FERMENTATION STUDIES AND GENETIC MODIFICATION OF CELLOBIOHYDROLASE I GENE OF MUTATED *ASPERGILLUS NIGER* AND *PENICILLIUM CITRINUM* ISOLATED FROM SAWDUST (*MITRAGYNA CILIATA*)

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES OF THE UNIVERSITY OF LAGOS, LAGOS NIGERIA, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY (Ph.D.) DEGREE IN BIOCHEMISTRY

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DECLARATION

The study titled "Fermentation Studies and Genetic Modification of Cellobiohydrolase I Gene of Mutated *Aspergillus niger* and *Penicillium citrinum* Isolated from Sawdust (*Mitragyna ciliata*)", submitted to the School of Postgraduate Studies, University of Lagos, Lagos, Nigeria, for the award of Doctor of Philosophy (Ph.D.) Degree in Biochemistry, is an original research carried out by **BABALOLA**, **Oladayo Musa**, in the Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine of the University of Lagos under the supervision of **Prof. (Mrs.) O.A. Magbagbeola**, **Prof. O.A.T. Ebuehi** and **Prof. (Mrs.) V.I. Okochi.** It is hereby declared that this work has not been submitted previously (in whole or in part) to any institution for the purpose of awarding of any academic degree.

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CERTIFICATION

DEDICATION

This thesis is dedicated to Almighty Allah, The Omniscient, The All Knowing for His grace and inspiration**…Surely, my prayers, sacrifices, efforts and death are for the Lord of the worlds… (Q6: 162).**

To my late parents…

a journey we started at a time I was naive

I felt secured when I look back and see you

…but you left the race quite early enough without notice

leaving me behind to my fate

the journey became longer yet the road was rough

the Good Lord was there who saw me through

the task is accomplished but you are AWOL

notwithstanding, I present to you the report

May Allah increase His mercy on your souls

"*Ameen*"

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ABSTRACT

The need for utilizing renewable resources to meet the future demand for fuel and other value added products has increased the attention on lignocellulose, the most abundant and renewable resource in the world. Lignocellulose is degraded by lignocellulolytic enzymes produced by fungi and bacteria. Mutagenic agents can be used to achieve improvement of these strains. However, there has been a challenge in improving and optimizing fermentation process which in most cases has been carried out independently. The aim of this study was to hyper produce cellulase from fungi in order to enhance lignocellulosic wastes biodegradation. Raw Abura sawdust (*Mitragyna ciliata*) was collected from Okobaba sawmill, Ebute-meta, Lagos. It was pretreated mechanically and chemically using ammonium hydroxide. The lignocellulosic and proximate compositions of both the raw and pretreated sawdust types were determined. Fungi were isolated from decomposing wood wastes. They were genotyped by amplifying the internally transcribed spacer (ITS) regions on their DNA, sequencing the amplicons and analyzing the sequences. Two fungal strains with best cellulolytic potential were selected and co-cultured. They were genetically modified for enhanced cellulase production using ultraviolet rays. The optimal conditions for the effective production of cellulase by both wild and mutant strains of the fungi were investigated. The solid-state fermentation of these mutants was optimized in order to further enhance the production of cellulase. The enzyme from both the wild and mutant strains were partially purified and characterized. RNA of the fungal mutants was extracted from their mycelia. The extracted RNA was reverse transcribed to complementary DNA (cDNA). Selected genes coding for cellulase were amplified and the amplicons were sequenced. The points of mutation were identified from the sequence and the sequences of the

mutants were aligned and compared with referenced sequences which included those of *Trichoderma reesei* and *Phanerochaete chrysosporium* using MEGA5 and Jalview softwares. The sequences were also translated to amino acid and in-silico X-ray crystallography structure of the active site of the enzyme was constructed. The hemicellulose content of the sawdust reduced significantly upon pretreatment (32.70 ± 2.20% to 19.80 ± 1.30%) while its cellulose content increased (48.11 ± 1.60% to 64.94 ± 1.20%). Proximate analysis revealed that moisture (6.30 ± 0.60% to 3.60 ± 1.30%) and crude fibre contents (62.20 ± 3.40% to 52.80 ± 2.20%) reduced significantly upon pretreatment. *Aspergillus niger* and *Penicillium citrinum* were selected out of four fungi genotyped which included *Trichosporon asahii* and *Penicillium corylophylum*. Co-culturing was more efficient in the biodegradation of sawdust. *Aspergilus niger* mutant strain had a 2.1-fold and 2.4-fold increase in carboxymethylcellulase (CMCase) and filter paper cellulase (FPase) production respectively more than the wild strain while *Penicillium citrinum* had a 1.8-fold and 2.1-fold increase in CMCase and FPase production. However, optimized fermentation of *A. niger* mutant produced a 7.4-fold and 7.6-fold higher increase in CMCase and FPase production more than the wild strain while *P. citrinum* mutant produced a 5.3-fold and 5.8-fold increase (CMCase and FPase). Purified cellulase from wild *A. niger* had a catalytic efficiency of $0.305M^{-1}s^{-1}$ while its mutant had a catalytic efficiency of $0.429M^{-1}s^{-1}$. Purified cellulase from wild *P. citrinum* had a catalytic efficiency of $0.858M^{-1}s^{-1}$ while its mutant had a catalytic efficiency of $1.036M^{-1}s^{-1}$. Bioinformatics analysis of the sequence of the cbh1 gene of the *A. niger* mutant showed that it had strong similarity with compared industrially beneficial fungi; *Trichoderma reesei* and *Phanerochaete chrysosporium*. Amino acid residues in its active site had low hydropathy index. Predicted structure of cellulase from *A. niger* mutant revealed that alteration occurred in the β-pleated sheets of the enzyme. Genetic modification of *Aspergillus niger* and *Penicillium citrinum* and optimizing their solid-state fermentation resulted in enhanced cellulase enzyme production.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF STUDY

Technological advancement and industrial revolution have resulted in immense urbanization of major cities in the world. This has led to attendant increase in commercial activities and ultimately in generation of large volumes of solid wastes. These wastes usually accumulate in the environment causing environmental menace. Lignocellulosic wastes are major renewable natural resources of the world and represent a major source of renewable organic matter. Lignocellulosic biomass can be grouped into four main categories: agricultural residues (corn stover and cobs, sugar cane bagasse, banana peels and stalks etc), dedicated energy crops, wood residues (sawmill

and paper mill discards) and municipal paper waste. The plant biomass regarded as wastes are biodegradable and can be converted into valuable products (Dashtban *et al.,* 2009). The bioconversion of lignocellulosic materials rather than its management is now a subject of intensive research which will not only solve aesthetic problem they create, but generate wealth by development of primary industries and creation of value added products. Lignocellulosic wastes can be utilized not only for ethanol production but industrial chemicals. Howard *et al.,* (2003) reported that many organic chemicals can be produced from ethylene, propylene, benzene, toluene and xylene. Benzene, toluene and xylene can be obtained from lignin, being aromatic while ethylene and propylene can be obtained from ethanol derived from fermentation of glucose obtained from cellulose biodegradation.

Biodegradation of lignocellulosics can be achieved with the use of ligninocellulolytic enzymes produced by bacteria and fungi (Kaur *et al.*, 2007). Enzymes that act on cellulose are generally referred to as cellulases, those that degrade hemicellulose and lignin are called hemicellulases and ligninases respectively. Cellulases can be classified into three broad categories. Endoglucanases (endo-1,4-β-D-glucanases); cellobiohydrolases or exoglucanases (exo-1,4-β-D-glucanases); and β-glucosidases (1,4-β-D-glucosidases) (Gao *et al*., 2008). Cellulose hydrolysis is a simultaneous and synergistic action of these three types of enzymes. The hydrolysis process starts with endoglucanases that randomly hydrolyze internal β-1,4-glycosidic linkages in the amorphous region of cellulose microfibrils, reducing significantly its degree of crystallinity and opening new terminal ends. Simultaneously, the accessible reducing and non-reducing ends of cellulose chains are attacked by types I and II cellobiohydrolases, respectively. The cellobiose units released are then hydrolyzed into glucose units by β-glucosidases (Textor *et al*., 2013). Cellulases can be classified into glycosyl hydrolase (GH) families based on their amino acid

sequence and folding similarities. Together with some endoglucanases, type I cellobiohydrolases belong to the GH family 7 (Textor *et al*., 2013). The exoglucanase, Cel7A, is the most important single enzyme component for cellulose depolymerization and conversion into cellobiose in fungi (Lynd *et al*., 2002). It is considered to be the key enzyme in the hydrolysis process because it is able to hydrolyze crystalline cellulose extensively, although at a slow rate (Textor *et al*., 2013).

The performance of microbes for hyper-production of lignocellulolytic enzymes can be improved by different methods of mutation (Pradeep *et al.*, 2012; De Nicolas Santiago *et al.*, 2006). Previously used physical mutagens include Ultraviolet light, microwaves and high energy ionizing radiations (Li *et al*., 2010; Xu *et al.*, 2011). Chemical mutagenesis had been carried out by treatment with Nitrosoguanidine, Ethyl methane sulphonate, Diethyl sulphonate and colchicines (Bhargavi and Singara, 2010). On the other hand, enhanced enzyme production can be achieved by supplementations with salts (Junior *et al.*, 2009), metals (Nikolic *et al.*, 2009) and optimization of fermentation conditions (Acharya *et al*., 2008).

Modification of fungi for enhanced performance by mutation and optimization of their fermentation conditions are the major approaches for enhanced ligninocellulolytic enzymes production. In view of the utilization of sawdust and agro-wastes in general, the present research was carried out with the aim of hyper producing cellulase from fungi by strain improvement in order to enhance lignocellulosic wastes biodegradation

1.2 STATEMENT OF PROBLEM

The recognition that environmental pollution is a worldwide threat to public health has given rise to new massive industries for environmental restoration (Milala *et al.,* 2009). The activities of these industries have ultimately led to generation of large volumes of wastes. Waste generation is an inevitable aspect of living which, at least can only be managed. The problems posed by these wastes are many: they degrade the urban environment, reduce its aesthetic value, produce offensive odours during the rains and pollute the air with smoke when the wastes are burnt uncontrollably. They also constitute health hazards in themselves if they are not timely disposed (Ogunbode *et al*., 2013). Agricultural wastes represent the largest class of cellulosic wastes which are grossly underutilized leaving a large proportion to constitute source of environmental

pollution (Rahman *et al.*, 2000). In Nigeria, agricultural wastes constitute over 60% of cellulosic wastes which are currently underutilized (Abu *et al.*, 2002).

A huge volume of wood residue is generated annually from timber processing activities around sawmills, plank markets, and furniture-making factories in cities within Nigeria. For instance, about 294,798 tons of wood waste is generated yearly in the city of Lagos (Dosunmu and Ajayi, 2002), while about 2288 m^3 is generated daily in Abeokuta, while estimated 31,324.3 tons annually in Ilorin and a total of 104,000 m^3 of wood waste is generated daily in Nigeria (Aina, 2006).

Lignocellulosic wastes alone account for over 80% urban refuse in Lagos, Nigeria (LAWMA report, 2004). Nwankwo (2004) observed that wood wastes constitute the largest class of industrial wastes which have been reported to have caused enormous environmental pollution. It was estimated that 5,666.19 tons of wastes are generated in one week by the saw mills in Lagos in 2011. This produces about 6.6 tons of Sulfure (IV) oxide and 3331.7 tons of ash per annum if it gets burned (Ogunbode *et al*., 2013).

Enzyme cost is estimated to represent approximately 50% of the total hydrolysis process cost. The cost of enzyme on the economics of lignocellulosic waste bioconversion has been a subject of controversy. There have been arguments that the cost of enzyme production itself is more expensive than the product of the bioconversion (Klein-Marcuschammer *et al*., 2012). At 20% solids loading during saccharification, a typical enzyme loading is 10 FPU/g cellulose, equivalent to approximately 20mg enzyme/g cellulose (Gusakov, 2011). The typical yield for the saccharification of cellulose at this enzyme loading is 70% after 5 days (Roche *et al*., 2009). It must be noted that the optimal value of enzyme loading varies depending on feedstock, solids

loading, and pretreatment technology, among other variables (Kazi *et al*., 2010; Kristensen *et al*., 2009).

A thorough understanding of the mechanisms guiding the expression of the genes coding for cellulase is imperative for the development of the genetic improvement programme for cellulase producing organisms. There is paucity of data on studies on tropical fungi and their mutants.

1.3 AIM OF STUDY

The aim of this study is to hyper-produce cellulase from fungi in order to enhance lignocellulosic wastes biodegradation.

1.4 OBJECTIVES OF STUDY

The specific objectives of this study are to:

1. determine the lignocellulosic composition of raw and pretreated sawdust.
2. isolate and genotype cellulolytic fungi.

3. mutate isolates and optimize their solid-state fermentation.
4. sequence cDNA amplicons obtained from wild and mutant fungi strains.
5. analyze the sequence using bioinformatics tools and predict the structure of cellulase from mutant fungi.

1.5 SIGNIFICANCE OF STUDY

Generally, sawdust as well as other lignocellulosic wastes are disposed by burning, thus constitutes a source of greenhouse gases and other forms of environmental pollution. This study will enhance the transformation of sawdust and other lignocellulosic wastes from pollutant to raw materials. By implication, the massive turnover of wood wastes which is estimated to be about hundreds of tons per day will lead to the creation of primary and secondary industries producing valuable products thus creating jobs for the teeming unemployed youths of the nation.

This will inevitably contribute to Nigeria's Gross Domestic earnings. Simultaneously, the environment will be salvaged from further deterioration.

This study will also provide alternative for energy crops, which itself is uneconomical for hydrolysis to bioproducts.

The lignocellulosic composition of a number of biomass wastes has been determined. These include sugarcane chaffs, paper, corn-cobs, and banana peels, to name a few. There is however, no data on sawdust. This study will provide data on the composition of sawdust and the implication of chemical pretreatment its composition.

The traditional method of identifying a microorganism is from its distinct feature on plates and under the microscope. These observations are comfirmed using biochemical tests. However, it has been revealed that some organisms share similar features macroscopically and microscopically which makes it difficult to distinguish organisms belonging to the same phyla. DNA barcoding is now in use in the past two decades to identify microorganisms most especially fungi, thus solving the problems of misidentification of microorganisms. This study seeks to add to the already existing bank of fungal gene sequences in public databases. Thus further enhancing the identification of fungi isolated from our environment.

The kinetic parameters of the cellulase obtained shall be studied. This will elucidate the catalytic mechanism of hydrolysis by cellulase from both wild and mutant strains of different fungi.

Strain improvement has been the target of researchers in the field of lignocellulosic biotechnology in this decade. Several attempts have been made which proved auspicious albeit delimited by cost of the procedure, safety and generation of harmful mutants. This study set out to create strains which are efficient yet cheap to produce. The efficiency of these mutants shall be complemented by improving their solid-state fermentation. The improved solid-state

fermentation procedure shall be premised on independent experiments which this study set to carry-out.

Based on the emerging trend in solving problems in lignocellulosic biotechnology using recombinant DNA technology and protein engineering, this study seeks to propose a sequence which can be cloned and expressed in suitable vectors for hyper production of cellulase. The efficiency of an enzyme largely depends on its structure. This study will provide a model structure of cellulase enzyme from mutant fungi which will be an addition to the database needed in studying the structure and function relationship in enzyme biotechnology.

1.6 OPERATIONAL DEFINITION OF TERMS

Lignocellulosic Wastes (LCW): are plant biomass wastes that are composed of cellulose, hemicellulose, and lignin. They are majorly agricultural residues and municipal solid wastes.

Pretreatment: Preparation of lignocellulosic materials for enzymatic degradation. It involves the alteration of structural and chemical composition of lignocellulosics to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars.

Cellulases: are enzymes responsible for the hydrolysis of cellulose with specificities to hydrolyze glycosidic bonds.

Endoglucanase (CMCase): are type of cellulases which initiate attack randomly at multiple internal sites for subsequent attack by cellobiohydrolase. The are also known as carboxymethylcellulase.

Solid State Fermentation (SSF): Growth of microorganisms in the absence or near absence of free water with inert natural substrates as solid support.

Gene Mutation: Permanent change in DNA sequence that makes up a gene.

Mutagenesis: Conscious introduction of mutation in the genome of an organism which is achieved with physical or chemical agents called mutagens.

Polymerase Chain Reaction (PCR): Primer specific in-vitro enzymatic amplification of a target segment of DNA from a complex mixture of starting material usually termed the template.

Complementary DNA (cDNA): DNA molecule generated from RNA by reverse transcription.

DNA Barcoding: Molecular identification and phylogenetic classification of organisms from bioinformatic analysis of sequence of specific segment of their DNA.

1.7 LIST OF ABBREVIATIONS

ace1 – transcriptional activating gene class1

aep1 - aldose epimerase gene class1

ANM - *Aspergillus niger* (mutant strain)

ANW- *Aspergillus niger* (wild strain)

BLAST – Basic Local Alignment Search Tool

cbh - cellobiohydrolase gene class 1

CBH1- cellobiohydrolase enzyme class 1

CBM – cellulose binding module

cDNA – complimentary deoxyribonucleic acid

DNA- deoxyribonucleic acid

FPase – filter paper cellulase

k(off) – dissociation rate

Lacc1 – laccase gene class 1

LCW – lignocellulosic wastes

PCM - *Penicillium citrinum* (mutant strain)

PCW – *Penicillium citrinum* (wild strain)

RNA – ribonucleic acid

UV- ultraviolet

X – times

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 MICROFUNGI

Microfungi are diverse group of fungi consisting of yeasts and moulds. Microfungi belong to three major phyla; Ascomycota, Deuteromycota and Zygomycota. The fungal body consists of microscopic threads called hyphae, extending through the substrate through which they grow

(Carlile *et al.*, 2001). Typically only the "fruiting body" of the fungus is visible, producing thousands of tiny spores that are carried by the air, spreading the fungus to new locations. Spores are produced in a variety of ways and occur in a bewildering array of shapes and sizes. In spite of this diversity, spores are quite constant in their shapes, sizes (about 2–20 μm), colour and form and as such these characteristics are very useful for identification of microfungi. The basic difference between spores lies in their method of initiation, which can be either sexual or asexual (Carlile *et al.*, 2001).

2.1.1 CHARACTERISTICS AND IMPORTANCE

Microfungi are well adapted to extreme environmental conditions. They tolerate a wide range of temperature, pH, dryness, oxygen concentrations and ultraviolet radiation better than the wood-rotting basidiomycetes called white or brown rot fungi. In addition they are found in all climatic zones ranging from the poles to the tropics (Blanchette, 2000). Generally, fungi prefer an acidic environment although microfungal activities occur within a broad pH range of between 3.7 and 8.6 (Daniel and Nilsson, 1998). Microfungi can protect themselves by relatively quick growth in natural niches and by the production of antibiotics and toxic substances (mycotoxins). Microfungi play important role in carbon cycling, and are also involved in many biotechnological processes. These processes include: brewing, wine making, baking, cheese-making and the preparation of other fermented food (e.g. soy sauce) together with edible mushroom production are the most important microfungal applications. Production of enzymes (amylase, cellulase, invertase, lipase, pectinase, proteinase, rennin and xylanase), organic acids (citric, itaconic and lactic acids), antibiotics and other pharmaceuticals (penicillin, mevinolin, cephalosporin, griseofulvin and cyclosporine) by fungi are common processes that have been reviewed (Demain *et al*., 2004).

2.1.2 *Aspergillus niger*

Aspergillus niger or ***A. niger*** is a ubiquitous fungus and one of the most common species of the genus *Aspergillus*. It causes a disease called black mould on certain fruits and vegetables. It is characteristic with black colonies which can be confused with those of *Stachybotrys* (Samson *et al*., 2001). Studies have proved that some true *A. niger* strains do produce ochratoxin A. It also produces the isoflavone orobol (Samson *et al*., 2001; Schuster *et al*., 2002). The black aspergilli are among the most common fungi causing food spoilage and deterioration of other materials. They have also been used for diverse biotechnological purposes, not limited to production of organic acids and enzymes (Schuster *et al*. 2002). The taxonomy of *Aspergillus* section *Nigr*i has been studied by many taxonomists and was recently reviewed by Abarca *et al*., (2004). Al-Musallam (1980) did a comprehensive revision of the taxonomy of the *A. niger* group based on morphological features. Seven species (*A. japonicus, A. carbonarius, A. ellipticus, A. helicothrix, A. heteromorphus, A. foetidus,* and *A. niger*) were recognized. *A. niger* was described as an aggregate consisting of seven varieties and two formae. Samson *et al*., (2004) reported that Kozakiewicz (1989) distinguished *A. japonicus, A. helicothrix, A. atroviolaceus, A. heteromorphus, A. ellipticus* and *A. carbonarius* as species exhibiting echinulate conidial ornamentations distinct from the remaining black Aspergillus taxa, which produce verrucose conidia. Within the verrucose category, *A. acidus, A. fonsecaeus, A. niger* var. *ficuum, A. niger* var. *phoenicis, A. niger* var. *niger, A. niger* var. *awamori, A. niger* var. *pulverulentus, A. niger* var. *tubingensis, A. citricus (A. foetidus)* and *A. citricus* var. *pallidus* were recognized. *Aspergillus niger* is the most frequently reported species in this section and has often been included in biotechnological processes that are Generally Regarded as Safe (GRAS). However,

species concepts are uncertain in this complex and occasionally the name *A. niger* has been used for any member of the section. Taxonomic studies using molecular methods have divided the *A. niger* complex into two species, *A. niger* and *A. tubingensis* (Abarca *et al*., 2004).

2.1.2.1 Pathogenicity

A. niger is less likely to cause human disease than some other *Aspergillus* species. In extremely rare instances, humans may become ill, but this is due to a serious lung disease, aspergillosis, that can occur. Aspergillosis is, in particular, frequent among horticultural workers that inhale peat dust, which can be rich in *Aspergillus* spores. *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss and in severe cases, damage to the ear canal and tympanic membrane. In plants, *A. niger* causes a common postharvest disease of onions, in which the black conidia can be observed between the scales of the bulb. The fungus also causes disease in peanuts, grapes and other fruits (Samson *et al*. 2001).

2.1.2.2 Industrial Uses

A. niger is cultured for the industrial production of many substances. Various strains of *A. niger* are used in the industrial preparation of gluconic acid (E574) and citric acid (E330) and have been assessed as acceptable for daily intake by the World Health Organisation (WHO). *A. niger* fermentation is "generally recognized as safe" (GRAS) by the United States Food and Drug Administration under the Federal Food, Drug, and Cosmetic Act (FDA, 2008). Enzymes of economic importance are produced using industrial fermentation of *A. niger* . These include alpha-galactosidase used in the food industry, pectinases used in cider and wine clarification, glucoamylase used in the production of high fructose corn syrup. Another use for *A. niger*

within the biotechnology industry is in the production of magnetic isotope-containing variants of biological macromolecules for nuclear magnetic reasonance (NMR) studies. The enzyme protease is derived from *Aspergillus niger* and used to produce the supplement Clarity-Ferm. This product is being used in the brewing industry to reduce gluten content of wheat and barley based beers. A Clarity-Ferm treated beer made from barley or wheat usually tests below 20 ppm of gluten, the current international standard for gluten free (Mitea *et al*., 2008).

2.1.2.3 Other Uses

A microbial-derived enzyme, prolyl endoprotease, which cleaves gluten has been found to be producible by *A. niger* . This enzyme has strong implications in the treatment of coeliac disease or other metabolic gluten sensitivity disease processes (Mitea, 2008). A placebo controlled, double blind study was initiated in December 2008 to determine the efficacy of this enzyme in treating humans with coeliac disease (Mulder, 2008). *A. niger* is also cultured for the extraction of the enzymes glucose oxidase (GO) and alpha-galactosidase (AGS). Glucose oxidase is used in the design of glucose biosensors, due to its high affinity for β-D-glucose (Staiano *et al*., 2005). α-galactosidase can be produced by *A. niger* fermentation and is used to hydrolyze α-1-6 bonds found in melibiose, raffinose, and stachyose.

2.1.3 *Penicillium citrinum*

Penicillium citrinum is an anamorph, mesophilic fungus species of the genus of *Penicillium* which produces tanzawaic acid, Mevastatin, Quinocitrinine A, Quinocitrinine B, and nephrotoxic citrinin (John *et al*., 2009; Mossini and Kemmelmeier, 2008). *Penicillium citrinum* is often found on moldy citrus fruits and occasionally occurs in tropical spices and cereals. This Penicillium

species is also mortally to the mosquito *Culex quinquefasciatus* (Maketon, 2014). In view of its mesophilic character, *Penicillium citrinum* occurs worldwide (John *et al*., 2009). The first statin (*Mevastatin*) was isolated from this species in 1970.

2.2 BIOMASS AND ITS POTENTIALS

Any mass of biological material is termed Biomass. These include whole or plant parts, plant constituents and byproducts, animal byproducts, municipal and industrial wastes (Howard *et al*., 2003). Bioproducts can be generated from these materials following a thorough knowledge of techniques to be used in manipulating its constituents to obtain the desired product.

Biomass of plant origin is composed mainly of cellulose, hemicelluloses and lignin, hence, called lignocellulosic biomass. In addition, small amounts of other components can be found in them depending on source (Sanchez, 2008). These components may include pectin, protein, and ash.

Agricultural resources of lignocellulosic waste are quite abundant as estimated by the Food and Agriculture Organization (FAOSTAT, 2006). Around $2.9x10^3$ million tons from cereal crops and $1.6x10^2$ millions tons from pulse crops, 1.4x10 million tons from oil seed crops and $5.4x10^2$ million tons from plantation crops are produced annually worldwide (Kumar *et al*., 2008). The various types of lignocellulosic raw materials include wheat straw, rice straw, palm, corncobs, corn stems and husk etc., have varying amounts of cellulosic components. It has been estimated that the yearly biomass production of cellulose is 1.5 trillion tons, making it an essentially inexhaustible source of raw material for environmentally friendly and biocompatible products (Kim and Yun, 2006). Therefore, the bioconversion of large amounts of lignocellulosic biomass into fermentable sugars has potential application in the area of bioenergy generation. Although extensive studies have been carried out to meet the future challenges of bioenegy generation,

there is no self-suficient process or technology available to convert the lignocellulosic biomass for bioenegy generation (Kim and Yun, 2006).

2.3 STRUCTURE OF THE PLANT CELL WALL

The plant cell wall consists of three types of layers, namely middle lamella, primary wall, and secondary wall. In the primary wall, the main structure is a skeleton of cellulose cross-linked with glycans, and there are two types according to the cross-link types. Type I walls are found in dicotyledonous plants and consist of equal amounts of glucan and xyloglucan embedded in a matrix of pectin. Type II walls are present in cereals and other grasses having glucuronoarabinoxylans as their cross-linking glucans, but lacking of pectin and structural proteins (Mohnen *et al*., 2008).

The secondary wall usually has three sub-layers, which are named S1, S2 and S3. S1 is the outer layer, S2 is the middle layer while S3 is the inner layer (Chundawat *et al*., 2011a).

The cellulose microfibrils of secondary wall are embedded in lignin, (Sticklen, 2008). Cellulose, hemicellulose, and lignin have different distribution in these layers. In wood fibers, it has been found that cellulose concentration is increased from middle lamella to the secondary wall. S2 and S3 lamellaes have the highest cellulose concentration. Most of the hemicellulose distributes in the secondary wall (Zhao *et al*., 2012). Lignin is found to be the dominant composition in the outer portion of the compound middle lamellae. The percentage of lignin in the lignocellulosic matrix decreases with increasing distance into the middle lamella. The percentages of lignin in the primary wall and in the S1 layer of the secondary wall are much higher than those in the S2 and S3 sections (Zhao *et al*., 2012). However, the plant cell wall is indeed a complex nanocomposite material at the molecular and nanoscales (Ding *et al*., 2008). Although much is

known of the structure of the plant cell wall, more information should be gained to understand the microstructure and even anostructure of plant cell wall and how these structures build the recalcitrance of biomass. Many modern analytic and simulation methods have been employed to deeply understand the molecular mechanism of biomass recalcitrance (Zhao *et al*., 2012) and the molecular dynamics of lignin (Petridis and Smith, 2009).

2.4 CHEMICAL COMPOSITION OF LIGNOCELLULOSE

Plant cell wall consists mainly of monosaccharides which could be structural or storage. The structural form could be celluloses, hemicelluloses, or pectins (Himmel *et al*., 2010). Their predominance is as enumerated. The most abundant weight fraction cell wall type in plant tissue is the secondary cell wall, produced after the cell has stopped growing. Secondary cell walls contain structural polysaccharides, strengthened further with lignin covalently cross-linked to hemicellulose. Cellulose is the most abundant. It exists as microfibrils and is embedded in matrix of hemicellulose and lignin as shown in Fig. 1 below.

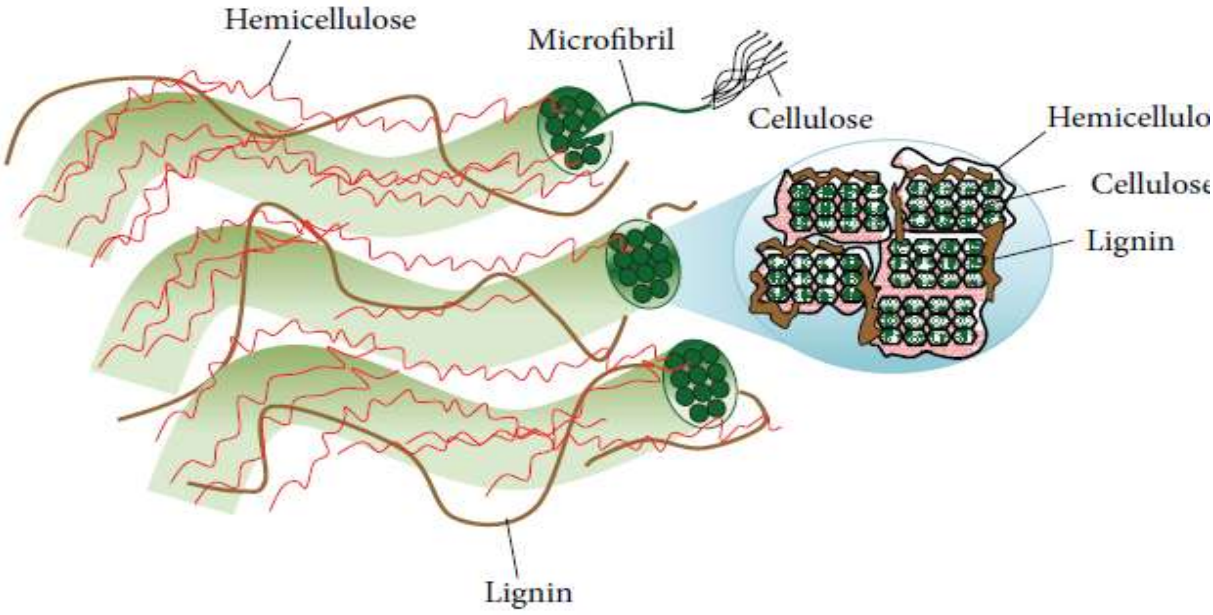


Fig. 1: Plant Cell Wall Structure and Microfibril Cross Section (Lee *et al*., 2014)
(Strands of cellulose embedded in matrix of hemicellulose and lignin)

2.4.1 Cellulose

Cellulose is a linear unbranched polymeric chain, consisting solely of β-(1,4)-linked D-glucose residues. Cellulose is synthesized by cellulose synthase 'rosettes', which contain 36 enzyme units located in the cell membrane. After synthesis, the cellodextrin chains are directly deposited into the cell wall as elementary fibrils that coalesce to form successively larger microfibrils and in some cases, macrofibrils (Ding and Himmel, 2006).

Cellulose of plant origin is classified into two; cellulose Iα and cellulose Iβ. Cellulose Iα is a triclinic form with one chain per unit cell and is of higher energy compared with the more stable monoclinic Iβ form (Himmel *et al*., 2010). The Iα cellulose can be easily hydrolysed, but stable Iβ cellulose is predominant in plant cell wall.

In cellulose, all of the glucosyl hydroxyl groups are in the equatorial position, whereas all of the axial positions are occupied by nonpolar (and nonhydrogen-bonding) aliphatic protons. This means that the 'sides' of the elementary microfibrils are polar and hydrogen bonding and the 'tops and bottoms' are hydrophobic. The relatively extended solution structure of cellodextrins permits them to aggregate with a regular crystalline packing, matching up hydrophobic faces as well as allowing hydrogen bond formation between chains (Nishiyama *et al*., 2003) as shown in Fig. 2. Currently, the detailed mechanisms of cellulase attack on these cellulose allomorphs and their respective crystal faces remain unknown.

Cellulose consists of a linear chain of several hundred to over ten thousand β (1→4) linked D-glucose units. It makes up 15–30% of the dry mass of primary and up to 40% of secondary cell walls, where it is found in the form of microfibrils (Sticken, 2008). It is the β-1,4-polyacetal of cellobiose (4-O-β-D-glucopyranosyl-D-glucose). Cellulose is more commonly considered as a polymer of glucose because cellobiose consists of two molecules of glucose. The chemical formula of cellulose is $(C_6H_{10}O_5)_n$ (Harmsen *et al*., 2010).

They are composed of about 36 hydrogen-bonded glucan chains, each of which contains between 500 and 14 000 β-1,4-linked glucose molecules (Mohnen *et al*., 2008). Cellulose can exist in several crystalline polymorphs. Native or natural cellulose has a polymorph structure of cellulose I. However, cellulose I can be converted to other polymorphs (II, III, and IV) through a variety of treatments (Agbor *et al*., 2011). Cellulose also has amorphous or soluble regions, in which the molecules are less compact and more easily hydrolyzed by cellulases.

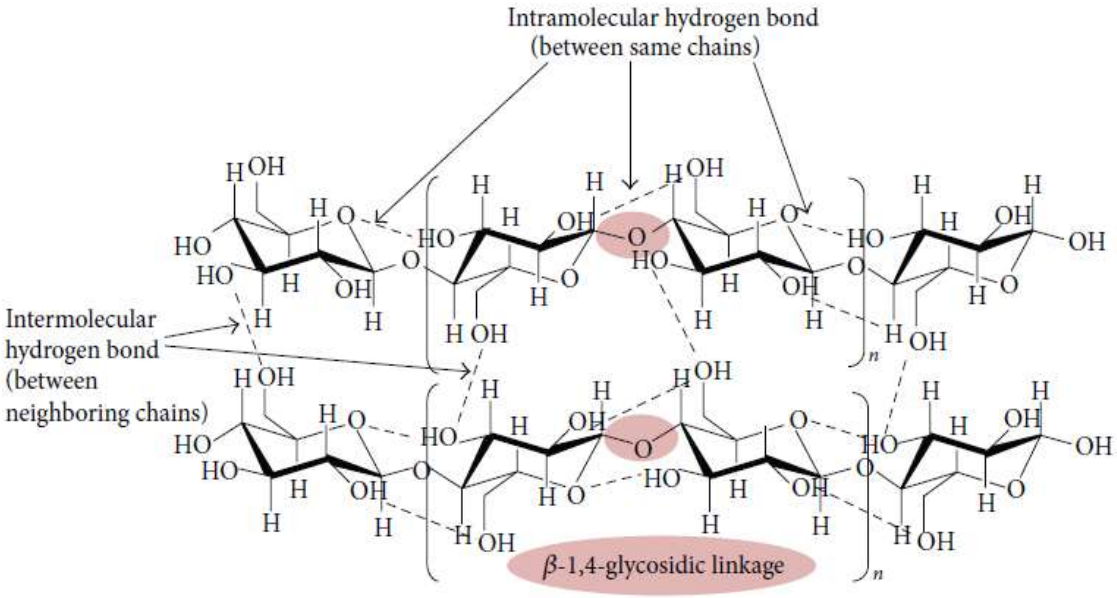


Fig. 2: Structure of Cellulose Molecule (Lee *et al.*, 2014)

Many properties of cellulose depend on the number of glucose units that make up one polymer molecule. This is referred to as its degree of polymerization. It can extend to a value up to 17000, although, 800-10000 units is usually encountered (Harmsen *et al*., 2010).

The nature of bond between the glucose molecules (β-1,4 glucosidic) allows the polymer to be arranged in long straight chains. The latter arrangement of the molecule, together with the fact that the hydroxides are evenly distributed on both sides of the monomers, allows for the formation of hydrogen bonds between the molecules of cellulose. The hydrogen bonds result in the formation of a structure that is comprised of several parallel chains linked to each other (Harmsen *et al*., 2010).

The resulting structure is as shown in the Fig. 3.

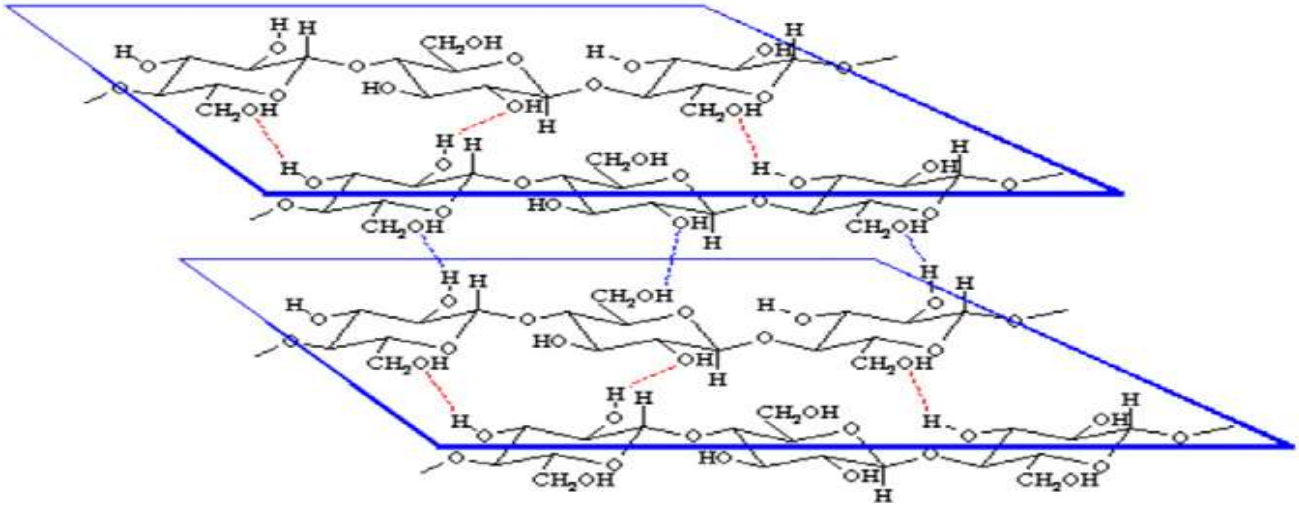


Fig. 3: **Demonstration of Hydrogen Bonding that Allows the Parallel Arrangement of the Cellulose Polymer Chains** (Harmsen *et al*., 2010)

2.4.2 Hemicellulose

In addition to cellulose, the plant cell wall matrix contains two additional major types of cell wall polysaccharides; the hemicelluloses and the pectins. Unlike cellulose, both are synthesized in the Golgi apparatus, delivered to the cell membrane via small vesicles and secreted into the cell wall. Hemicelluloses are generally complex, branched carbohydrate polymers that are formed from different monomeric sugars attached through different linkages (Chundawat *et al*., 2011a). Carbohydrate substituents and noncarbohydrate components occur in hemicelluloses on either the main chain or on the carbohydrate branches. The complex structures of the hemicelluloses are thought to confer a wide range of biophysical and biomechanical properties on the plant tissues in which they occur, as well as on products made from these tissues. The principal pentose sugar in the major plant cell wall hemicellulose is β-D-xylopyranose, which has only the position 2- and 3-carbons available for *O*-linked substitution by substituent sugars when β-(1,4) linked as in xylan. Hemicelluloses also include xyloglucan, arabinoxylan and glucomannans, which contain other sugars including the pentose arabinose and the aldohexoses glucose,

mannose and galactose. The hemicelluloses can also be esterified by acetylation and/or cross-linked to lignins via p-coumaroyl and feruloyl groups (Chundawat *et al*., 2011a).

