

**THE EFFECTS OF MUTAGENIC TREATMENT ON
THE RATE OF CELLULASE PRODUCTION BY
ASPERGILLUS NIGER ISOLATED FROM THE SOIL**

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DECLARATION

I hereby declare that this dissertation titled “The effects of mutagenic treatment on the rate of cellulase production by *Aspergillus niger* isolated from the soil” is a record of my own research work and has not been submitted for the award of a degree in any other university. All quotations are indicated and sources of information are specifically acknowledged by means of reference.

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CERTIFICATION

This dissertation titled “The effects of mutagenic treatment on the rate of cellulase production by *Aspergillus niger* isolated from the soil” by *DAMISA* Duro meets the regulations governing the award of the Degree of Doctor of Philosophy (PhD) in Microbiology of Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

Dedicated to my Mother, Adisetu Damisa, for your splendid ideas.

“ONLY THOSE WHO HAVE NEVER TRIED IT... CAN SUPPOSE THAT THE
PURSUIT OF KNOWLEDGE DOES NOT DEMAND A STRENGTH AND
DETERMINATION, A RESOLVE ... THAT IS A SPECIAL KIND OF ENERGY”

- Robert Davies, *THE REBEL ANGELS*, 1987

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ABSTRACT

Cellulases present great and exciting possibilities for use in several biotechnological processes especially lignocellulosic bioconversion. Since microbial sources of cellulases appear to be the cheapest option, the search for strains of microorganisms with robust characteristics is open ended. Conidia of parental strains of *Aspergillus niger* isolated from soil with cellulolytic capabilities on Mandel's medium were mutagenised with U.V irradiation, hydroxylamine and acridine orange independently. Visual agar plate detection system using Mandel-Weber screening medium was used to identify mutants with increased cellulase activity. The selected mutant coded *Aspergillus niger AH3*, obtained after treatment with hydroxylamine was tested for the production of cellulase on carboxymethylcellulose, bagasse, corn cob and corn straw in shake flask incubated at 32⁰C. The crystalline lignocelluloses were milled and fractionated into two particle sizes of 210 μ and 500 μ for testing cellulase activity. Portions of unscreened ground crystalline lignocellulosics were pretreated in two concentrations (0.5M and 2M) of both acid (sulphuric acid) and alkali (sodium hydroxide) independently and were left for varying residence time of one hour or three hours in the digester. Incubation temperature, pH, inoculum concentration, soluble protein and proximate constituents were all investigated. Component cellulase enzymes: exoglucanase, endoglucanase and β glucosidase were evaluated and compared with *Trichoderma reesei* cellulase preparation (ECONASE CEP)

as control. Aseptic conditions were used in all the growth studies. Supernatants of crude enzyme were taken and assayed at 24 hours interval. The optimum pH of 4.8 was found to have strong influence on enzyme production. Maximum enzyme activity was at 96 and 120 hours for the mutant and wild strains respectively. Optimum spore concentration for cellulase expression was 1.0×10^6 spores/ml. Higher spore concentration was not advantageous while lower concentration resulted in prolonged lag. Optimum incubation temperature of the crude cellulase from the mutant was 50°C . Soluble protein recovery correlated with cellulase production and cellulose material utilization by the organism. Particle size and compositional variance of the lignocellulosics were found to have a substantial influence on the enzyme expression by the organism. Larger particle size (500μ) gave low yield of cellulase whereas the smaller size fraction (210μ) gave a corresponding high yield of cellulase. Generally, enzyme expression in the lignocellulosics was in the order: bagasse > corn cob > corn strain. Alkali pretreated residues gave significantly higher cellulase yield than acid treated residues. Non-pretreated residues gave only low enzyme titers. Maximal activity of exoglucanase and endoglucanase was reached at 96 hours. Mutant strain showed improved production of the cellulase in the pretreated lignocellulosics by 51%, 40% and 16% for bagasse, corncob, and corn straw respectively. Exoglucanase, endoglucanase and β -glucosidase activities of the mutant increased by 48%, 35% and 64% respectively. The mutant was found to be an efficient producer of the β -glucosidase; 25% higher than the ECONASE. Maximal cellulase yield, 0.068IU/ml/min, was obtained from bagasse digested in 2M NaOH for one hour and fermented with the mutant. This translated to 39% increase in enzyme expression when compared with non-treated bagasse of 0.049IU/ml/min. Mutations using sub-lethal concentrations of

hydroxylamine for repeated bouts produced an elite strain of *Aspergillus niger* with improved rate of cellulase production. Sugarcane bagasse therefore has a great potential as a raw material for commercial production of cellulases.

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CHAPTER ONE

1.0 INTRODUCTION

The problems of Nigeria's import dependence has gained the attention of the Federal Government in recent years through the introduction of measures aimed at attaining economic independence, self reliance and the strengthening of the productive base of the economy. In March 2004, the federal government of Nigeria launched a new economic reform programme tagged "national economic empowerment and development strategy" (NEEDS) aimed at catalyzing economic growth, poverty reduction, import restrictions and value re-orientation in Nigeria. Other areas of expansion include technology for sustainable development which is growing very fast in the country. Therefore in line with this new economic agenda, biotechnological exploitation of agricultural residues into value added commodities could provide a missing link to Nigeria's economic prosperity.

Crop production is associated with waste generation and the exploitation of these waste materials as a renewable resource for bio-product development could be a major challenge

for biotechnology (Bahrim, 2004). Large quantities of agricultural residues accumulate every year which results not only in the deterioration of the environment, but also in the loss of potentially valuable materials which can be processed to yield a number of value added products such as food, fuel, feed and a variety of chemicals (Someet *et al.*, 2001). Agricultural wastes are wastes of organic origin particularly cellulosic or lignocellulosic matter which are available on a renewable or recurring basis which can be grown, collected and replenished fairly quickly without permanently depleting the natural resources (Geoffroy *et al.*, 2006)

The amount of organic agricultural wastes such as corn stalks, leaves and straw from produce processing facilities, sawdust and other residues from wood mills could be a principal resource for bio-development (VanWyk, 2001).

THE PROBLEM

Traditional methods and popular ways to deal with cellulosic waste disposals in our setting have been incineration and incorporation into the soil. Incineration option promotes particulate matter discharge into the atmosphere, thereby increasing concentrations of carbon dioxide and other green house gases in the earth's atmosphere. There is now a general scientific consensus that observed trends in climatic change and global warming are caused by anthropogenic emissions of green house gases (Lovett *et al.*, 2006). Lignocellulosic waste disposal by burning is not restricted to developing countries alone, but is considered as a global phenomenon (VanWyk, 2001). A way of addressing this

sensitive issue could be through the development of biowaste and scientific innovation as a means of converting these wastes into feedstocks

JUSTIFICATION

In Nigeria ,the outrageous cost of procuring chemicals and gas for domestic and industrial energy generation relative to cost of gas elsewhere in the sub-region is a potent disincentive for the domestic and industrial needs for economic growth. Also, increased concern for the security of the oil supply and the negative impact of fossil fuels on the environment has put pressure on society to find renewable fuel alternatives. The most common renewable fuel today is ethanol produced from sugarcane; however, this raw material base will not be sufficient and consequently future large scale use of ethanol will most certainly have to be based on production from lignocellulosic materials (Hahn-Hagerdal *et al.*, 2006).Therefore the development of a national biomass and biofuels programme in Nigeria, similar to the Brazil's success story, cannot be over-emphasised. Such programme would be based on use of agricultural wastes as raw materials in the production of chemical feedstocks.

Agricultural biowastes and virtually all lignocellulosics can be converted into products of economic magnitude (Solomon *et al.*, 1999). Successful utilization of cellulose materials as renewable carbon source is dependent on the development of economically feasible process technologies for cellulase production, and for the enzymatic hydrolysis of cellulosic materials to low molecular weight products such as hexoses and pentoses. Although technically feasible via numerous thermo-chemical and biological processes,

lignocellulosic conversion to ethanol on a commercial scale depends on improving conversion process economics and the ability to produce ethanol from low cost biomass is essential to making ethanol competitive with gasoline and the benefits are clear: reducing the amount of petroleum fuel used, energy efficiency and replacing it with cleaner burning ethanol will decrease air pollution reduction in green house gases. Currently, there are two major ways of converting cellulose to glucose which can then be fermented to ethanol: chemical versus enzymatic. Although acid cellulose hydrolysis is well known, the enzymatic process has a major advantage because it produces less inhibitor during the process (Panagiotou and Olsson, 2006). The biodegradation of cellulose to soluble sugars before ethanol can be fermented is a process which is only possible after the action of multienzyme system of cellulases produced by cellulolytic microorganisms (Singh *et al.*, 2003). A diverse spectrum of cellulolytic microorganisms, mainly fungi (Baldrian and Gabriel, 2003) and bacteria (Mawadza *et al.*, 2000) have been isolated and identified over the years and this list continues to grow. Despite the impressive collection of cellulolytic microorganisms, only a few have been extensively studied. The most popular is *Trichoderma reesei* and its mutants which are widely used for commercial production of hemicellulases and cellulases (Jorgensen *et al.*, 2003). Though a large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell-free enzymes capable of degrading crystalline cellulose *in-vitro* (Bhat and Bhat 1997).

Up to date, the production of cellulase, which is the key enzyme for cellulose biodegradation, has been found to be the most expensive step. This step according to

Yinbo *et al.* (2006) is quite expensive during ethanol production from cellulosic biomass. One of the different approaches to overcome this hindrance is to make continuous search for organisms with secretion of cellulase enzymes in copious amounts and to optimize enzyme production with them. Agricultural biomass such as corn cob and straw are abundantly available in maize production fields while bagasse can be assessed at sugar processing sites. They appear to be a favourable substrate, being cheaply available in the tropical and subtropical countries. These biowastes accumulate in the agro-industrial yards, with no significant and commercial use, but constitute serious environmental problems. The use of biowaste as a raw material can therefore broaden the options of the chemical industry, giving it more flexibility and a broader application range (Wymann and Goodman, 1993) because it would rely on more biodegradable products and processes that create less pollution and generally have fewer harmful environmental impacts; develop less expensive products; and use less expensive raw materials.

In the light of the issues discussed above, the general aim of this research was to utilize agricultural biomass readily available in our locality for the growth of *Aspergillus niger* and cellulase production thereafter.

The specific objectives included:

1. To isolate cellulase producing *Aspergillus niger* strains from soil.
2. To carry out growth studies on the performance of the isolates on different lignocellulosic substrates (corn cob, corn straw and bagasse) and amorphous cellulose (carboxymethylcellulose) in Mandel's medium.

3. To determine the effects of mutagenic treatments on the isolates using U.V. irradiation and chemicals (hydroxylamine and acridine orange) with a view to obtaining an elite strain that would have high cellulase activity.
4. To investigate the effects of alkali or acid pretreatment of the cellulose materials and determine the optimum conditions of the cellulose hydrolysis.
5. To determine the effects of particle size of substrate, inoculum size, pH and temperatures of incubation on enzyme yield.

CHAPTER TWO

2.0 LITERATURE

2.1 Background

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing natural as well as modified celluloses to smaller sugar components like glucose units. True cellulase activity is defined as the ability of the cellulase preparation to degrade crystalline cellulose extensively (Johnson *et al.*, 1992). Cellulolytic enzymes play an important role in nature's biodegradation processes where plant lignocellulosic materials are efficiently degraded by cellulolytic fungi and bacteria. There are several applications of cellulases in various industries, including food, brewery and wine, animal feed, pollution treatment, textile and laundry, pulp and paper, agriculture waste management, protoplast production, genetic engineering as well as in research and development (Da-Silva *et al.*, 1997; Poutanen, 1997; Bajpai, 1999; Anish *et al.*, 2006).

Cellulase production has attracted a worldwide attention due to the possibility of using this enzyme complex for conversion of abundantly available renewable lignocellulosic biomass for the production of carbohydrates for numerous industrial applications (Hayward *et al.*, 2000). Economical production of cellulase is a key for feasible chemical production from lignocellulosic biomass using cellulase based processes (Lynd *et al.*, 2002). Cellulases are produced by a range of microorganisms including bacteria, actinomycetes, fungi, and yeast, but fungi appear to be the most efficient producers of this extracellular enzyme (Viikari *et al.*, 1994; Ratanakhanokchai and Kyu, 1998). According to Laurent *et al.* (2000) the amount of cellulases produced by microorganisms depend on the growth conditions as substrate type, concentration and temperature. Majority of microbial cellulase studied have been shown to be acidic proteins with significant carbohydrate content (Rabinovich *et al.*, 2000b). A complete cellulase system consists of three classes of enzymes which act synergistically in the degradation process (Rabinovich *et al.*, 2002a). They act to break down the cellulose to glucose. These include the endoglucanase (EC.3.2.1.4; 1, 4- β -D-glucan 4- glucanohydrolase) or carboxymethylcellulose cellulases; or endocellulase. Exoglucanases (EC.3.2.1.91; 1,4- β -D-glucan cellobiohydrolase) or avicelase or exocellulase and β -glucosidase (EC.3.2.1.21) or cellobiase (Maheshwari *et al.*, 2000). Synergism between the cellulase components exists when hydrolysis by a combination of two or more components exceeds the sum of the activities expressed by the individual components (Valjamae, 2002). Strictly speaking, β -glucosidase enzyme is not a cellulase since this enzyme does not act on water insoluble cellulose, however, it shares a common feature with the endo- and exo-glucanases, with specificity being similar to α -1,4-glucosidic bonds. In most of the cellulases produced by cellulolytic fungi such as in

Trichoderma cellulases, the amount of β -glucosidase is lower than needed for the efficient cellulose to glucose hydrolysis. Therefore the main product of the hydrolysis is cellobiose (Yue *et al.*, 2004). Cellobiose is a strong inhibitor of endo and exoglucanases, and the accumulation of cellobiose will slow down the hydrolysis process significantly (Gruno *et al.*, 2004). *Aspergillus* strains are known for their ability to produce β -glucosidase with significantly higher yields than *Trichoderma* species (Kang *et al.*, 1999). It has been widely accepted that enzymatic degradation of native cellulose occurs preferentially first at amorphous regions by a random attack of endo type cellulase resulting in exposure of crystalline regions to attack by exo-type cellulase (Wood, 1991). Exoglucanase is the major component of the fungal cellulose system accounting for 40-70% of the total cellulase proteins and can hydrolyse highly crystalline cellulose (Esterbauer *et al.*, 1991).

2.2 Enzymes

Enzymes were discovered in the latter part of the century and have been in use since then. They have attracted the attention of scientists world over due to their wide range of physiological, analytical and industrial applications (Chand and Mishra, 2003). Several classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases are known (IUBMB, 1992). The majority of enzyme products to date have been derived from mesophilic and to a lesser extent, alkaliphilic microorganisms (Hough and Danson, 1999). Although many of the enzymes that are of value in some applications can be found in nature, the levels produced by wild type organisms are relatively low making the economics unsuitable for most commercial applications. Among the methods that have been adopted for treatment of cellulosic wastes, enhanced biodegradation by genetically

engineered microorganism that secretes the enzyme, cellulase, has been regarded as the most appealing one (Klysov, 1990).

The development of enzyme products often rely on screening a large number of organisms for an enzyme activity with a specific set of biochemical and physical characteristics that suit the targeted applications (Miyoko and Henrik, 2000). Traditionally, microbial screening processes have involved evaluation of purified enzymes isolated from pure cultures obtained from in-house or accessible culture collections throughout the world; or microorganisms isolated from environments rich in the substrate of interest or microorganisms that have a history of being good enzyme producers such as *Trichoderma*, *Aspergillus* and *Bacillus* species (Atkinson and Mavituna, 1991).

Screening literally means searching for a particular character in an organism. This is referred to as 'classical microbial screening'. The classical method of screening natural microbial isolates involves subjecting an environmental sample, for example, soil to selective pressure using the principle of culture enrichment established in the 20th century by Beijerinck and Winogradsky (Madigan and Martinko, 2006). By manipulating the growth and culture conditions, only those microorganisms expressing the enzyme that we desire are able to survive and grow. The selection of microorganisms is a very critical stage in the screening process and can be quite subjective especially if the numbers of microorganisms involved are large.

2.2.1 Cellulolytic Enzyme Systems in Microorganisms

Naturally, the conversion of plant or tree cellulose into biomass is mediated by a complex set of microorganisms which produce cellulases. Cellulases hydrolytically and synergistically convert crystalline cellulose to small oligosaccharides and finally to glucose (Ilmen *et al.*, 1997). Cellulase enzymes are by far less common in nature than do the amylase enzymes, because cellulose itself confers strength and structure to plants, it would therefore be undesirable if there were a plethora of organisms in nature that could break down the cellulose.

2.2.2 Cellulase Producing Strains

Cellulase biosynthesis is a property of many microorganisms (Cai, 2000). Many microorganisms are able to use cellulose as their sole carbon and energy source and produce the cellulase enzyme at the same time, but only a few of them produce significant quantities of cell-free cellulase capable of completely hydrolyzing crystalline cellulose *in vitro* (Kotchoni *et al.*, 2003). Among microorganisms, fungi are of great interest for enzyme production because they excrete their enzymes extracellularly (Bollok and Reczey, 2000). In the fungal family, *Trichoderma reesei* is regarded as the most efficient producer of cellulase (Miettinen-Oinonen and Suominen, 2002). However, the fungus does not excrete sufficient amount of β -glucosidase for efficient enzymatic hydrolysis (Grohmann, 1993), for which *Aspergillus* strains are known to be good producers (Juhasz *et al.*, 2003). The wide range of catabolic diversity among microorganisms is one of the distinguishing features of the microbial world. The range of this diversity varies widely among individual species from highly specialized ones that can utilize only one or a few substrates as energy

source to highly versatile species that can utilize over one hundred compounds as the sole carbon and energy source. In general, cellulolytic microorganisms are found near the specialist end of this continuum. They are primarily carbohydrate degraders and generally are unable to use proteins or lipids (or their components) as energy source for growth. Cellulolytic microorganisms native to soil (like the bacteria *Cellulomonas* and *Cytophaga* and most fungi) can generally utilize a variety of other carbohydrates in addition to cellulose (Rajoka and Malik, 1997). Anaerobic cellulolytic species (like *Fibrobacter*, *Ruminococcus* and *Clostridium*) are more limited in their carbohydrate range, growing on cellulose and its hydrolytic products but often not on monosaccharides, oligosaccharide and polysaccharides based on sugars other than glucose. *Clostridium thermocellum* does not grow well on glucose (Zhang and Lynd, 2005) and both *C. thermocellum* and *Ruminococcus albus* use cellobiose in preference to glucose when both substances are present (Thurston *et al.*, 1993). The specialist nature of the anaerobic cellulolytic microorganisms probably results mainly from the specialized enzymatic machinery for cellulose hydrolysis, and significant metabolic effort devoted to its synthesis and other features peculiar to cellulose utilization. These characteristics along with the high calorie value and natural abundance of cellulose itself, apply a significant selective pressure on microorganisms for its utilization particularly if the organism develops a strategy for positioning itself in such a way as to gain earlier access to the products of cellulose hydrolysis. A species of microorganism sufficiently well adapted to cellulose utilization is unlikely to starve in any habitat (natural or man-made) receiving periodic input of plant biomass (Simankova *et al.*, 1993). The nutrient requirements for growth of cellulolytic species include available nitrogen, phosphorus, and sulphur, plus standard macro and

microminerals and various vitamins. Although additional nutrients present in complex media (like peptones and yeast extract) are not usually required, they often stimulate the growth of individual strains (Pedersen and Nielson, 2000).

