acids. Urea may also be used as nitrogen source. Other organic nitrogen sources are peptone and yeast extracts but they are relatively expensive. Phosphorus is a key element in the regulation of cell metabolism. Inorganic phosphate salts: $KH_2PO_4$, $K_2HPO_4$ and organic phosphate:

glycerophosphates are the most common phosphate sources. Sulphur forms proteins and some coenzymes. Common sulphur sources are sulphate salts e.g. $(NH_4)_2SO_4$ and sulphur-containning amino acids. Molecular oxygen is required as terminal electron acceptor in the aerobic metabolism of carbon compounds. Oxygen is present in all organic cell components. Hydrogen is derived mainly from carbon compounds e.g. carbohydrates. Potassium is a cofactor for some enzymes and is required in carbohydrate metabolism. The most commonly used potassium salts are $KH_2PO_4$, $K_2HPO_4$ and $K_3PO_4$. Magnesium is a cofactor for some enzymes and required by ribosome. Common sources are $MgSO_4.7H_2O$ and $MgCl_2$.

Lack of essential trace elements increases the lag phase and may decrease the specific growth rate and the yield. The major trace elements are Ar, Fe, Zn, and Mn.

Chelating agents keep ions such as $Mg^{2+}$, $Fe^{3+}$, and $PO_4^{3+}$ which precipitate in nutrient medium soluble to make them available to cells. They are added in media in very low concentrations. Common chelating agents are citric acid, ethylenediaminetetracetic acid (EDTA), polyphosphates, histidine, tyrosine and cysteine.

Growth factors stimulate the growth and synthesis of some metabolites. Vitamins, hormones, and amino acids are major growth factors. Some commonly required vitamins are thiamine, riboflavin, pyridoxine, biotin, cyanocobalamine, folic acid (Shuler and Kargi, 1997; Atlas, 1995; Todar, 2007; Jennings and Lysek, 1996; Ingold, 1984).

**2.6 Culture media**

The substance on which a microorganism is grown in the laboratory is called medium. It can be liquid or solid. In preparation of solid medium, agar, a hydrocolloid derived from red algae is used as gelling agent. Agar has unique properties: it melts at 100C and gels at 40°C and cannot be metabolized by most microorganisms. Previously gelatin was used as gelling agent but it was found to serve as source of protein for some microbes and cause liquefaction of the medium. Solid media are widely used for isolation of pure cultures, estimation of viable microbe population, and identification purposes whilst liquid media are for physiological studies (Malloch, 1997, Todar, 2007; Nester *et al.*, 2001; Sirockin and Gullimore, 1969).

Media are classified into synthetic or defined, semi-synthetic and complex or natural media. Synthetic media are composed of pure chemical compounds with known composition and concentration. The primary advantage of defined media is that results are more reproducible so they are useful in physiological and descriptive studies. Few fungi grow well on synthetic media but many fungi easily produce the sporulating structures necessary for identification (Todar, 2007; Malloch, 1997: Shuler and Kargi, 1997; Nester *et al.*, 2001). Semi-synthetic media contain some chemical compounds of known composition and some of unknown or variable composition. Semi-synthetic media are widely used in routine work. Complex media contain natural compounds whose chemical composition is not exactly known. They are often very good and allow sporulation of fungi that may otherwise remain sterile. Their major disadvantage is that they may differ considerably from batch to batch and thus not yield reliable experimental results (Malloch, 1997; Todar, 2007; Shuler and Kargi. 1997; Nester *et al.*, 2001).

Media can be described as selective, differential or enrichment. A selective medium is one which has a component(s) added to inhibit or prevent the growth of certain species and/or promote the growth of desired species. Physical condition of a medium such as pH or temperature can also be adjusted to render the medium selective. A differential medium is one which makes species distinguishable based on some observable traits in their growth pattern. An enrichment medium contains some components that permit the growth of specific type of species, usually because they alone can utilize the component from their environment (Malloch, 1997; Todar, 2007; Nester *et al.*, 2001).



**2.7 Isolation of celluloytic fungi**

There are two main strategies for isolating cellulolytic fungi. All fungal species in the sample can be isolated using a general media and then screened for cellulolytic activity. The other alternative is to isolate on cellulose enrichment media so that the ones which form colonies are cellulolytic species. Some common general media are potato dextrose agar (PDA), Sabouraud Agar, Czapek Dox Agar or water agar and some cellulose enrichment media are Mandel's agar medium, Eggins and Pugins (E and P) medium and Czapek-Dox broth medium with filter paper as carbon source (Malloch, 1997; Kader and Omar, 1998).

**2.8 Screening for cellulolytic fungi**

Detection of cellulase activity can be based on the formation of halo or clearing zone around a colony when the isolate is grown on cellulose enrichment agar medium or by the ability to release dye from dye-cellulose complex. The former is relatively inexpensive but has certain limitations. Clearing zones or halos are difficult to detect

even in well dispersed medium, it is very laborious and needs a lot of handling which increase the risk of contamination (Montenecourt and Eveleigh, 1977; Kader and Omar, 1998). Dye-cellulose complex assay is relatively expensive but is less laborious, fast and very sensitive especially when combined with spectrophotometer (Smith, 1977; Lai *et al.*, 2006; Poincelot and Day, 1972; Correia *et al.*, 1998).



**2.9 Assay for cellulase activity**

Enzyme assays are carried out mostly on pure cellulose or cellulose derivatives. Enzyme unit are based on limited conversion of the substrate. Cellobiose and carboxymethyl cellulose are used as substrates when quantifying β-glucosidase and endoglucanase activity, respectively. Cotton and cellodextrins can be used as the substrate when measuring exoglucanase activities. Filter paper is normally used when the saccharifying cellulase activity is of interest (Lai *et al.*, 2006). Reducing sugar released is then quantified by HPLC, GC or colourimetric methods such as the Neslon-Somogyi method or the Dinitrosalicylic acid (DNS) method (Lai *et al.*, 2006).

DNS assay tests for the presence of free carbonyl group (C=O) of reducing sugars. This involves simultaneous oxidation of the aldehyde or ketone functional group present and reduction of 3, 5-dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid (dark red coloured product) under alkaline conditions:

Aldehyde group $\xrightarrow{\text{oxidation}}$ carboxyl group

3,5-dinitrosalicylic acid $\xrightarrow{\text{reduction}}$ 3-amino, 5-nitrosalicylic acid + gluconic acid

The intensity of the colour formed is then quantified with spectrophotometer (Wang, 2008). DNS reagent consists of phenol, Rochelle salt, sodium hydroxide and sodium bisulfite. Phenol increases the colour intensity (it increases the intensity of the colour of 1 mg of glucose by 300%). The Rochelle salt (sodium potassium tartrate) reduces the oxygen concentration before heating, avoiding degradation of the sugar by the action of the oxygen in alkaline medium; therefore, the intensity of colour increases due to higher DNS reduction. Sodium bisulfite stabilizes the product after heating (Wang, 2008; Canizares-Maicas *et al*., 2001; Jeffries, 1987; Ghose, 1987; Adney and Baker, 1996).



**2.10 Methods of identification**

Morphological, biochemical and molecular methods are the main methods used for fungal identification. The traditional method is morphological identification and it is still useful and it employs other techniques such as microscopy, fluorescence cytochemistry and staining. Modern techniques such as electronic microscopy (transmission and scanning), automated image analyzers, electronic particle sizing and fractal geometry have improved its accuracy. Its major drawbacks are instability and dependency of morphological features on environment, and high subjectivity on the part of the observer. Molecular method is universally applicable and is very useful at differentiating strains.

The molecular methods widely used are sequencing and electrophoresis (RFLP and RAPD (Atlas, 1995; Guarro *et al*., 1999; Collins *et al*., 1989; Webster and Weber, 2007).

**2.11 Taxonomy of isolated fungi**

**2.11.1** ***Aspergillus spp***.

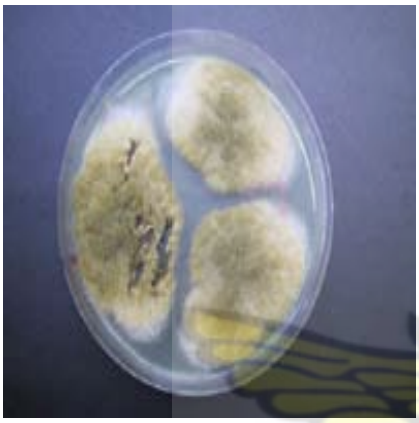
*Aspergillus spp.* Micheli ex Link belong to Kingdom Fungi, Phylum Ascomycota, Order Eurotiales, Family Trichocomaceae and Genus *Aspergillus* (www.doctorfungus.org, 2008; Webster and Weber, 2007). They are commonly isolated from soil, plant debris, and indoor air. *Aspergillus spp.* are filamentous, cosmopolitan and ubiquitous fungi. The teleomorphic states of few species are known. Others are accepted to be mitosporic with no known sexual spore production. This genus includes over 185 species notable among them are *A. fumigatus*, *A. flavus*, *A. niger*, *A. clavatus*, *A. glaucus*, *A. nidulans*, *A. oryzae*, *A. terreus*, *A. ustus*, and *A. versicolor* (www.doctorfungus.org, 2008; Webster and Weber, 2007). Plate 2.1 below shows some of the species.



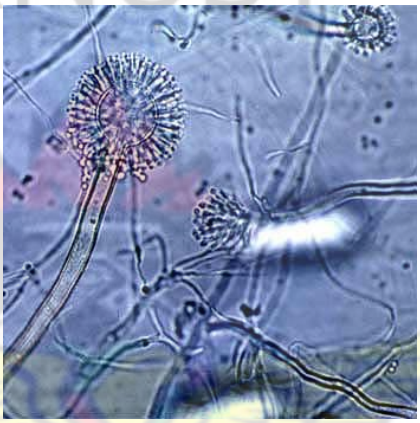
The major macroscopic features remarkable in species identification are the growth rate, colony appearance and thermotolerance. *Aspergillus* colonies are usually fast growing except *A. nidulans* and *A. glaucus* which reach maximum colony diameter of 0.5-1 cm. The remaining species reach colony diameter 1-9 cm on czapek-dox agar at incubation temperature of 25˚C for 7 days (www.doctorfungus.org, 2008). *Aspergillus* colonies have downy to powdery texture and their surface colours range from white, yellow, yellow-brown, brown to black and different shades of green depending on the species. The reverse of their plates are uncoloured to pale yellow for most isolates. It is olive and purple in some strains of *A. nidulans and A. versicolor* respectively.

*A fumigatus* has a unique thermotolerance: its optimum growth temperature is above 40˚C (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008).
Microscopically, their hyphae are septate and hyaline. Conidiophores are dense and erect. Conidiophores terminate in a vesicle covered with a single palisade-like layer of

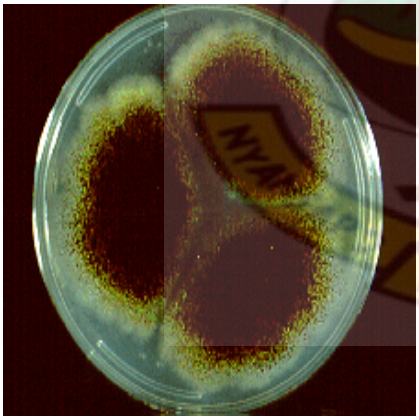
phialides (uniseriate) or a layer of subtending cells (metulae) which bear small whorls of phialides (biseriate). The vesicle, phialides, metulae (if present) and conidia form conidial head. Conidia are one-celled, smooth-or –rough-walled, hyaline or pigmented and basocatenate (form long chains) which may be divergent (radiate) or aggregated in compact columns (columnar). Certain species have unique structures such as sclerotia, cleistothecia, aleuriconidia and hulle cells (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008; Burnett and Hunter, 1972).



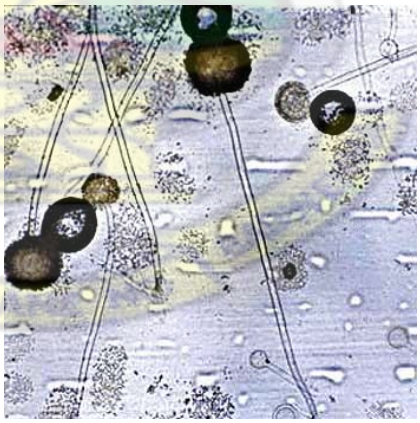
(a) *A. flavus colony*



(b) Conidial head of *A. flavus*

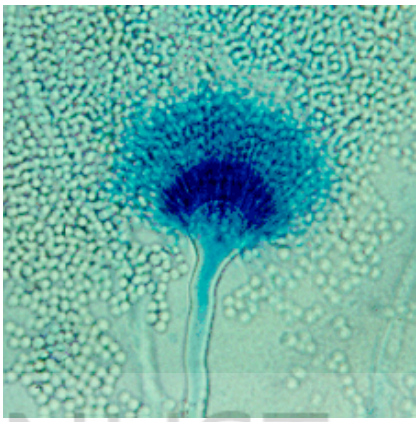


(c) *A. niger*



(d) Conidial head of *A. niger*

(d) *A. terreus*



(e) Conidial head of *A. terreus*



Sources: www.doctorfungus.org (2008) and www.mycologyonline.adelaide.edu.au (2008)

**Plate 2.1: Colonies of some *Aspergillus spp.* on Czapek-dox media and their conidial heads**

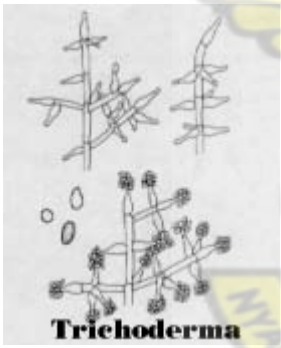
### 2.11.2 *Trichoderma spp.*

*Trichoderma* spp. (Anamorphic Hypocra) Persoon ex Gray belong to Kingdom Fungi, Phylum Ascomycota, Class Euascomycetes, Order Hypocreales, Family Hypocreaceae and Genus *Trichoderma* (www.doctorfungus.org, 2008, Webster and Weber, 2007).

*Trichoderma spp.* are filamentous fungi widely distributed in the soil, plant material, decaying vegetation, and wood. *Hypocrea* spp. are the anamorphic states of some *Trichoderma* species. The prominent species of this genus are *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma pseudokoningii*, and *Trichoderma viride*. Two other species, *Trichoderma asperelum* and *Trichoderma citrinoviride* have been proposed (www.doctorfungus.org, 2008; Webster and Weber, 2007).

*Trichoderma* colonies are fast growing, white and downy at early stage, later developing to yellowish-green to deep green compact tufts, often only in small areas or in concentric ring-like zones on the agar surface (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008; Burnett and Hunter, 1972).

Conidiophores are hyaline, repeatedly branched, irregularly verticillate, bearing clusters of divergent, often irregularly bent, flask-shaped phialides. Conidia are mostly green, but sometimes hyaline, 1-celled, ovoid with smooth or rough walls (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008; Burnett and Hunter, 1972). The species of *Trichoderma* are defined on the basis of their morphology but it is not effective (Guarro *et al.*, 1999; www.doctorfungus.org, 2008; Webster and Weber, 2007).




Source: Malloch, 1997

**Figure 2.6: Conidiophores and conidia of *Trichoderma sp.***

### 2.11.3 *Penicillium spp.*

*Penicillium spp.* Link belong to Kingdom Fungi, Phylum Ascomycota, Class Euascomycetes, Order Eurotiales, Family Trichomaceae and Genus *Penicillum* (www.doctorfungus.org, 2008; Webster and Weber, 2007). The taxonomy of *Penicillium*

is complex due to its great number of species (nearly 250) having very few differences (Guarro *et al*., 1999). Notable among them are *P. chrysogenum*, *P. cirinum*, *P. janthinellum* *P. marneffei*, *P. purpurogenum*, *P. italicum and P. digitatum*, *P. notatum* and *P. roquefortii* . They are filamentous fungi except *P. marneffei* which is thermally dimorphic. *P. chrysogenum*, is classified as a psychrotrophic. They are widespread and found in soil, decaying vegetation and the air (www.doctorfungus.org, 2008).



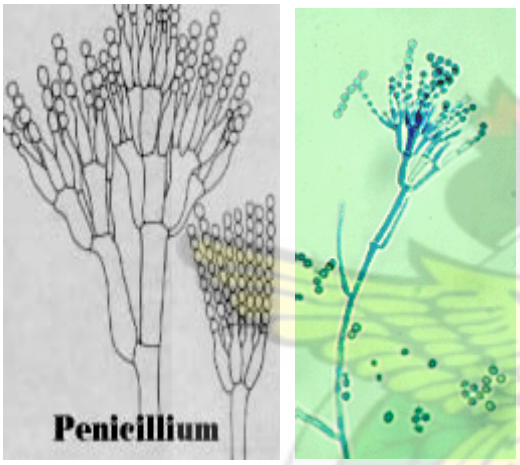
*Penicillium spp.* colonies except *P. marneffei* are fast growing, flat, filamentous, and have velvety, woolly, or cottony texture. The colonies are initially white and become blue green, gray green, olive gray, yellow or pinkish with time. The reverse plates are usually pale to yellow (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008).

*P. marneffei* is thermally dimorphic and produces filamentous, flat, radially sulcate colonies at 25C. These colonies are bluish -gray-green at centre and white at the periphery. It also produces rapidly diffusing red soluble pigment observed on the plate. At 37°C, its colonies are slightly pink in colour and glabrous to convolute in texture (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008).

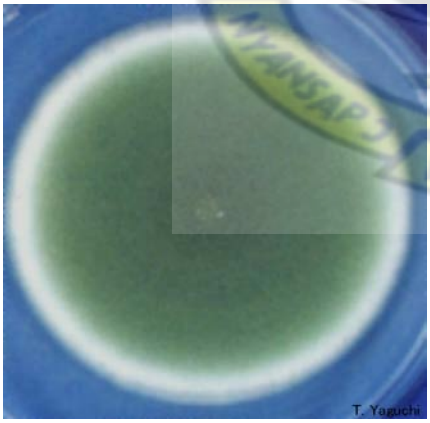
Microscopically, chains of single-celled conidia (ameroconidia) are produced in basipetal succession on the phialides. The phialides are produced singly, in groups or form branched metulae, giving a brush-like appearance known as a penicillus. The penicillus may contain both branches and metulae (penultimate branches which bear a whorl of phialides). All cells between the metulae and the stipes of the conidiophores are referred to as branches. The branching pattern may be either simple (non-branched or monoverticillate), one-stage branched (biverticillate-symmetrical), two-stage branched

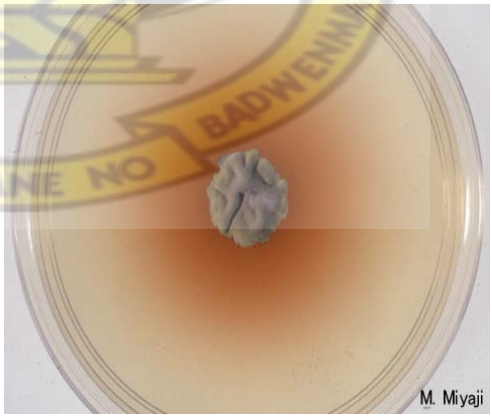
(biverticillate-asymmetrical) or three- to more-staged branched. Conidiophores are hyaline and have smooth or rough-walls. Phialides are usually flask-shaped (consisting of a cylindrical basal part and a distinct neck) or lanceolate (with a narrow basal part tapering to a somewhat pointed apex). Conidia are globose, ellipsoidal, cylindrical or fusiform; hyaline or greenish; and smooth- or rough- walled. Sclerotia may be produced by some species (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008; Burnett and Hunter, 1972). Plate 2.2 shows colonies and pennicillates of *Penicillium spp.*






(a) Penicilliates of *Penicillium sp.*




(b) *Penicillium chrysogenum* colony (c) *P. marneffei*

Source: Mollach, (1997); www.mycologyonline.adelaide.edu.au

**Plate 2.2: Pennicilliates and colonies of some *Penicillium spp.***

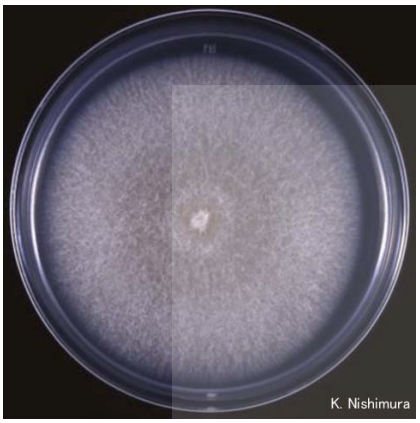
### 2.11.4 *Fusarium spp.*

*Fusarium spp.* Link ex Gray belong to Kingdom Fungi Phylum Ascomycota Order Hypocreales, Family Hypocreaceae, and Genus *Fusarium* (www.doctorfungus.org, 2008; Webster and Weber, 2007). Teleomorphs of some *Fusarium* species occur in the genera *Gibberella* and *Nectria* (Guarro *et al.*, 1999; Webster and Weber, 2007).