Hemicellulose consists of polysaccharides other than cellulose. Its structure reveals that either type of bonds is the main one that forms its molecule. The main difference with cellulose is that the hydrogen bonds are absent and that there is significant amount of carboxyl groups. The carboxyl groups can be present as carboxyl or as esters or even as salts in the molecule (Harmsen *et al*., 2010). Hemicellulose is a diverse group of short-chain branched, substituted polymer of sugars with a degree of polymerization ~70 to 200, and is usually characterized as the heterogeneous polysaccharides being soluble in strong alkali (.Scheller and Ulvskov, 2010). It has a backbone composed of 1, 4-linked *β*-D-hexosyl residues and may contain pentoses, hexoses, and/or uronic acids. Other sugars, such as *α*-L-rhamnose and *α*-L-fucose, may also be present in small amounts and the hydroxyl groups of sugars can be partially substituted with acetyl groups (Zhao *et al*., 2009). Hemicelluloses comprises a family of polysaccharides such as arabinoxylans, glucomannans, galactans, and others that are found in the plant cell wall and have different composition and structure depending on their source. The most common type of polymers that belongs to the hemicellulose family of polysaccharides is xylan. As shown in Fig. 3, the molecule of a xylan involves 1->4 linkages of xylopyranosyl units with α-(4-O)-methyl-D-glucuronopyranosyl units attached to anhydroxylose units (Fig. 4). The result is a branched polymer chain that is mainly composed of five carbon sugar monomers, xylose, and to a lesser extent six carbon sugar monomers such as glucose. Important aspects of the structure and composition of hemicellulose are the lack of crystalline structure, mainly due to the highly branched structure and the presence of acetyl groups connected to the polymer chain (Harmsen *et al*., 2010).

Unlike cellulose, hemicellulose composition varies depending on cell tissue and plant species and differs in type of glycosidic linkages, side-chain composition and degree of polymerization (Chundawat *et al*., 2011a). The principal hemicellulose of hardwoods is an O-acetyl-4-O-methylglucuronoxylans with amounts between 15% and 30% depending on the species. However, the main hemicellulose of soft woods are an O-acetylgalactoglucomannan with contents between 10% and 25%, and in Gramineae such as cereal straws, the main hemicelluloses is arabinoxylans, which are similar to harwoods xylan but the amount of L-arabinose is higher (Peng *et al*., 2011).

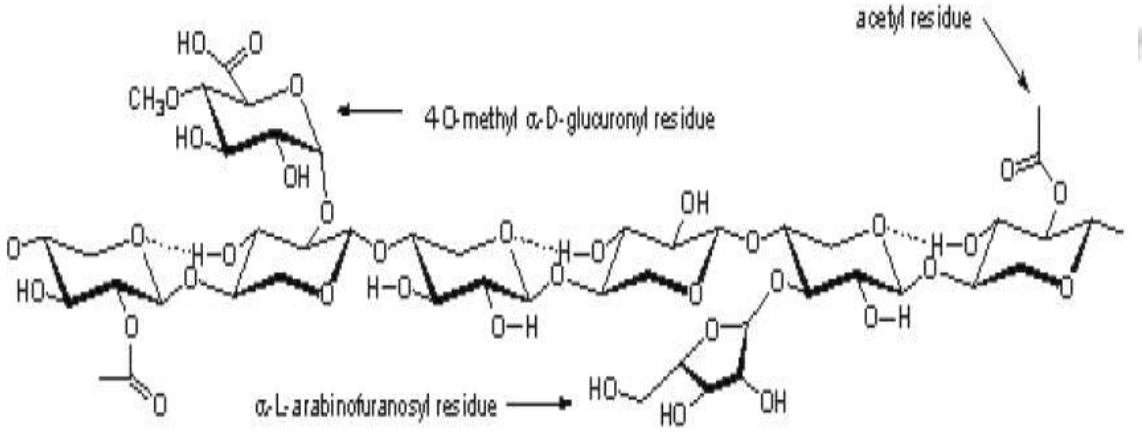


Fig. 4: Structure of Xylan: a Typical Hemicellulose (Harmsen *et al*., 2010)

Hemicellulose extracted from plants possesses a high degree of polydispersity, and po-lymolecularity (a broad range of size, shape and mass characteristics). However, the degree of polym-erization does not exceed the 200 units whereas the minimum limit can be around 150 monomers. Hemicellulose is insoluble in water at low temperature. However, its hydrolysis starts at a temperature lower than that of cellulose, which renders it soluble at elevated temperatures

(Thermowoodhandbook, 2003). The presence of acid highly improves the solubility of hemicellulose in water.

2.4.3 Lignin

As a complex phenolic polymer, lignins exist widely in cell walls of plants and some algae. There are three types of functional groups in lignins, including p-hydroxyphenyl, guaiacyl and syringyl, from which the monolignols, 4-hydroxycinnamyl, coniferyl and sinapyl alcohols, respectively are comprised. The complex and highly variable chemical heterogeneity of lignin is due to the diversity of substitution patterns and intermolecular linkages utilized during polymerization. Although lignins enable critical functions for the plant, including mechanical support, water transport and defense, lignin is also an undesirable component in the biomass conversion process, due to its ability to shield polysaccharides from enzymatic hydrolysis and generally impede diffusion into plant tissue by chemicals and enzymes. In native plant cell walls, lignins are covalently linked to hemicellulose, which forms a matrixing layer around the cellulose comprising the microfibril core that further hinders cellulolytic and hemicellulolytic enzymes. Moreover, many of the lignin degradation products are either inhibitory or generally detrimental to the plant cell polysaccharide-degrading enzymes. It is therefore necessary to take this into account when designing an enzymatic process for degradation of lignocellulosic biomass (Harmsen *et al*., 2010).

Lignin is the most complex natural polymer (Calvo-Flores and Dobado, 2010). It is an amorphous three-dimensional polymer with phenylpropane units as the predominant building blocks. These building blocks are mainly (1) sinapyl alcohol, (2) p-coumaryl alcohol and (3) coniferyl alcohol as shown in Fig. 5a. As an organic substance binding the cells, fibers, and vessels, lignin has an important role in protecting the plants against invasion by pathogens and

insects (Sticken, 2008). Lignin in wood behaves as an insoluble three-dimensional network. It plays an important role in the development and stability of the cell. This is because it affects the transport of water, nutrients and metabolites in the plant cell.

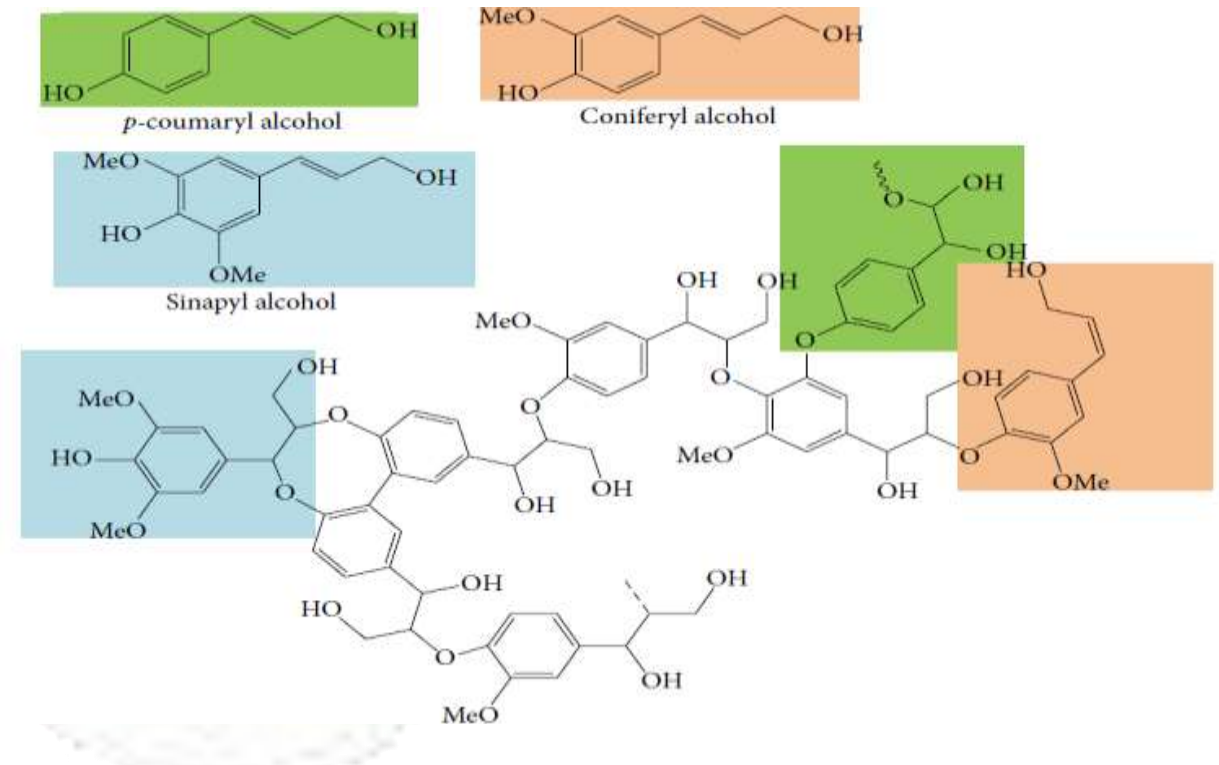


Fig. 5a: Dominant Building Blocks of Polymer Lignin (Lee *et al*., 2014)

The three basic monomeric units constituting lignin are *p*-hydroxyphenyls, guaicyls, and syringyls which vary between species and cell tissue type. Hardwood lignins are predominantly guaicyls and syringyls monolignols with trace amounts of *p*-hydroxyphenyls units. Soft wood lignins are composed of mostly guaicyls units, whereas herbaceous plants contain all three units in significant amounts with different ratios (Chundawat *et al*., 2011a). The cell wall also contains some other substances such as pectin, proteins, and ashes. However, these compositions are not important in the biomass refining.

Lignin from softwood is made up of more than 90% of coniferyl alcohol with the remaining being mainly p-coumaryl alcohol units. However, lignin found in hardwood is made up of varying ratios of coniferyl and sinapyl alcohol type of units. Low molecular alcohols significantly dissolve lignin, likewise di-oxane, acetone, pyridine, and dimethyl sulfoxide. Also, it has been found out that at elevated temperatures, lignin softens, and this allows depolythmerization reactions of acidic or alkaline nature to accelerate (O'Connor *et al.*, 2007).

The property of polydispersity, just as with hemicellulose, characterizes lignin as well. Different branching and bonding in otherwise similar molecules are encountered (Harmsen *et al*., 2010). Fig. 5b shows a model structure of lignin from spruce pine.

2.4.4 Bonds in the Lignocellulosic Complex

There are four main types of bonds identified in the lignocellulose complex. These four bonds are the main types of bonds that provide linkages within the individual components of lignocellulose (intrapolymer link-ages) and connect the different components to form the complex (interpolymer linkages). The position and bonding function of the latter linkages is summarized in the Table 1 below.

Table 1: Bonds Linking the Different Components of Lignocellulose (Harmsen *et al*., 2010)

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

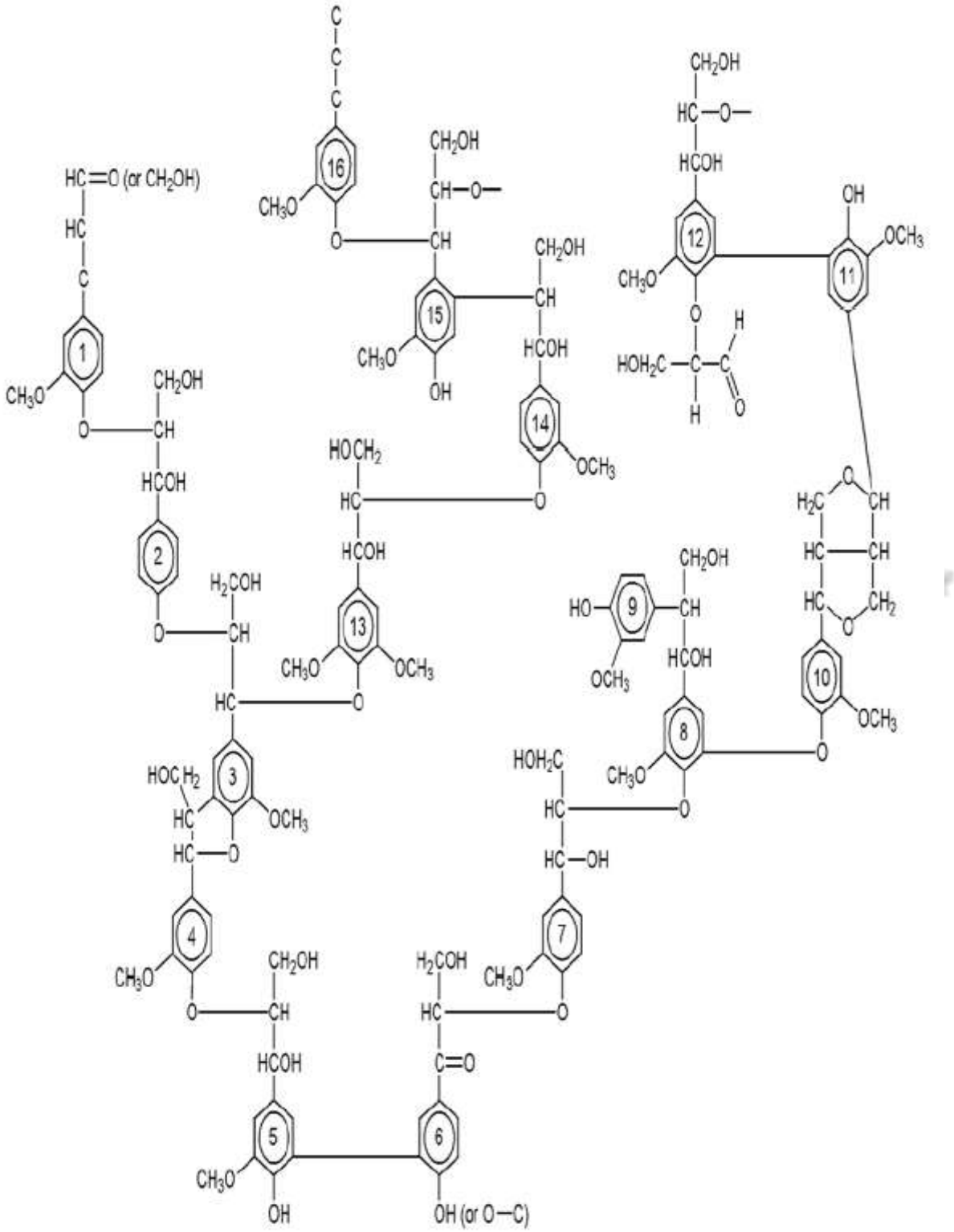


Fig. 5b: Structure of Lignin (Harmsen *et al*., 2010)

2.4.5 Interactions between the Lignocellulosic Components

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

1. The glucosidic linkage is the one that forms the initial polymer chain. More specifically, it is a 1-4 β D-glucosidic bond that binds the glucose units together. The glucosidic bond can also be considered as an ether bond, since it is in fact the connection of two carbon atoms with an elementary oxygen interfering (Harmsen *et al*., 2010).
2. The hydrogen bond is considered to be responsible for the crystalline fibrous structure of cellulose. The arrangement of the polymer in long straight parallel chains together with the fact that the hydroxyl groups are evenly distributed in both sides of the glucose monomer, allow the formation of hydrogen bond between two hydroxyl groups of different polymer chains (Harmsen *et al*., 2010).

2.5 ENZYMES INVOLVED IN THE BIODEGRADATION OF LIGNOCELLULOSICS

Biological degradation of lignocellulosics is achieved with enzymes usually termed lignocellulolytic enzymes. Theses enzymes are produced by species of fungi from the phylum Basidiomycetes, Ascomycetes and some members of the Orpinomycetes (Dashtban *et al*., 2009) and some of bacteria. Consortiums of enzymes that degrade cellulose are called cellulases, while those that degrade hemicelluloses are called hemicellulases and those that degrade lignin are called ligninases. Celluases and majority of hemicellulases belong to a group of enzymes called Glycoside hydrolases (GH) (Dashtban *et al*., 2009). It has been estimated that 2500 glycoside hydrolases exists and they have been classified into 115 families (Cantarel *et al*., 2009).

However, fungal cellulases are found within glycoside GH 5-9, 12, 44, 45, 48, 61 and 74 (Dashtban *et al*., 2009).

According to the International Union of Biochemistry and Molecular Biology's Enzyme Nomenclature and Classification (http://www.chem.qmul.ac.uk/iubmb/enzyme/ Sweeney and Xu, 2012), enzymes involved in the bioconversion of lignocellulosics may be classified according to their structural, evolutionary relationship and specificity, although other criteria for classification are available. Based on these, they have been classified to belong to glycosidases (EC 3.2.1), lyases (EC 4.2.2), esterases (EC 3.1.1), peroxidises (EC 1.11.1), carbohydrate oxidases (EC 1.1.3), phenol oxidase (EC 1.10.3) and other EC classes, according to their main reactions.

Based on Carbohydrate-Active EnZYmes (http://www.cazy.org/) and Fungal Oxidative Lignin Enzymes (FOLy) (http://foly.esil.univ-mrs.fr/ Sweeney and Xu, 2012) databases, enzymes involved in biodegradation of lignocellulosics belong to Glycoside Hydrolases (GH), Carbohydrate Esterases (CE), Polysaccharide Lyases (PL), Lignin Oxidases (LO), and Lignin Degrading Auxiliary enzymes (LDA) families according to their sequence and structural homology. Each family has shared three-dimensional structure and catalytic mechanism. This feature may facilitate bioinformatic analyses of genomic data. It has been found out that enzymes from families which do not share structural homology and evolutionary relationship may however, catalyze the same reaction.

Enzymes degrading lignocelluloses possesses some structural features. Some of these features may not be involved in their catalytic activities. They possess a catalytic core for catalysis and carbohydrate binding modules which Guillen *et al*., (2010) claimed anchors host enzymes to targeted carbohydrate substrates. Other modules include dockerins, fibronectin 3-like modules, immunoglobulin-like domains, or functionally unknown "X" domains (Sweeney and Xu, 2012).

These enzymes also disrupt cellulose microfibrils to enhance cellulase enzymes in hydrolysis (Moser *et al*., 2008). Through affinity to cohesion, dockerin attaches host enzymes onto scaffoldin to assemble a cellulosome consisting a clustering of different though synergistic enzymes. Modularity equips lignocellulololytic enzymes with enormous flexibility (Sweeney and Xu, 2012).

In most cases, lignocellulosic enzymes degrade celluloses and hemicelluloses by hydrolytic reactions while they degrade lignin by oxido-reduction reactions. Virtually all cellulases and hemicellulases fall into carbohydrate hydrolases family. They utilise two mechanisms in their hydrolytic reactions. First, is the "retaining" mechanism which leads to product of the same anomeric configuration after cleaving a glycosidic bond with a "double-displacement" hydrolysis. Also an "inverting" mechanism, which leads to a product of the opposite anomeric configuration after cleaving a glycosidic bond with a "single nucleophilic-displacement" hydrolysis. In both cases two acidic amino acid residues (Glu or Asp) as a proton donor and as a nucleophile are involved (Vocaldo and Davies, 2008)

2.5.1 Cellulases

Hydrolytic cleavage of the β(1→4) glucosidic bond in cellulose, leading to the release of glucose (Glc) and short cellodextrins, is carried out mainly by cellulases, a group of enzymes comprising cellobiohydrolase (CBH), *endo*-1,4-β-D-glucanase (EG), and β-glucosidase (BG). Although cellulose is relatively simple in terms of composition and morphology, there is a vast natural diversity of cellulases with catalytic modules belonging to about fourteen GH families to accommodate four major reactions modes and different synergisms (Sweeney and Xu, 2012).

2.5.1.1. Cellobiohydrolase

Cellobiohydrolases hydrolyze β-1,4-glycosidic bonds from chain ends, producing cellobiose as the main product. CBHs create a substrate-binding tunnel with their extended loops which

surround the cellulose (Dashtban *et al*., 2009). Cellobiohydrolases are monomers with no or low glycosylation with pH optima between 4.0 and 5.0, but the temperature optima are wider, from 37 to 60 °C. Studies have shown that some CBHs can act from the non-reducing ends and others from the reducing ends of the cellulosic chains, which increases the synergy between opposite-acting enzymes (Dashtban *et al*., 2009).

Degradation of crystalline cellulose is carried out mainly by Cellobiohydrolases (CBHs), thus the enzymes are essential for industrial enzymatic lignocellulose degradation. Archetypical CBHs are found in GH6 and 7, as well as 48, families. GH7 CBH is found in all known cellulolytic fungi. GH6 CBH is also found in many cellulolytic fungi. Chundawat *et al*., (2011b) observed that about 70% secreted proteins and enzymes of cellulolytic fungi may be CBHs. Also known as CBH-I, GH7 CBH has specificity towards the reducing end of a cellulose chain. In contrast, GH6 CBH, also known as CBH-II (EC 3.2.1.91), can be specific towards the non-reducing end of a cellulose chain. Such "opposing" specificities render GH7 and 6 CBHs highly synergistic and cooperative in degrading their common substrate (Sweeney and Xu, 2012).

The CBH catalytic core features tunnel-like active sites, a topology that equips CBH with the ability to hydrolyze cellulose: it threads into the end of a cellulose chain through its active site, cleaves off a cellobiosyl unit, glides down the chain, and starts the next hydrolysis step (Liu *et al*., 2011). A CBM may assist the catalytic core with processivity (Beckham *et al*., 2010). Processive CBH movement can be obstructed by kinks or other impediments on the cellulose surface and as such it has been suggested that $k_{(off)}$ values may be a major factor in CBH efficiency (Praestgaard *et al*, 2011). GH7 CBH-I may have approximately ten anhydro-Glc-binding subsites within its active tunnel, in which a cellulose segment or cellodextrin is bound and activated via H-bonding and π-stacking with key

amino acid residues. In addition to the catalytic core, many CBHs also have CBMs, which is believed key in CBH's action on crystalline cellulose (Sweeney and Xu, 2012).

2.5.1.2 Endo-1,4-β-Glucanase

Endoglucanases (EG) are also referred to as carboxymethylcellulases (CMCase). Initiate cellulose degradation by attacking the amorphous regions of the cellulose. This makes cellulose more easily reached for cellobiohydrolases by providing new free chain ends. Fungal Endoglucanases are generally monomers with no or low glycosylation and have an open binding cleft. They mostly have pH optima between 4.0 and 5.0 and temperature optima from 50-70 °C (Dashtban *et al*., 2009). There is a significant synergism between CBH and EG, this synergism is inevitable for efficient enzymatic systems of industrial biomass-conversion.

Different EGs have a catalytic core belonging to over ten GH families, of which GH5, 7, 9, 12, 45, and 48 are representative. Typical cellulolytic fungi secrete EGs at approximately 20% wt level in their secretomes (Sipos *et al*., 2010). Also known as EG-I, II, III, and V, respectively, GH7, 5, 12, and 45 EG are most common in natural fungal cellulase mixes. Most cellulolytic fungi and bacteria produce numerous EGs. Although they all act on the same cellulose substrate, they do so through differing mechanisms ("inverting" for GH6, 9, 45, and 48 EGs; "retaining" for GH5, 7, 12 EGs). Such EG "plurality" may relate to different EGs' side-activities on hemicellulose in degrading complex lignocelluloses (Vlasenko *et al*., 2010), or synergism between processive and conventional EGs (Wilson, 2008).

The active sites of most EGs are cleft- or groove-shaped, inside which a cellodextrin or a cellulose segment may be bound and acted on by EG. In addition to the catalytic core, EGs may possess CBMs or other domains. CBMs may direct host EG, but is not a pre-requisite, for EG's action.

2.5.1.3 β-Glucosidases

β-glucosidases hydrolyze soluble cellobiose and cellodextrins to glucose. They are competitively inhibited by glucose. β-glucosidases have been placed in families 1 and 3 of glycoside hydrolases based on their amino acid sequences (Dashtban *et al*., 2009). β-glucosidases from fungi, bacteria, and plants are classified as family 3, while β-glucosidases of bacterial, plant and mammalian origins which have galactosidase activity in addition to β-glucosidase activity are classified as family 1.

Degradation of cellobiose, as well as other cellodextrins, is carried out by β-Glucosidases (BG) or cellobiose hydrolase (EC 3.2.1.21). Unlike CBH and EG, BGs in general are not modular (lacking distinct CBMs), and have pocket-shaped active sites to act on the non-reducing sugar unit from cellobiose or cellodextrin (Langston *et al*., 2006). BGs belong to the GH1, 3, and 9 families, with GH1 and 3 BGs being archetypical (Eyzaguirre *et al*., 2005). Unlike the majority of biomass degrading enzymes, the activity of BG, which acts upon soluble rather than insoluble substrate, can be studied using traditional kinetic models (Jeoh *et al*.,2005).

Many cellulolytic fungi produce one or more BGs at levels of about 1% of total secreted proteins, significantly lower than that of CBH and EG (Sipos *et al*., 2010; Chundawat *et al*., 2011b). However, BG plays a key role in the efficiency of an enzymatic lignocellulose-degrading system, because its

action on cellobiose mitigates product inhibition on CBH and EG. For industrial biomass conversion targeting high feedstock loads, supplementing BG to common microbial cellulolytic enzyme preparations can be imperative, because of high cellobiose level during the enzymatic conversion (Kristensen *et al*., 2009).

GH1 BGs tend to be more resilient to product inhibition, as well as more active on different di- or oligosaccharides, than GH3 BGs do. Thus having GH1 BG might enable a cellulolytic enzyme system to be more potent in degrading complex lignocellulose.

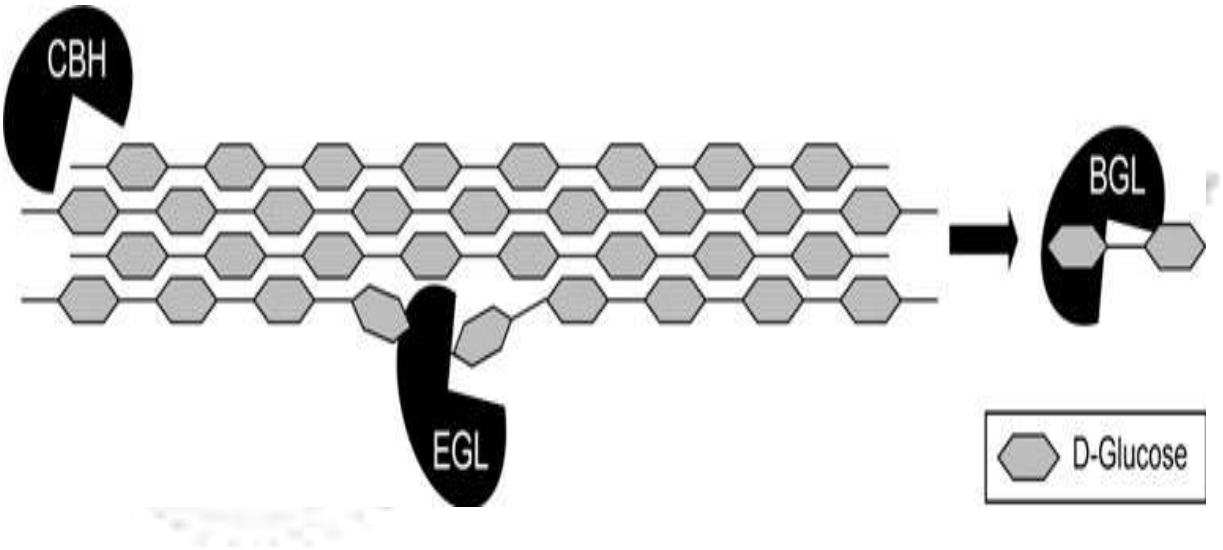


Fig. 6: Schematic Illustration of Cellulose Degradation by Cellulase Enzymes (Van den Brink and deVries, 2011)

2.5.2 Hemicellulases

In plant cell walls, cellulose is entangled with and shielded by hemicellulose, a group of complex polysaccharides made by different glyco-units and glycosidic bonds. Degradation of hemicellulose, which not only "liberates" cellulose for cellulases but also converts hemicellulose into valuable saccharides, is carried out mainly by an array of interdependent and synergistic hemicellulases (Scheller and Ulvskov, 2010).

Common hemicelluloses include β-glucan, xylan, xyloglucan, arabinoxylan, mannan, galactomannan, arabinan, galactan, polygalacturonan, *etc*., which are targets of β-glucanase, xylanase, xyloglucanase, mannanase, arabinase, galactanase, polygalacturonase, glucuronidase, acetyl xylan esterase, and other enzymes (Van den Brink and de Vries, 2011; Scheller and Ulvskov, 2010).

Among hemicellulases, glycoside hydrolases hydrolyze glycosidic bonds, carbohydrate esterases hydrolyze ester bonds, polysaccharide lyases cleave glycosidic bonds, *endo*-Hemicellulases cleave backbone glycosidic bonds, whereas other glycosidases remove mainly the chain's substituents or side chains. Cellulolytic microbes produce many hemicellulases along with cellulases for effective lignocellulose degradation (Sipos *et al*., 2010).

Different plants have different hemicelluloses: acetylated (galacto)glucomannan (as well as arabinoglucuronoxylan), glucuronoxylan, and arabinoxylan are major hemicellulose in softwood, hardwood, and grass, respectively (Scheller and Ulvskov, 2010). Hence different hemicellulase combinations are needed for different biomass feedstocks in industrial biomass conversion. Synergism of hemicellulases is found both amongst hemicellulases themselves and between hemicellulases and cellulases (Banerjee *et al*., 2010; Gottschalk *et al*., 2010; Couturier *et al*., 2011). Fig. 7 below depicts degradation of typical hemicelluloses.

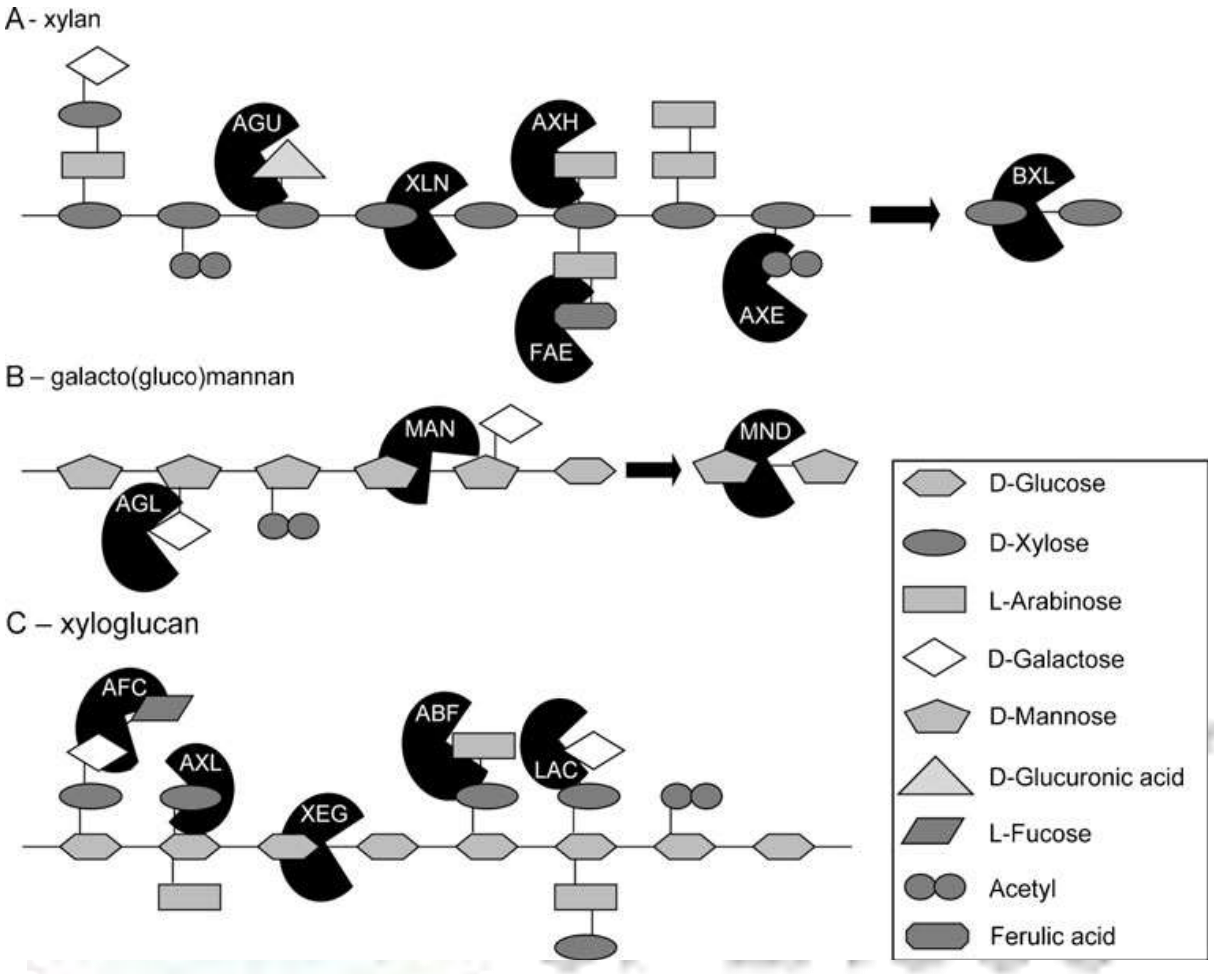


Fig. 7; Schematic Illustration of Hemicellulose Degradation by Hemicellulases (van den Brink and deVries, 2011)

Schematic structure of three hemicelluloses, xylan, galacto(gluco)mannan, and xyloglucan, with hemicellulolytic enzymes. ABF α- arabinofuranosidase, AFC α-fucosidase, AGL α-1,4- galactosidase, AGU α-glucuronidase, AXE acetyl (xylan) esterase, AXH arabinoxylan α-arabinofuranohydrolase, AXL α-xylosidase, BXL β-1,4-xylosidase, FAE feruloyl esterase, LAC β-1,4-galactosidase, MAN β-1,4-endomannanase, MND β-1,4-mannosidase, XEG xyloglucan-active β-1,4-endoglucanase, XLN β-1,4-endoxylanase

2.5.3. Ligninase

Lignin degradation is inevitable for industrial enzymatic biomass-conversion because it not only increases hemicelluloses and cellulose accessibility for their respective enzymes but also diminishes hemicellulase and cellulase inactivation caused by lignin adsorption (Sweeney and Xu, 2012). Fungi degrade lignin by secreting enzymes collectively termed "ligninases". These include two ligninolytic families; (i) phenol oxidase (laccase) and (ii) peroxidases [lignin peroxidase (LiP) and manganese peroxidase (MnP)] (Martinez *et al*., 2005). Interestingly, LiP is able to oxidize the non-phenolic part of lignin, but it was not detected in many lignin degrading fungi. In addition, it has been widely accepted that the oxidative ligninolytic enzymes are not able to penetrate the cell walls due to their size. Thus, it has been suggested that prior to the enzymatic attack, low-molecular weight diffusible reactive oxidative compounds have to initiate changes to the lignin structure (Dashtban *et al*., 2009).

Lignin peroxidase (EC 1.11.1.14), Manganese peroxidase (EC 1.11.1.13), and versatile peroxidase (EC 1.11.1.16) are extracellular fungal heme peroxidases (belonging to LO2 family) with high potency to oxidatively degrade lignin. Upon interaction with H_2O_2, these enzymes form highly reactive Fe(V) or Fe(IV)-oxo species, which abstract electrons from lignin (to cause oxidation or radicalization) either directly or via Mn(III) species. Laccase (EC 1.10.3.2) is a multi-copper oxidase (belonging to LO1 family) secreted by numerous lignocellulolytic fungi. This enzyme can directly oxidize phenolic parts of lignin, or indirectly oxidize non-phenolic lignin parts with the aid of suitable redox-active mediator (Sweeney and Xu, 2012).

2.5.4 Emerging Cell Wall Degrading Enzymes

Besides the major lignocellulolytic enzymes classifications, the microbes producing lignocellulolytic enzymes secrete some proteins, which co-induced and secreted with cellulases and hemicellulases, with more mysterious functions. Some of these molecules, when added to cellulases, enhance their performance, while others has little or no stimulatory effects on cellullase activity (Sweeney and Xu, 2012). A proper understanding to how these enzymes function will lead to improvements in industrial lignocellulose degradation,

2.5.4.1 Cellulase-Enhancing Proteins

Analysis of the polysaccharide cleavage products formed by GH61 and CBM33 molecules reveals that these enzymes release oxidized cellooligosaccharides, though significant quantities of non-oxidized oligosaccharides are also detected in some studies (Forsberg *et al*., 2011; Quinlan *et al*., 2011; Westereng *et al*., 2011). Unlike other glycoside hydrolases, which are more active on cellooligosaccharides than crystalline cellulose, GH61 and CBM33 enzymes appear inactive upon cellooligosaccahrides (Forsberg *et al*., 2011; Quinlan *et al*., 2011). The position of the oxidation on the oligosaccharide products has been reported on the reducing end (Langston *et al*., 2011; Phillips *et al*., 2011), non-reducing end (Langston *et al*., 2011; Phillips *et al*., 2011), or both (Quinlan *et al*., 2011), which could suggest differences amongst these enzymes. GH61 proteins also require a metal for this cellulose cleaving activity, specifically copper which binds tightly to the protein in a type-2 copper site geometry (Vaaje-Kolstad *et al*., 2010). Interestingly, despite the large potential of this enzyme class to promote lignocellulose breakdown, the precise cleavage mechanism of GH61 and CBM33 enzymes is unclear and is the subject of ongoing study by multiple groups.

