Isolation, Purification and Characterization of Cellulase Produced by *Aspergillus niger* Cultured on *Arachis hypogaea* Shells

By

SULYMAN, Abdulhakeem Olarewaju

(05/55EH216)

B.Sc., M.Sc. (Ilorin)

Isolation, Purification and Characterization of Cellulase Produced by *Aspergillus niger* Cultured on *Arachis hypogaea* Shells

By

SULYMAN, Abdulhakeem Olarewaju

(05/55EH216)

B.Sc., M.Sc. (Ilorin)

A Thesis Submitted to the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, in Fulfilment of the Requirement for the Award of Doctor of Philosophy (Ph. D.) Degree in Biochemistry

June, 2018

DECLARATION

I, SULYMAN, Abdulhakeem Olarewaju with matriculation number 05/55EH216, declare that this thesis entitled **"Isolation, purification and characterization of cellulase produced by *Aspergillus niger* cultured on *Arachis hypogaea* shells"** is the result of a study carried out by me under the supervision of Prof. Sylvia O. Malomo in the Department of Biochemistry, University of Ilorin, Nigeria. I confirm that this thesis presents the results of my findings and that its contents are entirely my ideas unless otherwise stated with appropriate referencing. This thesis has neither been submitted to any University nor under consideration for the award of Doctor of Philosophy (Ph.D.) degree in Biochemistry at any other University.

SULYMAN, Abdulhakeem Olarewaju

CERTIFICATION

I certify that this work was carried out by Mr. Sulyman, Abdulhakeem Olarewaju in the Department of Biochemistry, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria, under my supervision.

…… ……..

Prof. Sylvia O. Malomo Date

Supervisor

APPROVAL PAGE

This thesis has been read and approved as having met the requirement of the Department of Biochemistry, Faculty of Life Sciences and Postgraduate School, University of Ilorin, Ilorin, Nigeria, for the award of Doctor of Philosophy (Ph.D.) degree in Biochemistry.

_____ _____

Prof. Sylvia O. Malomo Date

Supervisor

---------- ----------

Prof. M. T. Yakubu Date

Head of Department

_____ _____

Date

External Examiner

DEDICATION

This thesis is dedicated to Almighty Allah, the most Compassionate, and the most Merciful,

His noble Prophet (SAW), the best among mankind,

My father, Alhaji Sulyman Aremu,

My mother, Hajia Memunat AbdulRaheem,

My wife, Abdulhakeem Rukayat Ibrahim,

and

My sons and co-computer operators, AbdulRahman Olamilekan and AbdulMaliq Opeyemi.

ACKNOWLEDGEMENTS

All thanks and praises are due to Almighty Allah, I seek His help and forgiveness, I seek refuge in Allah from the evil within myself and the consequences of my evil deeds. Whoever Allah has guided will never go astray, and whoever Allah has leads astray will never find guidance. I bear witness that there is no God but He, alone without partners, and I bear witness that Prophet Muhammad (SAW) is His messenger.

My sincere appreciation goes to my supervisor, Prof. Sylvia O. Malomo whose contributions and constructive criticism has pushed me to expend the kind of efforts I have exerted to make this work original as it can be. Through her, I have acquired the knowledge and the very best training I may not have gotten from anywhere else. I have learnt the written and communication skills through her. God Almighty will bless, reward her and I wish her long life. Also, I duly acknowledge Dr. Adedoyin Igunnu, he is such a wonderful lecturer and a role model. His untiring efforts at ensuring that I achieved this feat is highly appreciated; may he always find favors in his life.

I appreciate the support of Prof. M. T. Yakubu (Head of Department and Departmental Postgraduate Coordinator), I pray that Almighty Allah will continue to elevate him. Special thanks to Prof. O.B. Oloyede, Prof. Bewaji, Prof. (Mrs.) E. O. Balogun, Prof. (Mrs.) A. T. Oladiji, Dr. R. O. Arise, Dr. M.O. Salawu, Dr. M. O. Nafiu, Dr. (Mrs.) F. A. Sulaiman, Dr. (Mrs.) R. A. Oyegoke, Mrs. H. F. Muritala and Mrs. F. A. Abubakar for their moral support, I am indeed very grateful.

I sincerely thank the entire management and staff of Kwara State University, Malete, under the leadership of the Vice Chancellor and Chief Executive, Prof. AbdulRasheed Na'Allah for granting me permission to undertake a Ph.D programme and also provide an enabling environment to carry out my research work. God bless Kwara State University. I recognized the staff and students of Department of Biochemistry, Kwara State University, Malete, under the stewardship of Dr. E. O. Ajani. I acknowledge the efforts of my students most especially Maliki, Adenike Monsurah, Aliyu Rukayat and Ajadi, Ramat Omowunmi, Almighty Allah will reward them abundantly. I acknowledge the technical support of Mrs. R. O. Amosun, Mrs. D. O. Gabriel and Mrs. A. H. Muhammed of Department of Microbiology, Kwara State University, Malete.

My utmost regards go to my parents, Alhaji Sulyman Aremu and Hajia Memunat AbdulRaheem, who painstakingly laid the good foundation for my education, giving it all it takes. I pray that Almighty Allah grant them long life so that they can reap the fruit of their labour. Also, I appreciate all my siblings, I love them all.

I am indebted to my brother from another mother and a friend Dr. Akolade, Jubril Olayinka (Abu Bilal). If there is someone who loves for his brother what he loves for himself, Dr. Akolade is the person, I love him so much and I pray that Almighty Allah will continue to elevate him and reward him in multiple folds. I appreciate my coursemates, Dr. Adigun Nurudeen Oluwaseun, Dr. Nurain Ismail Olarewaju, Mr. Babalola Nurudeen, Mr. Balogun AbdulAzeez, Mrs. Asiata Na'Allah, Mrs. AbdulAzeez Azima among others and to my colleagues at Kwasu, Dr. Saheed Sabiu, Dr. (Mrs) R. A. Aladodo, Mr. Rasheed Ibrahim whose immeasurable contributions has led to the successful completion of this work. I say jazakummullahu khairan to them all.

Finally, to the best wife in the world, my baby mama, Abdulhakeem Rukayat Ibrahim and my lovely sons (Co-computer operators), AbdulRahman Abdulhakeem Olamilekan and AbdulMaliq Opeyemi Abdulhakeem, their endurance, perseverance, understandings are highly acknowledged.

SULYMAN, Abdulhakeem Olarewaju

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The substantial amounts of waste materials such as shells, tatters, trunks, peels and seeds are produced as a result of agricultural practice. Large quantities of these agrowastes obtained are from heavy consumptions of agricultural products. These wastes particularly groundnut shells are abundant because once the nuts have been removed, the shells are always discarded. Accumulation of these shells constitutes what we called "wastes" and consequently lead to environmental pollution. Transformation of these wastes to expedient products will not only combat environmental pollution arising from unnecessary discarding of shell but also boast the economy of our country. The uses of different agricultural wastes such as corn cob, rice bran, bagasses, wheat bran, banana trunk for production and characterization of cellulase have been studied in the past. Therefore, this study addressed the possible use of *Arachis hypogaea* shells as a substrate for cellulase production from *Aspergillus niger*. The *A. hypogaea* shells were dried and subjected to acid and alkali pretreatment. Proximate analysis was carried out on the treated and untreated substrates. The remains of pretreated substrates were then used as substrates in a shake-flask containing enriched media preparation and *A. niger* was inoculated. Fermentations were carried out in flasks containing the enriched media inoculated with *A. niger* for 168 hours. Optimization parameters for maximum cellulase production were determined by varying the fermentation conditions. The crude cellulase was precipitated at 80% ammonium sulphate saturation, followed by dialysis and gel filtration using Sephadex G-100 as stationary phase in chromatographic column. The molecular weight was estimated using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Cellulase activity was assayed by determining the concentration of glucose produced from the hydrolysis of carboxymethylcellulose catalysed by cellulase. The effects of pH and temperature on the activity of purified cellulase were

determined. The kinetic parameters (K_m and V_{max}) were also evaluated. The effects of some cations (Na^+, K^+, Mg^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}) and anions (CO_3^{2-}, Cl^-, and SO_4^{2-}) on the activity of purified cellulase were investigated. Also, the effects of some surfactants (DMSO, Triton X, Tween 20 and Mercaptoethanol) on the activity of purified cellulase as well as detergent compatibility of purified cellulase were determined. The results obtained from proximate analysis of pretreated *A. hypogaea* shells showed that crude fiber content, alkaline pretreated substrates had the highest percentage of crude fibre with 87.0% compared to acid treated and untreated with 85.5 and 82.0% respectively. There was a significant increase ($p < 0.05$) in the content of crude protein following the treatment of *A. hypogaea* shell with alkali compared to acid treated and untreated shells. The alkaline pretreated substrates had the highest percentage of cellulose with 2.2 folds increase when compared to untreated substrate. Also, the percentage of cellulose in the acid treated substrate was higher with a 1.7-fold increased, compared to untreated *A. hypogaea*. This study also revealed that the: optimal production of crude cellulase was achieved at incubation period of 120 hours, pH of 4, temperature of 40 ^oC, and inoculum size of 13 cfu/ml; enzyme was purified to 68.12 fold with a yield and specific activity of 3.87% and 484.3 U/mg respectively; V_{max} for the cellulase was 9.26 U/ml while the K_m was 0.23 mg/ml; molecular weight of the cellulase was 13.5 kDa; optimum pH and temperature for the cellulase activity were 4 and 40 ^oC respectively; CO_3^{2-}, Cl^- and SO_4^{2-} decreased the activity of cellulase. Na^+ activated the cellulase activity at 1 to 5mM while K^+ did not affect the cellulase activity; Mg^{2+} competitively inhibited cellulase activity while Zn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} non-competitively inhibited the activity of the enzyme. Mn^{2+} and Co^{2+} enhanced the cellulase activity; and cellulase activity was inhibited by dimethyl sulfoxide, triton X, tween 20 and mercaptoethanol. The study concluded that *A. hypogaea* shells can be used as a source of

carbon by *A. niger* for the production of cellulase. Therefore, the purified cellulase produced may be explored for the various industrial applications of the enzyme.

Chapter One

1.0 Introduction

The agricultural wastes are composed mainly of cellulosic or lignocellulosic matter. These materials are considered to be the inexpensive basis for the production of different utilizable products throughout the world (Karmakar and Ray, 2011). Agricultural wastes are form of plant products that are not considered to be useful either because they do not have economic significance or they are plant derived products that are not known for any important purpose (Adeyi, 2010). Large amount of wastes is generated as a result of agricultural practice and industrial processing of agricultural materials, predominantly from industries. These wastes need to be managed and if left untreated, largely amass in the surroundings as environmental pollutants (Abu *et al.,* 2000). Waste management is a worldwide concern which has become progressively provoking with increase in population and growth in a country's economy, especially in developing countries. All the tiers of Nigeria government is confronted by the foremost task of managing waste. Efforts by several organizations to tackle these menaces have yielded little or no result at all. The amount of solid and agricultural wastes produced in various parts of Nigeria depend largely on the population growth, level of development, socio-economic status of the people living in a particular environment as well as the varieties of commercial activities that is predominant in that settlement. Babayemi and Dauda (2009) reported that an

individual generates an average of 0.58Kg solid waste per day. It was projected by Yusuf and Oyewumi (2008) that by year 2020, an individual will generate approximately 0.43Kg in Ilorin, the Kwara State capital. The inability of these wastes to be treated results in environmental pollution. A huge percentage of wastes generated either from wood product industries or as a result of agricultural practices (Lin and Tanaka, 2006) is composed chiefly of lignocellulosic materials which under normal circumstances are indigestible and as a result add no value to animal feed compositions. Efforts must be intensified by individuals, corporate bodies or Government to convert these wastes into useful products.

Presently, the conversion of cellulosic materials to commodity chemicals and fermentable sugars offer important technical and economic challenges, and its accomplishment hang on the development of extremely resourceful and cost-effective enzymes for degradation of pretreated lignocellulosic substrates to fermentable sugars. Naturally, lignocellulosic materials can be fermented by microorganisms which may results in a product whose activities will be very low and inefficient. Therefore, these residual components of lignocellulosic materials can serve as superior substrates for the growth of microorganisms that may produce enzymes such as lipase, glucoamylase, pectinase, xylanase and cellulase either through submerged fermentation or solid-state fermentation. Generally, microorganisms of the genera *Trichoderma* and *Aspergillus* are understood to have cellulase synthetic ability, and enzymes synthesized by this class of organisms are readily available for industries (Sukumaran *et al*., 2005; Kuhad *et al*., 2010).

Cellulases are a conglomerate of enzymes that hydrolyze cellulose and oligosaccharide derivatives that relate to cellulose. The three major components of cellulase are endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and *β*-glucosidase (EC 3.2.1.21) (Kaur *et al.,* 2007; Thongekkaew *et al.,* 2008). Cellulases as enzyme can be got from different sources. Cellulases

from different sources possess distinctive features since they exhibit specific pH optima, solubility depending on the amino acid composition. The stability of cellulase to heat and the ability of cellulase to recognize different substrate may also vary with the origin (Bhat, 2000; Parry *et al.,* 2001). The importance of cellulase to industries are too numerous to mention. Cellulases are used in the production of beers, production of animal feed. The major limitation in the use of cellulases in industry is the exorbitant production cost. The cost of cellulase production has been considered to be important to the success of bioethanol industry. Accordingly, a highly stable, efficient and also economic cellulase or hemicellulase for the biofuel industry has generated interest among scientists. Therefore, there is need to find a very cheap and eco-friendly alternative for cellulase production. This research work addressed the use of agrowastes especially *Arachis hypogaea* shells for cellulase production.

1.1 Justification

Groundnut is produced throughout the world with Brazil, China, India, Nigeria, USA, Indonesia and Sudan as major producers. Nigeria accounts for about 96% of the worldwide groundnut area and production of about 92% (Taru *et al*., 2010). It was estimated in 1999 by Okolo and Utoh that approximately 3 million hectares of land in Nigeria are being used for groundnut plant production and between 500 – 3000 kg/ha of groundnut are being produced. Despite the amount of this groundnut yield, the shells have not been put into use as they are always discarded once the nuts have been removed. These shells can be employed for cellulase production when they are used as a substrate for the growth of microorganisms. Also, cellulases have wide range of applications in biotechnology and industry (Ito, 1997; Azevedo *et al.,* 2000; Caf and Arikan, 2017). Inaddition, this enzyme can be used to hydrolyze renewable cellulosic biomass to commodity chemicals that are very useful (Himmel *et al.,* 1999; Ha *et al.,* 2011). Due

to growing anxiety for the greenhouse effect, diminishing oil reserves and rising oil prices, as well as interest in renewable fuels, such as bioethanol, cellulases are increasingly becoming important for contributing to the green environment through conversion to biofuel. Therefore, countless energies have been made to convert various cellulosic biomasses into fermentable sugar using cellulases for further conversion to ethanol, which is the most common renewable fuel so far (Sánchez and Montoya, 2013). This work seeks to use submerged fermentation as a simpler and less sophisticated method of converting lignocelluloses of *A. hypogaea* shells to produce cellulase using *A. niger*. Analyses of the optimization parameters may lead to maximum production of cellulase enzymes. Finally, more value will be added to groundnut plant production when the shells are exploited in cellulase production. Hence, this research intends to look into the possibility of isolating and purifying cellulase from *A. niger* cultured on groundnut shell and characterize it for possible commercial utilizations.

1.2 Objective of the Study

The overall objective of this study was to isolate, purify and characterize cellulase produced by *A. niger* using *A. hypogaea* shells.

1.3 Specific Objectives

The specific objectives of this study include:

i. isolation and purification of cellulase from *A. niger* cultured on *A. hypogaea* shells;
ii. estimation of the molecular weight of the purified cellulase;
iii. determination of kinetic parameters of cellulase using carboxymethylcellulose as substrates;
iv. studying the effect of pH and temperature on the activity of the purified cellulase;

v. determination of the influence of some cations (Na^{+}, K^{+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Co^{2+}) and anions (CO_3^{2-}, Cl^{-}, and SO_4^{2-}) on the activity of the purified cellulase;

vi. investigation of the effect of some surfactants (Dimethyl sulphuroxide (DMSO), Triton X, Tween 20 and Mercaptoethanol) on the activity of purified cellulase; and

vii. determination of the detergent compatibility of the purified cellulase.

Chapter Two

2.0 Literature Review

2.1 Distribution of Cellulase

Cellulases are enzymes that are produced by different microorganisms including fungi and bacteria during their growth on cellulosic materials (Kubicek, 1993; Koo, 2001). The microorganisms of the genera *Clostridium, Cellulomonas, Thermomonospora, Trichoderma,* and *Aspergillus* have been documented to produce large amount of cellulase (Kuhad *et al*., 1999; Sun and Cheng, 2002; Sukumaran *et al*., 2005; Kuhad *et al*., 2010). Cellulases produced by fungi are simpler in structure compared to those produced by bacteria (Bayer *et al*., 1994, Bayer *et al*., 1998; Zhang *et al*., 2006). In fungi cellulase, there are two domains. These are catalytic domain (CD) and a cellulose binding module (CBM). These two domains are linked together by a short polylinker at the N-terminal. The CBM is made up of approximately 35 amino acids, and the linker region is composed of serine and threonine in abundance.