The genus *Fusarium* has cosmopolitan distribution and has been implicated as plants, animals and human pathogen of interest to scientists. Numerous taxonomic schemes have been proposed in an attempt to make identification easier (Guarro *et al.*, 1999; www.doctorfungus.org, 2008; Webster and Weber, 2007).

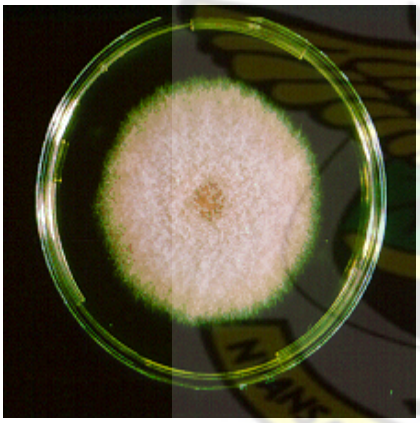
*Fusarium spp.* colonies are cottony, often with tinge of pink, purple or yellow, in the mycelium or medium. Conidiophores are varied, either slender and simple or stout and short, branched irregularly or bearing a whorl of phialide, single or grouped into sporodochia (dense cushion-like structure). Conidia are hyaline and are of two types often held in small moist head. Microconidia are single-celled, ovoid or oblong and are borne singly or in chains. Macroconidia are hyaline, two- to several-celled, fusiform- to sickle-shaped, mostly with an elongated apical cell and pedicellate basal cell. Chlamydoconidia may be present (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008; Burnette and Hunter, 1972). Many species form conidia in the aerial mycelium on phialidic or blastic conidiogenous cells called mesoconidia (Guarro *et al.*, 1999; www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008).

Some *Fusarium* species are placed in Tuberculariaceae because of the sporodochia they produced (www.mycologyonline.adelaide.edu.au). Plate 2.3 shows colonies of some *Fusarium* species and macroconidia.




(a) *Fusarium solani* colony


(b) *Fusarium solani* macroconidia



(c) *Fusarium sp.* colony

Source: www.mycologyonline.adelaide.edu.au (2008)

**Plate 2.3: Colonies and macroconidia of some *Fusarium spp.***

### 2.11.5 *Mucor spp.*

*Mucor spp.* Micheli ex Saint-Amans belong to the Kingdom Fungi, Phylum Zygomycota, Order Mucorales, Family Mucoraceae and Genus *Mucor*

(www.doctorfungus.org, 2008; Webster and Weber, 2007). *Mucor* is a filamentous fungus found in soil, plants, decaying fruits and vegetables. The genus *Mucor* contains several species. The most common ones are *Mucor amphibiorum*, *Mucor circinelloides*, *Mucor hiemalis*, *Mucor indicus*, *Mucor racemosus*, and *Mucor ramosissimus*. *Mucor* colonies are very fast growing, cottony to fluffy, white to yellow, becoming dark grey with the development of sporangia (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008).

Sporangiophores are erect, simple or branched, forming large (60-300 µm in diameter) terminal, globose to spherical, multi-spored sporangia, without apophyses and with well developed subtending columellae. A conspicuous collarette (remnants of the sporangial wall) is usually visible at the base of the columella following sporangiospore dispersal. Sporangiospores are hyaline, grey or brownish, globose to ellipsoidal and smooth-walled or finely ornamented. Stolons and rhizoids are absent, however, chlamydoconidia and zygospores may be present (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008 Burnett and Hunter, 1972).

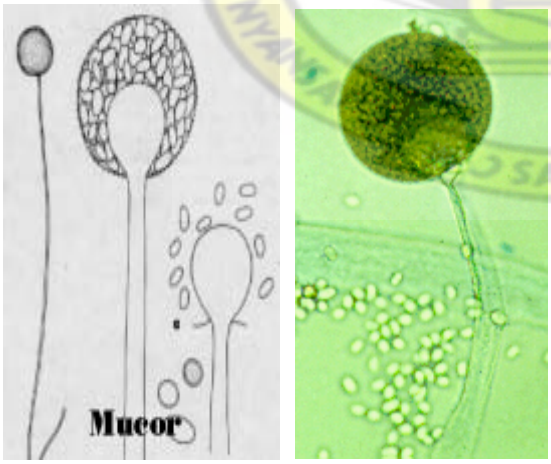



**Plate 2.4: Sporangiophores of *Mucor sp.***

Source: Mollach (1997); www.mycologyonline.adelaide.edu.au

### 2.11.6 *Rhodotorula spp.*

*Rhodotorula spp.* Harrison belong to Kingdom Fungi, Phylum Basidiomycota, Class Urediniomycetes, Order: Sporidiales, Family: Sporidiobolaceae and Genus: *Rhodotorula* (www.doctorfungus.org, 2008; Webster and Weber, 2007).

*Rhodotorula* is yeast found in air, soil, lakes, sea water, and dairy products and may colonize plants, humans, and other mammals (www.doctorfungus.org, 2008). The genus *Rhodotorula* includes three active species; *Rhodotorula glutinis*, *Rhodotorula minuta*, and *Rhodotorula mucilaginosa*. *Rhodotorula mucilaginosa* is the current name for the species formerly known as *Rhodotorula rubra* (www.doctorfungus.org, 2008).

On Sabouraud's dextrose agar *Rhodotorula* forms coral pink, usually smooth, sometimes reticulate, rugose or corrugated, moist to mucoid yeast-like colonies. Microscopic morphology shows spherical to elongate budding yeast-like cells or blastoconidia, 2.5-6.5 x 6.5-14.0 µm in size (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au).

**Plate 2.5: *Rhodotorula sp.* colony on Sabouraud's agar**

### 2.11.7 *Coccidiodes spp.*

*Coccidiodes spp.* Rixford and Gilchrist belong to Kingdom Fungi, Phylum Ascomycota, Class Euascomycetes, Order Onygenales, Family Onygenaceae and

Genus: *Coccidioides* (www.doctorfungus.org, 2008; Webster and Weber, 2007).



*Coccidioides spp.* are found in soil particularly at warm and dry areas with low rainfall, high temperatures, and low altitude. *Coccidioides immitis* and *C. posadasii* are the only species included in this genus. They are thermally dimorphic fungi. The two species are morphologically identical but genetically and epidemiologically distinct. They have no known teleomorphs (www.doctorfungus.org, 2008)).

*Coccidioides* colonies grow rapidly. The macroscopic morphology varies. At 25 or 37°C on Sabouraud dextrose agar, the colonies are moist, glabrous, membranous, and grayish initially, later producing white and cottony aerial mycelium. With age, colonies become tan to brown in colour (www.doctorfungus.org, 2008).

Microscopic appearance of the fungus depends on the temperature of isolation. At 25°C, hyphae and arthroconidia are produced. Hyphae are hyaline, septate and thin. Racquet hyphae may occasionally be observed on slides prepared from young cultures. Arthroconidia are thick-walled, barrel-shaped, and 2-4 x 3-6 µm in size. Typically, these arthroconidia alternate with empty disjunctor cells. When arthroconidia are released, annular frills, remnants of the disjunctor cells are observed (www.doctorfungus.org, 2008). At 37°C, Large, round, thick-walled spherules (10-80 µm in diameter) filled with

endospores (2-5 μm in diameter) are observed. Production of spherules *in vitro* requires inoculation into a special synthetic medium, such as converse liquid medium, an incubation temperature of 37-40°C and presence of $CO_2$ at a concentration as high as 20% (www.doctorfungus.org, 2008).

*Coccidioides* continues to grow as a mould and does not produce spherules at any temperature unless special growth medium is provided *in vitro*. This finding indicates that temperature is not the only variable that controls the spherule formation. Thus, some authorities prefer not to classify this fungus as thermally dimorphic (www.doctorfungus.org, 2008). The definitive identification of an isolated *Coccidioides* strains require demonstration of spherule production *in vitro*, use of DNA probes or demonstration of spherule production *in vivo* by animal experiments. Molecular typing appears useful in identification (www.doctorfungus.org, 2008).

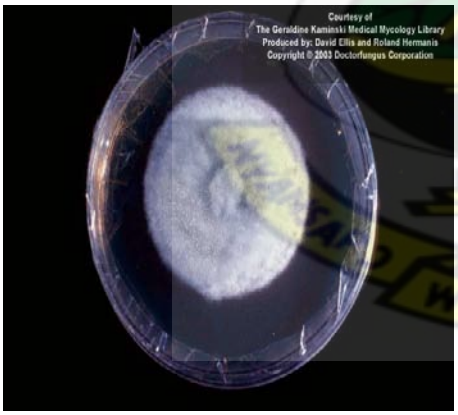
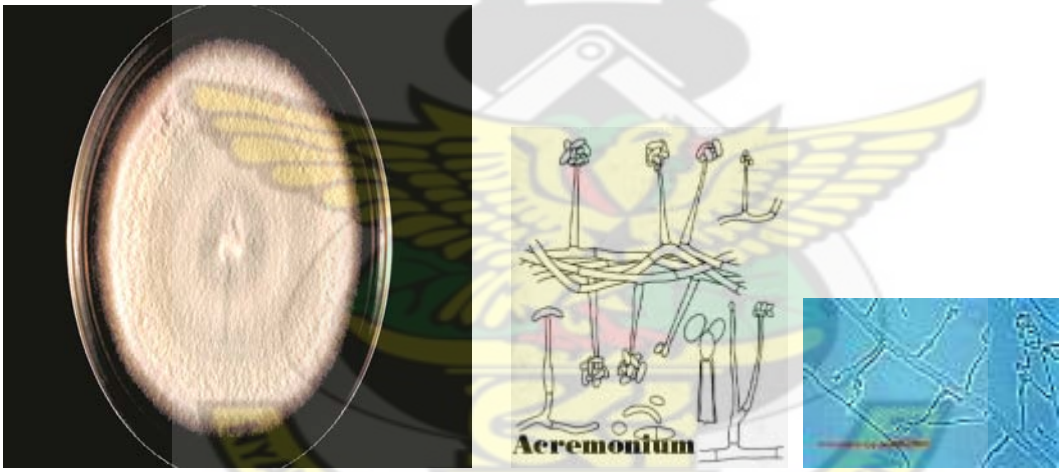



**Plate 2.6: *Coccidioides sp.* colony**

### 2.11.8 *Acremonium spp.*

*Acremonium spp.* Link ex Fries belong to Kingdom Fungi, Phylum Ascomycota, Class Ascomycotina, Order Hypocreales, Family Hypocreaceae and Genus *Acremonium* (www.doctorfungus.org, 2008; Webster and Weber, 2007).

*Acremonium spp.* are filamentous, cosmopolitan fungi commonly isolated from plant debris and soil. The sexual state of *Acremonium* is not well-defined (www.doctorfungus.org, 2008).




(a) *Acremonium sp.* colony (b) *Acremonium sp.* mycelia and conidia

**Plate 2.7: *Acremonium sp.* colony and its microscopic features.**

The growth rate of *Acremonium* colonies is moderately rapid, maturing within 5 days. The diameter of the colony is 1-3 cm following incubation at 25°C for 7 days on potato dextrose agar. The texture of the colony is compact, flat or folded, and occasionally

raised in the center. It is glabrous, velvety, and membrane-like at the beginning. Powdery texture may also be observed. By aging, the surface of the colony may become cottony due to the overgrowth of loose hyphae. The colour of the colony is white, pale grey or pale pink on the surface. The reverse side is either uncolored or a pink to rose coloured pigment production is observed (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008)



*Acremonium* spp. possesses hyaline, septate hyphae which are typically very fine and narrow. Vegetative hyphae often form hyphal ropes. Unbranched, solitary, erect phialides are formed directly on the hyphal tips, the hyphal ropes, or both. The phialides are separated from hyphae by a septum and taper towards their apices. At the apices of the phialides are the hyaline conidia 2-3 x 4-8 µm in size. They usually appear in clusters, in balls or rarely as fragile chains. The conidia are bound by a gelatinous material. They may be single or multicellular, fusiform with a slight curve or resemble a shallow crescent. These structural properties of conidia vary depending on the species. *Acremonium falciforme* usually produces crescentic, nonseptate conidia. Sometimes, 2- or 3-celled conidia may also be observed. *Acremonium kiliense*, on the other hand, has short straight conidia and the conidia of *Acremonium recifei* are usually crescentic and nonseptate (www.doctorfungus.org, 2008; www.mycologyonline.adelaide.edu.au, 2008).

### 2.12 Ethanol

Ethanol or ethyl alcohol is a clear, colourless, flammable, oxygenated hydrocarbon with the chemical formula $CH_3CH_2OH$. It is volatile, miscible in both water and non-polar

solvents at ordinary conditions and has density of 0.792 g/cm$^3$ at 15.5°C (Purwadi, 2006; Thomsen *et al.*, 2008).

Ethanol is less hygroscopic, contains a reasonable heat of combustion, has lower evaporation heat and, most importantly, is not toxic like methanol (Purwadi, 2006). Acetaldehyde, a product of partial oxidation of ethanol in the exhaust gas of vehicles is much less toxic than formaldehyde, which is formed when using methanol. Ethanol is harmless to the environment. In ground water and soil mixtures, ethanol can be rapidly degraded both aerobically (100 ml/L in 7 days) and anaerobically (100 mg/L in 3-25 days). Ethanol in surface water is also rapidly degraded. Exposure of humans to ethanol is harmless (Purwadi, 2006).

Ethanol production and its use as a fuel date back to the invention of automobiles. For instance, internal combustion engine invented by Nikolas Otto in 1897 and Henry Ford's Model T built in 1908 ran on ethanol (Demirbas, 2005; Purwadi, 2006). This continued until the advent of cheap petroleum in early part of 20$^{th}$ century. Interest in ethanol fuel was renewed in 1970s due to energy crisis. In this 21$^{st}$ century interest has once again been awakened due to ever increasing demand for fuel against anticipated dwindling crude oil reserves in the future (Demirbas, 2005).

### 2.12.1 Ethanol Production

Ethanol is produced by two routes: synthetic (chemical) and biological (fermentative). The synthetic production is by catalytic hydration of ethylene in gaseous phase. Synthetically produced ethanol is mostly used as a solvent (60%) and chemical intermediate (40%). Fermentative ethanol (bio-ethanol) production accounts for 93% of

world total ethanol production of which 92% is used as fuel, 4% as industrial solvents and chemicals, and 4% in beverages (Taherzadeh and Karimi, 2007; Demirbas, 2005; Purwadi, 2006; Badger, 2002).

The leading bio-ethanol producing countries are Brazil (major feedstock is sugar cane) and USA (major feedstock is corn starch) account for more than 65% (40 billion litres) of global ethanol production (Bon and Ferrara, 2007; Purwadi, 2006; Badger, 2002). Their major feedstock has been food crops and this is contributing to rising food prices. However lignocellulosic materials are rich in carbohydrates, are abundant, renewable, fall outside the food chain and can serve as alternate feedstock. (Purwadi, 2006; Fujita *et al*., 2002). The first attempt at commercializing a process for ethanol production from wood was in Germany in 1898. The process involved dilute acid hydrolysis of cellulose to glucose, and was able to produce 18 gallons of ethanol per tonne of wood waste. In April 2004, Iogen Cooperation, a Canadian Biotechnology firm, became the first to commercially sell cellulosic ethanol, though in very small quantities (www.iogen.ca/index.html, 2008).

### 2.12.2 Ethanol as fuel

Ethanol can be used as a transport fuel in at least four forms: anhydrous ethanol (100% ethanol), hydrous ethanol (95% ethanol and 5% water), anhydrous ethanol-gasoline blends (10–20% ethanol in gasoline) and as raw material for ethyltert-butyl ether (ETBE) (Demirbas, 2005; Purwadi, 2006). As an oxygenated compound, ethanol provides additional oxygen in combustion which increases the completeness of combustion. The emission of carbon monoxide is reduced by 32.5% while the emission

of hydrocarbons is decreased by 14.5%. Emission of nitrogen oxides is also reduced by using ethanol as additive (Demirbas, 2005; Purwadi, 2006; Badger, 2002).

As a high-octane additive, ethanol has disadvantages. It emits higher amount of acetaldehyde than gasoline; it is highly corrosive due to the water content and this has negative effect on rubber and plastic; and the blend with gasoline tends to separate in the presence of traces of water (Purwadi, 2006). These drawbacks have been overcome as follows: An addition of 5% water in a blend of ethanol and gasoline can reduce the emission of acetaldehyde. Some corrosion inhibitor such as hydroxyethylated alkylphenols and alkyl imidazolines can attain essential anticorrosion resistance. The polymer industries have also developed special materials that are resistant to alcohols. Stabilizers like higher alcohols, fusel oil, aromatic amines, ethers or ketones are useful in preventing separation (Purwadi, 2006).

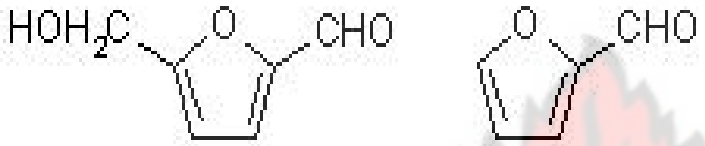
### 2.12.3 Cellulosic ethanol production processes

There are two methods of producing ethanol from lignocellulosics namely cellulolysis (enzymes based) and gasification (thermochemical). Steps involved in cellulolysis are (1) pretreatment (2) hydrolysis (3) separation of the sugar solution from the residual materials (4) microbial fermentation and (5) distillation.

Pretreatment refers to the solubilization and separation of one or more of the four components of lignocellulosics – hemicellulose, cellulose, lignin, and extractives – to make the remaining solid biomass more accessible (Graf and Koehler, 2000; Demirbas, 2005). Pretreatment is achieved by physical or chemical means. Current leading

pretreatment technologies include the use of dilute acid, hot water, wet oxidation (alkali and oxygen), ammonia fiber explosion (AFEX), ammonia recycle percolation, lime, microwave, steam explosion and "organsolv" (ethanol/water mixture) (Rajesh *et al.*,2008; Olofsson *et al.*, 2008). The type of feedstock strongly affects the choice of pretreatment method (Olofsson *et al.*, 2008). Most of these techniques form products which act as inhibitors prominent among them are furfural and hydroxyl methylfurfural (HMF) as shown in Fig 2.7.






HMF Furfural

**Figure 2.7: Major inhibitors produced in pretreatment of lignocellulose**

AFEX is the sole pretreatment which has no inhibitory effect on resulting hydrolysate. Pretreatment using alkali and oxygen (wet oxidation) effectively removes lignin and produces less toxic compounds and seems to give the best performance at the enzyme treatment stage when treating annual crops like wheat straw (Olofsson *et al.*, 2008).

Hydrolysis is chiefly achieved by acid or enzymatic mean (Graf and Koehler, 2000; Demirbas, 2005). Hydrolysis can also be achieved by gamma-ray, electron-beam irradiation, or microwave irradiation but these are not used commercially (Demirbas, 2005; Taherzadeh and Karimi, 2007). There are two acid hydrolysis processes commonly used: dilute acid and concentrated acid. The dilute acid process is conducted under high temperature and pressure, and has a reaction time in the range of seconds or

minutes, which facilitates continuous processing. The concentrated acid process occurs at relatively mild temperatures, and the only pressures involved are those created by pumping materials from vessel to vessel. Reaction times are much longer than for dilute acid (Graf and Koehler, 2000; Taherzadeh and Karimi, 2007; Badger, 2002).

Enzymatic hydrolysis has comparative advantage in that hydrolysis occur at relatively mild condition (50˚C and pH 5) which prevents formation of inhibitory by-products. This process gives higher sugar yield from pretreated lignocellulosics (Bon and Ferrara, 2007; Olofsson *et al.*, 2008). There are two technological developments: enzymatic and direct microbial conversion methods (Demirbas, 2005; Graf and Koehler, 2000).

*Saccharomyces cerevisiae* is the most popular microorganism used for ethanol fermentation from hexoses due to its high ethanol yield, high tolerance to rather high ethanol and inhibitor concentration, and ability to grow at low pH values which prevents bacterial contaminations (Olofsson *et al.*, 2008). However, yeast strains used cannot withstand highly toxic hydrolyzates or ferment pentoses and minor hexoses efficiently (Taherzadeh and Karimi, 2007; Shi and Jeffries, 1998). Because lignocellulosics contain considerable amount of pentoses namely xylose and arabinose, attempts had been made to genetically engineer *Saccharomyces cerevisiae* capable of utilizing both pentoses and hexoses. National Renewable Energy Laboratory (NREL) and its partners developed yeast capable of fermenting arabinose which constitutes up to 20% of the fermentable sugars in corn fiber in 2000. NREL has also developed the bacterium *Zymomonas mobilis* which gives a high ethanol yield, tolerates high ethanol concentrations and can ferment arabinose and xylose (www.nrel.gov, 2008). Yeasts

*Pichia stipitis*, *Candida shehatae* and *Pachysolen* are capable of fermenting xylose efficiently into ethanol (Okur and Saracoglu, 2006; Bon and Ferrara, 2007; Sreenath *et al.*, 2001; Taherzadeh and Karimi, 2007; Shi and Jeffries, 1998). Recombinant *Saccharomyces cerevisiae* TMB3400 capable of co-fermented glucose and xylose with relatively high ethanol yields has been developed (Ohgren *et al.*, 2006). *Escherichia coli* has also been targeted through metabolic engineering for cellulosic ethanol production (Olofsson *et al.*, 2008).