2.5.4.2 Cellulose Induced Proteins

CIP1 and CIP2 (cellulose induced protein-1 and -2,) were first found in a transcriptional analysis of *T. reesei*. Both contain a carbohydrate binding module and are co-regulated with known cellulases (Foreman *et al*., 2003). The function of CIP1 is unknown, though it is claimed that CIP1 from *T. reesei* has weak activity on *p*-nitrophenyl β-D-cellobioside (Foreman *et al*., 2011) and some synergistic activity with both GH61 and swollenin (Scott *et al*., 2011). CIP2, found in both *T. reesei* and *Schizophyllum commune*, has recently been shown to be an esterase that cleaves the methyl ester of 4-*O*-methyl-D-glucuronic acid (Li *et al*., 2007). This enzyme, now classified as the first member of CE15 family, likely acts in the cleavage of hemicellulose-lignin crosslinks. Further investigation of both the functions and the potential of these enzymes in industrial applications are needed.

2.5.4.3. **Expansin, Swollenin, and Loosinin**

Expansins are of plant proteins which interact with cell walls and/or cell wall components by an unknown activity. This interaction causes expansion or lengthening of cell wall structures. These proteins have two domains. A domain is homologous to the GH45 endoglucanase catalytic core and the other domain is homologous to Group II grass pollen allergens besides both domains have no known catalytic function and display no detectable hydrolytic activity on lignocellulosic or model substrates (Sweeney and Xu, 2012). Swollenin is also a protein causes a disruptive effect on cellulosic substrates. It has sequence homology to plant expansions. It has been expressed in *T. reesei* (Sweeney and Xu, 2012). Loosinin, like swollenin and expasin, increases cellulose activity (Quiroz-Casterneda *et al*., 2011). They are produced by the basidiomycete fungus *Bjerkandera adusta.* Bacterial species, including *Bacillus subtilis* (Kerff *et al*., 2008) and *Hahella chejuensis* (Lee *et al*., 2010), also produces expansin-like molecules. Similar to fungal swollenins, bacterial expansins modifies cellulose fiber structure and promotes the hydrolysis of cellulose without showing detectable direct hydrolase activity (Kim *et al*., 2009)

2.5.4.4 Cellulosomes

Cellulosomes multiple cellulase and hemicellulase proteins, assembled by specific connections between dockerin domains on the enzyme and cohesins bound to structural scaffoldins on the microbial surface (Bayer *et al*., 2008; Fontes and Gilbert, 2010). These proteins are usually produced by anaerobic organisms. This spatial clustering of multiple lignocellulose degrading enzymes results in an increased synergy between lytic activities (Morais *et al*., 2011). It has been shown that recombinant cellulosomes can be transplanted to other industrially useful organisms, such as *S. cerevisiae* (Tsai *et al*., 2010) and *B. subtilis* (Anderson *et al*., 2011).

2.6 IMPROVING ENZYME PRODUCTION

There has been increasing interest in hyper-producing enzymes that have the potential to hydrolyze lignocellulose. This is influenced by many factors which include the large potential market for these enzymes, especially cellulase in the production of many products including bioethanol, glucose syrups, animal feed, extractives etc. These have provided the platform and incentives for the development of genetic improvement programmes which are based on mutagenesis and selection (Chand *et al.*, 2005) which produce new strains (Dillion *et al.*, 2006) and genetic transformants (Meittinen-Oinonem and Suominen, 2002).

There are a number of ways of improving enzyme production. These vary from co-culturing, mutagenesis, genetics and recombinant gene technology to name a few. The techniques to be used depend on the desired change in the organism.

2.6.1 Mutagenesis

Mutagenesis is a process by which the genetic information of an organism is changed in a stable manner, resulting in a mutation. It may occur spontaneously in nature or as a result of exposure to mutagens. It can also be achieved experimentally using laboratory procedures. Major producers of fermentation products usually make use of mutation and selection. The production of cellulases by the microbial cell is regulated by controls that include induction and catabolite repression, or end product inhibition (Kumar *et al*., 2008). These controls are operative under cellulase production conditions, thus resulting in limited yields of the enzymatic constituents.

There have been several attempts to improve enzyme yield using different mutagenic agents combined or alone. Different techniques including UV-light and chemicals were used to induce point mutations in fungi to enable them produce large amounts of degradative enzymes (Dashtban *et al.,* 2009).

Strain improvement has been achieved using chemical mutagens (Parekh *et al.*, 2000), selection and genetic recombination (De Nicolas-Santiago *et al.,* 2006). Chand *et al.* (2005) used simultaneous treatment of fungi with N-methyl-N'-nitro-N-nitrosoguanidine (NTG), ethidium bromide and UV light to enhance cellulase production more than wild type fungi. Abo-State *et al.,* (2010) isolated twenty-nine fungal strains and carried out solid-state fermentation (SSF) on agricultural wastes and compared with *Trichoderma reesei*. They were able to enhance CMCase, FPase, and β-glucosidase production by mutating with 0.5KGy dose of Gamma radiation. Pradeep *et al.*, (2012) subjected *A. niger* to mutations involving treatment with ethidium bromide for the production of cellulases. Mutant strains were found to show increase in activities of cellulase.

Mutagenic treatments of *Trichoderma reesei* Qm 6a, led to the development of mutants with higher cellulolytic activity (Kumar *et al*., 2008). Vu *et al*. (2011) reported that a hypercellulolytic

mutant from *Fusarium oxysporum* was developed by ultraviolet treatment followed by chemical mutagenesis using NTG (100 µg ml^{-1}). The resultant mutant strain had substantially higher (80%) cellulolytic activity than its parent strain. NTG treatment of *Cellulomonas Xavigena* also produced four mutants with improved xylanolytic activities. A mutant creAd30 with the end product inhibition resistance showed improved levels of D-glucose metabolism and was constructed from *Aspergillus nidulans*. However, this effort did not result in robust strains that consistently produce ethanol at high yields under a broad range of conditions and in the hands of different investigators (Kumar *et al*., 2008).

Ultraviolet (UV) light is one of the most utilised physical mutagenic agents. This may not be unconnected to its high success rate. It has strong genotoxic effects to produce DNA damage and induce mutations. UV produces specific DNA damage such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone photoproducts (64PPs) at dipyrimidine sites, where two pyrimidine (Py) bases are juxtaposed in tandem in the nucleotide sequence of DNA. These UV lesions are formed through a photochemical reaction, whose efficiency depends on the wavelength, following direct UV energy absorption by DNA bases. The yields of CPD and 64PP are highest at around 260 nm, and the action spectra of their formation are in parallel to the absorption spectrum of DNA. It is also known that 64PP can change into an isomeric secondary product, Dewar valence isomer, after subsequent absorption of ultraviolet light band A (UVA) with wavelengths around 325 nm (Matsunaga *et al*., 1991). These photolesions, CPD, 64PP and Dewar, are assumed to cause UV-specific mutations (Ikehata and Ono, 2011).

2.6.2 Co-Culturing

Conversion of both cellulolytic and hemicellulosic hydrolytic products can be achieved by co-culturing of two or more hydrolytic microorganisms. Co-culturing enhances hydrolysis of lignocellulosic residues, and also enhances product utilization which diminishes the need for use of additional enzymes in the bioconversion process. In the case of cellulose degradation, all three enzymatic components have to be present in large amounts. Unfortunately, none of the fungal strains, not even the best mutants, are able to produce high levels of the enzymes at the same time (Dashtban *et al*., 2009). Conversion of both cellulosic and hemicellulosic hydrolytic products in a single process can be achieved by co-culturing two or more compatible microorganisms with the ability to utilize the materials. In fact, in nature, lignocellulosic residues are degraded by multiple co-existing microorganisms. Co-culturing of two or more fungal strains in fermentation is widely used in many biological processes including the production of antibiotics, enzymes and fermented food (Ahamed and Vermette, 2008). Mixed fungal cultures have many advantages compared to their monocultures, including improving productivity, adaptability and substrate utilization.

Terijan and Xu (2011) was able demonstrate that co-culturing of various fungi could be important in the complete biodegradation of lignocellulosic biomass since cellulase hydrolytic reactions are prone to inhibition by some oligomeric phenolics contained in lignin molecules.

The main drawback of co-culturing however is the complexity of growing multiple microorganisms in the same culture (Lynd *et al*., 2002). Alternative, to co-culture, microorganisms can be metabolically engineered which and this enable one microorganism to complete an entire task from beginning to end. This can be done by altering metabolic flux by blocking undesirable pathway(s) and/or enhancement of desirable pathway(s).

2.7 HETEROLOGOUS CELLULASE EXPRESSION

2.7.1 Change of AT-Rich Sequences in Desired Gene

Partial processing of pre-mRNA can lead to limitation of heterologous genes expression at the transcriptional level. It has been suggested that AT-rich sequences in the coding regions of heterologous genes act as internal polyadenylation sequences and produce short and incomplete transcripts. It has been reported that premature termination of transcription can be overcome by replacing an AT-rich sequence in gene with a more GC-rich sequence or changing codon usage (Te'o *et al*., 2000). Also, the partial processing of transcripts has been overcome by increasing the GC-content in the AT-rich area without changing the amino acid sequences of protein.

2.7.2 The Use of Strong Promoters in Desired Gene

Strong promoters have a high affinity for RNA polymerase. The strength and specificity of promoter are characteristics of regulated endogenous gene expression and are inevitable for genetic engineering of heterologous gene expression. Limited numbers of promoters allow high level of transgenic expression, usually by ubiquitous and constitutive expression in all tissue types. A promoter for transgenic expression in fungal strains should have certain characteristics to be selected as a suitable promoter for a high-level expression of the interested gene. It must be strong (30% or more of the total gene products), exhibit a minimal level of basal transcriptional activity, be easily transferable to other vectors genomes and to be induced via simple, natural or cost-effective manner (Koushki *et al*., 2011). In transformation constructs, promoters to be used should be induced in a simple and natural condition. Gene expression of cell wall-degrading enzymes (CWDEs) in *Trichoderma* spp. frequently has been reported to be induced by fungal cell wall components and repressed by carbon catabolite repressors, such as glucose (Donzelli *et*

al., 2001). In some cases, starvation conditions alone could trigger CWDEs production, while in others, cell walls or cell wall components were needed (Ramot *et al*., 2000).

2.7.3 The Construction and Use of Protease-Deficient Fungal Strains

Proteases are accountable for the degradation of many heterologous proteins (Van den Hombergh *et al*., 1997). Fungi can secrete extracellular proteases. Extracellular proteases of *Aspergillus* are responsible for the degradation of many heterologous proteins (Broekhuijsen *et al*., 1993). Intracellular or cell wall localized proteases have been implicated for low yields of secreted heterologous proteins as in the case of hIL-6 in *A. awamori* (Gouka *et al*., 1996a &b). So far, most researchers focused their efforts on selecting and using protease deficient strains as hosts for recombinant DNA to overcome protease degradation problems. However, such strains are usually incapacitated and unsteady for large-scale bioreactor and biocontrol applications. Therefore, it is often vital to match the protease loss of function strain to the desired protein and identify genes involved in regulating extracellular protease secretion, since knocking out all native proteases would likely be dangerous (Katz *et al*., 2000). Fungal strains deficient in extracellular proteases have been constructed by random mutagenesis (Mattern *et al*., 1992; van den Hombergh *et al*., 1997) or molecular genetic approaches (Berka *et al.,* 1990; van den Hombergh *et al*., 1997). The use of these protease deficient strains has resulted in the enhancement of production level of heterologous proteins (Berka, 1991; Roberts *et al*., 1992; Broekhuijsen *et al*., 1993). The use of strains deficient in vacuolar proteases has led to increased level of heterologous proteins by *Saccharomyces cerevisiae* (Wingfield and Dickinson, 1993).

2.7.4 Optimization of Codon Usage of Desired Gene

The use of synonymous codons varies between different genes and organisms, the codon usage of the desired gene can be optimized to perk up expression in each used transformants. Genes can then be manipulated with rare codons replaced by their optimal synonyms and then reconstructed by overlap extension of synthetic oligonucleotides. When the native family 11 xylanase gene (*xynB*) from *Dictyoglomus thermophilum* was introduced into *T. Reesei,* enzyme was not produced (Hazell *et al*., 2000; Te'o *et al*., 2002). It was explained that excessive differences in the codon usage between the heterologous gene and the expression host could prevent expression at the transcriptional level. They overcame this problem by constructing a synthetic xynB gene carrying changes in 20 codons which was generated by advanced primer extension PCR using the codon preference of highly-expressed *T. reesei* genes (Te'o *et al*., 2000).

2.7.5 Glycosylation of Produced Heterologous Proteins

Wrong glycosylation of glycoproteins has been suggested as a problem for heterologous protein processing. Engineering the protein glycosylation sites for enhanced usage *in vivo* has made huge improvements in yield of several glycoproteins (van den Brink *et al*., 2006). N-glycosylation has been shown to improve the amount of secreted chymosin in *A. awamori*, although, the specific activity of the chymosin was reduced (Ward, 1989). It has been reported that the overexpression of mannosylphospho dolichol synthase encoding gene from *S. cerevisiae* in *T. reesei*, which is needed for O-glycan precursor synthesis, improved the production of CBHI (Kruszewska *et al*., 1999). Overexpression of a yeast glycosylating enzyme has improved protein yields in an *A. nidulans* mutant impaired in glycosylation (Perlinska-Lenart *et al*., 2005). These observations could indicate that the problems encountered in glycoprotein production could be reduced by manipulating glycosylation. Earlier studies with endogenous *Trichoderma* cellulases have

indicated that N-glycosylation of cellulases of *T. reesei* was not required for enzyme activity and secretion (Kubicek *et al*., 1987), but had an effect on their thermostability and resistance to proteolysis (Merivuori *et al*., 1985; Wang *et al*., 1996).

2.7.6 Use of Native or Artificial Intron-Containing Genes in Fungal Strains

In several eukaryotic genes, introns are necessary for efficient mRNA cytoplasmic accumulation (Nesic *et al*., 1993). It has been suggested that introns regulate gene expression at transcription and post-transcription (Morello *et al*., 2002; Sivak *et al*., 1999) levels. Introns might also protect pre- mRNA from undergoing degradation in the nucleus, facilitate polyadenylation or transport mRNA to the cytoplasm (Liu and Mertz, 1995). Jonsson *et al*., (1992) also concluded that certain genes cannot be expressed without their introns. Two possible mechanisms for intron dependent mRNA accumulation are commonly accepted. One is that introns protect the pre-mRNA from degradation, either by accepting a stable secondary structure or by providing binding sites for factors protecting the pre-mRNA, such as oligo-U binding proteins (Kurachi *et al*., 1995; Gniadkowski *et al*., 1996) or heterogeneous nuclear ribonucleoproteins (Krecic and Swanson, 1999). Other mechanism is that spliceosome assembly onto the introns in pre-mRNA facilitates an association with enzymes involved in other aspects of RNA maturation (such as polyadenylation) and transport of the mRNA to the cytoplasm. Koziel *et al*., (1996) proposed the phenomenon of intron-dependent mRNA accumulation in mammalian and plant cells. It also proposed that the small introns in the genes of filamentous fungi may not only act as intervening elements, but may also play vital roles in gene expression by increasing the stability of the mRNA or by assisting the export of mRNA (Xu and Gong, 2003). In a trial, after introducing the coding genes of *ABH1* (*Agaricus bisporus*), *SC3*, *SC6* (*S. commune*) and *GFP* (*Aequorea victoria*), without introns, no or very low expression level were seen contrasting with the

expression of intron containing genes (Scholtmeijer *et al*., 2001). Xu and Gong (2003) showed that introns are required for AFP gene (a gene encoding antifungal protein in *A. giganteus*) expression in *T. atroviride* transformants, as demonstrated by the level of mRNA and confirmed by analysis of AFP synthesis. However, in *S. cerevisiae* only 2 to 5% of the genes contain introns and no obligation for introns has been observed for vertebrate and plant genes expression (Hiraiwa *et al*.,1997; Lin *et al*., 1997).

2.7.7 Desired Gene Fusion with Well-Expressed Genes

A low mRNA stability can affect heterologous gene expression at the transcriptional level. Many of mRNAs in eucaryotic cells are unstable, because they contain specific sequences (AU-rich) in the 3' untranslated region (UTR) that induce their degradation (Koushki *et al*., 2011). mRNA degradation is facilitated by AU-rich sequence. This is done by stimulating the removal of the poly-A tail found at the 3' end of almost all eucaryotic mRNAs. Other unstable mRNAs contain recognition sites in their 3' UTR for specific endonucleases that cleave the mRNA. Low mRNA stability can be partly overcome by fusing the desired gene to the 3' end of a homologous gene. Fusion of the gene to be expressed behind a highly expressed homologous gene does not only increase stability of the mRNAs, but can also resolve limitations at early stages in the secretion pathway (ER) of protein metabolites. The production of antibody fragments in *T. reesei* as a CBHI fusion resulted in more than 150-fold increase in the yield (Koushki *et al*., 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Source of Sawdust

Abura (*Mitragyna ciliata*) sawdust was collected from Okobaba Saw Mill, Ebute-metta (N06^{o} 28. 714' E003^{o} 23. 426'). Lagos. The sawdust was pulverised and sieved by Jayant Test Sieves (B. S. S. Mesh No. 60 and 0.250mm) to make uniform particle size and to obtain the respective fine powder which was used for the study.

3.1.2 Chemicals

Media used were obtained from Biomark, India; yeast exract from Oxoid, England and other chemicals were obtained from Fluka, Germany.

3.2 Pretreatment of Sawdust

Mechanical pretreatment was done by pulverizing the sawdust. The cellulosic substrate was milled in a communitor and stored in polythene bags (De Sousa *et al.*, 2004). Modified chemical pretreatment methods using NH_4OH as described by Abu *et al.,* (2002) were used. Ammonia solution (2.9M) was prepared to steep Abura sawdust. The substrates were left to steep for five

days after which they were properly washed and sun-dried. The pretreated substrates were maintained to constant weight at 80^{o}C in an oven.

3.3 Determination of Lignocellulosic Content of Sawdust

3.3.1 Determination of Cellulose Content of Sawdust

To 1g of pretreated and unpretreated dried sawdust samples, 15 mL of 80 % acetic was added followed by 1.5 mL of concentrated nitric acid. It was refluxed for 20 min. It was filtered, the residue was washed with ethanol, dried in the oven at 100-105 ^{o}C and weighed. Then it was incinerated at 540 ^{o}C. Cellulose content was determined in accordance with the method of Abdullah *et al.,* (2006) using the formula below.

$$\% \text{ cellulose} = \frac{\text{dried material} - \text{incinerated material}}{\text{weight of sample}}$$

3.3.2 Determination of Hemicellulose Content of Sawdust

Pretreated and unpretreated dried sawdust samples (1g) were added to 15ml of 80% acetic acid and 1.5mL of concentrated nitric acid and refluxed for 20 mins. It was filtered, the residue was washed with ethanol, dried in the oven at 100-105^{o}C and weighed. Then it was incinerated at 540^{o}C. Cellulose content was determined in accordance with the method of Abdullah *et al.,* (2006) using the formula below:

$$\% \text{ hemicellulose} = \frac{\text{dried material} - \text{incinerated material}}{\text{weight of sample}}$$

3.3.3 Determination of Lignin Content of Sawdust

Pretreated and unpretreated samples (1g) were dispensed into 2 different conical flasks. A 70ml portion of 1.25 % H_2SO_4 (sulphuric acid) was added and the mixture was refluxed for 120min,

filtered and washed with water. Thirty mL of 72% H_2SO_4 was added to this material and allowed to stand for 4hr with occasional stirring. It was dried at 105^oC after previously filtered and dried, then incinerated at 540^oC.

Lignin content for both pretreated and unpretreated samples was calculated in accordance with the method of Abdullah *et al.*, (2006) using the formula below:

$$\% \text{ lignin} = \frac{\text{dried material} - \text{incinerated material}}{\text{weight of sample}}$$

3.4 Proximate Analysis

The methods of the Association of Official Analytical Chemists (A.O.A.C., 2005) was used to determine the proximate composition of the unpretreated and pretreated sawdust.

3.4.1 Determination of Moisture Content

Dishes with lids were oven dried to remove traces of moisture and weighed. A 2 g portion of each of the samples was weighed into dishes. The samples were dried in an oven at 100^oC for 5hr without the lid. The samples were removed from the oven after drying and the lid replaced. The dishes were placed in a dessicator containing silica, cooled to room temperature and weighed. The processes of drying, cooling and weighing were continued until a constant weight for each sample dish was obtained.

Calculation: $\% \text{ moisture} = t\text{-}u/s \times 100$

Where s = weight of sample for the analysis

t= weight of sample + dish before drying

u= weight of sample + dish after drying

t - u = moisture content or weight loss

3.4.2 Determination of Crude Protein Content

Finely ground sample (1g) was introduced into the Kjeldahl digestion flask. Concentrated H_2SO_4 (20ml) and 8 g of the catalyst (10 g K_2SO_4 and 0.5g HgO) were added to the flask and mixed to speed up the digestion. Flask was placed on digestion unit and the sample digested in a fume cupboard until a clear pale green solution was obtained and the time noted. The solution was allowed to cool and 150mL of distilled water added, mixed and the content transferred to distillation flask. Saturated 0.1M NaOH (80mL) was added to the distillation flask. The solution was distilled with 50 ml of 2% Boric acid using methyl red indicator. The ammonium borate formed was titrated with standard HCl and titre value noted.

Calculation:

0.01M HCl ≡ 1.40 mg of Nitrogen

Therefore the nitrogen content = titre value × 1.40

$$\%N = \frac{1.40 \times XY}{10^5}$$

Where X is the titre value when the digest is distilled with Boric acid, Y is the weight of the sample used. Crude protein (%) = $1.40XY/10^5 \times 6.25$ (a factor derived from the fact that protein contains 16% Nitrogen).

Equation of the reaction:

Nitrogen in sample $\xrightarrow{\text{conc. } H_2SO_4 \text{ catalyst}}$ NH_3

The ammonia is produced in the form of ammonium tetraoxosulphate (vi), i.e.,

$2NH_3 + H_2SO_4 \rightarrow NH_4(SO_4)_2$

When the ammonium tetraoxosulphate (VI) is reacted with an alkali, such as NaOH, NH_3 is evolved and then trapped into boric acid.

$(NH_4)_2SO_4 + NaOH \rightarrow NH_2SO_4 + 2H_2O + 2NH_3$

$H_3BO_3 + NH_3 \rightarrow NH_4^+H_2BO_3^-$

When the complex is steam-distilled, the NH_3 comes out as NH_4OH which was titrated with HCl. Thus,

$NH_4OH + HCl \rightarrow NH_4Cl + H_2O$

3.4.3 Determination of Ash Content

Four dry porcelain dishes were weighed using an analytical balance. A 2g portion of each the samples was weighed separately into the dish and dried in an oven for 3 hr. The dishes were heated on a bunsen burner until the content turned black. The dishes and contents were placed into a muffle furnace and heated for 7hr until greyish white residue was obtained. The dishes were removed and some drops of water added to expose unashed carbon still present. The dishes were dried again in oven for 3hr and re-ashed in muffle furnace for 45min. The dishes were removed and cooled. The cooled dishes were placed in a desiccator to cool to remove traces of moisture. The dishes with the contents were weighed to determine the level of ash using the calculation below.

Calculation

$$\text{Ash (\%)} = \frac{100(X-Y)}{Z}$$

Where X= weight (g) of dish and content after drying

Y= weight (g) of empty dish

Z= weight of sample (g) used for the analysis

3.4.4 Determination of carbohydrate content

Samples (2 g) were weighed on filter paper and transferred to a measuring cylinder containing 10 ml of distilled water. Fifty two percent cold perchloric acid (10 mL) was added to the dissolved sample and stirred for 30 min. The solution was further diluted to 100mL with distilled water. The solution was filtered into a 250 mL graduated flask and filled to mark. A 45 mL portion of the extract was diluted to 450 mL with distilled water. 1mL of the diluted extract was pipetted into test tube using 1mL of distilled water as blank and glucose as standard (0.15 mg/mL). A 5 mL portion of anthrone reagent was added to the tubes and incubate in a water bath for 12 min; cooled and absorbance was read at 630 nm with a spectrophotometer.

Calculation

% glucose = $25\ A_1/\ A_2 \times 100$

Where A_1 = Absorbance of diluted sample

A_2 = Absorbance of diluted standard

3.4.5 Determination of Crude Fat

Samples (2 g) were weighed on Whatman 542 filter paper and transferred to extraction thimble. The thimble was placed in a beaker and put in an oven for 7 hr to expel traces of moisture. The beakers were removed from the oven and placed into the soxhlet apparatus and the solvent mixture (chloroform : methanol 2:1) was added. The lipid content was extracted at condensation rate of 240 drops/min for 7hr. The extract was transferred to an already weighed evaporating dish. The solvent was allowed to evaporate in a fume chamber and dried in an oven at 100^0C for 25min. The dish was removed and taken to a dessicator to cool and weighed.

Calculation

Crude fat (%) = $\underline{(X-Y)}$

$$Z \times 100$$

Where X = weight (g) of dish and content after drying

Y = weight (g) of empty evaporating dish

Z = weight of sample taken for analysis

3.4.6 Crude Fiber Content

This was determined by subtracting the proximate components from 100% as shown below as shown below:

%Crude fiber = 100 - (%ash +%protein+%moisture+%CHO+%crude fat)

3.5 Isolation of Fungi

3.5.1 Organisms and Culture Conditions

Fungi were isolated from decomposing wood wastes obtained from Okobaba Sawmills, Ebute-metta, south-west Nigeria, using different selective and differential media (Guarro *et al*., 1999). They were maintained on potato dextrose agar, PDA (Biomark Laboratories, Pune, India) and modifed Czapek Dox agar (CDA) under ambient laboratory conditions (28 ± 2 ^{o}C, 12hr light and 12hr darkness). Morphological studies of their mycelia were done by inoculation of sample from pure culture into PDA broth and Czapek Dox broth into 100ml conical flasks and were maintained at 28 ± 2 ^{o}C for 5 days. The fungi were identified from their cultural and morphological characteristics by Prof. Adejare Adekunle at the Department of Botany, University of Lagos, Nigeria.

3.5.2 Macroscopic and Microscopic Study

Macroscopic study was done by studying the growth rate, texture, pigmentation of both top and reverse plates, and topography of colony using PDA, yeast extract agar and Czapek Dox agar.

Microscopic study was done by preparing slide mount with lactophenol cotton blue stain and observed under light microscope.

3.6 Genotyping of Selected Isolates

3.6.1 Harvesting of Mycelia

Mycelia of isolates were harvested from Czapek Dox media grown for 36 hr in 100 mL flask containing 30 mL of modified Czapek Dox media which contained: 3 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4$, 1 g KH_2PO_4, 0.01 g $FeSO_4$, 20 g glucose, 20 g agar per litre (Nwodo-Chinedu *et al.*, 2007). The mycelia were collected by filtration/decantation through muslin cloth, washed with cold sterile water and cold acetone on a Buchner funnel. The shrunken mycelia material was then washed for 3hr under cold acetone. The dehydrated mycelia so obtained were desiccated under vacuum (to remove vapour) for 20 min. The dried mycelia were stored at 4 ^{o}C in the refrigerator for use.

3.6.2 DNA Extraction

The extraction was achieved using the modified method of Keb-Llanes *et al.,* (2002). Mycelia of isolates were harvested from Czapek Dox media grown for 36 hr in 100 mL flask containing 30 mL of Czapek Dox media (per litre). The mycelia were collected by filtration through muslin cloth, washed with cold sterile water and cold acetone on a Buchner funnel. The shrunken mycelia material was washed for 3 hr under cold acetone. The dehydrated mycelia so obtained were dessicated under vacuum for 20 min. The dried mycelia obtained from each (500mg) isolate was washed twice with sterile Ca^{2+}/Mg^{2+} free phosphate buffered saline (PBS) at pH 7.4. 200µL of N-Cetyl-N,N,N- trimethylammonium bromide (CTAB) buffer (2X) containing 55 mmol/L CTAB, 1.4 mol/L NaCL, 20 mmol/L EDTA and 0.1 M Tris (pH 7.0) was added to the

washed pellets. Pellets were crushed with a sterile dounce homogenizer and incubated at 65 ^{o}C for 2h r with occasional vigorous shaking. A 120 µL portion of chloroform/isoamylalcohol (24:1) was added and the mixture was placed on an orbital shaker at room temperature for 20min. Mixture phases were resolved by centrifugation at 4000 rpm for 30 min. The aqueous phase was carefully pipetted into a fresh microcentrifuge tube and 300 µL of pre-chilled isopropanol was added and incubated at -80^{o}C for 1hr. DNA pellets was collected by centrifugation at 3000 rpm for 5min, the supernatant was discarded and the pellet was washed with 70 % ethanol twice and dried. Pellets were dissolved in 100µL TE buffer (10 mM Tris: 1 mM EDTA, pH 8) after extraction by heating to 56°C for 10min.

3.6.3 DNA Quantification

The integrity of the isolated DNA was confirmed by running agarose gel electrophoresis (0.8% gel strength) alongside a negative control. Staining was done by mixing 5µL of DNA template with 2µL (each) of loading dye and SyberGreen. Electrophoresis was carried out at 120V, 50Hz, 300mA for about 30min. Bands were viewed using BioRad^{TM} Gene Viewer. DNA quantification was done using nanodrop spectrophotometric measurement of UV absorption of 260nm and 280nm (Tiwari *et al.*, 2011). DNA concentration was calculated using the formula:

DNA concentration (µg/mL) = OD at 260nm X dilution times X standard value (1nm =50µg).

3.6.4 PCR Amplification of ITS1, 5.8S, and ITS2 fragments

PCR amplification of the internal transcribed spacer regions ITS1, ITS2, and 5.8S of the rRNA gene cluster (Bellemain *et al.*, 2010) was performed in a 25µL reaction containing 5µL total genomic DNA template, 1X buffer, 0.2mM dNTP mix, 0.25 µM of each primer pair ITS1(5'-TCC GTA GGT GAA CCT GCG G-3'), ITS4 (5'- TCC TCC GCT TAT TGA TAT GC-3') and

0.04 U/µL High Yield taq polymerase (Jena Biosciences, Germany). The PCR conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles for 30sec at 94°C, 30sec at 52°C, and 30 sec at 72°C. A final extension step was conducted at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1.2% agarose gel containing ethidium bromide in tris-acetate EDTA buffer at 120V for 25min. Gel Images were captured and analyzed using Gel analyzer version 2010a.

3.6.5 Sequencing of PCR Amplified Fungal ITS fragments

PCR amplicon bands of approximately 648 bp on the 1.2 % agarose gel were excised and purified using the NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Germany) as specified by the manufacturer. Purified PCR amplicons were sequenced in one direction using the forward primer (ITS1 primer) on an ABI377 DNA sequencer (Jena Bioscience, Germany).

3.6.6 Molecular Phylogenetic Analysis

The 18S rDNA and 5.8S rDNA sequences were compared to sequences received from GenBank database by using BLASTN algorithm version 2.2.28+ (http://www.ncbi.nlm.nih.gov/BLAST/). Other known sequences of similar fungal species were downloaded and aligned using CLUSTAL W of the MEGA-5 Software (Tamura *et al.*, 2011). Aligned sequences were pruned and edited with Jalview software 2.8 (Waterhouse *et al.*, 2009). Phylogenetic analyses were conducted in MEGA-5 (Tamura *et al.*, 2011); evolutionary history was inferred using the Maximum

Likelihood method based on the Tamura-Nei model with 1000 replicates of bootstrap cycles (Tamura and Nei, 1993).

3.7 Co-culturing of Fungi for Biodegradation of Sawdust

Cellulolytic activity of the isolates was determined and two fungi with lead activities were selected. The effect of biodegradation using single cellulolytic organism or synergistic combination of organisms was assessed. The effect of pretreatment was also determined using these two organisms on the substrate. Ten discs (8mm size) of fungal spores which were freshly sub-cultured on PDA were used to inoculate 5g of the pretreated and the unpretreated sawdust submerged in mineral salt media earlier described. Control was set up using pretreated and unpretreated sawdust submerged in flasks containing the media without the organisms. The pH of the medium, released reducing sugar, enzyme activity, and protein were monitored using modified methods of Nwodo-Chinedu *et al*., 2007.

3.8 Determination of Optimum Fermentation Conditions

The modified method of Acharya *et al.,* (2008) was adopted for the optimization studies. Optimum substrate concentration was determined by carrying out fermentation with different amounts of pretreated sawdust (2.4-12 % w/v) as substrate in the fermentation media. Determination of optimum pH (4.0-6.0) was carried out. The different nitrogen sources optimized were peptone, $NaNO_3$, $(NH4)_2SO_4$ and urea. Peptone was used in range from 0.05-0.15% w/v. $(NH4)_2SO_4$ was used in range from 0.1-0.18 % w/v while sodium nitrate was used in the range 0.05-0.15% respectively. However, urea was used in range from 0.01-0.05 % w/v. All the flasks were inoculated with 10 discs of 8 mm size of *A. niger* and *P. citrinum* and incubated

at 28 ± 2°C in a 250ml Erlenmeyer flask in an orbital shaker incubator at 120 rpm. At regular intervals, enzyme assays were performed.

3.8.1 Media Preparation and Enzyme Production

Medium composition described by Mandels and Weber as reported by Acharya *et al.*, (2008) was used for fermentation. The media contained 0.3 g Urea, 1.4 g $(NH4)_2SO_4$, 2.0 g KH_2PO_4, 0.3 g $CaCl_2$, 0.3 g $MgSO_4.7H_2O$, 1.0 g protease peptone, 5.0 mg $FeSO_4.7H_2O$, $MnSO_4.7H_2O$ 1.6 mg, $ZnSO_4.7H_2O$ 1.4 mg, $CoCl_2$ 2.0 mg per litre. The pH of media was adjusted to 5.0 ± 0.2.

3.8.2 Reducing Sugar Assay

Extract from the fermentation crude was centrifuged and the supernatant (1mL) was collected into test tubes. 1mL of prepared DNS reagent was added. It was boiled for 5min and allowed to cool before the absorbance was read at 540nm against blank (Singh *et al.*, 2009).

Enzyme assay was carried out according to the methods described by Singh *et al.* (2009). Carboxyl-methyl cellulase (CMCase) activity was determined at 40^oC by using carboxy-methyl cellulose as substrate. For total cellulase/filter paper (FPase) activity, Whatman No. 1 filter paper strip of dimension 1.0 x 6cm (50mg) was placed into each assay tube. The filter paper strip was saturated with 1.0ml of Na-Citrate buffer (0.05M, pH 4.8) and was heated for 10min at 50^OC. Half milliliter of an appropriate diluted enzyme (in Na-citrate buffer, 0.05M; pH 4.8) was added to the tube and incubated at 50^OC for 60min. Cellulase assay was determined using the method of Singh *et al.*, (2009).

3.8.3 Protein Content Determination

Five microlitre each of bovine serum albumin (BSA) standard and tests were pipetted into the appropriate microplate wells (Thermo Scientific Pierce™ 96-Well Plates). 250µL of Coomassie blue reagent was added to each well and was mixed with plate shaker for 30min. It was removed from plate shaker and incubated at room temperature for 10min in order to obtain a consistent result. The absorbance was measured at 595nm with a plate reader. The average measurement for the blank replicates was subtracted from all other measurements (standard and unknown samples). Standard curve was plotted from which protein concentration of the tests were determined.

3.9 Mutagenesis of Fungi for Hyper-Production of Cellulase

3.9.1 UV Mutation

The mutagenesis step involved finding the exposure time at which 50% of the fungal spores were inactivated by ultraviolet radiation (LD_{50}) at different distances. Four days old spores of fungi were harvested, counted in a Neubauer chamber and their concentration adjusted to $1x10^6$spores/mL. Fifteen mL of the spore suspension were poured into sterile petri dish and agitated during the procedure. The ultraviolet radiation source was placed at different distances above the surface of the spores suspension (10-50cm), radiation intensity was delivered at 300µW/cm^2 (modified method of De Nicholas-Santiago *et al.*, 2006) at different times ranging from 10-60 mins. Samples were taken at intervals of 5 min, kept in the dark and concentration adjusted to $1x10^4$spores/mL and inoculated in petri dishes containing mineral salt agar at 30 ^oC for 72 hr.

3.9.2 Selection of Hyper Producing Mutants

The selection of mutants was based on the modified method of De Nicholas-Santiago *et al.*, (2006). The parent and mutated strains were inoculated (0.5mL) separately in flasks containing mineral salt medium (MSM) which contained 10g glucose, 0.3g Urea, 1.4g $(NH_4)_2SO_4$, 2.0g KH_2PO_4, 0.3g $CaCl_2$, 0.3g $MgSO_4.7H_2O$, 1.0g protease peptone, 5.0mg $FeSO_4.7H_2O$, 1.6mg $MnSO_4.7H_2O$, 1.4mg $ZnSO_4.7H_2O$, 2.0mg $CoCl_2$ and 1.0g 2-deoxy-D-glucose. The pH of media was adjusted to 5.0 ± 0.2, and incubated at 30^oC for 72hr. The contents of the flasks were harvested by centrifugation at 4000 x g for 10min. The clear extract was used as enzyme source.

The crude enzyme was subjected to screening using the cup-plate method. Two grams of ammonium oxalate, 2g of agar and 1g of cellulose were dissolved in 100ml of water in a 150ml conical flask. The mixture was autoclaved at 121^oC for 15min. It was then poured into sterile petri dishes and allowed to solidify. Holes were made into the agar which were filled with 1ml of the crude enzyme and sealed with cellophane paper. The plates were incubated at room temperature for 24hr. The zone of hydrolysis was detected with potassium iodide-iodine solution which contained 1.0g Iodine, 5.0g potassium iodide and 330ml H_20/Litre. Potency index was calculated as the ratio of diameter of clearance (DC) to diameter of cup (DC_0).

3.10 Improvement of Fungal Cellulase Production by Sequential UV Mutation and Optimizing Solid State Fermentation

The effect of various physicochemical parameters required for maximal production of cellulase by the *A. niger* and *P. citrinum* mutants were investigated using solid state fermentation (SSF). The parameters optimized were moisture (10-60 % v/w), incubation temperature (25-50^0C), pH (3-6), fermentation time (1-8 days), size of inoculum (%, w/w), and age of culture medium

(days). Studies were also done to investigate the effect of various supplementations into the culture on cellulase production. The supplementations include carbon source (glucose, lactose, sucrose, maltose, corn starch), surfactants (sodium dodecyl sulfate SDS, ethylene-diamine-tetra acetic acid EDTA, Tween 20, Tween 80), metal salts ($ZnSO_4$, $FeSO_4$, $CuSO_4$, $CaCl_2$, $MgCl_2$, $CoCl_2$, KCl), and nitrogen supplements (malt extract, yeast extract, ammonium nitrate, ammonium chloride, urea).