2.2.3 Induction and Regulation of Cellulase Production

Cellulase is an inducible enzyme system (Kubicek *et al.*, 1993). Cellobiose, lactose and sophorose are known to facilitate the production of either complete or incomplete cellulase system by few microorganisms (Ryu and Mandels, 1980). Synthetic compounds such as palmitate and acetate esters of disaccharide and thiocellobiose have also been shown to function as inducers of cellulases (Reese *et al.*, 1969). However, cellulose has been found to be the best carbon source for the production of high levels of cellulase by many micro-organisms (Wood, 1985).

The most generally accepted view of induction process is that the low levels of cellulase constitutively produced by the microorganism first hydrolyze cellulose to soluble sugars (Benguin and Aubert, 1994). These sugars are presumably converted into true inducers, which enter the cell and either directly or indirectly influence DNA binding protein and promote cellulase gene expression (Kubicek *et al.*, 1993). Carle-Urioste *et al.* (1997) predicted that cellobiose, the repeating unit of cellulose, could be the natural inducer of cellulase in *Trichoderma reesei*. However, it has been shown that in *Trichoderma viride* cellobiose could induce cellulase production only at a high concentration (1% or more) or in the presence of a surfactant such as Tween 80 (Eriksson *et al.*, 2002). Bhat *et al.* (1997) demonstrated that cellobiose at a concentration as low as 0.2% induced the production of both carboxymethyl cellulase and true cellulase activities by *Clostridium thermocellum*.

Therefore, it can be argued that cellobiose is the natural inducer of cellulase system at least in some microorganisms.

It has also been shown that sophorose, a contaminant in glucose preparation, is an effective inducer of cellulase in *T. reesei*, and in other species of *Trichoderma* even at a concentration of 0.3µg/ml (Sternberg and Mandels, 1979). However, sophorose did not induce the production of cellulase in other fungi and in a mutant of *T. reesei*, QM 9414 (Moloney *et al.*, 1985). Thus, sophorose is not a universal inducer of cellulase.

Carbon catabolite repression is another regulatory mechanism known to control cellulase production in bacteria and fungi. In this case, the end-product of cellulose hydrolysis interacts with a cellular protein to form a complex which interacts with a particular gene at the transcription level and represses cellulase synthesis (Suto and Tomita, 2001). The proof for carbon catabolite repression is based on the fact that no cellulase is formed during the growth of a microorganism on glucose, glycerol and other carbon sources related to glycolytic metabolism. The efficiency of cellulosic materials as enzymatic inductors depend on its susceptibility to microbial attack. In general, crystalline cellulose has been found to be a superior carbon source for induction of cellulase enzymes in thermophilic fungi than were its amorphous or impure forms (Fracheboud and Canevascini, 1989).

2.2.4 Mechanism of Cellulolytic Reaction

Although the types of enzymes expressed by these microorganisms are very similar, the actual mechanism of degradation of crystalline cellulose may be different for the different enzyme systems. The enzymatic hydrolysis of cellulosic materials is a slow and complex reaction which correlates with the level of cellulose crystallinity (Kotchoni *et*

al., 2003). Native cellulolytic microorganisms produce a variety of products, some of which like soluble sugars, ethanol, lactic acid, acetic acid and acetone are potentially useful industrially and others are not. Only a few fungal and bacterial species have been demonstrated that produce cellulases with activity high enough to extensively degrade insoluble cellulose to soluble sugars *in – vitro*. Endoglucanases hydrolyze cellulose chains at random to produce principally short chain oligosaccharides. They attack β -1,-4-glucosidic bond at random positions in the cellulose chain. The substrates are amorphous cellulose, such as carboxymethyl cellulose, phosphoric acid or alkali-swollen cellulose, instead of crystalline cellulose. Hydrolysis of amorphous cellulose by endoglucanase produces a rapid change in degree of polymerization. Exoglucanases act by removing glucose or cellobiose from the non-reducing end of the chain. Cellobiases are very important components of the cellulase system in that they complete the hydrolysis to glucose of short-chain oligosaccharides and cellobiose which are released by the other enzymes (Wood and Bhat, 1988). These enzymatic components act synergistically in the hydrolysis of crystalline cellulose (Kim and Kim, 1994). Most cellulose degrading enzymes have a two-domain structure that consists of a catalytic and a cellulose binding domain (CBD) connected to a linker region (Linder *et al.*, 1996). CBDs are widespread in all types of cellulases.

2.2.5 *Methods for Measuring Cellulase Activity*

The physical heterogeneity of cellulosic substrates together with the complexity of cellulase system produced by different microorganisms have led to the development of several assay procedures for the measurement of cellulase activities. The considerable

difference in the nature of the substrates used, variation in assay procedures adopted for measuring different cellulase components and the synergistic action of cellulase components have made the comparison of results among laboratories difficult. Therefore, in 1984, the IUPAC commission and Biotechnology published standard assay procedures for measuring cellulase activities (Ghose, 1984). Some of the recommendations have been readily accepted by biotechnologists, but many enzymologists feel that these procedures are quite restricted and not satisfactory for understanding the mechanism of action and substrate specificities of cellulases in detail.

Most of the methods used to quantify the different enzymes are based on measuring the enzyme activity. One common assay to measure total cellulase activity is the 'Filter Paper Assay' (FPA), where the production of reducing sugars from the filter paper is measured (Miller, 1959; Adney and Baker, 1996). One problem with using enzymatic assays is how to distinguish between endoglucanase activity from exoglucanase. Specific assays to measure endoglucanase activity involve the use of modified cellulose e.g., carboxymethyl cellulose (Singh *et al.*, 1995). Other approaches have been to use dyed cellulose derivatives to develop fast methods for determining endo and exoglucanase activity (Biswas *et al.*, 1988). At present, no substrate allowing specific determination of only exoglucanase activity is available, however synthetic chromogenic substrates like O- or P- nitrophenyl – b- D – cellobioside or 20 – chloro, 40– nitrophenyl – b – D – glycosides of either lactose, cellobiose or cellotriose or salicin or esculin (Kuhad, 1993) have been used for measuring cellobiohydrolase activity in the presence of endoglucanase.

2.3 Aspergillus niger

Soil is perhaps the most complex and heterogeneous of natural systems, being composed of a solid, liquid, and gaseous phase of changing proportions in time and space. Soil is also a medium for plant growth and a habitat for myriads of micro-organisms. Although the microbial biomass constitutes only a very small proportion (3%) of the total organic carbon in soil (Martin and Haider, 1986), it is the most active and dynamic fraction of the living organic pool and home to many fungi including *Aspergillus niger*. *Aspergillus niger* is a member of the genus *Aspergillus*, which includes a set of fungi that are generally considered asexual, although perfect forms (reproduce sexually) have been found. *Aspergillus* is filamentous, cosmopolitan and ubiquitous fungus found in nature. They are geographically widely distributed and have been observed in a broad range of habitats because they can colonize a wide variety of substrates. Filamentous fungi such as *Aspergillus niger*, have the capability to secrete large amounts of proteins into their growth medium (Gouka *et al.*, 1997a). *Aspergillus niger* is commonly isolated from soil, plant debris, and other decaying vegetation as saprophytes (U.S. EPA, 1997). The primary uses of *Aspergillus niger* are for the production of enzymes and organic acids by fermentation. Fermentations to produce these enzymes may be carried out in vessels as large as 100,000 litres (Finkelstein, 1989). *A. niger* is also used to produce organic acids such as citric and gluconic acids. The history of safe use of *A. niger* comes primarily from its use in the food industry for the production of many enzymes such as α -amylase, amyloglucosidase, cellulase, lactase, invertase, pectinase and acid proteases (Ward 1989). Products of this species have obtained a GRAS (Generally Regarded as Safe) status, which allows them to be used in food and feed applications (Archer, 2000). *A. niger* has some uses itself in addition to its products of fermentation. For example, due to its ease of visualization and

resistance to several antifungal agents, *A. niger* is used to test the efficacy of preservative treatments (Jong and Gantt, 1987). The fungus is also used to perform certain enzymatic reactions that are very difficult to accomplish by strictly chemical means, such as specific additions to steroids and other complex rings. (Jong and Gantt, 1987; Radzio and Kuck, 1997)

2.3.1 Identification and Taxonomy

As is the case of many fungi, the taxonomy of *Aspergillus* is primarily based on morphological rather than the physiological, biochemical features and genetic characteristics often used to classify bacteria (U.S. EPA, 1997). The genus *Aspergillus* is usually defined as asexual saprophytic fungi that produce large black or brown conidia phialides that are arranged in a globose head radiating from a vesicle or spherical conidiophore (Raper and Fennel, 1965). *Aspergillus niger* is both a species and a group within the genus *Aspergillus* (Bennett, 1985b). The concept of retention of the *A. niger* group based on black conidia seems to be dominant in the definition of the *A. niger* group (Kusters-van Someren *et al.*, 1990). A more sophisticated means of treating the classification of *Aspergillus* from *Penicillium* have been the use of molecular technique that determines the molar percent of G + C and DNA complementarily (measuring rate and extent of re-association of single stranded DNA from two isolates) (Tsuchiya *et al.*, 2002).

While morphology provides a reasonable means of classification and assignment within the *A. niger* group, it is not a reliable means for identifying a given isolate from the field (U.S. EPA, 1997). The major distinction currently separating *A. niger* from the other species of *Aspergillus* is the production of carbon black or very dark brown spores from

biseriate phialides (Raper and Fennell, 1965). Other features include the smooth and generally colourless conidiophores and spores that are 5 μm , globose and have conspicuous ridges or spines not arranged in rows. *A. niger* isolates grow slowly on Czapek agar (Raper and Fennell, 1965).

Generally, the genus includes over 150 species but the major macroscopic features remarkable in *Aspergillus* species identification are the growth rate, colour of the colony and thermotolerance (Larone, 1995; St. Germain and Summerbell, 1996). The surface colour may vary depending on the species. The reverse is uncoloured to pale yellow in most of the isolates. However, the reverse colour may be purple to olive (*Aspergillus nidulans*); and orange to purple (*Aspergillus vesicolor*). Some of the colour of the colonies in various *Aspergilli* is shown in Plates I - III.

2.3.2 Growth in Submerged Culture

The most common feature of filamentous microorganisms is their polarized growth pattern in the form of filaments called hyphae. A typical fungal hypha grows out of a single cell-spore as a multinucleate tube containing cytoplasm which moves within a hypha towards the hyphal tip, where it grows. New tips are grown out of the main hyphae and produce branches which result in a network of hyphae, called mycelium. The hyphae of *Aspergillus* have numerous crosswalls or septa and are able to fuse to adjacent hyphae and thereby establish protoplasmic continuity in a reticulate mycelium (Znidarsic and Pavko, 2001). The life cycle of filamentous fungi starts and ends in the form of spores, the dormant state of the microorganism providing the dispersal or the survival of the species. At the required physical and chemical conditions, spores enlarge (swell) due to intensive

formation and accumulation of new active cell material. After a specific period, polarization of growth occurs, resulting in germ-tube formation. The fraction of spores that will germinate and the dynamics of swelling and germ tube formation in submerged culture mainly depend on the state of the inoculum and medium composition and have been suggested to be regulated separately (Spohr *et al.*, 2000). Sporulation at the end of the development cycle of fungi is rarely achieved during submerged cultivation, partially due to the physical nature of the hyphal wall (Pazouki and Panda, 2000). Chemostat experiments have indicated that the main trigger for fungal sporulation is nitrogen limitation and has mainly been observed at low specific growth rates (Calvo *et al.*, 2002).

2.3.3 Pelleted Growth Form

Pellets may be classified as stable spherical or oval agglomerates consisting of branched and intertwined networks of hyphae (Znidarsic and Pavko, 2001). Pellet morphology exhibits some advantages over the filamentous growth form – improvement in culture rheology and possibility of biomass re-use. Also the problems associated with the tendency of the filamentous form of fungi to grow on fermenter walls are overcome (Wang *et al.*, 2003; Gibbs *et al.*, 2000). Pellets may be loose or compact. *A. niger* produces coagulative type of pellet formation. Spores essentially conglomerate at an early stage of development (Nielsen and Carlsen, 1996). The medium pH value has been shown to have a decisive influence on spore coagulation (Vecht-Lifshitz *et al.*, 1990) and strong agitation has been found to be unfavourable for coagulative pellet formation (Nielson, *et al.*, 1995). Pellets have become an interesting growth form of filamentous microorganisms due to better culture rheology and pellets include several physiological states of the

microorganism with the specific metabolite productions which may not occur in the filamentous form (Znidarsic and Pavko, 2001).

2.3.4 Enzymes Produced by *Aspergillus niger*

Aspergillus niger produces a spectrum of extracellular enzymes. Selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. *Aspergillus niger* produces 19 types of enzymes (Coulibaly and Agathos, 2003). Thus, the selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions. Some of the major industrial enzymes produced by *A. niger* is discussed below.

2.3.4.1 Amylases

The amylase family of enzymes has been well characterized through the study of various microorganisms. Presence of two major classes of starch-degrading enzymes have been identified in *A. niger*, viz. alpha-amylase which cleaves glucosidic linkages between the adjacent glucose units in linear amylose chain and glucoamylase which hydrolyses single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner. Amylolytic enzymes produced by a strain of *Aspergillus niger* cultivated on cassava starch in liquid or solid culture were found to be mainly glucoamylase (Wallis *et al.*, 2001). Amylases and glucoamylases have found applications in processed food industry, fermentation technology, textile and paper industries (Pandey, *et al.*, 2000).

2.3.4.2 Galactosidases

There has been considerable interest to produce alpha and beta-galactosidases in solid state fermentation processes because these enzymes have applications in the pharmaceutical and food industries. Alpha galactosidase has an application in medicine for the treatment of fabry disease (Golubev *et al.*, 2004). Somiari and Balogh (1995) used a strain of *A. niger* for alpha-galactosidase production on wheat bran or rice bran. Srinivas *et al.*, (1994) described the use of Plackett – Burman design for rapid screening of several nitrogen sources, growth/product promoters, minerals and enzyme inducers for the production of alpha-galactosidase by *A. niger* MRSS 234 in solid state fermentation. Mutant strains of *Aspergillus niger* producing elevated levels of Beta-galactosidase has been characterized (Nevalainen, 1981).

2.3.4.3 Lignases

Lignin is a three-dimensional phenylpropanoid polymer which is considerably resistant to microbial degradation in comparison to polysaccharides and other naturally occurring biopolymers. Biological delignification processes using microbial cultures producing lignolytic enzymes – the lignases – can have applications in delignification of ligno-cellulosic materials which can be used as the feedstock for the production of biofuels or in paper industry or as animal feedstuff (Bhat, 2000). Lignin peroxidase, (manganese peroxidase and laccase) are the most important lignin-modifying enzymes collectively termed lignases (Malherbe and Cloete, 2003). Both basic and applied studies of the ligninases have been hampered by the difficulty of producing them in sufficient quantity (Sun *et al.*, 2004). Identifying lignin degrading microorganisms has also been hampered because of the lack of reliable assays, but significant progress has been made through the use of a C-labelled lignin assay (Freer and Detroy 1982).

2.3.4.4 Pectinases

Studies have been made to compare the production of pectinase by *Aspergillus niger* CH4 in solid state (SSF) and submerged (SMF) fermentations supplemented with different carbon sources like glucose, sucrose and galactouronic acid, (Solis–Pereira *et al.*, 1993). Polygalactouronase production increased in SSF system but decreased in SMF system. The production of a pectolytic enzyme complex in a submerged fermentation was studied using the *Aspergillus niger* mutant A 138 varying the aeration and agitation regimes. Increase in aeration and agitation regimes gave a double depectinizing activity and the time required for fermentation was reduced from 95 to 65 hours (Friedrich *et al.*, 1994). Exopectinase production by *A. niger* has also been compared in submerged and solid state fermentation with the purpose of studying the effect of sucrose addition and water activity on the level of enzyme activity. Sucrose addition in submerged fermentation resulted in catabolite repression of exopectinase activity but in solid substrate fermentation an enhancement of enzyme activity was observed (Diaz-Godinez *et al.*, 2001). Media acidity plays a significant role on pectinases production by *Aspergillus niger* in SSF system (Solis – Pereira, *et al.*, 1996). Pectinase hyperproducing mutants of *Aspergillus niger* C28B25 have been isolated for solid state fermentation of coffee pulp (Antier *et al.*, 1993). Pectinase is utilized in treatment of softwoods to improve preservative efficiency making it more permeable for chemical preservative (Gregorio *et al.*, 2002).

2.3.4.5 Proteases

Proteolytic enzymes account for nearly 60% of the industrial market in the world. They find application in a number of biotechnological processes, viz. in food processing

and pharmaceuticals, leather industry, detergent industry etc. In recent years, there have been increasing attempts to produce different types of proteases (acid, neutral, alkaline) through SSF route using agro industrial residues (Sumantha *et al.*, 2005). The regulation of two major acidic extracellular proteases, pepA and pepB in *Aspergillus niger* has been investigated using Northern analysis and both genes have been found to be under complex regulatory control as they could be turned off by the presence of good nitrogen or carbon sources in the media (Jarai and Buxton, 1994). The pH of the medium also played a major role as the expression of both genes was completely turned off under alkaline conditions. A new strain of *Aspergillus niger* Tieghem 331221 produced large quantities of an extracellular acid protease when grown in SSF system using wheat bran as the sole substrate. (Chakraborty *et al.*, 1995).

2.3.4.6 Xylanases

Strains of *Aspergillus niger* and *Aspergillus terreus* known to produce xylanase with undetectable amounts of cellulase have been studied for xylanase – production on various lignocellulosic substrate using solid state fermentation (Gawande and Kamat, 1999). Xylanase has been produced by *Aspergillus niger* via solid state fermentation of palm kernel cake (Kheng and Omar, 2005). Wheat bran stands out to be the best for xylanase production. Xylanase production from the two strains selectively removed the hemicellulose fraction from all lignocellulosic materials tested. Park *et al.* (2002) optimized the initial moisture content, cultivation time, inoculum size and concentration of basal medium in SSF for the production of xylanase by an *Aspergillus niger* mutant using statistical experimental designs. The researchers found out that cultivation time and

concentration of basal medium were the most important factors affecting xylanase activity. Enzymatic hydrolysis of xylan to xylose is catalyzed by endo-1,4, β -xylanase and β -xylosidase, the former randomly hydrolyzing xylan to xylo-oligomers and the latter producing xylose from xylo-oligomers.

2.3.4.7 Cellulase Production by *Aspergillus*

Cellulases are fundamental for the degradation of amorphous and crystalline cellulose in lignocellulosic material, but unfortunately, they have a low catalytic efficiency on their substrates when compared to similar enzymes like amylases (Escovar-Kousen *et al.*, 2003), therefore there is a strong interest in improving their activities. Cellulases are currently sold to the textile industry for cotton softening and denim finishing and to detergent markets for colour care, cleaning and anti-redeposition in washing powders (Cherry and Fidantsef, 2003).