2.2 Cellulase Enzyme Complex

Cellulase is a conglomerate of enzymes that comprises three major components: Endo-ß-glucanase (EC 3.2.1.4), Exo-ß-glucanase (EC 3.2.1.91) and *β*-glucosidase (EC 3.2.1.21) (Kaur *et al.,* 2007; Thongekkaew *et al.,* 2008). Endoglucanase breaks internal β-1,4-glycosidic bonds in cellulose and as a result, the polymer swiftly reduces in length, but the amount of the reducing sugar increases steadily (Robson and Chamblish, 1989). Exoglucanase hydrolyse cellulose by

removing the cellobiose unit from the non-reducing end of cellulose; the reducing sugars are rapidly increased, but there is little change in polymer. β-glucosidase hydrolyze cellobiose and oligosaccharides to liberate glucose. Cellulases can be obtained from different sources and as a result have distinct features as they exhibit different optimum conditions such as pH optima, and solubility. The solubility depends on amino acid compositions. The stability of the cellulase to heat and precise requirement for each substrate may also vary with the origin (Bhat, 2000; Parry *et al.*, 2001). Cellulases have been found applicable in different industries (Kirk *et al.*, 2002; Cherry and Fidantsef, 2003) but the major obstacle against the industrial uses of cellulase is the high cost of cellulase production. Presently, most available cellulases for commercial purposes are produced mainly by microorganisms of the genera *Trichoderma* and *Aspergillus* species (Kirk *et al.*, 2002).

2.2.1 Endoglucanase

Endoglucanase catalyzes the cleavage of internal β-1,4-glycosidic bonds in cellulose and this leads to a decrease in the length of polymer with an upsurge of the reducing sugar produced (Robson and Chamblish, 1989). The endoglucanase obtained from *Thermoascus aurantiacus* is an example of endoglucanase (Figure 1). After post-translational modification, it was revealed that endoglucanase from *T. aurantiacus* contains 335 amino acid long peptide chain molds into a functional enzyme which has eight-fold (β/α) barrel architecture (Juturu and Wu, 2014). The substrate binding site is shallow and long, ranging from subsites -4 to -3. This substrate binding site always stays in an active conformation. (Leggio and Larsen, 2002).

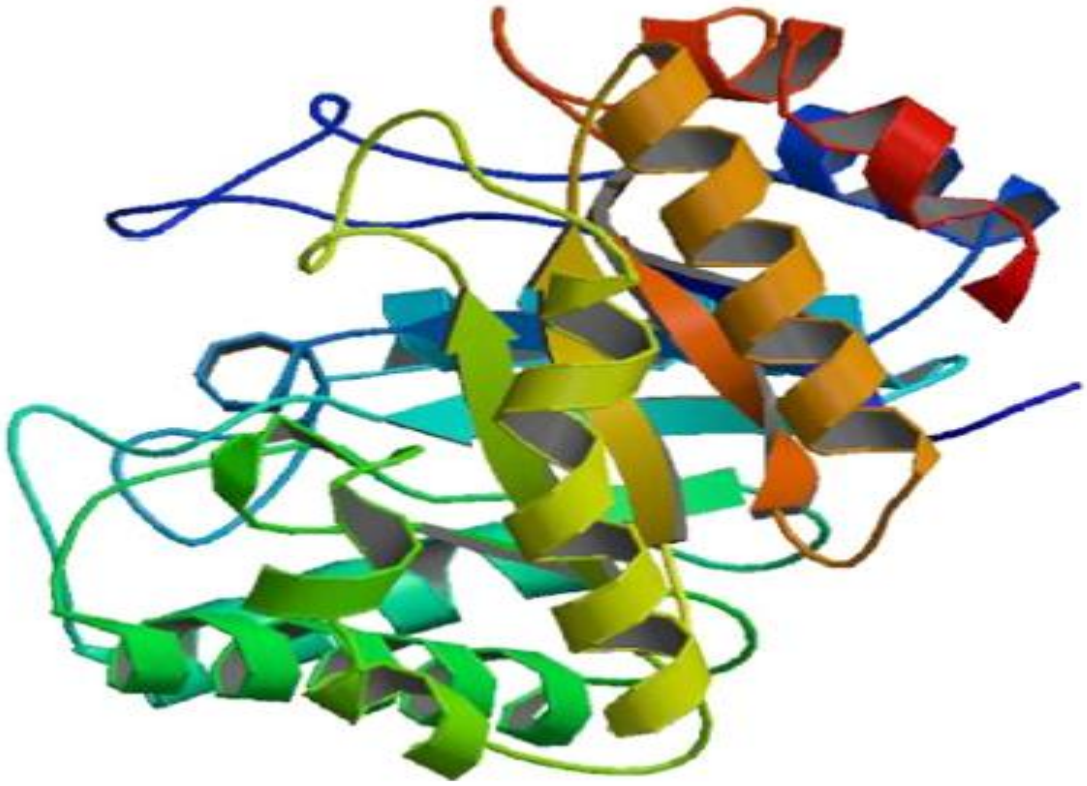


Figure 1: Crystal structure of *T. aurantiacus* endoglucanase (PDBID:1GJH) showing eight-fold (β/α) 8-barrel architecture which is regular and compact with short loops.

Source: Leggio and Larsen (2002)

2.2.2 Exoglucanase

Exoglucanases, a component of cellulase cleaves both the reducing or non-reducing ends of cellulose polysaccharide chains, releasing either glucose or cellobiose as main products (Saranraj *et al*., 2012). The exoglucanase obtained from *P. chrysosporium* is an example of exoglucanase (Figure 2). The exoglucanase from *P. chrysosporium* has a β-jelly roll structure formed by two large antiparallel β-sheets packing one over other. These enzymes are specific for crystalline substrates like Avicel, amorphous celluloses and cellooligosaccharides. Exoglucanase have been shown to create a substrate-binding tunnel with their extended loops which surround the cellulose (Gilbert, 2014; Moroz *et al.,* 2015). The pH of exoglucanase is usually between 4.0 and 5.0 while the optimum temperature is usually from 37 to 60 °C. The actions of exoglucanase from non-reducing and reducing end of cellulosic chains have been reported in several studies. For example, exoglucanase from *T. reesei* has two exoglucanases that act from non-reducing and reducing end (Sandgren *et al.,* 2005; Baldrian and Valaskova, 2008; Hobdey *et al.,* 2016).

2.2.3 β-glucosidase

β-glucosidase breaks down cellobiose and oligosaccharides to β-glucose (Gottschalk *et al*., 2010). Generally, *Trichoderma* and *Aspergillus* have been reported to produce β-glucosidase (Gottschalk *et al*., 2010). β-glucosidases breaks down soluble cellobiose and cellodextrins to glucose and the enzyme is inhibited by glucose. β-glucosidases belongs to families 1 and 3 of glucoside hydrolases. They are so placed because of their amino acid sequence. (Levasseur *et al.,* 2013). β-glucosidases produced from fungi and bacteria and plants belongs to family 3 while those produced from bacterial, plant and mammal belong to family 1. Family1 β-glucosidases contain galactosidase activity in addition to β-glucosidase activity. Irrespective of the family that β-glucosidase belongs, they both decompose β-1,4-glycosidic bonds using the retaining

mechanism (Dan *et al.,* 2000). β-glucosidase show the most inconsistency among the cellulolytic enzymes due to their structure and localization. For example, the molecular structure of β-glucosidase from *Pleurotus ostreatus* is monomeric with approximately 35 kDa molecular mass (Dashtban *et al.,* 2009), β-glucosidase produced from *Sporobolomyces singularis* are dimeric with 146 kDa) (Ishikawa *et al.,* 2005) while β-glucosidase from *Pisolithus tinctorius*) is trimeric with a molecular weight of 450 kDa (Cao *et al.,* 1993).

2.3 Structure of Cellulase

The cellulases obtained from fungi normally possess a very simple architecture and usually contained a catalytic domain (CD) and a cellulose binding domain (CBD). Linker peptide connect CD and CBD. CBD is not involved in hydrolysis but its removal significantly reduces the enzyme activity towards the substrate (Bayer *et al*., 1998).

Figure 2: Structure of *P. chrysosporium* exoglucanase (PDBID:1GPI). The protein has a β-jelly roll structure formed by two large antiparallel β-sheets packing one over other.

Source: Muñoz *et al*. (2001)

2.4 Substrate Specificity of Cellulase

Cellulase can act on several cellulose containing materials and hydrolyze them to yield glucose. Examples include cellulose, hemicellulose, lignins, xylans e.t.c. The extent of hydrolysis of each of the materials depends on the affinity and specificity that cellulase have for each substrate.

2.4.1 Cellulose

Cellulose is the most renewable organic polymer found in the universe, and it represents about 1.5×10^{12} tons of total wastes generated from agricultural derived products annually, through photosynthesis especially in the tropics (Kamiya *et al*., 2008). It is the most copious and renewable biopolymer on earth and the leading solid waste derived from agriculture (Bhat and Bhat, 1997). A favorable approach for proficient exploitation of this renewable resource is the microbial hydrolysis of lignocellulosic wastes and fermentation of the resultant reducing sugars for production of useful metabolites or biofuel. Cellulose is a crystalline polymer, an uncommon property among biopolymers. Cellulose chains in the crystals are thickened by inter and intra chain hydrogen bonds and the adjacent sheets which superimpose one another, are held together by weak bonds. In a normal flora, cellulose is present in a nearly pure state in a few instances whereas in most cases, the cellulose fibers are entrenched in a matrix of other polymers and lignin. The advantage of crystallinity is the relative impenetrability of not only higher molecular weight molecules like enzymes but also in some cases of small molecules like water. There are crystalline and amorphous regions, in the polymeric structure and in addition there exists several types of surface irregularities (Chang and Holtzapple, 2000; Taherzadeh and Karimi, 2008). The crystalline and amorphous nature of fibers makes it possible for enzymes and other large molecules to penetrate it. At the molecular level, cellulose is a linear polymer of glucose joining

together in a β (1 4) linkage. There is variation in the number of glucose units present in the cellulose molecules and degree of polymerization ranges from 250 to well over 10, 000. (Klemm *et al.*, 2005). The uses of enzyme to hydrolyze cellulosic biomass depends on the nature of cellulosic substrates and its physical state. Though, lignocellulosic biomass is usually obstinate to microbial action, suitable pretreatments result in the removal of lignin content and increase enzyme accessibility. (Lynd *et al.*, 2002). Microbial conversion of lignocellulosic waste to useful products can be achieved by a combined action of numerous enzymes, the most prominent of which are the cellulases. These cellulolytic microbes can hydrolyze cellulose into fermentable sugars either by acid or enzymatic hydrolysis. Thus, microbes can secrete cellulase when cultured on cellulose containing materials as substrates.

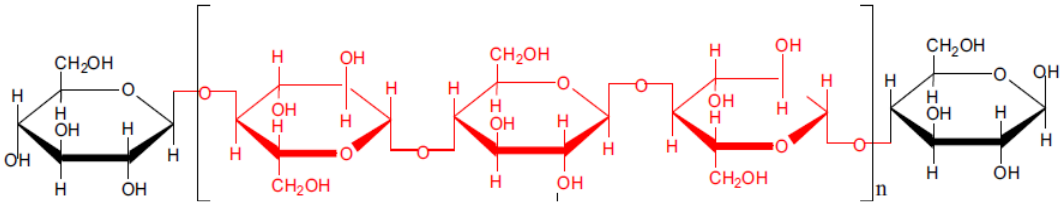
2.4.2 Hemicellulose

Hemicelluloses are a diverse class of polymers accounting for about 15–35% of plant biomass and the composition may be pentoses (b-D-xylose, a-L-arabinose), hexoses (b-D-mannose, b-D-glucose, a-D-galactose) and /or uronic acids (a-D-glucuronic, a-D-4-O-methylgalacturonic and a-D-galacturonic acids) (Gírio *et al*., 2010). Other sugars that can also be found in minute quantity are L-rhamnose and α-L-fucose. The hydroxyl groups of such sugars can be replaced with acetyl groups (Gírio *et al*., 2010). Xylans and glucomannas are the most important hemicellulose with xylans being the most abundant of the two. Xylans are core component of hemicellulose and are comprising about 20–30% of the biomass of hardwoods and herbaceous plants (Gírio *et al*., 2010). Xylans account for about 50 % in some tissues of grasses and cereals (Ebringerova *et al*., 2005). Xylans are available in large quantities and it is one of the chief products of agriculture. Contrary to the secondary by-products of hardwoods,

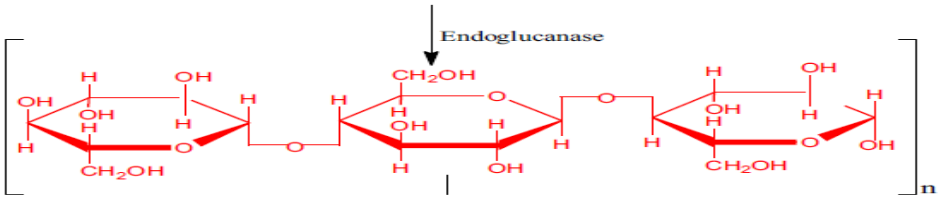
hemicellulose containing mannans like glucomannans and galactoglucomannans are the main hemicellulosic components of the secondary wall of softwoods (Gírio *et al*., 2010).

2.5 Mechanism of Cellulolysis by Cellulase

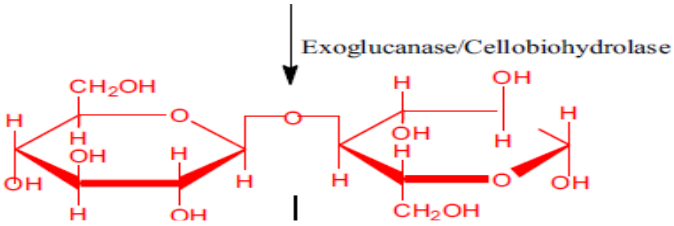
Cellulosic enzymes act in a synergistic manner and consist of three major components: endoglucanase, exoglucanase and ß-glucosidase. The manner in which these three enzymes hydrolyze cellulose or related cellu-oligosaccharide derivatives are as follow: (i) Endoglucanase, randomly cuts cellulose chains leading to the production of glucose and cello-oligosaccharides. (ii) Exoglucanase, on the other hand attacks on the non-reducing end of cellulase producing primarily cellobiose and (iii) ß-glucosidase, converts cellobiose to ß-glucose (Lynd *et al.,* 2002) (Figure 3). Endoglucanase acts randomly on the inner cellulose chain, while exoglucanase acts on outer chains ends by splitting off cellobiose or glucose (Saranraj *et al*., 2012). Cellobiose is subsequently acted upon by ß-glucosidase to glucose. The enzymatic breakdown of the β-1, 4-glucosidic bonds in cellulose involves acid hydrolysis mechanism, using a proton donor and nucleophile or base (Lynd *et al.,* 2002).



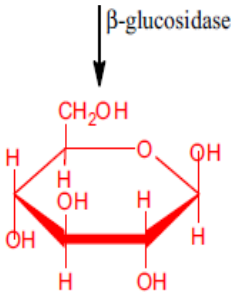
Cellulose



Cello-oligosaccharide



Cellobiose



β-glucose

Figure 3: Mechanism of action of cellulase complex

Source: Juturu and Wu (2014)

2.6 Cellulase Production

Cellulases are produced majorly by microorganisms when cultured on carbon-containing substrates. These microorganisms degrade cellulosic materials and release the enzyme into the solution. The production of cellulase from bacteria and fungi have been documented (Immanual *et al*., 2006). However, filamentous fungi are more preferable to bacteria for commercial enzyme production, because the enzymes produced by these cultures are generally regarded as harmless (Bakri *et al*., 2003).

2.6.1 Cellulase Producing Microbes

Microorganism that produce cellulase are primarily degrade carbohydrate and are unable to utilize either proteins or lipids as sources for growth (Lynd *et al.,* 2002). Cellulolytic microbes particularly the bacteria *Cellulomonas* and *Cytophage* and most fungi can make use of a diversity of carbohydrates in addition to cellulose (Poulsen and Petersen, 1988; Sambusiti *et al.,* 2014), while the anaerobic cellulolytic microbes are only constrained to cellulose and cellobioses (Parisutham *et al.,* 2017). The capacity to secrete enormous amounts of enzymes is typical of certain fungi and such strains are most appropriate for production of higher levels of extracellular cellulases. Some of the best studied fungi are *T. reesei* and *Aspergillus niger* which decompose cellulose to beta glucose. Although lots of fungi can use cellulose as carbon source not all are able to secrete a complex of cellulase enzymes, which could be used for the hydrolysis of lignocellulose wastes. Apart from *T. reesie,* other notable fungi species that can secrete cellulase enzyme are *Humicola, Penicillium* and *Aspergillu*s (Hayashida *et al.,* 1988; Chaabouni *et al.,* 1995; Ong *et al.*, 2004; Festersen *et al.,* 2016). As the microorganisms such as *Aspergillus niger* break down the nutrients, they release the desired enzymes and other products into solution.