3.11 Partial Purification and Characterization of Cellulase

The modified method of Kolawole *et al.,* (2006) was followed in the partial purification and characterization of the cellulase enzyme. The crude enzyme preparation was obtained from 72h solid-state fermentation.

3.11.1 Cellulase Production

The medium composition described by Mandels and Weber as reported by Acharya *et al.*, (2008) was used for fermentation. The media contained 20g glucose, 0.3g Urea, 1.4g $(NH4)_2SO_4$, 2.0g KH_2PO_4, 0.3g $CaCl_2$, 0.3g $MgSO_4.7H_2O$, 1.0g protease peptone, 5.0mg $FeSO4.7H_2O$, $MnSO4.7H_2O$ 1.6 mg, $ZnSO_4.7H_2O$ 1.4 mg, $CoCl_2$ 2.0 mg per litre. The pH of media was adjusted to 5.0 ± 0.2. After sterilization at 121°C for 15min, flasks were cooled, inoculated (1·105 spores/mL) and incubated at 30°C for a period of 72hr. The content of the flask was harvested by adding 100mL of 50mM Na-Citrate buffer pH 5 and centrifuged at 5000 x g for 10 min, the clear filtrate was used as crude enzyme. The clear filtrates were pooled and freeze dried at 4^oC, to about 120mL.

3.11.2 Ammonium Sulphate Precipitation

The concentrated fractions of the crude enzyme (50mL) were precipitated using 10-80% $(NH_4)_2SO_4$ precipitation with gentle agitation until final dissolution of the salt. The mixture was centrifuged at 1000 x g for 20 min. The precipitate was reconstituted in 50 mM Na-Citrate buffer pH 5 and dialyzed over-night at 4^oC against the same buffer.

3.11.3 Anion Exchange Chromatography

Gel chromatography was carried out using anion exchanger; Sephadex A25-120 and Whatman DE-52. 10 g of Sephadex A25-120 was suspended in Tris-HCl buffer pH 8.3, to equilibrate and allowed to swell for 10hr. After swelling it was packed in a vertical glass tube chromatography column of 1.5 × 70cm. The column was eluted several times with 0.05M acetate buffer (pH 5.0). Whatman DE-52 suspension was prepared using the same buffer but allowed to swell for 5hr. The void volume was determined with blue dextran. Ten millilitres of the ammonium sulphate-dialysate enzyme concentrate was applied to the column eluted with buffer and fractions of 5mL at a flow rate of 20mL h^{-1} were collected. The bound proteins were eluted with 0-1.0M NaCl in the same eluting buffer. The pooled bound cellulase was dialyzed for 24hr with four changes of the acetate buffer (0.05M, pH 5.0) and stored at -5^oC. All purifications were carried out in the cold laboratory.

3.11.4 Determination of Protein

Protein was determined spectrophotometrically at 280 nm using the Microplate method (Thermo Scientific) and cellulase activity was assayed using the method of Singh *et al.*, (2009).

Five microlitre each of Bovine Serum Albumin (BSA) standard and tests were pipetted into the appropriate microplate wells (Thermo Scientific Pierce^{TM} 96-Well Plates). Two hundred and fifty microliter of Coomassie blue reagent was added to each well and was mixed with plate shaker for

30min. It was removed from plate shaker and incubated at room temperature for 10min in order to obtain a consistent result. The absorbance was measured at or near 595nm with a plate reader. The average measurement for the blank replicates was subtracted from all other measurements (standard and unknown samples). Standard curve was plotted from which protein concentration of the tests were determined.

3.11.5 Effect of Substrate Concentration

The effect of various concentrations of carboxymethylcellulose CMC (2mg/mL-20mg/mL) on the enzyme activity was studied at optimum temperature and pH. The maximum velocity (Vmax) and Michaelis Menten constant (Km) of the enzyme were obtained using the Line-weaver Burk-plot.

3.12 GENETIC STUDIES

3.12.1 RNA extraction

Spores from the wild and mutated fungi were grown on PDA for four days. The spores were inoculated in mineral salt media (MSM) and allowed to grow for another four days. Fungal mycelia were harvested and 50μg was transferred aseptically into sterile eppendorf tubes containing the lysis buffer.

The Direct-zol RNA MiniPrep kit (Zymo Research, USA) was used according to manufacturer's instruction for the extraction.

3.12.2 RNA Quantification

This was done using the Nanodrop spectrophotometric technique as applied by Aranda *et al.*, (2012). RNA sample (0.1µL) was introduced into the probe pedestal of the spectrophotometer and the absorbance read at 230nm, 260nm and 280nm. The quality of the extracted RNA was confirmed by running the samples in 1% agarose gel electrophoresis using 1X TAE buffer.

3.12.3 Reverse Transcription

This polymerase chain reaction based technique was accomplished using SCRIPT reverse Transcriptase (Jena Bioscience, Germany) kit. The following components were mixed gently in a nuclease-free microtube on ice: 5µg RNA template, 50-100ng primer, 1X SCRIPT RT complete buffer, 500nM dNTP mix, 5mM DTT stock solution, 40 units RNase inhibitor, 100-200 units SCRIPT reverse transcriptase, the content of the tube was made up to 20µl. The mixture was incubated at 65-67^{o}C for 5min after which it was placed at room temperature. Thermal conditions applied were 42^{o}C for 10 min, 50^{o}C for 10 min, 70^{o}C for 10 minutes and finally left at 4^{o}C for an extended period.

3.12.4 Amplification of cDNA

This was carried out using the SCRIPT DNeasy kit (Jena Biosciences, Germany) according to the procedure described by Bellemain *et al.*, (2010).

PCR amplification of the cbh1 gene was performed in a 25µL reaction containing 2µL cDNA template, 1X buffer, 0.2mM dNTP mix, 0.25 µM of each primer pair forward (5'-GTC ATT GAC GCC AAC TGG CGC TGG-3'), reverse (5'- ACG CTC CCA GCC CTC AAC GTT GG-3') and 0.04 U/µL High Yield taq polymerase (Jena Biosciences, Germany). The PCR conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles for 30sec at 94°C, 30sec at 52°C, and 30sec at 72°C. A final extension step was conducted at 72°C for 10 min. The PCR

products were analyzed by electrophoresis on 1.2% agarose gel containing ethidium bromide in tris-acetate EDTA buffer at 120V for 25min. Gel images were captured and analyzed using Gel analyzer version 2010a.

3.12.5 Agarose Gel Electrophoresis

Amplifcation was confirmed by electrophoresing amplicons in 1% agarose. Staining was done by mixing 5µL of cDNA template with 2µL of each of loading dye and SyberGreen. Electrophoresis was carried out at 120V, 50Hz, 300mA for about 30min. On completion of electrophoresis, bands were viewed using PrepOne^{TM} Sapphire Gene Viewer (Embi Tec).

3.12.6 cDNA Clean-Up

Gel slice containing the cDNA fragment of interest was excised using sterile sharp scalpel. The Gel cleanup kit (MCLAB, USA) was used. The protocol for spin column chromatography as described by manufacturer was followed. A 200mg portion of gel was transferred into sterile 2ml centrifuge tube. 1ml of QG buffer (provided by manufacturer) was added onto it. It was incubated at 37^{o}C for 10min until the gel was completely dissolved. The column was placed in a collection tube and 0.7ml of dissolved gel mixture was loaded into the column and centrifuged for 60sec at 8000rpm. The flow-through was discarded. This step was repeated for the rest of the mixture. The column was washed twice with PE buffer by centrifuging for 30sec. The flow through was discarded. The column was centrifuged at full speed for 5 min to remove residual ethanol. The column was placed in a new 1.5ml centrifuge tube and 30µl of Elution Buffer added to the centre of the membrane. The column was centrifuged at maximum speed for 2min after allowing it to stand for 10min. Eluted cDNA was stored at -20^{o}C for further use.

3.12.7 cDNA Sequencing

Purified PCR amplicons were sequenced in one direction using the forward primers (lacc1, ace1, aep1 and cbh1 primers) using Sanger dideoxy method on an ABI377 DNA sequencer (MCLAB, USA).

3.12.8 Bioinformatics and Prediction of Cellulase Structure

The sequences of the mutant fungi amplified using primers designed for lacc1, ace1, aep1 and cbh1 genes were compared to those in the NCBI GenBank database by using BLASTN algorithm version 2.2.28+ (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences of wild species of the same organism were aligned using CLUSTAL W of the MEGA-5 Software (Tamura *et al.*, 2011). Aligned sequences were pruned and edited with Jalview software 2.8 (Waterhouse *et al.*, 2009). Regions where mutation has occurred were identified.

3.13 Statistical Analysis

All experiments were carried out in triplicates, unless otherwise stated. The results were expressed as mean ± standard error of mean. The treatment means were tested for significant difference and compared with the control using the Dunnet interval test. The Student's t-*test* was used to compare between two groups. One-way analysis of variance (ANOVA) with Turkey's test was used to compare the level of significant difference between the samples more than two; $p<0.05$ was considered to be significant. These were done using the GraphPad Prism 5 statistical package.

CHAPTER FOUR

4.0 RESULTS

4.1 Analysis of Lignocellulosic Content of Sawdust

The lignin, cellulose and hemicellulose content of both unpretreated and pretreated sawdust are shown in Table 2. After the pretreatment process, the lignin content and the hemicellulose content reduced showing the removal of part of hemicelluloses and lignin components as a result of the pretreatment process. The cellulose and hemicellulose content of both types of sawdust are however, significantly different from each other ($p<0.05$). This consequently makes cellulose more accessible for microbial hydrolysis.

4.2 Proximate Composition of Sawdust

The sawdust was found to be high in crude fibre but low in carbohydrate, lipid, ash, protein and moisture content (Table 3). However, when it was chemically pretreated, the moisture content and crude fibre reduced significantly.

Table 2: Lignocellulosic Composition of Unpretreated and Pretreated Sawdust

Sawdust Type	Lignin Content (%)	Cellulose Content (%)	Hemicellulose Content (%)	Ash Content (%)
Unpretreated	19.10±0.30	48.11±1.60*	*32.70±2.20	0.09±0.10
Pretreated	15.20±0.20	64.94±1.20*	*19.80±1.30	0.06±0.10

*The values were significantly different from each other ($p < 0.05$). Values are expressed as Mean±SEM; N=3

Table 3: Proximate Composition of Unpretreated and Pretreated Sawdust

Sawdust type	Moisture (%)	Protein (%)	Ash (%)	Lipid (%)	Crude (%)Fibre	CHO (%)
Unpretreated	6.30±0.60*	2.50±0.20	1.70±0.10	6.40±0.40	62.20±3.40*	24.40±1.80
Pretreated	3.60±1.30*	4.00±0.20	2.00±0.10	5.70±0.20	52.80±2.20)*	29.80±1.60

*Values were statistically different from each other ($p < 0.05$). Values are expressed as Mean±SEM; N=3

4.3 Isolation of fungi

Four foremost cellulase producers were retained for further studies. Selected fungi were *Penicillium corylophilum, Penicillium citrinum, Aspergillus niger, and Trichosporon asahii*.

Colonies of *Penicillium corylophilum* were green in colour on PDA plate. The reverse side of plate was yellow. Microscopic feature include septate hyphae (Fig.s 8A, 8E, and 8I). Colonies of *Penicillium citrinum* were powdery and purplish-green in colour **o**n PDA plates. The reverse side of the colony was pale yellow in colour, similar characteristics were observed on Czapek Dox

media. Hyphae were septate and rough (Fig.s 8B, 8F, and 8J). *Aspergillus niger* was black in colour on PDA plate. The reverse view of the plate was slightly yellow. Hyphae were septate and rough with very dense spores (Fig.s 8C, 8G and 8K). Colonies of *Trichosporon asahii* on PDA were white, creamy with a wide margin. The reverse side of the plate was also creamy in colour. Hyphae were arial and smooth (Fig.s 8D, 8H, and 8L).

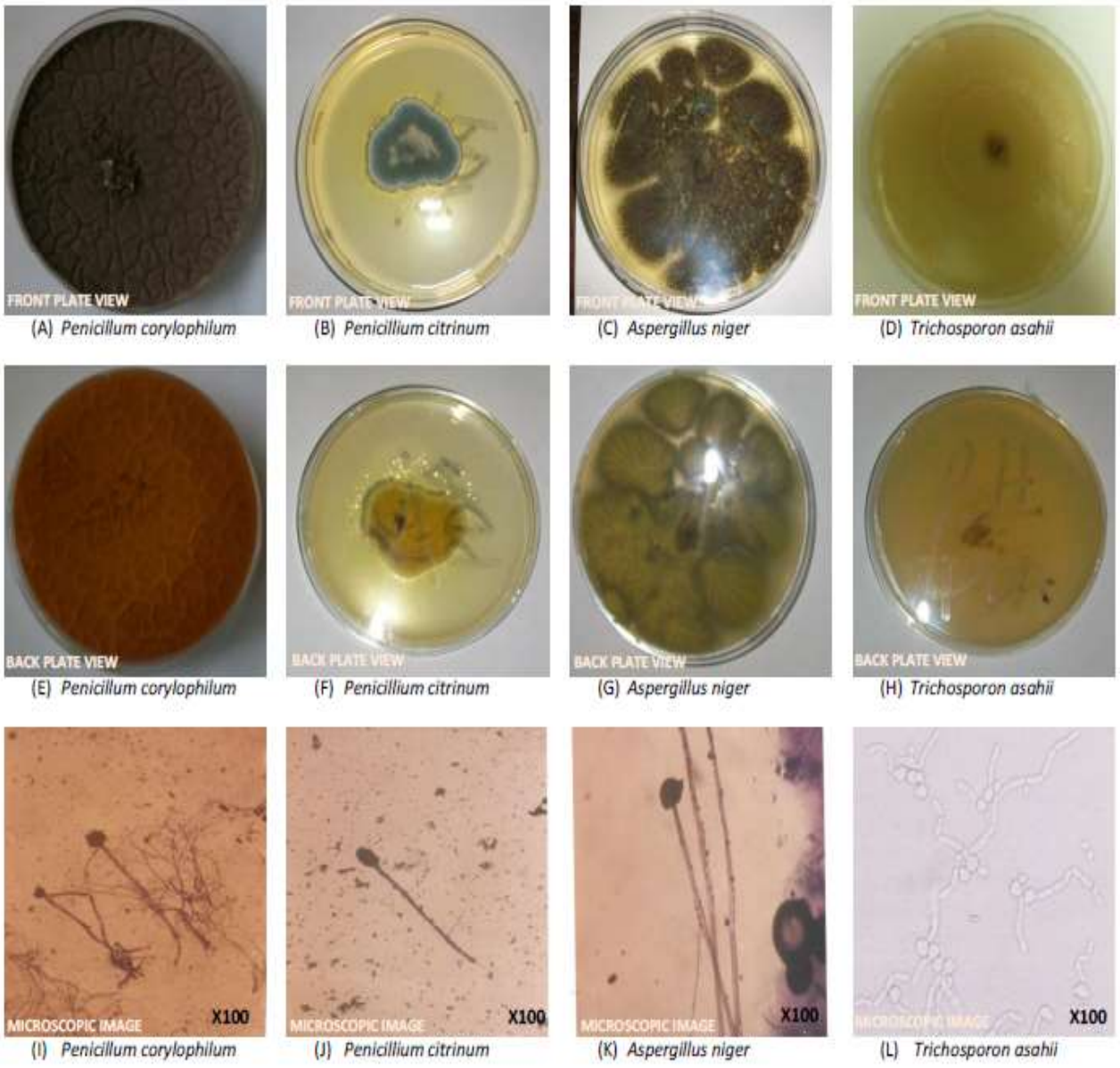


Fig. 8: Isolated Fungi from Sawdust Waste Cultured On PDA and Czapek Dox Agar

4.4 GENOTYPING OF SELECTED ISOLATES

4.4.1 Integrity and Quantification of Extracted DNA

The sharp bands show that extracted DNAs were intact with less degradation as shown in Fig. 9. Lane S3 is negative control. The non-appearance of band on lane S3 confirms that there was no contamination. The quantities of the extracted DNA are stated in Table 4.

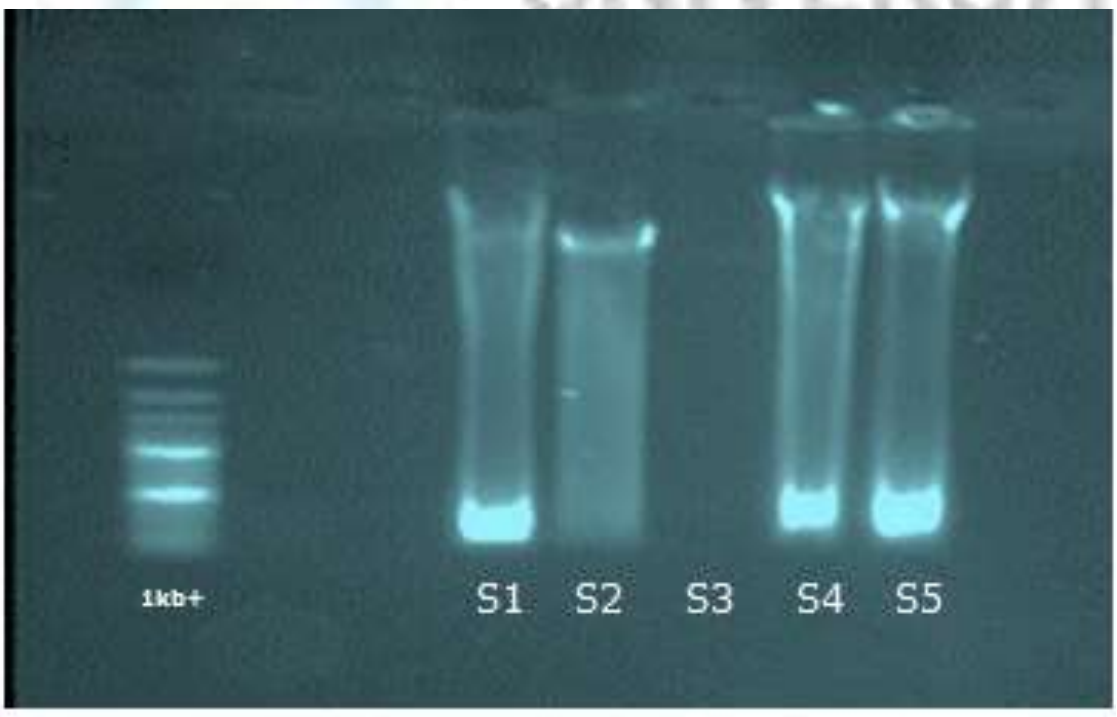


Fig. 9: Agarose Gel Electrohoregram of DNA of Selected Fungi

S1, S2, S4 and S5 represent the DNA of *Penicillium citrinum*, *Penicilium corylophylum*, *Meyerozyme quillamondii, Aspergillus niger, and Trichosporon asahii* respectively. To the extreme left is the DNA ladder (1Kb+). S3 is a negative control, hence, no band shown on the lane.

Table 4 below depicts the spectrophotometric measurements of the DNA samples. A260/280nm measurements above 1.00 shows integrity of extracted DNA while the A260/A230nm measurements below 0.50 shows no contamination in the extraction.

Table 4: Quantification of Extracted DNA

DNA Sample	Quantity (g/mL)	A260/280 nm	A260/230 nm
S1	1.93	1.45	0.25
S2	1.75	1.87	0.47
S4	2.31	2.08	0.65
S5	2.53	2.41	0.23

4.4.2 Amplification of DNA of Isolates

ITS1/ITS4 primers amplified the selected genes with all the samples with band size of about 648bp. NS7/LR3 primers also amplified the selected genes with band size of 1642bp (Fig. 10). Amplification of the rDNA regions with primers ITS1 and ITS4 yielded products of approximately 700bp as estimated by agarose gel electrophoresis (Fig. 10). The sequences used for the final phylogenetic analysis were 482 to 533 bp after manual counting trimming and corresponded to the ITS 1 and 2 complete regions; the 5´ portion of the 18S gene, 5.8S completes sequence and the 3´ end of the 28S gene. In addition to ITS 1, 5.8S rDNA and ITS 2, such sequences contained the last base of the 28S rDNA and the first bases of the 18S rDNA.

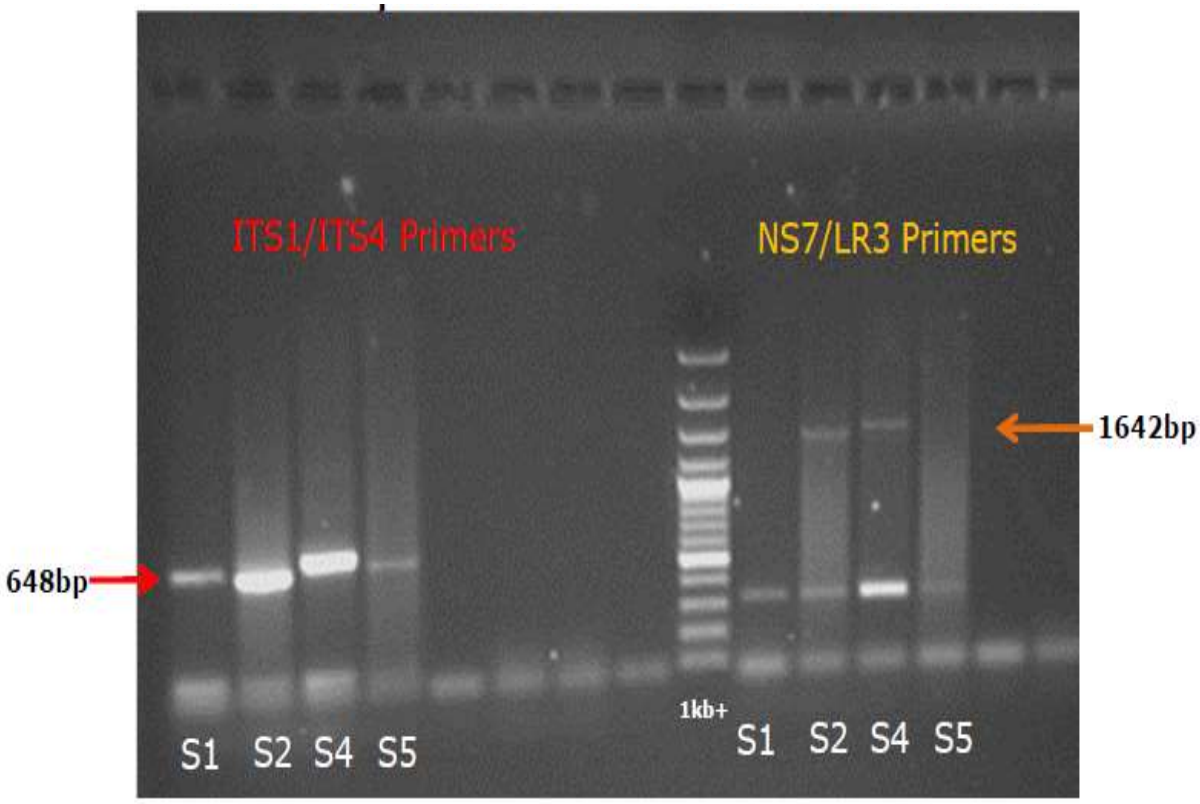


Fig. 10: Banding Pattern Produced by ITS1/ITS4 and NS7/LR3 Primers

S1,S2, S4 and S5 represents the amplicons of *Penicillium citrinum, Penicilium corylophylum, Meyerozyme quillamondii, Aspergillus niger, and Trichosporon asahii* respectively. To the extreme left is the DNA ladder (1Kb+).

4.4.3 Molecular Phylogenetic Analysis

Analysis revealed that fungi isolates were *Aspergillus niger* (Fig. 11), *Penicilium citrinum, Penicillium corylophilum* (Fig. 12) and *Trichosporon asahii*. They all had boostrap values which confirm their evolutionary and phylogenetic relationship with compared sequences.

4.5 Co-culturing of Cellulolytic Fungi in the Biodegradation Of Sawdust

An enzyme activity of 0.92 U/mL was attained in 96 hr when both fungi were co-cultured (Fig. 13). Pretreated sawdust fermented with co-cultured *A. niger* and *P. citrinum* released more reducing sugar (2.72 mg/mL) from the sawdust at 72 hr (Fig. 14). Likewise, pretreated sawdust fermented with co-cultured *Aspergillus niger* and *Penicilium citrinum* released more cellobiohydrolase (0.58 U/mL) from the sawdust at 100 hr (Fig. 15).

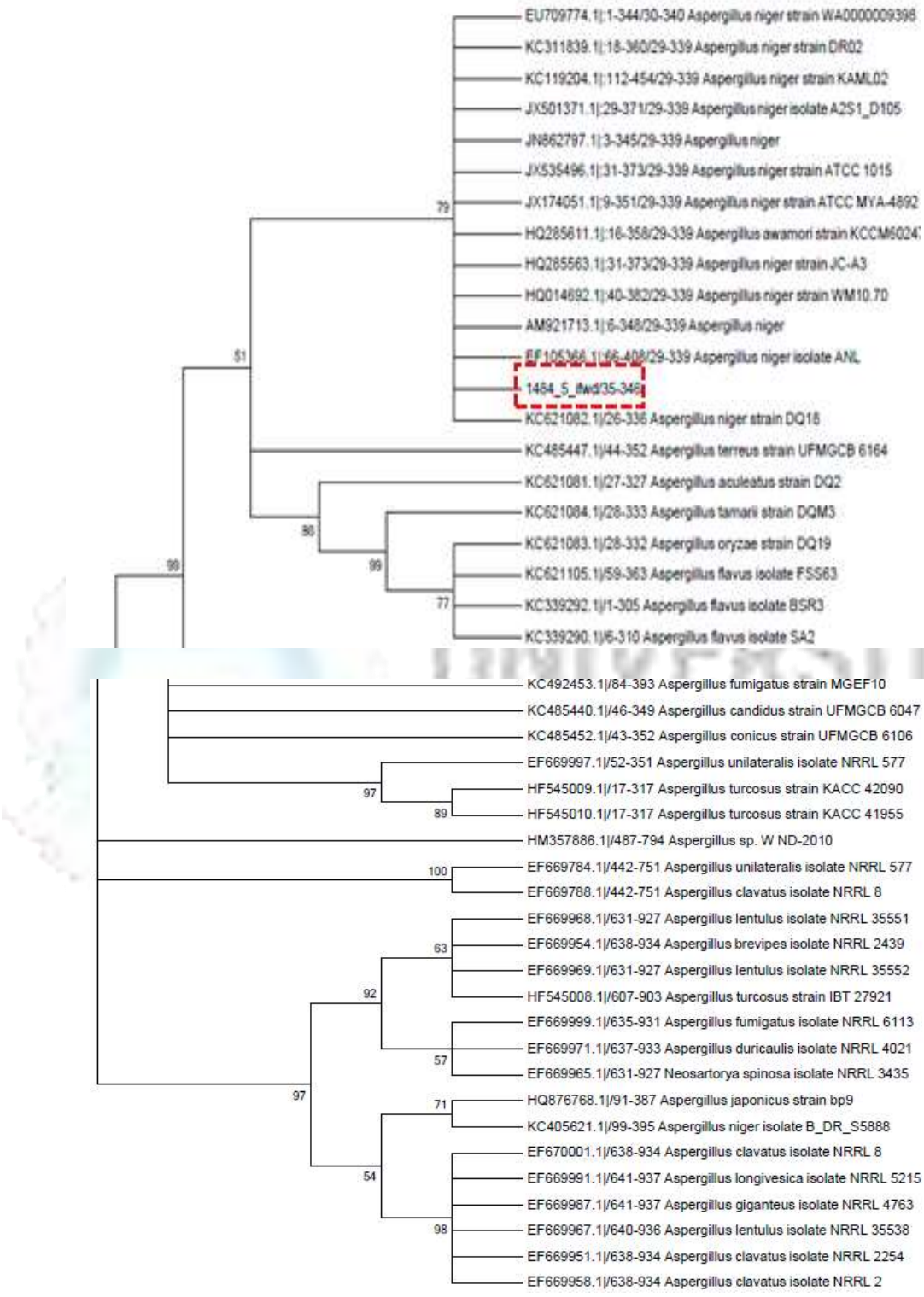


Fig. 11: Phylogenetic Tree Showing Relationship Between Sample and Reference *Aspergillus* species

The highlighted sequence represents that of the fungal isolate while forty four others are sequences of reference Aspergillus spp downloaded from NCBI. After subjecting all to BLAST, sequence denoted 1484_5_1fwd/47_346 had very close homology with reference *Aspergilus niger* strain DQ 18 thus confirming its identity.

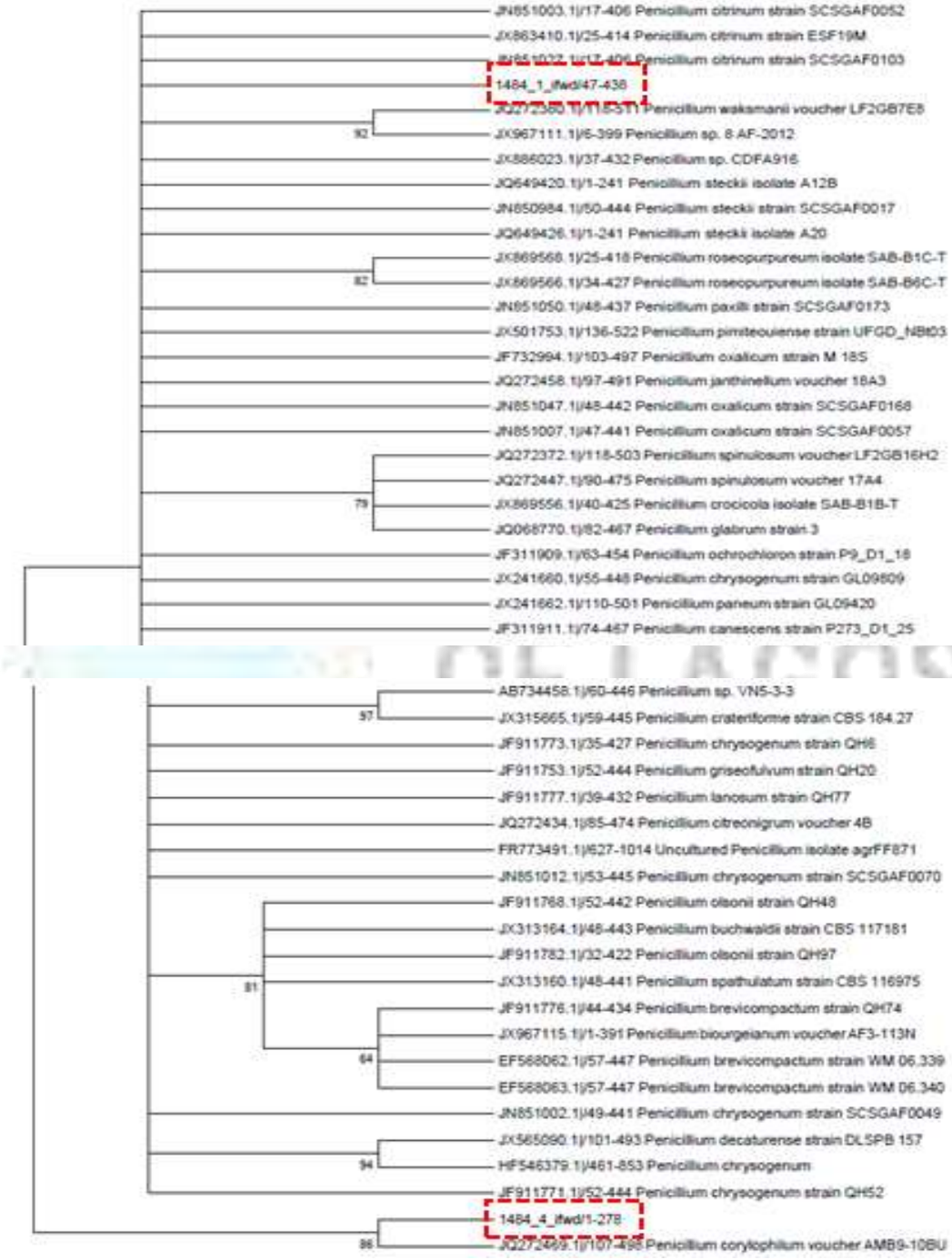


Fig. 12: Phylogenetic Tree Showing Relationship Between Sample and Reference *Penicillium* species

The highlighted sequences represent that of the fungal isolates while forty six others are reference sequences downloaded from NCBI. After subjecting all to BLAST, sequence denoted 1484_1_1fwd/47_438 had very close homology with reference *Penicillium citrinum* strain SCSGAF0103 and sequence 1484_4_1fwd/1-278 had very close homology with *Penicillium corylophilum* strain AMB9-1034 thus confirming their identities.

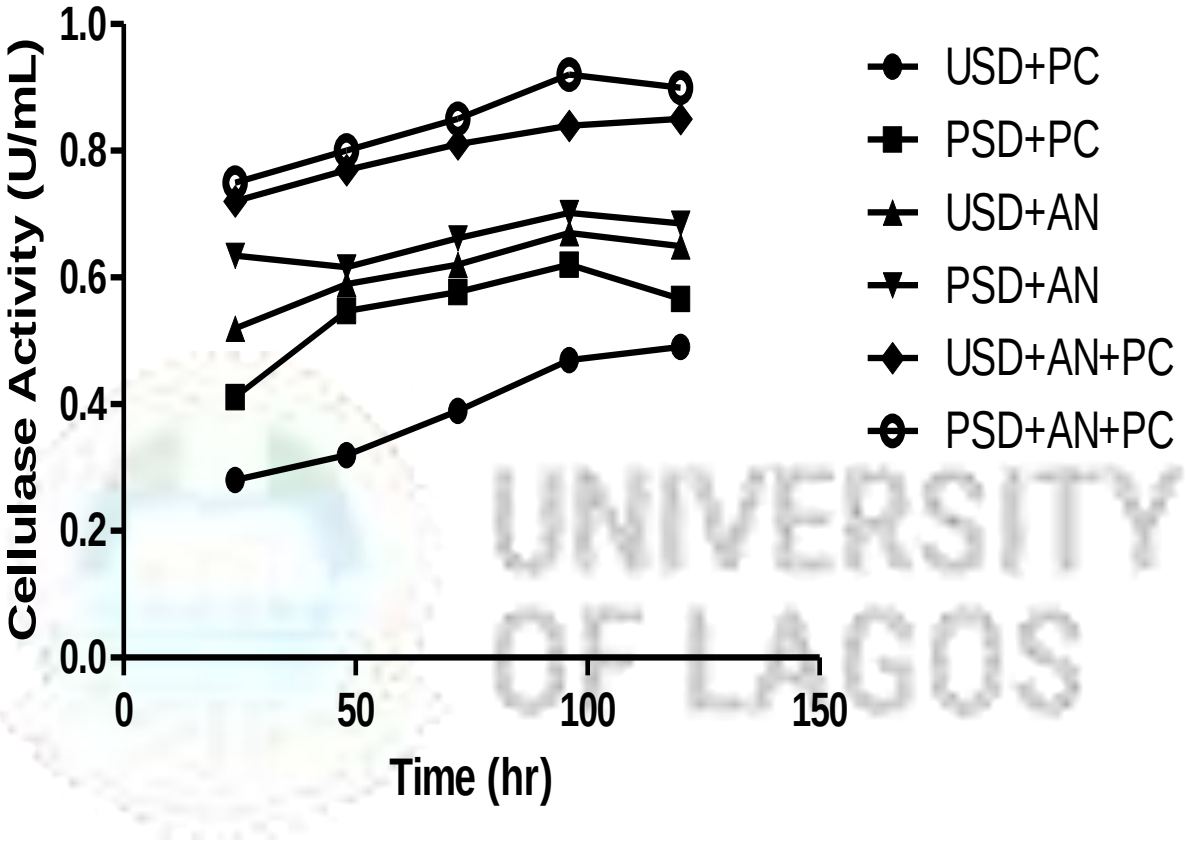


Fig. 13: Effect Co-culturing on Cellulase Activity

There was no significant difference ($p < 0.05$) in the treatment groups. (USD= untreated sawdust; PSD= pretreated sawdust; AN= *Aspergillus niger*; PC= *Penicillium citrinum*)

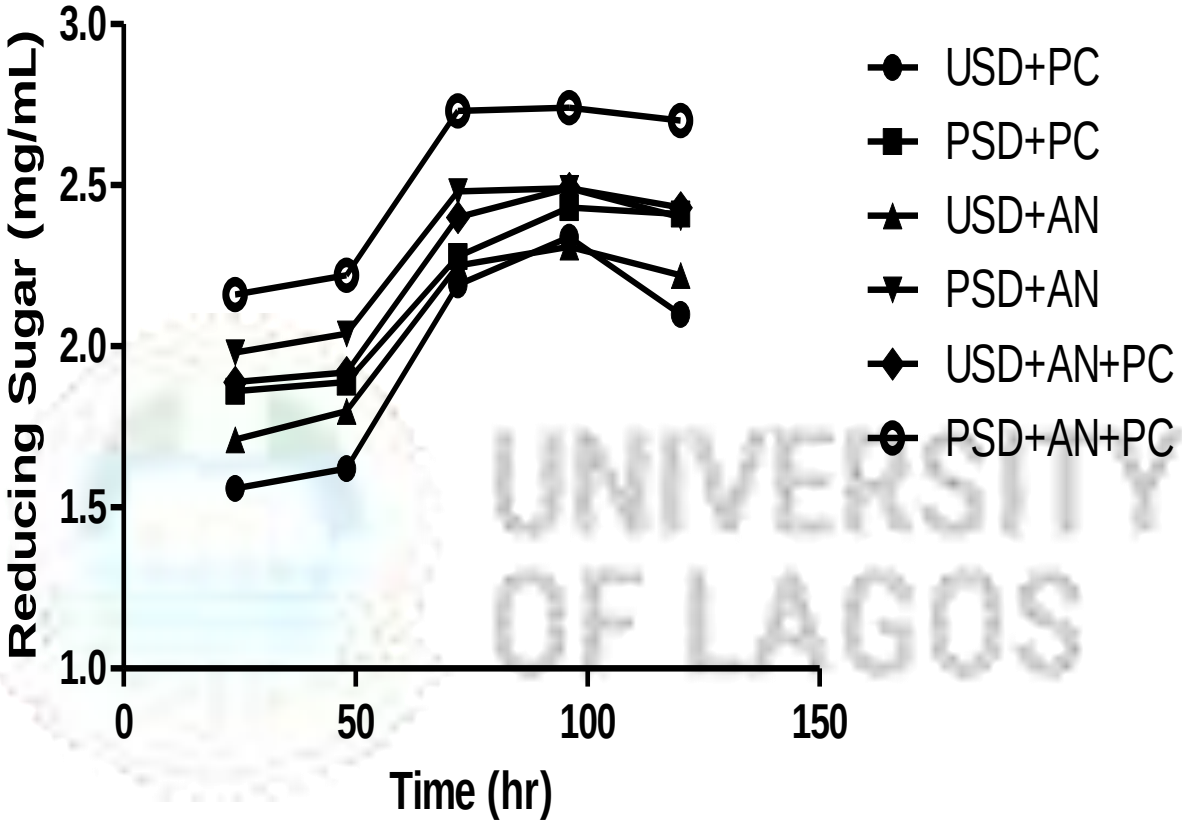


Fig. 14: Effect Co-culturing on Released Reducing Sugar

There was no significant difference ($p < 0.05$) in the treatment groups. (USD= untreated sawdust; PSD= pretreated sawdust; AN= *Aspergillus niger*; PC= *Penicillium citrinum*)

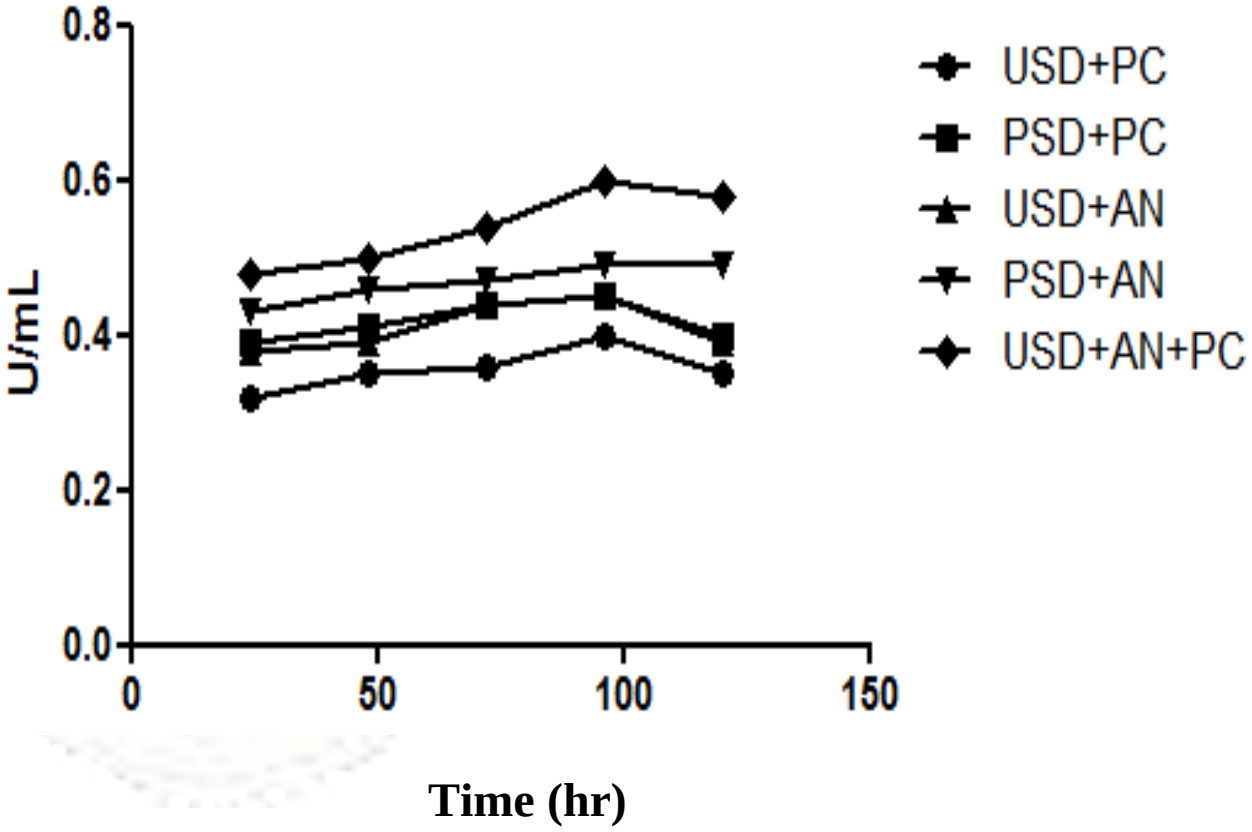


Fig. 15: Effect Co-Culturing on Cellobiohydrolase Activity

There was no significant difference ($p < 0.05$) in the treatment groups. (USD= untreated sawdust; PSD= pretreated sawdust; AN= *Aspergillus niger*; PC= *Penicillium citrinum*)

4.6 Mutagenesis of Fungi for the hyperproduction of cellulase

Eleven mutants of *A. niger* were selected after the irradiation. Fig. 16 below shows the comparison of their cellulase activities with the wild strain. All had cellulase activities higher than the wild strain. Mutant ANM202 was retained for further studies. Likewise, eleven mutants of *P. citrinum* were selected after the irradiation (Fig. 17). All mutants had cellulase activity more than the wild except PCM403, PCM502 and PCM504. Mutant PCM505 was retained for further studies.