In gasification, the carbon in lignocellulosics is converted into carbon monoxide, carbon dioxide and hydrogen gas (syngas) by partial combustion. The syngas is then fermented into ethanol by *Clostridium ljungdahlii* or chemical catalysis followed by distillation (Badger, 2002).

### 2.12.4 Conversion of lignocellulose into bio-ethanol

Four biologically mediated steps occur in the course of converting lignocellulosic materials into bio-ethanol: (i) cellulase production, (ii) hydrolysis of the cellulose and other insoluble polysaccharides, (iii) fermentation of soluble cellulose hydrolysates and (iv) fermentation of soluble hemicellulose hydrolysates. There are four types of process configuration namely separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and cofermentation (SSCF) and consolidated bioprocessing (CBP) based on the level of consolidation between these steps. In SHF, four all the steps occur discretely. For SSF, three discrete steps are involved: hydrolysis and fermentation of cellulose hydrolysates are combined into a single step, and production of cellulase and fermentation of

hemicellulose hydrolysates occurring in two additional discrete steps. Simultaneous saccharification and cofermentation (SSCF) involves two process steps: production of cellulase and a second step in which hydrolysis and fermentation of both cellulose and hemicellulose hydrolysates. In consolidated bioprocessing (CBP), production of cellulase, hydrolysis, and fermentation of both cellulose and hemicellulose hydrolysates are accomplished in a single step (Lynd *et al.*, 2002).



SSF has comparative advantages over SHF which include increase in glucose yield, reduced cost of investment and avoidance of lost of sugars (Olofsson *et al.*, 2008).
Removal of glucose and cellobiose by the fermentation in SSF, reduces inhibition and increase the yield of sugars. The sugars are not separated from the lignin fraction as in SHF, and this prevents lost of sugar. The combination of hydrolysis and fermentation decreases the number of vessels needed and thereby reduces investment costs (Karimi *et al.*, 2006; Olofsson *et al.*, 2008).

The principal drawbacks are (1) the need to find favourable conditions (*e.g.* temperature and pH) for both the enzymatic hydrolysis and the fermentation and (2) the difficulty to recycle the fermenting organism and the enzymes (Olofsson *et al.*, 2008). To satisfy the former, the temperature is normally kept below 37°C, whereas the difficulty to recycle the yeast makes it beneficial to operate with a low yeast concentration and at a high solid loading (Karimi *et al.*, 2006; Olofsson *et al.*, 2008).

**2.12.5 Bio-ethanol market**

Fuel ethanol is the largest market for total ethanol production, accounting for 60%. The Freedonia Group has predicted that world demand for biofuels will expand nearly 20% a

year to 92 million tonnes in 2011(www.biofuel-news.com, 2008). The rapid expansion of the market is due to combination of factors such as politics, rising energy demand in transportation, rising oil prices, increasing concerns about global warming, and potential economic opportunity. The USA is the world's fastest-growing ethanol fuel market, representing a twofold increase in the last 4 years (Purwadi, 2006). Two important markets standing out for their potential impacts on fuel ethanol demand are the EU and China. In the EU recently, only a small fraction of ethanol is used as fuel and the demand is growing rapidly. The EU biofuel directive aims at replacing up to 5.75% of all petrol and diesel for transport purposes with biofuels by the year 2010 (Kallioinen *et al.*, 2008). Chinese automobile industries are fast-growing and hence it is quite certain that China will become a big player in the fuel ethanol market (Purwardi, 2006). Brazilian alcohol is the cheapest and more sustainable in the world, with a production cost of U$ 0.16-0.20 / L (Bon and Ferrara, 2007).



## CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1.1 Source of corncobs and soil samples

Corncobs were obtained from a local farmer at Bekwai and the soil was sampled from Ejura farms at Ejura in Ashanti Region.

### 3.1.2 Preparation of media

Preparation of Potato dextrose agar (PDA), Mandel's agar medium (MAM), and corncob based broth (CCB) media 1 and 2 were according to procedure described by Jeffries (1987).



**(a) Preparation of PDA**

One litre of quarter strength PDA was prepared by weighing 50 g of peeled, diced and washed Irish potato. The weighed quantity was boiled with 250 ml distilled water to tender, mashed with wooden spoon and strained through cheese cloth. The filtrate was added to 20 g of melted agar in a water bath and topped up with distilled water to one litre mark. Dextrose was omitted from the preparation. Exactly 500 mg of chloramphenicol was added to it and autoclaved at 121°C for 15 minutes.

**(b) Preparation of MAM**

MAM consisted of these chemicals per litre: $(NH_4)_2SO_4$ 1.4 g; $KH_2PO_4$ 2.04 g; $CaCl_2$ 0.3 g; $MgSO_4.7H_2O$ 0.3 g; Urea 2.1g; Citric acid 0.25 g; Tween 80 2 ml; Cellulose (Avicel) 10 g; Peptone1 g; and Trace metal stock solution, 1 ml.

MAM was prepared by dissolving the above chemicals except cellulose, peptone and trace metal stock in a litre volumetric flask containing about 750 ml distilled water on a hot plate/ magnetic stirrer. The rest was added after the former had dissolved.

The trace metal stock solution consisted of the following chemicals per 500 ml: $FeSO_4$ 2.55 g; $MnSO_4.H_2O$ 0.93 g; $ZnSO_4.H_20$ 1.78 g; $Co(NO_3)_2.6H_2O$ 1.25 g; and concentrated HCl 5 ml.

Trace metal stock solution was prepared by dissolving the above chemicals in 500 ml volumetric flask containing about 300 ml distilled water on a hot plate/ magnetic stirrer. The solution was topped to 500 ml when the reagent had completely dissolved.

**(c)Preparation CBB1**

Preparation of CBB was similar to MAM except that cellulose (Avicel) was substituted with corncob powder. Corncobs were sun dried and pounded. It was then oven dried at 120°C for 3 days after which it was milled, and sieved (Mesh series 70, Endecott filter) to obtain the powder. Some of this powder was used for other experiments.

CBB1 differs from CBB2 in that peptone was substituted with yeast extract.

**(d) Preparations of yeast extract peptone dextrose media (YPD)**

Two hundred and fifty milliliter of YPD was prepared by dissolving 2.5 g of yeast extract, 5 g peptone, and 5 g of glucose in 500 ml conical flask containing about 150 ml distilled water on hot pate/ magnetic stirrer. Exactly 5 g of agar (melted) was added and topped up to 250 ml mark and autoclaved at 121°C for 15 minutes.

Preparation of YP with 5% dextrose broth was similar to YPD except no agar was added.

### 3.2 Isolation of fungi

Soil samples were taken from 3 plots of Ejura farms. Ten samples each weighing 5 g were taken from each plot with auger and put into a sterile polythene bags. The samples were then bulked together. Ten grams of the bulked soil was weighed into a sterile 500 ml conical flask containing 100 ml of sterile distilled water and the suspension was shaken vigorously for about a minute. Different dilutions ($10^{-3}$ to $10^{-9}$) were made. One ml of each dilution was pour plated and incubated at 25°C for 4-5 days under asceptic conditions.

The different colonies observed aided by their appearances (pigmentations, textures, conformations and elevation) were subcultured on PDA incubated inverted at 25°C until pure cultures were obtained.

### 3.3 Identification of isolates

Burnett, *et al.* (1972), Malloch, (1997), Guarro *et al.*, (1999), www.doctorfungus.org, 2008; and www.mycologyonline.adelaide.edu.au, 2008 were employed for identification of the isolates. Morphological characteristics such as colony colour, texture, conformation, elevation, hyphal structure, conidial structure, condiophores structure etc were looked examined. Light microscope was employed to observe microscopic features. Distilled water was used as mounting media for slides preparation.

### 3.4 Screening of isolates for cellulase activity

About 1 $mm^2$ of mycelium of each isolate was inoculated onto MAM plate using inoculating needle under laminar flow and incubated at 25˚C for 6 days. Colony diameter and clearing zone diameter of the isolates colonies were measured on the third, fifth and sixth day after inoculation.



### 3.5 Production of cellulases by the cellulolytic isolates on CBB1

Hundred millilitres of CBB1 media was dispensed into each 250 ml conical flasks. The flasks were plugged with cotton wool and autoclaved at 121˚C for 15 minutes. Inoculum of each isolate was prepared by flooding a 4-days old slant of that isolate with 10 ml sterile distilled water. With the aid of flamed inoculating needle, conidia were scratched and the tubes were shaken vigorously to break the clumps of conidia and obtain homogenous suspension. The spores of each isolate were estimated with hyaemocytometer (Improved Neubauer Haemocytometer) count. Approximately $1\times10^7$ spores of each isolate were inoculated under laminar flow into a conical flask containing 100 ml of autoclaved CBB1. The inoculated conical flasks were incubated on shaker at 150 rpm at room temperature (28˚C) for 6 days. Five millilitres aliquots were taken from each inoculated media on third and sixth days. The aliquots were centrifuged at 10000g for 10 min at 4˚C, the supernatants decanted into new tubes and stored frozen for assay.

### 3.6 Preparation of citrate buffer

One litre of 1 M citrate buffer was prepared by dissolving 210 g citric acid monohydrate in 750 ml distilled water. NaOH pellets (50-60 g) were added until pH reached 4.3. The solution was topped up to 1litre with pH of 4.5. 50 mM citrate buffer was prepared by diluting the 1M buffer and pH adjusted to 4.8.

### 3.7 Dinitrosalicylic acid Assay



**(a) Preparation of DNS reagents**

DNS reagent was prepared as described by Wang, 2008. It consisted of two solutions, (i) 1% DNS reagent solution, and (ii) 40% potassium sodium tartrate (Rochelle salt) solution.

One percent of DNS reagent solution was prepared by dissolving 10 g of Dinitrosalicylic acid in a beaker containing about 500 ml distilled water on magnetic stirrer, after which 10 g NaOH was added gradually followed by 0.5 g sodium sulphite. The solution was then topped up to 1 litre.

Hundred millilitres of 40% potassium sodium tartrate (Rochelle salt) solution was prepared by dissolving 40 g of Rochelle salt in a beaker containing about 50 ml distilled water. The solution was then poured into 100 ml volumetric flask and topped up to the graduation mark. Rochelle salt solution was stored at -20°C until it was used. Phenol was omitted because it intensifies the coloured product.

**(b) Procedure**

Three millitres of DNS reagent was added to each assay tube. The contents of the tubes were boiled in vigorously boiling water bath containing sufficient water to cover the portions of the tubes occupied by reaction mixture plus reagent for 5 minutes. One ml

potassium sodium tartrate solution was then added to each tube and transferred to cold ice-water bath. Intensity of colour formation was determined by measuring absorbance at 540 nm with spectrophotometer. Amount of reducing sugars released was determined from glucose standard curve (glucose mg/0.5ml) against absorbance at 540 nm).

### 3.8 Assay for cellulase activity



Cellulase activities were determined by filter paper assay (FPA) and carboxymethyl cellulose assay (CMCA). The procedures for these assays were adopted from the studies of Jeffries, 1987; Ghose, 1987 and Adney and Baker, 1996. Whatman No.1 filter paper and carboxymethyl cellulose (Degree of Substitution = 0.7) were substrates in FPA and CMCA respectively. Reducing sugars released from these assays were quantified by DNS assay.

**(a) Filte paper assay**

The assay consisted of (i) glucose standards, (ii) reagent blank, (iii) controls and (iv) assay mixtures.

(i) Glucose standards: a working glucose stock solution (10 mg/ml) was prepared by dissolving 1 g of anhydrous glucose in distilled water in 100 ml volumetric flask and topped up to the graduation mark. The stock was tightly sealed and stored at -20°C. The stock was thawed and vortexed prior to use. Dilutions were prepared from the stock in the following manner:

1.0 ml + 0.5 ml buffer = 1:1.5 = 6.7 mg/ml (3.35 mg/0.5 ml).

1.0 ml + 1.0 ml buffer = 1:2 = 5 mg/ml (2.5 mg/0.5 ml).

1.0 ml + 2.0 ml buffer = 1:3 = 3.3 mg/ml (1.65 mg/0.5 ml).

1.0 ml + 4.0 ml buffer = 1:5 = 2 mg/ml (1.0 mg/0.5 ml).

Half of a millilitre was of each of the above dilutions was added to 1 ml of 50 mM citrate buffer in each test tube.

(ii) Reagent blank consisted of 1.5 ml of 50 mM citrate buffer.

(iii) Controls consisted of enzyme controls and substrate control. Enzyme controls consisted of 1.0 ml of 50 mM citrate buffer and 0.5 ml of isolate filtrate.

(iv) assay mixtures: a rolled 1.0 x 6.0 cm filter paper strip was placed in each test tube and 1 ml of 50 mM citrate buffer pH 4.8 added to saturate the strips. The substrates were equilibrated at 50°C and 0.5 ml of each isolate filtrate was added to a substrate.

The glucose standards, blanks, controls and assay mixtures were incubated at 50°C for exactly 60 minutes and stopped by addition of 3 ml of DNS reagent. Amount of glucose released was determined by DNS method.

FP activity as estimated as: Filter paper unit, FPU = mg glucose released ×0.185.

**(b) Carboxymethyl cellulose assay**

The assay consists of (i) glucose standards, (ii) reagent blanks and (iii) assay mixtures.

(i) glucose standards: glucose stock solution (2 mg/ml) was prepared by dissolving 0.2 g anhydrous glucose in distilled water and poured into 100 ml volumetric flask and topped up to the graduation mark. Aliquots of 5 ml were stored at -20°C. Aliquot was thawed and vortexed before used. Dilutions were prepared from the stock in the following manner:

Undiluted = 2.0 mg/ml (1.0 mg/0.5 ml)

1.0 ml + 0.5 ml buffer = 1: 1.5 = 1.33 mg/ml (0.67 mg/0.5 ml)

1.0 ml + 1.0 ml buffer = 1: 2 = 1.0 mg/ml (0.5 mg/0.5 ml)

1.0 ml + 3.0 ml buffer = 1: 4 = 0.5 mg/ml (0.25 mg/0.5 ml)

Half of a millilitre of the substrate solution was placed in each test tube and incubated at 50°C for 30 minutes after which 3.0 ml DNS reagent was added. Half of a millilitre of each standard solution was then added.

(ii) Reagent blank consisted of 0.5 ml of substrate solution was placed in a test tube. Enzyme blank consisted of 0.5 ml of filtrate of each isolate in a test tube.

(iii) Assay mixtures: Two percent carboxymethyl cellulose solution was prepared by dissolving 10 g of carboxymethyl cellulose powder (degree of substitution = 0.7) in 300 ml of 50 mM citrate buffer in 500 ml conical flask on hot plate/ magnetic stirrer. After it had dissolved, the viscous mixture was topped up to 500 ml and stored at -20°C.

Test tubes containing 0.5 ml of each isolate filtrate was equilibrated at 50°C. Half of a millilitre of the substrate solution was added to each isolate filtrate and incubated at 50°C for 30 minutes after which 3.0 ml DNS reagent was added. Amount of glucose released was determined by DNS method.

CMC activity as estimated as: CMC Unit = mg of glucose released × 0.37.

### 3.9 Estimation of total filtrate protein concentration by Biuret method

#### (a) Preparation of Biuret reagent

Biuret reagent contains the following chemicals per litre: $CuSO_4.5H_2O$ 1.5 g; potassium sodium tartrate 6.0 g; and NaOH 30 g. $CuSO_4.5H_2O$ and potassium sodium tartrate

were put in a volumetric flask and 500 ml distilled water added to dissolve the chemicals with the aid of a magnetic stirrer. While stirring the flask content vigorously, 300 ml of 10% (w/v) NaOH solution was added gradually. The stirring bar was removed and the solution was topped to the litre mark with distilled water. The reagent was transferred into a plastic bottle and stored at room temperature.



**(b) Procedure**

Biuret assay consists of (i) egg albumin standards, (ii) reagent blank and (iii) assay mixture.

(i) Egg albumin standards: egg albumin stock solution (10 mg/ml) was prepared by dissolving 1 g egg albumin in distilled water in 100 ml volumetric flask. The solution was topped up to the 100 ml graduation mark. Dilutions were prepared from the stock in the following manner:

Undiluted = 10 mg/ml

0.7 ml stock solution + 0.3 distilled water = 7 mg/ml

0.4 ml stock solution + 0.6 distilled water = 4 mg/ml

0.2 ml stock solution + 0.8 distilled water = 2 mg/ml

One millilitre of the undiluted stock and each of the above dilutions were dispensed into test tubes.

(ii) Reagent blank consisted of 1 ml distilled water

(iii) For the assay mixtures, 1 ml of each isolate filtrate was dispensed into a test tube.

Four millilitres of Biuret reagent was dispensed into each tube (standards, reagent blank and assay mixtures) and swirled to ensure uniform mixing. All the tubes were incubated at 37°C for 20 minutes after which their absorbances were determined with spectrophotometer at 540 nm. The spectrophotometer was zeroed with the reagent blank before all the other readings were carried out.

Standard graph was obtained by plotting the concentrations of the egg albumin stock solutions against their absorbance at 540 nm. The total protein present in a millilitre of each isolate filtrate was determined from the graph.



### 3.10 Cellulase production by *Aspergillus niger* on CBB2

Two hundred and fifty millilitres of CBB2 was inoculated with 3.02 $\times 10^7$ spores and incubated on shaker at 120 rpm at room temperature for 6 days period. The broth was centrifuged at 10000g for 10 minutes and the supernatant was used as crude enzyme in saccharification tests. To determine enzyme activity over the period, 5 ml aliquots were sampled at 24 hours interval.

### 3.11 Saccharification tests

Method was adopted from Baig *et al.*, (2004). One percent corn cob suspension was prepared by suspending 10 g of the substrate in 1 litre 50 mM citrate buffer (pH 5.5). The suspension was autoclaved at 121°C for 20 min for sterilization. Exactly 15 ml of the substrate suspension was poured into 100-ml Erlenmeyer flasks. Five ml of culture filtrate obtained from *A. niger* was added to the suspension in each flask.

Saccharification was performed in a water bath shaker at 27±2°C for 24 h. The resultant supernatants following centrifugation (2500 g, 15 min) were assayed for total reducing sugars using DNS method. The sugars released after subtracting controls is expressed as equivalent to glucose. To determine the optimum temperature of saccharification, the reaction mixture was incubated at different temperature ranging from 25°C to 60°C.

The optimum pH was determined by adjusting the pH of the reaction mixture within the range of 3.5 to 6.5. The optimum substrate concentration was determined by preparing substrate suspensions ranging from 1 to 6 percents.



### 3.12.1 Preparation of *Saccharomyces cerevisiae* inoculum

The procedures were adopted from Scholar and Benedikte (1999) and Suh *et al.*, (2007). One gram of dry baker's yeast purchased from Kumasi central market was suspended in 10 ml of sterile distilled water to form slurry. A loop full of the slurry was streaked on Yeast Peptone Dextrose (YPD) agar plate and incubated at 30˚C for 48 hours to activate the yeast and check for contaminates. A loopful of the yeast colony was transferred from the agar plate into 100 ml of 5% YPD broth and incubated at room temperature on a shaker at 130 rpm for 48 hours. Seven millilitres of the broth (from calculation) was centrifuged at 4500 rpm for 5 minutes. The supernatant was decanted, resuspended in sterile distilled water twice, centrifuged and the supernatant decanted. The precipitant was resuspended in $1/10^{th}$ of 50 mM citrate buffer working for each flask.

### 3.12.2 Shake flask simultaneous saccharification and fermentation

The procedures were adopted from Dowe and McMillian (2008). Fermentation was carried out in 250 ml Erlenmeyer flasks. The fermentation lock or bubble trap consisted of rubber stopper (with two holes) through which a glass tube is inserted. A cotton plug was inserted in the tube and the tube connected to silicone tubing. The other end was submerged in a test tube containing water.