Industrial bioprocesses with filamentous fungi embrace the production of a majority of commercially important products of biotechnology, in the sense of quality as well as the diversity of metabolites (Znidarsic and Pavko, 2001). These are mainly the submerged culture processes, where a dynamic relationship exists between the environmental conditions and the growth pattern of these microorganisms (Znidarsic and Pavko, 2001).

Hypercellulolytic mutant strains secrete large amounts of cellulases, the largest published amounts being 40g/litre (Durand *et al.*, 1988). Isolation and screening of a hyper producer strain therefore plays a key role in the production of the cellulase enzyme. Only a few organisms have the ability to hydrolyze native cellulose. The black *Aspergilli* have a

number of characteristics which make them ideal organisms for industrial applications, such as good fermentation capabilities, and high levels of protein secretion (De-Vries and Visser, 2001); ability to assimilate various organic substrates and suppress development of other micro-organisms and high sporulation capacity (Millner *et al.*, 1994). *Aspergillus* strains are known for their ability to produce β -glucosidase with significantly higher yields than *Trichoderma* species (Kang *et al.*, 1999). The integrity of the three – dimensional structure of active site is essential to the maintenance of the activity and any other factor that influence the integrity of this structure (secondary, tertiary and or quaternary) will affect the enzymatic activity. The individual enzymes that make up the cellulase complex often display strong synergy in the hydrolysis of cellulose. 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 or cellobiose as the major products. β -glucosidase hydrolyze soluble cellodextrins and cellobiose to glucose (Teeri, 1997).

Currently industrial demand for cellulases is being met by production methods using submerged fermentation processes, employing generally, genetically modified species of *Trichoderma*. There are several reports describing co-culturing of two cultures for enhanced enzyme production. Gupta and Madamwar, (1997) cultivated two species of *Aspergillus*: *Aspergillus ellipticus* and *Aspergillus fumigatus* and reported improved hydrolytic and β -glucosidase activities compared to when they were used separately using SSF system. Ojumu *et al.* (2003) reported the production of cellulase from some

lignocellulosic substrates in a submerged fermentation using *Aspergillus flavus*. Microbial production of cellulases by *Aspergillus fumigatus* using wheat straw as a carbon source has been documented in a work by Dahot and Noomrio (1996).

2.4 Cultivation conditions

2.4.1 Carbon Sources and Inducers

In most studies involving the production of cellulase, cellulosic materials have been used as the substrate for fungus growth (Suto and Tomita, 2001). The rate of enzyme synthesis depends on the hydrolysis of the substrates (Oashima *et al.*, 1990). Soluble substrates like amorphous cellulose have some advantages compared to the cellulosic materials (crystalline cellulose). A series of different carbohydrates have been studied for *T. reesei* growth. In general, lactose has been used as a common carbon source and inducer of industrial enzyme production (especially cellulases) in *T. reesei* (Olsson *et al.*, 2003). Hydrolysates of many bulk materials have been used in cellulose synthesis. Wastepaper hydrolysates have a similar cellulose-inducing strength as cellulose and it induces a wide set of cellulases (Ju and Afolabi, 1999). Since cellulose is unable to enter the microbial cell, it has been suggested that low molecular weight degradation products of cellulose hydrolysis penetrate into the cells and induce the production of the enzyme (Nikolev and Vinetski, 1998). Cellulases are induced by monosaccharides and disaccharides. L-sorbose has been considered to be the only monosaccharide found to induce cellulase formation, and sophorose is regarded as the most efficient inducer of cellulases. Cellobiose is thought to be the inducer in natural conditions (Nogawa *et al.*, 2001).

2.4.2 Nitrogen Sources

Typical nitrogen sources in *Aspergillus* species cultivation are ammonium sulphate or ammonia water solution. Trace peptone and yeast extract can stimulate an increase in enzyme production (Oshoma and Ikenebomeh, 2005), and same is true for *T. reesei* (Pedersen and Nielsen, 2000) but nitrate or urea are not suitable for *T. reesei* cultivation (Lieckfeldt *et al.*, 2000). The Maillard reaction may have a bad influence on the cultivation when using plant hydrolysates as the carbon source. The preparation of plant hydrolysates to produce saccharides and proteins involves high temperatures, and the colour-forming Maillard reaction plays a significant role in this process. The Maillard reaction consumes nutrients such as amino acids and saccharides, and the colour products can be harmful for microorganisms and cell growth (Jing and Kitts, 2000). Yeast extract has remained a better nitrogen source and nutrient material than other common organic or inorganic nitrogen sources (Purkathofer *et al.*, 1993a).

2.4.3 pH and Temperature

pH is an important parameter in the production of enzymes by *T. reesei* (Denison, 2000). Good production of cellulases was found at low pH (4.0) (Bailey *et al.*, 1993a). A high pH (7.0) was essential for high xylanase production by *Trichoderma longibrachiatum* in cellulose medium (Royer and Nakas, 1990). During the course of the fermentation, the nitrogen source can significantly influence the pH of the medium (Haapala *et al.*, 1994). The pH of *T. reesei* culture both decreased during the cultivation when ammonium salts were used as the nitrogen source, whereas the pH increased when urea was the nitrogen source.

The cultivation temperature does not only affect the growth rate of an organism, but it can also have a marked effect on the level of cellulase production. *T. reesei* RUT. C-30 grew well at 17⁰, 28⁰ and 37⁰C when cultivated on lactose substrate, but cellulase production was reduced. An initial phase of cultivation at 37⁰C followed by a shift to 28⁰C in the beginning of the enzyme production phase was advantageous for the amount of cellulase activity (Smits *et al.*, 1998).

2.4.4 Other Nutrients and Surfactants

Beside carbon and nitrogen sources, several other factors also have to be considered in designing the optimum cultivation conditions for cellulase production. The metal ions, organic nitrogen and surfactants most often used are KH_2PO_4 - 2.0g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.3g/L; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ - 0.3g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.005g/L; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ - 0.0016g/L; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.0014g/L; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ - 0.002g/L; Tween 80 - 0.1ml/L; yeast extract - 0.3g/L and peptone - 1g/L. (Krishna *et al.*, 2000).

Tween 80 is beneficial for the secretion of enzymes; its optimal concentration is close to 0.2ml/L, while a higher concentration is harmful for the production of cellulases (Arnesen *et al.*, 1998). The ion mechanism for the enhanced enzyme production by Tween-80 may be related to the increased permeability of the cell membrane, allowing a more rapid excretion of the enzyme, which leads to greater enzyme synthesis (Eriksson *et al.*, 2002).

2.4.5 Aeration and Agitation

In the cultivation of filamentous fungi for industrial enzyme production in fermenters, the agitation rate and aeration levels influence the fungal growth and secretion of enzymes. Too strong agitation and aeration have been shown to be harmful for the production of hemicellulases (Reddy *et al.*, 2002). Enzyme production by *T.reesei* QM 9414 has been found to be affected seriously by agitation (Lejeune and Baron, 1995). When using lactose as the substrate in fifteen litre fermentation, the optimal agitation rate was found to be 200rpm. Low xylanase activities were obtained at 130rpm, most probably due to oxygen or mass transfer limitations, while at 400rpm, almost no xylanase was produced. When cellulose powder was used as the substrate, the effect of agitation rate was less pronounced than with lactose, because optimum speed was 300rpm and significant xylanase production occurred even with the highest agitation of 400rpm. The particles of the insoluble substrate appeared to have a protective effect on the mycelium (Lejeune and Baron, 1995). The effect of oxygen saturation for *T.reesei* grown on 1% cellulose has been studied. Enzyme and extracellular protein levels were not affected by oxygen levels of 20% or above, but were severely reduced at 10% oxygen saturation (Schafner and Toledo, 1992). On the contrary, low levels of dissolved oxygen or even oxygen limitation did not adversely influence xylanase production by *T.lanuginosus* (Purkarthofer *et al.*, 1993b).

2.4.6 Batch Fermentation

The workhorse of the bioprocess industries is still the batch fermentation that is often carried out in batch fermenter (also spelled fermentor). A batch fermentation can be considered to be a “closed system”, wherein at time T=0, the sterilized nutrient solution in the fermenter is inoculated with microorganisms and incubation is allowed to proceed under optimal physiological conditions. (Pumphrey and Julien, 1996). In submerged batch

culture, enzyme is released into the culture supernatant and then the supernatant is harvested and the mycelium is discarded. In submerged culture, the morphology of filamentous microorganisms vary between two extreme forms, pellets and free filaments, depending on culture conditions and the genotype of the strain (Papagianni and Mattey, 2006). In view of the time that it takes to grow the productive fungal culture, batch production of enzymes may not be the most efficient and cost effective means, yet only a few studies have been carried out on continuous enzyme production by fungi (Lambert, 1983; Linko *et al.*, 1986). The composition of the culture medium, the biomass concentration and the metabolite concentration generally change constantly as a result of the metabolism of the cells (Crueger and Crueger, 1984). After the inoculation of a sterile nutrient solution with microorganisms and cultivation under physiological conditions, four typical phases of growth; lag, log, stationary and death phases are observed. Control of mycelial morphology in fermentations is often a prerequisite for industrial application. In some processes, free mycelia are required for increased productivities as in the production of penicillin from *Penicillium chrysogenum*, whereas in other processes, pellets are required according to reports on some fungal enzymes such as α -glucosidase (Wang *et al.*, 2005).

2.4.7 Solid State Fermentation

Besides submerged fermentation, solid state fermentation (SSF) is a popular method to produce cellulases by fungi. SSF means that the micro-organism grows on moist solid substrates in the absence of free-flowing water. Filamentous fungi grow typically in nature on solid substrates such as wood, seeds, stems, roots and leaves of plants in symbiotic associations. Compared to the submerged fermentation, SSF possesses several

advantages such as higher fermentation productivity, higher end-product concentration, higher product stability, lower catabolite repression, lower demand of sterility due to the low water activity used in SSF and mixed cultivation of various fungi (Holker *et al.*, 2004). However, SSF is currently used only to a small extent for enzymes and secondary metabolites production because of severe process engineering problems.

2.5 Strain Development

The vast reservoir of genetic resources of microorganisms is increasingly being tapped and harnessed for greater productivity. Strain improvement is a vital part of process development in most fermentation industries. In strain development, classical mutagenesis is one of the most powerful techniques used to increase enzyme yield from microorganisms (Okonko *et al.*, 2006). Mutagenesis effects mutation on the target cell. Mutation is an error during DNA replications that results in a change in the sequence of deoxyribonucleic bases in the DNA and an organism that exhibits a new phenotype as a result of mutation is called a mutant. Effects of mutation on phenotype range from alterations, so minor that they can only be detected by special techniques, to gross modifications of morphology or function, to lethality. Conditional lethal mutations are the most useful for genetic studies. These mutations are lethal in one environment, the restrictive condition, and viable in a second environment, the permissive condition. The three major classes of mutants with conditional lethal phenotypes are; auxotrophic mutants – those unable to synthesis an essential metabolite; temperature sensitive mutants-those that will grow at one temperature but not at another and suppressor sensitive mutants – those that are viable only when a certain genetic factor, a suppressor is present.

Classical mutagenesis techniques are induced mutations originally developed by the pharmaceutical industry to improve the yields of antibiotics (Chiang, 2004). The same techniques are used to improve enzyme yield. It requires mutation in the DNA of an organism to bring about changes in genetic traits. Cells or spores are treated with mutagens (ultra-violet, gamma ray or chemicals) reactive with DNA. A typical process of enzyme development by mutagenesis is to conduct a lot of cycles of mutagenesis selecting the best variant in each cycle which becomes the starting point in the next cycle until no further improvement is observed (Miyoko and Henrik, 2000). Random mutation can create a high degree of artificial diversity by repeating these cycles and is a veritable tool in creating a new and improved enzyme. After treatment with mutagens, a diverse population of cells is obtained (Monaghan and Koupal, 1990). A screening process is used to identify cells having mutations conferring desirable traits. Traditionally strain development meant a laborious approach with regard to identification of superior isolates from a mutagen treated population. Rational selection procedures are more efficient and usually have a biochemical basis (Fiedurek *et al.*, 2000). In early or primary screening prior to laboratory fermentations, rational selection is achieved by the used of techniques allowing visual identification of superior mutations. The selection of alpha-amylase producers using the size of the zone of hydrolysis of starch is an example. Invariably, strain development of cellulase producers employs the same criterion for selection. Enhancement of beta-galactosidase productivity of *A. niger* NCIM-616 has been achieved using nitrosoguanidine and U.V radiation by Rasouli and Kulkarni, (1994). While many mutations are lethal or detrimental to an organism, a few can lead to increased enzyme

productivity. Classical mutagenesis and screening of a wild type strain will result in the isolation of a more productive and thus commercially viable strain (Ogawa and Shimizu, 1999).

The amount of mutagenesis caused by a mutagen is determined by three factors: the chemical reactivity of the mutagen with DNA; the concentration of the mutagen and the DNA target; and the amount of time the DNA is exposed to the mutagen (Cleaver, 1994). It is typically difficult to control the chemical reactivity of the mutagen, so the dose of a mutagen is usually controlled by varying the concentration of the mutagen or the time of exposure (Maloy, 2002).

One of four things can happen as a result of mutation. This could stem from:

- i. Misense: results in single substitution mutation to cause one wrong codon and one wrong amino acid.
- ii. Nonsense: results in the transcription of a stop codon and protein terminated there.
- iii. Sense: change in DNA base sequence resulting in a new codon still coding for same amino acid.
- iv. DNA nucleotides not divisible by three is added or deleted.

Induced mutations are caused by mutagens such as U.V and chemicals

2.5.1 Ultra Violet Radiation

Ultraviolet radiation is one example of a physical agent that causes random mutagenesis. Random mutagenesis introduces mutations at random along the entire length of a gene (Miyoko and Henrik, 2000). The ultraviolet portion of the light spectrum

includes all radiations with wavelengths from 100nm to 400nm (Crow, 1993), and are non-ionizing; unlike gamma and x-rays that are ionizing, this means that it has enough energy to remove electrons from a target molecule – causing it to form ions (Kowalski, *et al.*, 2000). UV radiations though less energetic, but its wavelength are preferentially absorbed by bases of DNA and by aromatic amino acids of proteins, and so has important biological and genetic effects. The microbicidal activity of the U.V. light depends on the length of exposure: the longer the greater the cidal activity. It also depends on the wavelength of U.V used; the most cidal lie in the 260nm – 270nm range, where it is absorbed by nucleic acids (Harkki *et al.*, 1991). U.V light damages DNA and creates covalent linkage between these bases that prevent replication and transcription. Primarily, U.V light is absorbed by DNA and causes adjacent thymine bases on the same DNA strand to covalently bond together forming “thymine-thymine dimmers” (Kowalski *et al.*, 2000). As the DNA replicates, nucleotides do not complementarity base pair with thymine dimers and this terminates the replication of that DNA strand. U.V irradiations have been used by several workers to improve enzyme productions from *Aspergillus* species for the production of beta-glucosidase (Kang *et al.*, 1999), glucoamylase (Ghosh *et al.*, 1991); amyloglucosidase (Ul-Haq *et al.*, 2002); Xylanase (Butt *et al.*, 2002); Glucose-6-phosphate dehydrogenase (Weng and Ji, 2003); glucose oxidase (Khattab and Bazaraa, 2005).

2.5.2 Chemical Mutagens

A variety of chemicals are known to be mutagenic and these may be classified into three groups according to their modes of action:

- i. Mutagen which affect non-replicating DNAs.

- ii. Base analogs, which are incorporated into replicating DNA due to their structural similarity with one of the naturally occurring bases.
- iii. Frameshift mutagens, which enter into DNA during replication or repair and through this intercalation causes insertion or deletion of one or a few nucleotide pairs (Ayala *et al.*, 2000).

Chemicals which affect non replicating DNA include nitrous acid (HNO_2) which deaminates adenine to hypoxanthine and cytosine to uracil (Budke and Kuzminov, 2006). Through the changed pairing properties of the deamination products, (hypoxanthine pairs with cytosine, uracil with adenine) $\text{AT} \rightarrow \text{GC}$ and/ or $\text{GC} \rightarrow \text{AT}$ transitions occur. Hydroxylamine (NH_2OH) reacts with pyrimidines, but only the reaction with cytosine is mutagenic, whereby the amino group is replaced with a hydroxylamino group (Budowsky, 1976). The hydroxylamine derivate from cytosine shows tautomerization and pairs then with adenine so that through hydroxylamine action, $\text{GT} \rightarrow \text{AT}$ transitions are caused. Hydroxylamine is a very specific mutagen and acts by specifically adding a hydroxyl group to cytosine, producing hydroxylaminocytosine which undergoes the tautomeric shift (Hong and Ames, 1971). Another group of chemicals affecting non replicating DNAs are the alkylating agents: ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), diethylsulfate (DES), N-methyl-N-nitro-N-nitrosoguanidine (NTG) and others. Mutagenesis with alkylating agents occurs via various pathways. These compounds cause the formation of a whole spectrum of alkylated bases in DNA, along with phosphotriester, purine-free sites and single strand breaks. The alkyl groups are transferred to nucleophilic sites on purine and pyrimidine rings especially the N-7 and O-6 positions of guanine and the O-4 position of thymine (Drake and Baltz, 1976).

Base analogs such as 5-bromouracil (BU) or 2-aminopurine (AP) have structural similarities with the natural DNA bases and are therefore incorporated into replicating DNA instead of the corresponding bases thymine and adenine. The analogs tautomerize more frequently than the natural DNA bases. If the keto form of BU is incorporated (BU in Keto form pairs with adenine, whereas BU in enol form pairs with guanine) there is AT → GC transition; and if enol form BU is incorporated, GC → AT transition is observed (Auerbach and Kilbey, 1971).

Frameshift mutagens intercalate into the DNA molecule and cause errors which result in an alteration of the reading frame resulting in the formation of faulty protein or no protein at all (Maki, 2002). The most commonly used frameshift mutagens are the acridine dyes such as acridine orange, proflavine and acriflavine. The induction of insertions or deletions is dependent on the ability of an acridine dye to become inserted between two neighbouring bases of a DNA strand (Wang and Ripley, 1998).

Chemical mutagens have been used to induce enhanced biosynthesis of lipase in *Rhizopus* (Bapiraju *et al.*, 2004), increased glucose-6-phosphate dehydrogenase production in *Aspergillus niger* (Liu *et al.*, 2003), β -glucosidase in *Aspergillus niger* (Kang *et al.*, 1999).

2.6 Lignocellulosic Substrates

Different regions of the world have excess agricultural or forest waste products with high potential for conversion into value added products. Nigeria like many other developing countries in sub-Saharan Africa is basically an agricultural country endowed with huge amount of bioresources and crop residues which can be harnessed for

sustainable development in the countryside. The crop residues are essentially lignocellulosic. Lignocellulose is the major component of biomass that makes up about half of the matter produced by photosynthesis (Perez *et al.*, 2002). It consists of three types of polymers – cellulose, hemicellulose and lignin – that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross - linkages (Rowell, 1995). These lignocelluloses constitute wastes and waste management is one of the biggest problems faced by the agricultural sector considering the amount generated annually. Much of the lignocellulose wastes in our farms and industries are often disposed of by incineration which is a global phenomenon (Levine, 1996). Biomass resources available in Nigeria are generally residues from rice, maize, timber and sugarcane. These resources can be used to create new biomaterials and thus requires indepth understanding of the composition of the raw materials so that the desired functional elements can be obtained for bioproduct production. Different potential bioproducts and their many applications have been discussed (Bhat, 2000; Beg *et al.*, 2001; Subramamiyan and Prema 2002; and Beauchemin *et al.*, 2003).