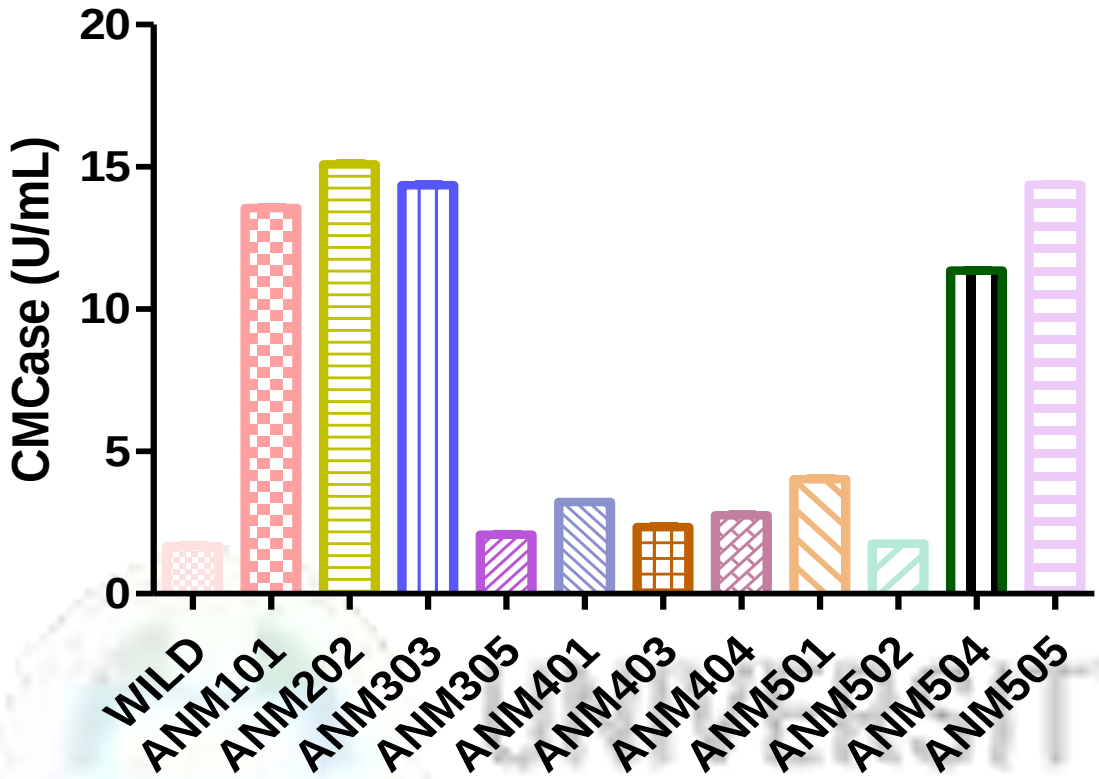


Fig. 16: Cellulase Activity of UV Mutants of *A. niger*

All eleven mutants selected had cellulase activities which were significantly higher ($p < 0.05$) than the wild except ANM502.

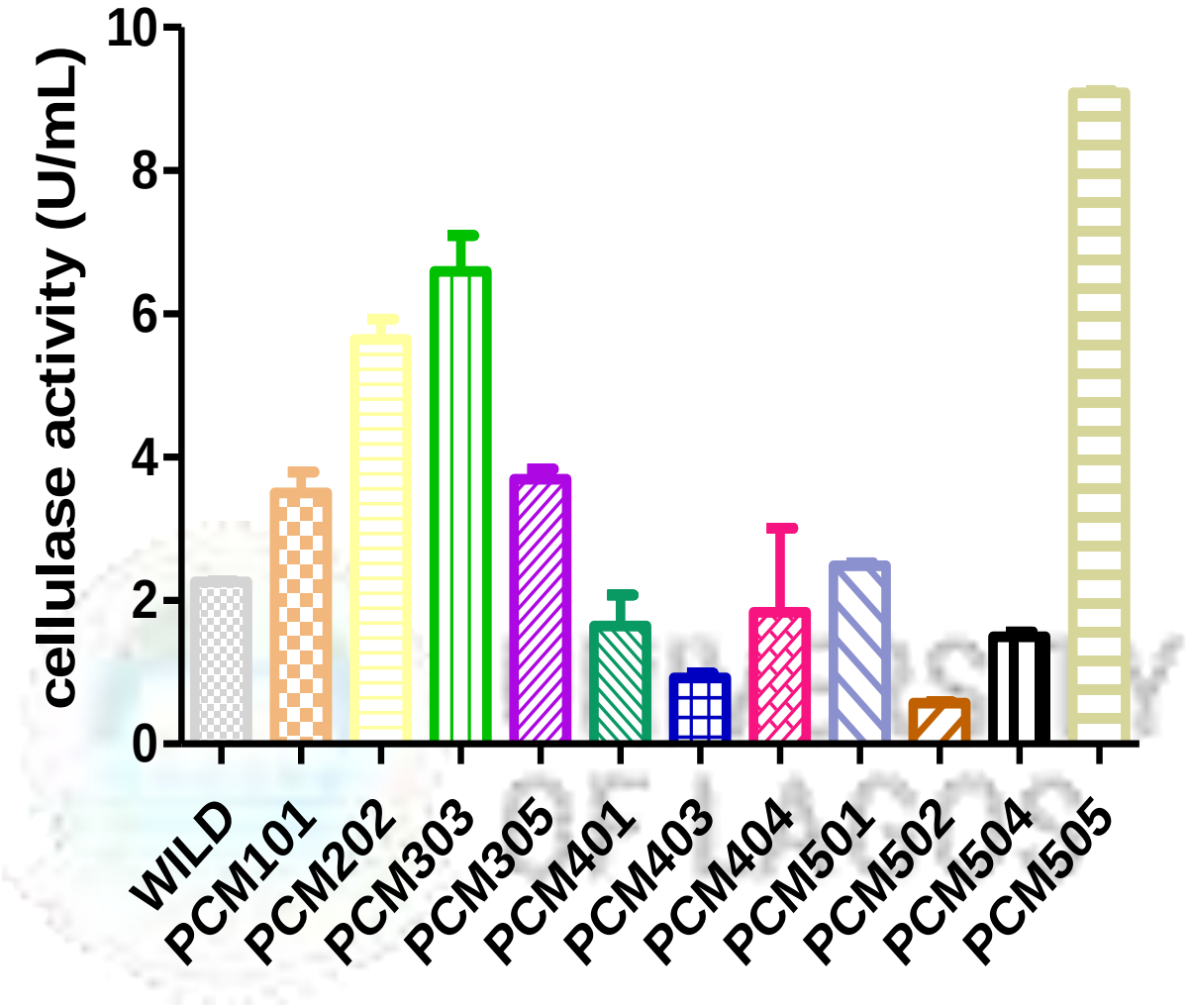


Fig. 17: Cellulase Activity of UV Mutants of *P. citrinum*

Only mutants PCM202, PCM303 and PCM501 had cellulase activities which were significantly higher ($p < 0.05$) than the wild.

4.7 Improvement of *A. niger* Cellulase Production by Sequential UV Mutation and Optimizing of Solid State Fermentation

The *A. niger* mutant had a 2.1-fold and 2.4-fold increase in CMCase and FPase more than the wild (Fig. 18a and b). Optimum moisture for high enzyme yield was found to be 20 % (Fig. 19a), temperature of 37 ^{o}C (Fig. 19b), pH of 3.5 (Fig. 19c) and fermentation time of 4 days (Fig. 19d), for the *A. niger* mutant. Other culture condition parameters include age of seeding culture of 3 days (Fig. 20a) and inoculum size of 20 % w/w (Fig. 20b). Corn starch was found to enhance the production of cellulase more than glucose, lactose, sucrose and maltose (Fig. 21a and b). EDTA enhanced more enzyme production more than SDS, Tween 20 and Tween 80 (Fig. 22a and b). $FeSO_4$, supported cellulase production more than $ZnSO_4$, $CuSO_4$, $CaCl_2$, $MgCl_2$, $CoCl_2$ and KCl (Fig. 23a and b). Urea was the best nitrogen supplement compared to malt extract, yeast extract, NH_4NO_3 and NH_4Cl (Fig. 24a and b).

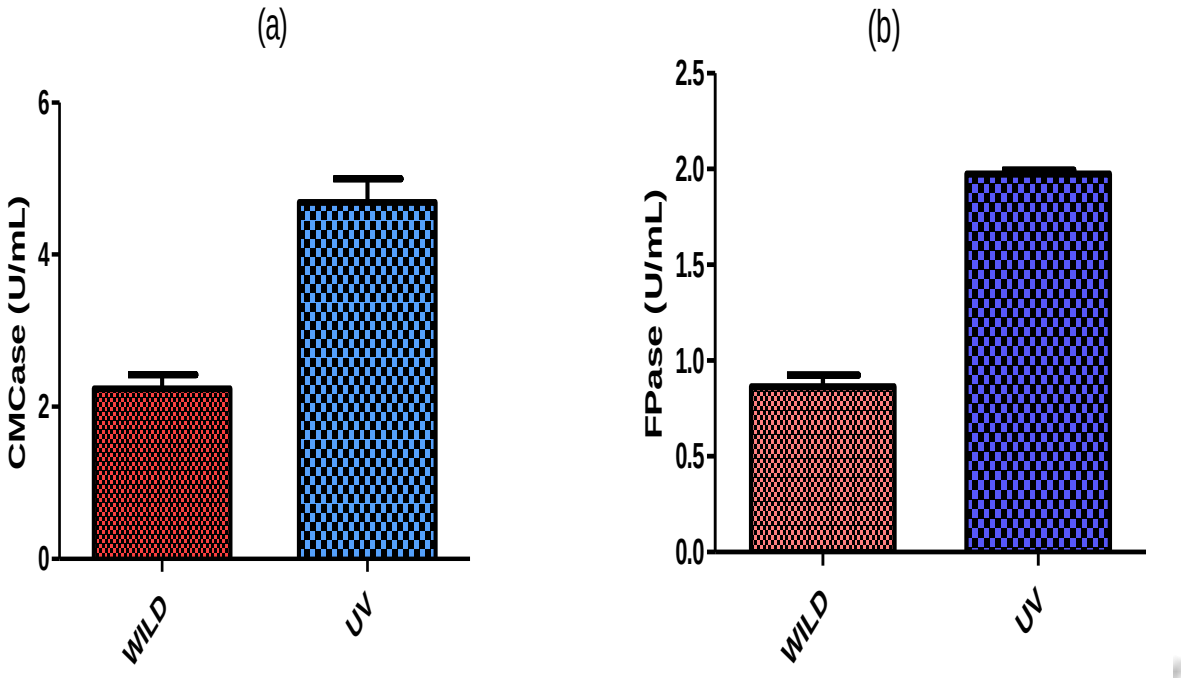


Fig. 18: Comparison of Cellulase (CMCase and FPase) Production by Wild and UV Mutated Strain of *A. niger*

Cellulase production by mutant *A. niger* was significantly ($p < 0.05$) higher than the wild

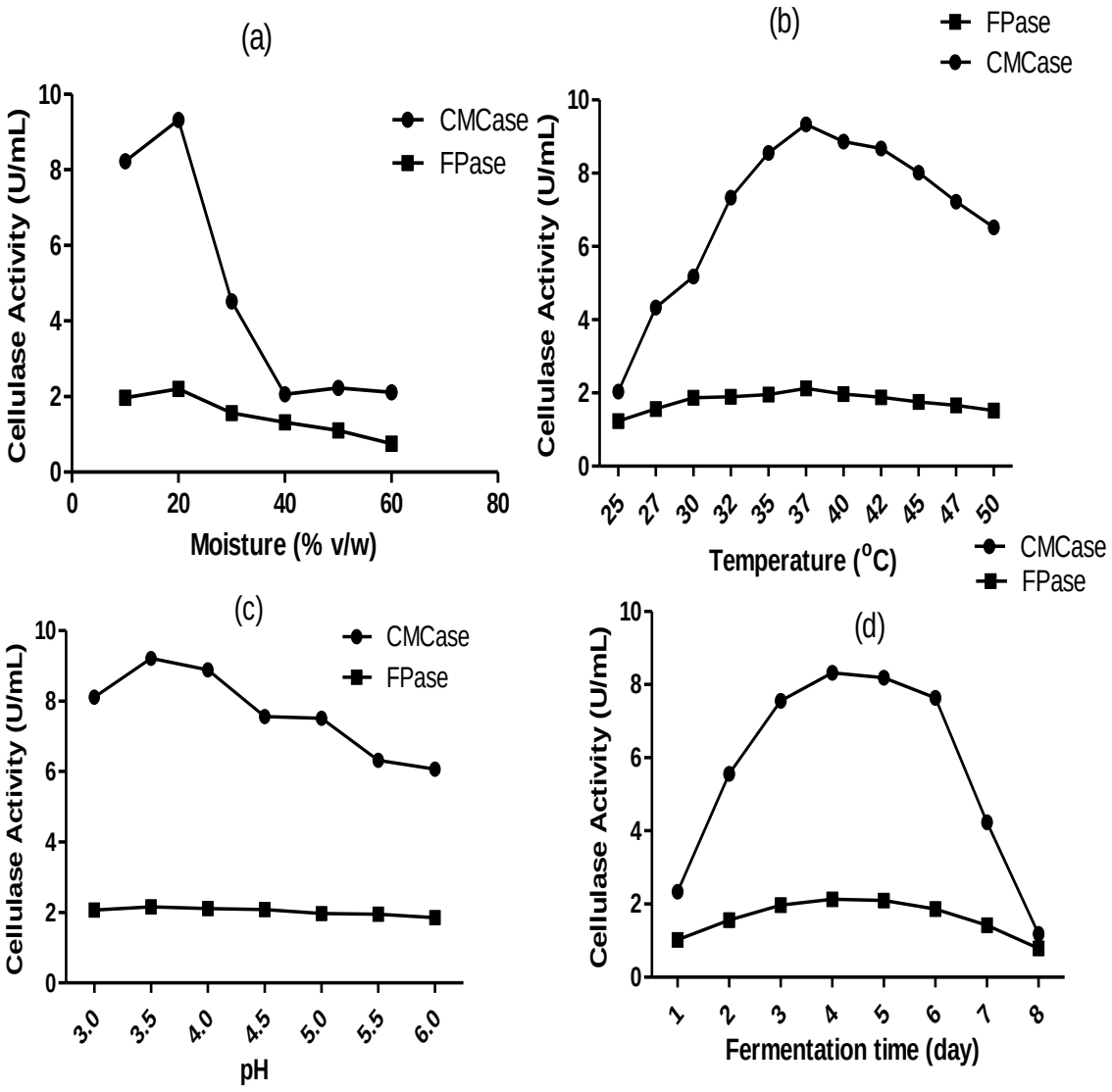


Fig. 19: Effect of Various Culture Conditions (Moisture (a), Temperature (b), pH (c) And Fermentation Time (d)) On Cellulase (CMCase and FPase) Production by *A. niger* Mutant

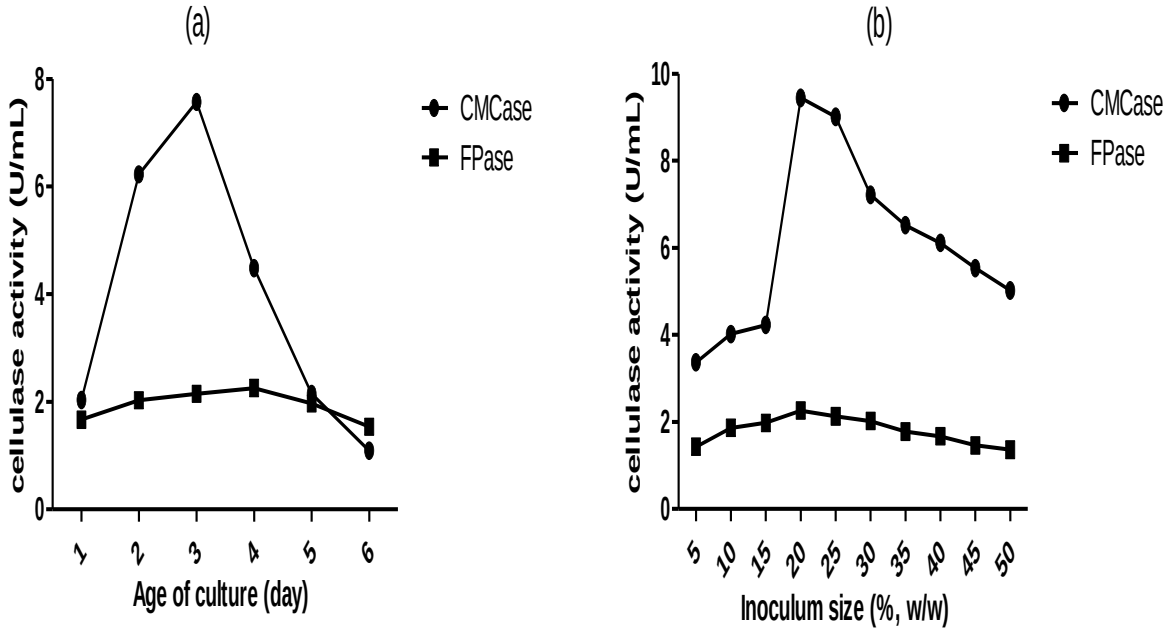


Fig. 20: Effect of Age of Culture Medium and Inoculum Size on Cellulase (CMCase and FPase) Production by *A. niger* Mutant

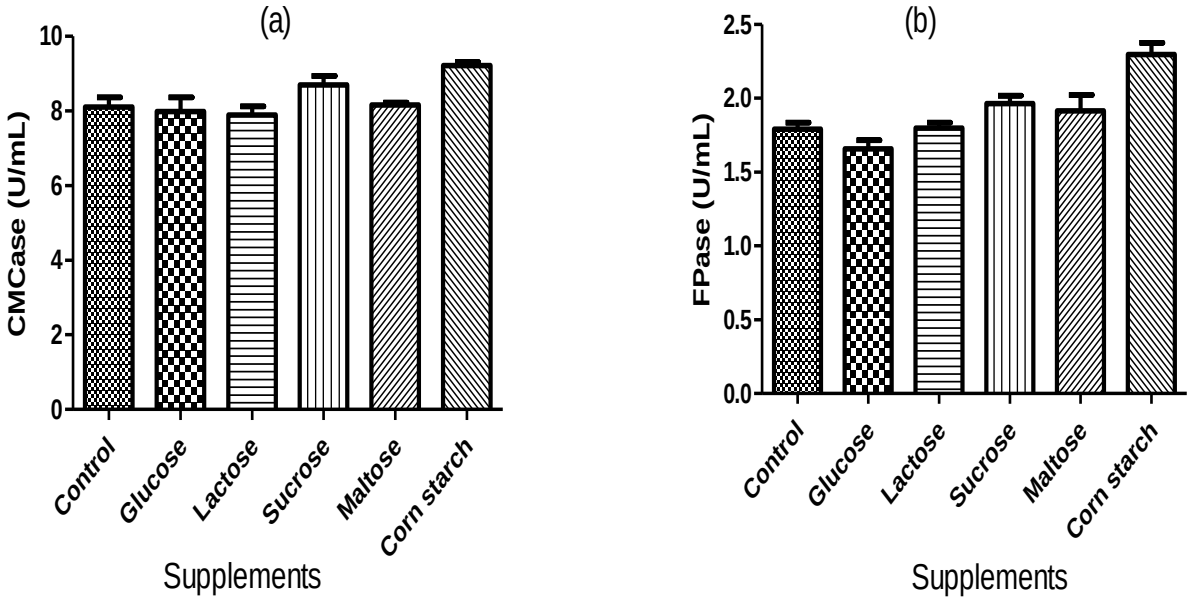


Fig. 21: Effect of Carbon Supplement on Cellulase (CMCase and FPase) Production by *A. niger* Mutant

Only supplementation with corn starch produced cellulase which was significantly higher ($p < 0.05$) than the control

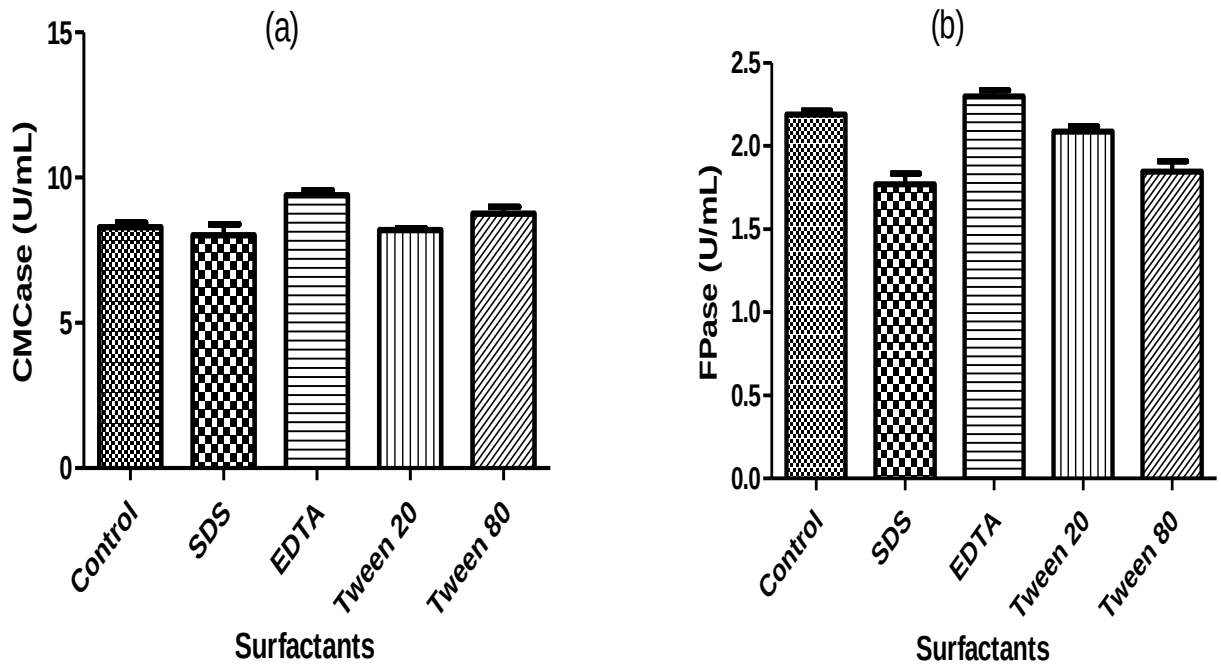


Fig. 22: Effect of surfactants on cellulase (CMCase and FPase) production by *A. niger* Mutant

Only supplementation with EDTA produced cellulase which was significantly higher ($p < 0.05$) than the control

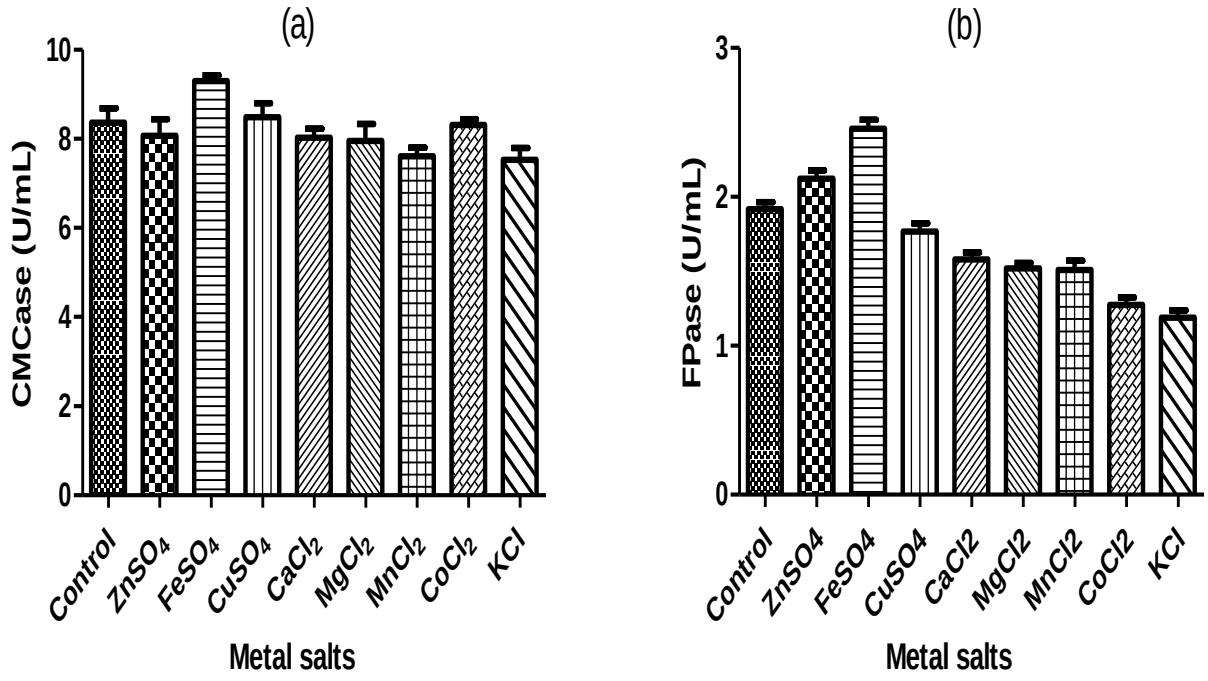


Fig. 23: Effect of Metal Salts on Cellulase (CMCase and FPase) Production by *A. niger* Mutant

Only supplementation with $FeSO_4$ produced cellulase which was significantly higher ($p < 0.05$) than the control

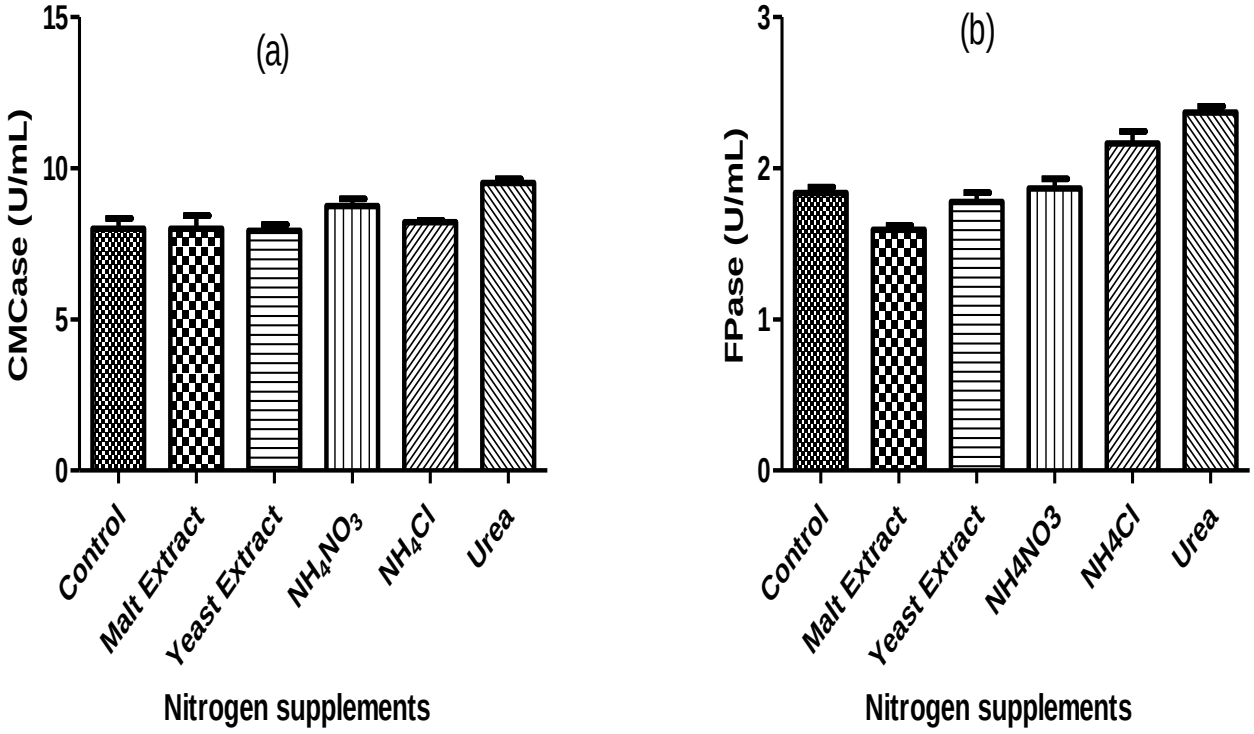


Fig. 24: Effect of Nitrogenous Salts on Cellulase (CMCase and FPase) Production by *A. niger* Mutant

Only supplementation with Urea produced cellulase which was significantly higher ($p < 0.05$) than the control.

Optimizing the solid state fermentation of mutant *A. niger* resulted in a 7.4-fold and 7.6-fold higher yield in cellulase (CMCase and FPase respectively) more than the wild strain (Fig. 25a and b).

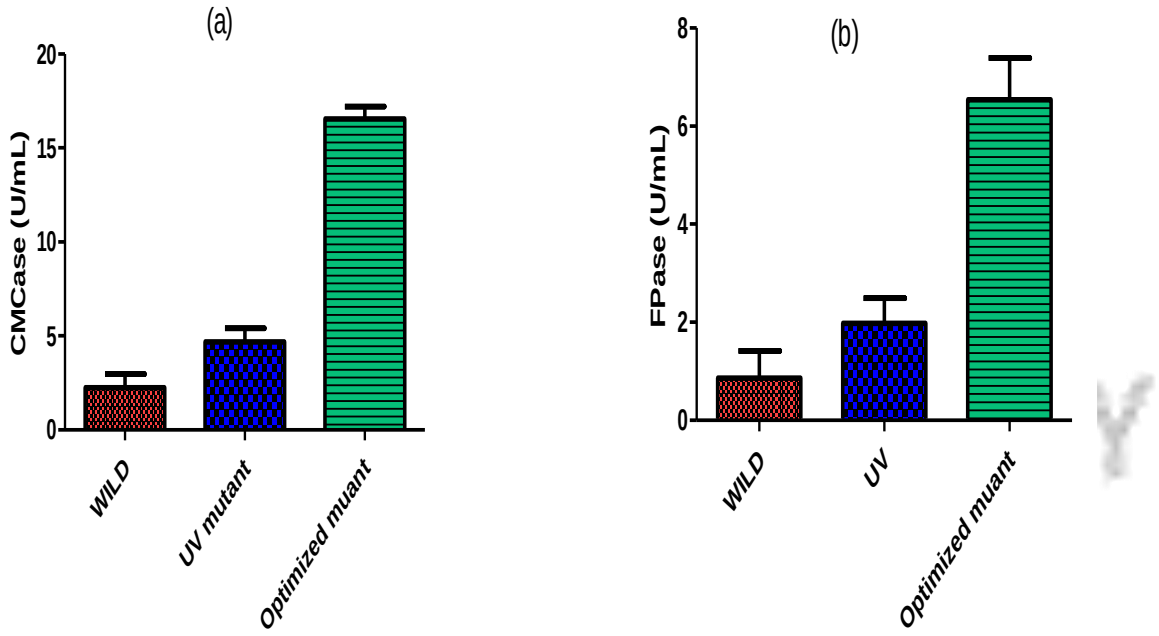


Fig. 25: Comparison of the Cellulase (CMCase and FPase) Produced by Wild *A. niger* Strain, its UV Mutant and the Optimized UV Mutant

Optimized mutant strain of *A. niger* produced cellulase which was significantly higher ($p < 0.05$) than ordinary UV mutant as well as the wild strain.

4.8 Improvement of *P. citrinum* Cellulase Production by Sequential UV Mutation and Optimization of Solid State Fermentation

Fig. 26 a and b shows that mutant *P. citrinum* had a 1.8-fold and 2.1-fold increase in CMCase and FPase more than the wild. Optimum moisture for high enzyme yield was found to be 20 % (Fig. 27a), temperature of 37 ^{O}C (Fig. 27b), pH of 5.5 (Fig. 27c) and fermentation time of 7 days (Fig. 27d) for the *P. citrinum* mutant. Other parameters include age of seeding culture of 5days (Fig. 28a) and inoculum size of 30% w/w and (Fig. 28b). Maltose was found to enhance the production of cellulase more than corn-starch, glucose, lactose, sucrose (Fig. 29a and b). SDS enhanced more enzyme production than EDTA, Tween 20 and Tween 80 (Fig. 30a and b). $FeSO_4$ supported cellulase production more than $ZnSO_4$, $CuSO_4$, $CaCl_2$, $MgCl_2$, $CoCl_2$ and KCl were used (Fig. 31a and b). Urea was the best nitrogen supplement compared to malt extract, yeast extract, NH_4NO_3 and NH_4Cl (Fig. 32a and b).