2.6.1.1 *Aspergillus niger*

The genus *Aspergillus* are filamentous fungi which are abundant and of great significance in medical and industrial microbiology. They are made up of different species and strains such as *Aspergillus niger* that secrete large quantities of cellulolytic enzymes. Production of cellulolytic enzymes using *Aspergillus* and *Trichoderma* have been reported (Coral *et al.,* 2002; Acharya *et al.,* 2008; Omojasola *et al.,* 2008; Sridevi *et al.,* 2009). Other *Aspergillus* species that have been reported to be cellulase producers include *A. terreus* (Garg and Neelakantan, 1982; Vyas *et al.,* 2005; Shahriarinour *et al.,* 2011), *A. flavus* (Ojumu *et al.,* 2003), and *A. fumigatus* (Soni *et al.,* 2010).

2.6.1.2 Taxonomy of *A. niger*

As can be observed in many fungi, the nomenclature of *Aspergillus* is predominantly centered on morphological characteristics, rather than the physiological and biochemical features. Genetic features are frequently used to classify bacteria. The use of morphological approach to identify *Aspergillus* had resulted to the presence of numerous synonyms for the genus *Aspergillus*. These include: *Streptomyces corda; Spermatoloncha spegazzini; Sphaeromyces montagne; Sterigmatocystis cramer;* and *Stilbothamnium hennings* (Baker and Bennett, 2007).

2.6.1.3 Pathogenicity of *A. niger*

A. niger causes black mold disease in onions and some ornamental plants. Infection of onion seedlings by *A. niger* can become general and can only spread under a favorable condition. The fungus also causes disease in peanuts and in grapes.

2.6.1.4 Human Diseases Caused by *A. niger*

A, niger is not likely to cause any disease to human when compared with other species of *Aspergillus*. In exceptionally rare instances, humans may fall sick, but this is due to a severe lung disease called aspergillosis that can occur. Aspergillosis is in particular, common among horticultural workers that gulp peat dust, which can be rich in *Aspergillus* spores (Kulshrestha, 2010). Aspergillosis is the term used to describe the growth of *Aspergillus* within air-containing space of the body of humans (Herbrecht *et al.,* 2002). Patients suffering from aspergillosis are usually immunocompromised, and thus vulnerable to otherwise common and usually harmless microorganisms. Immunosuppression of the patients suffering from aspergillosis may be due to factors like debilitating disease, chemotherapy, and the use of supraphysiological doses of adrenal corticosteroids (Bennett, 1985). Cough that is chronic and hemoptysis are the most common symptoms of pulmonary aspergillosis. Colonization is generally a consequence of a prolonged inflammatory process, such as tuberculosis, bronchiectasis, histoplasmosis, or sarcoidosis. The fungus does not attack the wall of the cavity, cyst, or bronchus in such patients" (Herbrecht *et al.,* 2002). It is still not clear the major role played by *Aspergillus* in non-invasive lung disease. Plugs of hyphae may block bronchi; maybe allergic or toxic reactions to *Aspergillus* antigens could cause bronchial constriction and damage (Bennett, 1980). Both the sternness of aspergillosis and the patient's diagnosis are reliant on the physiologic status of the patient. In a case of non-severity, intravenous amphotericin B has been employed to arrest or

cure of invasive aspergillosis especially when immunosuppression is not severe (Saddiq, 2014). Pleural aspergillosis often responds well to surgical drainage alone (Dreizen *et al.,* 1985).

2.6.1.5 Application of *A. niger* in Enzyme Production

Virtually all fungi of genus *Aspergillus* has a potential to produce enzymes especially cellulase, therefore this genus has the prospective to dictate the enzyme industry. *Aspergillus* and *Trichoderma* spp. have been studied for their proficient production of cellulases (van Peij *et al.,* 1998). Most of the enzymes that have been used in industries have been produced through submerged fermentation (SmF) because it is very easy to handle. However, solid state fermentation (SSF) technique can be preferred to submerged fermentation, because it is cost effective and more yield can be gotten (Ghildyal *et al.,* 1985; Hui *et al.,* 2010). The cellulase production by fungus using SmF and SSF has been documented (Fadel, 2000; Acharya *et al.,* 2008). Numerous researchers have reported the use of agro industrial wastes for the production of cellulase such as wheat straw, wheat bran and rice straw as substrates (Goyal *et al.,* 2008; Alegre *et al.,* 2009; Singhania *et al.,* 2010; Mrudula and Murugammal, 2011).

2.6.2 Substrates for Cellulase Production

2.6.2.1 Carboxymethyl Cellulose

Carboxymethyl cellulose (CMC) is a cellulose derivative with carboxymethyl group (-CH_2-COOH) bound to some of the hydroxyl groups of the glucopyranose monomers that make up the cellulose backbone. CMC has also been used widely to depict enzyme activity of cellulase especially endoglucanases which is part of the cellulase complex. CMC is an ideal substrate for endo-acting cellulases, as its structure has been contrived to decrystallize cellulose and create amorphous sites that are ideal for endoglucanase action. CMC is desirable because the catalytic product (glucose) can easily be measured using a reducing sugar assay, such as 3,5-dinitrosalicylic acid (Silveira *et al*., 2014).

2.6.3 Agricultural Wastes

Large amount of wastes is generated from agricultural practice and this results in environmental imbalance. Agricultural wastes or simply called agrowastes are any type of wastes that are derived from agricultural practice and are considered as non-competitive with food crops. Examples of such wastes include forage, wood, weed, forest residues, etc. These wastes when left unattended to, constitute a hefty danger to the life of humans and animals at large. In Nigeria for instance, the percentage of wastes production due to agricultural practice is very high and most of these wastes are not being put to use but rather discarded or later burnt as an alternative disposal method to get rid of them (Adeyi, 2010). Majority of food crops that were consumed in Nigeria and the world at large generate wastes in one way or the other. Such food crops include cashew, orange, yam, cassava, millet, banana, pineapple, wheat, rice and groundnut, just to mention a few (Usman and Said, 2012). It is the waste of these food crops

produced as peels, chaff, brans, cobs, pods or shells that constitute wastes that eventually cause environmental pollution.

2.6.4 *Arachis hypogaea*

The peanut, which also is referred to as groundnut (USDA, 2016) and scientifically referred as *A. hypogaea*, is a legume crop cultivated mostly because of its edible seeds. It is widely produced in the tropics and subtropics, being significant to subsistence and commercial agriculture. Peanut is an oil crop because the lipid content is very high. It was projected in 2014 that the annual production of shelled peanuts was 42 million tons (Abdul-Rahim, 2014). Unlike other crops, peanut pods ripen underground instead of above ground as in the case of other crops. It is based on this underground development that made botanist Linnaeus to assign the specific name *hypogaea*, which means "under the earth" (Abdul-Rahim, 2014). As a legume, the peanut belongs to the family fabaceae; and also called leguminoseae. Legumes have a characteristic of harboring symbiotic nitrogen-fixing bacteria in their root nodules and peanuts are not an exception. This nitrogen fixing ability of peanuts makes them require little or no fertilizer for germination. The scientific meaning of a "nut" is a fruit whose ovary wall becomes very hard at maturity. Therefore, peanut is not actually a true nut, but rather a legume. However, for culinary purposes and in common English language usage, peanuts are normally referred to as nuts (Abdul-Rahim, 2014).

2.6.4.1 Botany of *A. hypogaea*

Peanut is herbaceous crop that is produced yearly. As a legume, it belongs to the family Fabaceae. It is about 30 to 50 cm (1.0 to 1.6 ft) tall. The leaves of peanuts are two opposite pairs with no terminal leaflet; each leaflet is 1 to 7 cm (⅜ to 2¾ in) in length and 1 to

3 cm (⅜ to 1 in) wide. Like many other legumes, the leaves are nyctinastic, that is, they have "sleep" movements, closing at night. Peanut pods have a unique feature called geocarpy (Kamboj, 2000). The flowers are 1.0 to 1.5 cm (0.4 to 0.6 in) across, and yellowish orange with reddish veining (Krapovickas *et al.*, 1994; 2007). They are borne in axillary clusters on the stems above ground and last for just one day. The ovary is found at the base of what appears to be the flower stem but is actually a highly elongated floral cup. After fertilization, a short stalk at the base of the ovary (termed a pedicel) increased in length and lead to the formation of a "peg". This thread-like structure grows down into the soil, and the tip, which contains the ovary, develops into a mature peanut pod (Smith, 1950). The pods are 3 to 7 cm (1.2 to 2.8 in) long, normally harbor between one to four seeds (Krapovickas *et al.*, 1994; 2007).

2.6.4.2 Cultivation of *A. hypogaea*

Groundnut germinates best in light, sandy loamy soil with approximate pH of 5.9–7. Peanuts are nodulated and they do not require fertilizer for germination (Dotray *et al.*, 2012). Therefore, they are very important in crop rotations. The crop rotation of peanut increases its percentage yield, reduce diseases, pests and weeds. Adequate levels of macronutrients are required for high production of peanuts (Dotray *et al.*, 2012). Groundnut is produced in nearly 100 countries globally and the leading countries in its productions are Brazil, China, India, Nigeria, USA, Indonesia and Sudan. In 2014, about 43.9 million tons of groundnuts were produced with China having about 38% of the global total production followed by India (15%). Food and Agricultural Organization reported in 2013 that, India exported 32% of world total exports and United States exported 19% of total exports of groundnut. The percentage of groundnut exported by European Countries 52% with Netherland alone accounting for 40%.

2.6.4.3 Nutritional Values of *A. hypogaea*

Groundnut is very rich in essential nutrients and provide about 570 calories. It is an excellent source of many B vitamins, vitamin E, dietary minerals like manganese (95% DV), magnesium (52% DV) and phosphorus (48%), and dietary fiber. Researchers have reported that consumption of groundnut regularly, reduces the risk of mortality especially from certain diseases (Bao *et al.*, 2013). According to the US Food and Drug Administration, "Scientific confirmation proposes but does not prove that consumption of 1.5 ounces of peanuts per day of may reduce the risk of heart disease (Bao *et al.*, 2013)".

2.6.4.4 Applications of *A. hypogaea*

Groundnuts have a wide range of applications in industries. Paint and varnish industry, lubricating oil, leather dressings, furniture polishing, insecticides production, and nitroglycerin industries utilize groundnut oil for manufacture of various products. Soap is produced from saponification of oil. Also, groundnut oils can be found in many cosmetics. Groundnut shells are used in the production of plastic, wallboard, abrasives, biofuel, cellulose (used in rayon and paper), and mucilage (glue). Groundnut shells can also serve as inducer for cellulase production. Like other agrowastes, groundnut shells need to be pretreated before it can be used as substrates for cellulase production. This is necessary to make the groundnut shells amenable to enzyme hydrolysis.

2.6.5 Pretreatment of Substrates for Cellulase Production

Prior to the application of cellulose suspension with higher efficacy, many approaches have been suggested to alter cellulose in both product quality and process control. Pretreatment of cellulose enabled the fragmentation of cellulose from wood fiber pulp, effectually resulting in increased growth in water. The role of pretreatment was vehemently considered for cellulose reform due to consumption of little or no energy. It is imperative to note that mechanical isolation process for cellulose required high energy consumption (Chinga-Carrasco, 2011).

Different categories of pretreatment of substrates are known. These are: (i) physical pretreatment, which involves milling and grinding, (ii) physicochemical pretreatment that involves steam pretreatment/autohydrolysis, hydrothermolysis, and wet oxidation, (iii) chemical pretreatment that involves alkali, dilute acid, oxidizing agents as well as organic solvents, and biological and electrical. The following pretreatment techniques have been shown to be economical for the conversion of cellulosic wastes to fermentable sugars (Kumar *et al.*, 2009).

2.6.5.1 Mechanical Pretreament

Pretreatment of lignocellulosic materials through a combination of chipping, crushing, and/or milling can be used to make cellulose amenable to enzyme hydrolysis. The size of the cellulosic materials varies between 10-30 mm after chipping and 0.2-2 mm after crushing (Sun and Cheng, 2002). Ball milling is more preferable to ordinary milling for reduction of crystallinity of cellulose and this improves the digestibility of cellulose (Gunaseelan, 2016). The energy required for size reduction of agricultural wastes as a function of final particle size was quantified by Adekunle *et al*. (2016). The irradiation of cellulose by X-ray has been tested (Takacs *et al.*, 2000). This method has been found to be too costly for use in large-scale process (Galbe and Zacchi, 2007).

2.6.5.2 Physicochemical Pretreatment

The most frequently used method for the pretreatment of cellulosic biomass is steam explosion (McMillan *et al.,* 1994). High-pressure saturated steam is normally used for the hydrolysis of wastes, and then the pressure is abruptly lowered, which makes the materials undergo an explosive decompression. Steam explosion is usually carried out at a high temperature (160-260 °C) and left to stand for a while before being exposed to atmospheric pressure (Sun and Cheng, 2002). The process converts hemicellulose and transforms lignin, thus increasing the potential of cellulose degradation. It has been reported that when steam explosion method of wastes pretreatment was used for popular chip, 90% efficiency of hydrolysis was achieved in 24 h (Chen *et al.,* 2014). Treatment of hemicelluloses with steam explosio exposes the cellulose surface and increases enzyme availability to the cellulose microfibrils (Kabel *et al.,* 2007). Lignin is not totally removed during the pretreatment of wastes and as a result redistributed because of melting and depolymerization/repolymerization reactions (Li *et al.,* 2007). Addition of H_2SO_4 (or SO_2) or CO_2 in steam explosion can save time and lowers the temperature, effectively improve hydrolysis, decrease the production of inhibitory compounds (Ballesteros *et al.,* 2006; Olsen *et al.,* 2015). The factors that hinder the effective utilization of steam-explosion pretreatment are time, temperature, material size, and moisture content (Agbor *et al.,* 2011; Brosse *et al.,* 2017). The advantages of steam-explosion pretreatment over the mechanical method include requirement of little amount energy as well as little or no environmental costs. The steam explosion pretreatment has been verified comprehensively for a variety of cellulosic biomass (Brosse *et al.,* 2017).

2.6.5.3 Chemical Pretreatment

Pretreatment of lignocellulosic materials with organic chemicals involves the uses of concentrated acid or alkali.

2.6.5.3.1 Acid Pretreatment

Concentrated acids such as H_2SO_4, HCl or HNO_3 have been employed in treating cellulosic biomass. Pretreatment of agrowastes with acid can result in substrates being amenable to enzyme hydrolysis to release fermentable sugars. After pretreating lignocellulosic materials, the acid must be recovered in order to make the process cost effective (Sun and Cheng, 2002; Anwar *et al.,* 2014). Pretreatment of agrowastes with dilute acid has been successfully carried out. Dilute H_2SO_4 has been employed during the production of furfural from lignocellulosic materials (Antal *et al.,* 1991; Zeitsch, 2000). The pretreatment of cellulosic materials with dilute H_2SO_4 leads to high reaction rates and considerably improved cellulose hydrolysis (Greenwood *et al.,* 2015). Cellulosic material pretreatment with acid requires high temperature (McMillan *et al.,* 1994). Recently, acid pretreatment has been employed on a variety of feedstocks and agricultural residues. The uncertainty on whether porosity affects enzymatic digestibility of lignocellulose following pretreatment with acid has been reported (Ishizawa *et al.,* 2007). It is very important to carefully select the type of acid pretreatment to be used for cellulosic materials. This is because materials subjected to acid pretreatment are always very difficult to ferment because of the presence of toxic substances, such as ethanol and lactic acid (Galbe and Zacchi, 2007).

2.6.5.3.2 Alkaline Hydrolysis

Some alkalis can be employed in the pretreatment of lignocellulosic materials, and their effect is governed by the lignin content of the material (McMillan *et al.,* 1994). Alkali pretreatment processes exploit reduced temperatures and pressures compared to other pretreatment approaches (Mosier *et al.,* 2005). Alkali pretreatment can be carried out easily, but the time of pretreatment times are in hours rather than minutes or seconds. Alkaline processes result in less sugar degradation when compared with acid processes. Sodium, potassium, calcium, and ammonium hydroxides are suitable alkaline pretreatment agents. Of these four, sodium hydroxide has been studied the most (Mamo *et al.,* 2013; Kataria and Ghosh, 2014; Ding *et al.,* 2016; Zhang *et al.,* 2016). However, calcium hydroxide (slake lime) has been shown to be an effective pretreatment agent and is the least expensive.