Each flask was loaded with 6.0 g of the substrate, 1.0 g yeast extract and 2.0 g peptone and mixed with 50 mM citrate buffer, pH 5.0 up to 80 or 85 ml. The flasks were plugged with the rubber stoppers in which a rubber tube and glass tube plugged with cotton wool were inserted. The mouths of the flasks were wrapped with aluminum foil. The rest of the fermentation lock device was also wrapped with aluminium foil and they were autoclaved together with the flasks at 121˚C for 20 minutes. After cooling to room temperature, 5 ml and 10 ml of the *A. niger* culture supernatant were introduced into flask A and B respectively through the rubber tube with sterile 5 ml syringe. The 10 ml yeast inoculum was also introduced into each flask through the rubber tube. Sterile syringe was inserted into each rubber tube and the rest of fermentation lock devices were fixed to the glass tubes. The flasks were incubated at room temperature on shaker at 110 rpm for 24 hours. Five millitres aliquots were drawn from the flasks at interval with inserted sterile syringes. The syringes were disposed off after every sampling. The aliquots were centrifuged at 10000g for 10 minutes and the supernatant kept frozen for gas chromatography analysis for ethanol.

### 3.12.3 Determination of ethanol concentration by gas chromatography

Ethanol concentration was determined using a Perkin Elmer, Autosystem XL, Gas Chromatograph (USA) equipped with a flame ionization detector (FID), coupled to a Yokogawa 3021 Pen recorder. A chromopak K 80/100 CRS column was used. The flow rate of the carrier gas, $N_2$, was 42 ml/min. $H_2$ and air were the fuel used. The oven temperature, injector temperature and detector temperature were 130˚C, 200˚C and 200˚C respectively. The injected volume was 1 µl and the retention time was 8.5 minutes. Identification and quantification were based on direct comparison of the gas chromatogram response to ethanol standards.



# CHAPTER FOUR

## 4.0 RESULTS AND DISCUSSION

### 4.1 Results

#### 4.1.1 Isolated species

Twelve species from 8 different genera were isolated from the soil. These were *Aspergillus terreus*, *Trichoderma sp. Aspergillus versicolor*, *Penicillium sp.9*, *Mucor sp. Aspergillus flavus*, *Aspergillus niger*, *Fusarium sp. Penicillium sp. 7*, *Rhodotorula sp. Acremonium sp.* and *Coccidiodes sp.* The morphological features and pictures of the isolates are below.

##### 4.1.1.1 *Aspergillus terreus*

*A terreus* formed chocolate sand brown colonies on PDA as shown on Plate 4.1. Conidiophores were upright, simple terminating in compact, columnar and biseriate conidial heads. Conidiophores were hyaline and smooth-walled. Conidia were globose, hyaline and smooth-walled.




**Plate 4.1: *Aspergillus terreus* colony on Mandel's agar.**

#### 4.1.1.2 *Aspergillus niger*

*Aspergillus niger* mycelia formed dark-brown to black, powdery colonies on PDA as shown in Plate 4.2. Conidiophores were upright and simple terminating in dark-brown to black vesicle. Conidiophores were smooth-walled and hyaline turning dark towards the vesicle. Conidial heads were large, dark-brown to black in colour, radiate and biseriate with the phialides borne on brown metulae. Conidia were globose, dark brown to black and rough-walled.

**Plate 4.2: *Aspergillus niger* colony on PDA.**

#### 4.1.1.3 *Aspergillus flavus*

*Aspergillus flavus* colonies were granular, flat and green on PDA as shown in Plate 4.3. Conidiophores were hyaline and coarse. Conidiophores were simple and upright terminating in globose vesicle. Conidial heads were radiates and biseriate. Some bear loosely formed columns. Conidia were globose and pale green in colour.


**Plate 4.3: *Aspergillus flavus* colony on Mandel's agar and PDA.**

#### 4.1.1.4 *Aspergillus versicolor*

*A. versicolor* formed granular and white colony which turned tan with age. Plate 4.4 shows *A. versicolor* colony on PDA 4.4. Conidiophores were simple and upright terminating in globose swelling. Conidiophores were hyaline and smooth walled. Conidial heads were large, round, radiate and biseriate. Conidia were hyaline and globose.

**Plate 4.4: *Aspergillus versicolor* colony on PDA.**



#### 4.1.1.5 *Penicillum sp.* 7

*Penicillum sp.* mycelia formed flat, bright green patch with white margins after 4-5 days on PDA as shown in Plate 4.5. The reverse of culture plates was purple to red. Mycelia produced red rapidly diffusing pigments when in culture in different fungal species. Conidiophores arose from mycelium singly and branched near the apex, into penicillates ending in phialides. Conidia were hyaline, 1-celled, and globose and were in dry basipetal chains.

**Plate 4.5: *Penicillium sp.* 7 colony on PDA.**

#### 4.1.1.6 *Penicillium sp.* 9

*Penicillium sp.* 9 colonies were tufty and had lime to teal colour on PDA as shown in Plate 4.6. Conidiophores arose from mycelium singly and branch near the apex into penicillates ending in phialides. Conidia were hyaline, single-celled, globose and in dry basipetal chains.

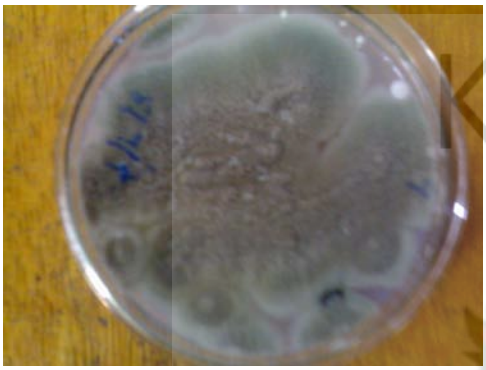


**Plate 4.6: *Penicillium sp.* 9 colony on PDA**

#### 4.1.1.7 *Fusarium sp.*

*Fusarium sp.* colonies were cottony and had tan colour on PDA as shown in Plate 4.7. Conidiophores varied from slender and simple to stout and short, branched irregularly to bearing a whorl of phialide. Conidia were hyaline and often held in small moist head. Microconidia were single-celled, ovoid and were borne singly or in chains.

Macroconidia were hyaline, two- to several-celled, sickle-shaped, mostly with an elongated apical cell and pedicellate basal cell.

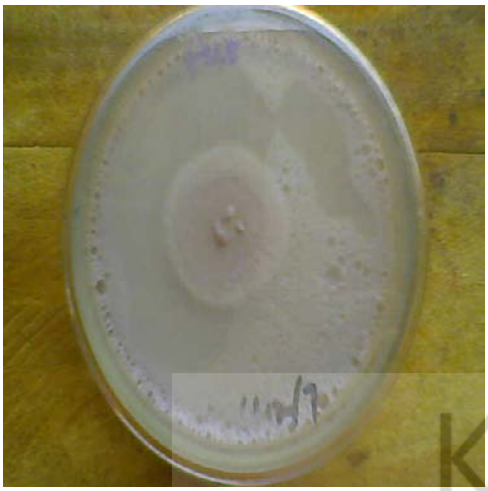
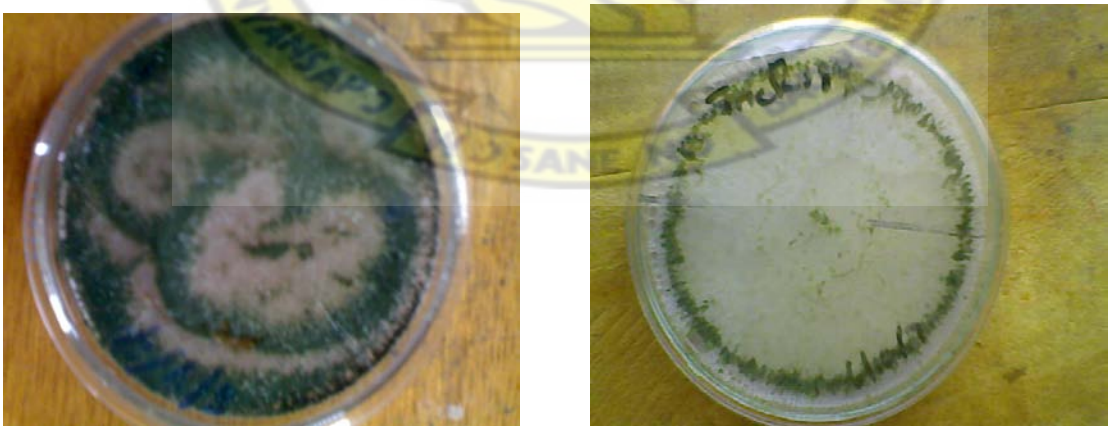


**Plate 4.7: *Fusarium sp.* colony on Mandel's agar.**

#### 4.1.1.8 *Trichoderma sp.*

*Trichoderma sp.* colonies were fast growing, white at early stage and later developing into deep green compact tufts in concentric ring-like zones on the PDA as shown in Plate 4.8. 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.



(a) colony on PDA (b) colony on Mandel's agar

**Plate 4.8: *Trichoderma sp.* colonies on PDA and Mandel's agar.**

#### 4.1.1.9 *Mucor sp.*

*Mucor sp.* colonies were very fast growing. Their mycelia formed white, cottony colonies becoming dark grey with the development of sporangia as shown in plate 4.9. Sporangiophores were erect, simple or branched, forming large terminal, globose to spherical, multi-spored sporangia, without apophyses and with well developed subtending columellae. A conspicuous collarette (remnants of the sporangial wall) was usually seen at the base of the columella, a sign of sporangiospore dispersal. Sporangiospores were hyaline, grey or brownish, globose to ellipsoidal and smooth-walled or finely ornamented. Stolons and rhizoids were absent.

**Plate 4.9: *Mucor sp.* colony on PDA.**

#### 4.1.1.10 *Rhodotorula sp.*

*Rhodotorula sp.* colonies were rose coloured, smooth and had moist to mucoid yeast-like appearance as shown in Plate 4.10. Microscopically, it consists of spherical to elongated budding yeast-like cells.

**Plate 4.10: *Rhodotorula sp.* colony on Mandel's agar.**



#### 4.1.1.11 *Coccidiodes sp.*

*Coccidiodes spp.* Colonies had white, wooly and moist appearance on PDA as shown in Plate 4.11.

Hyphae were hyaline, septate and thin. Arthroconidia were present.

**Plate 4.11: *Coccidioides sp.* colony on PDA.**

#### 4.1.1.12 *Acremonium sp.*

*Acremonium spp.* colonies were compact, flat or folded, glabrous and occasionally umbonate as shown in Plate 4.12. Hyphae were hyaline, septate, fine and narrow.

Unbranched, solitary, erect phialides are formed directly on the hyphal tips. The phialides were separated from hyphae by a septum and taper towards their apices. Conidia were borne at the apices of the phialides. The conidia were hyaline, single-celled or multicellular, fusiform with a slight curve.

**Plate 4.12: *Acremonium sp.* colony on PDA.**

**Table 4.1: Number of species isolated in each genus**

| Genera | No. of species |
|---|---|
| *Aspergillus* | 4 |
| *Trichoderma* | 1 |
| *Penicillium* | 2 |
| *Mucor* | 1 |
| *Fusarium* | 1 |
| *Rhodotorula* | 1 |
| *Acremonium* | 1 |
| *Coccidiodes* | 1 |

**Table 4.2: Mean mycelial diameter of the isolates on MAM over 6 days period.**

| Isolates | Mean diameter at 3 days (cm) | Mean diameter for 6 days (cm) |
|---|---|---|
| *A. terreus* | 3.4[b] | 4.22[b] |
| *Trichoderma* | 7.26[a] | 8.31[a] |
| *A. versicolor* | 1.50[d] | 2.13[d] |
| *Penicillium sp.9* | 2.50[c] | 3.00[c] |
| *Mucor sp.* | 1.23[d] | 0.41[f]* |
| *A. flavus* | 3.5[b] | 4.36[b] |
| *A. niger* | 2.4[c] | 3.93[b] |
| *Fusarium sp* | 1.57[d] | 2.49[cd] |
| *Penicillium sp.7* | 1.30[d] | 2.220[d] |
| *Rhodotorula sp.* | 0.13[e] | 0.21[f] |
| *Acremonium sp.* | 1.10[d] | 1.18[e] |

The alphabets represent the order of the means within a column which are significantly different at $P = 5\%$ by Duncan's Multiple Range Test (DMRT). Means in a column with different superscripts are significantly different.

* Measurement was not taken after 3 days

**Table 4.3: Clearing zone to mycelial diameter ratios for the isolated fungal species**

| Isolates | Ratio at 3 days (cm) | Ratio at 6 days (cm) |
|---|---|---|
| *A. terreus* | 1.16$^{bc}$ | 1.28$^{ab}$ |
| *Trichoderma* | 1.18$^{bc}$ | 1.08$^{cd}$ |
| *A. versicolor* | 1.09$^{bc}$ | 1.12$^{bc}$ |
| *Penicillium sp.9* | 1.11$^{bc}$ | 1.00$^{d}$ |
| *Mucor sp.* | 1.97$^{a}$ | 1.32$^{ab*}$ |
| *A. flavus* | 1.34$^{bc}$ | 1.33$^{ab}$ |
| *A. niger* | 1.43$^{b}$ | 1.47$^{a}$ |
| *Fusarium sp* | 1.39$^{b}$ | 1.2$^{ab}$ |
| *Penicillium sp.7* | 1.09$^{bc}$ | 1.14b$^{c}$ |
| *Rhodotorula sp.* | 1.00$^{c}$ | 1.00$^{d}$ |
| *Acremonium sp.* | 1.09$^{bc}$ | 1.10$^{cd}$ |

The alphabets represent the order of the means within a column which are significantly different at $P = 5\%$ by Duncan's Multiple Range Test (DMRT). Means in a column with different superscripts are significantly different.

* Measurement was not taken after 3 days

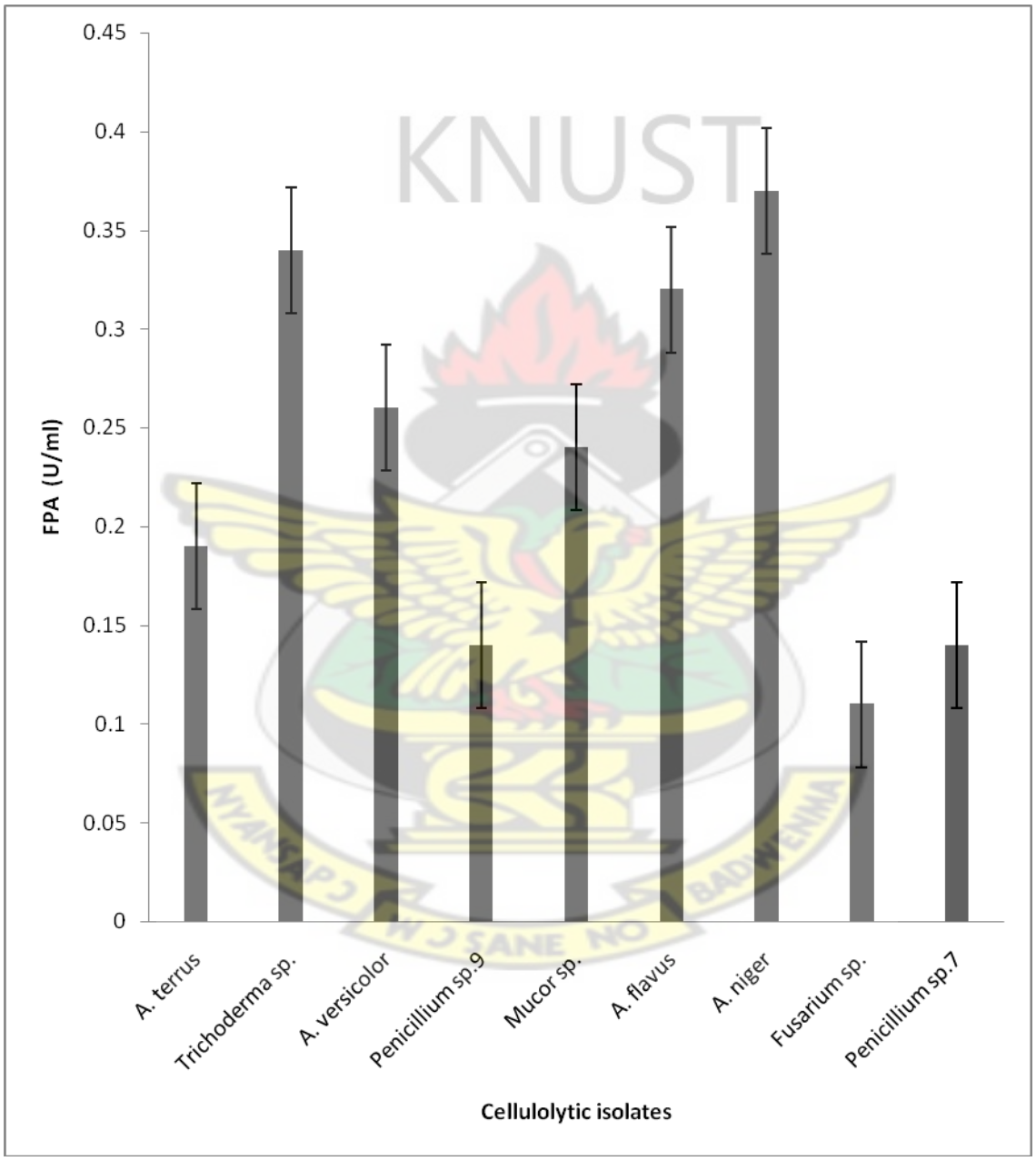

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
FPA (U/ml)
A. terrus
Trichoderma sp.
A. versicolor
Penicillium sp.9
Mucor sp.
A. flavus
A. niger
Fusarium sp.
Penicillium sp.7
Cellulolytic isolates