Bioconversion of lignocellulosic wastes could make a significant contribution to the production of organic chemicals (Howard *et al.*, 2003). Lignocellulosic materials represent an underutilized renewable resource available in huge amounts, approximately 3,720 million tones per annum (Wayman and Parekh, 1990), and are continually being replenished by the carbon cycle (Ojumu *et al.*, 2003). Cellulose, hemicellulose and lignin occur in agricultural residues in an average ratio of 4:3:3: respectively (Brauns and Brauns,

1960) although the exact percentage of these components varies from source to source (Rowell, 1992).

To understand the biological potential of lignocellulosic materials, it is important to understand the complex structure of the cell walls and the physico-chemical characteristics of the three most important cell wall components: cellulose, hemicellulose and lignin.

2.6.1 Cellulose

Cellulose is the main polymeric component of the plant cell wall and is the most abundant polysaccharide on earth. It is composed of repeating disaccharide units of cellobiose. Cellulose fibers are highly stable homopolymer chains of up to 12,000, beta-1-4 linked glucose units (a six-carbon sugar). In its native state, cellulose chains are held together laterally by intermolecular hydrogen bonds while intramolecular hydrogen bonds and Vander Waal forces also form between glucose units of the same chain (Dey and Brinson, 1984).

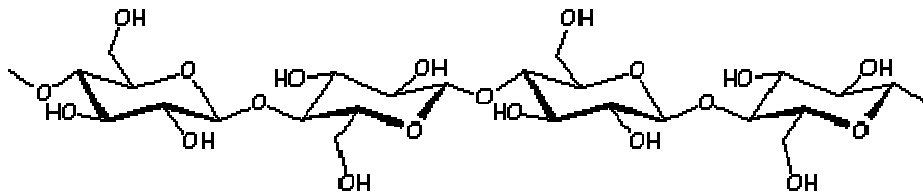


FIG. 4.0 Structure of the Cellulose Chain (Atalla, 1993)

The additive effect of the bonding energies of the hydrogen bonds increases the rigidity of cellulose and causes it to be highly insoluble as well as highly resistant to most organic solvents. The cellulose chains further aggregate into alternating highly ordered regions and amorphous regions to form fibrils (Sjostrom, 1993). Thirty to forty parallel cellulose

chains associate to form microfibrils with crystalline and amorphous part. Various purified forms of celluloses such as avicel, solka floc, carboxymethylcellulose, have been used as carbon sources for cellulase production (Esterbauer *et al.*, 1991; Persson *et al.*, 1991; Domingues *et al.*, 2000), but their price is too expensive to be applied in large scale processes. The simplicity of the cellulosic structure using repeated identical bonds, means that only a small amount of enzymes are required to degrade this material. The structure of the cellulose will be partly dependent on the source of the cellulose. These long non-branched chains are deposited in an anti-parallel arrangement and bound laterally through numerous hydrogen bonds. The stereo chemistry of the glucose units in cellulose chains, with each glucose moiety being translated 180° to its neighbour permits three hydrogen bonds per residue between each adjacent chain (McAloon *et al.*, 2000). This highly ordered three dimensional structure confers the mechanical strength to cellulose and also results in its low susceptibility to chemical and enzymic attack (Walker and Wilson, 1991).

2.6.2 Hemicellulose

Hemicellulose is an amorphous biopolymer consisting of a mixture of 5-carbon and 6-carbon sugars such as xylose, mannose, glucose, arabinose, galactose and uronic acids (Magge and Kosaric, 1985). Xylose, mannose and galactose are found in the hemicellulose backbone while arabinose, glucuronic acid and galactose form the side chain. This polymer is more soluble than cellulose with a degree of polymerization of less than 200 (Cowling and Kirk, 1976). Hemicelluloses that contain acids in their side chains are slightly charged and water soluble. Hemicelluloses cross-link with lignin creating a complex web of bonds which provide structural strength, but also challenge microbial degradation (Lynch, 1992).

There are many different types of hemicelluloses like xylans, mannans, glucans, galactans, and galacturonans in plants depending upon the main sugar residues in the backbone (De Vries and Visser, 2001). Xylans and mannans are the two most important groups of hemicelluloses present in lignocellulosic materials. Xylans are the most common hemicellulose and the main non-cellulose polysaccharides of monocotyledons.

2.6.3 Lignin

Lignin is the third main component of the biomass cell wall and is the non-carbohydrate of the fibre (Anderson *et al.*, 1994). It is difficult to isolate because of its highly variable nature (Dekker, 1988). As part of the microfibrillar structure, lignin acts like a glue by filling the spaces between and around cellulose and hemicellulose and complexing with the polymers. One of the primary functions of lignin is to provide structural support for the plant. Grasses have lower lignin contents than trees which make them more vulnerable to biodegradation because the lignin which contains no sugars ironically encloses the cellulose and hemicellulose molecules of the tree (Perez *et al.*, 2002). The presence of lignin therefore greatly limits accessibility to the cellulose and hemicellulose molecules. Lignin is a complex polymer of phenylpropane units cross-linked with each other-by carbon – carbon and ether bonds (Sodhi, 2005). Degree of lignification and crystallinity coupled with the capillary structure of cellulose are intrinsic features known to inhibit their degradation or and bioconversion (Solomon *et al.*, 1999). Lignin is insoluble in water and has hydrophobic binding capacity, nonetheless, some organisms particularly fungi such as white rot fungus and Actinomycetes have developed the necessary enzymes to break the lignin apart (Basaglia *et al.*, 1992). Because lignin is the most recalcitrant component of the plant cell wall, the higher the proportion of lignin, the

lower the bioavailability of the substrate (Haug, 1993). In order to depolymerize the lignin 'core' of any lignocellulosic, the chemical bonds like C-C and C-O-C (ether) bonds between lignin precursors needed to be disrupted by, for example high temperature chemical treatment (Chua and Wayman, 1979b) or oxidative treatments (Gould, 1984) or. Lignin degradation is primarily an aerobic process and in an anaerobic environment can persist for very long periods (Van-Soest, 1994).

2.6.4 Lignocellulosic Pretreatment

The purpose of pretreatment is to break down the lignocellulosic structure to its monosaccharide components for use as fermentation substrates. Pretreatment of cellulose opens up the structure and removes secondary interaction between glucose chains (Tang *et al.*, 1996). Pretreatment refers to the solubilization and separation of one or more of the four major components of biomass – hemicellulose, cellulose, lignin and extractives to make the remaining solid biomass more accessible to further chemical or biological treatment. The three main factors on the ease of lignocellulose breakdown to fermentable monosaccharides are the cellulose crystallinity (Goldstein, 1983), pore size (Grous *et al.*, 1986), and the removal of lignin (Dekker, 1988). According to McMillan (1994), enhanced cellulose accessibility can be achieved by hemicellulose removal because the relative ease of hemicellulose hydrolysis provides an ideal avenue for creating larger pores in the microfibrils.

Many physical, chemical and enzymic pretreatment methods for enhancing bioconversion of cellulosic materials have been reported (Wu and Lee, 1997; Kansoh *et al.*, 1999; Solomon *et al.*, 1999). Among all the methods that have been adopted for treatment of

cellulosic wastes, enhanced biodegradation by genetically engineered microorganism has been regarded as the most appealing one (Lynd *et al.*, 2002). Although the types of enzymes expressed by these microorganisms are very similar, the actual mechanism of degradation of the crystalline cellulose may be different for the different enzyme systems. Only a few fungal and bacterial species have been demonstrated to produce cellulases which bear activity high enough to extensively degrade insoluble cellulose to soluble sugars *in-vitro*. So many researchers have studied cellulose degradation to glucose, as glucose could easily be converted to many chemicals. Catabolism of cellulose involves both enzymic depolymerization of insoluble cellulose and cellular utilization of the hydrolytic products. Cellulose hydrolysis requires prior binding of enzymes to cellulose, either as an enzyme – substrate binary complex or as a cellulose – enzyme – microorganism ternary complex (Bae *et al.*, 2004) as displayed by *Cellulomanas* species strain NRCC 2406. Considerable cellulase activity is located at the surface of growing hyphae of *T. reesei* (Busto *et al.*, 1996) and growth on corn cobs resulted in the formation of a biofilm which presumably maintained the contact between the fungal hyphae and the cellulose (Tengerdy *et al.*, 1991). In one study, cellulolytic activity and the production of $^{14}\text{CO}_2$ from carbon -14 of cellulose decreased with soil depth (Vardavakis, 1989), suggesting that cellulose utilization is largely an aerobic process. While cellulolytic species may compete directly for cellulose (Odenyo *et al.*, 1994; Shi *et al.*, 1997), both cellulolytic and non-cellulolytic species can compete for cellodextrin products of cellulose hydrolysis, in cross-feeding nutrients and in the production of inhibitory compounds. In nature, cellulose, hemicellulose and lignin are indispensable in the carbon cycle. Each polymer is

degraded by a variety of microorganisms which produce a battery of enzymes that work synergistically (Perez *et al.*, 2002).

Physical pretreatment breaks down the feedstock size by milling and thus opening up the material for enzymatic hydrolysis (Fahey *et al.*, 1992).

The most common chemical pretreatment methods employed during the production of cellulosic feed stocks make use of dilute acids, alkaline and ammonia in order to make the biomass more digestible by the enzymes (Tang *et al.*, 1996; Ladisch and Lee, 2003). Biological pretreatments (enzymic) are sometimes used in combination with chemical treatments to solubilize the lignin in order to make cellulose more accessible to hydrolysis and fermentation (Malherbe and Cloete, 2003). Pretreatment of cellulosic biomass in a cost effective manner is a major challenge of cellulase technology research and development.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Samples

Soil samples were taken from six locations in Zaria. The samples were collected from rice growing field, compost soils, street soil, fallow farm land, flower beds and maize farm. Sterile hand trowel was used to dig to the depth of about 5-10cm from the soil surface. A weight of 10 gram of each soil sample was collected with the aid of sterile spatula and sterile polypropylene bag and labeled appropriately. Razak *et al.* (1999) reported the genus *Aspergillus* to be very abundant in this region.

3.2 The Test Organism

Strains of *Aspergillus niger* employed for this work were isolated from the various soil environments collected from the six experimental locations. The *Aspergillus niger* strains were obtained using the dilution plate count technique described by Dhingra and Sinchair (1995). Ten gram (10g) quantities of the soil samples were suspended in 90ml of sterile distilled water (SDW) and a series of ten fold dilution were made. From the dilutions, 0.5ml volumes were pipetted onto Czapek Dox Agar (CDA) (Oxoid) plates. A volume of 30µg/ml of streptomycin sulphate solution was added to each plate and the plates were swirled and then incubated at ambient temperature for ten days. The streptomycin was added in order to suppress bacterial growth and allow the easy development of *Aspergillus* species with characteristic colonies with pigmentation and conidial heads which fitted the descriptions of Raper and Fennel (1965). The mixed cultures obtained were purified on fresh CDA plates by single spore inoculation technique. Colonies on CDA that appeared closely velutinous with thin white basal mycelium, almost completely submerged with conidial structure in dark brown shades and which appeared black *en-masse* to the un-aided eye were selected. Slide culture technique according to the method of Johnson and Case (1986) was performed to confirm the isolates. Only those possessing biseriate sterigmata with upright conidiophore and phialides at the apex (unique for *Aspergillus niger*) microscopically were selected based on the current universal keys of identification (Hawksworth *et al.*, 1995). Further confirmation of the identities of the isolates was done by the Department of Crop Protection, Institute of Agriculture Research, I.A.R; Ahmadu Bello University, Zaria. Pure stock cultures of the *Aspergillus niger* isolates were stored on slants of Potato Dextrose Agar (PDA) and kept at 4⁰C. Subcultures were made onto fresh PDA slopes monthly.

3.3 Lignocellulosic Materials

The lignocellulosic biomass, or substrates, used for the study were corn cob (CC), corn straw (CS) and sugarcane bagasse (SB). The materials were quite abundant, readily sourced and renewable. The corn cob and corn straw were obtained from harvest waste dump site of IAR, A.B.U. research farm Shika. The bagasse was obtained from the streets in Samaru-Zaria. The dried substrates were chopped into bits, pulverized into coarse particle sizes and then washed in several changes of hot water in order to remove the residual sugars as described by Rezende *et al.* (2002). The said experimental substrates were sun-dried for a period of three weeks and subsequently oven-dried slowly at 50⁰C for two days. One kilogram (1Kg) of each of the dried substrates was ball milled for ten minutes using Christy Laboratory Mill (Christy and Norris, Ltd England) in the Department of Food Technology, Kaduna Polytechnic, Kaduna. The ball milled substrates were individually screen analyzed to pass through 0.8mm (850 μ) sieve. The pulverized materials were packaged in sterile polypropylene bags and labeled appropriately.

3.4 Preparation of Standard Reagents

3.4.1 Preparation of Dinitrosalicylic Acid Regent

The preparation was based on the original protocol by Miller, (1959) using the following steps:

1. The dinitrosalicylic acid (DNS) was prepared by adding 10.6g of 3, 5-dinitrosalicylic acid powder to 1416ml of sterile distilled water. A weight of 19.8g of sodium hydroxide pellet was then added and the whole contents were subsequently mixed thoroughly.

2. Rochelle salts, weighing 306g was measured while 7.6ml of phenol crystal melted at 50⁰C and 8.3g of sodium metabisulphite were added in sequence to 1 above. The solution was mixed thoroughly and kept in an amber bottle wrapped with aluminium foil paper and stored away from light at room temperature of 32⁰C.

3.4.2 Preparation of 0.05m Citrate Buffer, pH 4.8

To one liter of deionised water were dissolved 10.5g quantities of citric acid monohydrate in one litre of deionised water and labeled as (1). To another liter of deionised water were dissolved 14.71g quantities of sodium citrate and labeled as (2). The pH of (2) was adjusted to 4.8 with (1) above (about 667ml of citric acid monohydrate solution was required per litre of sodium citrate). The pH was checked using the digital pH meter (Hanna, U.K).

3.4.3 Preparation of Somogyi-Nelson Reagent

The method of Nelson (1944) was adopted which include:

SOMOGYI REAGENT

a. Somogyi I: The Somogyi I reagent was made by dissolving 288g quantities of anhydrous sodium sulphate in one liter of boiled distilled water. To this solution was dissolved 24g of Rochelle salts, 48g of sodium carbonate and 30g of sodium bicarbonate in that order. The solution was made up to 1600ml with boiled distilled water and the preparation was stored in a labeled amber bottle at room temperature of 32⁰C.

b. Somogyi II: The Somogyi II reagent was prepared by dissolving 72g of sodium sulphate and 8g of copper sulphate in 300ml of boiled distilled water. The solution

was made up to 400ml with boiled distilled water and stored in a labeled amber bottle at room temperature of 32⁰C

NELSON REAGENT

This reagent was made up of 0.04M ammonium molybdate (49.43g of salt in 480ml of distilled water); 0.02M of sodium hydrogen arsenate (62.4g of the salt in 480ml of distilled water) which were mixed together to give a volume of 960ml. Aliquots of 40ml of concentrated sulphuric acid was added to the preparation to make up one litre and mixed thoroughly to give a homogeneous solution

3.4.4 Preparation of 0.003 M Hydroxylamine Solutions

The method of Freese *et al.*(1961) was adopted which is as follows:

Hydroxylammonium chloride solution of 1M was obtained by dissolving 69.49g of the salt in one litre of distilled water. Therefore, to obtain 0.003M solution, 0.2085g of the hydroxylammonium chloride salt was dissolved in a buffer containing: 17.7g of Na₂HP0₄.12H₂O ; 3g of KH₂PO₄ ; 0.001M of MgSO₄.7H₂O (0.2463g salt per litre), and one litre of distilled water. The pH was adjusted to 7.5 with concentrated (1M) sodium hydroxide solution using Hanna digital pH meter as the guide. The preparation was labeled and stored at refrigeration temperature of 4⁰C.

3.4.5 Preparation of Acridine Orange Solution, 200µg/ml.

The modified method of Zaldivar *et al.*, (2001) was used in the preparation of this solution which follows thus: Aliquots of 0.2g of acridine orange dye (Sigma) was weighed and dissolved in 10ml of sterile deionised distilled water. This solution whose

concentration is expressed as 200 μ g per milliliter was stored in a medical bottle, labeled appropriately and stored at refrigeration temperature of 4⁰C.

3.4.6 Preparation of Bradford Reagent

The method of Bradford (1976) was adopted which was as follows: Aliquots of 0.3g of Coomassie brilliant blue G was weighed and dissolved in 150ml of 95% ethanol and 300ml of 85% phosphoric acid was added to the mixture and mixed thoroughly. This stock reagent solution was stored in an amber bottle and kept at refrigeration temperature. To obtain working solution, 15ml of the stock was always measured out and diluted to 100ml with sterile distilled water. The solution was always filtered through Whatman No. 1 filter paper into an amber bottle and stored at room temperature of 32⁰C.

3.4.7 Preparation of the Various Concentrations of Sulphuric Acid and Sodium Hydroxide Used for the Chemical Pretreatment

Aliquots of 0.5M sulphuric acid solution (H₂SO₄) was prepared by adding gradually 1 litre of distilled water to 27.1ml of concentrated H₂SO₄ (BDH) contained in a two litre flask, while for the sodium hydroxide solution (NaOH), 0.5M was obtained by dissolving 20 g of NaOH (BDH) pellets in one litre of distilled water. To prepare 2M concentrations of H₂SO₄ one litre of distilled water was gradually added to 108.7ml of concentrated H₂SO₄ contained in a two litre flask while 2M NaOH solution was prepared by dissolving 80g of the pellets in 1 litre of distilled water

3.5 Qualitative Determination of Cellulolytic Activity

From the various isolates obtained, screening for cellulolytic *A. niger* strains was made in a synthetic modified Mandels mineral agar medium (Mandels *et al.*, 1974) which served as the selective and expression medium. The screening medium consisted of (gL⁻¹) yeast extract 0.2, carboxymethyl cellulose 10, peptone 1, (NH₄)₂SO₄ 4, KH₂PO₄ 2, Urea 0.3, MgSO₄·7H₂O 0.3, CaCl₂ 0.3, FeSO₄·7H₂O 0.5, MnSO₄·4H₂O 0.16, ZnSO₄ 0.14, CoCl₂ 2, L-Sorbose 6, Congo red 0.025, Tween -80 0.1%, Triton – X100 0.1% and Agar 17.5. The pH was adjusted to 6 using 0.05M sodium hydroxide, and then sterilized at 121⁰C for 15 minutes. The medium was dispensed into petri plates, allowed to gel and then seeded with spores from the isolates. The cultures were incubated at ambient temperature of 32⁰C for 90 hours; thereafter they were transferred to an incubator set at 50⁰C for 18 hours. The cultures were flooded with 1M NaCl solution. Cellulolytic species had a clear zone around the colony on a reddish pink agar background in form of a halo.