2.6.6 Cellulase Purification

Cellulase can be purified by applying different techniques of enzyme purification. Some of these techniques include ammonium sulphate precipitation (salting out), dialysis, gel-filtration chromatography, etc (Scopes, 2013).

2.6.6.1 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation is the most used methods for large scale protein purification and fractionation based on how soluble the proteins are in the presence of a high salt concentration. Ammonium sulphate is an inorganic salt which is very soluble in water and decomposes into ammonium (NH_4^+) and sulphate (SO_4^{2-}) in aqueous environments. Ammonium sulphate is widely used as a precipitating agent because it can easily be dissolved in water and readily available with a low density. The concentration of ammonium sulphate salt determines

the solubility of proteins. At very low ion concentration (<0.5 M), the solubility of proteins directly proportional to salt concentration, an effect termed "salting in". Addition of more salt will result in decrease in solubility of protein. When the ionic strength of the salt is high enough, the protein will precipitate out of the solution, a process called "salting out" (Lorsch, 2014).

The ionic strength of the salt determines whether solubility of the protein will be high or low, therefore, "salting out" is an important procedure to in the isolation and purification of a desired protein. Ammonium sulphate is frequently used for precipitation processes because of its solubility in water. The ammonium sulphate solubility performance for a protein is generally expressed as a function of the percentage of saturation. A solubility curve can be prepared by calculating the log of the practically determined solubility, expressed as mg/mL and plotted against percentage saturation of ammonium sulphate (Burgess and Deutscher, 2009). This is normally carried out by progressive addition of solid ammonium sulphate. Though, knowing the exact amount of ammonium sulphate to be added to a solution in order to achieve the desired concentration may not be easy because the addition of more ammonium sulphate progressively increases the volume of the solution. The protein contents in each of the precipitated proteins can be determined by using a standard method of assaying for proteins. The quantity of ammonium sulphate concentration present in a solution must increase so as to be able to remove the protein of interest while leaving the undesirable ones in the solution. The desired protein can therefore be separated from contaminants by centrifugation. The desired protein is thereafter dissolved in small quantity of buffer and kept for further analysis.

2.6.6.2 Dialysis

Dialysis is the process by which biological molecules that differ in the rate of diffusion are segregated through a dialysis bag. The major importance of using dialysis in life science research is to remove some extra salts, undesirable and bigger molecules that may interfere with the enzyme of interest (Berg, 2007). Dialysis is also found applicable in the exchange of buffer as well as in studying drugs binding ability.

In dialysis, where a semipermeable membrane is required, the protein sample and a buffer solution (called the dialysate) are alienated by a dialysis bag that causes change in diffusion patterns, thereby allowing the separation of proteins from small molecular weight molecules present in the solution. Dialysis bag is not permeable to higher molecular weight compounds and as a result such molecules cannot diffuse across the membrane. However, small molecular weight compounds can diffuse freely and by so doing create equilibrium across the entire solution volume, thereby changing the overall concentration of these molecules in the sample and dialysate. Upon attaining equilibrium, the dialysis bag can be emptied to determine the volume of protein. Since small molecular weight molecules can diffuse freely in and out, dialysis can be used to either add some low molecular weight to the protein of interest or remove unwanted material from desired samples. This makes dialysis important tools in a wide range of applications.

2.6.6.3 Gel-Filtration Chromatography

Gel filtration chromatography also known as size-exclusion chromatography (SEC). It can also be termed molecular sieve chromatography (Garrett *et al.,* 2013), is a type of chromatographic technique that is based on size / molecular weight of the molecules (Paul-Dauphin *et al.,* 2007). It is typically employed to separate higher molecular weight compound such as proteins and industrial polymers. The uses of an aqueous solution as a medium of transport in a column is called gel-filtration chromatography. SEC is a technique that is extensively used the molecular weight of the polymer can be determined easily. The main application of gel-filtration chromatography is the fractionation of proteins and other water-soluble polymers.

There is distinction between gel-filtration chromatography and gel electrophoresis, because in electrophoresis the samples are passed through an electric field. SEC operates by running samples in a column containing the stationary phase. The factor to be considered when carrying out SEC is that the sample containing protein of interest must be chemically unreactive with the stationary phase. Thus, a small molecular weight compound that can diffuse through the pores system can enter a total volume equal to the sum of the entire pore volume and the interparticle volume (Paul-Dauphin *et al.,* 2007).

On the other hand, a large molecular weight compound that cannot diffuse through the pores can enter only the interparticle volume (~35% of the column volume) and elutes first when this volume of mobile phase has penetrated through the column. The fundamental principle upon which SEC is based is that particles with different sizes diffuse through stationary phase at different speeds and are eluted at different rates. This give rise to separation of samples based on size. Molecules with approximately the same size and molecular weight will elute almost

together if they are loaded to the column together. Also, particles with different sizes will elute at different rates (Paul-Dauphin *et al.*, 2007).

2.6.7 Cellulase Characterization

Charaterization of cellulase involves the determination of molecular weight via electrophoresis, western blotting etc.

2.6.7.1 Electrophoresis

Electrophoresis is the process of separating charged particles under the influence of an electric field (Jouniaux and Pozzi, 1995; Elzbieciak-Wodka *et al.*, 2014; Ma *et al.*, 2014; Nägele, 2014; Tucker *et al.*, 2015). This electrokinetic phenomenon was first discovered by Russian scientists Professors Peter Ivan Strakhov and Ferdinand Frederic Reuss in Moscow State University (Dukhin and Goetz, 2002), who observed that the application of a constant electric field triggered the spread of clay particles in water. This technique is generally applied for separating molecules having different size, charge and binding affinity. In this method, the negatively charged particles will migrate towards a positive pole and positively charged particles will migrate towards a negative pole. This technique can be used for both DNA and RNA analysis. Polyacrylamide gel electrophoresis (PAGE) has a better resolution compared to agarose and hence more preferable for the determination of molecular weight of proteins. It is applicable in plasmid analysis, which has developed understanding of bacteria resistance to antibiotics.

2.6.7.2 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (PAGE), describes a method broadly used in life sciences and medicine to isolate biological macromolecules, usually proteins or nucleic acids, with the help of an electric field. Polyacrylamide gel electrophoresis (PAGE) may be SDS or native depending on whether a detergent is used to denature the protein or not. If SDS is used to distort the architectural structure of protein, it is called SDS PAGE. But if molecules are run in their native state, it is called "Native PAGE".

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a method of separation that rely on the difference in molecular weight of different proteins to be separated. The SDS which is a negatively charged molecule binds to protein in a ratio 1:2 (Ninfa *et al.*, 2010). In this way, SDS offers all proteins the same charge to mass ratio, independent of their original charge. SDS binds to proteins and denature the tertiary and quaternary structures by conferring a negative charge on them (Kindt *et al.*, 2007). Molecular weight of a polypeptide can be estimated by monitoring the distance travelled by the polypeptides (Kumar *et al.*, 2009). Urea is used to denature nucleic acids while SDS is used for proteins (Benore, 2010). Mercaptoethanol can also be used to break the disulfide bonds found between protein complexes, which further leads to denaturation of proteins.

Proteins that are more hydrophobic and those that react with detergents in their native form are basically difficult to treat accurately using this method, due to the variation in the amount of SDS that bind (Rath *et al.*, 2009). Native-PAGE keeps the oligomeric form intact and displays a band on the gel that shows the extent of purity. SDS-PAGE denatures the proteins and separates the oligomeric forms into its monomers, showing different bands that actually shows the molecular weight of each subunit. These bands can be used to determine the extent of purity

and can as well be used to identify the protein (Ninfa *et al.*, 2010). Different samples may be analyzed using SDS PAGE.

A tracking dye is usually added to monitor the movement of the proteins during electrophoresis. Two types of gel can be identified: these are stacking gel and separating gel. Stacking gels have relatively higher porosity compared to the separating gel and permits proteins to move in a concentrated area. In addition, the pH of the stacking gel and separating gel are not the same. Stacking gel operate at a pH 6.8 while separating gel operate at a pH of 8.8. The lower the pH of the buffer in solution, the faster the movement of proteins during electrophoresis. Commonly used buffers in SDS PAGE include Tris and Bis-Tris. Gel polymerization requires mixing of chemicals together. Most of these chemicals such as acrylamides are carcinogenic (Tareke *et al.*, 2000) and also toxins (LoPachin, 2004). It is very important to keep acrylamide in a cool dark and dry place to reduce autopolymerisation and hydrolysis.

Bisacrylamide (*N*,*N*′-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$; mW: 154.17) is the most commonly used cross linker for polyacrylamide gels. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in a gel. Sodium dodecyl sulphate (SDS) having the molecular formula $C_{12}H_{25}NaO_4S$ and and molecular weight of 288.38 is a detergent used to denature native proteins to individual subunits. When a protein mixture is heated alongside SDS to 100 °C, the detergent wraps around the polypeptide backbone. SDS binds to polypeptides in a constant weight ratio of 1.4 g SDS/g of polypeptide. In this process, the inherent charges of polypeptides become insignificant when compared to the negative charges contributed by SDS. PAGE systems are usually loaded from the top of the wells. To ensure that the sample descends to the bottom of the gel, some non-reactive additives must be added to the sample. These additives serve as carrier that drives down the samples. Examples

include glycerol and sucrose. After the entire electrophoretic processes, the sample is stained with a dye, e.g coomassie Brilliant Blue R-250 (CBB)($C_{45}H_{44}N_3NaO_7S_2$; mW: 825.97), the most popular staining reagent. In order to make the bands more visible, excess dye are removed by destaining of the gel.

2.7 Applications of Cellulase

Microbial cellulases have a diverse range of applications in industries such as pulp and paper, textile, biofuel, wine and brewery, food processing, animal feed, agricultural and detergent as well as in the management of waste materials.

2.7.1 Application of Cellulase in Pulp and Paper Industry

The use of cellulase in pulp and paper industry have increased tremendously over the last decade (Trache *et al*., 2017). The refining and grinding of raw material, an example of mechanical pulping processes may result in pulp with high content of fines, bulk, and stiffness. Whereas the uses of cellulase in pulping requires less energy and also bring about improvement in the quality of the products (Pere *et al.*, 2001; Rawat *et al.*, 2014). The improvement in biomodification of fibers with the sole aim of making the paper beatable is possible when the mixture of cellulases and hemicellulases are employed (Dienes *et al.*, 2004). Hébert-Ouellet *et al*. (2017) reported the activity of cellulase prepared on different fractions of Douglas from kraft pulp and detected that the cellulase treatment dwindled the defibrillation, thus tumbling the fibre stiffness. While endoglucanases possess the capacity to decrease the pulp viscosity with a lower degree of hydrolysis (Pere *et al.*, 1995), cellulases produce a better result compared to xylanase when used to bleach softwood (Kuhad *et al.*, 2011). Cellulase is also used in de-ink of different types of paper wastes. The release of ink as result of partial hydrolysis of carbohydrate present at the surface of fiber using the combination of cellulase and hemicellulase has been documented (Kuhad *et al.*, 2010). The advantage of cellulase and other enzymes in deinking process are reduction in the use of alkali, improvement of fiber brightness, higher pulp freeness and cleanliness, and compact fine particles in the pulp (Kuhad *et al.*, 2010 (a); Kuhad *et al.*, 2010 (b)). Application of cellulase in deinking tends to lower the hostile environmental influences that paper industry might cause (Stork and Puls, 1996). Interestingly, the routine of cellulases in taming the drainage has also been tracked by several mills with the objective to increase the production rate. Treatments with cellulase eliminate some of the fines or peel off fibrils on the fiber surface and colloidal substances, which often result in stark drainage problems in paper

mills. In this aspect, cellulases have shown substantial progress in the overall performance of paper mills (Sharmeen *et al.,* 2016). Enzymatic treatment also subverts the lipophilic extractives in the filtrates and eases their attachment to thermomechanical pulping fibers. These enzymes are also used in preparation of easily biodegradable cardboard (Imran *et al.,* 2016), manufacturing of soft paper including paper towels and sanitary paper (Hsu and Lakhani, 2002), and removal of adhered paper (Sharyo *et al.,* 2002).

2.7.2 Application of Cellulase in Textile Industry

Cellulases are the most effective enzymes found applicable in textile industry, especially in finishing of cellulose-based textiles, with the objective of developing a soft textile material with good appearance (Uhlig, 1998; Karmakar and Ray, 2011). Cellulases have efficacies in stoning and polishing of jeans and cotton of cellulosic material. The use of cellulase as biopolishing is generally undertaken during the wet processing stages. The cellulase in acidic environment usually increases smoothness and absorbance property of fibers, powerfully lower the propensity for pill formation, and afford a cleaner surface structure with less fur (Sreenath *et al.,* 1996). Endoglucanase, a component of cellulase is best appropriate for biopolishing of fabrics because it improves fabric look, texture, and color without adding any other chemicals (Sreenath *et al.,* 1996). Equally, endoglucanase seems to be the best for biofinishing. The use of cellulase normally removes the fluffy dull-like appearance that appears as result of repeated washing of most cotton or cotton blended garments. Therefore, using cellulase removes these stains and restores the garment to its original colour (Hebeish and Ibrahim, 2007; Ibrahim *et al.,* 2011). Also, cellulase helps in removing some dirt that is trapped in the garment. Efforts have been made to ensure that cellulase improves the dimensional firmness of cellulosic fabrics (Cortez *et al.,* 2002; Ibrahim *et al.,* 2011).

2.7.3 Application of Cellulase in Bioethanol Industry

Enzymatic hydrolysis of agrowastes such as bagasse, wheat straw, banana peel, groundnut shells, corncob, rice bran, and saw dust by cellulases for conversion to sugar and subsequently to ethanol is the most significant application currently under investigation (Sukumaran *et al.,* 2005; Kuhad *et al.,* 2010; Gupta *et al.,* 2011). The hydrolysis of cellulosic materials into products that are useful and other value-added products requires diverse processes (Wyman *et al.,* 2005; Kuhad *et al.,* 2010; Smith *et al.,* 2016). These processes comprise; pretreatment of agrowastes, conversion of these agrowastes to fermentable sugars and the use of fermentative microorganisms to ferment reducing sugar to produce ethanol. The cost of enzyme production is very high compared to when agrowastes are subjected to pretreament because bioconversion of lignocellulose wastes is usually conducted at slight acidic conditions (pH 4–6 and temperature 45–50◦C) (Kuhad *et al.,* 2010; Gupta *et al.,* 2011). All the machineries required for the conversion of cellulosic biomass to ethanol are currently available (Mosier *et al.,* 2005; Sreena *et al.,* 2016). However, some of the equipment required for the production of ethanol must be upgraded to yield renewable biofuel. There are lots of factors that affect the efficacy of cellulase; among these are the presence of lignin and inhibitors (Yang and Wyman, 2008). To reduce the high cost of enzyme production especially cellulase, two factors must be put into consideration. These are optimization parameters for enzyme production and improvement of a more effective cellulase-based catalysis structure. Approaches for reutilizing enzymes may also be applied to reduce enzymatic hydrolysis costs (Mosier *et al.,* 2005; Saini *et al.,* 2016; Sharmeen *et al.,* 2016). Among diverse approaches to improve and recycle the cellulases are concentration of the cellulose fraction by ultrafiltration to eliminate sugars and other small compounds that may thwart the action of the enzymes (Tu *et al.,* 2007) and reprocessing of

immobilized enzymes, which permits parting of the enzymes from the process flow (Dourado *et al.,* 2002; Mosier *et al.,* 2005).

2.7.4 Applications of Cellulase in Wine and Brewery Industry

Microbial cellulase and other related cellu-oligosaccharide derivatives play a significant role in the process of fermentation to produce alcoholic soft drinks (Sukumaran *et al.,* 2005; Singh *et al.,* 2007; Bamforth, 2009). The request of cellulase can increase both quality and the amounts of the fermented products (Bamforth, 2009). Cellulases are added either during crushing of cellulosic wastes to lessen the viscosity of wort and improve the permeability (Bamforth 2016). Other enzymes such as protease, xylanase, pectinases, amylases and hemicellulases play a central role in wine and brewery production as they help in the colour development (Singh *et al.,* 2007). The key profits of using these enzymes during wine production include improved softening, enhanced colour removal, easy amplification, easy permeability and enhanced wine quality (Singh *et al.,* 2007). Beer brewing rely on the action of enzymes activated during malting and fermentation. Some macerating enzymes such as α- and β-amylases, carboxypeptidase, and β- glucanase are mainly produced during the germination of seed (Bamforth, 2009). These enzymes produce an important and reproducible progress in grape pressability and the rate at which the juice settle. The total amount of juice that is produced would be high if a combination of macerating enzymes is used compared to when a single macerating enzyme is used. Such enhancements of wine production would only be visible if only the correct combination of pectinases, cellulases, and hemicellulases were used. An array of enhanced enzymes like cellulase and pectinase that would be exogenously added to the process are anticipated to boost the efficiency of current brewing processes in future (Bamforth, 2009).