**Figure 4.1: Mean FPA (U/ml) of the cellulolytic isolates in CBB1 over 6 days period.**

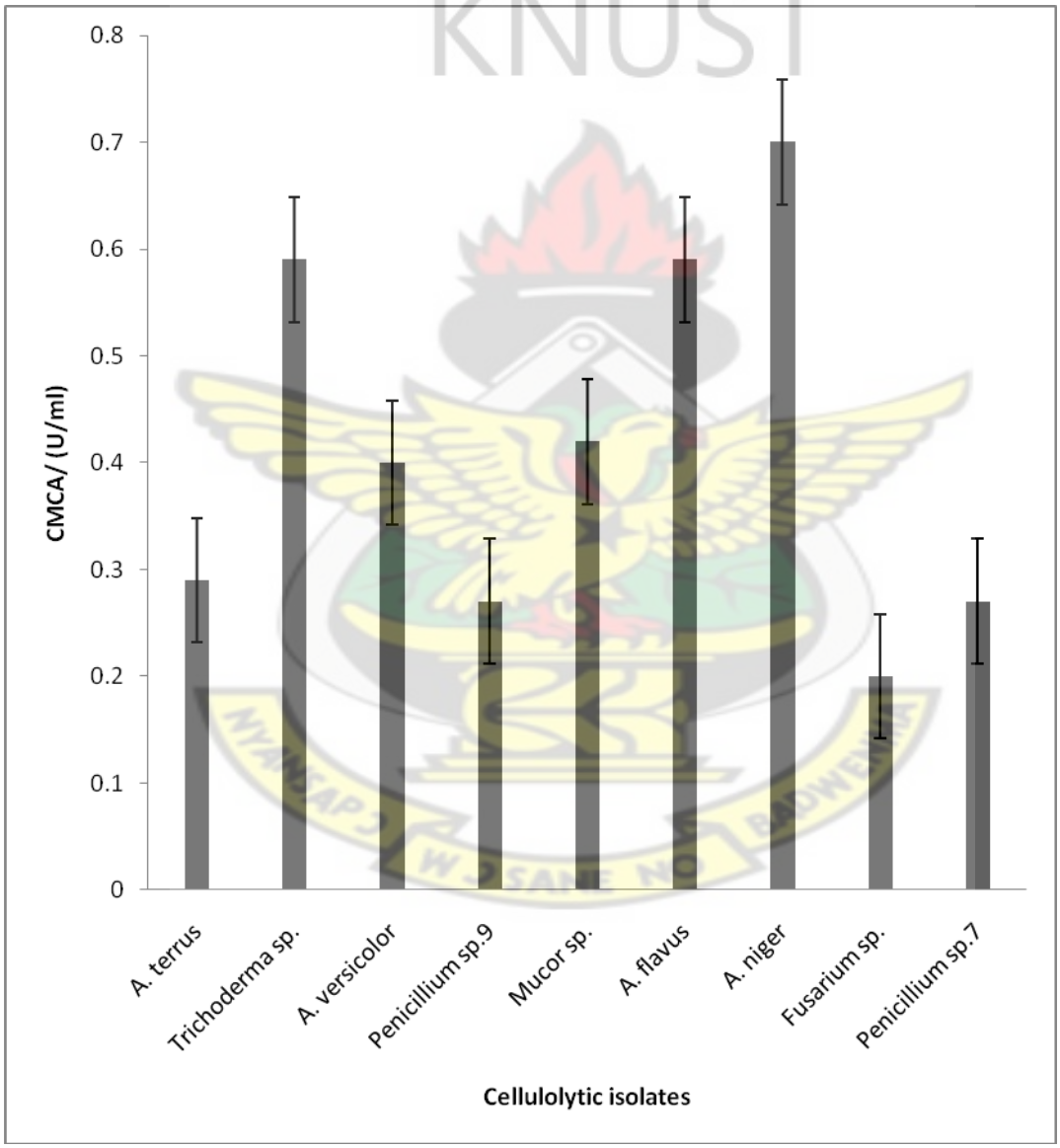


**Figure 4.2: Mean CMC activity (U/ml) of cellulolytic isolates in CBB1 over 6 days period.**



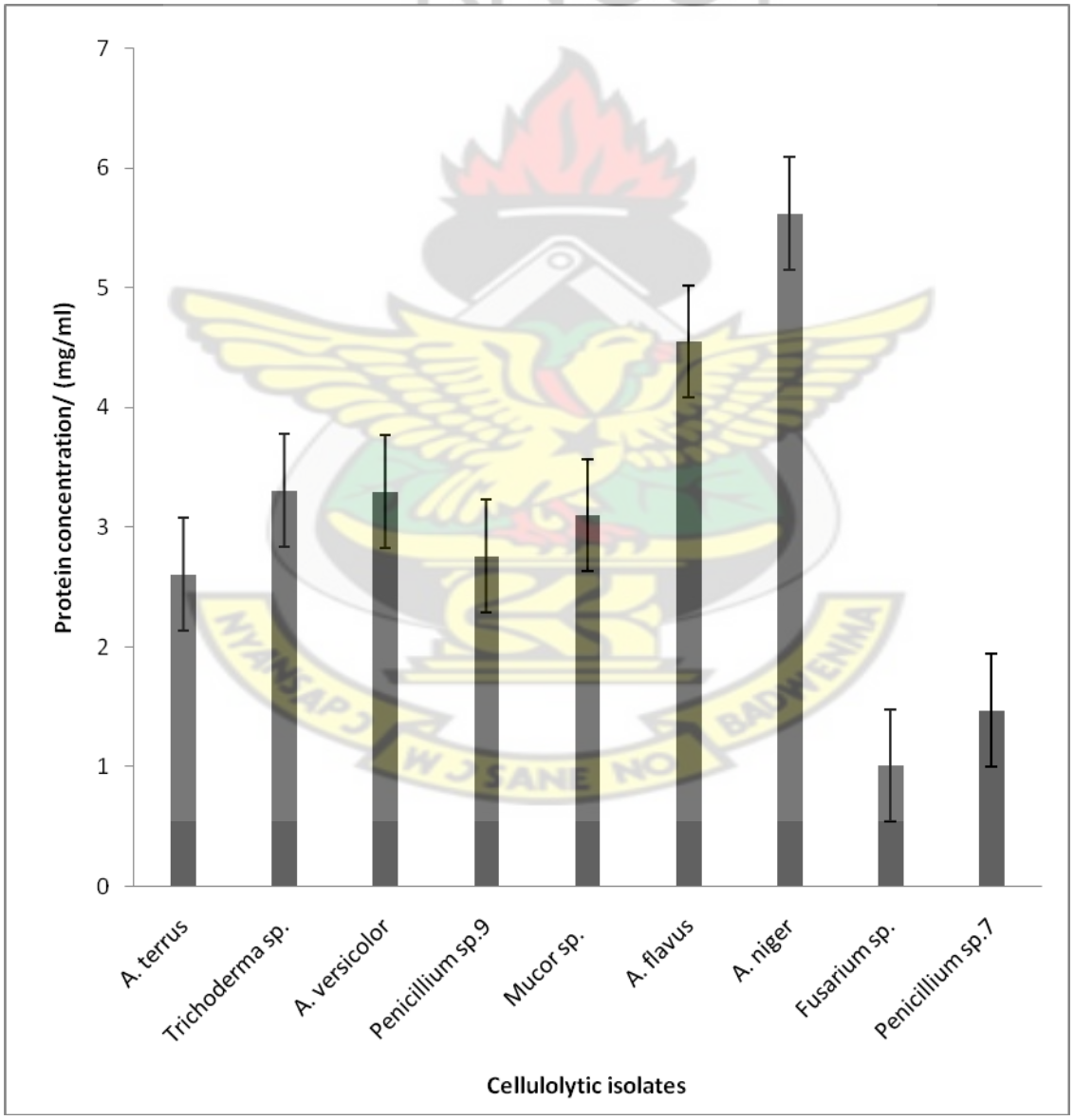


**Figure 4.3: Mean protein concentration (mg/ml) of the cellulolytic isolates in CBB1 over 6 days period.**

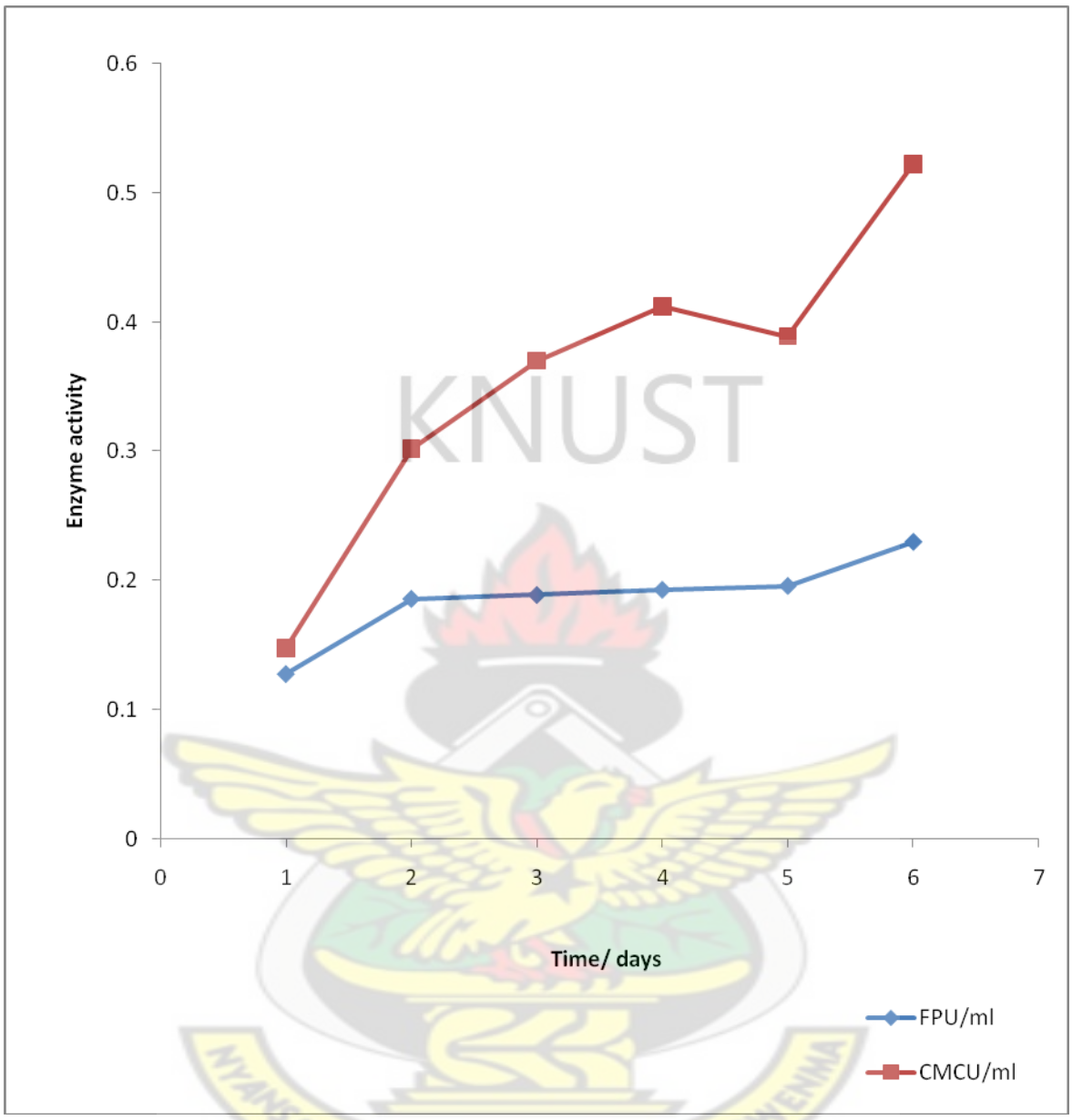



**Figure 4.4: Enzyme activity of *Aspergillus niger* in CBB2 over 6 days period.**

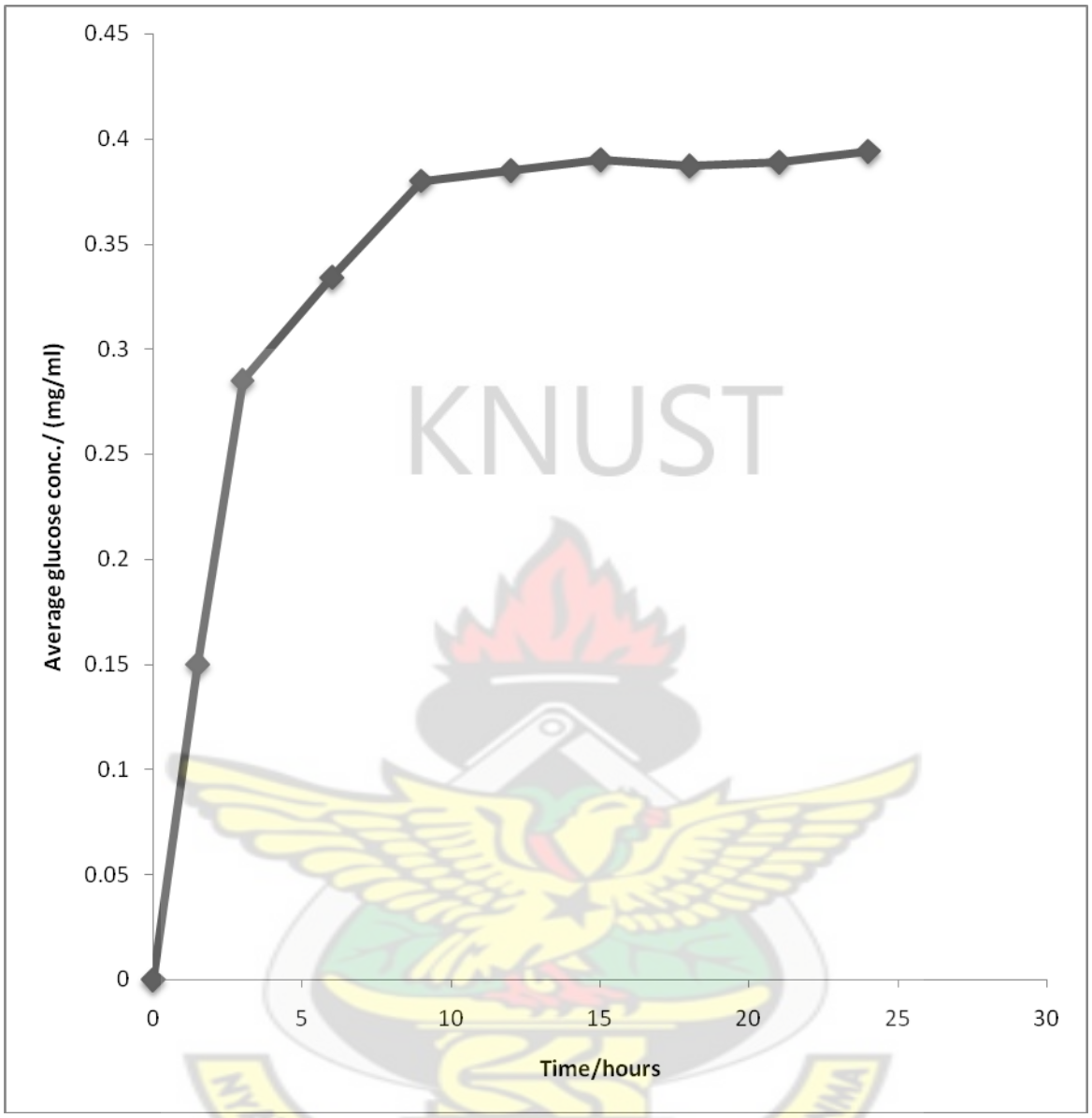



**Figure 4.5: The rate of saccharification of corncobs powder at room temperature for 24 h period.**

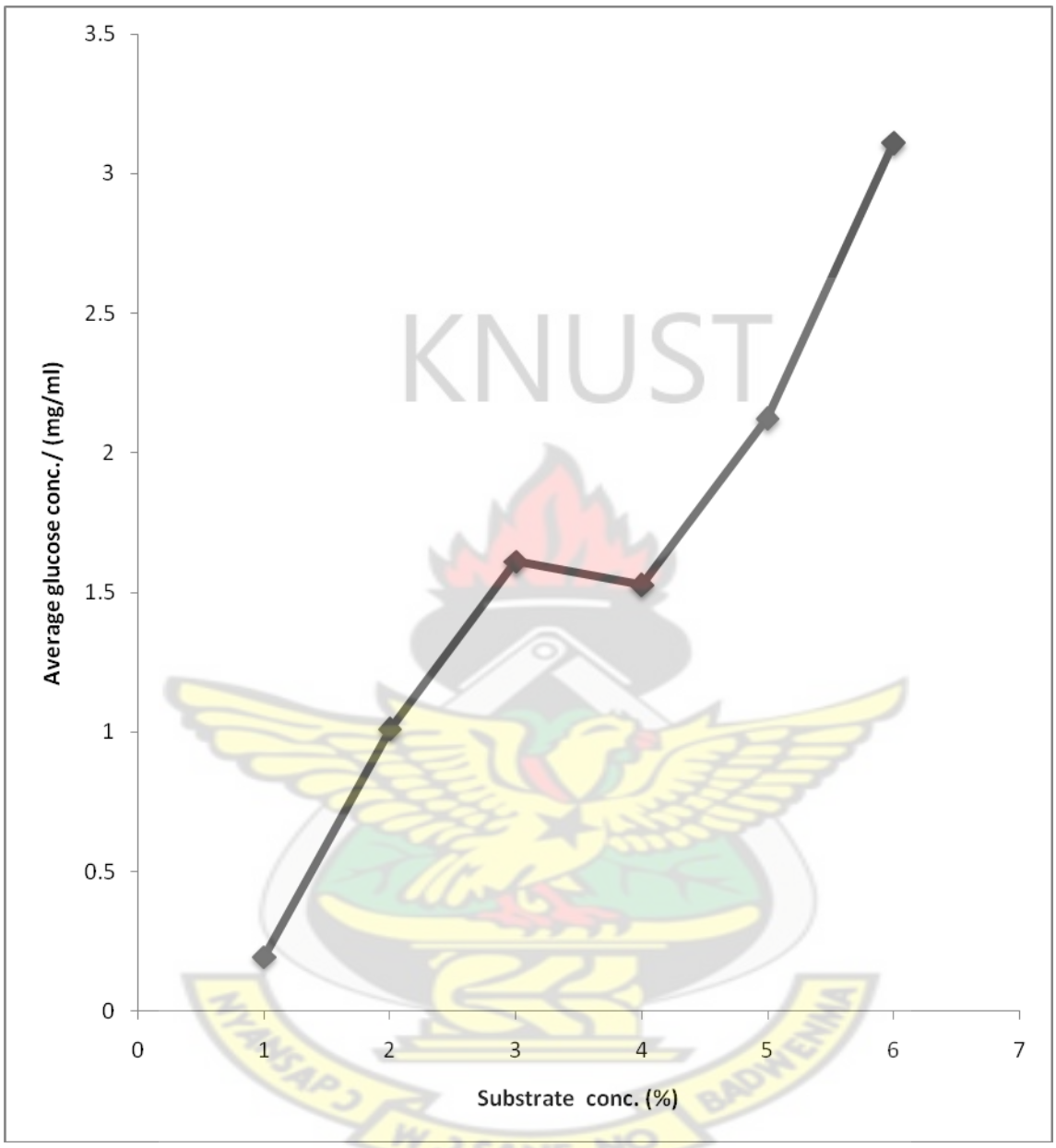



**Figure 4.6: Effect of substrate concentration on glucose concentration over 24 h period.**

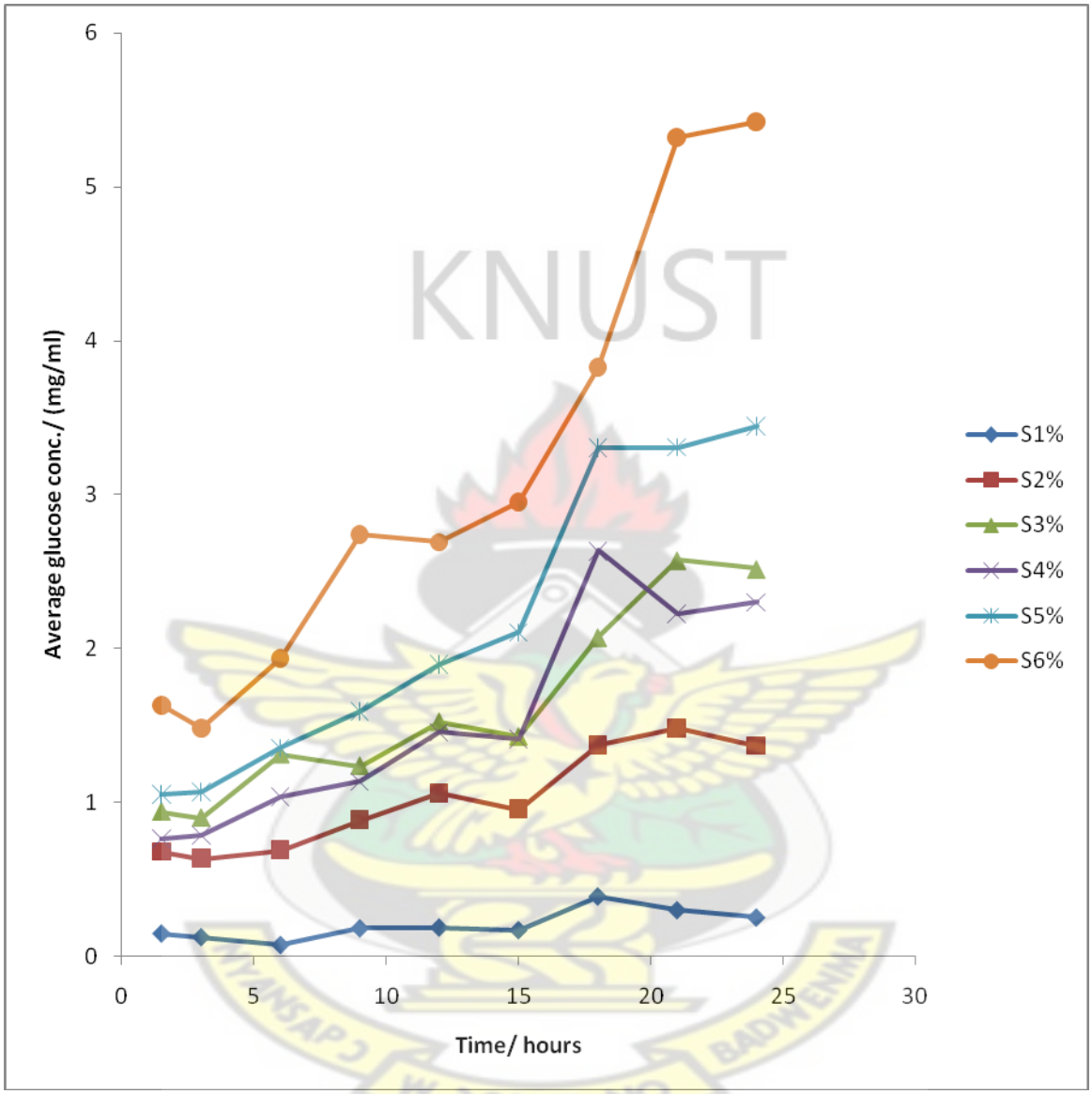



**Figure 4.7: Influence of substrate concentration on saccharification over 24 h period at room temperature.**

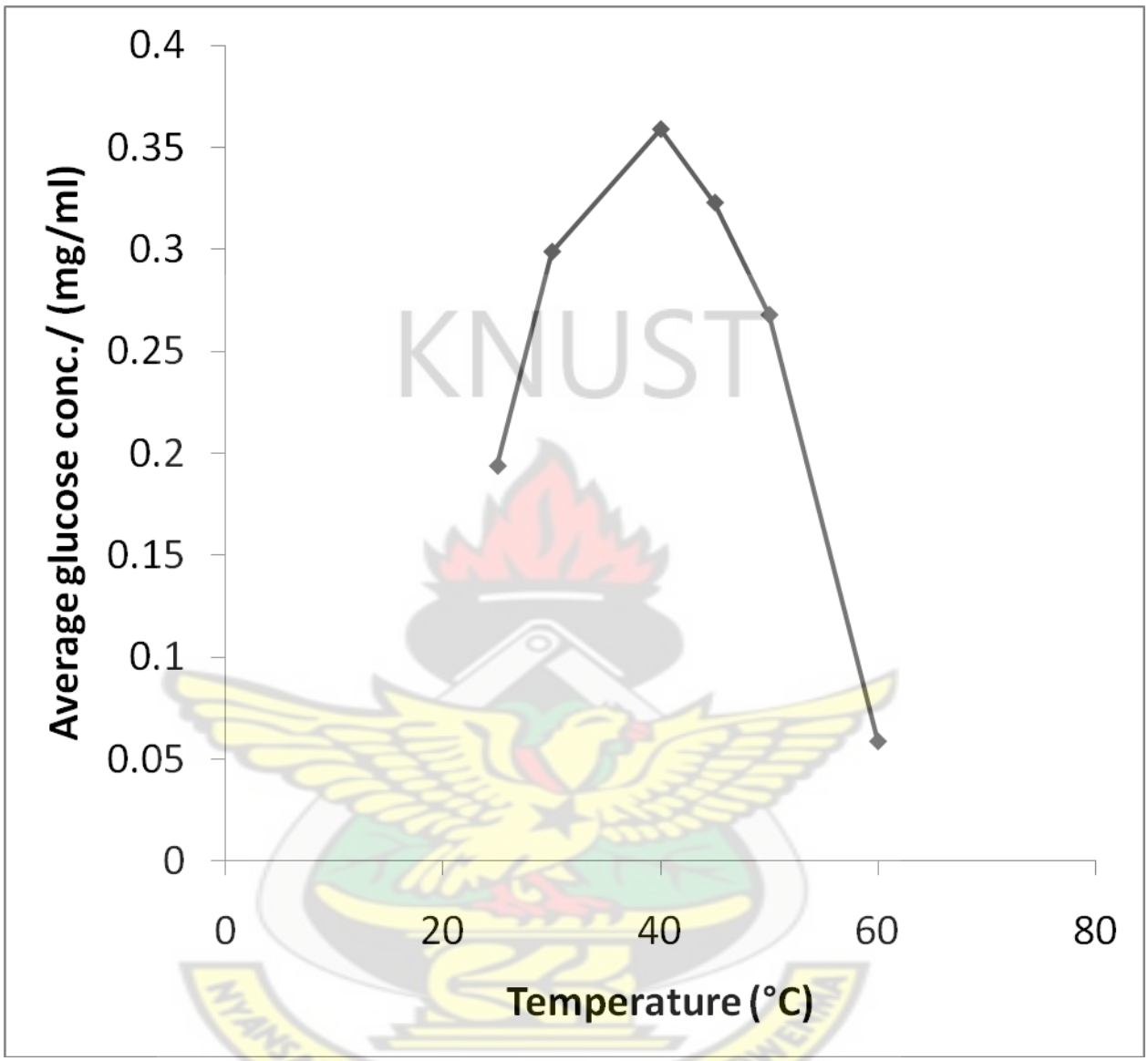



**Fig 4.8: Effect of temperature on glucose concentration over 24 h period.**

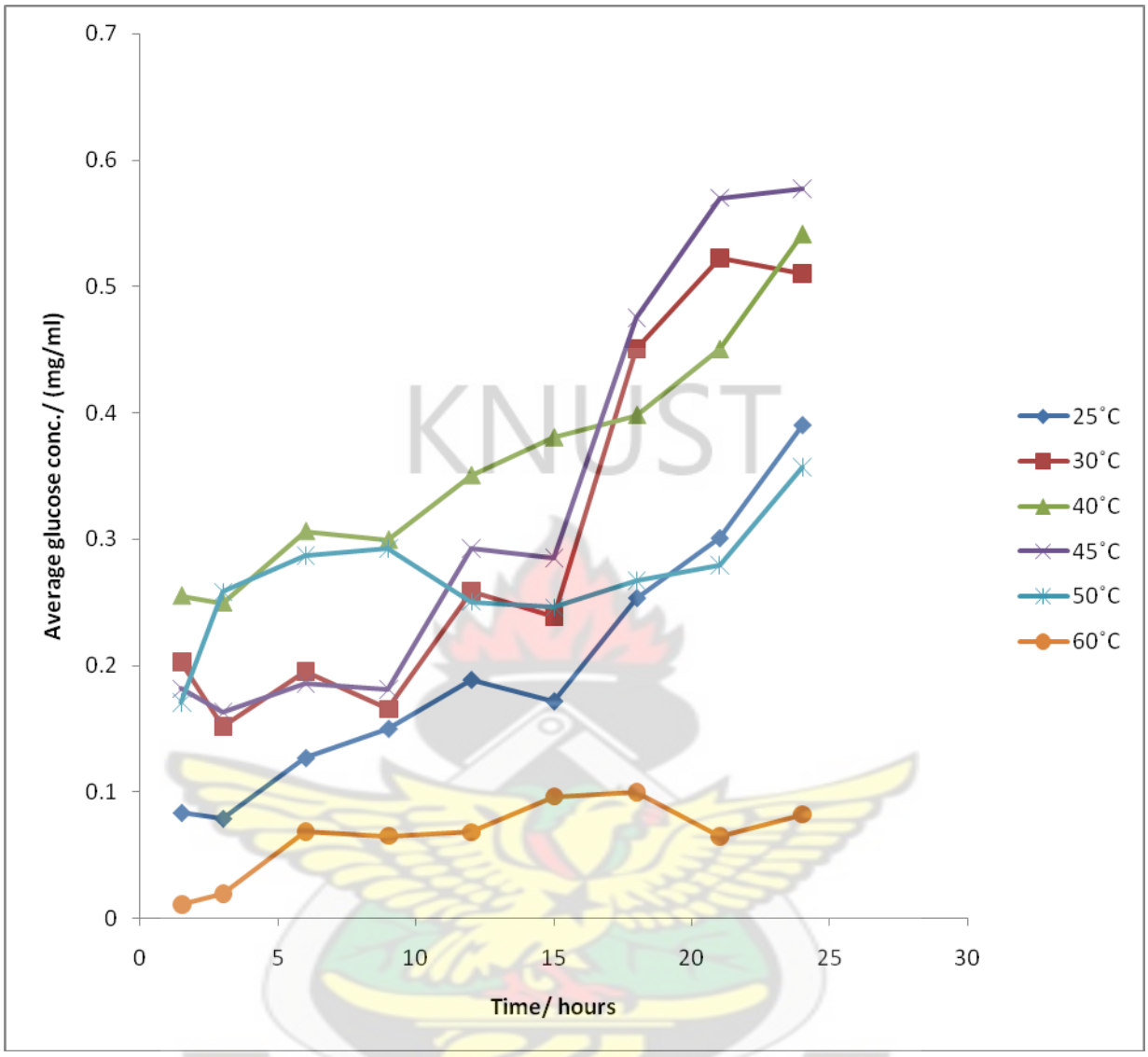



**Fig 4.9: Influence of temperature on saccharification of corncobs powder over 24 h period.**

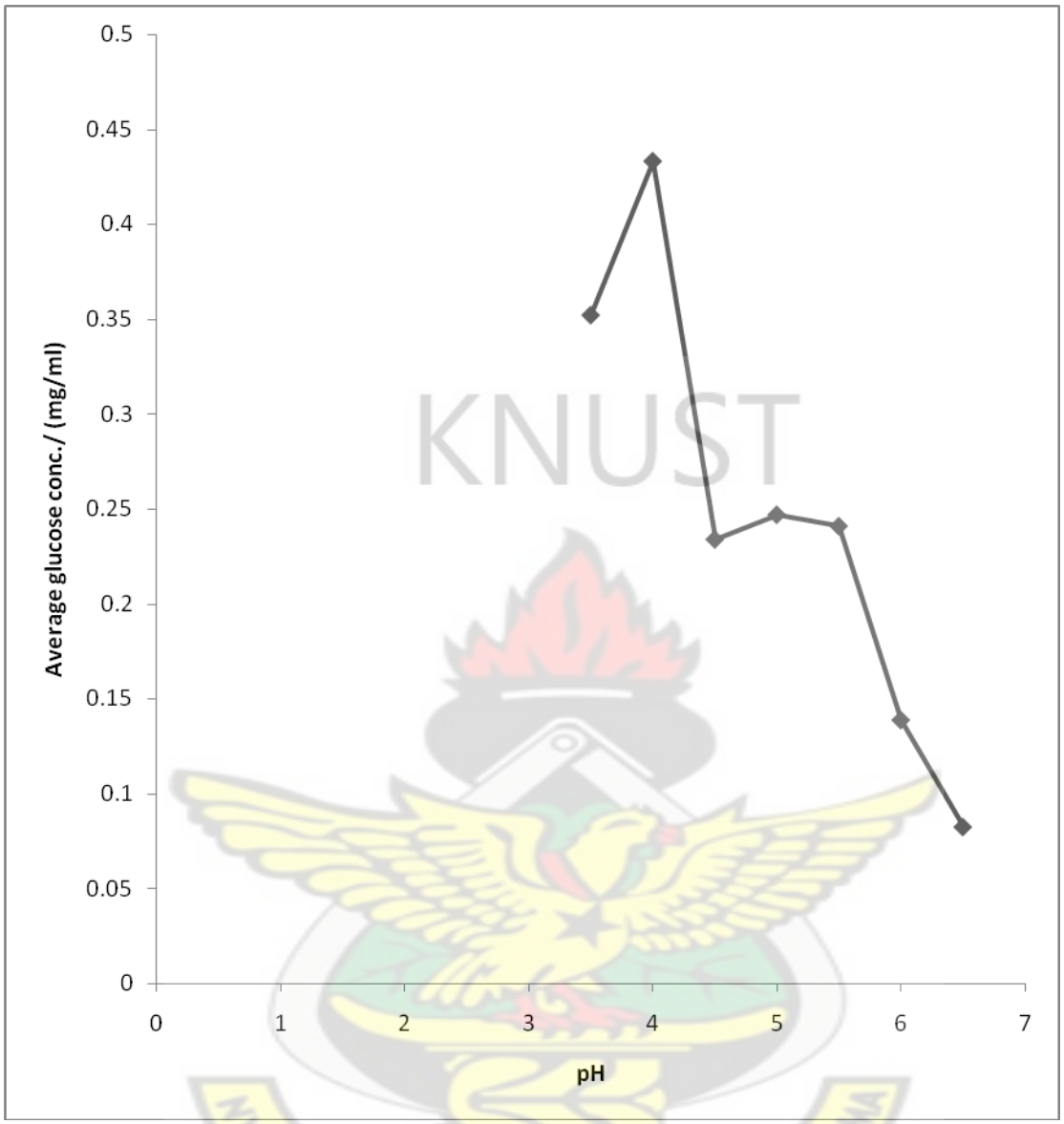



**Fig 4.10: Effect of pH on glucose concentration at room temperature over 24 h period.**

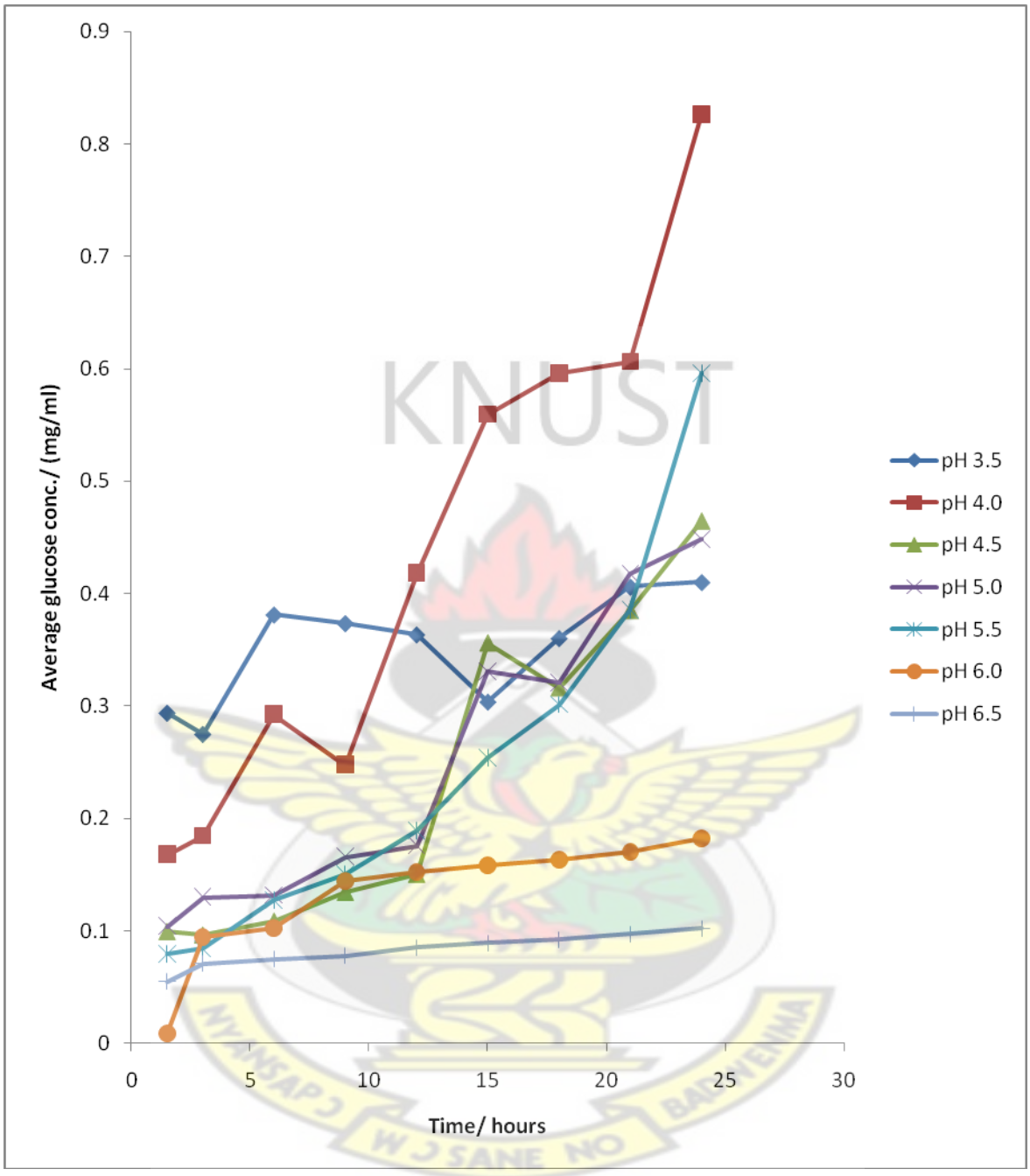



**Fig 4.11: Influence of pH on saccharification of corncobs powder at room temperature over 24 h period.**

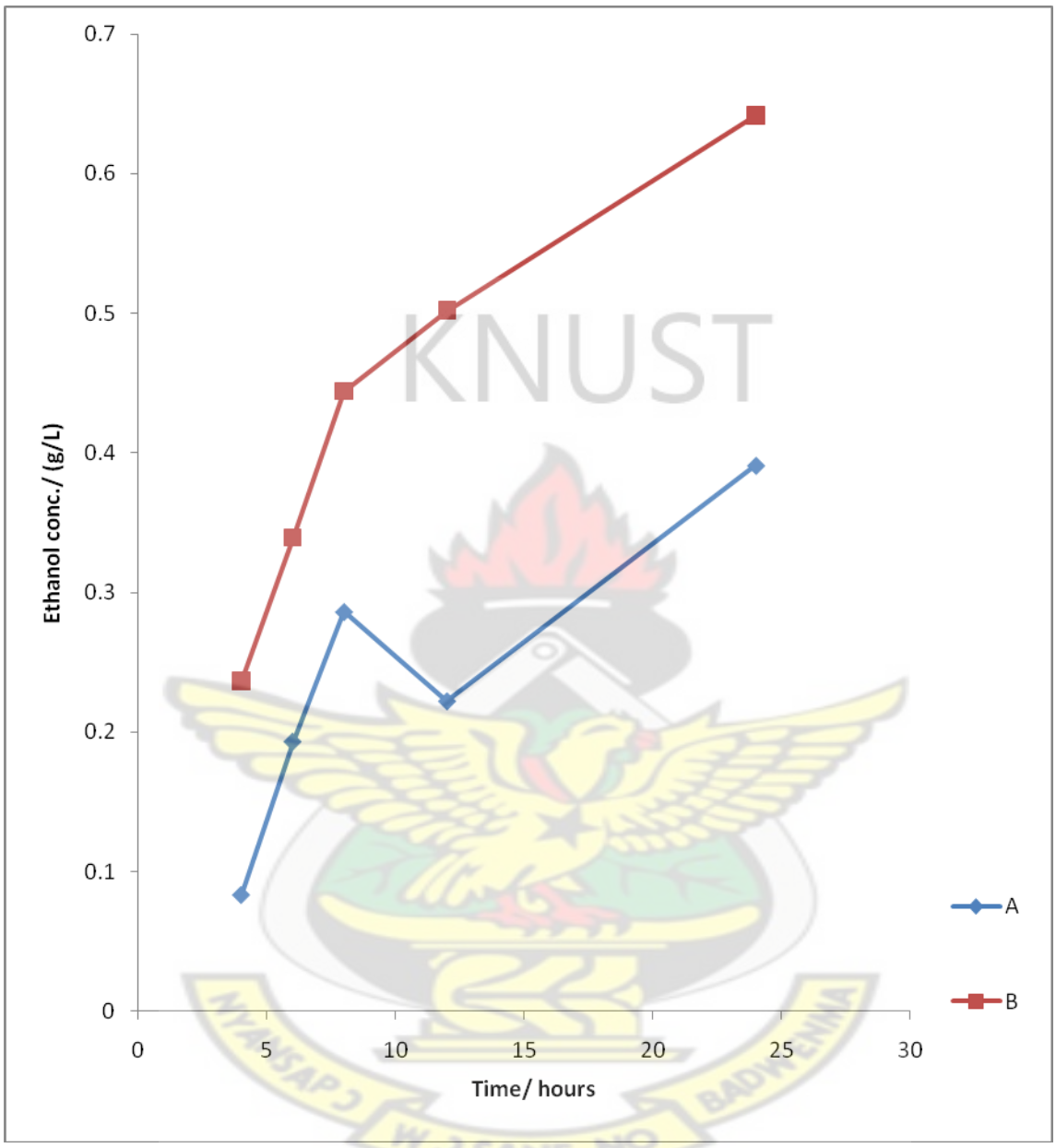



**Fig 4.12: Effect of enzyme loading on ethanol concentration over 24 h period.**

## 4.2 Discussion

The number of species from each genus is illustrated in Table 4.1. The genus *Aspergillus* was the most diverse followed by *Penicillium* species.