Further confirmation of the cellulolytic species was done using the Congo red assay technique as described by Teather and Wood (1982). The medium comprised (w/v): ball-milled filter paper 0.5%, Congo red powder 0.1%, Agar 2%, pH adjusted to 6. The cellulolytic species showing zone of clearing around the colony on red background were selected and stored on slopes of PDA at 4⁰C.

3.6 Induction of Mutants

Conidia of cellulolytic *Aspergillus niger* strains were grown for one week on PDA plates and used for the mutation studies.

3.6.1 Ultra-Violet (U.V) Mutagenesis

A modification of the methods of Duru and Uma (2003) was used for the irradiation. Five millilitres of 0.1% Tween 80 in sterile distilled water (Jorgensen, *et al.*, 2003) was added onto the surface of the wild cellulolytic cultures and sterile inoculating loop was used to tease the spores to form a suspension, which was collected into a sterile test tube. The spore suspension was concentrated in the test tube with the aid of the centrifuge at 8000rpm for five minutes. The concentrated spores were re-suspended in 10ml of the sterile Tween 80 solution and 4ml quantities aseptically pipetted into sterile petri plates of 100mm diameter.

The exposure to U.V was done in a dispensing cabinet fitted with 50Hz, U.V germicidal lamp at short wavelength of 254nm. The exposure was carried out at a distance of 20cm away from the center of the U.V light source. The exposure times were varied for 5, 10, 15, 20, 25, 30, 35 and 40 minutes (Plate IV). Controls were left un-irradiated. Each U.V exposed spore suspension was stored in dark overnight to avoid photo-reactivation. Surviving conidia were grown on plate cloning medium described by Morikawa *et al.*, (1985). It consisted of PDA with 0.1% Triton-X100, and 4g/Litre of L-Sorbose added as colony restrictors. The inoculated plates were incubated at room temperature for four days. Mutant clones were subjected to three bouts of mutations using surviving conidia from previous U.V treated cells. To determine the success or otherwise of the mutations by ultra-violet irradiation, mutated conidia were replicated on plate screening medium described by Mandels *et al.*, (1974) (section 3.5), and the plates incubated at ambient temperature for three days, followed by 18 hours at 50⁰C. The cultures were removed from the incubator and flooded with 1M NaCl solution to remove unbounded excess dye (Howard *et al.*, 2003). Hypercellulolytic mutants were selected based on the diameter of the zone of

hydrolysis (measured in millimeter) surrounding the colonies in comparison with the controls (wild). Mutants were stored in slopes of PDA and kept at 4⁰C until required for further analysis. To avoid photo reactivation, mutants were wrapped in aluminum foil paper.

3.6.2 Chemical Mutagenesis Using Acridine Orange and Hydroxylamine

The acridine mutagen solution was prepared as described in 3.4.5 to give a concentration of 200µg per milliliter. A volume of 5ml of 0.1% Tween 80 in sterile distilled water was added onto the surface of the wild cellulolytic cultures on the PDA plates. With the aid of a sterile inoculating loop the spores of the culture were loosened to form a suspension, which was collected in a test tube and concentrated using centrifuge at 8000 rpm for five minutes. The concentrated spores were re-suspended in 5ml of the mutagen solution and then transferred into sterile bijou bottles. The mixtures were incubated at 37⁰C overnight and then subsequently cloned on plate cloning medium described by Morikawa *et al.* (1985). The inoculated plates were incubated at room temperature of 32⁰C for four days. Conidia from the mutant clones were further subjected to three cycles of mutation using surviving conidia from previous acridine orange treated cells. To determine the success or otherwise of the mutations using acridine orange, cloned conidia were replicated on modified plate screening medium described by Mandels *et al.* (1974) in 3.5 above and the plates were treated the same way as that described for mutations using U.V. radiation. Controls (wild) were not introduced into the mutagen solution. Mutants were stored on PDA slopes and stored at 4⁰C until required for further analysis.

Hydroxylamine does come in the form of hydroxylammonium chloride. Therefore to obtain hydroxylamine from hydroxylammonium chloride, the salt was reacted with 1M NaOH solution using the method of Freese *et al.* (1961). The hydroxylamine mutagen solution was prepared to give a concentration of 0.003M as described earlier in section 3.4.4 above. Five milliliters of 0.1% Tween 80 in sterile distilled water was added onto the surface of the wild cellulolytic cultures on PDA plates with sterile inoculating loop was used to loosen the spores to form a suspension which was collected in a sterile test tube and concentrated using digital centrifuge at 8000rpm for five minutes. The concentrated spores were re-suspended in 5ml of the 0.003M mutagen solution and then transferred aseptically into sterile bijou bottles. The mixtures were incubated at 37⁰C for 18 hours and then subsequently cloned on plate cloning medium described by Morikawa *et al.* (1985). The inoculated plates were incubated at room temperature of 32⁰C for four days. Conidia from the mutant clones were further subjected to three cycles of mutation using conidia from previous hydroxylamine treated cells. To determine the success or otherwise of the mutations using hydroxylamine, cloned conidia were replicated on modified plate screening medium described by Mandels *et al.* (1974) as in 3.5 above and the plates incubated at ambient temperature (32⁰C) for three days, followed by 18 hours at 50⁰C. The cultures were removed from the incubator and flooded with 1M NaCl. Hypercellulolytic mutants were selected based on the diameter of the zone of hydrolysis (in mm) surrounding the colonies in comparison with the controls (wild) (Plate V). Controls were not introduced into the mutagen solution. Mutants were stored on slopes of PDA and kept at 4⁰C until required for analysis.

3.7 Substrate Pretreatment

3.7.1 Particle Size Screen Analysis

Fifty grams of each of the pulverized materials (corn cob, corn straw and sugarcane bagasse) that were sifted through 0.8mm (850 μ) sieve were further screen analyzed into two particle sizes (210 μ and 500 μ) using Endecotts test sieve (Griffin and George, Edinburgh). The purpose was to evaluate the effect of particle size of each of the substrate on the yield of cellulase in both wild and mutant strains of *Aspergillus niger* strains used for this work.

3.7.2 Chemical Pretreatment of Substrates

The balled milled, 850 μ sieved, samples were used as the standard to assess the effect of both acid and alkali pretreatments on the cellulase yield.

3.7.2.1 Acid and Alkali Pretreatment of the Cellulosic Substrates

The effect of acid pretreatment of the lignocellulosics on the enzyme yield was tested by soaking the pulverized samples in 0.5M and 2M sulphuric acid solution for varying residence time in conical flasks which served as the digester. The procedure was repeated for alkali pretreatment using sodium hydroxide solution at 0.5 M and 2M concentrations. Four types of treatment were applied for each substrate and the summary is presented in Table 3.0

Table 3.0. Summary of the Pretreatments Using Sulphuric Acid and Sodium Hydroxide

Serial Number	Type of Agricultural Wastes	Physical State and Quantity	Concentrations of NaOH or H ₂ SO ₄ used (100ml)	Treatment Exposure Time (Hours)
1	CC	P(850μ), 20g	0.5M	1
2	CC	P(850μ), 20g	0.5M	3
3	CC	P(850μ), 20g	2M	1
4	CC	P(850μ), 20g	2M	3
5	CS	P(850μ), 20g	0.5M	1
6	CS	P(850μ), 20g	0.5M	3
7	CS	P(850μ), 20g	2M	1
8	CS	P(850μ), 20g	2M	3

9	SB	P(850 μ), 20g	0.5M	1
10	SB	P(850 μ), 20g	0.5M	3
11	SB	P(850 μ), 20g	2M	1
12	SB	P(850 μ), 20g	2M	3

CC = corn cob

CS = corn straw

SB = sugarcane bagasse

P(850 μ) = pulverized and screened to 850 μ particle size

After the soaking period had lapsed, sufficient water was added to each Erlenmeyer flask to dilute the acid or the alkali as the case may be and the digested samples recovered by filtration. The particles were returned into the flasks and repeatedly washed with sterile distilled water until the pH of the wash water was neutral, then dried overnight at 60⁰C using the method described by Fan *et al.* (1982) and Rajoka and Malik (1997). The samples were packaged in polypropylene bags and labeled appropriately. A comparison was also made with undigested samples of each substrate.

3.8 Proximate Analyses of the Substrate Samples

Each of the pulverized samples that had been screened with sieve to 850 μ particle size was analyzed proximately using the AOAC (1990) method, for dry matter, crude protein, crude fiber, crude fat, ash content and nitrogen free extracts as given below:

3.8.1 Dry Matter Content

Five grams of each sample was weighed onto a flat aluminium dish and dried to constant weight at 100⁰C in a draught air oven (Memmert GmbH, Germany). The moisture content percent was determined by the relation:

$$\frac{\text{Weight of fresh sample} - \text{Weight of dry sample} \times 100}{\text{Weight of fresh sample}} = \% \text{ moisture content}$$

Therefore the dry matter percent was evaluated by subtracting the moisture content % from 100. (100 – Percentage moisture content)

3.8.2 Crude Protein

One gram of each sample was weighed into a digestion flask and potassium sulphate, 10g; mercuric oxide, 0.7g and concentrated sulphuric acid, 20ml added. The flask was heated gently at an inclined angle until frothing subsides and then boiled until the solution clears. It was continued for half an hour. When frothing was excessive, a small amount of paraffin wax was added. On cooling, 90ml of distilled water was added, and mixed. A small piece of pumice was added to prevent ‘bumping’ and 80ml of 2M sodium hydroxide solution added while tilting the flask so that two layers were formed. The condenser unit was rapidly connected, heated and the distilled ammonia was collected in

50ml boric acid / methyl red indicator. Fifty milliliters of the distillate was collected and titrated against 0.1M hydrochloric acid solution. The nitrogen content percent was calculated by using the relation:

$$\frac{(\text{Volume of acid} \times \text{Molarity of standard acid}) \times 0.014 \times 100}{\text{Weight of sample (g)}}$$

$$\text{Crude protein content \%} = \text{nitrogen content} \times 6.25$$

3.8.3 Ash Content

Two grams of the dry sample was weighed into a dry porcelain dish and then placed in a muffle furnace at 600⁰C for 6 hours. It was cooled in a desiccator and then weighed. The percent ash content was calculated using the relation:

$$\frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

3.8.4 Crude Fibre

Two gram of the dried sample was weighed into a 600ml beaker, and 200ml of hot sulphuric acid was added. The beaker was placed under the condenser and brought to boiling within 1 minute. It was boiled gently for exactly 30 minutes using distilled water to maintain volume and to wash down particles adhering to the sides. Antifoam (octyl alcohol) was used where necessary. It was filtered through Whatman No. 541 paper in a Buchner funnel using suction and subsequently washed well with boiling water. The residues were transferred back into the beaker and 200 ml of hot sodium hydroxide added. The condenser was replaced again and brought to boil within one minute. After exactly 30

minutes of boiling, it was filtered through porous crucible and then washed with boiling water, 1% hydrochloric acid and then again with boiling water. It was washed twice with alcohol and dried overnight at 100⁰C, cooled and then weighed to determine the quantity. The dried material was ashed at 500⁰C for three hours, cooled and then weighed. The crude fiber (% free-fat dry matter) was calculated by the formula:

$$\frac{(\text{Wt. of crucible + dried residue}) - (\text{Wt. of crucible + ashed residue})}{\text{Weight of sample}} \times 100$$

3.8.5 *Crude Fat*

Two gram of the dried sample was weighed into an extraction thimble and the thimble placed inside the soxhlet apparatus with a dry tared solvent flask placed in position beneath. The required quantity of ether solvent was added and then connected to the condenser. The heating rate was adjusted to give a fast condensation rate per second and was extracted for 6 hours. On completion, the thimble was removed and the ether reclaimed. Complete removal of the ether was accomplished by boiling on a water bath and subsequently drying at 105⁰C for 30 minutes. The preparation was cooled in a desiccator and weighed. The crude fat (% of dry matter)

$$= \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

3.8.6 *Nitrogen Free Extract*

The nitrogen free extract (NFE) was calculated from the relation:

100 – (% crude protein - % crude fat - % crude fiber - % ash) all on a dry matter basis

3.9 Inoculum Preparation

Pure cultures of *Aspergillus niger* strains of both wild and the best mutant from slopes were grown on PDA plates at ambient temperature for 5 days and 100ml of 0.1% Tween 80 was used to wash and resuspend the spores in sterile 100ml capacity Erlenmeyer flasks. The spore suspension was diluted to concentrations of 1.0×10^4 , 1.0×10^6 and 1.0×10^8 cells per ml by counting in Neubauer chamber using the method of Adhikari and Shrestha (1989). The inoculum ratio was 10% v/v of the entire volume. Inoculations into the fermentation flasks containing the mash were achieved using sterile pipettes.

3.10 Preparation of Substrate for Fermentation

The production of extracellular enzymes in all treatments using the carboxymethyl cellulose and crystalline cellulose (corn cob, corn straw and bagasse) for all fermentation runs were carried out in submerged cultures using the wild type isolate and the selected mutant strains in batch units based on the method of Liming and Xueliang (2004). The substrates were fermented in Mandel's medium as proposed by Mandels *et al.* (1981) with the addition of ten gram per litre of the appropriate carbon source. The medium consisted of (g L^{-1}): yeast extract, 0.2; peptone, 1; $(\text{NH}_4)_2 \text{SO}_4$, 4; KH_2PO_4 , 2; Urea, 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3, CaCl_2 , 0.3; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{Mn SO}_4 \cdot 4\text{H}_2\text{O}$, 0.16; ZnSO_4 , 0.14; CoCl_2 , 2; Tween-80, 0.1% and pH adjusted to 6 using 0.05M sodium hydroxide solution. The medium was divided into batches of 100ml into 250ml Erlenmeyer flasks and cotton plugged which served as the fermentors. The media were sterilized at 121°C for 15 minutes, allowed to cool and inoculum ratio as obtained in 3.9 seeded aseptically into the flask. Fermentations were performed in a rotary shaker (Bellco Glass Inc. USA) at ambient

temperature of 32⁰C and 350rpm. During the growth process, samples were withdrawn for analysis of cellulase every 24 hours throughout the growth phase using the supernatant of centrifugates until enzyme activity peaks off. The pH was also recorded at each time.

3.11 Effect of Inoculum Size, pH and Temperature on Extracellular Cellulase Enzyme Production by Strains of the Test Organism in Submerged Shake Flask.

3.11.1 Effect of Inoculum Size on the Cellulase of the Test Organism

Baseline study of the effect of inoculum size on cellulase yield from the carboxymethyl cellulose was evaluated by seeding 10ml each of the inoculum obtained by dilutions (1.0×10^4 , 1.0×10^6 and 1.0×10^8 cells/ml) as described above into the already sterilized fermentation medium (Mandels *et al.*, 1981). Cultivations were carried out in a 250ml capacity Erlenmeyer flask to which has been dispensed 100ml volume of the medium and 10g per litre of the carboxymethyl cellulose added.

3.11.2 Effect of pH on the Cellulase of the Test Organism

Preliminary study on the effect of pH on the enzyme yield from both wild and mutant strains were determined by using carboxymethyl cellulose. This was to determine the pH at which cellulase yield would be optimum. Into 250ml Erlenmeyer flask to which has been dispensed 100ml volume of the Mandels medium (Mandels *et al.*, 1981), 10g/L of the carboxymethyl cellulose was added to each of the six flasks (three each for the wild and the mutant) and the pH adjusted to either 4.8, 5.0 or 7.4 using either base (0.1M NaOH) or acid (0.05M H₂SO₄) as the case may be. The medium was sterilized at 121⁰C for 15 minutes, allowed to cool and 10ml volume of inoculum seeded and fermentation started

as described above. Samples were withdrawn for analysis of cellulase and pH measurement at every 24 hours.

3.11.3 Effect of Temperature on the Cellulase of the Test Organism

The effect of different incubation temperatures (30⁰C, 40⁰C, 50⁰C, 60⁰C, 70⁰C, and 80⁰C) on the residual activity of the crude enzyme obtained from the carboxymethyl cellulose was studied by incubating 0.5ml of the four day old fermenting culture supernatant with a rolled (1cm by 1cm) Whatman filter paper strip in a preset waterbath and for each temperature allowed to equilibrate for one hour after which the appropriate assay was applied.

3.12 Cellulase Production on Amorphous Cellulose Using Wild and Mutant Strains

Having obtained the optimum pH value for cellulase production, growth studies were then carried out using carboxymethyl cellulose as sole carbon source for both the wild strain and the best mutant selected using ANOVA statistical analysis. The initial pH value of the medium was adjusted to the optimum, the medium sterilized and the inocula seeded into the flasks and growth begun at ambient temperature of 32⁰C. Samples were withdrawn for analysis of cellulase at 24 hour interval.

3.13 The Effects of Substrate Particle Size on the Rate of Cellulase Production by the Strains of the Test Organisms

To assess the cellulase production level on the corncob, corn straw and bagasse, the pulverized carbon sources screened to 210 μ , 500 μ and 850 μ particle sizes were added

individually to batches of basal medium (Mandels *et al.*, 1981) to give a cellulose level of 10g per litre. The pH was adjusted to optimum, the flasks autoclaved and thereafter inoculated with the wild and the mutant strains and then incubated at the appropriate temperature for growth to take place. Samples of each flask were taken aseptically at 24 hours interval for a period of 170 hours, as mentioned above, throughout the growth phase, centrifuged and the clear supernatant was used for enzyme measurement.

3.14 Cellulase Production by Strains of the Test Organism on Pre-Treated Crystalline Cellulose

The effect of the substrate pre-treatment using acid and alkali at two different concentrations (0.5M and 2M) for two different residence timings (1hr and 3hrs) was studied. The wild and the mutant strains were each inoculated into 10g/L of the processed pre-treated lignocellulosic substrates previously added to batches of the basal medium (Mandels *et al.*, 1981) as described earlier. The pH of the medium was adjusted to optimum and the flasks with the contents autoclaved, thereafter fermentation begun as described earlier. Samples of each flask were taken aseptically at regular interval (24hours) throughout the growth phase until the enzyme activity peaks off (between 110 and 170 hours) centrifuged and the clear supernatant was used for the enzyme assay.