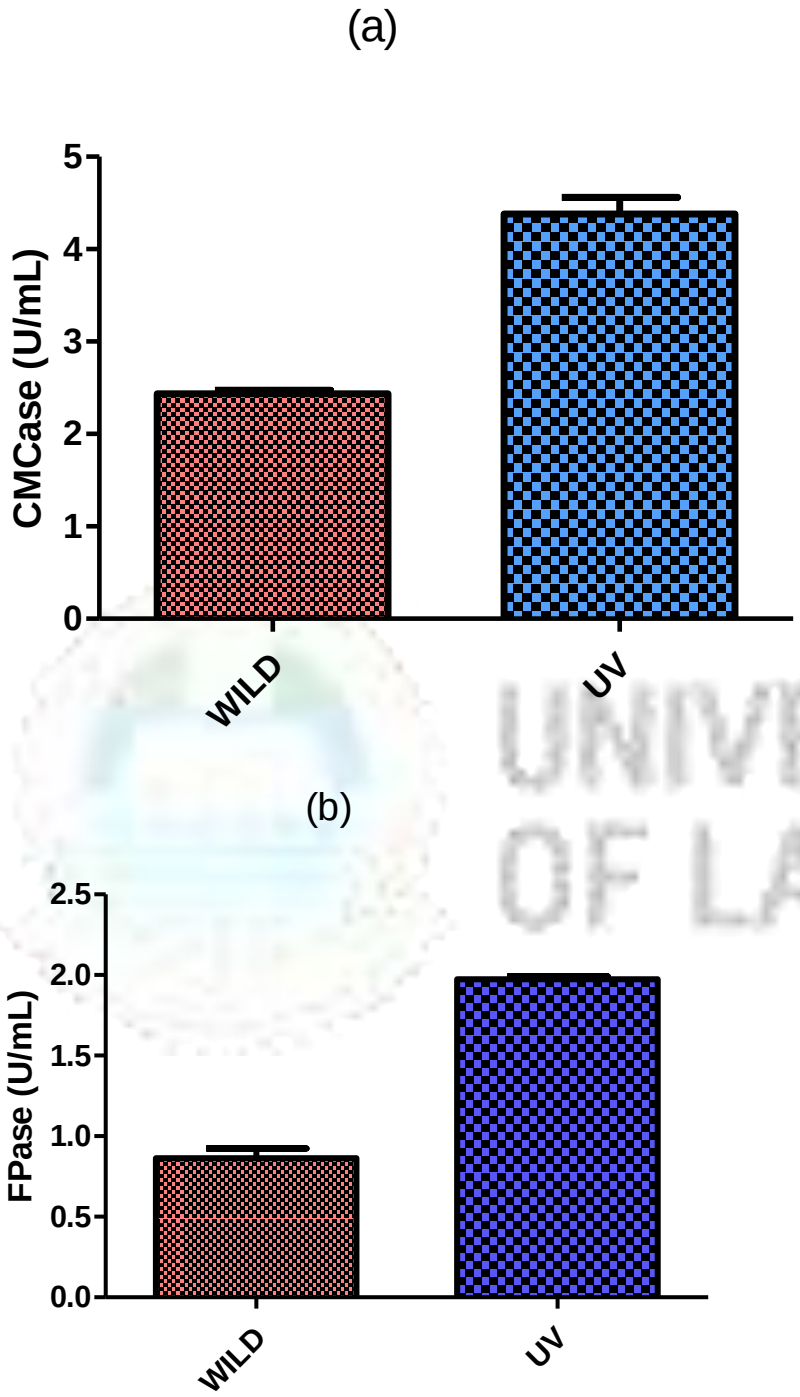


Fig. 26: Comparison of cellulase (CMCase and FPase) Production by Wild and UV Mutated Strains of *P. citrinum*

Cellulase production by mutant *P. citrinum* was significantly higher ($p < 0.05$) than the wild.

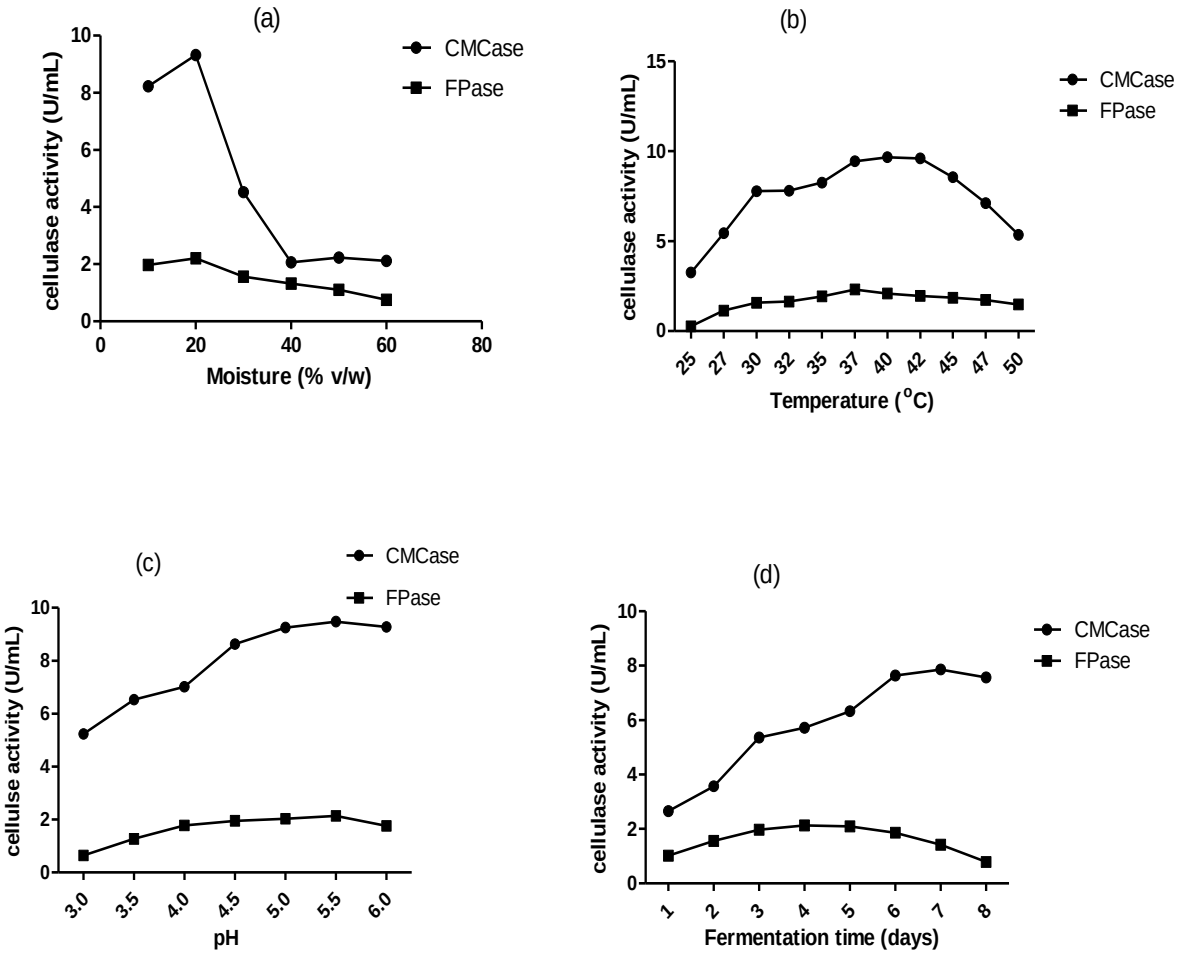


Fig. 27: Effect of Various Culture Conditions (Moisture (a), Temperature (b), pH (c) and Fermentation time (d)) on Cellulase (CMCase and FPase) Production by *P. citrinum* Mutant

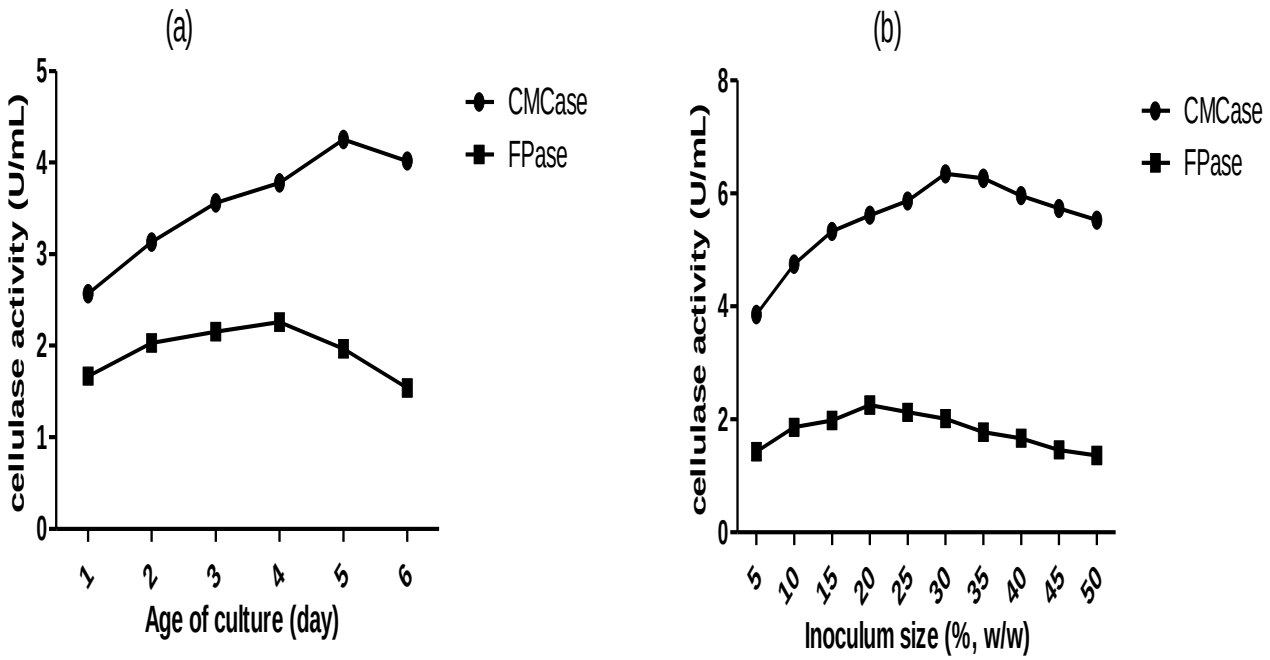


Fig. 28: Effect of Age of Culture Medium (a) and Inoculum Size (b) on Cellulase (CMCase and FPase) Production by *P. citrinum* Mutant

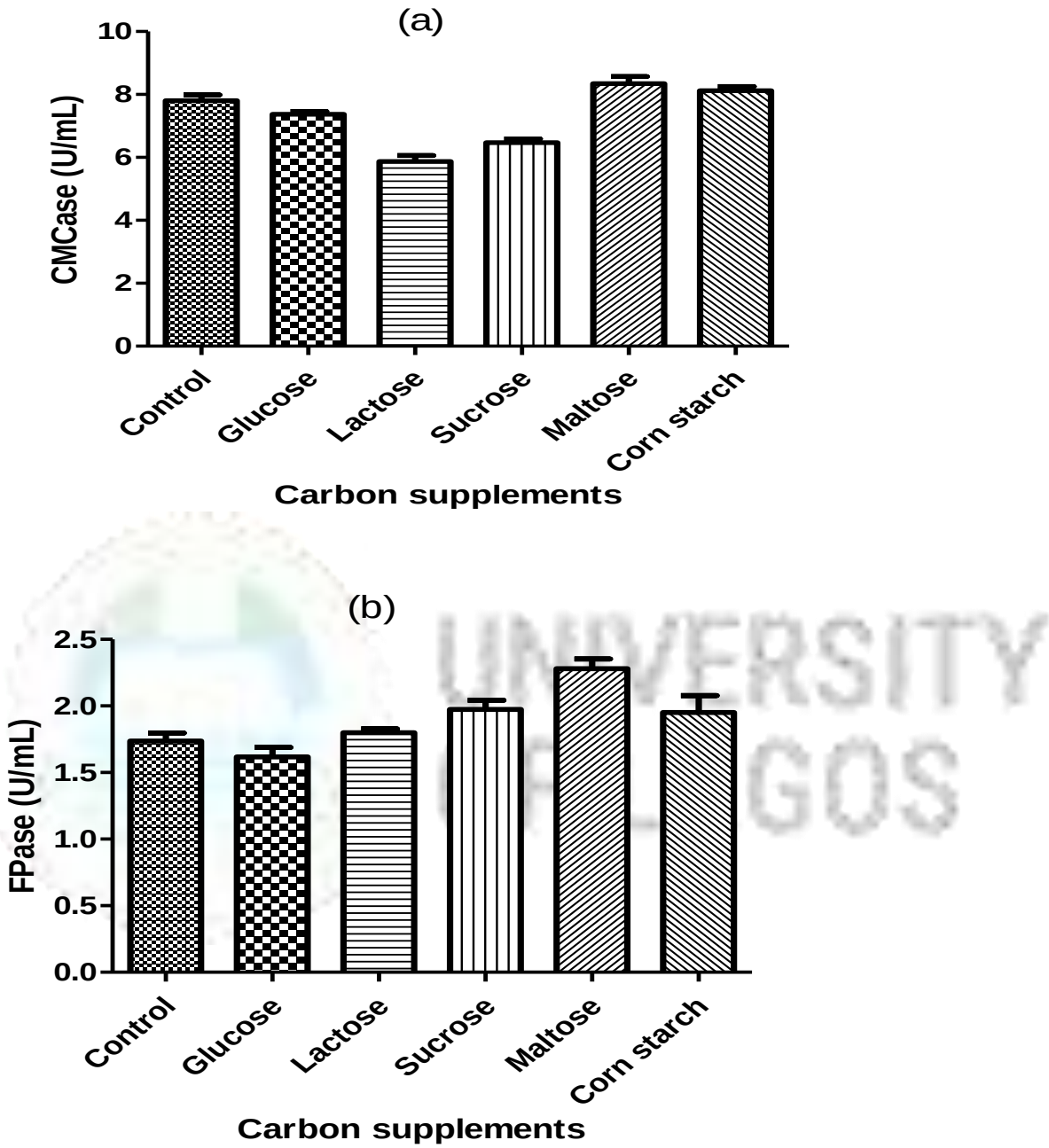


Fig. 29: Effect of Carbon Supplement on Cellulase (CMCase and FPase) Production by *P. citrinum* Mutant

Only supplementation with Maltose produced cellulase which was significantly higher ($p < 0.05$) than the control

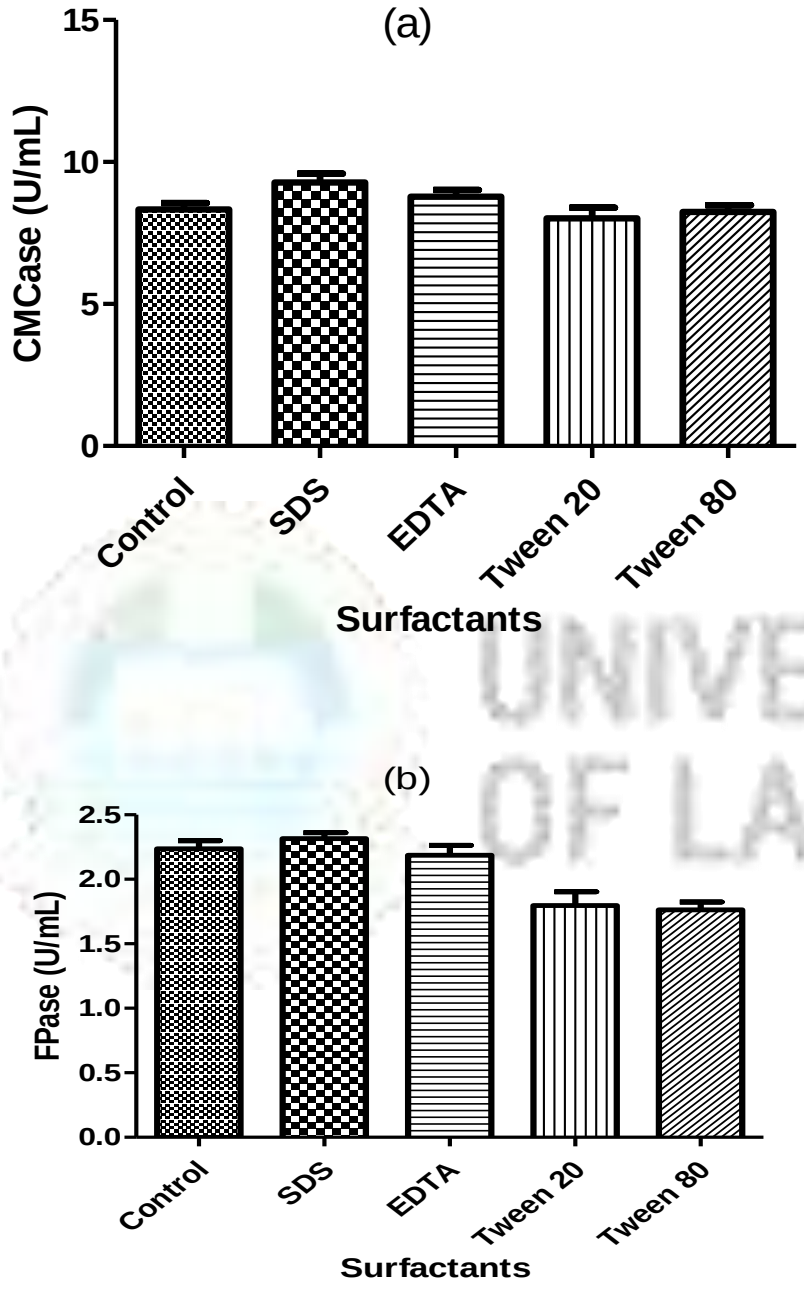
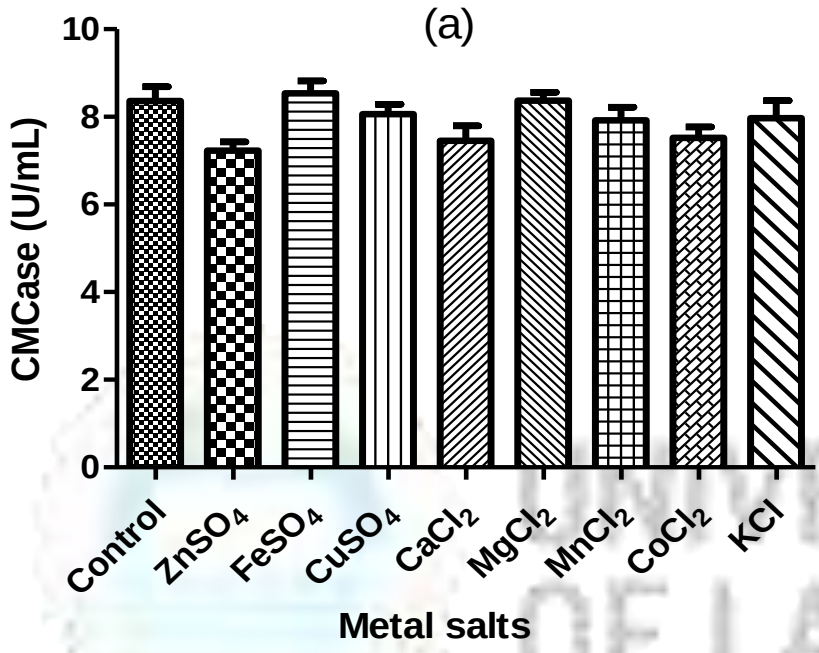


Fig. 30: Effect of Surfactants on Cellulase (CMCase and FPase) Production by *P. citrinum* Mutant

Supplementation with SDS produced higher cellulase than the control, although not significantly ($p < 0.05$).



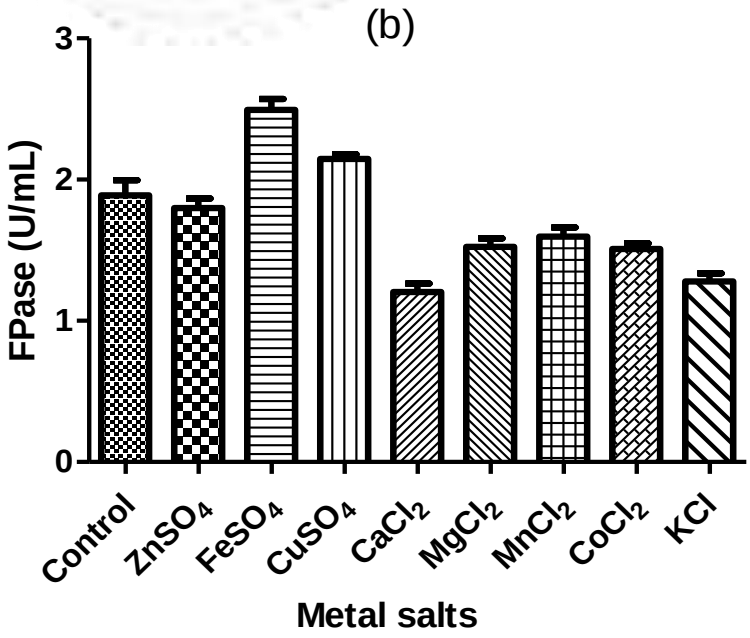
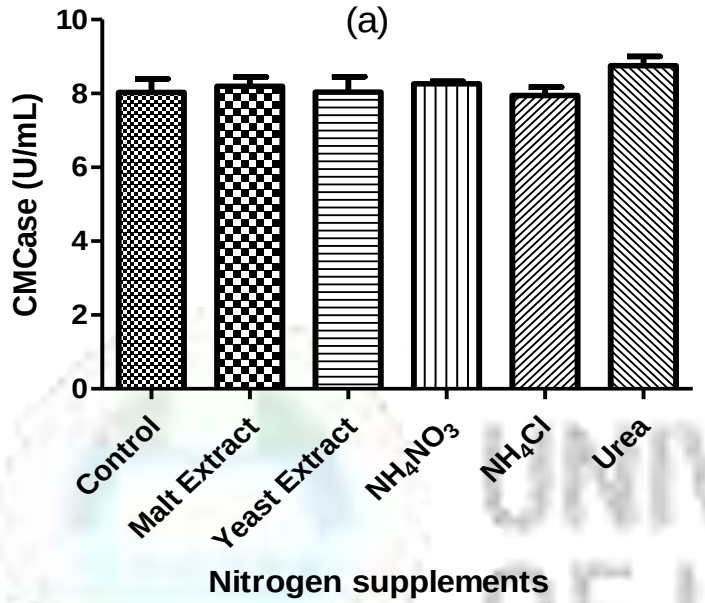


Fig. 31: Effect of Metal Salts on Cellulase (CMCase and FPase) Production by *P. citrinum* Mutant

Only supplementation with $FeSO_4$ produced significantly higher ($p < 0.05$) cellulase than the control with FPase but not with CMCase.



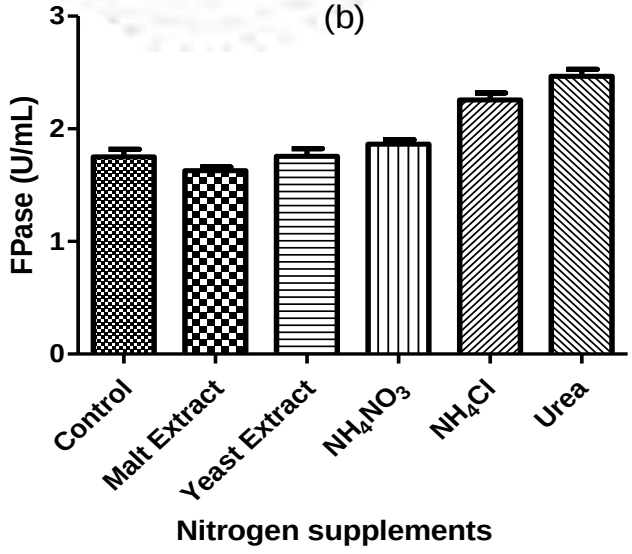
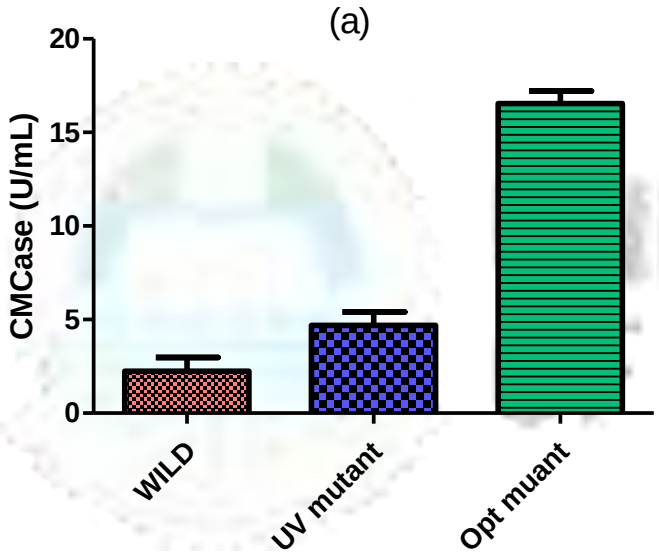


Fig. 32: Effect of Nitrogen Supplement on Cellulase (CMCase and FPase) Production by *P. citrinum* Mutant

There was no significant difference ($p < 0.05$) in the cellulase production in media supplemented with the different nitrogen supplements with CMCase. However, yeast extract and NH_4Cl produced FPase which was significantly higher ($p < 0.05$) than the control
Optimizing the solid state fermentation of mutant *P. citrinum* resulted in a 5.3-fold and 5.8-fold higher yield in cellulase (CMCase and FPase respectively) more than the wild strain (Fig. 33a and b).



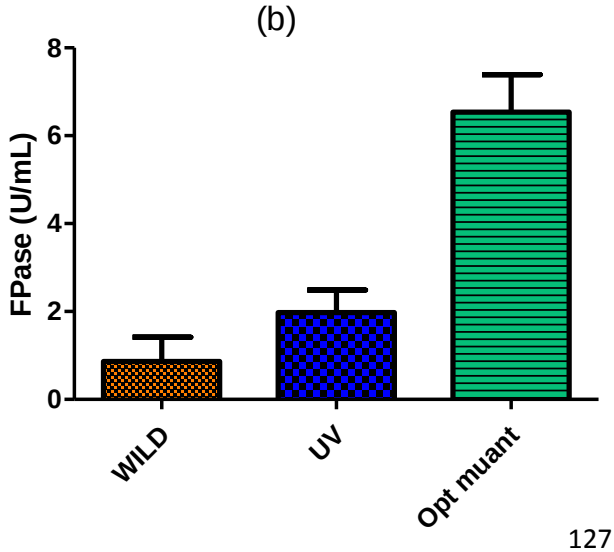


Fig. 33: Comparison of the Cellulase Produced by wild *P. citrinum* strain, its UV Mutant and the Optimized UV Mutant

Optimized mutant strain of *P. citrinum* produced cellulase which was significantly higher ($p < 0.05$) than ordinary UV mutant as well as the wild strain.

4.9 Partial Purification and Characterization of Cellulase

A purification fold of 6.36 and an enzyme yield of 54.0 % were achieved with wild *A. niger* after anion exchange chromatography. However, a purification fold of 9.10 and enzyme yield of 54.2% was achieved with *A. niger* mutant. Details are shown in Table 5.

In the case of wild *P. citrinum*, a purification fold of 7.89 and an enzyme yield of 36.03 % were achieved after anion exchange chromatography. However, a purification fold of 13.55 and enzyme yield of 45.12 % was achieved with its mutant. Details are shown in Table 6.

Anion exchange chromatography elution profile of cellulase from wild *A. niger* revealed eight peaks with two major peaks. The first peak has a total protein content of 313.32 µg/mL with an enzyme activity of 2.0 U/mL while the second peak has a total protein of 304.76 µg/mL with an enzyme activity of 1.8 U/mL (Fig. 34). Elution profile of the cellulase from mutated *A. niger* revealed eight peaks with two major peaks. The first peak has a total protein content of 116.55 µg/mL with an enzyme activity of 3.74 U/mL while the second peak has a total protein of 244.11 µg/mL with an enzyme activity of 3.26 U/mL (Fig. 35).

Cellulase purified from wild *P. citrinum* had five peaks with three major peaks. Their total protein contents were 352.48 µg/mL, 396.99 µg/mL and 400.55 µg/mL respectively. Their enzyme activities were 4.0 U/mL, 3.41 U/mL and 2.47 U/mL respectively (Fig. 36). However,

mutated *P. citrinum* had nine peaks with one major peak. Its total protein content was 167.34 µg/mL with an enzyme activity of 4.50 U/mL (Fig. 37).

Table 5: Purification of Cellulase of Wild and Mutated *A.niger*

Organism	Purification Step	Total Activity (U/mL)	Total Protein (mg)	Specific Activity (U/mg protein)	Purification Fold	Enzyme Yield (%)
Wild *A. niger*	Crude enzyme	72.8±1.3	129.4±2.5	0.56±1.1	1.0	1.0
	$(NH_4)_2SO_4$ (70-80 %)	46.4±0.3	56.3±0.2	0.82±0.2	1.46	64.0
	Whatman DE-52	38.5±1.4	10.8±3.2	3.56±2.4	6.36	53.0
Mutant *A. niger*	Crude enzyme	82.5±1.2	138.4±0.3	0.60±1.2	1.00	100.0
	$(NH_4)_2SO_4$ (70-80 %)	56.4±0.1	64.4±0.2	0.87±0.2	1.45	68.4
	Whatman DE-52	44.7±1.3	8.2±2.1	5.45±2.4	9.10	54.2

Table 6: Purification of Cellulase of Wild and Mutant *P. citrinum*

Organism	Purification Step	Total Activity (U/mL)	Total Protein (mg)	Specific Activity (U/mg protein)	Purification Fold	Enzyme Yield (%)
Wild *P.*	Crude enzyme	60.5±5.1	93.2±2.2	0.65±2.6	1.0	100
citrinum	$(NH_4)_2SO_4$ (70-80 %)	33.4±0.2	38.5±1.3	0.87±0.6	1.34	55.21
	Whatman DE-52	21.8±1.1	4.2±0.8	5.19±1.0	7.98	36.03
Mutant *P.*	Crude enzyme	72.3±2.1	104.1±1.1	0.69±1.0	1.00	100.0
citrinum	$(NH_4)_2SO_4$ (70-80 %)	45.4±0.3	44.7±1.1	1.02±0.5	1.48	63.00
	Whatman DE-52	27.3±2.1	3.1±0.2	8.81±1.5	13.55	45.12

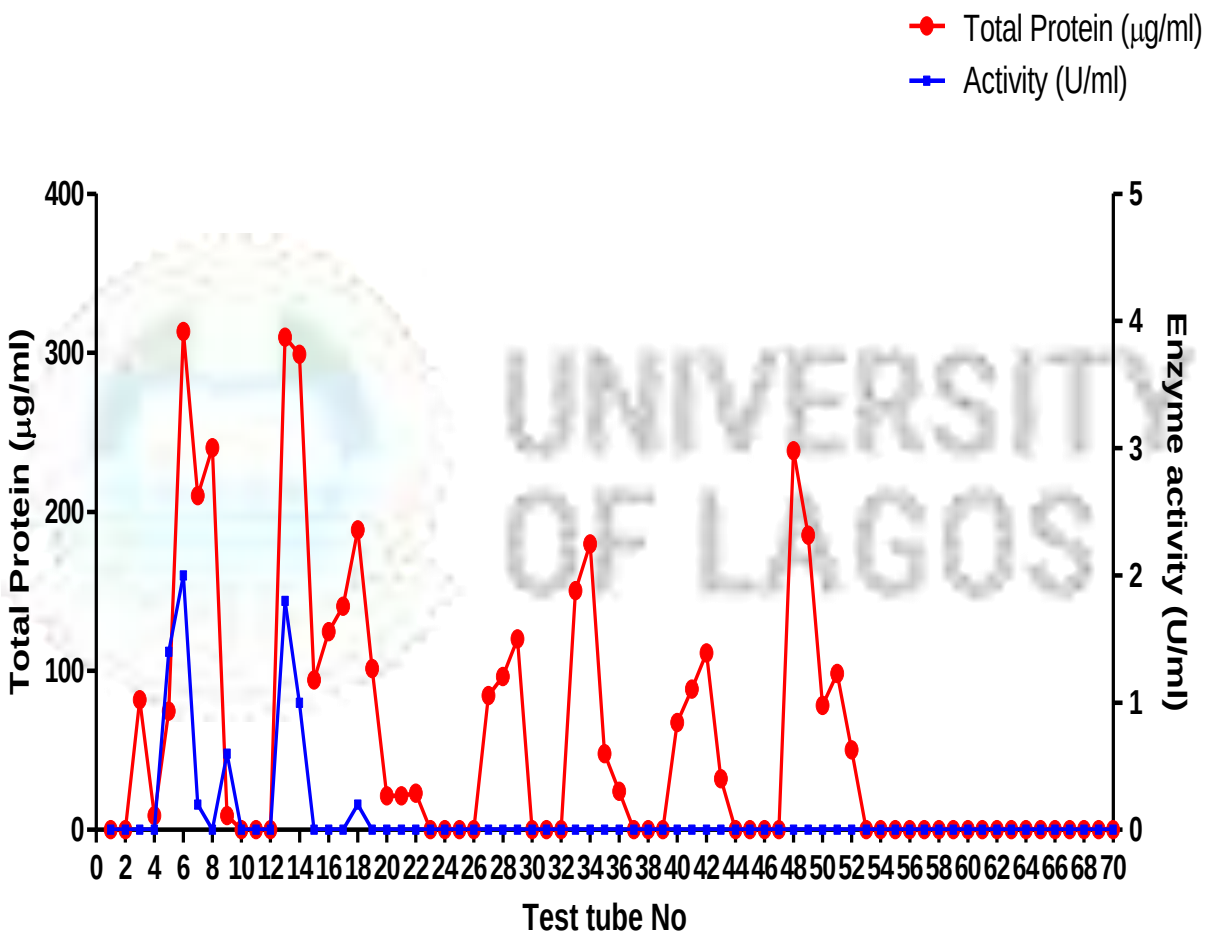


Fig. 34: Elution profile of Anion Exchange Chromatography of Cellulase Produced by Wild *Aspergillus niger*

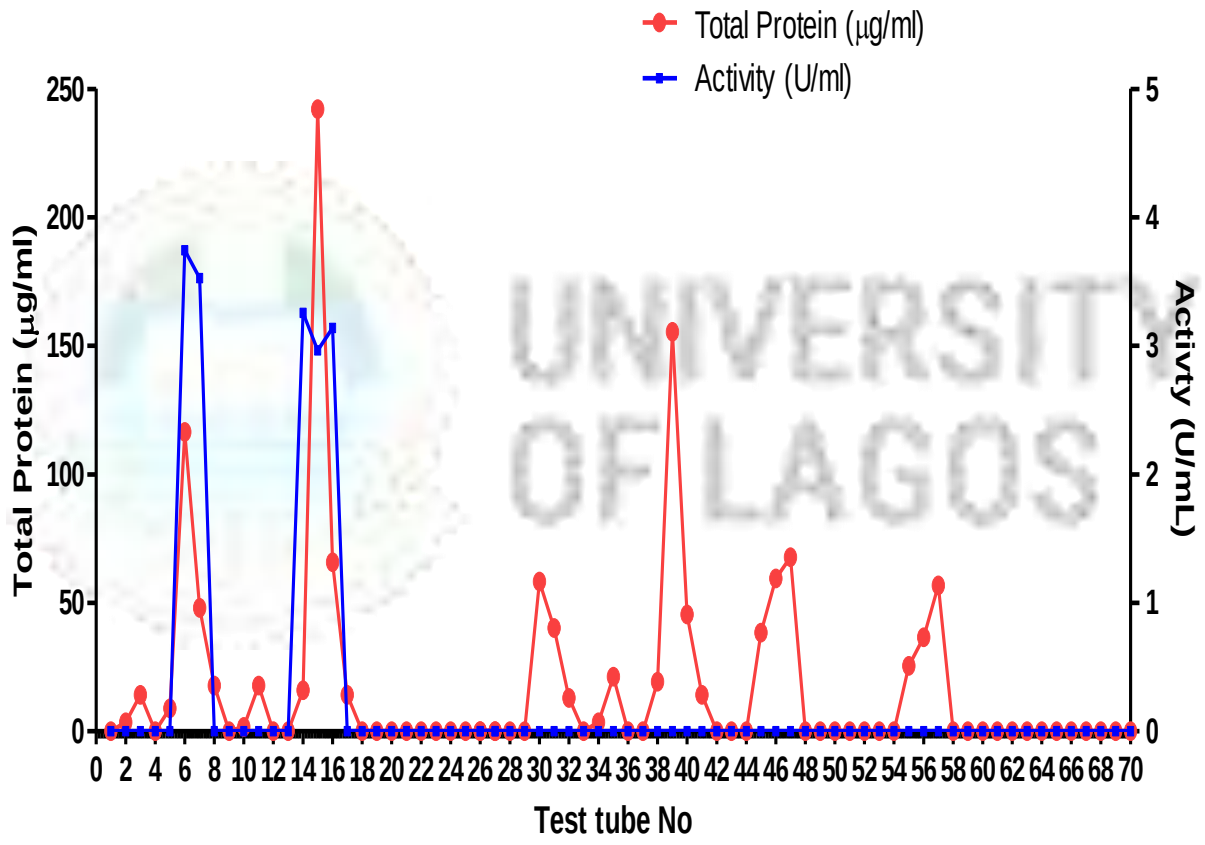


Fig. 35: Elution Profile of Anion Exchange Chromatography of cellulase Produced by UV Mutated *Aspergillus niger*

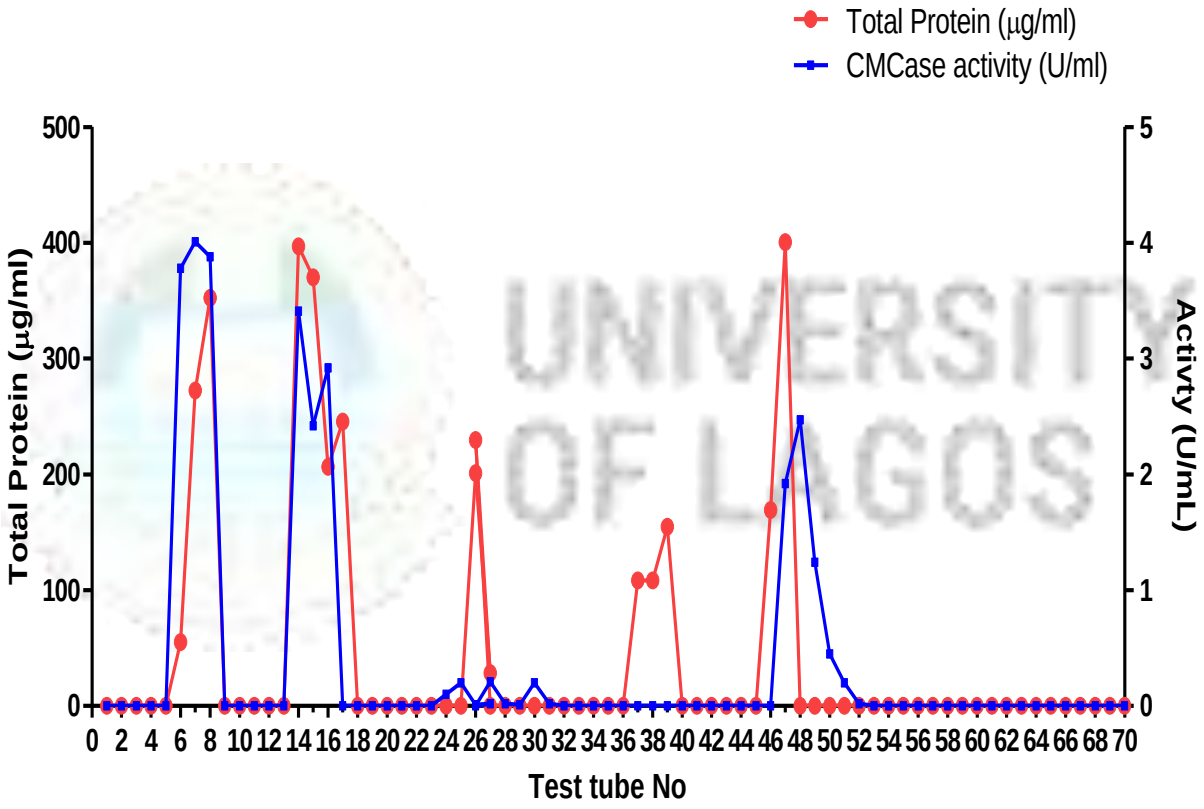


Fig. 36: Elution Profile of Anion Exchange Chromatography of Cellulase Produced by Wild *Penicillium citrinum*

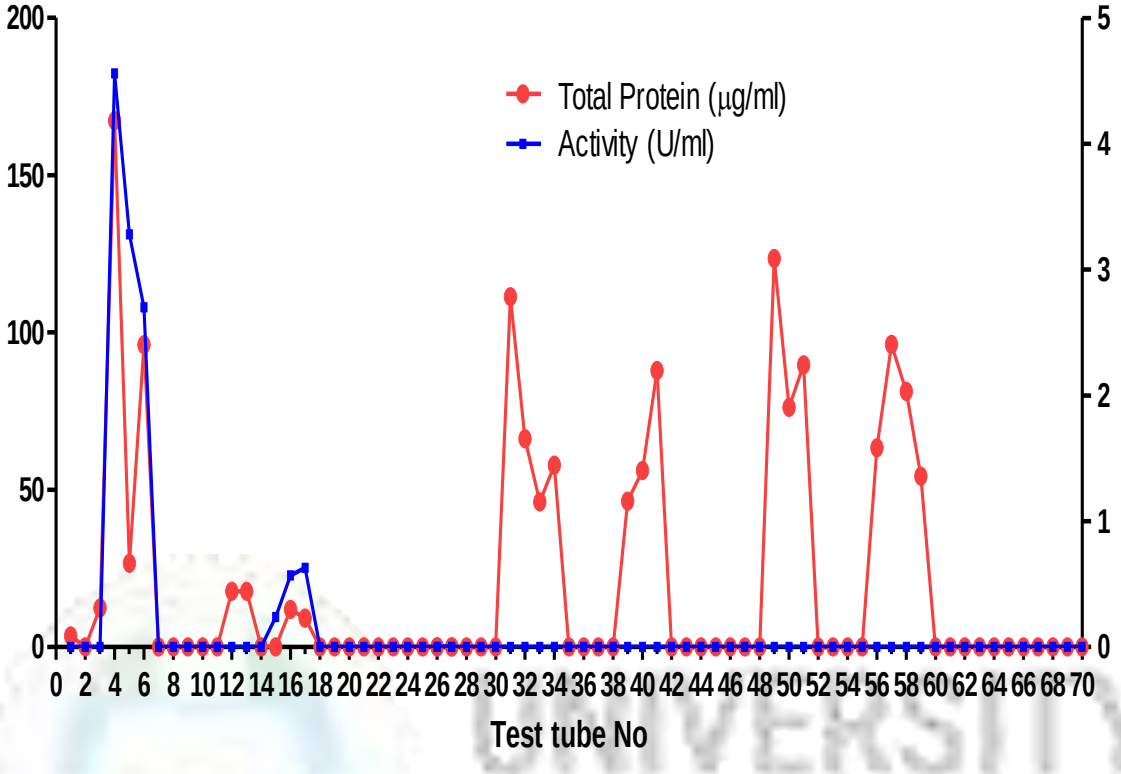


Fig. 37: Elution Profile of Anion Exchange Chromatography of Cellulase Produced by UV Mutated *P. citrinum*

4.10 Effect of Substrate Concentration

The Michaelis-Menten constant (Km) and Maximum velocity (Vmax) for wild *A. niger* were 15.31 g/L and 4.67 g/L respectively while for the mutant *A. niger*, they were 7.9g/L and 3.39g/L respectively (Fig. 38).