Ten out of the 12 isolated species exhibited cellulolytic activity. The number of cellulolytic fungi isolated is comparable to those cited in literature. Kader and Omar (1998), in a scientific expedition to Sayab-Kinabalu Park at Sabah in Malaysia isolated 16 cellulolytic fungi species. Baig *et al*., (2003), in a similar work isolated 12 cellulolytic fungi from a banana farm at Maharashtra in India. *Coccidiodes sp.* was not tested for cellulolytic activity because it is highly infectious as human pathogen (www.mycology.adelaide.edu.au/Mycoses/Dimorphic_systemic, 2008).

The isolates were cultured on Mandel agar media (MAM). Mycelial and clearing zone diameters were measured on the 3, 5 and 6 days after inoculation. The average was calculated as mean (Table 4.2). The mean for 3 days mycelia diameter is of importance because *Mucor sp.* exhibited profuse aerial growth which made measurement after third day impracticable. The mean of mycelial diameter on the agar is an indication of the ability of the isolates to hydrolyze Avicel cellulose (pure crystalline cellulose).

There were significant differences ($p<0.05$) between the mean mycelial diameters for both the 3 and 6 days period. *Trichoderma sp.* had the highest mean mycelial diameter for 3 days period and was significantly different from other species, followed by *A. flavus*, *A. terreus*, *Penicillium sp.9 and A. niger* whilst *Rhodotorula sp.* recorded the least. For the mean mycelial diameter over the 6 days period, *Trichoderma sp.* recorded the highest followed by *A. flavus*, *A. terreus* and *A. niger*, in that order.

All the 10 cellulolytic species isolated had been reported to exhibit cellulase activity with *Trichoderma sp.* and *Aspergillus sp.* being prominent (Lynd *et al.*, 2002;

Miyamoto,1997; Bon and Ferrara, 2007; Rajesh *et al.*, 2008). Of the ten cellulolytic species, four were *Aspergillus* species and 2 were *Penicillium* species. *Aspergillus* and *Penicillium* species were the abundant cellulolytic species isolated from Rawalpindi district in Pakistan by Khalid *et al.* (2006).