3.15 Enzyme Assay

3.15.1 Saccharifying Cellulase Activity Assay

The total cellulase activity, termed saccharifying cellulase, was determined for all the substrates used using filter paper as the substrate as proposed by Ghose (1987). This

method estimates overall cellulolytic activity. It was assayed by incubating 0.5ml of each culture supernatant with a rolled '1cm by 6cm' filter paper strip (Whatman No. 1) (Whatman, UK) in one millilitre (1ml) of 0.05M citrate buffer (pH 4.8) contained in test tube at 50⁰C for 60 minutes. Whatman No.1 filter paper was used as a substrate in this regard because it is widely available and very similar to 'real' process substrate – not too resistant, and not too susceptible (Mandels *et al.*, 1976). Each assay tube was removed from the 50⁰C water bath (Grant J.B. Series, U.K) and the enzyme reaction stopped by addition of 3.0ml Dinitrosalicylic acid (DNS) reagent prepared earlier. The tubes were boiled vigorously for five minutes in a boiling water bath containing sufficient water to cover the portions of tubes occupied by the reaction mixture plus the reagent. Thereafter, the tubes were transferred to cold water bath and each diluted subsequently with 2.5millilitre of sterile distilled water. The absorbance was determined using U.V spectrophotometer (Cecil 1000 series, Cambridge England) against the reagent blank at 540nm and the quantity of reducing sugar read from a glucose standard (Appendix 1). Cellulase activity was calculated and expressed in International Units (IU) as described by Janas *et al.* (2002); and Ul-Haq *et al.* (2002) using Whatman No 1 filter paper and applying the following formula:

$$\text{IU/ml/min} = \frac{\text{Activity of enzyme}}{\text{Molecular weight of glucose} \times \text{incubation time.}} \times 100$$

One unit of cellulase corresponded to the amount necessary to form 1 milligram (1mg) of glucose per minute at 50⁰C.

3.15.2 Endoglucanase, Exoglucanase and Beta-Glucosidase Activity Assays

The endoglucanase activity assay (or carboxymethyl cellulase test) was determined from the wild and mutant strains by the method of Mandels *et al.* (1976) using carboxymethyl cellulose. To 0.5ml of 0.05M citrate buffer (pH 4.8) in a test tube was added 0.5ml of 4 gram carboxymethyl cellulose per litre (0.4% W/V) (Zaldivar *et al.*, 2001). Thereafter 0.5ml of the centrifuged culture supernatant was added and incubated at 37⁰C for 6 hours. The test tubes were transferred to water bath and equilibrated at 50⁰C for 10 minutes. The Somogyi-Nelson method (Nelson, 1944) was applied to assay for the reducing sugars. 2ml mixture of Somogyi I and II (that is, 4ml Somogyi I + 1ml Somogyi II and mixed immediately before use) was added to stop the reaction and the solution boiled in a water bath for 15 minutes and then subsequently cooled in a cold bath. Two millilitres of Nelson reagent was added and the contents of the tubes mixed carefully. Each tube was diluted with 4ml of distilled water and further mixed by inversion. The absorbance was determined using U.V. spectrophotometer (Cecil 1000 series) against the reagent blank at 520nm and the quantity of reducing sugar read from a standard curve (Appendix II). The activity was calculated and expressed in International Units (IU), using the relations in 3.15.1. One unit of endoglucanase corresponded to the amount necessary to form 1 milligram (1mg) of glucose per minute at 50⁰C.

The exoglucanase activity assay (or filter paper assay) was achieved by the method described by Mandels *et al.* (1976) using filter paper. To 0.5ml of 0.05M citrate buffer (pH 4.8) in a test tube was immersed 0.5g rolled filter paper. The buffer saturated filter paper was added 0.5ml of the centrifuged culture supernatant and incubated in water bath at 50⁰C for 60 minutes, after which the Somogyi – Nelson method (Nelson, 1944) was applied to

assay the reducing sugar as described. One unit of exoglucanase corresponded to the amount necessary to form one milligram (1mg) of glucose per minute at 50⁰C.

The determination of β -glucosidase activity (cellobiase assay) was achieved by the method proposed by Warzywoda *et al.*, (1983). To 0.5ml of 0.05M citrate buffer in a test tube was added 0.1ml of 2.5mg cellobiose (Sigma) corresponding to 0.2% weight per volume. Salicin or esculin may be used in place of cellobiose (Cai, 2000). To the substrate and buffer in the test tube was added 0.5ml of the centrifuged culture supernatant and allowed to equilibrate to 50⁰C for 10 minutes and thereafter the Somogyi – Nelson method (Nelson, 1944) was applied to assay the reducing sugar as described earlier. One unit of β -glucosidase corresponds to the amount necessary to form one milligram (mg) of glucose per minute at 50⁰C.

3.15.3 Protein Concentration Determination

Protein in the culture filtrate was determined by the method of Bradford (1976) using bovine serum albumin (BDH). In the presence of orthophosphoric acid, the Coomassie Brilliant Blue G-250, an organic dye, binds to the proteins to give colour change. The change was quantified by measuring at maximum absorption of 595nm. The protein standard curve (Appendix III) was established by plotting OD₅₉₅ against bovine serum albumin. The amount of absorption was proportional to the protein concentration. The standard 3.1ml assay protocol was used. Aliquot of 0.1ml of the sample was measured into test tube and 3ml of the Bradford reagent, was added to each tube and vortex-mixed. The samples were incubated at room temperature for ten minutes and then the absorbance taken at 595nm using spectrophotometer (Cecil 1000, Cambridge England). The quantity

of protein in sample, expressed in mg/ml, was determined by extrapolation on protein standard curve.

3.16 Statistical Analyses

Statistical Analysis System (SAS) software was used to perform analysis of variance (ANOVA) to test the quality of performance of the different mutagens. Paired sample test was used to compare a number of parameters between the wild and the mutant, while the correlation and least significant difference test was performed to determine the effect of chemical pretreatment and residence time on enzyme yield from the various lignocellulosic substrates. **CHAPTER FOUR**

4.0 RESULTS

4.1 Isolated *Aspergillus*

Ten (10) different *Aspergillus* species from the sixteen pool were isolated and characterized from the different soil samples studied (Plates I - IV). Seven were confirmed to be *Aspergillus niger* (Table 4.1) and were coded as A, B, F, I, J, L and S respectively. Other *Aspergillus* species isolated include *A. ochraceous*, *A. terreus*, *A. fumigatus*, *A. flavus*, *A. clavatus*, *A. nidulans*, *A. versicolor*, *A. clavatus* and *A. candidus* .Soil compost gave the highest number of different Aspergilli strains (5), followed by the rice field (4), then maize farm (3), and fallow farmland (2). *Aspergillus niger* was isolated as single entity in street soil and flower bed. The isolates were identified based on the structural morphologies as observed under the light microscope and cultural characteristics on the agar medium. Distinct conidiophores terminated by a swollen vesicle bearing flask – shaped biseriate phialides were seen. The spores showed black colonization in long chains from the ends of the phialides. Early growth on Czapek Dox Agar showed white mycelia which gradually turned yellow and finally to black colouration upon maturation (Plate 1).

The conidial heads showed black colouration and based on these descriptions, the isolates were identified to be *Aspergillus niger* (Pitt and Samson, 2000).

4.2 Cellulolytic Activity Determination (Qualitative) of the Wild Isolates

The result of the cellulolytic ability determination of the *A. niger* isolates is presented in Table 4.2. All isolates designated as A, B, F, I, J, L and S were able to grow on the modified Mandels mineral Agar medium. The seven isolates were selected judging from the clearing zone diameter exhibited on the medium. The diameter of zone of clearing is expressed in millimeter (mm). Isolates B and S exhibited the highest cellulolytic ability while 'A' and 'I' were the least.

Table 4.1 Species of *Aspergillus* Isolated from the Experimental Locations

Locality	Number of other <i>Aspergillus</i> isolates	<i>A. niger</i>	Designation code for <i>A. niger</i>
Rice field	4	2	J, S
Compost soil	5	1	F
Street soil	1	1	I
Fallow farmland	2	1	A
Flower beds	1	1	L

4.3 Qualitative Performance of the Mutagenised Strains.

The cellulolytic performance of the mutagenised *Aspergillus niger* strains using hydroxylamine, acridine orange and ultra-violet radiation in relation to the wild strains is presented in Table 4.2. The results are expressed as zone of clearing in millimeter (mm). Treatment of the 'F' strain with hydroxylamine resulted in decreased enzyme activity in which case it was deleterious to it., while in acridine orange and U.V. treatments, there was an increase in their activity as evidenced by the zones of clearing. Exposure of isolates I, J, B, A, L, S to the three mutagens resulted in appreciable increase in the enzyme activity of the isolates, though to varying degrees. Highest zone of clearing (26mm) was observed in isolate 'S' treated with hydroxylamine, which translates to 160% increase in activity. Isolate 'A' treated with hydroxylamine gave a 325 % fold increase in activity.

Table 4.2. Zone of Clearing on Mandel's Medium Exhibited by the Wild and Mutant Strains of *A. niger* on the Screening Medium (in mm)

Isolate	Wild	M u t a n t s		
	Untreated strain	Hydroxylamine (0.003M) treated	Acridine orange (200µg/ml) treated	U.V. (254nm) irradiation treated
F	08	07	12	10
I	04	16	13	13
J	09	11	14	16
B	10	20	21	19
A	04	17	12	16

L	06	11	09	12
S	10	26	21	25

Results are average of triplicate analyses

The U.V treated cells that gave the best mutations was obtained at 254nm when the conidia suspensions were treated for between 20 and 30 minutes exposure at a distance of 20cm from the U.V source at 50Hz. Mutants from U.V source that gave appreciable zones of clearing were obtained at either second or third exposure (Appendix V). Among the hydroxylamine treated cells, isolate 'A' mutant gave good zone of inhibition on the Mandel's screening medium, and so was chosen for the rest of the work. It was designated *Aspergillus niger* AH3 (*A. niger* AH3) meaning *Aspergillus niger* mutant strain 'A' obtained through hydroxylamine (H) mutagen at the third (3) cycles of exposure of the variant to the chemical. The wild strain was designated *A. niger* AW i.e. *Aspergillus niger* strain 'A', wild type (W).

4.4 Influence of pH, and Cultivation Time on Cellulase Production by Strains of the Test Organisms in Submerged Shake Flask

The effect of pH, strain type and cultivation time is presented in Table. 4.3.

Preliminary studies on the influence of these parameters on cellulase yield in submerged growth was studied using the natural substrate, carboxymethyl cellulose, for both the wild

(*A. niger* AW) and the mutant strains (*A. niger* AH3) and is presented in Figure 4.1. All mutants showed increased activity of the cellulase than the wild irrespective of the pH, although increasing the pH towards the neutral region showed corresponding marked decrease in enzyme expression by both the wild isolates and the mutant strains. The optimum pH observed was at 4.8 for both wild and mutant strains. Comparatively the wild type cellulase displayed the same optimum pH (4.8) as that of the mutant strains except that for the mutant strain, maximum cellulase expression was at day 4 (0.07162 IU/ml/min) while it was at day 5 for the wild (0.05143 IU/ml/min). With increasing pH value, cellulase expression is diminished. In contrast the *T. reesei* cellulase (ECONASE CEP used as standard) had cellulase activity of 0.0897 IU/ml/min (1mg/10ml) - about 25% activities higher than the enzyme from the mutagenised *A.niger*

TABLE 4.3. Effect of pH, and Period of Growth on Cellulase Yield in Submerged Shake Flasks of the Test Strains

Fermentation days	Wild, pH 4.8	Mutant, pH 4.8	Wild, pH 5.5	Mutant, pH 5.5	Wild, pH 7.4	Mutant, pH 7.4
1	2.128	2.420	1.201	2.010	0.902	1.917
2	2.490	2.940	1.765	2.736	1.510	2.274
3	2.589	3.558	2.000	3.154	1.522	2.639
4	2.690	4.156	2.195	3.753	1.844	2.880
5	2.986	3.704	2.404	2.230	1.923	2.020
6	1.931	2.980	1.412	1.982	1.324	1.773

7	1.211	2.653	0.837	1.400	0.468	1.506
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Absorbance value measurement at 540nm

4.5 Effect of Inoculum Size of the Mutant Strain on Cellulase Yield

The effect of inoculum size on cellulase yield was determined using the mutant strain on the natural substrate at the optimum pH of 4.8 that had been obtained and the result is presented in Figure 4.2. Increase in inoculum size resulted in a rapid increase in cellulase production. The time taken to achieve maximum cellulase production for the different inoculum levels varied. Cellulase yield was highest on the seventh day (0.06995 IU/ml/min) for inoculum size of 1.0×10^4 cells/ml; fourth day (0.07196 IU/ml/min) for 1.0×10^6 cells/ml and third day (0.05909 IU/ml/min) for 1.0×10^8 cells/ml. From the figure, cellulase expression was in the order $1.0 \times 10^6 > 1.0 \times 10^4 >$ for 1.0×10^8 cells/ml. Based on the cellulase production level observed using the different inoculum sizes, the inoculum level of 1.0×10^6 spores/ml was selected for the rest of the work.

Table 4.4. Proximate Composition of the Cellulose Residues.

Residue	%DM	%CP	%CF	% oil	% ash	%NFE
Corn straw	86.56	4.13	36.30	15.15	7.02	37.40
Corn cob	95.68	6.69	22.27	7.90	2.75	60.31
Bagasse	96.19	5.38	23.14	8.93	2.35	60.18

DM = dry matter

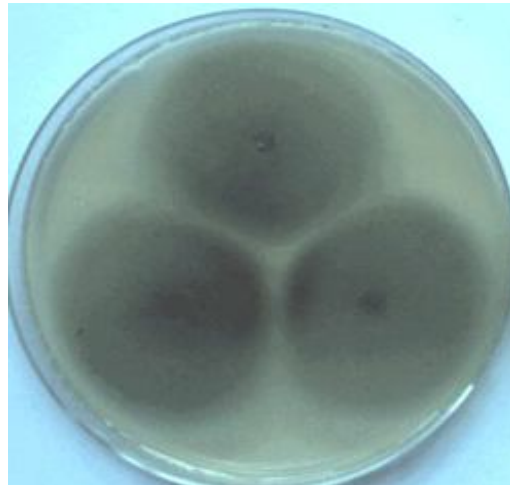
CP = crude protein

CF = crude fiber

NFE = nitrogen free extract



Aspergillus terreus



Aspergillus fumigatus



Aspergillus ochraceus



Aspergillus niger

PLATE I. Typical isolates of *Aspergillus* species on Potato Dextrose Agar



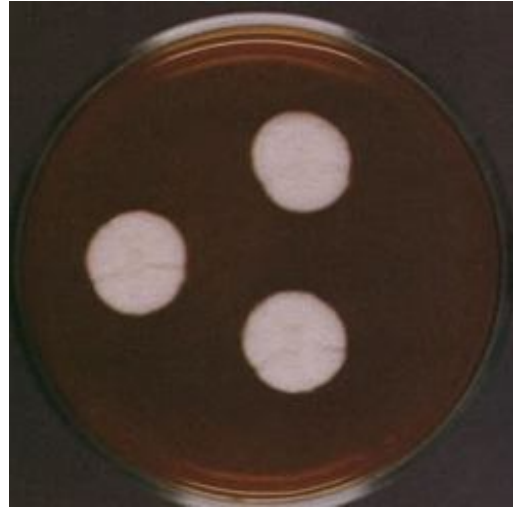
Aspergillus versicolor



Aspergillus flavus



Aspergillus clavatus

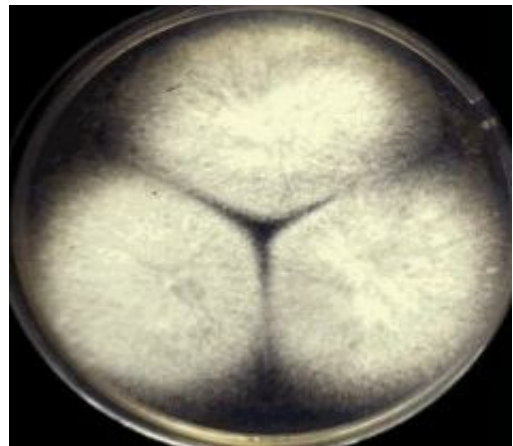


Aspergillus candidus

PLATE II. Typical isolates of *Aspergillus* species on Potato Dextrose Agar



Aspergillus niveus



Aspergillus nidulans

PLATE III. Typical isolates of *Aspergillus* species on Potato Dextrose Agar

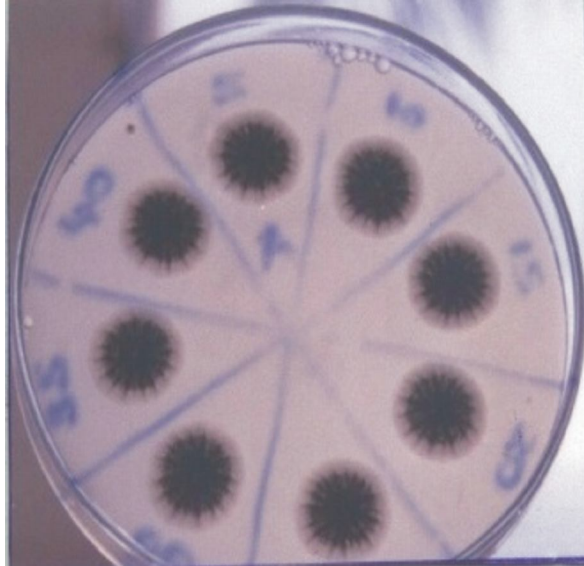


PLATE IV. Typical *Aspergillus niger* colonies growing on potato dextrose agar (PDA that had been exposed to U.V. treatment at varied time)

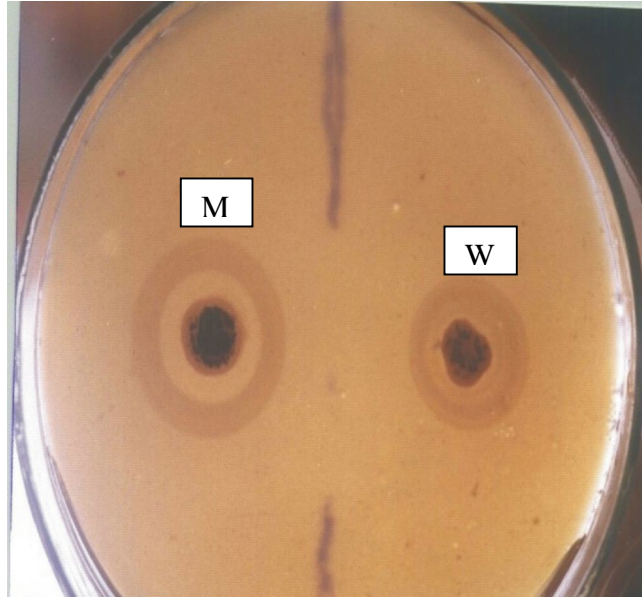


PLATE V. Typical Zone of Hydrolysis on Mandel's Medium Exhibited by the Mutant (M) and the Wild (W) Strains

4.6 Effects of Different Incubation Temperatures on the Performance of the Crude Cellulase from the Mutant.

The effect of different incubation temperatures studied: 30⁰, 40⁰, 50⁰, 60⁰, 70⁰, and 80⁰C on the performance of the crude enzyme obtained from the mutant are presented in Figure 4.3. Results showed that the cellulase from the mutant had high activity between 50⁰C and 60⁰C, and optimum activity at 50⁰C. The reduced activity at low (30⁰C and 40⁰C) and high (70⁰C and 80⁰C) temperatures shows that the enzyme optimum activity is outside of these values. At low temperature (30⁰C), the cellulase activity was low , but with

increasing temperature, there was a corresponding increase in enzyme activity until it peaked off at about 50⁰C and thereafter began to decline gradually. Increase in temperature from 50⁰C to 60⁰C resulted in a 3% decline in the cellulase activity while further increase from 60⁰C to 70⁰C gave a 36% fold decline in activity.