2.7.5 Application of Cellulase in Food Processing Industry

Cellulases have a widespread potential use in food biotechnology as well. The production of fruit and vegetable juices necessitates an improved method for better production. Cellulases are also part of macerating consortium of enzymes used for extraction and amplification of fruit and vegetable increases the amount of juices produced (Minussi *et al.,* 2002; de Carvalho *et al.,* 2008). The use of consortium of enzymes in macerating improved both the number of products produced, as well as reducing the cost of production. The use of pectinases and *β*-glucosidases in food processing industry enhanced some properties of fruit and vegetable by decreasing unwarranted bitterness of citrus fruits (Youn *et al.,* 2004; Rai *et al.,* 2007; Galindo and Yusof, 2015). The combination of cellulase with other enzymes such as pectinase and hemicellulase in maceration improves the cloud stability as well as decreases the viscosity rapidly (Stein-Chisholm *et al.,* 2017). Thus, the complex enzymes used in maceration plays an important role in food biotechnology. Thus, there will be an upsurge in need for cellulase for juice production from varieties of fruits and vegetables (Dourado *et al.,* 2002). In addition, the introduction of pectinases and *β*-glucosidases have also been shown to change the texture, flavor, aroma and volatile features of fruits and vegetables (Bhat, 2000; Singh *et al.,* 2007; Karmakar and Ray, 2011; Wen *et al.,* 2014; Cui *et al.,* 2016).

2.7.6 Application of Cellulase in Animal Feed Industry

The application of enzymes in animal feed came into limelight after the rejection of some nutritive ionophore antibiotics, which were used hitherto in some European Countries (Ali *et al.,* 1995). The utilization of cellulases and hemicellulases in the feed industry have generated lots of attention because of their ability to increase feed value and overall concert of animals (Dhiman *et al.,* 2002). The hydrolysis of agrowastes using cellulase or xylanase brings about improvement in

the nutritional value of wastes (Behera *et al.,* 2016). The infusion of cellulases eliminates some antinutritional factors that may be present in the feed grains thereby improving the nutritional value of the feed. For example, crude fibers which are mainly undigested carbohydrates contain celluloses, lignins, hemicelluloses and cellodextrins which do not have any nutritional values (Ali *et al.,* 1995). Enzymes particularly the hydrolytic enzymes, have been employed to convert the crude fibers to digestible disaccharides or monosaccharides. The hydrolysis of agrowastes using cellulase improves the quality of the feed as this reduces the concentration of the undigested carbohydrates present in the feed (Meale *et al.,* 2014). The accumulation of large amount of undigested roughage in the digestive tract may support the growth of some hazardous pathogens which may be harmful to the system (Pascual, 2001; Pazarlioglu *et al.,* 2005). Cellulase is very important in the caecal fermentation processes; because it increases the production of propionic acid, which decreases the colonization of pathogenic microorganisms by acting as a bacteriostatic material (Fortun-Lamothe *et al.,* 2001; Pazarlioglu *et al.,* 2005).

2.7.7 Application of Cellulase in Agricultural Industries

Cellulose in conjuction with other enzymes like cellulases, hemicellulases, and pectinases have been reported to be very momentous in agriculture for enlightening growth of crops and control of plant diseases (Bhat, 2000; Kuhad *et al.,* 2011). Many cellulolytic microorganisms have been shown to play a vital role in agro-industries by aiding improved seed germination, quick plant growth flowering, as well as increased crop production (Harman, 1998; Harman *et al.,* 2004; Martins and Hayes, 2017). Though the microorganisms that are cellulase producers have both direct and indirect effects on plants (Harman, 1998; Martin and Hayes, 2017), it is still poorly understood how these fungi producing enzymes facilitate the enhanced plant performance. Lorito *et al.* (1994) reported that *β*-1,3-glucanase in conjuction with N-

acetylglucosaminidase produced by *T. harzianum* strain P1 thwarts spore germination and germ tube elongation of *B. cinerea*. The exoglucanase, one of the components of cellulase complex has been used for the expression of chymosin (Zhang *et al.,* 2016), glucoamylase, lignin peroxidase, and laccase (Saloheimo and Niku-Paavola, 1991; Penttila *et al*., 1998; Orth and Tien, 2013). Cellulases have also been studied for usage in the development of soil quality. Conventionally straw integration is considered a significant approach to improve soil quality and lessen reliance on mineral fertilizers (Escobar and Hue, 2008; Tejada *et al.,* 2008). Cellulase produced by cellulolytic microbes such as *Aspergillus*, *Chaetomium*, *Trichoderma* (Sharada *et al.,* 2014; Bokhorst *et al.,* 2017), and actinomycetes (Abdulla and El-Shatoury, 2007) have a wide range of applications and have revealed promising results in agricultural industries. Fontaine *et al*. (2004) reported that exogenous cellulase supplementation fast-tracked hydrolysis of cellulose in soil. Therefore, applying supplemented exogenous cellulase to the soil may improve the fertility of the soil as well as be a means of accelerating decomposition of agrowastes (Han and He, 2010).

2.7.8 Application of Cellulase in Detergent Industry

Application of cellulases in amalgamation with other enzymes such as protease and lipase in detergents is a topical discovery in the industry to advance the value of the product (Singh *et al.,* 2007). Cellulase complex helps in the modification of cellulose fibrils and as a result improves some properties of detergents like colour, texture and also remove dirt from cotton garments. Currently, liquid laundry detergent containing anionic or nonionic surfactants, citric acid or a water-soluble salt, protease, cellulose, and a mixture of propanediol and boric acid or its derivative has been employed to surge the stability of cellulases.

2.7.9 Application of Cellulase in Waste Management

The wastes produced from forests and agricultural practices when left untreated cause environmental pollution (Abu *et al.,* 2000; Milala *et al.,* 2005). Currently, these so-called wastes should no longer be considered as wastes becauses they harbour valuable materials that when harnessed properly can give rise to products like enzymes, monosaccharides which can undergo fermentation to yield biofuels and some other products that can improve animal feeds, and human nutrients (Humpf and Schreier, 1991; Gupta *et al.,* 2009; Gupta *et al.,* 2011; Karmakar and Ray, 2011).

For the past decade, there has been a tremendous interest in the conversion of lignocellulosic biomass to simple sugar (Clanet *et al.,* 1988). This attention stems from the advantages that such a process would offer, namely, the conversion of lignocellulosic and cellulosic wastes to a useful energy source (Coughlan, 1992; Lagerkvist and Chen, 1993). However, until now, the major limitation against the production of sugar from cellulose is the high cost of enzyme that will hydrolyze the cellulose (Clanet *et al.,* 1988; Coughlan, 1992). There has been a considerable improvement in the enzymatic hydrolysis of cellulose to yield sugar and consequently ethanol (Clanet *et al.* 1988). Sugar concentrations as high as 45 g sugar dm~3 for substrate È water ratios of 400 È 600gdm^3 were produced in a packed-column reactor and the hydrolyzates obtained could repeatedly be used in anaerobic fermentations.

Chapter Three

3.0 Materials and Methods

3.1 *Arachis hypogaea* Shells (Substrates)

A, hypogaea shells were collected from Oja-Tuntun, Ilorin, Kwara State, Nigeria. This was identified and authenticated at the Herbarium Unit of the Plant Biology Department, University of Ilorin, where a voucher specimen number (UILH/001/156) was deposited.

3.2 Microbial Strain

Pure culture of *Aspergillus niger* was obtained from Microbial Culture Bank, Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Ilorin, Nigeria and it was used throughout the study.

3.3 Growth Substrates

Potato dextrose agar (PDA) used for the cultivation of *A. niger* fungus stock was a product of Biotec Laboratory, United Kingdom.

3.4 Other Chemicals and Reagents

Carboxymethyl cellulose (CMC), Avicel and *p*-Nitrophenol- β-D-glucopyranoside (*p*-NPG) were products of BDA Chemicals Ltd., Poole, England. Sephadex G-100 was obtained from Superfine, India. 3,5-Dinitrosalicyclic acid (DNS) was obtained from Lab. Tech. Chemicals, Avighkar, India. Sodium hydroxide, sodium potassium tartarate (Rochelle salt), sodium dihydrogen phosphate, disodium hydrogen phosphate and Bovine Serum Albumin (BSA) were products of Santa Crux Biotechnology (Germany). Other reagents used were of analytical grade and prepared in all glass apparatus using distilled water and stored appropriately.

3.5 Preparation and Pretreatment of *A. hypogaea* Shell Substrate

Pretreatment of cellulose opens up the structure and removes secondary interaction between glucose chains (Fockink *et al.,* 2016). For enzymatic processes to be operative, some kind of pretreatment process is thus needed to open up the crystalline structure of the lignocellulose and remove the lignin. The *A. hypogaea* shells were first washed with distilled water in order to get rid of dust and other contaminants that may be attached to it. The shells were then dried, subsequently pulverized with an electric blender (Philip Comfort Blender, mode HR1727, Holland) and sieved to make the substrates susceptible to enzyme hydrolysis. It was later stored in a polypropylene bag until further use.

3.5.1 Acid Pretreatment

Acid pretreatment solubilize the hemicellulose portions of the agrowastes and make the cellulose more available to enzymes (Saritha *et al.*, 2012). This entails the utilization of concentrated or diluted acid (Knappert *et al.*, 1981) but the major constraint against the utilization of concentrated acid is the high operational and maintenance costs as well as equipment corrosion (Wyman, 1996). The method described by Schell *et al*. (2003) was adopted for acid pretreatment of *A. hypogaea* shells.

Procedure:

The raw *A. hypogaea* shell was crushed and grinded into powder using electric blender (Phillip Comfort Blender, mode HR 1727, Holland). 10g of powdered *A. hypogaea* shell was weighed and added to 1000 ml of 0.25M H_2SO_4 in a beaker, left for two hours at room temperature. The pretreated solid was washed with distilled water and tested with litmus paper

until the pH of the washed water became neutral. The pretreated *A. hypogaea* shell was air-dried and stored in a polypropylene bag until further use.

3.5.2 Alkaline Pretreatment

The treatment of substrates with alkali provide a way of extracting lignin and other acetate from lignocellulosic biomass thereby make the remaining polysaccharides digestible. One of the key important of alkaline pretreatment is the ability to recover the loss carbohydrates that had been lost due to treatment with alkali.

Procedure:

The raw *A. hypogaea* shells were air-dried individually to reduce the moisture content, crushed and grinded into powder using an electric blender (Philip Comfort Blender, mode HR1727, Holland). 10g of powdered *A. hypogaea* shell was weighed and added to 1000 ml of 0.1M NaOH solution in a beaker. The mixture was left to stand for two hours at room temperature and later autoclaved at 121 ^{o}C for one hour. The pretreated solid was washed with distilled water and tested with litmus paper until the pH of the washed water became neutral. The pretreated *A. hypogaea* shell was air-dried and stored in a polypropylene bag until further use (Gharpuray *et al.,* 1983; Solomon *et al.,* 1999).

3.6 Proximate Analysis of the Pretreated *A. hypogaea* shell

Proximate analysis is a pertitioning of the pretreated *A. hypogaea* shell into six categories based on the chemical properties found in *A. hypogaea* shell. The six categories are: (i) moisture, (ii) ash, (iii) crude protein (iv) lipid, (v) crude fibre, and (vi) carbohydrates.

3.6.1 Determination of Moisture Content

Moisture content gives the amount of water that may be present in a sample. The method described by Gafar *et al*. (2011) was followed for the determination of moisture content.

Procedure:

The powdered *A. hypogaea* shell was weighed (W_1) into a crucible of known weight (W_o) and placed in a hot-air oven at 105 ^oC for 4 hours. The crucible was detached, allowed to cool in a desiccator, and weighed again. The processes of drying, cooling and re-weighing were repeated until a constant weight (W_2) was obtained.

Calculation:

The weight loss due to moisture was obtained thus:

Moisture (%) = $\frac{W1-W2}{W1-Wo}$ x 100

Where:

W_o= Weight of the empty crucible (g) W_1= weight of the powder sample + empty crucible(g), and W_2= weight of dried sample + empty crucible(g)

3.6.2 Determination of Ash Content

This is a measure of the residue remaining after combustion of the dried sample in a furnace at a temperature of 600 ^oC for 5 hours. The method of Sales (1996) was used to determine the percentage ash content present in the pretreated *A. hypogaea* shell.

Procedure:

The dried pretreated *A. hypogaea* shell was weighed (W_1) into a pre-weighed empty crucible (W_o) and placed in a furnace and heated for 600 ^oC at 5hrs. The ash was allowed to cooled in a desiccator and re-weighed (W_2).

Calculation:

Percentage ash was obtained using the formula:

Ash (%) = $\frac{W2-Wo}{W1-Wo}$ x 100

Where:

W_o = weight of empty crucible(g) W_1 = weight of crucible + powdered sample(g), and W_2= Weight of empty crucible + ash sample(g)

3.6.3 Determination of Lipid Content

The Crude lipid content in the sample was extracted by soxhlet extraction procedure, as described by Gafar *et al*. (2011). The principle is based on the extraction of fats with diethyl ether which dissolves fats, oils and other fatty soluble substances.

Procedure:

The pulverized *A. hypogaea* shell (5g) was weighed (W_o) into a porous thimble and covered with clean white cotton wool. Petroleum ether (200 ml) was poured into a 250 ml extraction flask, which was previously dried in the oven at 105 ^oC and weighed (W_2). The porous thimble was placed into the soxhlet and the rest of the apparatus assembled. Extraction was done for 5hr. The thimble was carefully detached and the extraction flask was placed in a water bath to eliminate the petroleum ether and then dried in the oven at a temperature of 105 ^oC to completely free the solvent and moisture. It was cooled in a desiccator and reweighed (W_1).

Calculation:

The percentage crude lipid was calculated using the equation below:

Crude lipid (%) = $\frac{W1-W2}{Wo}$ x 100

Where:

W_o= weight of sample (g) W_1= weight of flask + oil(g), and W_2= weight of flask(g)

3.6.4 Determination of Crude Fibre Content

Crude fiber is a measure of the quantity of undigested carbohydrates such as cellulose, pentosans, lignin, pectins and other components of this type in food sampls (Van Soest and Robertson, 1979). Percentage crude fibre was determined by the method of Gafar *et al*. (2011).

Procedure:

The pulverized *A. hypogaea* shell (5g) was weighed (W_o) into a 1dm^3 conical flask. 100 ml of water and 20 ml of 20% H_2SO_4 were added. The mixture was boiled gently for 30 minutes. The content was sieved through a Whatmann No.1 filter paper. The residue was scraped back into the flask with a spatula. 100 ml of water and 20 ml of 10% NaOH were added and allowed to boil gently for 30 minutes. The content was filtered and the residue washed thoroughly with hot distilled water, then rinsed once with 10% HCl and twice with ethanol and finally, thrice with petroleum ether. It was allowed to dry and scraped into the crucible and dried overnight at 105^oC in an air oven. It was then removed and cooled in a dessicator. The sample was weighed (W_1), and ashed at 600 ^oC for 90 minutes in a muffle furnace. It was finally cooled in a desiccator and re-weighed (W_2).

Calculation:

The percentage crude fibre was calculated thus:

Crude fibre (%)= $\frac{W1-W2}{Wo}$ x 100

Where:

W_o= weight of sample (g) W_1=weight of dried sample (g) W_2=weight of ash sample (g)

3.6.5 Determination of Crude Protein Content

Crude protein is a measure of nitrogen in the sample. The crude protein of the sample was determined using the micro-Kjedahl method described by Horwitz (1975).

Principle:

The principle relied on conversion of protein and other nitrogen containing organic compounds (other than nitrites and nitrates), into ammonium sulphate. The ammonia is distilled from an alkaline medium and absorbed in homogenous mineral acid. The ammonia is determined by back titration with a standardized mineral base. The equations for the reaction are as follows:

Sample nitrogen + $H_2SO_{4(aq)} \longrightarrow (NH_4)_2SO_{4(aq)}$

$(NH_4)_2SO_{4(aq)} + 2NaOH_{(aq)} \longrightarrow 2NH_{3(aq)} + 2H_2O + Na_2SO_{4(aq)}$

$NH_{3(aq)} + H_3BO_{3(aq)} \longrightarrow NH_4^+{}_{(aq)} + H_2BO_3^-{}_{(aq)}$

$H^+{}_{(aq)} + H_2BO_3^-{}_{(aq)} \longrightarrow H_3BO_{3(aq)}$

Procedure:

The sample (5g) was weighed along with 20 ml of distilled water into a micro-Kjedahl digestion flask. It was shaken and allowed to stand. One selenium boiling granule was added followed by the addition of 20 ml concentrated tetraoxosulphate (vi) acid. The flask was heated on the digestion block at 100 ºC for 4 hours until the digest became clear. The flask was removed

from the block and allowed to cool. The content i. e the mixture was transferred into 50 ml volumetric flask and diluted to the mark with water. An aliquot of the digest (10 ml) was transferred into another micro-Kjedahl flask along with 20 ml of distilled water and placed in the distilling outlet of the micro-Kjedahl distillation unit. A conical flask containing 20 ml of boric acid indicator was placed under the condenser outlet. Sodium hydroxide solution (20 ml, 40%) was added to the content in the Kjedahl flask by opening the funnel stopcock. The distillation starts and the heat supplied were regulated to avoid sucking back. When all the available distillates were collected in 20 ml of boric acid, the distillation was stopped. The nitrogen in the distillate was determined by titrating with 0.01M of H_2SO_4; the end point was obtained when the colour of the distillate changed from green to pink.