Clearing zone diameter to mycelial diameter ratio indicates the ability of the isolates to exude extracellular enzymes into the media. The ratios for 3 and 6 days periods are shown in Table 4.3. The ratios for 3 days are preferred to 6 days period because the petri plate size restricted the clearing zone diameter of fast growing *Trichoderma* species. Profuse aerial growth of *Mucor sp.* made measurement after 3 days impracticable. *Mucor sp.* had the highest ratio whilst *Rhodotorula sp.* recorded the least ratio of 1 implies that there was no difference between its clearing zone diameter and mycelial diameter.

The ability of a fungus to hydrolyze pure crystalline cellulose does not necessarily translate into ability to hydrolyze lignocellulosics (Lynd *et al.*, 2002). Nine of the cellulolytic isolates were cultured in CBB at pH 5.0 at 25°C. The mean enzyme activities and protein concentrations of the isolates were determined. The unit of activity (U) is defined as the amount of enzyme liberating 1 µmol glucose per minute in a standard assay.

Filter paper activity (FPA) of the cellulolytic isolates measures the overall activity of all the cellulase components (exoglucanase, endoglucanase and **β– glucosidase**). There was significant difference ($p<0.05$) between FPA of the isolates. Fig. 4.1 illustrates the mean FPA of the isolates. *A. niger* had the highest mean FPA of 0.37 FP U/ml and was

significantly different from the rest. This compares favourably to similar studies by Immanuel *et al.* (2007) who reported that *A. niger* strain with maximum FPA of 0.26 U/ml on sawdust-based medium. Baig *et al.*, (2003) also reported that *Trichoderma lignorum* isolated from banana field yielded maximum FPA of 0.45 U/ml on banana-based medium. *A. niger* was followed by *Trichoderma sp.* and *A. flavus* with 0.34 U/ml and 0.32 U/ml respectively. *Fusarium sp.* and *Penicillium sp.7* recorded the least.



Carboxymethyl Cellulose (CMC) activity measures endoglucanase activity. There was significant difference ($p<0.05$) between the CMC activity of the isolates and the mean CMC activities are illustrated in Fig. 4. 2. *A. niger* had the highest mean CMC activity of 0.70 U/ml and was significantly different from the rest. It was followed by *A. flavus* and *Trichoderma sp.* with 0.59 U/ml and 0.59 U/ml respectively. *A. terreus*, *Penicillium sp. 9*, *Fusarium sp.* and *Penicillium sp. 7* recorded the least significant CMC activity.

Most enzymes are proteins so protein concentration can indicates enzyme concentration. There was significant difference ($p<0.05$) in the mean protein concentration of the isolates. The mean protein concentration of the isolates is illustrated in Fig 4.3. *A. niger* had the highest protein concentration of 5.62 mg/ml followed by *A. flavus* with 4.55 mg/ml though the difference between them is not significant. *Fusarium sp.* was the least significant.

*A. niger* had the highest enzyme activities (FPA and CMCA) and protein concentration. The higher FPA may be due to superior composition of its cellulase component (Olofsson *et al.*, 2008) or possession of other complementary enzymes such as lignase

and hemicellulase (Baig *et al.*, 2004). The effective hydrolysis of lignocellulosics is determined by the constitution of the three components of cellulase.

The genus *Aspergillus* is noted for its higher endoglucanase activity measured as CMC activity (Miyamoto, 1997). Since there is a direct link between protein concentration and enzyme concentration, it might account for the higher protein concentration of *A. niger*.



Although *Trichoderma sp.* had the highest significant mean mycelial diameter on Mandel agar medium: an indicator of cellulase activity (Maheshwari *et al.*, 2000), its enzyme activities (FPA and CMCA) on CBB came next to *A. niger*. The structure of the corncobs powder and its crystallinity might have posed impediments to hydrolysis by *Trichoderma sp.*. The optimal composition of cellulase determines lignocellulosic hydrolysis (Olofsson *et al.*, 2008). The cellulase constituents in this case were predetermined by the source of the enzyme (isolate).

*A niger*, the best cellulolytic fungi isolated from soil sampled from Ejura farms was cultured on CBB for 6 days on shaker at 120 rpm. Aliquots of 5 ml were sampled at 24 h interval and assayed for enzyme activities. Figure 4.4 illustrates the enzyme activities over the 6 days period.

There was progressive increase in enzyme activity from one to 6 days after incubation. Cellulase is an induced enzyme and its production increased with increase in fungal biomass over the incubation period and as simple sugar in the substrate diminished (Lynd *et al.*, 2002).

Saccharification of corncob powder over the 24 h is illustrated in Figure 4.5. There was increase in saccharification from 0 to 24th h. The increase was steeper up to 9th h (phase 1) than from 9 to the 24th h (phase 2). The slow down in rate for hydrolysis must be due to the action of the enzymes been slowed down by obstacles that interfere with their path or a loss in activity and/or processivity making them less effective (Yang *et al.*, 2006).



The effects of substrate concentration, temperature and pH on release of reducing sugars were also carried out. The rate of sacccharifficatiion is directly proportional to substrate concentration up to the optimal substrate concentration. This is because random collisions between the substrate and enzyme active sites happen more frequently. Beyond the optimum, the active sites are saturated so higher substrate concentration has no effect on rate of saccharification (www.canacad.ac.jp:3445/BiologyIBHL/541). Saccharification increased with substrate concentration as shown in Figure 4.6. There was increase in reducing sugars with increase in substrate concentration. The highest mean glucose concentration of 3.11 mg/ml was recorded for substrate concentration of 6% and was significantly different ($p<0.05$). Substrate concentration of 1% released the least reducing sugars concentration. The glucose concentration for 6% substrate concentration was higher and significantly different from 5% substrate concentration, which suggests that 6% is or below optimum substrate concentration. Time course for different substrate concentrations is shown on Fig. 4.7. Generally, there was progressive increase in saccharification for all the different concentrations over the period. There were

increasing rate of saccharification for 5% and 6% substrate concentration from 0 to 24th h. This may due to availability of less recalcitrant carbohydrates during the period.

Temperature has complex effect on enzyme activity and hence saccharification. It affects the speed of molecules; the activation energy of the catalytic reaction and thermal stability of the enzyme. Generally saccharification increased with temperature up to the optimum after which it declines. The increase with temperature is due to corresponding increase in kinetic energy of the molecules involved and lowering of the activation energy. The decline in saccharification after the optimum temperature might be due enzyme denaturation caused by thermal instability (Shuler and Kargi, 1997; Chaplin and Bucke, 1990a; www.blurtit.com/science/chemistry). Effect of temperature on saccharification is shown on Figure 4.8. Saccharification increased from 25°C to maximum at 40°C after which it decreased up to 60°C. Temperature (40°C) recorded the highest release of reducing sugars and it was significantly different ($p<0.05$). Thus the optimum temperature is 40°C, implying that the isolate is mesophile (Shuler and et al. (2007) reported of *A. niger* with optimum temperature of 40°C on coir waste and sawdust. Fewer studies of enzymes from mesophilic fungi are available (Baig *et al.*, 2003). Saccharification was least significant ($p<0.05$) at 60°C. The decrease from 50 to 60°C was sharp due to the fact that enzyme denaturation is much faster (Shuler and Kargi, 1997; Chaplin and Bucke, 1990a; www.blurtit. com/science/chemistry). Time course for saccharification at different temperatures is shown on Fig 4.9. Generally, there was progressive increase in saccharification over the 24 h period at all the temperatures except 60C, where glucose concentration did not change significantly with time.

The pH of a solution has several effects on the structure and activity of enzymes and hence saccharification. Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly situated on their surface. The charges on these groups vary, according to their acid dissociation constants, with the pH of the solution. Thus pH affects the reactivity of the catalytically active groups (Chaplin and Bucke, 1990b). Figure 4.10 illustrates the effect of pH on release of reducing sugars from the substrate. Saccharification increased from pH 3.5 to a maximum of 4.0 after which it decreased up to 6.5. The highest saccharification which was significantly different ($p<0.05$) was recorded at pH 4.0. This makes the isolate acidophile (Nester *et al.*, 2001). Saccharification was least significant ($p<0.05$) at pH 6.5, thus the optimum pH was 4.0. The relatively low pH provides acidic condition which prevents bacterial contamination during fermentation (Olofsson et al., 2008). Time course for saccharification at different pH is illustrated in Fig. 4.11. Generally, there was progressive increase in saccharification over the 24 h period at all pH except at pH 6.5.

SSF was carried out over 24 h period at 25C and 110 rpm. SSF was carried out with 2 different enzyme loadings: 5 ml (A) and 10 ml (B) of *A. niger* culture filtrate. Concentrations of ethanol of the fermentations were determined at 4, 6, 8, 12 and 24 h after fermentation. Fig 4.12 shows the trend for ethanol production over the period. The mean ethanol concentrations were 0.24 g/l and 0.43 g/l for A and B respectively. Though there was no significant difference ($p<0.05$) in mean ethanol concentration over the period between the 2 enzyme loadings but concentration for B higher. This might be that B yielded higher glucose for fermentation. There was increase in ethanol production over the period for B. There might be increase in saccharification over the

period making glucose available to *Saccharomyces cerevisae* for fermentation. Olofsson *et al*., (2008) reported that enzymatic hydrolysis of the solid fraction has a large control over the total rate of ethanol production in SSF. Also the ethanol concentration and other by-products in the broth might not have reached the inhibitory level of the yeast over the period. The highest concentration of 0.642 g/l was recorded for B on 24$^{th}$ hour. The drop in ethanol concentration for A at 12 h was not significant. Comparison to similar works in litreature is difficult because they differ in either in raw material, type of pretreatment if any and detoxification, substrate concentration, fermentation strain, temperature or mode of operation which affects the final ethanol concentration as reported in work by Olofsson *et al*., (2008).



# CHAPTER FIVE

## 5.0 CONCLUSION AND RECOMMENDATIONS

### 5.1 Conclusion

Bio-ethanol was produced from maize agrowastes collected from a farmer at Bekwai-Ashanti. Soil samples from Ejura farms harbour cellulolytic fungi *Aspergillus niger*,

which produced the greatest amount of cellulase used to hydrolyze maize agro-waste (corncobs) into reducing sugars. The reducing sugars were converted onto bio-ethanol by *Saccharomyces cerevisae*. The mean FP activity of 0.3648 U/ml on corncob based medium was appreciable especially against the background that no strain improvement has been carried out. As mesophilic enzyme, with optimum temperature 40°C, it is easier to find favourable temperature for SSF. The optimum pH of 4.0 provided acidic condition which prevented bacterial contamination of SSF. The highest mean ethanol concentration from shake flask fermentation is 0.64 g/l and can be increased significantly by including pretreatment and detoxification processes.



### 5.2 Recommendations

1. Strain improvement of *A. niger* by mutation to increase its cellulase activity should be considered.

2. Different pretreatment must be tested on the maize agrowastes to find the one which will yield high reducing sugars with less inhibitor concentration.

3. SSF with *Saccharomyces cerevisae*TMB3400 capable of co-fermenting glucose and xylose should also be used in future experiment.

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# APPENDICES

**Appendix A**: **List of Equipments**

Balance: AdventurerPro., Ohaus; Gram precision electronic balance.

Centrifuge: Centrikon T-42K, Kontron instruments.

Colony counter: Gallenkamp.

Dry oven: Gallenkamp.

Haemocytometer: Improved Neubauer Haemocytometer.

Magnetic stirrer/hot plate: Staurt scientific UK.

Micropipette: Accupette (0650012).

Microscope Reichert Neova (serial No. 372112) and Olympus (401458) Tokyo.

pH meter: Basic pH meter (840087) Spec Scientific Ltd.

Shaker: G24 Environmental incubator shaker, New Brumswick scientific company inc. NJ, USA.

Shaking incubator/waterbath: Dubnoff metabolic shaking incubator/water bath. Premium instruments- chicaga, USA.

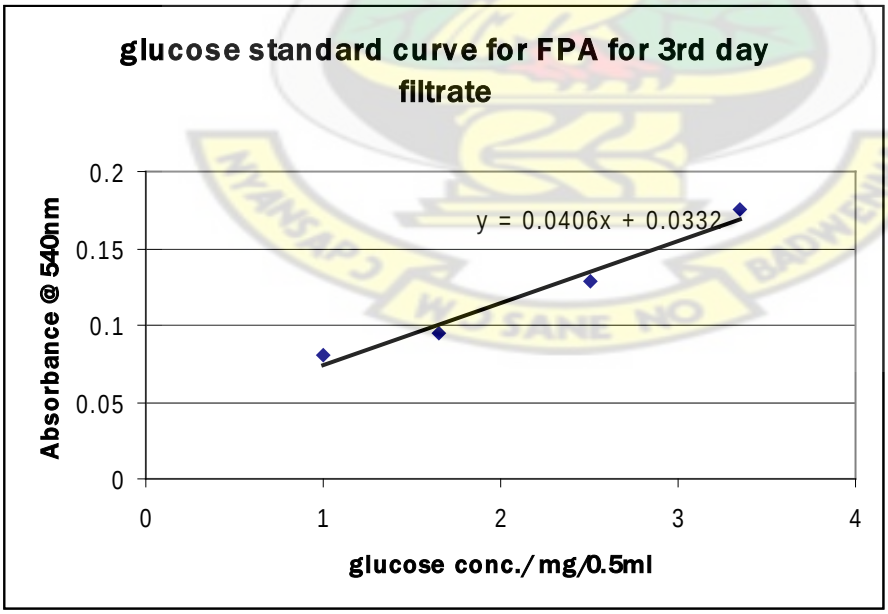
Spectrophotometer: Helios UV Visible Spectrometer. Thermospectronic UVG 121108. Thermo Electron Cooperation.

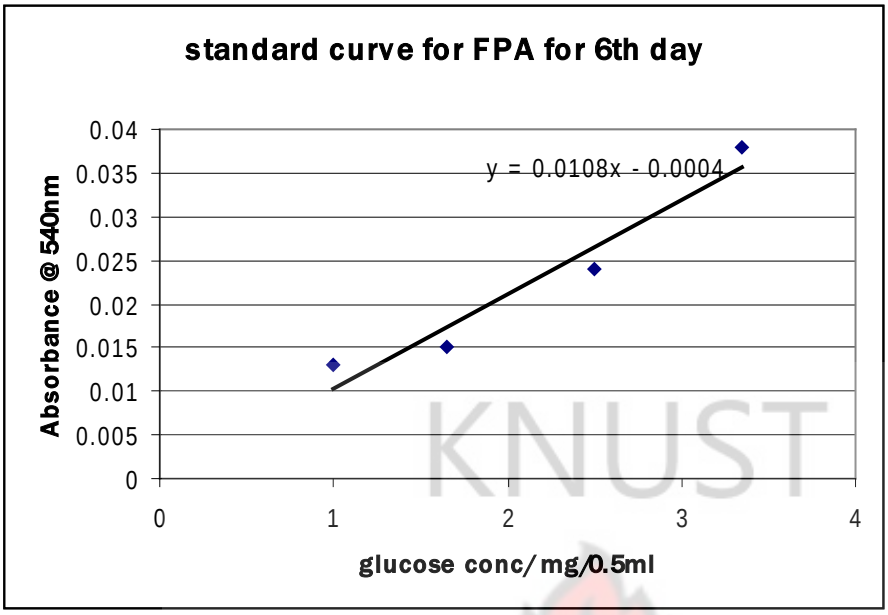
UV lamp: Gallenkamp.

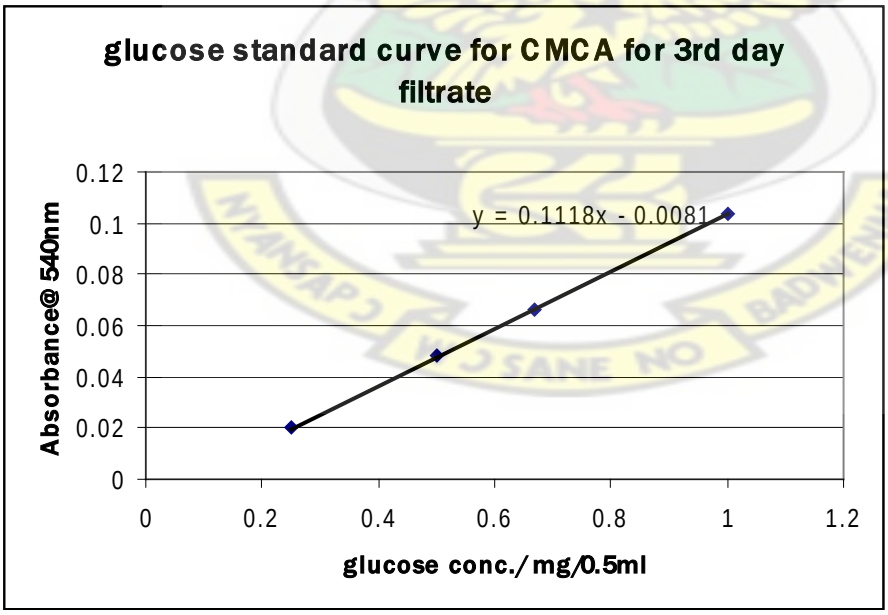
Water bath: Grant Instruments (Cambridge) Ltd.

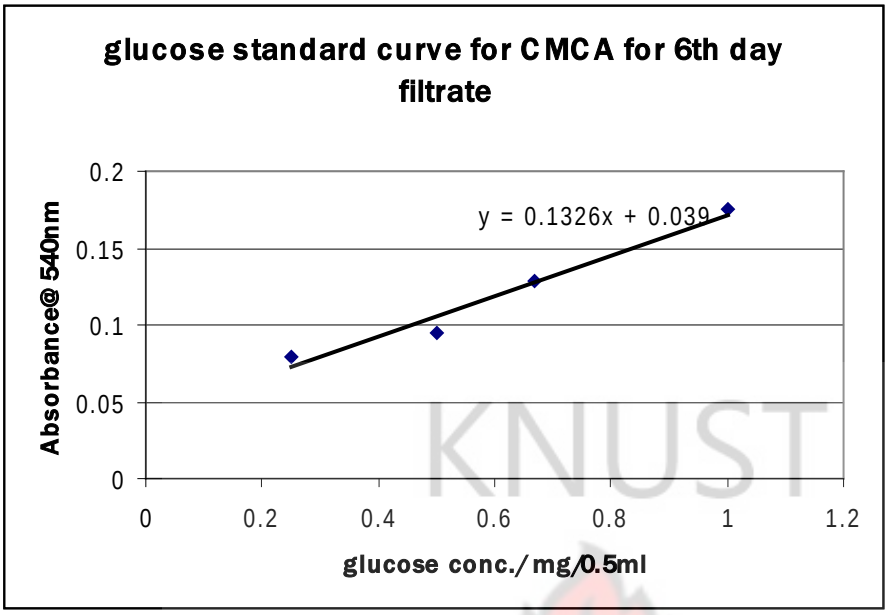


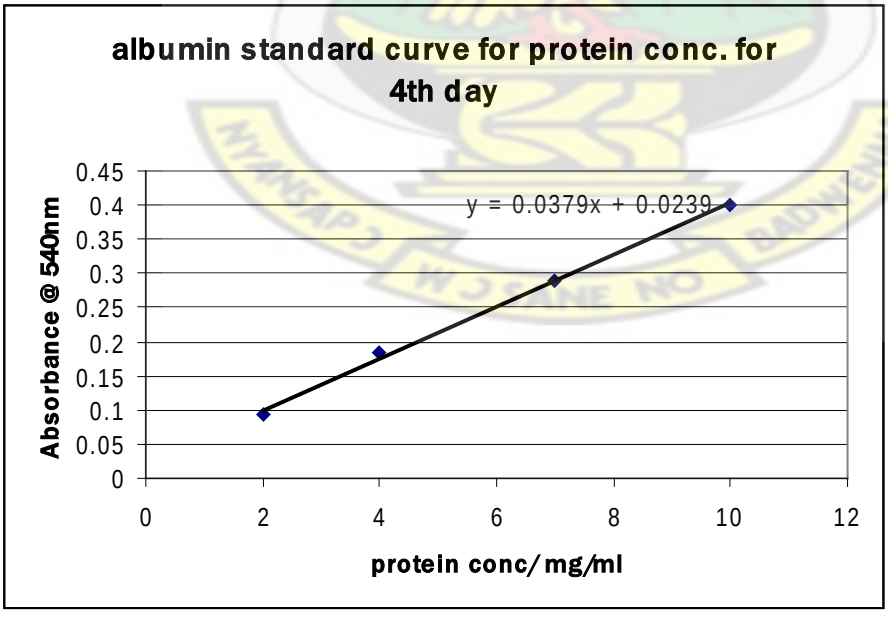
**APPENDIX B: Standard curves for enzyme activities and protein concentration assays.**

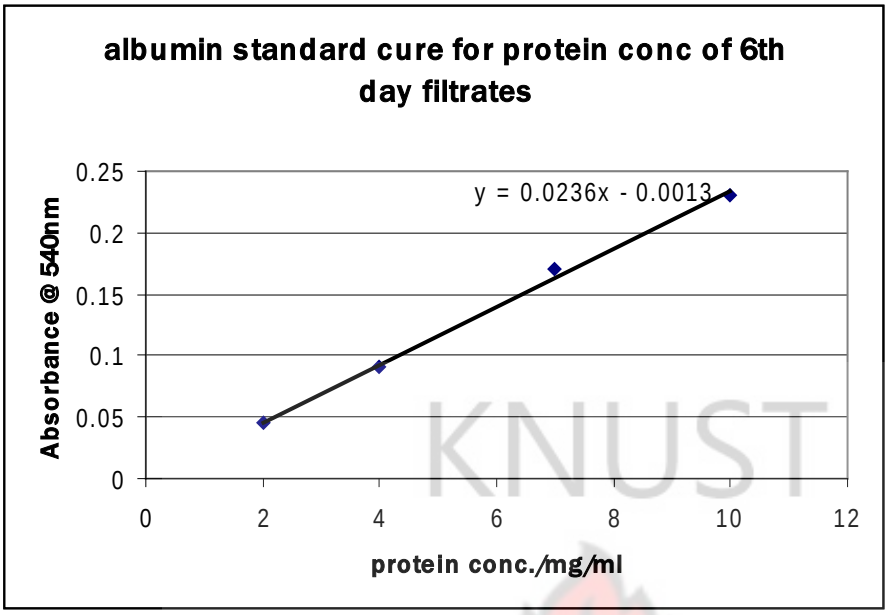



standard curve for FPA for 6th day
y = 0.0108x - 0.0004
Absorbance @ 540nm
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
1
2
3
4
glucose conc/ mg/0.5ml


glucose standard curve for CMCA for 3rd day filtrate
y = 0.1118x - 0.0081
Absorbance@ 540nm
0.12
0.1
0.08
0.06
0.04
0.02
0
0
0.2
0.4
0.6
0.8
1
1.2
glucose conc./ mg/0.5ml


glucose standard curve for CMCA for 6th day filtrate
y = 0.1326x + 0.039
Absorbance@ 540nm
0.2
0.15
0.1
0.05
0
0
0.2
0.4
0.6
0.8
1
1.2
glucose conc./ mg/0.5ml


albumin standard curve for protein conc. for 4th day
y = 0.0379x + 0.0239
Absorbance @ 540nm
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
protein conc/ mg/ml






**APPENDIX C: Data and ANOVA analysis**

All the works experiments were conducted in triplicates except the fermentation. ANOVA analyses were carried out with Assistant 7.5 beta. Graphs were plotted with Microsoft Excel.