4.7 Influence of Cultivation Time on the pH and Soluble Protein Content of Cellulase from Wild and Mutant Strains

The pH change per time course of the fermentation for the wild and mutant strains is illustrated in Figure 4.4. Drop in pH from the original was observed after 48 hours of cultivation for the strains cultivated at pH of 7.4; while for the strains at pH 4.8 and 5.5, no drop in pH was observed, but rather a gradual rise until decline was observed at day 6 (wild) and day 5 (mutant). At pH 7.4 for both the wild and the mutant strains, there was a decline in activity of the enzyme (6% drop in mutant and 9% drop in the wild) in the first twenty-four hours and thereafter began to rise steadily until it peaked off at day five (120 hours) for the mutant and day six (144 hours) for the wild, and then their activities began to decline. For the strains fermented at pH 5.5, steady increase in cellulase activity was observed from day one and peaked off at day five and six for the mutant and wild strains respectively and thereafter began to decline in activities. Similar pattern was observed for the strains fermented at pH 4.8 although peak activity for the wild and mutant was at day six and thereafter decline in activity was observed.

The soluble protein produced in the medium as a function of time is presented in Figure 4.5 for the wild and mutant strains. For all the pH values studied, the cultures presented an initial decrease of soluble protein, indicating utilization of available nitrogen sources. In

the later stage of growth the cultures displayed increased protein liberation into the medium. The soluble protein content for both the wild and the mutant strains present the same pattern for all the pH studied. For the mutant strain, the soluble protein content of the enzyme declined sharply from day one through day three (except for the mutant at pH 7.4 that declined further in soluble protein content till day four) and thereafter rose steadily again, peaking off at day seven. The mutant used at pH 7.4 showed an exception to it because it peaked off at day six. The wild isolates displayed tremendous decline in the soluble protein content from day one through day four and rose steadily to day seven (except for the one at pH 7.4) before peaking off. Generally, the enzyme obtained from the mutant at pH 4.8 showed the highest soluble protein content of 1.09mg/ml, while the least (0.80mg/ml) was obtained from the wild at pH 7.4

4.8 Effects of Particle Size on Cellulase Yield from Wild and Mutant Strains.

The effect of the particle size of corn-cob, bagasse and corn straw on yield of cellulase from wild and mutant strains of the *Aspergillus niger* as presented in Figures 4.6, 4.7 and 4.8 respectively shows that the highest cellulase level (0.06376 IU/ml/min) obtained was on the fifth day from the mutant grown on bagasse with particle size of 210 μ (Figure 4.7), and the least (0.03016 IU/ml/min) was from the wild that acted on the corn straw with particle size of 500 μ (Figure 4.8). For the corn cob, cellulase activity rose (though unsteadily for the particle size of 210 μ) from day one and peaked off at day five for both particle sizes (210 μ and 500 μ) before decline in cellulase activity was observed. The highest cellulase activity, 0.05092 IU/ml/min, was expressed by the mutant at 210 μ in the corncob. The wild strain presented a similar pattern although for both particle sizes, enzyme expression peaked off at day six. For the bagasse, rise in cellulase expression was

slow for the wild as against the dramatic increase in enzyme activity for the mutant fermented using both particle sizes from day one. Enzyme expression peaked off at day five for the mutant, while it was at day six for the wild in both particle sizes investigated. The highest cellulase expression, 0.06376 IU/ml/min, was displayed by the mutant at 210 μ particle size of the bagasse. Similarly, in corn straw, enzyme expression from day one was progressively slow for the first 48 hours before rising steadily and peaking off at day five for both particle sizes. Enzyme activity peak was obtained at day six for the wild

4.9 Effects of Chemical Pretreatment on Cellulase Yield From Wild and Mutant Strains

The effect of chemical (alkali and acid) pre-treatment on cellulase yield from the biomass residues fermented with both wild and mutant strains supplemented with Mandels mineral medium is presented in Figures 4.9 to 4.16. Generally for the alkali treated residues irrespective of residence time, maximum cellulase yield was at day 5 while for the acid treated residues, maximum cellulase yield was at day 6. Residues treated for longer period (3 hours) using alkali and fermenting with the mutant (Figure 4.12) when compared to those using acid (Figure 4.16) under the same condition of fermentation (using mutant strain) is highly significant (0.001) using the corn cob as example. Generally, the alkali treated residues showed higher cellulase yield than the acid treated residues. Enzyme expression in biomass pretreated with 0.5M NaOH for one hour increased steadily from day one and peaked off at day five for the wild and mutant strains (Figure 4.9) except for the bagasse fermented with the mutant strains whose activity peaked off at day four. Highest cellulase activity of 0.05041 IU/ml/min was displayed by the mutant fermented on

bagasse, followed by the mutant on corn cob (0.04922 IU/ml/min) and then the corn straw (0.04692 IU/ml/min) by the mutant.

In comparison with the biomass pretreated with 0.5M NaOH for three hours, enzyme expression rose steadily from day one and peaked off at day five for both strains (Figure 4.10) except for the wild strain fermented on bagasse whose activity peaked off at day four. Highest cellulase activity of 0.05985 IU/ml/min was observed on bagasse substrate inoculated with the mutant strain.

Figure 4.11 presents the effect of biomass pretreatment using 2M NaOH of the alkali for one hour on the yield of cellulase from the wild and mutant strains. Very high cellulase expression of 0.06777 IU/ml/min was exhibited by the mutant strain fermented on bagasse. Activity of the enzyme for the mutant strains peaked off at day five but varies from day four to six for the wild on all the substrates.

The cellulase yield from the wild and mutant strains grown on the biomass pretreated with 2M NaOH for three hours is illustrated in Figure 4.12. The unique feature observed in this treatment is that cellulase expressions by the mutants in the pretreated biomass are in the same range (0.04922 to 0.04973 IU/ml/min), the difference between them accounting for only one percent. Enzyme activity peaked off on the fifth day for the strains except for the wild fermented in bagasse with low cellulolytic activity (0.03206 IU/ml/min).

The cellulase yield of the wild and the mutant strains grown on the biomass pretreated with 0.5M H₂SO₄ for one hour is illustrated in Figure 4.13. There was a gradual increase in

enzyme expression from day one and peaked off at day six for both strains, and thereafter sharp decline in activity was observed. The highest cellulase activity (0.05024 IU/ml/min) for this treatment was expressed by the mutant strain fermented in bagasse. The lowest (0.03271 IU/ml/min) was observed in the wild fermented in corn cob

The effect of biomass pretreatment using 0.5M H₂SO₄ for residence time of three hours on cellulase yield from the wild and mutant strains in Figure 4.14 shows that there was a sharp increase in enzyme expression from day one and peaked off at day six for both strains irrespective of the biomass. Highest enzyme activity, 0.04724 IU/ml/min, was observed in the mutant fermented in bagasse. The lowest (0.03016 IU/ml/min) was observed in the wild fermented in the corn cob.

Figure 4.15 presents the cellulase yield by the wild and mutant strains on pretreated biomass digested in 2M H₂SO₄ for a residence time of one hour. For the wild strain, there was a gradual increase in enzyme expression from day one which peaked off at day six, whereas the mutant strain fermented in the straw showed a decline in activity between days two and three and thereafter began to show increase in enzyme activity which peaked off at day six. Highest (0.04676 IU/ml/min) and lowest (0.03203 IU/ml/min) expression of the activity was exhibited by the mutant fermented in bagasse and straw respectively. This is a deviation from previous observations where the mutant had always showed improved activity than the wild.

Enzyme expression by the wild and mutant strains grown on the biomass pretreated with 2M H₂SO₄ solution in the digester for three hours is illustrated in Figure 4.16. Increase in enzyme activity was observed in both strains irrespective of the biomass and they all peaked off at day six. Highest enzyme expression, 0.04953 IU/ml/min, was observed however in the straw rather than bagasse as observed in other treatments. The lowest (0.03339 IU/ml/min) was observed in corn cob. Activity began to decline at day seven for both strains.

4.10 Effect of Supplementation of the Different Cellulose Sources with Mandel's Salt Solution on Cellulase Yield from Wild and Mutant Strains

The Enzyme production on the different untreated cellulosic substrates is illustrated in Figures 4.17 and 4.18 for the mutant and wild strains respectively. The substrates were supplemented with Mandel's mineral salt medium and fermented at pH 4.8. Figures 4.17 and 4.18 illustrate the effect of addition of Mandel's salt solution to the different (untreated) biomass in relation to enzyme expression by the mutant and wild strains respectively. Enzyme synthesis increased from day one and attained maximum by day five and thereafter their activities began to decline. The mutant showed elevated level of enzyme expression (0.04888 IU/ml/min) than the wild (0.04158 IU/ml/min) for untreated bagasse. For the corn cob, activity of the mutant was 0.04192 IU/ml/min while that of the wild was 0.02936 IU/ml/min; whereas the least expression was observed in straw (0.03373 IU/ml/min for the mutant and 0.02678 IU/ml/min for the wild). Peak of activity for both strains was observed at day five.

4.11 Effect of Different Cellulose Sources without Supplementation on Cellulase Yield from Wild and Mutant Strains.

The raw substrates without Mandel's mineral salt supplementation were fermented with the mutant and wild strains of the *Aspergillus niger* and the result is presented in Figures 4.19 and 4.20. Untreated bagasse fermented with the mutant strains gave the highest cellulase yield of 0.03560 IU/ml/min compared to the wild strain (0.03373 IU/ml/min). The enzyme expression on corn cob was 0.03560 IU/ml/min and 0.03136 IU/ml/min by the mutant and wild strains respectively putting the substrate only second to bagasse; whereas enzyme activity on the straw was least (0.03150 IU/ml/min and 0.02572 IU/ml/min respectively) in this regard. The unique feature exhibited by both strains in all the biomass studied was the decline in enzyme synthesis in the first two days, followed by a sharp rise in activity that peaked off at day four, followed by gradual decline again in enzyme synthesis

4.12 Proximate Analysis of the Lignocellulosic Residues Used In the Work

The proximate composition which invariably depicts the nutrient content of the agricultural wastes used for this work is presented in Table 4.4. They were calculated on percent basis. The percent dry matter for all the residues was high. Highest (96.19%) was observed in bagasse, followed by corn cob (95.68%) and then corn straw (86.56%). Crude protein was low in all residues; corn cob had the highest (6.69%), followed by bagasse (5.38%) and corn straw (4.13%). Nitrogen free extracts for both bagasse and corn cob was high (60%) but very low in the corn straw (37%). The percent ash value was generally low though highest value (7.02%) was obtained in the corn straw, corn cob (2.75%) and 2.35% for the bagasse. Crude fiber determination revealed that corn straw had 36.30%, corn cob –

22.27% and 23.14% for bagasse. Percent oil content was low for all residues and is in the order corn straw > bagasse > corn cob i.e. 15.15%, 8.93% and 7.90% respectively

In summary bagasse contained substantial quantity of the dry matter (96.19%) with good content of nitrogen free extract (60.18%) which are contained in the biomass needed by the organism for fermentation.

4.13 Determination of the Activity Level of the Various Enzyme Components of the Cellulase

A complete cellulase system consists of three classes of enzymes: exoglucanase, endoglucanase and β -glucosidase which act synergistically together in the degradation process (Rabinovich *et al.*, 2002a). The result therefore of the exoglucanase, endoglucanase and β -glucosidase expression by the wild and mutant strains fermented in amorphous cellulose is presented in Figure 4.21. Both strains exhibited high level of β -glucosidase synthesis of 0.06358 IU/ml/min and 0.05952 IU/ml/min for the mutant and wild strains respectively. In relation to β -glucosidase, endoglucanase was moderately expressed – 0.03152 IU/ml/min and 0.02333 IU/ml/min for the mutant and wild respectively. A very low secretion of exoglucanase was exhibited by both strains: 0.00621 IU/ml/min and 0.00420 IU/ml/min for the mutant and the wild respectively. Enzyme synthesis was maximal on the fourth day for all strains except for the β -glucosidase obtained from the wild whose activity peaked on the second day and thereafter began to decline. Exoglucanase synthesis declined from day one to day three and increased to 78% and 68% expressions for both the mutant and wild respectively at day four before finally declining

in activity. In general the mutant showed increased secretion of the enzymes than the wild strain

CHAPTER FIVE

5.0 DISCUSSION

5.1 Influence of Habitat on the Isolation of *Aspergillus* Strains

According to Malik and Rajoka (1973), *Aspergillus* species is plentiful in rice fields. High recovery of the organism from rice field may not be unconnected with the fact that cellulose of rice straw has a medium lignin content (12 to 16%) when compared with other cereal stalks ($\geq 17\%$) so that the cellulose is easily metabolized (Youngquist and English 1993; Qingxiang, 2002). This makes *Aspergillus species* (even the least in cellulase expression) to easily colonize soil where stalks of rice have decayed and probably mineralized the soil. The degraded cellulose is utilized as source of carbon and energy. In the locations studied, *Aspergillus niger* was isolated in all instances. This confirms the ubiquity of *Aspergillus niger* in all soil environments. Montenegro *et al.* (1996) in their attempt to isolate *Paracoccidioides brasiliensis* from 887 soil samples cultured on brain heart infusion agar, potato dextrose agar and yeast extract agar – all supplemented with antibiotics isolated 581 species of *Aspergillus* in comparison to their target organism. The soil samples yielded only five of their target organism. One sample T-Test analysis of the

Aspergillus niger obtained from the different locations showed that the cellulolytic abilities of the strains were significantly different ($P < 0.05$) at 95% confidence interval (Appendix IV). It therefore implies that the strains varied in their capabilities to produce cellulase needed for the degradation of the cellulose incorporated in the Mandel's screening medium. Isolation and selection of suitable strain of the organism became very necessary for maximum production of the cellulase enzyme.

5.2 Proximate Analysis of the Lignocellulosic Residues.

Dry matter content is a measure of the quantity of matter in the sample when the moisture is completely removed. Since the dry matter contributes to the total fermentable constituents in the medium there is therefore a correlation between the bagasse and cellulase yield. The crude protein content in the different residues varies from one substrate to another and is generally low. The corn straw (4.13%) contained less crude protein than bagasse (5.38%) and corn cob (6.69%). One can therefore conclude that the high crude protein content in the corn cob together with the inorganic (ammonium sulphate) and organic (yeast extract) sources can account for increased protein content which served as nitrogen source required for growth and efficient enzyme expression. Crude fiber content describes the amount of cellulose content i.e. cell wall composition. The crude fiber for the corn straw was 36.30%, corn cob (22.27%) and bagasse (23.14%). Cell wall of corncob is characterized by a low content of cellulose. High crude fiber content correlates positively with increase in xylose content (Stombaugh *et al.*, 2000) but this is not so with the straw because of the waxy cuticle and silica content that is found in it. They are recalcitrant and

masks this biomass sugar (Hoskinson *et al.*, 2002). Xylose is a common sugar in hemicelluloses and prevents close packing of fibers seen in cellulose. The oil content of the corn straw was higher than corn cob and bagasse. Corn straw had 15.15%, bagasse 8.93%, and corn cob 7.90%. There is a negative correlation between the crude fiber content and crude protein content in all the residues, which suggests that an increase in oil content is associated with a reduction in crude protein content. The inorganic constituents also called the mineral content of the biomass referred to as the ash, which cannot be converted to energy is shown to vary enormously from 2.35% for bagasse to 7.02% for corn straw. The negative impact of high inorganic constituents in the biomass may be aggravated in biochemical conversion systems where the use of inorganic chemicals during pretreatment adds to the total amount of non-convertible inorganic residues. The wide variability in ash is in itself a potential bottleneck for biomass conversion (Demirbos, 2002). The Nitrogen Free Extract (NFE) consists of carbohydrates, sugars, starches and a major portion of materials classified as hemicellulose. High value in both corn cob (60.31%) and bagasse (60.18%) is an indication of high content of fermentable portion of carbohydrate in the biomass.

5.3 Qualitative Determination of the Cellulolytic Activity of the Wild Strains Before and After Mutagenic Treatment

The mutants obtained are either from the first exposure to the mutagen or in the second treatment or in the third step of mutation. Majority of the mutants obtained by hydroxylamine treatment were from the first treatment except for strains A and B whose activities were best at the third phase of the mutagenic treatment whereas majority of the mutants obtained by acridine orange treatment were from the second exposure of the same

organism to the mutagen. In order to determine the performance of each of the variously treated mutants in relation to the wild strains, the values obtained were transformed into percentage and ANOVA procedure applied (Appendix VI). The F value obtained was higher than the type 1 error rate and this implied therefore that there was variation in the performance of the mutants treated with the different mutagens. The least significant difference observed was from the culture treated with acridine orange. Paired samples correlation was applied at 95% confidence interval and results indicate that there was significant difference ($P > 0.05$) between the wild and the mutants obtained using hydroxylamine, acridine orange and U.V. irradiation which support the inference from ANOVA (Appendix VII). Paired sample test shows highly significant difference between the wild and the mutant strains. Hydroxylamine treated cells has the highest standard deviation (6.26) and was least correlated (0.259) in the paired samples correlation test with the wild strain. Based on the zone of clearance on Mandel's medium (Plate V) isolate S, B and J from maize farm and rice field gave better zones of inhibition (Table 4.2), but after mutagenic treatment with hydroxylamine, acridine orange and ultra violet irradiation, their relative performance were assessed and isolate 'A' treated with Hydroxylamine gave a robust characteristics. ANOVA analysis applied to bring out the mutagen with the most significant difference supports the choice (Appendix VI) of mutant A coded as *A. niger* AH3. Hydroxylamine is known to introduce point mutations in eukaryotes (Izumiya and Yamamoto (1995). According to Inoue and Schroeder (1988), hydroxylamine modifies bases and causes base pair substitution just like nitrous acid. Hydroxylamine is a very useful mutagen for localized mutagenesis since it specifically modify (hydroxylate) cytosines (Miyoko and Henrik, 2000). Hydroxylamine has been used in the mutagenesis of

E. coli to improve its resistance to sulphonyl urea chlorimuron ethyl (Hill and Duggleby, 1998). It is safe to admit that there is a linear relationship between the number of times of exposure of the conidia and mutation rate although it will reach a limiting value. This is based on the premise that mutant 'A' obtained using hydroxylamine treatment was obtained at the third phase of the mutagenic treatment. Same can also be described for the ultra violet treated cells that dosage effect is cumulative and that there is a linear relationship between the length of time of UV exposure and mutation rate.