Calculation:

% crude protein was calculated by multiplying the total nitrogen content by a constant, 6.60. This was based on the assumption that, protein contains about 16% N which includes both true protein and non-protein N and does not make a distinction between available or unavailable protein (Gafar *et al.*, 2011). The crude protein was calculated using

Crude protein (%) = %N x 6.60

The nitrogen content of the sample is given by the formula below:

$$N\ (\%) = \frac{Tv\ x\ Na\ x\ 0.014\ x\ V1}{G\ x\ V2}\ x\ 100$$

Where:

Tv= titre value of acid (ml) Na=concentration or normality of acid V_1= volume of distilled water used for distilling the digest (50 ml) V_2=volume of aliquot used for distillation (10ml) G=original weight of sample used (g).

3.6.6 Determination of Carbohydrate Content

The total carbohydrate content was determined by the addition of percentage (%) of protein, crude fibre, ash content, and moisture content and then subtract from 100% (AOAC, 2000).

Calculation:

% Total Carbohydrate = 100 – (% Moiture + % Ash + % Fat + % Protein + % Fibre)

3.6.7 Cellulose Quantification using Anthrone

This involves the determination of the amount of cellulose present in each of the treated and untreated *A. hypogaea* shells.

Procedure:

10g of the pretreated *A. hypogaea* shell was weighed into a beaker containing 100 ml of 75% ethanol. This mixture was warmed at 70 ^{o}C for 1 hour in boiling water. The mixture was filtered with muslin cloth to obtain the supernant (ethanol extract) and pellet. 100 ml of 75% ethanol was added to the pellet, left for another 1 hour in a boiling water. The mixture was separated using muslin cloth to obtain another pellet and supernant (ethanol extract). The two supernants obtained were mixed together and dried. 10 ml of acetone was added to the dried sample obtained from ethanol and it was subsequently dried overnight. The resulting dried sample was weighed on an analytical balance and 10ml of 0.05M acetic nitric reagent was added, heated at 98^{o}C for 30 minutes. The mixture was centrifuged at 14000rpm for 10 minutes after which the supernatant was drawn. 1ml of distilled water was added to the supernant and centrifuged further for another 10 minutes at 14000rpm to obtain pellet and supernant. The supernant obtained was divided into two portions and 1ml of acetone was added to the first

portion and centrifuged for 5 minutes. The mixture was separated. Supernatant was removed as much as possible without losing any of the part, and then dried overnight. 0.1ml of 67% H_2SO_4 was added to the dried sample, covered and mixed well. 0.4ml of anthrone solution was added to the resulting mixture and boiled in water bath for 5 minutes. The sample was allowed to cool. Absorbance was read at 540nm and the result obtained was extrapolated from glucose standard curve prepared using 0.1 to 1.0 mg glucose (Figure 29 in appendix 2).

3.7 Microbial Culture

The growth of microorganisms depends on available and a favourable growth environment. Culture media are nutrient reagents used in laboratories to support the growth of microorganisms. For the effective cultivation of microorganism, it is imperative to know its nutritional necessities and then supply the essential nutrients in the proper form and proportion in a culture medium.

3.7.1 Preparation of Potato Dextrose Agar

9.75g of potato dextrose agar was dissolved in 250 ml distilled water. The resulting solution was autoclaved at 121℃ and 15 psi for 15 minutes until no colloid was seen to ensure the solution was completely diluted. The solution was poured into petri-dish, it covered one-third of the height of the petri-dish. The agar was left to solidify.

3.7.2 Preparation of Enriched Media

The enriched media for fermentation was prepared by mixing the chemicals into 1l solutions. The list of chemicals for preparation of enriched media is shown in Table 1. 250 ml of enriched media was prepared in a 500 ml Erlenmeyer flask and autoclaved for 15 minutes at 121 ℃ and 15 psi. It was then cooled down to room temperature and 10 ml spores' suspensions was

added into the flask in sterilized condition. Erlenmeyer flask containing all the chemicals was incubated at 30 ℃ with shaking speed of 150 rpm in incubator for 60 to 72 hours to ensure inoculants in log phase.

Table 1: Chemicals used in Preparation of Enriched Media

Materials	Mass
$(NH_4)_2SO_4$	1.4g
KH_2PO_4	2.0g
$CaCl_2.2H_20$	0.4g
$MgSO_4.7H_2O$	0.6g
$MnSO_4.H_2O$	1.0 mg
$ZnSO_4.7H_2O$	1.4 mg
$FeSO_4.7H_2O$	5.0 mg
$CoCl_2.6H_2O$	3.7 mg
Protease peptone	0.75mg
Tween 80	2.0 mg
Carboxyl methyl cellulose (control)	2.0g
Substrates (groundnut shell)	1-5 g

Source: Saliu and Sani (2012)

3.7.3 Extraction and Concentration of Cellulase Enzyme

Cultivation of the *A. niger* was done in 250-ml Erlenmeyer flasks containing 100 ml of enriched medium. The medium composition (in gl^{-1}) used for growth and enzyme induction was determined and it composed of carboxyl methyl cellulose, 1; sucrose, 30; $NaNO_3$, 2; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl 0.5; $FeSO_4 \cdot 7H_2O$, 0.01. Inoculums size was 10^5 spores' ml^{-1}. Flasks were shaken on an orbital shaker at 120 rpm for 7 days at 30 °C.

The culture supernatant and pellet (mycelia mat) were separated by filtration. Supernatant was discarded and 1g of pellet was re-suspended in 100 ml of 0.05M citrate buffer (pH 4.8) and homogenized with hand grinder and kept in an ice bath. This supernatant was taken as crude enzyme solution and concentrated to five-folds by citrate buffer.

3.8 Assay of Cellulase Activity

3.8.1 Determination of Endo-β-1, 4-glucanase Activity

Principle:

The principle is based on the hydrolysis of cellulose to produce oligosaccharides. Endoglucanase randomly cuts the cellulose chains producing cello-oligosaccharides (Lynd *et al*., 2002). The free carbonyl group present in the oligosaccharide produced reduces 3,5-dinitrosalicyclic acid to 3-amino,5-nitrosalicylic acid.

$$\text{Cellulose} + H_2O \xrightarrow{\text{Endoglucanase}} \text{Oligosaccharide}$$

Procedure:

0.5 ml of substrate solution (1% CMC) prepared in 0.5M citrate buffer (pH 4.8) was added to 0.1 ml of enzyme source in a test-tube and mixed well. The mixture was incubated at 50 °C for 30 minutes. 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to stop the

reaction and the mixture was placed in boiling water for 5 min. The mixture was cooled and 5 ml of distilled water was added. The absorbance was measured at 540 nm. The amount of reducing sugar obtained was extrapolated from glucose standard curve. One unit of endo-β-1, 4-glucanase activity is defined as the amount of enzyme that could hydrolyze CMC and release 1 µg of glucose within 1minute reaction at 50 °C (Miller, 1959).

3.8.2 Determination of Exoglucanase Activity

Principle:

The principle involves hydrolysis of cello-oligosaccharides to produce cellobiose. Exoglucanase attacks the non-reducing end of cello-oligosaccharide produced by endoglucanase to yield cellobiose. The free carbonyl group present in the oligosaccharide produced change the colour of dinitrosalicyclic acid (DNS) from orange to purple by reducing 3,5-dinitrosalicyclic acid to 3-amino,5-nitrosalicylic acid.

$$\text{Oligosaccharide} + H_2O \xrightarrow{\text{exoglucanase}} \text{Cellobiose}$$

Cellobiose + 3,5-dinitrosalicylic acid ----------> 3-amino,5-nitrosalicylic acid

(Yellow) (Purple)

Procedure:

Exoglucanase activity was determined by incubating 0.5 ml of 1% avicel in 0.05M citrate buffer (pH 4.0) with 0.1 ml of appropriate concentration of enzyme. The reaction mixture was incubated at 50 °C for 30 minutes. 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to stop the reaction and the mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The change in colour from orange to brown was measured at 540 nm. The amount of reducing sugar obtained was extrapolated from

glucose standard curve. One unit of exoglucanase activity is defined as the amount of enzyme that could hydrolyze avicel and release 1 μg of glucose within 1 min reaction at 50 ℃ (Sharma *et al.*, 2015).

3.8.3 Determination of β-glucosidase Activity

Principle:

The principle involves hydrolysis of p-Nitrophenyl-β-D-glucopyranoside to produce β-D-glucose when acted upon by enzyme β-glucosidase. The appearance of p-nitrophenol is measured at 405nm by spectrophotometry.

ρ-Nitrophenyl-β-D-glucopyranoside (PNPG) $\xrightarrow{\text{β-glucosidase}}$ ρ-Nitrophenol (PNP) + D-Glucose

Procedure:

β-glucosidase activity was determined by the hydrolysis of *p*-Nitrophenol-β-D-glucopyranoside (*p*-NPG) in 0.05M citrate buffer (pH 4.0). 2 ml of the substrate (0.5g/l *p*-NPG) was added to 0.2 ml of enzyme source. The mixture was incubated at 37 ℃ for 30 min. The reaction was terminated by the addition of 1 ml of 0.25 M Na_2CO_3. The absorbance at 405 nm was measured. The amount of ρ-nitrophenol produced was extrapolated from PNP standard curve. One unit of β-glucosidase activity is defined as the amount of enzyme that could hydrolyze *p*-NPG and release 1 μmol of *p*-nitrophenol within 1 min reaction at 37 ℃ (Lowry *et al.*, 1951).

3.9 Optimization Parameters for Cellulase Production

In order to determine the optimum conditions for cellulase production, various parameters were varied. These includes: incubation period, substrate concentration, temperature, pH, inoculum sizes and nitrogen.

3.9.1 Effect of Incubation Period on Cellulase Production

Fermentation media was prepared using *A. hypogaea* shell. The media was autoclaved at 121 °C for 15 minutes. It was allowed to cool and *A. niger* inoculated. Fermentation was carried out for period of 0-192 hours. The samples were withdrawn every 24 hours for 192 hours. The cellulase activity was measured from the samples withdrawn every 24 hours after which a period of maximum cellulase activity was determined (Bansal *et al.,* 2012).

3.9.2 Effect of Varying pH on Cellulase Production

The fermentation media was prepared using *A. hypogaea* shell sample. The pH was set at different levels of 3, 4, 5, and 6 respectively by adding 1% NaOH or concentrated HCl. The media was autoclaved at 121 °C for 15 minutes. It was allowed to cool and *A. niger* was inoculated. The flask containing enriched media was placed in an orbital shaker maintained at 100 rpm. The pH for maximum cellulase activity was determined.

3.9.3 Effect of Varying Temperature on Cellulase Production

The fermentation media was prepared using *A. hypogaea* shell sample at optimal conditions of incubation period and pH for cellulase production. The media was autoclaved at 121 °C for 15 minutes. It was allowed to cool and *A. niger* inoculated. Incubation temperature was set at 35 °C, 40 °C, 45 °C, and 50 °C respectively for their period of maximum production. The temperature for maximum cellulase activity was determined.

3.9.4 Effect of Varying Substrate Concentration on Cellulase Production

The fermentation media was prepared using *A. hypogaea* shell sample at different concentrations of 2%, 4%, 6%, and 8% w/v respectively and was autoclaved at 121 ℃ for 15 minutes. It was later inoculated with *A. niger* and placed on an orbital shaker maintained at 100 rpm for their period of maximal production of cellulase while keeping incubation period, pH and temperature constant. The cellulase activity was measured at different concentrations, and the maximum cellulase activity was determined.

3.9.5 Effect of Varying Innoculum Size on Cellulase Production

The fermentation media was prepared using *A. hypogaea* shell sample at different innoculum sizes of 10, 12, 14, 16 cfu/ml respectively and was autoclaved at 121 ℃ for 15 minutes. It was then inoculated with *A. niger* and placed on an orbital shaker maintained at 100 rpm for their period of maximal production of cellulase while keeping incubation period, pH, temperature and substrate concentration constant. The cellulase activity was determined at different innoculum sizes.

3.9.6 Effect of Different Nitrogen Source on Cellulase Production

The effect of yeast extract, beef extract, protease peptone and urea were studied. Different nitrogen sources were added to the fermentation medium. The nitrogen sources were used to replace the prescribed nitrogen source of the fermentation medium and after which the cellulase activity was measured.

3.10 Determination of Protein Concentration

Concentration of protein was determined by following the method of Lowry *et al*. (1951) using Bovin Serum Albumin (BSA) as standard. The protein concentration was then estimated from a standard curve.

Principle:

This method of protein concentrations determination is based on the reactivity of the peptide nitrogen(s) with the copper [II] ions under alkaline conditions and the subsequent transformation of a mixture of phosphomolybdic acid and phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids such as tyrosine and tryptophan. The colour intensity is proportional to the protein content of the sample at wavelength of 650 nm.

Procedure

To 1.0 ml of the sample, 4.5 ml of reagent A (containing alkaline Na_2CO_3, sodium potassium tartarate and hydrated copper sulphate) was added and mixed thoroughly. It was allowed to stand for about ten minute and then 0.5 ml of reagent B (Folin reagent) was added and kept standing for about 30 minutes in order to develop colour. The absorbance was then read at 650 nm against the blank using SpectrumLab 725S UV-VIS spectrophotometer. The protein concentration was extrapolated from standard curve, prepared using 1.0 to 10.0 mg/ml of BSA (Figure 27 in appendix II).

3.11 Cellulase Purification and Characterization

3.11.1 Ammonium Sulphate Precipitation

Principle

Ammonium sulphate precipitation relies on the principle that most proteins are less soluble in solutions of high salt concentrations because the addition of salt ions buffer proteins with multi-ion charges.

Procedure:

The supernatant was subjected to 80% ammonium sulphate precipitation by mixing a 300 ml of 100% saturated ammonium sulphate solution with 120 ml of the supernatant. Details of the preparation of 100% ammonium sulphate saturated solution are provided in the Appendix I. The precipitate obtained was allowed to settle at 4 °C overnight and then centrifuged at 10000 Xg for 30 minutes. The pellet obtained was collected by gently slanting the container and decanting the supernatant, then re-dissolve with small amount of ice cold 0.05M citrate buffer, pH 4.0.

Calculation:

In order to obtain 80% ammonium sulphate precipitation, the following formula was used:

$Cv \times Vf = Cn \times Vn$

Where;

Cv = Final Concentration

Vf = Final Volume

Vn = Volume of $(NH_4)_2SO_4$ added

Cn = Concentration of the $(NH_4)_2SO_4$

That is:

To determine what amount of 100% saturated ammonium sulphate solution that would be needed to add to 50 ml supernatant to make 80% ammonium sulphate saturation

$(50 + x)$ x (80%) = (x) x (100%)

Where x is the volume of 100% saturated ammonium sulphate solution needed to be added to the supernatant.

$(50 + x)$ x (80) = (x) x (100)

$4000 + 80x = 100x$

$4000 = 20x$

$x = 200$.

Therefore, to make 50 ml supernatant 80% ammonium sulphate saturated 200 ml of 100% ammonium sulphate saturation was added to the supernatant.

3.11.2 Dialysis

Dialysis bag was cut 6 cm x 12 cm and pretreated before the dialysis. This was done by soaking the tube for 24 hours in distilled water to remove glycerol and to open the tube from both ends according to method described by Ibraheem *et al*. (2017). The tube was washed again with distilled water, and in order to remove metal traces, it was further placed in a solution of 2 % sodium bicarbonate and 1 mM EDTA for 3 to 4 hours. The tube was washed with distilled water.

Principle:

The principle of dialysis is based on selective and passive diffusion of proteins through a semi-permeable membrane (dialysis bag).

Procedure:

The dialysis tube (6 cm x 12 cm) was tied at one of the open ends with thread. The solution was gently poured into the tube ensuring that the tube was half-filled. The half-filled tube was then suspended in 1 litre-beaker filled with ice-cold 0.05M citrate buffer, pH 4.0. The suspension of the dialysis tube was achieved by attaching the ends of the tube to a glass rod and carefully placed across the beaker. The solution was then subjected to dialysis against the buffer for 24 hours with continuous stirring using magnetic stirrer and intermittent replacement of the buffer at 3-4 hours' interval.