Treatments:

T1 *A terreus*; T2 *Trichoderma*; T3 *A versicolor*; T4 *Penicillium sp.9*; T5 *Mucor sp.*; T6 *A flavus*; T7 *A niger*; T8 *Fusarium sp*; T9 *Penicillium sp. 7*; T10 *Rhodotorula sp.*; and T11 *Acremonium sp.*

Replications:

Day 3 = R1, R2, R3; Day 5 = R4, R5, R6; Day6 = R7, R8, R9

Notes:

** Significative at a level of 1% of probability ($p < .01$)

* Significative at a level of 5% of probability ($.01 =< p < .05$)

ns Non-significative ($p >= .05$)



The alphabets (superscripts) represent the order of the means within a column which are significantly different at $P = 5\%$ by Duncan's Multiple Range Test (DMRT). Means in a column with different superscripts are significantly different.

**Data for mycelia diameter (cm) over 3 days period on MAM**

| | R1 | R2 | R3 |
|---|---|---|---|
| T1 | 3.5 | 3.5 | 3.2 |
| T2 | 6.8 | 7.0 | 8.0 |
| T3 | 1.5 | 1.5 | 1.5 |
| T4 | 2.1 | 2.6 | 2.8 |
| T5 | 1.0 | 1.2 | 1.5 |
| T6 | 3.3 | 3.5 | 3.7 |
| T7 | 2.3 | 2.4 | 2.6 |
| T8 | 1.4 | 1.6 | 1.7 |
| T9 | 1.1 | 1.4 | 1.4 |
| T10 | 0.2 | 0 .1 | 0 .1 |
| T11 | 1.0 | 1.1 | 1.2 |

**ANOVA table for mycelia diameter (cm) over 3 days period on MAM**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 10 | 110.36727 | 11.03673 | 158.3530 ** |
| Error | 22 | 1.53333 | 0.06970 | |
| Total | 32 | 111.90061 | | |

**Data for mycelia diameter (cm) over 6 days period on MAM**

| | R1 | R2 | R3 | R4 | R5 | R6 | R7 | R8 | R9 |
|---|---|---|---|---|---|---|---|---|---|
| TI | 3.5 | 3.5 | 3.2 | 3.8 | 4.1 | 4.4 | 4.6 | 5.0 | 5.9 |
| T2 | 6.8 | 7.0 | 8.0 | 8.5 | 8.7 | 8.8 | 8.7 | 9.1 | 9.2 |
| T3 | 1.5 | 1.5 | 1.5 | 2.2 | 2.4 | 2.7 | 2.1 | 2.5 | 2.8 |
| T4 | 2.1 | 2.6 | 2.8 | 2.7 | 3.1 | 3.0 | 3.4 | 3.6 | 3.7 |
| T5 | 1.0 | 1.2 | 1.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| T6 | 3.3 | 3.5 | 3.7 | 4.2 | 4.5 | 4.8 | 4.5 | 5.3 | 5.4 |
| T7 | 2.3 | 2.4 | 2.6 | 3.8 | 4.2 | 4.3 | 5.0 | 5.3 | 5.5 |
| T8 | 1.4 | 1.6 | 1.7 | 2.8 | 2.9 | 2.9 | 2.9 | 3.1 | 3.1 |
| T9 | 1.1 | 1.4 | 1.4 | 1.6 | 1.8 | 2.0 | 3.4 | 3.6 | 3.7 |
| T10 | .2 | .1 | .1 | .2 | .2 | .2 | .3 | .3 | .3 |
| T11 | 1.0 | 1.1 | 1.2 | 1.1 | 1.1 | 1.3 | 1.2 | 1.2 | 1.4 |

**ANOVA table for mycelia diameter (cm) over 6 days period on MAM**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 10 | 466.22283 | 46.62228 | 83.3142 ** |
| Error | 88 | 49.24444 | 0.55960 | |
| Total | 98 | 515.46727 | | |

**Data for clearing zone diameter to mycelia diameter ratio over 3 days period**

| | R1 | R2 | R3 |
|---|---|---|---|
| T1 | 1.16 | 1.16 | 1.16 |
| T2 | 1.20 | 1.20 | 1.13 |
| T3 | 1.12 | 1.07 | 1.07 |

| | | | |
|---|---|---|---|
| T4 | 1.11 | 1.10 | 1.11 |
| T5 | 1.67 | 2.66 | 1.58 |
| T6 | 1.34 | 1.34 | 1.35 |
| T7 | 1.43 | 1.44 | 1.41 |
| T8 | 1.30 | 1.39 | 1.47 |
| T9 | 1.09 | 1.09 | 1.08 |
| T10 | 1.00 | 1.00 | 1.00 |
| T11 | 1.09 | 1.10 | 1.09 |

**ANOVA table for clearing zone diameter to mycelia diameter ratio over 3 days period**



| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 10 | 2.25222 | 0.22522 | 6.7109 ** |
| Error | 22 | 0.73833 | 0.03356 | |
| Total | 32 | 2.99055 | | |

**Data for clearing zone diameter to mycelia diameter ratio over 3 days period**

| | R1 | R2 | R3 | R4 | R3 | R6 | R7 | R8 | R9 |
|---|---|---|---|---|---|---|---|---|---|
| **T1** | 1.16 | 1.16 | 1.16 | 1.37 | 1.59 | 1.58 | 1.16 | 1.15 | 1.15 |
| **T2** | 1.20 | 1.20 | 1.13 | 1.07 | 1.04 | 1.02 | 1.04 | 1.00 | 1.00 |
| **T3** | 1.12 | 1.07 | 1.07 | 1.12 | 1.13 | 1.22 | 1.22 | 1.12 | 1.02 |
| **T4** | 1.11 | 1.10 | .11 | 1.12 | 1.11 | 1.11 | 1.12 | 1.11 | 1.07 |
| **T5** | 1.67 | 2.66 | 1.58 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| **T6** | 1.34 | 1.34 | 1.35 | 1.36 | 1.32 | 1.28 | 1.32 | 1.34 | 1.33 |
| **T7** | 1.43 | 1.44 | 1.41 | 1.39 | 1.43 | 1.41 | 1.41 | 1.43 | 1.40 |
| **T8** | 1.30 | 1.39 | 1.47 | 1.21 | 1.20 | 1.20 | 1.21 | 1.23 | 1.21 |
| **T9** | 1.09 | 1.09 | 1.08 | 1.09 | 1.11 | 1.11 | 1.11 | 1.26 | 1.33 |
| **T10** | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| **T11** | 1.09 | 1.10 | 1.09 | 1.10 | 1.10 | 1.09 | 1.10 | 1.11 | 1.09 |

**ANOVA table for clearing zone diameter to mycelia diameter ratio over 3 days period**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 10 | 1.84319 | 0.18432 | 4.0508 ** |
| Error | 88 | 4.00416 | 0.04550 | |
| Total | 98 | 5.84734 | | |

**Data for mean FPA (FPU/ml) of the isolates**

| | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|
| T1 | .140 | .135 | .152 | .223 | .231 | .240 |
| T2 | .243 | .235 | .231 | .433 | .460 | .445 |
| T3 | .167 | .182 | .172 | .337 | .343 | .377 |
| T4 | .158 | .168 | .177 | .137 | .101 | .118 |
| T5 | .190 | .210 | .220 | .274 | .264 | .276 |
| T6 | .201 | .210 | .204 | .428 | .437 | .445 |
| T7 | .262 | .251 | .267 | .468 | .479 | .462 |
| T8 | .094 | .086 | .090 | .120 | .128 | .132 |
| T9 | .154 | .159 | .156 | .121 | .113 | .120 |

**ANOVA table for mean FPA (FPU/ml) of the isolates**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 8 | 0.43591 | 0.05449 | 8.4542 ** |
| Error | 45 | 0.29003 | 0.00645 | |
| Total | 53 | 0.72595 | | |

**Data for mean CMCA (U/ml) of the isolates**

| | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|
| T1 | .259 | .251 | .265 | .311 | .319 | .313 |
| T2 | .456 | .463 | .456 | .719 | .726 | .707 |
| T3 | .375 | .378 | .380 | .423 | .423 | .427 |
| T4 | .292 | .312 | .284 | .241 | .246 | .250 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **T5** | .424 | .418 | .411 | .416 | .422 | .429 |
| **T6** | .540 | .530 | .534 | .651 | .652 | .656 |
| **T7** | .592 | .578 | .584 | .825 | .817 | .819 |
| **T8** | .181 | .194 | .205 | .208 | .211 | .216 |
| **T9** | .265 | .271 | .280 | .251 | .245 | .252 |

**ANOVA table for mean CMCA (U/ml) of the isolates**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 8 | 1.50620 | 0.18828 | 38.5680 ** |
| Error | 45 | 0.21967 | 0.00488 | |
| Total | 53 | 1.72588 | | |



**Data for mean Protein concentration (mg/ml) of the isolates**

| | R1 | R2 | R3 | R4 | R5 | R6 |
|---|---|---|---|---|---|---|
| T1 | 2.247 | 2.249 | 2.958 | 2.982 | 2.247 | 2.984 |
| T2 | 3.264 | 3.26 | 3.261 | 3.379 | 3.331 | 3.353 |
| T3 | 2.162 | 2.163 | 2.16 | 4.462 | 4.43 | 4.435 |
| T4 | 2.035 | 1.951 | 1.993 | 3.512 | 3.538 | 3.512 |
| T5 | 2.162 | 2.16 | 2.154 | 4.044 | 4.039 | 4.013 |
| T6 | 3.603 | 3.642 | 3.592 | 5.346 | 5.807 | 5.306 |
| T7 | 3.773 | 3.868 | 3.592 | 7.522 | 7.521 | 7.417 |
| T8 | 0.891 | 0.915 | 0.803 | 1.163 | 1.242 | 1.034 |
| T9 | 1.061 | 1.738 | 1.061 | 1.6 | 1.56 | 1.797 |

**ANOVA table for mean protein concentration (mg/ml) of the isolates**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| Treatments | 8 | 95.40202 | 11.92525 | 12.0485 ** |
| Error | 45 | 44.53986 | 0.98977 | |
| Total | 53 | 139.94188 | | |



**Mean enzyme activities and protein concentration of the cellulolytic isolates over 6 days**

| Isolates | FPA U/ml | CMC U/ml | PROTEIN CONC.(mg/ml) |
|---|---|---|---|
| *A terrus* | 0.1868$^{de}$ | 0.2863$^{d}$ | 2.6112b$^{c}$ |
| *Trichoderma* | 0.3412$^{ab}$ | 0.5878$^{b}$ | 3.3080$^{b}$ |
| *A versicolor* | 0.2630$^{bc}$ | 0.4010$^{c}$ | 3.3020$^{b}$ |
| *Penicillium sp.9* | 0.1432$^{ef}$ | 0.2708$^{d}$ | 2.7568$^{b}$ |
| *Mucor sp.* | 0.2390$^{cd}$ | 0.4200$^{c}$ | 3.0953$^{b}$ |
| *A flavus* | 0.3208$^{ab}$ | 0.5938$^{b}$ | 4.5493$^{a}$ |
| *A niger* | 0.3648$^{a}$ | 0.7025$^{a}$ | 5.6155$^{a}$ |
| *Fusarium sp* | 0.1083$^{f}$ | 0.2025$^{d}$ | 1.0080$^{d}$ |

| | | | |
|---|---|---|---|
| *Penicillium sp.* | 0.1372[f] | 0.2607[d] | 1.4695[cd] |

**Data for enzyme activities of *A. niger* on CBB2**

| Days | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| FPU/ ml | 0.1282 | 0.1859 | 0.1889 | 0.1926 | 0.1962 | 0.2304 |
| CMC/ ml | 0.1484 | 0.302 | 0.3703 | 0.4122 | 0.3889 | 0.5221 |



**Data for the effect of substrate concentration on saccharification**

| Time (h) | 1.5 | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 |
|---|---|---|---|---|---|---|---|---|---|
| S1% | 0.1502 | 0.127 | 0.076 | 0.1836 | 0.1889 | 0.1719 | 0.3904 | 0.301 | 0.2536 |
| S2% | 0.6751 | 0.6317 | 0.689 | 0.8825 | 1.0574 | 0.9537 | 1.3703 | 1.4823 | 1.3678 |
| S3% | 0.9383 | 0.8979 | 1.3113 | 1.237 | 1.5234 | 1.4275 | 2.0716 | 2.574 | 2.5168 |
| S4% | 0.7618 | 0.7881 | 1.0373 | 1.1364 | 1.4553 | 1.412 | 2.6361 | 2.2259 | 2.3029 |
| S5% | 1.0529 | 1.0682 | 1.3547 | 1.5916 | 1.8965 | 2.1041 | 3.3026 | 3.3052 | 3.4425 |
| S6% | 1.6304 | 1.4786 | 1.9322 | 2.7404 | 2.6903 | 2.951 | 3.8276 | 5.3196 | 5.4241 |

**ANOVA table for the effect of substrate concentration on saccharification**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 5 | 43.96441 | 8.79288 | 12.7731 ** |
| Error | 48 | 33.04277 | 0.68839 | |
| Total | 53 | 77.00718 | | |

**Mean reducing sugars concentration for different substrate concentration**

| Substrate Concentration (%) | Glucose concentration (mg/ml) |
|---|---|
| 1% | 0.1936[d] |

| | |
|---|---|
| 2% | 1.0122[c] |
| 3% | 1.6108[bc] |
| 4% | 1.5284[bc] |
| 5% | 2.1243[b] |
| 6% | 3.1105[a] |



**Data for the effect of temperature on saccharification**

| Time (h) | 1.5 | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 |
|---|---|---|---|---|---|---|---|---|---|
| 25˚C | 0.0836 | 0.079 | 0.127 | 0.1502 | 0.1889 | 0.1719 | 0.2536 | 0.301 | 0.3904 |
| 30˚C | 0.2029 | 0.1517 | 0.1951 | 0.1657 | 0.2585 | 0.2385 | 0.4501 | 0.5223 | 0.5098 |
| 40˚C | 0.255 | 0.2494 | 0.306 | 0.2993 | 0.3503 | 0.3802 | 0.398 | 0.4501 | 0.5412 |
| 45˚C | 0.1812 | 0.1627 | 0.1858 | 0.1811 | 0.2926 | 0.2849 | 0.475 | 0.5696 | 0.577 |
| 50˚C | 0.1707 | 0.2583 | 0.2871 | 0.2924 | 0.2505 | 0.2461 | 0.2672 | 0.2794 | 0.357 |
| 60˚C | 0.011 | 0.0197 | 0.0681 | 0.065 | 0.068 | 0.096 | 0.0995 | 0.0647 | 0.082 |

**ANOVA table for the effect of temperature on saccharification**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 5 | 0.53764 | 0.10753 | 8.5759 ** |
| Error | 48 | 0.60184 | 0.01254 | |
| Total | 53 | 1.13949 | | |

**Mean reducing sugars concentration for different temperatures**

| Temperature (˚C) | Mean Glucose Concentration (mg/ml) |
|---|---|

| | |
|---|---|
| 25 | 0.1940[b] |
| 30 | 0.2994[ab] |
| 40 | 0.3588[a] |
| 45 | 0.3233[a] |
| 50 | 0.2676[ab] |
| 60 | 0.0586[c] |



**Data for the effect of pH on saccharification**

| Time (h) | 1.5 | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 |
|---|---|---|---|---|---|---|---|---|---|
| pH 3.5 | 0.2938 | 0.2749 | 0.3813 | 0.3736 | 0.3636 | 0.3037 | 0.3603 | 0.4061 | 0.4102 |
| pH 4.0 | 0.1676 | 0.1842 | 0.2926 | 0.2477 | 0.418 | 0.5596 | 0.5958 | 0.6063 | 0.8265 |
| pH 4.5 | 0.0992 | 0.096 | 0.1083 | 0.1341 | 0.1501 | 0.3558 | 0.3159 | 0.3845 | 0.4641 |
| pH 5.0 | 0.1037 | 0.1301 | 0.1316 | 0.1656 | 0.1759 | 0.3308 | 0.3209 | 0.4178 | 0.4487 |
| pH 5.5 | 0.079 | 0.0836 | 0.127 | 0.1502 | 0.1889 | 0.2536 | 0.301 | 0.3852 | 0.5959 |
| pH 6.0 | 0.00836 | 0.094 | 0.102 | 0.144 | 0.152 | 0.158 | 0.163 | 0.17 | 0.182 |
| pH 6.5 | 0.055 | 0.071 | 0.075 | 0.078 | 0.085 | 0.089 | 0.092 | 0.097 | 0.102 |

**ANOVA table for the effect of pH on saccharification**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 6 | 0.76214 | 0.12702 | 7.2534 ** |
| Error | 56 | 0.98069 | 0.01751 | |
| Total | 62 | 1.74283 | | |

**Mean reducing sugars concentration for different pH**

| pH | Mean Glucose Concentration (mg/ml) |
|---|---|
| 3.5 | 0.3518[ab] |

| | |
|---|---|
| 4.0 | 0.4334[a] |
| 4.5 | 0.2342[bc] |
| 5.0 | 0.2472[bc] |
| 5.5 | 0.2405[bc] |
| 6.0 | 0.1387[cd] |
| 6.5 | 0.08267[d] |



**Time course for ethanol concentration (g/l) data**

| Time (h) | 4 | 6 | 8 | 12 | 24 |
|---|---|---|---|---|---|
| A | .083 | .193 | .286 | .222 | .391 |
| B | .236 | .339 | .444 | .502 | .642 |

**ANOVA table for ethanol concentration**

| V.S. | D.F. | S.S. | S.A. | F |
|---|---|---|---|---|
| Treatments | 1 | 0.09761 | 0.09761 | 5.2700 ns |
| Error | 8 | 0.14818 | 0.01852 | |
| Total | 9 | 0.24580 | | |

**Mean ethanol concentration for two enzyme loadings**

| A | B |
|---|---|
| 0.23500 [a] | 0.43260 [a] |

**Time course for saccharification**



| Time (h) | 0 | 1.5 | 3 | 6 | 9 | 12 | 15 | 18 | 21 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|
| Glucose conc. | 0 | 0.1502 | 0.2846 | 0.3341 | 0.3804 | 0.3852 | 0.3904 | 0.3871 | 0.389 | 0.3942 |