5.4 The Effect of pH, Strain Type and Cultivation Regime on Cellulase Yield

Statistically, ANOVA procedure was applied (Appendix VIII) to test their performance and result shows that there is a significant difference between the mutant and wild strains. Hydrogen ion concentration (pH) was found to have a strong influence in the performance of both strains because of the strong significant difference. Enzyme yield between the days also varied significantly. For all the pH values tested, the organism retained its full enzyme activity for both wild and mutant strains. The pH of 4.8 was therefore adopted for the rest of the growth experiments. Maximum enzyme yield was observed after 72 hours for the mutant, and after 96 hours for the wild. Deschamps and Huet, (1985) observed similar experience in their work with *Aspergillus niger* on a variety of substrates. The pH value of the culture medium may have affected the permeability of the cells (Mase *et al.*, 1996). Expression of enzyme activity at neutral pH generally decreases and it is very likely that at alkaline side, there may not be expression at all. The organism, *A. niger* AH3 seems to react to the pH of the growth environment by modifying its enzyme production patterns. According to Coral *et al.* (2002), carboxymethyl cellulase from *Aspergillus niger* Z10 wild type obtained from their work had optimum activity peak pH of 4.5 and 7.5. High yield cellulases have been produced by *Trichoderma reesei* at pH

of 4.8 (Liming and Xueliang, 2004). The influence of pH on cellulase production by *Trichoderma reesei* RUTC-30 was studied by Xiong (2004) and found that the highest activity was at pH 4.0. This shows that the cellulase from *Aspergillus niger* AH3 and its wild strains is an acidic protease. In a solid state fermentation (SSF) process demonstrated by Fadel (2001), *Trichoderma harzianum* was found to produce high level xylanase – a closely related enzyme to cellulase, at pH value of 4.5. The enzyme synthesis was reduced to half its optimal level when the initial pH was 8.0.

5.5 The Effect of Inoculum Size on Cellulase Yield

Enzyme production and declination were achieved much faster when 1.0×10^8 spores/ml was used due to the rapid degradation of the substrate whose products were used up by the growing culture rapidly. This made the level of cellulase produced to be lower than when 1.0×10^6 spores/ml was used even though enzyme production was faster. Increase in spore concentration was not an advantage. When lower inoculum size (1.0×10^4 spores/ml) was seeded, maximum production observed at day 7 was still lower than what was obtained at day 4. According to Sikyta, (1983), the spore concentration in fungi cultivation must be high enough to colonize the substrate particles, in order to achieve good enzyme activity. There can be a decline in this activity over a spore concentration range (Simoes and Tank – Torniselo, 2005). In process economics, longer periods of fermentation is quite uneconomical and such processes are prone to contamination. In this regard therefore, the inoculum size of 1.0×10^6 spore/ml caused maximum cellulase production level in comparison to other inoculum sizes used and is described as the best for this work.

5.6 The effect of different incubation temperatures on the performance of the crude cellulase from the mutant

The temperature profile of the enzyme suggests that the cellulase from *A. niger* AH3 was not thermostable. Its utilization in the industry therefore can only be enhanced when the process is carried out at temperatures between 50⁰ and 60⁰C. Similar findings have been reported by Coral *et al.*, (2002). Enzymes are easily denatured at high temperatures. Though the growth temperature of the fungus is at ambient, it does not always follow that it is best for enzyme activity (Kotchoni *et al.*, 2003).

5.7 Influence of Cultivation Time on the pH and Soluble Protein Content of Cellulase from Wild and Mutant Strains

Generally shift of pH to higher side was recorded during the growth of the *A. niger* W3 and *A. niger* AH3 and the finding is in consonance with the work of Khan *et al.*, (1992); Mackenzie *et al.*, (2000). The mutant however produced elevated levels of acid than the wild for all cases studied. The high pH value of 7.4 observed from day 5 for the mutants may have halted the enzymatic activities of *A. niger* AH3. The results suggest that pH be controlled during the cultivation of this strain for cellulase production. There was a corresponding decrease of 11% in cellulase yield for just the 2.5% increase in pH rise observed for the mutant culture cultivated at pH 4.8 between day 5 and 6. The result also shows that pH increase in the medium was growth associated and not enzyme associated because even when enzyme excretion began to decrease, pH rise was still observed. Enzyme expression was optimum between pH 5.1 and 5.2 for the mutant strain.

Enhancement of the enzyme production in the mutant is due only to the mutagenic effect of the chemical (and not growth) which affects enzyme synthesis by the strain.

Increase in protein content was not unconnected with the beginning of cellulase synthesis by the culture. Highest soluble protein (1.09mg/ml) was obtained from the mutant that was used at pH 4.8 and the least (0.79mg/ml) was obtained from the wild at pH 7.4. The study of the influence of pH on xylanase and cellulase production by *T. reesei* conducted by Xiong *et al.*, (2004) revealed that highest concentration of soluble protein was obtained at pH 4.5 and decreased with increasing pH concentration. One striking difference between them is that the mutant was able to consume the available nitrogen source the third day and cellulase synthesis had commenced the fourth day, while for the wild, the available nitrogen source consumption tailed off the fourth day and enzyme synthesis had started the fifth day. It can therefore be concluded that cellulase activity and soluble protein concentration is a function of pH during growth. This observation was similarly reported by Xiong *et al.*, (2004). The soluble protein observed for the standard was 0.74mg/ml which is within the same range with the wild strain.

5.8 Effect of Particle Size on Cellulase Yield

The smaller particles gave a larger surface area which would ease oxygen diffusion, mass transfer and nutrient absorption (Sharma *et al.*, 1988) and assimilation by the mycelia. Statistically the results obtained from the runs were pair tested using the student 'T' analysis and the result is presented in Appendix IX. Paired sample test of 210 μ and 500 μ for the mutant and wild strains shows that there was a strong association between the

particle size and the cellulase yield. However no association was observed between the wild and mutant strains. Generally, the mutants produced high cellulase level at day 5 while the wild produced their high cellulase yield at day 6. Since in all the runs the 210 μ particle size gave better cellulase yield than the corresponding 500 μ particle size, it therefore follows that the particle size reduction led to increase in available surface area and release of intracellular components. Similar observation was made by Ford (1983) on digestion of mature Pangola grass stems by cellulase that had been pulverized into different particle sizes. Decrease in particle size and increase in the surface area of the substrate facilitated the enzyme complex produced by the organism to penetrate the substrate particles easily and solubilize the cellulose and convert it to sugar monomers. The effect of time period of fermentation on the production of cellulase was determined in terms of the solubilization of the cellulose which was found to be maximal on the 5th day for the mutant and 6th day for the wild. Lag phase is often prolonged with larger particle sizes though with most substrates, there is a threshold value under which further reduction in particle size becomes uneconomical (Chynoweth *et al.*, 1993).

5.9 Effect of Chemical Pre-Treatment on Cellulase Yield

Statistical analysis using ANOVA procedure on the effect of chemical pretreatment on cellulase yield showed that there is a significant difference in the enzyme yield between the strain type (mutant and wild) for all the residues treated with chemical (Appendix X). There was a remarkable difference in the performance of the mutant against the wild for all the residues treated in 0.5M NaOH for 1 hour (Figure 4.9) except for the straw residues which showed very little difference in enzyme yield between the wild and the mutant under

this condition. There was a strong correlation between the enzyme yield and strain type for all pretreated residues (Appendix XI) at 95% confidence interval. It is not unlikely that the residence (soaking) time and the concentration of the alkali were not adequate enough to achieve good alkali pretreatment. Same goes for those that were held in the digester for 3 hours (Figure 4.10). Increase in the concentration of the alkali to 2M and residence time of one hour gave no significant difference between the mutant and the wild for the corn straw. Very low correlation (0.082) was displayed by the wild strain fermented in straw whether digested for one or three in 0.5M sulphuric acid. For the three residues studied, paired samples statistics show that there is no significant difference in enzyme yield between the wild strains whether treated with 0.5M or 2M sulphuric acid and irrespective of the residence time (whether 1 or 3 hours) too (Appendix XI). Cellulase yield of 0.068 IU/ml/min was obtained when bagasse digested with 2M NaOH for one hour was fermented using the mutant strain and 0.049 IU/ml/min for the wild strain under the same condition. This was equivalent to 39% increase in enzyme activity. The standard enzyme *Trichoderma reesei* ECONASE CEP from AB enzymes used resulted in 32% increase in activity than the mutant for the alkali treated bagasse; and about 25% increase for the mutant grown on carboxymethyl cellulose under the same condition. There was a significant difference in the performance of the strains fermented in the biomass pretreated for varying residence time (Appendix X); for example there was variation in enzyme activity for biomass treated in the digester for one hour and three hours under the same condition of growth. Paired sample analysis between corncob treated in 0.5M of the alkali for 1 hour (Figure 4.9) and 3 hours (Figure 4.10) respectively using the mutant is highly significant at $P < 0.05$ (Appendix XI). Same goes for the corncob treated in 2M of the

alkali for 1 hour (Figure 4.11) and 3 hours (Figure 4.12) respectively using the mutant was highly significant at $P < 0.05$ (Appendix XI). However, an exception was observed when corn cob treated in 0.5M acid for 1 hour (Figure 4.13) and the corn cob treated in 0.5M acid for 3 hours (Figure 4.14) were fermented with the mutant strains, where there were no significant difference ($P = 0.874$) in their performance in terms of cellulase yield. Chemicals used in the pretreatment were also a factor in the cellulase yield from the residues. A typical situation is depicted in Figures 4.11 and 4.15 where the alkali treated bagasse at 2M for 1 hour using the mutant strain was very significant (0.023) (Appendix XI) when compared to acid treated bagasse at 2M for 1 hour using the same mutant strain at $P < 0.05$. Pre-treating the substrates with sodium hydroxide may have resulted in the swelling of the particles and hence easy removal of the lignin and the cellulose fibers were depolymerized due to the separation of hydrogen bonds of cellulose (Chum *et al.*, 1985). The swelling of the substrate particles was due to the saponification of inter molecular ester bonds (Fan *et al.*, 1982). Acid pre-treatment of the residues yielded significantly lower cellulase activity than alkali pre-treated samples. Acid treatment does not remove lignin from the substrate but only modifies the lignin-carbohydrate linkage according to Ghosh and Singh, (1984). Pretreating with higher concentration (2M) of acid even for prolonged period as in Figure 4.16 resulted in the loss of polysaccharides and formation of secondary reaction. This may have led to the accumulation of degradation products of pentoses and hexoses like furfural and other complexes which act as inhibitors that hinder the microbial fermentation process in the subsequent steps (Kodali and Pogaku, 2001). Acid chemical pretreatment may permit cellulose to re-anneal leading to hornification of cellulose in micro fibrils (Houghton *et al.*, 2006).

5.10 Effect of Different Cellulose Sources (Supplemented with Mandel's Mineral Salt) on Cellulase Yield

The effect of different cellulose sources (supplemented with Mandel's medium at pH of 4.8) on cellulase yield presented in Figures 4.17 (mutant) and 4.18 (wild) indicates that the source of the substrate is a factor influencing the enzyme production. Bagasse treated with the mutant gave the highest enzyme yield of 0.04888 IU/ml/min while the straw gave (0.03407 IU/ml/min) at day 5 and 4 respectively. The cellulase standard gave an activity of 0.08968 IU/ml/min at 1mg/10ml concentration. Similarly the wild strain displayed the same behaviour although yield in both cases were relatively lower when compared to those obtained from the mutants. Da-Silva *et al.* (2005) studied the effect of different nutrient sources on the production of xylanase and carboxymethyl cellulase for four days and found that the different lignocellulosic substrates used perform at variance with one another. This is not unrelated to the content and sugar composition of the substrates.

5.11 Effect of Different Cellulose Sources (Without Mandel's Mineral Salt) on Cellulase Yield.

The effect of different cellulose sources without Mandel's mineral medium is presented in Figures 4.19 and 4.20 for the mutant and the wild respectively. The substrates used were screened through 210 μ sieves. Without mineral supplementation the substrates produced activity which may be attributed to the medium which provided the *Aspergillus* with an environment similar to its own natural habitat (decaying cellulosic materials in soil). The strains of the organism both wild and mutant produced the cellulase independent

of the cellulosic source. Highest cellulase expression was at day 4 for all the substrates irrespective of the strain. Further increase in incubation period may have resulted in the reduced secretion of the enzyme. The decrease in enzyme production observed may be explained on the premise that the susceptible portions of the cellulose molecules was rapidly digested and only the crystalline portion was left behind which could not be used for the production of enzyme by the organism. Bagasse gave the highest enzyme production of 0.03560 IU/ml/min. Low cellulase yield in all of the cases could be attributed to the absence of the mineral salt solution which the Mandels medium often provides. In the study of pectinase production by Martins *et al.* (2000), they observed that good growth and enzyme production on some agricultural residues was optimum when these media were supplied with nutrient solution containing ammonium nitrate, ammonium phosphate and magnesium sulphate. The unique observation here was that after 48 hours of growth, the cellulase activity for all the substrates declined sharply before picking up again at 72 hours. This sharp decline was occasioned by the drop in pH due to lack of buffering agents. Lack of Mandels mineral medium which contain buffers temporarily resulted in reduction in growth of the *Aspergillus*. The low pH subsequently induces the anaplerotic enzyme (Gupta & Maheshwari, 1985). It has been reported that for effective growth and enzyme expression even in solid substrates, thermophilic fungi requires buffering agent (Maheshwari *et al.*, 2000). The differences in cellulase level production exhibited by the different cellulosic substrates is determined by factors such as the surface area, composition of the substrate, presence of activator or inhibitor or catabolite repression (Damaso *et al.*, 2000).

5.12 Activity Level Determination of the Component Cellulase System of the Strains

Exoglucanase seems to be the major component of this cellulase system as it agrees with that of the standard (0.01319 IU/ml/min) using ECONASE cellulase from *Trichoderma reesei*. Similar results have been reported by Esterbauer *et al.* (1991). Endoglucanase activity of the standard was 0.04918 IU/ml/min in comparison to the mutant (0.03152 IU/ml/min). However the β -glucosidase, activity of the mutant is significantly higher (0.06358 IU/ml/min) than the ECONASE CEP (0.05081 IU/ml/min). According to Grohmann (1993), *T. reesei* does not excrete a sufficient quantity of β - glucosidase enzyme for efficient enzymatic hydrolysis, whereas *Aspergillus* strains have been found to be efficient producers of the β -glucosidase component as observed by Reczey *et al.* (1998).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Many researchers attention to generate microorganisms capable of hyper production of cellulase has been focused on *Trichoderma*. In this study, several *Aspergillus niger* strains were screened for their ability to produce cellulase using the Mandels mineral medium which offered a good screening assay. The mutant obtained by hydroxylamine treatment presents the highest activity compared to those from U.V and acridine orange treated cells. The mutant strain was able to grow on the three lignocellulosic residues (corn cob, corn straw and bagasse) under study although the expression of cellulase differs from one substrate to another due to differences in proximate composition. High dry matter and nitrogen free extracts correlated with high growth constituent. Since selection of a suitable carbon and energy source was a strong factor in the process of extracellular production of hydrolases (such as cellulase) by filamentous fungi the substrates appear to be inducers of the enzyme. The mutant grew and produced maximum cellulase activity

within 96 hours to 120 hours – which is a significant advantage in reducing risks of contamination. Acid treatment only modified the carbohydrate – lignin linkage thereby affecting the growth process. The presence of lignin in the acid pretreated samples of the different residues used for this work did not impede the mutant from expressing the cellulase although the quantity is low. Weak acids tend to remove lignin but result in poor hydrolysis of the cellulose. The alkali treatment swelled and modified the lignocellulosic into a more reactive amorphous form thereby delignifying the substrate. The alkali pretreatment caused an increased surface area due to swelling and disruption of the lignin. Between the treatments applied to the residues, steeping of pulverized bagasse in 2M NaOH for 1 hour is good for producing cellulase. Bagasse was found to be generally, the best carbon source when compared with other biomass tested. Alkali pretreatment therefore offers the possibility for pilot scale process development preparatory to cellulase production. However, the process is too expensive to be employed in the pretreatment process. Secondly, there is the need and additional cost for specialized equipment able to withstand corrosion. Extensive washing to remove the alkali before fermentations is also a laborious and process demanding. The cellulase produced was very sensitive to pH, therefore selection of optimum pH (4.8) is very essential for cellulase production using this mutant. Inoculum size was a significant factor in enzyme expression by the mutant. Too low or too high spore concentration would adversely affect the enzyme expression of the mutant, so optimum spore concentration of 1.0×10^6 cells/ml was required to achieve good cellulase synthesis. The mutant produced elevated levels of β -glucosidase – which is an advantage over the *Trichoderma reesei* strains whose low level of β -glucosidase synthesis causes cellobiose accumulation and subsequently catabolite repression.

In conclusion, the mutant generated from *Aspergillus niger* using hydroxylamine was the best candidate in this work. Cellulase production from both amorphous and crystalline cellulose in shake flask showed that the enzyme expression was better in the amorphous substrate. Hydrogen ion concentration (pH) value of 4.8, spore inoculum level of 1.0×10^6 spore/ml and ambient temperature of 32°C was optimum for enzyme production. Increased protein liberation in the medium coincided with cellulase synthesis and are both functions of pH in this work. With the crystalline cellulose, pretreatment, step whether physical or chemical influenced the cellulase expression level. Small particle size (210μ) gave significantly higher cellulase yield than the corresponding big particle size (500μ) of the different lignocellulosics. Alkali pretreated (2M NaOH) pulverized substrate gave higher cellulase yield (0.068 IU/ml/min) than the acid pretreated samples. Acid pretreated samples took 144 hours to obtain maximum cellulase expression whereas it was 120 hours for alkali pretreated samples. The lignocellulosic substrates supplemented with Mandel's medium gave a better performance than the unsupplemented type in terms of enzyme yield. Generally cellulase yield was in the order bagasse > corncob > corn straw. The mutant strain showed improved synthesis of the cellulase components than the wild whereas β – glucosidase secretion by the mutant was significantly higher than the *Trichoderma reesei* ECONASE CEP standard used for this work.

6.2 Recommendations

The catalytic efficiency of the enzyme can be further improved upon by site directed mutagenesis of the mutant. Studies may further be carried out to purify the protein and conduct extensive characterization of the purified enzyme product. Understanding the molecular basis of mutations lays the ground work for the mutational analysis. The goal and challenge are to identify all of the genes that contributed to the process and then to understand the nature of the gene products. Northern (RNA blot) analysis of the mutant and the wild could be used to locate the gene responsible for the elevated expression of cellulase. Pulsed field electrophoresis gels of the chromosome could reveal the size of the chromosome in both wild and mutant strains where mutation had occurred. Southern analysis of genomic DNA will indicate either addition or deletion at a particular locus and the exact size could be determined.

The use of genetically modified microorganisms (GEMs) for environment application has generated considerable debate and controversy. Hurdles to overcome before usage of GEMs in the environment are technical, health, gene transfer and regulatory issues. The technical include how well one can design the GEMs for environmental application. Questions relating to the survival of the introduced organisms, their genetic stability and

expression of desired activities in the environment need to be answered. If technical problems were overcome, problems including those of health, occupational or bystander exposure need to be answered. There is a general reluctance to grant permission to release any GEMs into the environment unless one is certain that it is safe to do so. In the natural environment, the cellulolytic strain can be immobilized by organic sorption to soil surfaces or entrapment in small pores *in – situ* or by binding and sequestration with clays and humus. Biostimulation of the cellulolytic microflora in the soil may be achieved by application of surfactants (nutrient amendment) or providing appropriate environmental conditions for example, oxygen as terminal electron acceptor or by modifications of the pH. Manganese is one of the trace elements required by all microorganisms. However, it exists at low level in the earth crust and because of their limited solubility in aerobic, aqueous environment it is less available to fungi. Since cellulolytic enzymes are well associated (extracellular) and require manganese, provision of this element as an ion in the environment may well stimulate their activity when used as microbial inoculants

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