3.11.3 Gel Filtration Chromatography

Principle

The principle involves separation of proteins, peptides and oligonucleotides on the basis of their size / molecular weight and shape.

3.11.3.1 Packing of Chromatographic Column

The retort stand was used to firmly hold the chromatographic column (2.0 x 120 cm) positioned vertically while a piece of cotton wool was carefully introduced to the bottom of the column. The running buffer (0.05M Citrate buffer, pH 4.0) was then introduced into the column to ensure that the cotton wool was wet and well positioned inside the column. The tap was thereafter opened to release the buffer and also to prevent the air from being trapped in the column. Sephadex G 100 gel slurry was prepared by mixing 20 g of the gel with 200 ml of 0.05M citrate buffer (pH 4.0) and quickly poured into the column. This chromatographic column was filled up to 80 cm with the prepared slurry. Another piece of cotton wool was introduced and

the top of the gel was layered with buffer. This is very essential to prevent the gel from cracking. The packed column was left for 24 hours so as to allow the gel to settle and well packed.

3.11.3.2 Elution of the Column

10 ml of dialyzed enzyme was cautiously layered on top of the well packed column with sephadex G-100 column. Another cotton wool was then placed on top of the dialyzed enzyme and then eluted with mobile phase (0.05M citrate buffer (pH 4.0)). A flow rate of 0.1 ml/2minutes was maintained. Twenty (20) fractions of 5 ml each were collected. Cellulase activity and protein concentration were determined in each of the fractions. The fractions with the highest cellulase activity were pooled together and used for characterization and further analysis.

3.11.3.3 Concentration of the Enzymes

The pooled fractions obtained from gel filtration chromatography were concentrated using a concentrator with molecular weight limit (MWL) of 10 kDa.

Principle:

The principle is based on the removal of contaminants from protein samples while retaining the desired protein.

Procedure:

About 0.5 ml of the enzyme was put in a concentrator with a molecular weight limit (MWL) of 10 kDa and placed in an ependorff tube. The enzyme in an eppendorff tube was centrifuged at 10,000 rpm for 10 minutes. The concentrated enzyme was collected by

overturning the concentrator in another tube and centrifuged 10,000 rpm for another 5 minutes. The concentrated cellulase was then stored for further analysis.

3.11.4 Estimation of Molecular Weight of Purified Cellulase

3.11.4.1 Gel Electrophoresis (Molecular Weight Determination)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) was carried out by following the method described by Singh *et al.* (2012) on vertical slab-gel unit Mini Protean II electrophoretic cell (Bio Rad Laboratories). The gel was run for about 50 minutes at 150 V.

3.11.4.2 Casting of Sodium Dodecyl Sulphate Polyacrylamide Gel

For enzyme separation, 12% separating gel and 4% stacking gel were prepared. 12% separating gel was prepared by adding 3.35 ml distilled water, 2.5 ml of 1.5 M Tris (pH 8.8), 0.1 ml of 10% SDS and 4.0 ml of 30% acrylamide together. 50 µl of freshly prepared 10% ammonium persulphate (APS) was then added and followed by 15 µl of tetramethylethylenediamine (TEMED). The total volume was 10 ml. The mixture was gently mixed and quickly introduced into the two short plates with in-built spacer mounted on the casting tray. 50% n-butanol was carefully layered on top of the gel to make its surface smooth and left to stand until the gel solidified. After the solidification of the gel, the n-butanol was carefully removed by gently slanting the short plate upside down. The 4% stackling gel which was formed by mixing 3.0 ml of distilled water, 1.25 ml of 0.5 M Tris (pH 6.8), 50 µl of 10% SDS, 665 µl of 30% acrylamide, 25 µl of 10% APS and 10 µl TEMED together was layered on top of the separating gel. The wells were created on the short plates by inserting 10-tooth comb prior to the solidification of gel. The whole set-up was left for about 30 min until the gel

solidified and after solidification of the gel, the comb was removed and the wells rinsed with buffer prior to loading of the samples.

3.11.4.3 Sample Preparation and Loading

80 µl of the protein sample was mixed with 20 µl of 5x loading buffer in a 1:4 ratios and vortexed for 2 minutes. The mixture was then boiled for 5 minutes, spun down and kept on ice until loading. About 20 µl of the prepared sample was loaded into each well starting alongside known molecular markers. The standard protein markers used are β-Galactosidase, 116 kDa; phosphorylase B, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa and trypsin inhibitor, 21 kDa. Native molecular weight of the purified cellulase was estimated by the relative mobility (Rf values) of the protein bands compared with the standards.

3.11.4.4 Staining and Destaining of the Gel

The gel was carefully removed after complete separation was achieved. The gel was carefully released into a beaker containing staining solution. The beaker containing staining solution and the gel was placed on an orbital shaker and left for 24 hours to ensure proper staining. After 24 hours, the gel was removed from the staining solution and washed with distilled water. It was then distained in a solution containing the mixture of methanol, glacial acetic acid and distilled water in the ratio 4:1:5 respectively with intermittent shaking. Destaining was continued until the bands were visible. The bands were visualized and photogragh taken using Infinix Hot 2 cell phone.

3.11.5 Determination of Substrate Specificity of Purified Cellulase

The ability of purified cellulase to hydrolyze different substrates such as CMC, avicel, glucose, sucrose, ρ-NPG and filter paper was determined.

Procedure:

0.5 ml of 1% substrate (CMC, avicel, glucose, sucrose, ρ-NPG and filter paper) solution prepared in 0.5M citrate buffer (pH 4.0) was added to 0.1 ml of purified cellulase in a test-tube and mixed well. One strip of filter paper was used as substrate. The mixture was incubated at 50 °C for 30 minutes. Then, 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added and the mixture was placed in boiling water for 5 min. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was read at 540 nm.

3.11.6 Effect of pH on the Activity of Purified Cellulase

The activity of purified cellulase was measured by varying the pH of 50 mM citrate buffer. The pH was varied between 3.0-6.0, after which cellulase activity was determined.

Procedure:

0.5 ml of 1% CMC prepared in 0.5M citrate buffer of varying pH (3.0 - 6.0) was added to 0.1 ml of purified cellulase in a test-tube and mixed well. The mixture was incubated at 50 °C for 30 minutes. Then 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to stop the reaction and the mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm.

3.11.7 Effect of Temperature on the Activity of Purified Cellulase

The purified cellulase was incubated with substrate at different temperatures of 30, 40, 50 and 60 °C. The activity of cellulase was determined by following the method described by Miller (1959).

Procedure:

0.5 ml of 1% CMC prepared in 0.5M citrate buffer (pH 4.0) was added to 0.1 ml of purified cellulase in a test-tube and mixed well. The mixture was incubated at various temperatures of 30, 40, 50 and 60 °C for 30 minutes. Then 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to terminate the reaction and the mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm.

3.11.8 Effects of Substrate Concentration on the Activity of Purified Cellulase

The Michaelis-Menten kinetic constants, K_m and V_{max} for purified cellulase were determined by varying concentration of carboxymethyl cellulose ranging from 0.01-5.0 mM. Lineweaver-Burke plot was also generated to determine the K_m and V_{max}.

Procedure:

Half a millilitre of varying concentrations of CMC (0.01-5.0 mM) in 0.5M citrate buffer (pH 4.0) was added to 0.1 ml of purified cellulase in a test-tube and was thoroughly mixed. The mixture was incubated at 50 °C for 30 minutes. 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to stop the reaction and the mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm. The double reciprocal transformation of the cellulase activity and substrate concentrations were determined.

3.11.9 Effect of Cations on the Activity of Purified Cellulase

The effect of various cations ion on activity of the purified cellulase was studied using the various concentrations of salts in the reaction systems. The salts that released the cations Na^+,

K^+, Mg^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, and Co^{2+} are NaCl, KI, $MgSO_4$, $ZnSO_4$, $CuSO_4.5H_2O$, $CaCl_2$, $FeSO_4$, $MnCl_2.H_2O$ and $CoCl_2.6H_2O$ respectively and were prepared in the concentrations of 0.05, 0.1, 0.2, 0.5, 0.75 and 1 mmol/L. The salts were incubated with purified cellulase and after which the activity was determined.

Procedure:

Half a millilitre of varying concentrations (0.05 - 1mmol/L) of each cation ion was incubated with 0.1 ml of purified cellulase. The mixture was incubated for 30 minutes at 50 °C and this was followed by addition of 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution. The mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm.

3.11.10 Effect of Anions on the Activity of Purified Cellulase

The effect of some anions like CO_3^{2-}, Cl^- and SO_4^{2-} were investigated on the activity of purified cellulase. The salts that released the anions into the reaction system are $CaCO_3$, ZnCl and $MgSO_4$ respectively. The salts were incubated at varying concentrations with purified enzyme and the cellulase activity was later determined.

Procedure:

Half a millilitre of varying concentrations (0.05 - 1mmol/L) of each salt of anion was incubated with 0.1 ml of purified cellulase. The mixture was incubated for 30 minutes at 50 °C. 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added and the mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm. The blank was set up by replacing the enzyme with equal volume of distilled water.

3.11.11 Effect of Some Surfactants on the Activity of Purified Cellulase

The effect of some surfactants such as DMSO, Triton X, Tween 20 and Mercaptoethanol at the concentrations of 0.05, 0.10, 0.20, 0.50, 0.75, and 1.0µL were investigated on the activity of purified cellulase.

Procedure:

Zero point one milliltre of varying concentrations (0.05, 0.10, 0.20, 0.50, 0.75, and 1.0µL) of each of the inhibitor was added to 0.1 ml of purified cellulase. The reaction mixture was incubated for 15 min at 55 °C. Then, 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added to stop the reaction and the mixture was placed in boiling water for 5 min. The mixture was then allowed to cool and 5 ml of distilled water was added. The absorbance was measured at 540 nm.

3.11.12 Determination of Detergent Compatibility of Purified Cellulase

For the possible commercial application of cellulase in detergent industry, the purified cellulase was tested for its compatibility with various detergent brands in use such as omo, klin, wow, ariel, sunlight, mama and bimbo. The preparation of detergent was done based on the directions given on their respective sachets.

Procedure:

Carboxymethylcellulose (CMC) solution (1%) was used as substrate and prepared in citrate buffer of pH 4. A reaction mixture comprising 2 ml of substrate solution, 1.9 ml, detergent solution and 0.1 ml, purified cellulase was incubated at 55 °C for 10 - 15 minutes. 3 ml of 3,5-dinitrosalicyclic acid (DNS) solution was added and the mixture was placed in boiling water for 5 minutes. The mixture was then allowed to cool and 5 ml of distilled water was added. The

absorbance was measured at 540 nm. A control sample was also incubated in parallel to reaction mixture solution.

3.12 Statistical Analysis

All experiments and enzyme assays were performed in triplicates and the results were expressed as mean ± SEM. Graphpad prism version 6.02 was used to plot all the graphs.

Chapter Four

4.0 Results

4.1 Proximate Composition of *Arachis hypogaea* Shells

The proximate composition of *A. hypogaea* shells (untreated and treated) used as substrate is presented in Table 2. The crude fibre content, alkaline pretreated substrates had the highest percentage of crude fibre with an increase of about 6.09% when compared to acid pretreated and untreated *A. hypogaea* shell (control). Also, there was a significant increase ($p < 0.05$) in the content of crude protein following the treatment of *A. hypogaea* shell especially with alkaline pretreatment, compared to the untreated substrate with about 4 folds increase. There was about 19% reduction in the content of carbohydrates, upon treating the *A. hypogaea* shell with alkali compared with the untreated *A. hypogaea* shell. The untreated *A. hypogaea* substrate had the highest percentage of moisture content when compared to acid and alkaline pretreated substrate. There was no significant difference ($p > 0.05$) in the ash content of untreated, acid and alkaline pretreated *A. hypogaea* shell. There was about 24% reduction in the lipid content of alkali pretreated substrate when compared to untreated and acid pretreated *A. hypogaea* shell.

Table 2: Proximate Analysis of *A. hypogaea* Shell Substrate

Composition	Untreated (% w/w)	Acid Pretreatment (% w/w)	Alkaline Pretreatment (% w/w)
Moisture	4.0 ± 0.005^{b}	2.0 ± 0.005^{a}	1.8 ± 0.005^{a}
Ash	3.0 ± 0.005^{b}	2.5 ± 0.005^{a}	2.2 ± 0.005^{a}
Lipid	8.4 ± 0.005^{b}	8.0 ± 0.005^{b}	6.38 ± 0.005^{a}
Crude Fibre	82.0 ± 0.005^{a}	85.5 ± 0.005^{ab}	87.0 ± 0.005^{b}
Protein	0.16 ± 0.005^{a}	0.17 ± 0.005^{a}	0.65 ± 0.005^{b}
Carbohydrate	2.44 ± 0.005^{b}	1.83 ± 0.005^{a}	1.97 ± 0.005^{a}

Values are expressed as mean ± SEM of three different determinations. Values with different superscripts along a row are significantly different ($p < 0.05$) from one another.

The content of cellulose present in untreated and treated *A. hypogaea* shells is shown in Table 3. The alkaline pretreated substrates had the highest percentage of cellulose with 2.2 folds increase when compared to untreated substrate. Also, the percentage of cellulose in the acid pretreated substrate was higher with a 1.7 folds increase, compared to untreated *A. hypogaea* shell.

Table 3: Cellulose Content of Untreated and Treated *A. hypogaea* Shells

Pretreatment	Percentage (w/w)
Unpretreated	23.7 ± 2.8^{a}
Acid	39.1 ± 3.3^{b}
Alkaline	52.5 ± 2.5^{c}

Values are expressed as mean ± SEM of three different determinations. Values with different superscripts along a column are significantly different ($p < 0.05$) from one another.

4.2 Optimization of Cellulase Production

4.2.1 Effect of Incubation Period on Cellulase Production

The effect of incubation period on cellulase production is presented in Figure 4. The endoglucanase, exoglucanase and β-glucosidase showed maximum activity at 120 hours. Cellulase production increased as the time of incubation increased and reached its maximum production at 120 hours followed by gradual decrease up till 168 hours. There was about 4 folds' increase in activities of endoglucanase and β-glucosidase while that of exolucanase was about 14 folds at 120 hours.

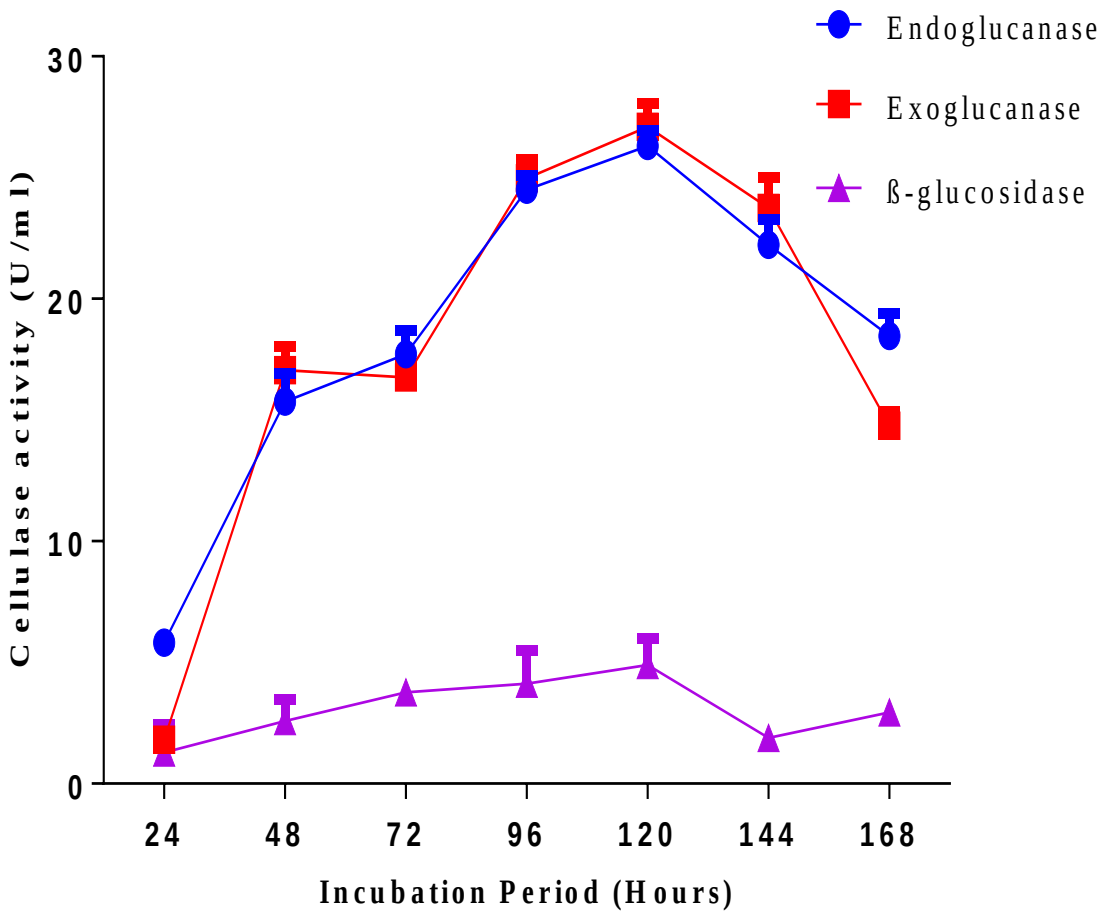


Figure 4: Cellulase activity of *A. niger* cultured on *A. hypogaea* shell at varying incubation times.