The Km and Vmax values for wild *P. citrinum* were 7.94 g/L and 6.81 g/L respectively while the mutant *P. citrinum* had Km and Vmax values of 6.60 g/L and 6.84 g/L respectively as shown in Fig. 39.

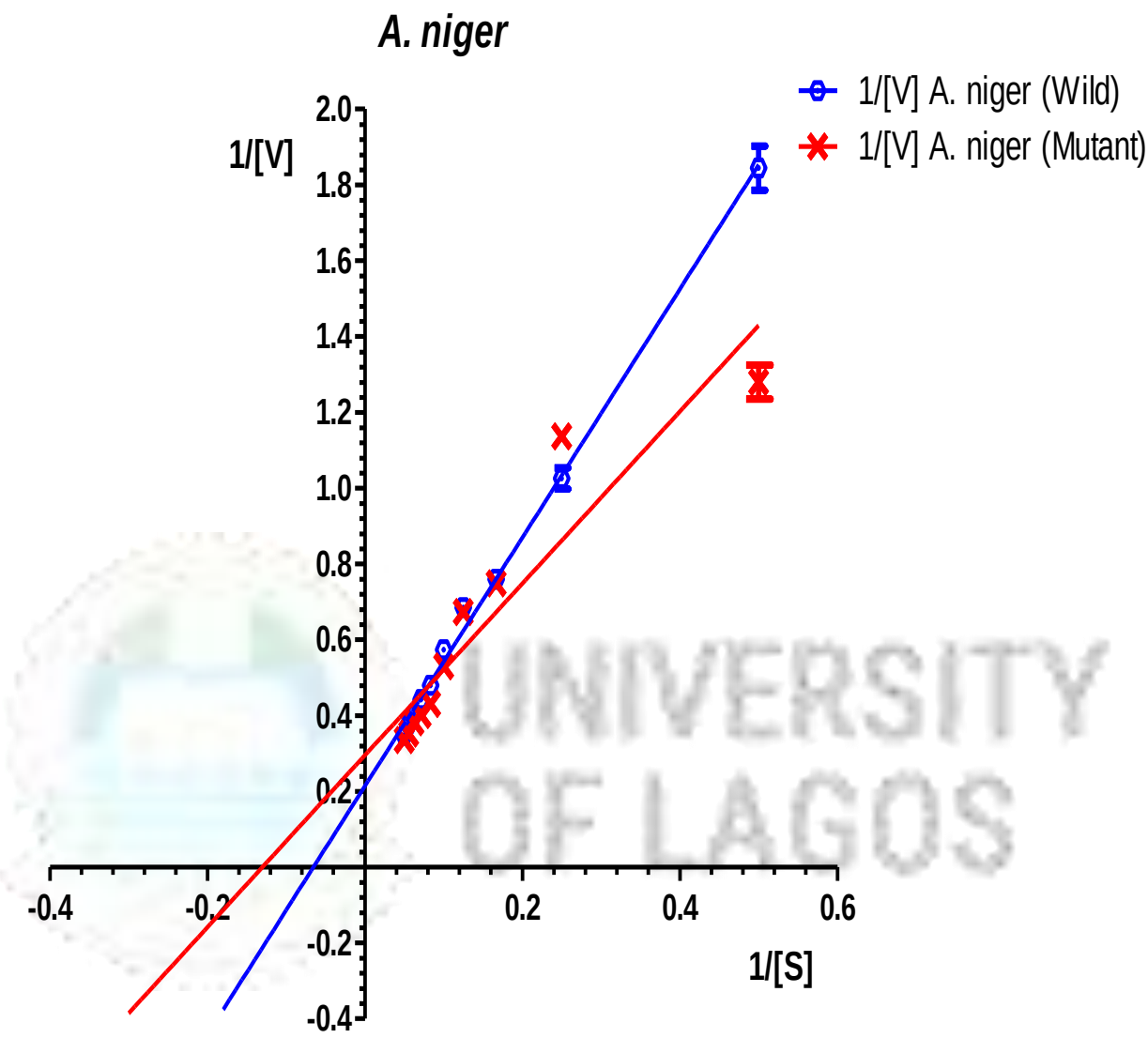


Fig. 38: Lineweaver-Burk plot of Cellulase Activity of Wild and Mutated *A. niger*

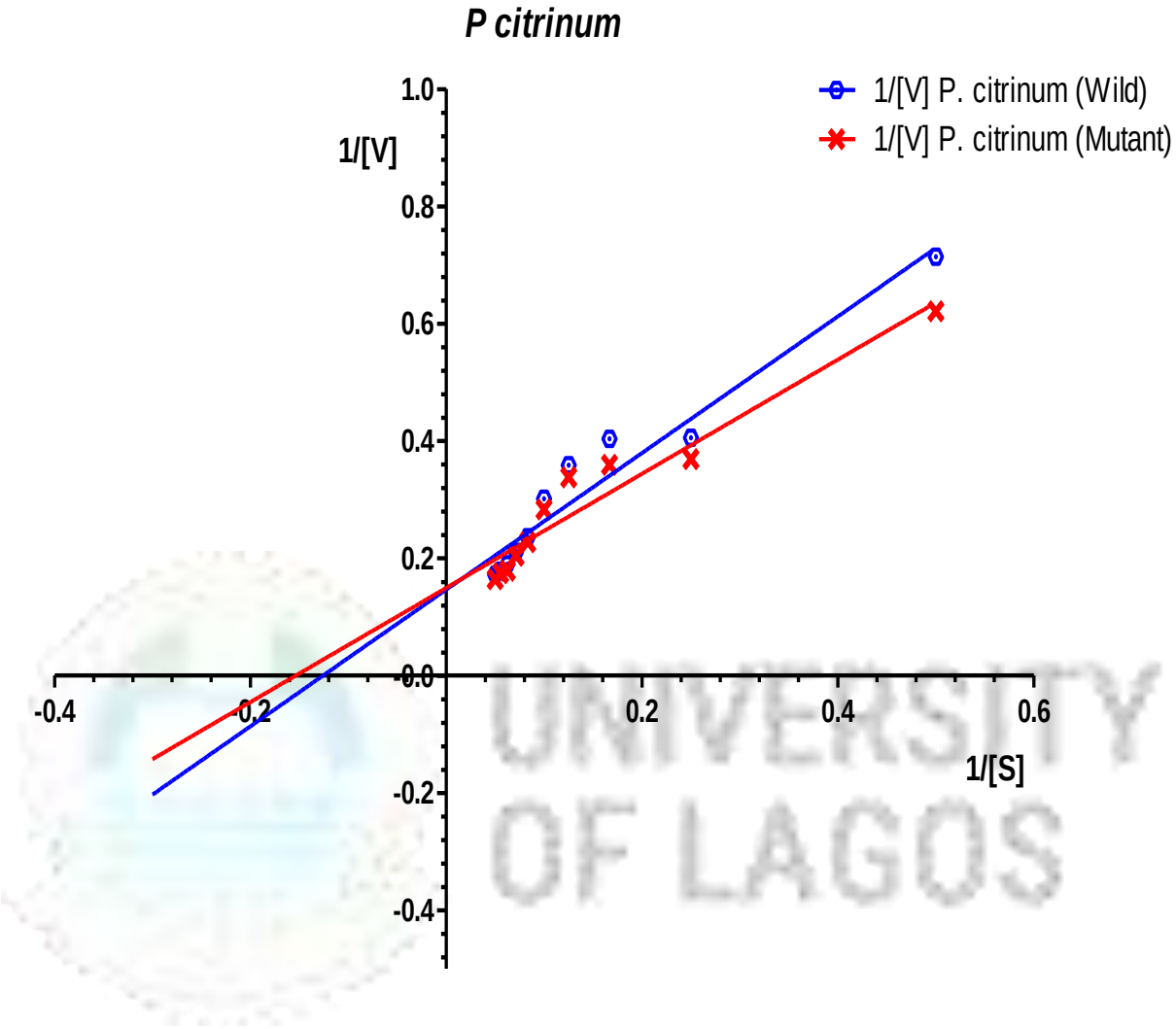


Fig. 39: Lineweaver-Burk plot of Cellulase Activity of Wild and Mutated *P. citrinum*

4.11 Genetic Studies

4.11.1 RNA Extraction

Bands of extracted RNA of wild and mutated strains of *Aspergillus niger* and *Penicillium citrinum* are shown in Fig. 40 below. Non-smearing of bands confirms quality of extraction. Quantities of extracted RNA are shown in Table 7. Measurement of the ratio of their absorbance at A260/A280 further confirmed the integrity of the extract while A260/A230 values show very minimal contaminations from extraction.

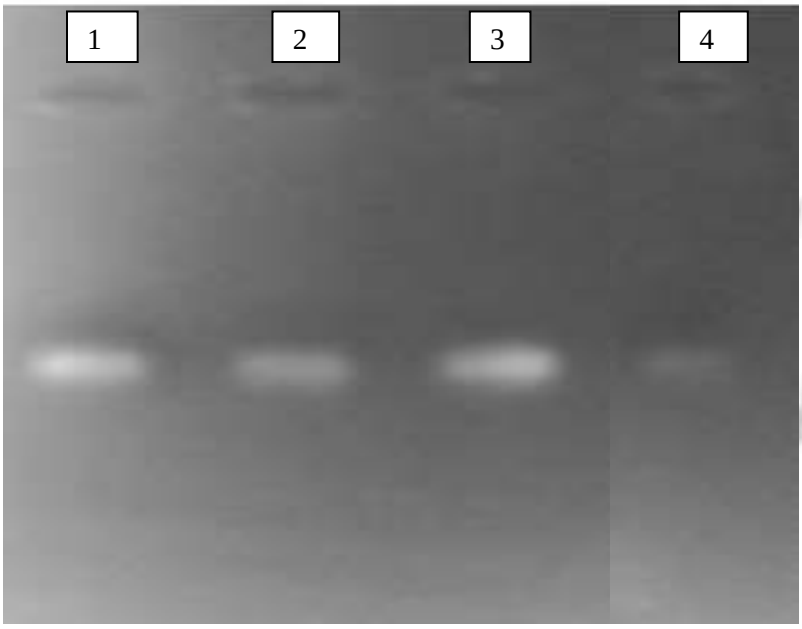


Fig. 40: Agarose Gel Electrophoregram of Extracted RNA Mutants

Lane1= ANW, Lane 2= PCW, Lane 3= ANM, Lane 4= PCM

Table 7: Quantification of Extracted RNA

RNA Sample	Quantity (µg/mL)	A260/A280	A260/A230
ANW	61.4	1.53	0.10
PCW	77.8	1.99	1.29
ANM	70.7	1.97	0.21
PCM	121.1	1.98	1.64

Key: ANW= Wild *A. niger* ; PCW= Wild *P. citrinum*; ANM= *A. niger* mutant; PCM= *P. citrinum* mutant

4.11.2 Amplification of cDNA

Bands of the amplified cDNA are shown in Fig. 41. Selected genes amplified in both wild and mutant strains of the two fungi are arranged in the order; Wild *A. niger*, Wild *P. citrinum*, *A. niger* Mutant, *P. citrinum* mutant. Lane 1-4 shows Lacc1 gene; Lane 5-8 is ace1 gene; Lane 9 is empty; Lane 10-13 shows aep1 gene; Lane 14-17 shows cbh1 gene; while Lane 18 shows the ladder. There were poor amplifications in the Lacc1 gene of the four fungi strains and the ace1 of Wild *Apergillus niger* (Lane 5). There was amplification of ace1 gene in wild *P.citrinum* and mutant *A. niger* (Lane 6 and 7). There were poor amplification of aep1 gene in all the fungi strains (Lane 10-13), but there was amplification of cbh1 gene in all the four fungi (Lane 14-17). It was found that the cbh1 gene which is about 730bp was amplified successfully.

4.12 Bioinformatic Analysis and Prediction of the Structure of Cellulase

The percentage identity between the active site region of uncultured fungus (JF347834), Wild *A. niger* strain nl-1 (HM769954.1), *Phanarechaete chrysosporium* (M22220.1), *Trichoderma reesei* (E00389) and *A. niger* mutant was very high (Fig. 42). This was also confirmed in their consensus region shaded in black (Fig. 42). Amino acid translation of their sequences using JPred suite of Jalview software showed high consensus as well (Fig. 43). Cladogram showed that *A. niger* mutant (cbh_AN10_5b_cbh_forward) had the closest value (6.81 and 7.17) with uncultured fungus (JF347834.1). However, values are not too different from other sequences analyzed (Fig. 44). X-ray crystal structure of the enzyme from the *A. niger* mutant revealed that the regions of the enzyme that have been altered as a result of the mutation were mainly in the β-pleated sheets of the enzyme structure which is represented in blue and white (Fig. 45). The translation

of the cbh1 gene (excluding introns) into the CBH1 protein was performed using the translate tool (http://web.expasy.org/translate/). The analysis revealed amino acid sequence similar to cellobiohydrolase of *T.reesei, P. chrysosporium* and other reference sequences downloaded from public databases (Fig. 43). There were changes in amino acids residues of the enzyme as a result of the mutation. Table 8 shows the amino acids that arose from the mutational change, their location in the amino acid chain and their hydropathy index.

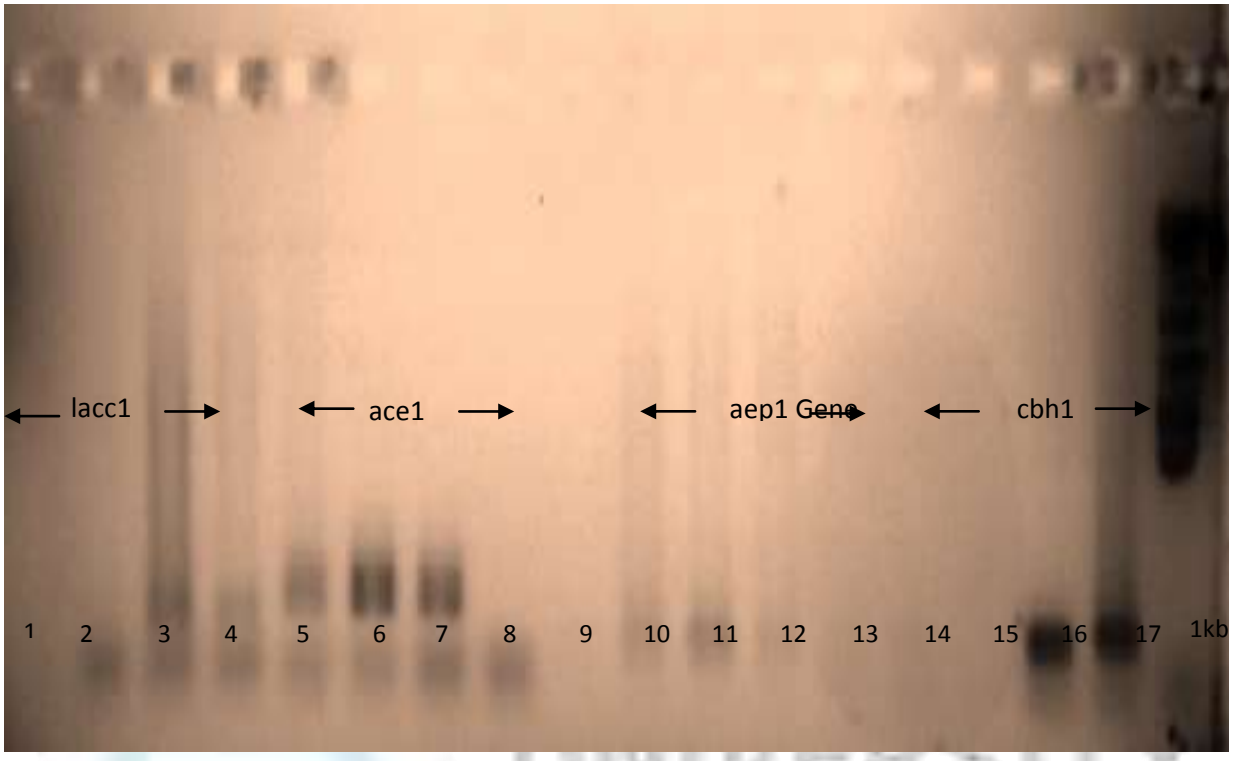


Fig. 41: Banding Pattern of Amplified Genes in cDNA

Lanes 1-4 shows laccase gene I (lacc1), lanes 5-8 shows transcriptional activating gene I (ace1), lane 9 is empty, lanes 10-13 shows aldose epimerase gene I (aep1), lane 14-17 shows cellobiohydrolase gene I (cbh1) and lane 18 shows the 1kb ladder.

Table 8: Amino Acid Changes in CBH1 of Mutated *A. niger* and their Hydropathy Index

Amino acid	Symbol	Position in the Protein Structure	Hydropathy Index
Lysine	K	8th	-3.9
Serine	S	18th	-0.8
Asparagine	N	27th	-3.5
Aspartate	D	28th	-3.5
Threonine	T	36th	-0.7
Glutamate	E	52nd	-3.5
Asparagine	N	53rd	-3.5
Lysine	K	56th	-3.5
Glutamine	Q	75th	-3.5
Asparagine	N	89th	-3.5
Serine	S	92th	-0.8

The amino acid on the 8th position on the chain has changed from Asparagine (N) to Lysine (K) while on the 18th position, there was a change from alanine (A) to serine (S). Similarly, there were changes 27th, 28th, 36th, up till the 92nd position where there was a change from alanine (A) to serine (S) (Fig. 43).

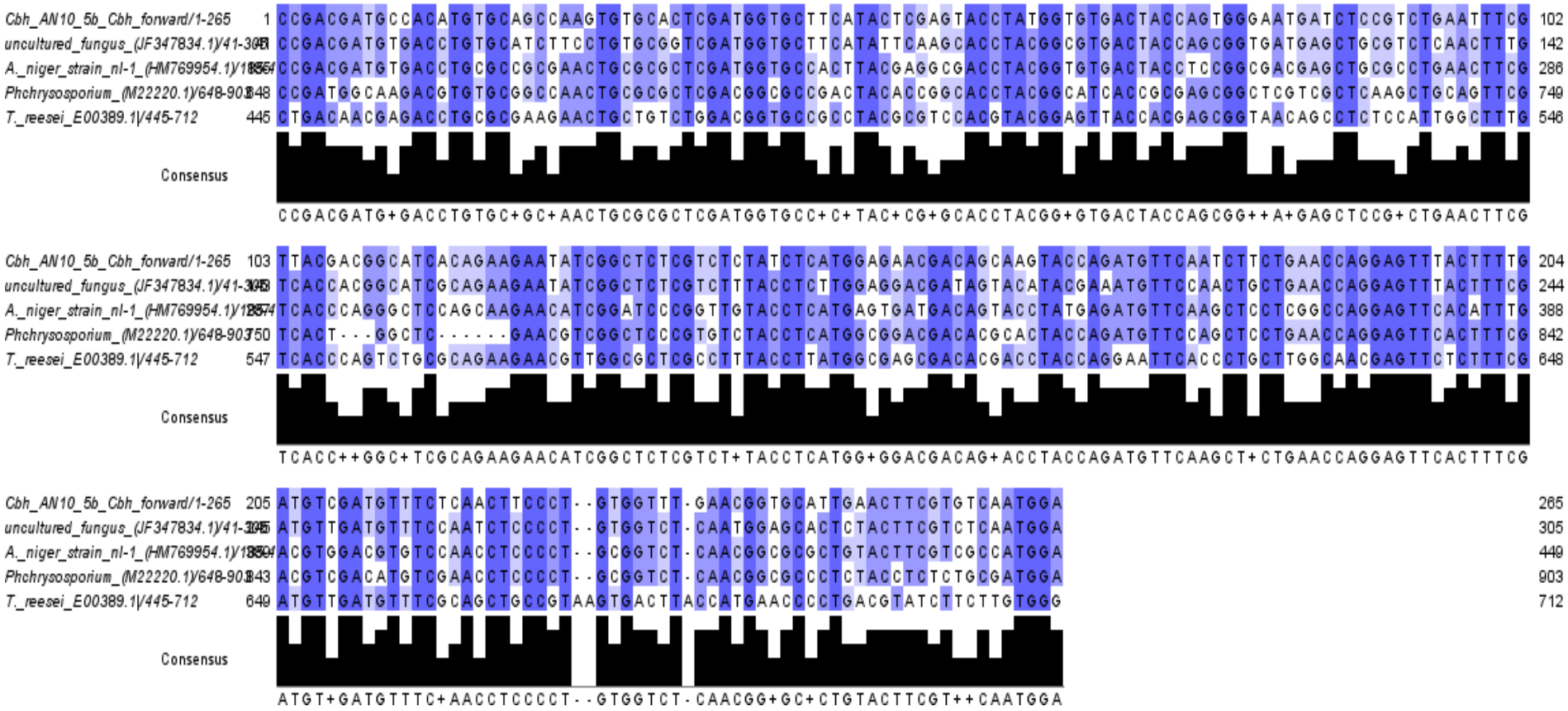


Fig. 42: Nucleotide sequence alignment of the active site region of cbh I gene from mutated *A. niger* (cbh_AN10) with other reference sequences

Dark blue shading indicates sequence region with highly conserved nucleotide sequence. Light blue shading indicates nucleotide sequence regions with slight variability while the unshaded regions indicate nucleotide regions with high level of variability.

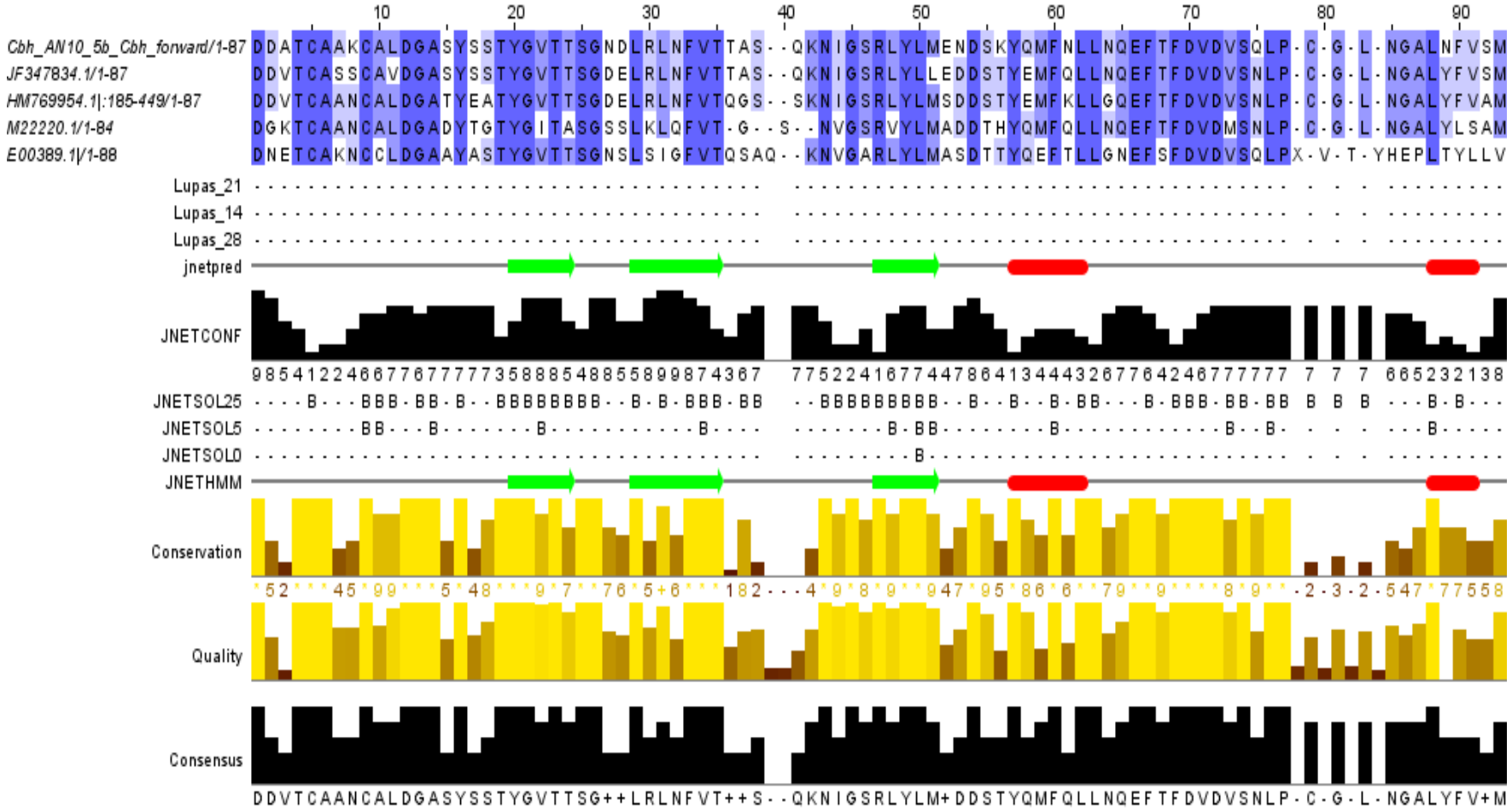


Fig. 43: Alignment of the amino acids of translated sequences showing regions of mutation in the CBHI of mutated *A. niger*

The translated amino acids were compared those obtained from referenced sequences. Secondary structure of CBH1 was predicted using JPred suite of Jalview software. The β-sheets are shown in green.

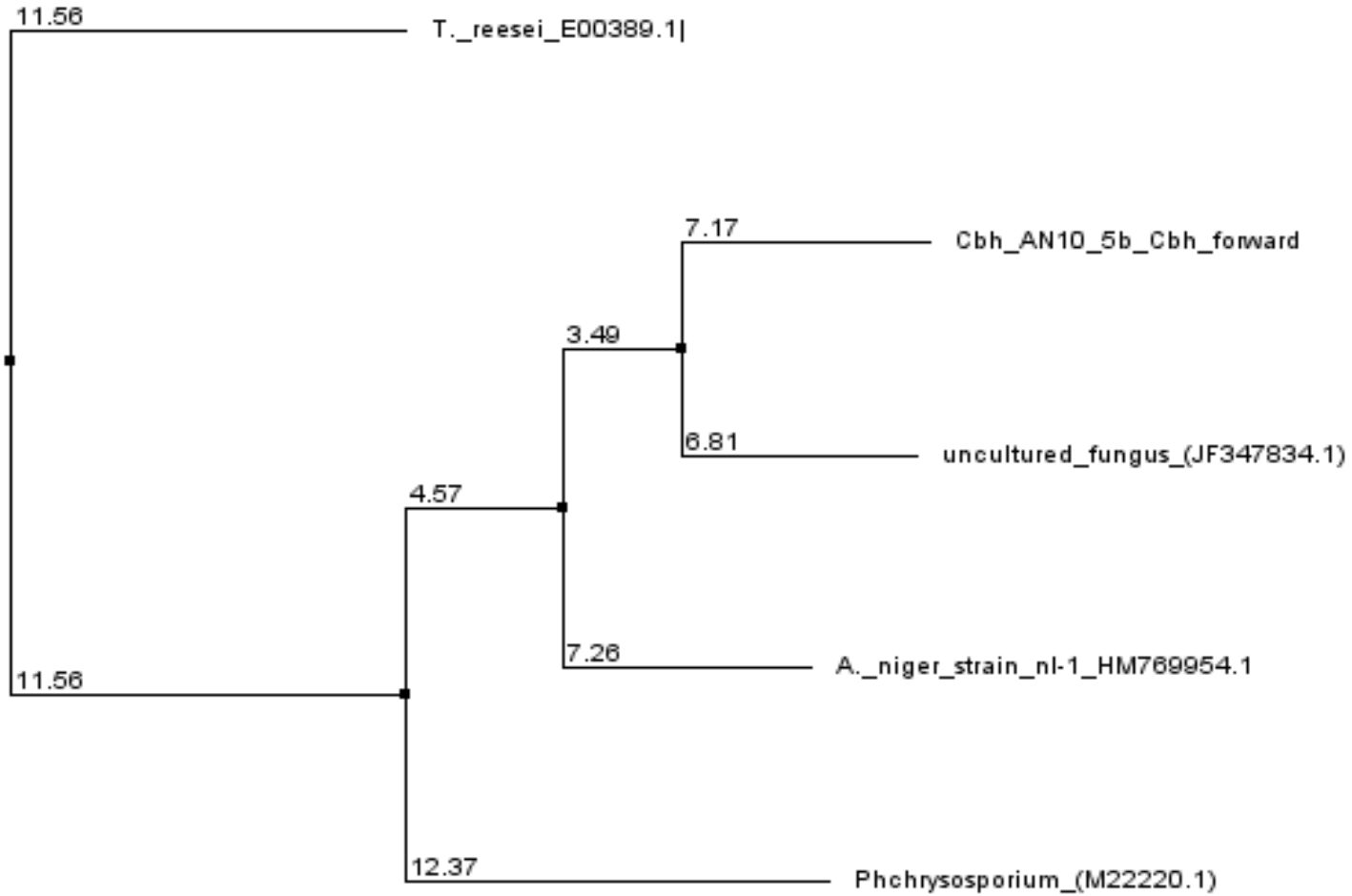


Fig. 44: Cladogram showing homology of mutated *A. niger* CBH1 active site region with those of reference fungi

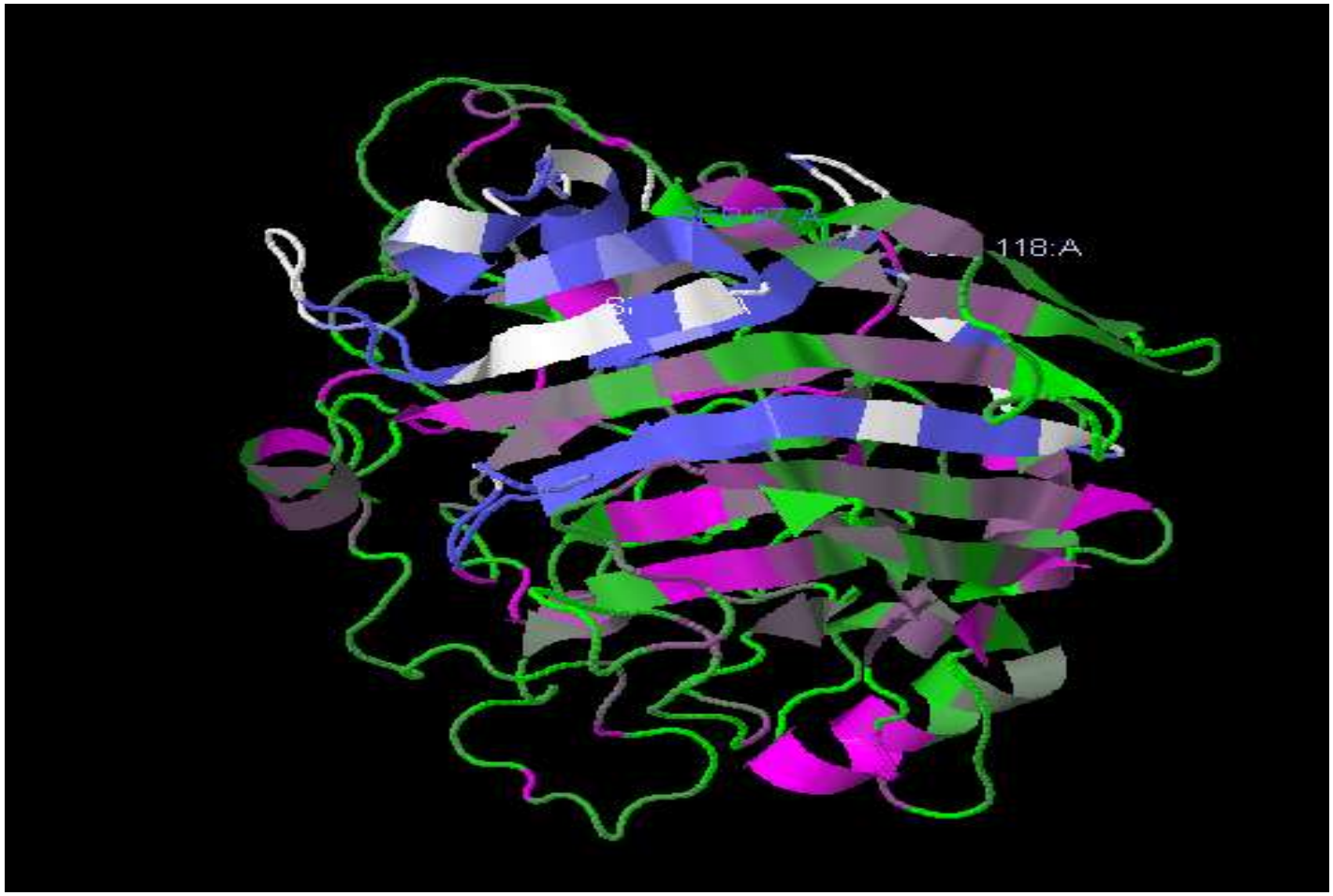


Fig. 45: In-silico X-ray crystal structure of chain-A of CBHI of mutated *A. niger*

Region and sites of mutation are shown in blue and white

CHAPTER FIVE

5.0 DISCUSSION

Microbes, most especially fungi posses the capability of producing a wide range of industrially important enzymes which find use in almost all industries including food, feed, textile, cosmetics and pharmaceuticals. These enzymes may however, not be available in commercial quantities. Hence, the need to modify these organisms for enhanced enzymes production.

The lignocellulose content (cellulose, hemicellulose and lignin) of Abura wood sawdust were comparable with the lignocellulose content of common agricultural residues and wastes compiled by Lee *et al*., (2014). Hardwood stem was said to be composed of 40-55% cellulose, 24-40% hemicelluloses, and 18-25% lignin while softwood contained 45-50% cellulose, 25-30% hemicelluloses and 25-35% lignin. This range for lignocelluloses composition is also similar to compilation of Pedersen and Meyer (2010). However, when the sawdust was pretreated, the cellulose content was 64.94%, hemicellulose was 19.80% and lignin was 15.20%. The sharp reduction was as a result of the removal of hemicelluloses and lignin due to the pretreatment process.

There was reduction in moisture and crude fibre content. This is unconnected to the fact that due to chemical pretreatment, lignin and hemicelluloses are removed. Pretreatment is considered to be the disruption of resistant carbohydrate-lignin shield that inhibits enzymes access to target substrates, which are cellulose and hemicelluloses (Yang and Wyman, 2008). Different methods have been used to achieve pretreatment. This varies from physical, mechanical, chemical, physico-chemical and even biological methods (Abu *et al*., 2000; Abu *et al*., 2002; Yang and Wyman, 2008; Harmsen *et al*., 2010). Cost plays a major role in

deciding the choice of pretreatment. In this study, ammonia steeping, otherwise called soaking in aqueous ammonia (SAA) was employed. It leads to about 60% reduction in lignin and hemicelluloses content. This was in agreement with observations of Abu *et al*., (2002). Kim *et al*., 2008 achieved 60% lignin solubilization and observed saccharification yields of 83% for glucan when destarched barley husk was treated with 15% aqueous ammonia at 75^{o}C during 48h. In this study, pretreatment was carried out at room temperature and comparable success was achieved.

The fungal isolates selected (*P. corylophilum, P. citrinum, A. niger* and *T. asahii*) had similar morphological characteristics with the species described by Tiwari *et al.,* (2011). Molecular identification of fungi by DNA barcoding has been described to be the most useful method of identification (Bellemain *et al.,* 2010). The internally transcribed spacer regions (ITS1 &2) were targetted for the amplification of the DNA fragments. It is located between the short sequence unit (SSU) and the long sequence unit (LSU) regions separated by the 5.8S gene in the rDNA repeat unit. It is specific for fungi and other organisms of plant origin. They had very close homology with reference sequence. The results of the genotyping were consistent with the findngs of Jang *et al.,* (2012) where isolates which included *Aspergillus* and *Penicilium* species where identified by amplification of the ITS region.

Alkaline pretreated sawdust fermented with co-cultured *A. niger* and *P. citrinum* released more cellulase enzyme more than unpretreated sawdust co-cultured with both organisms. However, when both sawdust types were fermented with either fungi, alkaline pretreated sawdust fermented with *A. niger* produced more enzyme more than unpretreated sawdust fermented with *A. niger*. The sawdust types fermented with *P. citrinum* did not release cellulase as much as those fermented with *A. niger*. This shows the efficiency of *A. niger*

over *P. citrinum* in the biodegradation of sawdust. However, synergism between both organisms was demonstrated when co-culturing of both organism in unpretreated sawdust could release more cellulase more than the use of either fungi on alkaline pretreated sawdust. Similar trend was observed with the release of reducing sugars and cellobiase (β-glucosidase) activity. This confirms the synergistic effect of both fungi.

The decrease in conidiospores of *A. niger* and *P. citrinum* mutant growing on mineral salt agar containing 2-deoxy glucose was due to the cytotoxity of the ultraviolet radiation as well as the presence of 2-deoxy glucose in the culture media. The glucose derivative is an anti-metabolite which could cause catabolite repression to non-mutant strains (Anwar *et al*., 1996; Farkas *et al*., 1981). Only mutated strains of fungi can survive this repression (Dillon *et al*., 2006).

The increase in cellulase activity was a confirmation of mutation in the genome of the organism. UV irradiation could lead to unspecific mutation which may be beneficial or not. In this case it has enhanced the production of cellulase in an appreciable manner. In most studies, mutation is achieved after exposure of organisms to repeated rounds of mutagenic agents and / or combination of different mutagenic agents. Vu *et al*., (2009) succeeded in improving carboxymethylcellulase, filter paper cellulase and β-glucosidase yield of *A. niger* by 2.03, 3.20, and 1.80 fold respectively by sequential treatments with two repeated rounds of μ-radaition of Co^{60}, ultraviolet treatment and four repeated rounds of treatment with a chemical mutagen, N-methyl-N'-nitro-N-nitrosoguanidine. In this study, a competent mutant was selected from different mutants created from fungi exposed to different doses of UV irradiation. *A. niger* mutant with CMCase of 4.69U/mL and FPase of 1.97U/mL and *P. citrinum* mutant with CMCase of 4.83U/mL and an FPase of 1.97U/mL.

Moisture is very essential for production of cellulase. It is pertinent that moisture level should be at a specific quantity required for optimum enzyme production. Too much moisture in a solid-state fermentation medium could affect porosity of substrate (Vu *et al*., 2010). The optimum moisture content obtained is comparable to the findings obtained by Vu *et al*., (2010) where moisture content higher than 50 % v/w (76.6 U/g) was said to be optimum for mutated *A. niger*.

The optimum temperature of 37 ^{o}C was found suitable for both CMCase and FPase of *A. niger* and *P. citrinum* mutants. The optimum temperature obtained quite agrees with the findings obtained in earlier works of Narasimha *et al*., (2006). A pH of 3.5 was found to be optimum for enzyme production of the *A. niger* mutant while a pH of 4.5 was optimum for *P. citrinum* mutant. There was a slight variation to findings of Acharya *et al*., 2008. They identified an optimum pH of 4.0 and 4.5 for wild *A. niger* species. The slight change could be due to the slight change in the make-up of the organism due to its mutation by UV. Vu *et al*., (2011) obtained 28.5 U/g for an optimum pH of 3.5. The rate of enzyme catalyzed reactions increase with temperature up to a certain limit. Above a certain temperature enzyme activity decreases with increase in temperature because of enzyme denaturation. The optimum pH for the growth of the organism leads to reduction in unwanted extra-cellular proteins other than the required product (Rajesh *et al*., 2012). Other parameters include fermentation time of 4 days was obtained for *A. niger* mutant while it was 7 days for *P. citrinum* mutant. Age of culture medium was 3 days for *A. niger* mutant while it was 7 days for *P.citrinum* mutant. Inoculum size was 20% w/w for *A. niger* mutant while it was 30% (w/w) for *P. citrinum*. In the studies conducted by Vu *et al.*, (2011) fermentation time of 3 days was obtained, age of culture medium of 2 days and inoculum size of 25 % for *A. niger* mutant.

Gilna and Khaleel, (2011) identified a pH of 6.5 to be optimum for enzyme production by *A. niger*, temperature of 32 ^{o}C, yeast extract as the best nitrogen source, and cellulose as a good inducer. The *A. niger* mutant used in this study had an optimum pH of 3.5, temperature of 37 ^{o}C and EDTA as an inducer. Changes in pH may also alter the structural architecture of the enzyme. This change in pH of the medium activates the active site of the enzyme and facilitates the reaction rate. Pradeep and Narasimha, (2011) mutated *A. niger* with ethyl methane sulfonate (EMS) and were able to obtain a CMCase of 18.09 U/mL and FPase of 19.73 U/mL after fermenting the mutant using pea seed husk as substrate. Pradeep *et al.*, (2012) was however, able to obtain CMCase of 3.15 U/mL and FPase of 3.15 U/mL after chemical mutagenesis of *A. niger* using ethidium bromide. Vu *et al.*, (2011) treated *A. niger* spores with Co^{60} rays, UV and NTG and was able to obtain a mutant. The solid state fermentation parameters of the mutant were optimized and a CMCase activity of 82.5 U/g was obtained using sawdust as substrate. Their findings represented an 8.5-fold increase in enzyme yield compared to the wild.

However, in this study, optimized fermentation of UV mutant of *A. niger* produced a CMCase of 17.2 U/mL and FPase of 6.5U/mL. This represented a 7.4-fold increase in enzyme yield compared to the wild. EDTA best enhanced cellulase production and this agrees with the findings of Acharya *et al.*, (2008). Corn-starch enhanced cellulase production which disagrees with the findings of Zhu *et al.*, (2011). The preference for natural carbon sources in enzyme production may be due to the presence of growth promoters in enough amounts covering the requirements of fungal growth and enzyme production (Rajesh *et al*., 2012). It could also be explained that the metabolic pattern of the organism may have been altered as a result of the mutation, thus making it to easily metabolize glucose biopolymers without hindrance. Increase in cellulase activity after UV mutation may be due to possible changes in the promoter regions of the genes (De Nicolas-Santiago *et al.*, 2006). During SSF,

the components of the cell wall are degraded and lignocellulolytic enzymes (LCEs) are produced and act optimally (Vu *et al*, 2011).

Solid-state fermentation of polysaccharide using *Paecilomyces cicadae* was carried out by Ren *et al*., (2014). The Box-Behnken design was used to optimize the final levels of culture conditions. Relative humidity of 56.07%, inoculums of 13.51mL/100g and temperature of 27.09^{o}C were found to be optimal parameters for polysaccharide production.

Solid State Fermentation (SSF) has been demonstrated to offer better hydrolytic enzyme yield more than submerged. It is fermentation in the absence or near absence of free water with inert natural substrates as solid support (Pandey *et al*., 1999). Additional carbon source is needed in the fermentation tank to enhance growth. It is expected that the supplemented carbon source should be easily metabolized (Zhu *et al*., 2011). In this study, cornstarch enhanced production of endoglucanase (CMCase) and total cellulase (FPase) activities more than glucose for *A. niger* mutant while maltose enhanced both enzymes in the fermentation by *P. citrinum* mutant. This was contrary to findings of Zhu *et al*., (2011) where supplementation of glucose and increased moisture content enhanced endoglucanase production with *Trametes versicolor* during solid-state fermentation of corn stover. It could be explained that the metabolic pattern of the organism may have been altered as a result of the mutation, thus enabling it to metabolize glucose biopolymers easily.

Surfactants have been used to enhance lignocellulolytic hydrolysis and when used, they reduce enzyme loadings. A number of mechanisms have been proposed for their action. Surfactants help the enzyme to adjust its structure to allow the substrate to fit into the active site of the enzyme. Yang and Wyman (2005) explained that Tween 80 bring cellulase in close proximity with cellulose. EDTA and SDS enhanced enzyme activity of *A. niger* and *P.*

citrinum mutants. The enhancement could be due to the ability of the surfactants to reduce unnecessary attachment of cellulase to substrates other than cellulose in the medium, thus enhancing its performance. The variation may however, be due to the physiological differences in the organisms. Yang and Wyman (2005) explained that bovine serum albumin, a biosurfactant, could attach to lignin, thus allowing non-specific attachment of cellulase to lignin when it is used in the hydrolysis of lignocellulosics.

Metal salts play significant roles in microbial metabolism. They act as cofactors or as co-enzymes. Some fermentation will not yield product without the inclusion of metal salts. $FeSO_4$ best enhanced the production of endoglucanase and total cellulase in both mutants. However, $MgSO_4$ was able to enhance endoglucanase production more than other salts when Vu *et al*., (2011) carried out solid-state fermentation after mutating *A. niger*.

Urea supplementation enhanced enzyme activity more than other nitrogen supplements. This corroborates the findings of Acharya *et al*., (2008). Optimization of nitrogen source for cellulase production was carried out on sawdust using wild *A. niger*. Urea was found to enhance optimum CMCase production more than peptone and sodium nitrite.

Optimization of the solid-state fermentation of mutant *A. niger* resulted in 7.4-fold and 7.6-fold higher yield (CMCase and FPase respectively) more than the wild strain while with *P. citrinum* mutant, it led to 5.3-fold and 5.8-fold higher yield. Vu *et al*., 2011 obtained 8.5-fold increase over wild strain after optimizing solid-state fermentation of mutant.

Cellulase enzyme from the mutated *A. niger* was purified to 9.10 purification fold. The enzyme yield was 54.2% with a specific activity of 5.42 ± 2.4 U/mg protein. Its activity was quite higher than cellulase from the wild which was 3.56 ± 2.4 U/mg protein (Table 5). The increase was due to the effect of the mutation. Ultraviolet mutation has been proven to impact

on cellulase activity (De Nicholas-Santiago *et al*., 2006; Vu *et al*., 2009; Vu *et al*., 2011). The Michaelis constant (K_M) of cellulase from *A. niger* (15.31g/L) reduced after mutation (7.9g/L). A low K_M indicates a high affinity between the enzyme and the substrate (Aehle *et al*., 2007). This probably explains the enhanced activity of the enzyme from the mutant. Substrates that are tightly bound to the enzyme has low $K_{M.}$ This affinity for the substrate will enhance its activity which was reflected in the elevated specific activity of the enzyme. The catalytic efficiency of cellulase from *A. niger* mutant also increased. Catalytic efficiency is the ability of an enzyme to release a product after its conversion almost instantaneously (Murray *et al*., 2012). It is a ratio of the turnover number (Kcat) of the enzyme to its Michaelis constant (K_M). Turnover number represents the µmoles of substrate converted per µmole of enzyme per second. Alternatively, the number of molecules of enzymes converted by one molecule of enzyme per second (Bugg, 2004). Since cellulase has one active site, at maximum substrate concentration, its turnover number is equivalent to its Vmax which was $7.9s^{-1}$. The catalytic efficiency (Kcat / K_M) of cellulase from *A. niger* mutant was calculated to be $0.429M^{-1}\ s^{-1}$ as compared to the wild which was $0.305M^{-1}\ s^{-1}$.

Cellulase enzyme from the mutated *P. citrinum* was purified to 13.55 purification fold. The enzyme yield was 45.12% with a specific activity of 8.81 ± 1.5 U/mg protein. Its activity was quite higher than cellulase from wild *P. citrinum* which was 5.19 ± 1.0 U/mg protein. The increase is also due to the effect of the mutation. The Michaelis constant (K_M) of cellulase from *P. citrinum* (7.94g/L) reduced after mutation (6.60g/L). Like it's *A. niger* counterpart, the catalytic efficiency of cellulase from *P. citrinum* mutant also increased.

Likewise, at maximum substrate concentration, its turnover number is equivalent to its Vmax which was $6.84s^{-1}$. The catalytic efficiency (Kcat / K_M) of cellulase from *P. citrinum* mutant was calculated to be $1.036M^{-1}\ s^{-1}$ as compared to the wild which was $0.858M^{-1}\ s^{-1}$. In the studies of Wang *et al*., (2012), two cellulases purified from *Trichoderma reesei* were found to

have a Km of 22.68 ± 0.28 g/Land Vmax of 0.269 ± 0.066 mg/min at 5.0mmol/L $AlCl_3$. It was however, inhibited at 10 mmol/L $MgCl_2$ when the Km increased to 50.0 g/L and Vmax of 0.434mg/min.

Studies on fungal cellulolytic system are only limited to a few key species such as *Trichoderma reesei* and *Phanerochaete chrysosporium* (Chukaetirote *et al.*, 2012). This data provides additional information on the presence of cbh1 gene in *A. niger* and *P. citrinum*. The cbh1 gene of *A. niger* showed in this study was distinct, although, it showed high homology to the cbh1 of *T. reesei* and *P. chrysosporium* as shown Figs. 42 and 45. This finding is in agreement with previous work of Chukaetirote *et al.,* (2012) in which several forms of the cbh1 gene has been reported. Cellobiohydrolases are classified as glycosyl hydrolases. Typical basic structures of fungal CBH are composed of three domains or regions: a cellulose binding domain which contains conserved cysteine residues, a catalytic domain which contains conserved glutamic and aspartic acid residues, and a linker region which is rich in serine, glycine and threonine residues (Chukaetirote *et al.*, 2012). *A. niger* CBH has been shown to consist solely a catalytic domain (Li *et al.*, 2009). The mutated *A. niger* in this study is rich in aspartic acid but not glutamic acid. In addition to the homologous amino acids it has with the compared sequence, it is rich in alanine. The nature of amino acid present in the active site of the enzyme determines the efficacy of the enzyme in performing its function. Hydrolysis of cellulose requires amino acids with low hydropathy index at the active site of the enzyme. The hydropathy index is a scale combining the hydrophobicity and hydrophilicity of amino acids functional groups. It is used to measure the tendency of an amino acid to seek an aqueous environment (- values) or a hydrophobic environment (+ values) (Nelson and Cox, 2008). Amino acids peculiar to the active site of the mutant *A.*

niger alone were found to have hydropathy index that favours aqueous environment which enhances cellulose hydrolysis. Lysine (K), which has a hydropathy index of -3.9 was found in the 8th position. Likewise, asparagine (N), glutamate (E), and glutamine (Q) and other amino acids with favourable hydropathy index are found in the enzyme amino acid chain. Almost all amino acids peculiar to the mutant have low hydropathy index which suggests the reason for their elevated activity.

The in-silico X-ray crystal structural elucidation of the active site of the enzyme revealed that the α-helices are not affected by the irradiation. However, mutation occurred in the β-strands (Fig. 44). Cladogram constructed based on relationship between the mutated *A. niger* and industrially important referenced fungi showed homology between the mutant and *Trichoderma reesei* and *Phanerochaete chrysosporium* with a bootstrap values of 11.56 and 12.37 respectively as compared with 7.17 of the mutated *A. niger*.

Homology in the sequence of the mutant *A. niger* and *Trichoderma reesei*, a model hyper producer of cellulase, signifies its potential in the industry for hyper production of cellulase. The analysis will allow the use of the exquisite tools of fungal genetics to further interrogate protein function and interactions (Bennett *et al.*, 2001).

6.0 SUMMARY OF FINDINGS

	OBJECTIVES	FINDINGS
1.	Determination of composition of raw and pretreated sawdust.	Cellulose was 48.11%, Hemicellulose was 32.70% and lignin was 19.10%.
2.	Isolation and genotyping of cellulolytic fungi.	Four cellulolytic fungi; *Penicillium citrinum, Aspergillus niger, Trichosporon asahii* and *Penicillium corylophylum* were genotyped.
3.	Mutation of isolates and optimizing their Solid-State Fermentation.	*A. niger* mutant required optimum moisture of 20%, temperature of 37 ^{o}C, pH of 3.5, fermentation time of 4 days, inoculum size of 25 % $^{w}/_{w}$, age of seeding culture of 3 days, corn starch as carbon supplement, EDTA as surfactant, $FeSO_4$ and urea as metal salt and nitrogen supplements respectively. However, *P. citrinum* mutant required an optimum moisture of 20 %, temperature of 37 ^{o}C, pH of 5.5, fermentation time of 7 days, inoculum size of 30 % $^{w}/_{w}$, age of seeding culture of 5 days, maltose as carbon supplement, SDS as surfactant, $FeSO_4$ and urea as metal salt and nitrogen supplements respectively. .*A. niger* mutant resulted in a 7.4-fold and 7.6-fold higher yield

		in CMCase and FPase respectively more than the wild strain. Likewise, *P. citrinum* mutant resulted in a 5.3-fold and 5.8-fold higher yield in CMCase and FPase respectively more than the wild strain.
4.	Sequencing of cDNA amplicons obtained from wild and mutant fungi strains.	Lacc1, ace1, aep1 and cbh1 genes were amplified but only cbh1 gene sequencing was successful.
5.	Analysis of sequence using Bioinformatics tools and prediction of the structure of cellulase from mutant.	Mutated *A. niger* had strong similarity with compared in□ustrially beneficial cellulolytic fungi. Amino acid residues in its active site had low hydropathy index. Predicted structure of cellulase from *A. niger* mutant revealed that alteration in structure occurred in the β-pleated sheets of enzyme.

6.1 CONCLUSION

Filamentous fungi are considered to be the most important and main group of microorganisms for the production of plant cell wall degrading enzymes in solid state fermentation. Mutagenesis using ultraviolet light and enrichment are cost effective yet efficient way of enhancing their performance. In this study, optimization of the solid state fermentation of mutated *A. niger* resulted in a 7.4-fold and 7.6-fold higher yield in CMCase and FPase respectively more than the wild strain while mutated *P. citrinum* resulted in 5.3-fold and 5.8-fold increase (CMCase and FPase). The cellulase produced by these modified fungi has high turnover rates. The amino acids in their active site has low hydropathy index which explains their superlative performance. This signifies their potential for use in the overproduction of enzymes for cellulosic wastes bioconversion. The huge tonnage of agricultural wastes produced everyday, which is an environmental threat, are raw materials for generation of valuable products and ultimately medium of wealth generation.

6.3 CONTRIBUTIONS TO KNOWLEDGE

1. The study sequenced cellobiohydrolase 1 gene from mutated *Aspergilus niger* and deposited its sequence in the National Center for Biotechnology Information, USA.

2. The study identified the region of alteration in the β-strands in chain-A of cellobiohydrolase I enzyme obtained from mutated *Aspergillus niger*.

3. DNA barcoding of four wild fungi; *Aspergillus niger* ANR-352L (KM192155.1), *Penicillium citrinum* PCM-254L (KJ425589), *Trichosporon asahii* TAS-150L (KM192153.1) and *Meyerozyma guilamondii* MQL-105L (KM192154.1) was achieved in the study. Their sequences were deposited in the the National Center for Biotechnology Information, USA.

4. The study determined the lignocellulosic content of Abura (*Mitragyna ciliata*) sawdust.

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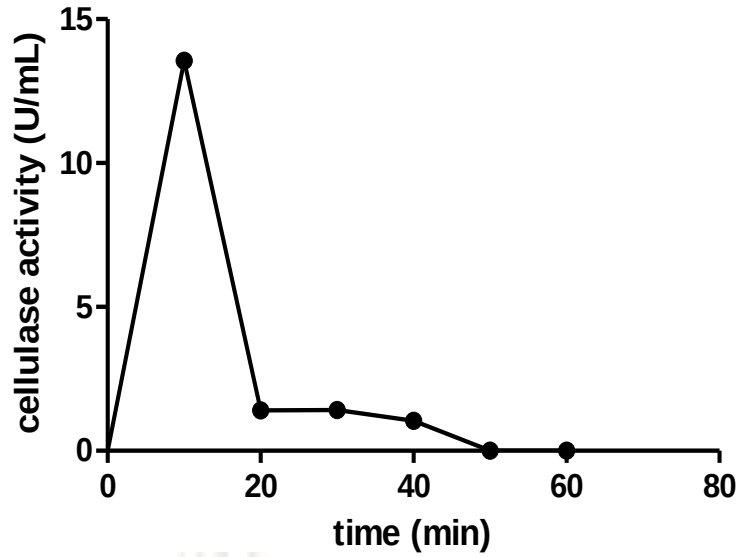
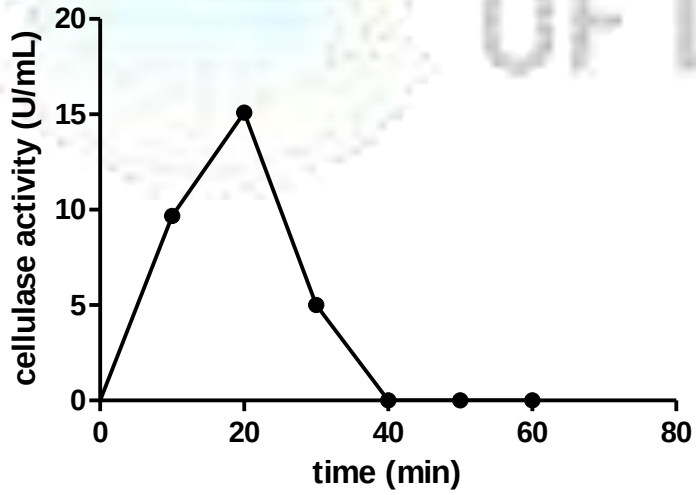
Appendix Ia
UV mutation of *Aspergillus niger*



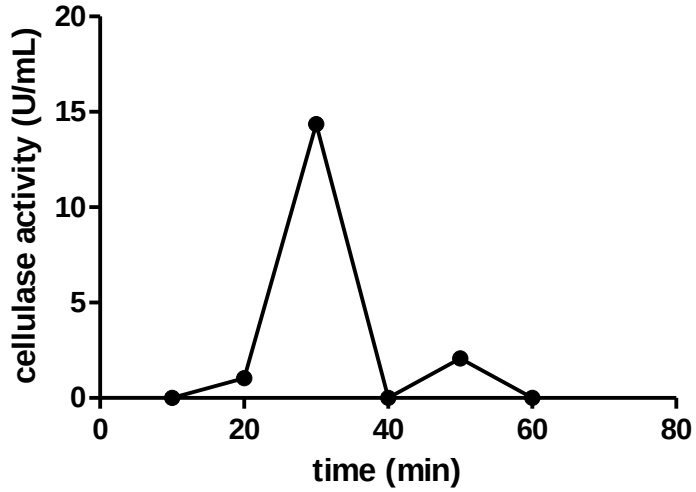
Cellulase activity of UV mutants of *Aspergillus niger* placed 10cm from source of irridiation

Mutant with high cellulase activity formed at ten minutes of irradiation.



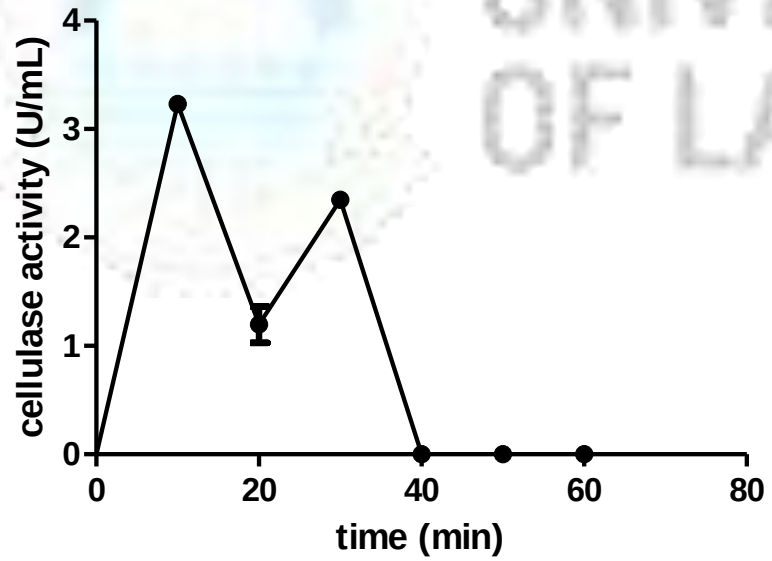
Cellulase activity of UV mutants of *Aspergillus niger* placed 20cm from source of irridiation

Mutant with high cellulase activity formed at twenty minutes of irradiation



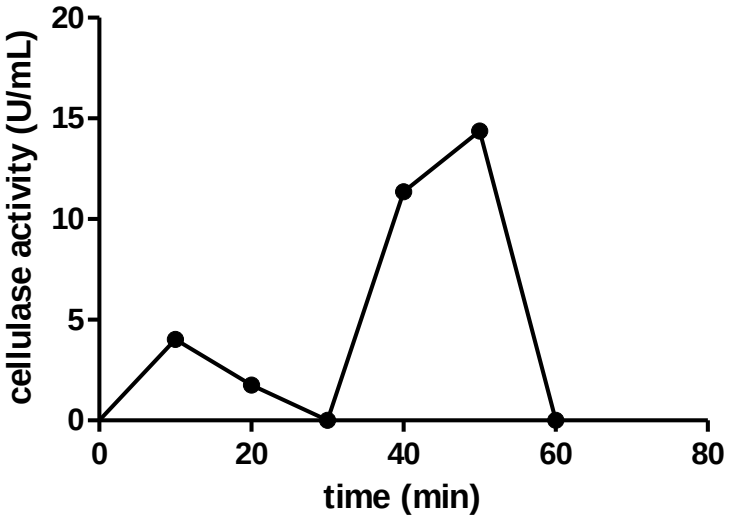
Cellulase activity of UV mutants of *Aspergillus niger* placed 30cm from source of irridiation

Mutant with high cellulase activity formed at thirty minutes of irradiation



Cellulase activity of UV mutants of *Aspergillus niger* placed 40cm from source of irridiation

Mutants with high cellulase activity formed at ten minutes and thirty minutes of irradiation



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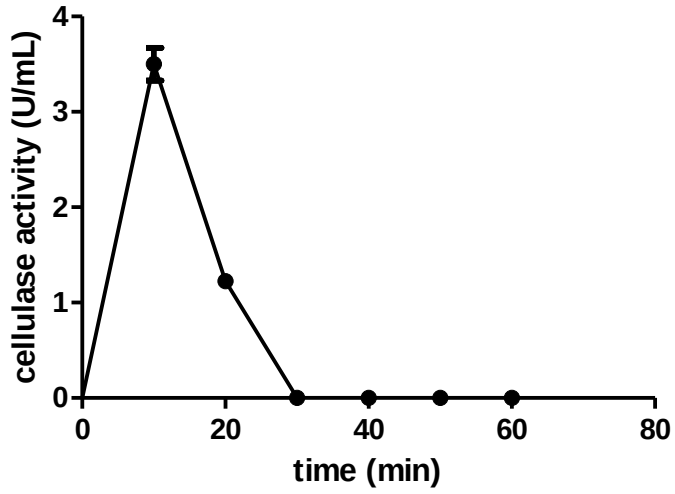
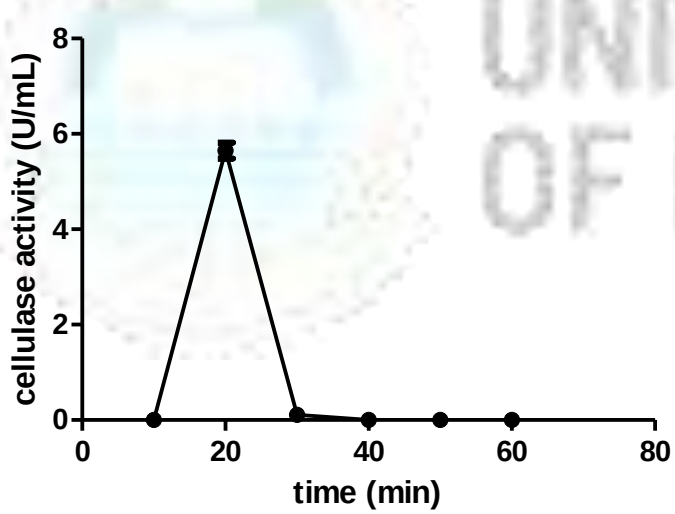
Cellulase activity of UV mutants of *Aspergillus niger* placed 50cm from source of irridiation

Mutant with high cellulase activity formed at fifty minutes of irradiation

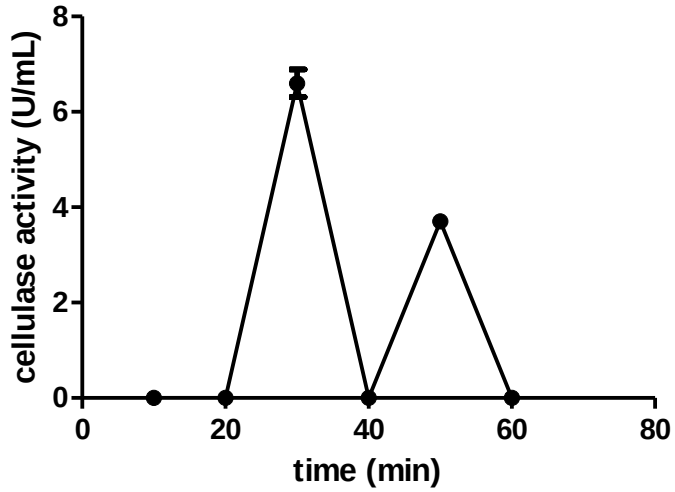
Appendix Ib
UV mutation of *Penicillium citrinum*



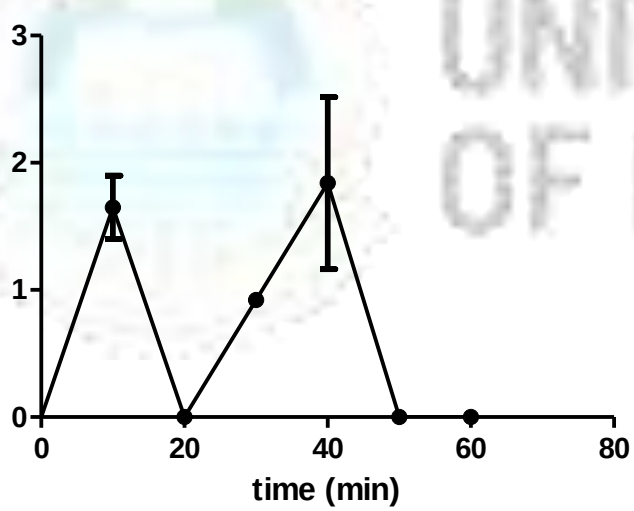
Cellulase activity of UV mutants of *P. citrinum* placed 10cm from source of irridiation
Mutant with high cellulase activity formed at ten minutes of irradiation



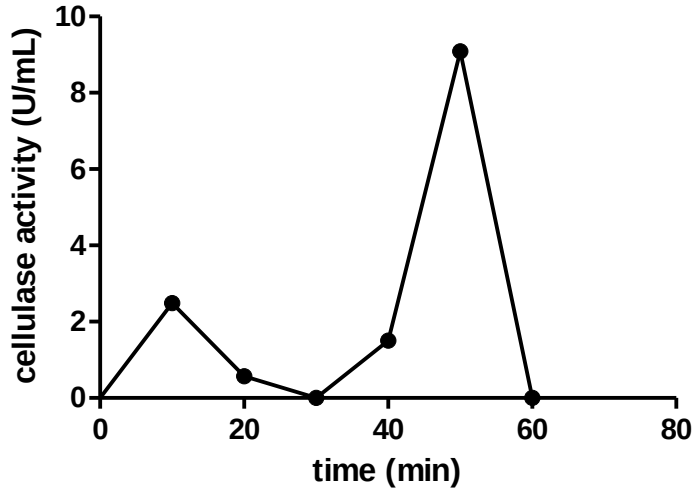
Cellulase activity of UV mutants of *P. citrinum* placed 20cm from source of irridiation
Mutant with high cellulase activity formed at twenty minutes of irradiation



Cellulase activity of UV mutants of *P. citrinum* placed 30cm from source of irridiation
Mutants with high cellulase activity formed at thirty minutes and fifty minutes of irradiation



Cellulase activity of UV mutants of *P. citrinum* placed 40cm from source of irridiation
Mutants with high cellulase activity formed at ten minutes and forty minutes of irradiation



Cellulase activity of UV mutants of *P. citrinum* placed 50cm from source of irridiation
Mutant with high cellulase activity formed at fifty minutes of irradiation

Appendix II

PREPARATION OF BUFFERS

ACETIC ACID - SODIUM ACETATE BUFFER:

Acetic Acid 0.2M: 1.5 ml of glacial acetic acid is made up to 100ml with distilled water.

Sodium Acetate Solution**:** 0.64g of sodium acetate or 2.72g of sodium acetate trihydrate was dissolved in 100mL distilled water.

PROCEDURE:

Exactly 36.2ml of sodium acetate solution was drawn into 100ml of standard flask and 14.8ml of glacial acetic acid was added. The volume was made up to 100ml using distilled water. This gave 0.2 M of acetic acid and sodium acetate buffer. The pH was measured with pH meter. Adjustments were made using 5N NaOH and 1N HCl.

CITRATE BUFFER (0.1M pH 2.5)

REAGENT S REQUIRED:

Citric acid: 2.101g of citric acid was dissolved in 100ml distilled water.

Sodium citrate solution 0.1 M: 2.941g of sodium citrate was dissolved in 100mL distilled water.

PROCEDURE:

Citric acid (46.5mL) was added to sodium citrate solution then made up to 100mL with distilled water. Adjustments were made using 5N NaOH and 1N HCl.

PHOSPHATE BUFFER pH 6.8

REAGENTS REQUIRED:

Monobasic: 2.78g of sodium dihydrogen phosphate was dissolved in 100mL of distilled water.

Dibasic sodium phosphate (0.2M): 5.3g of disodium hydrogen phosphate or 7.17g sodium hydrogen phosphate was dissolved in 100mL distilled water.

PROCEDURE:

Thirty nine mL of dihydrogen sodium phosphate was mixed with 61mL of disodium hydrogen phosphate. The solution was made up to 200ml with distilled water. This gave phosphate buffer of 0.2M. Adjustments were made using 5N NaOH and 1N HCl.

POTASSIUM PHOSPHATE BUFFER

Dipotassium hydrogen phosphate

Potassium dihydrogen phosphate

PROCEDURE:

Dipotassium hydrogen phosphate (174.18 g/mol) and Potassium dihydrogen phosphate (136.09 g/mol) were taken and made up to 200ml using distilled water. This gave the potassium buffer.

Standardised pH meter with standard buffer. Washed electrode with distilled water was introduced into potassium buffer prepared. The pH of the solution was 6.5.

Dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) solution were prepared and the pH was measured to be 9.87 and 4.23 respectively, the solution were made using 1N HCl and 5N NaOH respectively and the pH was found to be 6.5.

CTAB Buffer (100mL) preparation

1.1.1Materials: CTAB buffer, microfuge tubes, mortar and pestle, liquid nitrogen, microcentriguge, absolute ethanol (ice cold), 70% ethanol (ice cold), 7.5M Ammonium acetate, 55^{o}C water bath, chloroform : isoamyl alcohol (24:1), sterile water, agarose, 6x loading buffer, 1x TBE, agarose gel electrophoresis system, ethidium bromide solution.

CTAB buffer system 100ml

2.0g CTAB (Hexadecyl trimethyl-ammonium bromide)

10.0mL 1M Tris pH 8.0

4.0mL 0.5M EDTA pH 8.0 (EthylenediaminetetraAcetic acid Di-sodium salt)

28.0mL 5M NaCl

40.0mL H_2O

Adjust all pH 5.0 with HCl and make up to 100mL with distilled water

1M Tris pH 8.0

To 800mL of distilled water, 121.1g of Tris base was added. The pH was adjusted to 8.0 by adding 42mL of concentrated HCl. The solution was allowed to cool to room temperature before making the final adjustments to the pH. The volume was adjusted to 1L with distilled water and it was sterilized using an autoclave.

5X TBE Buffer

54g Tris base

27.5g boric acid

20mL of 0.5M EDTA (pH 8.0)

It was made up to 1L with distilled water

To make a 0.5X working solution, a 1:10 dilution of the concentrated stock was done.

1% Agarose gel: one gram agarose was dissolved in 100mL TBE

Appendix III

DNA quality confirmation

One percent solution of agarose was prepared by melting 1g of agarose in 100mL of 0.5X TBE buffer in a microwave for approximately 2min. It was allowed to cool for a couple of minutes before adding 2.5µL of ethidium bromide. It was was the stirred to mix.

The gel was casted using the supplied tray and comb. The gel was allowed to set for a minimum of 20min at room temperature on a flat surface.

The following was loaded into separate wells

- 10 µL 1Kb ladder
- 5 µL sample + 5 µL water +2 µL 6X loading buffer

The gel was run for 30min at 110V. Then it was exposed to UV light and photographed. The DNA quality was confirmed. Presence of highly resolved high molecular weight band indicates good quality DNA, presence of smeared band indicates DNA degradation.

Appendix IV

Primers used in the amplification of selected genes in cDNA

cbh1

Forward	5’ GTCATTGACGCCAACTGGCGCTGG 3’
Reverse	5’ ACGCTCCCAGCCCTCAACGTTGG 3’

ace1

Forward	5’ AGCGCGATCCATGCGGTCCATGGCCCGCCG 3’
Reverse	5’ AGCCGGAATTCGTAGCTGGGCGTGGAGGAAG 3’

aep 3

Forward	5’ CTGGCTGTCTTTGCTCTG 3’
Reverse	5’ TCCCACTCTGCTCAAACC 3’

lacc1

Forward	5’ GGTACATCCTAGCACCCAATG 3’
Reverse	5’ GACGAGATCAGTTTCCAAGAGG 3’

lacc5

Forward	5’ CGCATTTGCCGCTTTCTT 3’
Reverse	5’ GGTGACTAGGACTGAGTATCTC 3’

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