Each value is expressed as mean ± SEM of three different determinations.

4.2.2 Effect of pH on the Production of Cellulase

The effect of pH on cellulase production is shown in Figure 5. Cellulase production increased proportionately with increase in pH. Exoglucanase exhibited highest activity at a pH of 4.0 while endoglucanase and β-glucosidase had optimum pH in the range of 4.0 and 5.0. There was about 4, 5 and 4.5 folds increase in the activities of endoglucanase, exoglucanase and β-glucosidase respectively. A 2.0 increased in pH resulted in over 49%, 76.9% and 77% loss of endoglucanase, exoglucanase and β-glucosidase activities respectively.

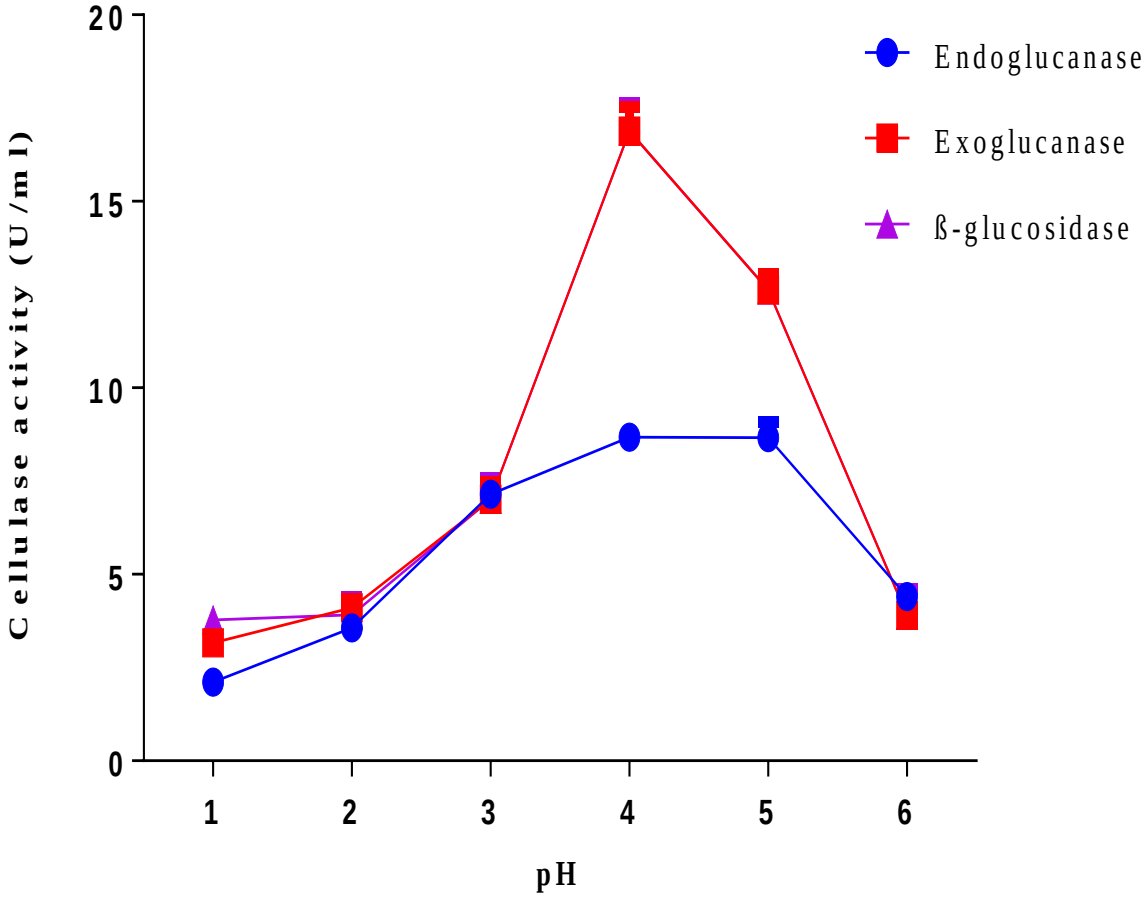


Figure 5: Cellulase activity of *A. niger* cultured on *A. hypogaea* shell at varying pH.

Each value is expressed as mean ± SEM of three different determinations.

4.2.3 Effect of Temperature on the Production of Cellulase

The effect of temperature on cellulase production is presented in Figure 6. Increase in temperature increased the activity of cellulase and thus increase the amount of cellulase production. Endoglucanase and β-glucosidase had an optimum temperature of 40 °C while exoglucanase had an optimum temperature of 50 °C. This result represents about 7 folds increase for endoglucanase and exoglucanase while there was about 1.5 folds increase in the activity of β-glucosidase. After optimum temperature, there was a stepwise decreased in the activities of three components of cellulase even with increased in temperature.

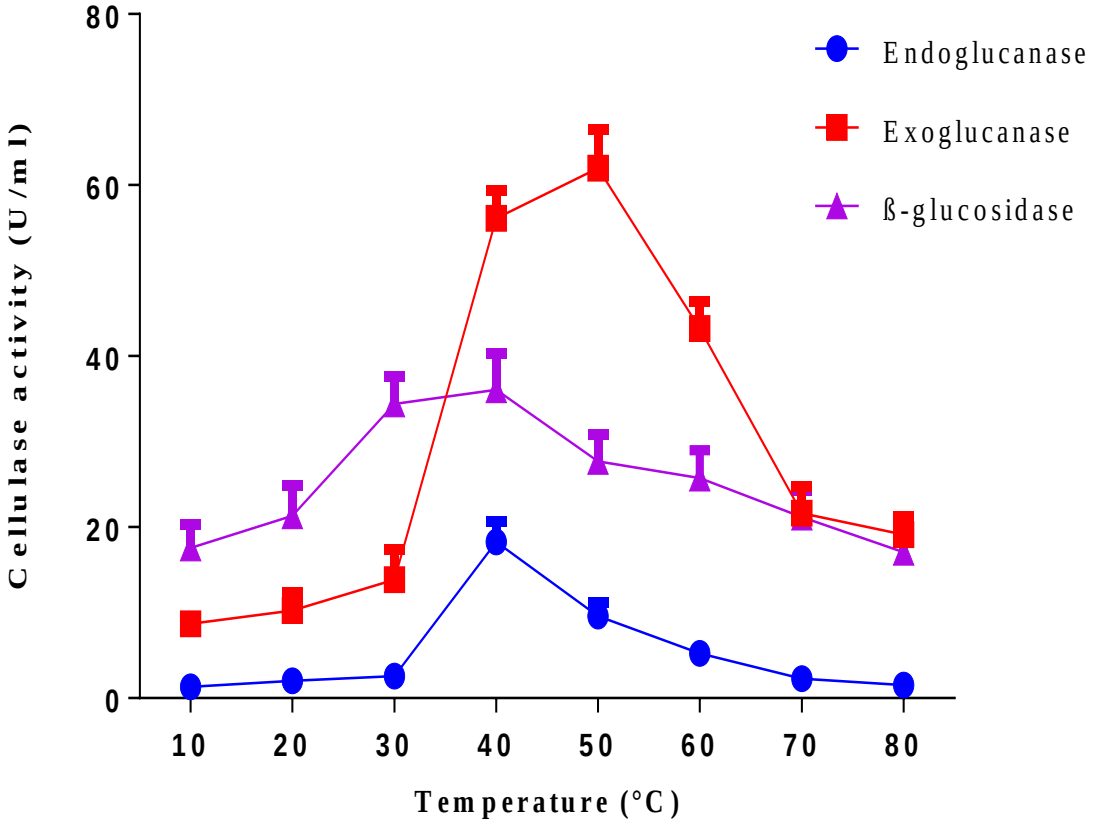


Figure 6: Cellulase activity of *A. niger* cultured on *A. hypogaea* shell at varying temperatures.

Each value is expressed as mean ± SEM of three different determinations.

4.2.4 Effect of Substrate Concentration on the Production of Cellulase

The activity of endoglucanase, exoglucanase and β-glucosidase increased with increase in substrate concentration (Figure 7). The activity of the three components measured followed Michaelis-menten hyperbolic curve with the substrate concentrations investigated. The activities of endoglucanase and β-glucosidase increased and reached maximum at 5% substrate concentration while exoglucanase activity dropped after reaching its maximum at 4% substrate concentration.

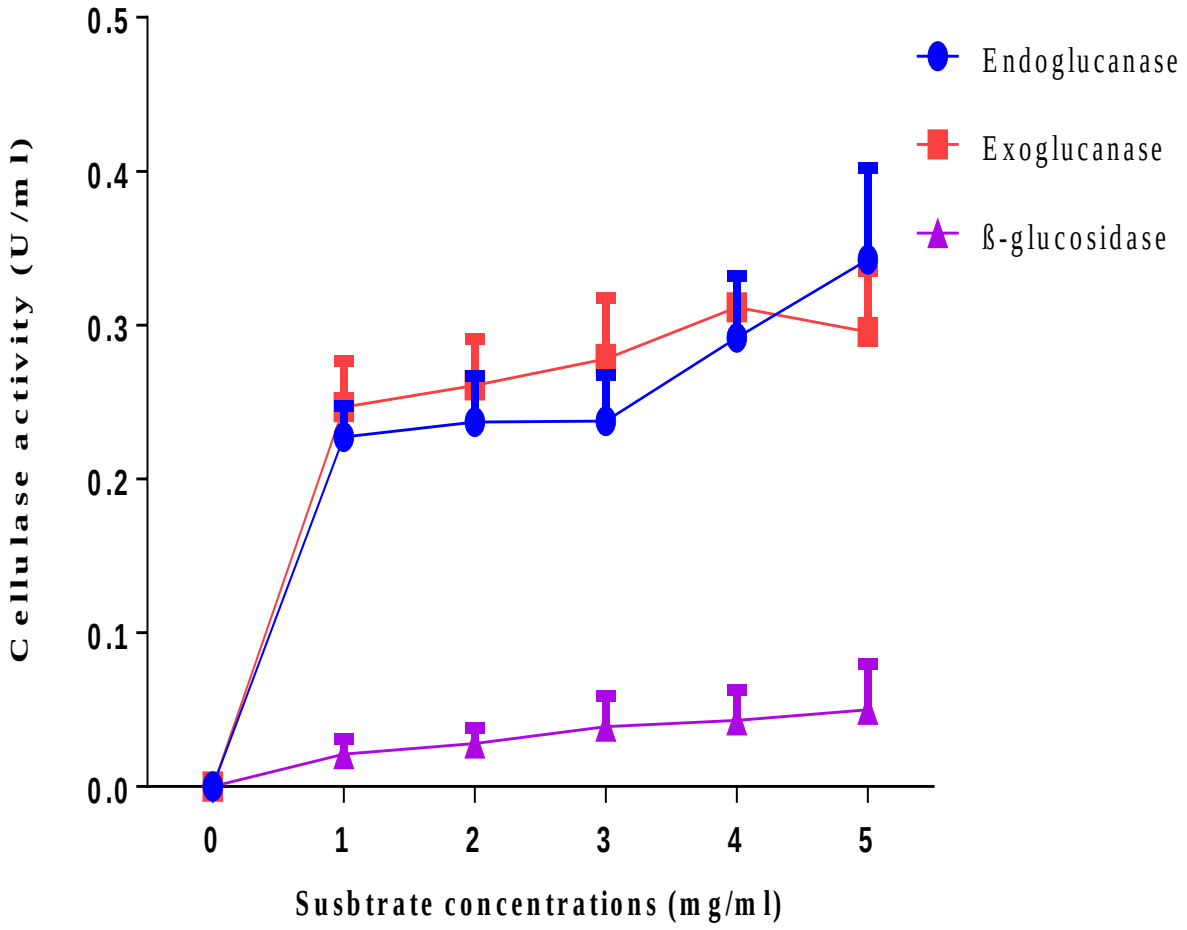


Figure 7: Cellulase activity of *A. niger* cultured on *A. hypogaea* shell at varying substrate concentrations.

Values are expressed as mean ± SEM of three different determinations.

4.2.5 Effect of Innoculum Size on Cellulase Production

The activities of endoglucanase, exoglucanase and β-glucosidase as a measure of cellulase production is shown in Figure 8. Increase in the size of inoculum resulted in increased activities of the three components of cellulase. The optimum innoculum size was between 10 to 13 cfu/ml for exoglucanase and endoglucanase while for β-glucosidase, optimum inoculum size was 13 cfu/ml.

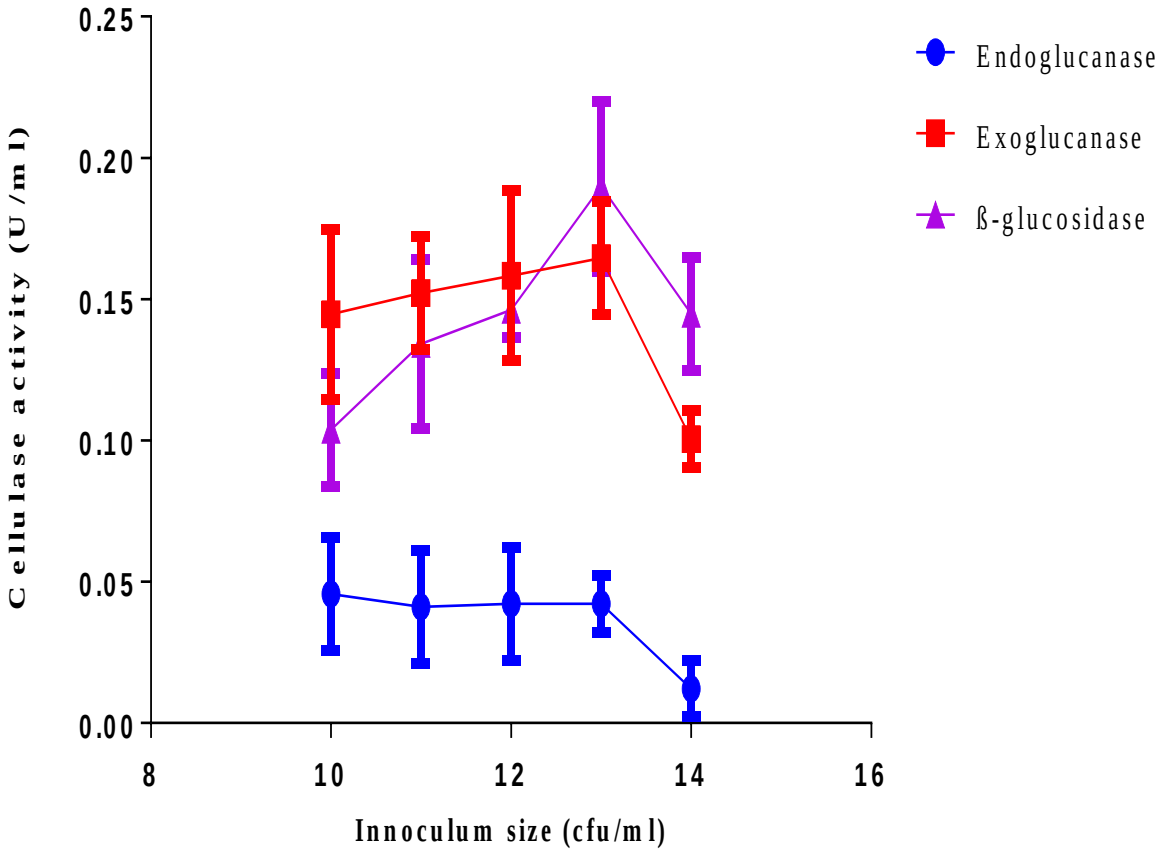


Figure 8: Cellulase activities produced by *A. niger* cultured on *A. hypogaea* shell at varying inoculum size.

Value are expressed as mean ± SEM of three different determinations.

4.2.6 Effect of Nitrogen Source on Cellulase Production

Figure 9 shows the activities of the three components of cellulase varied with different nitrogen source. Maximum endoglucanase and β-glucosidase activities were obtained in the presence of protease peptone as nitrogen source while exoglucanase showed maximum activity when yeast extract was used.

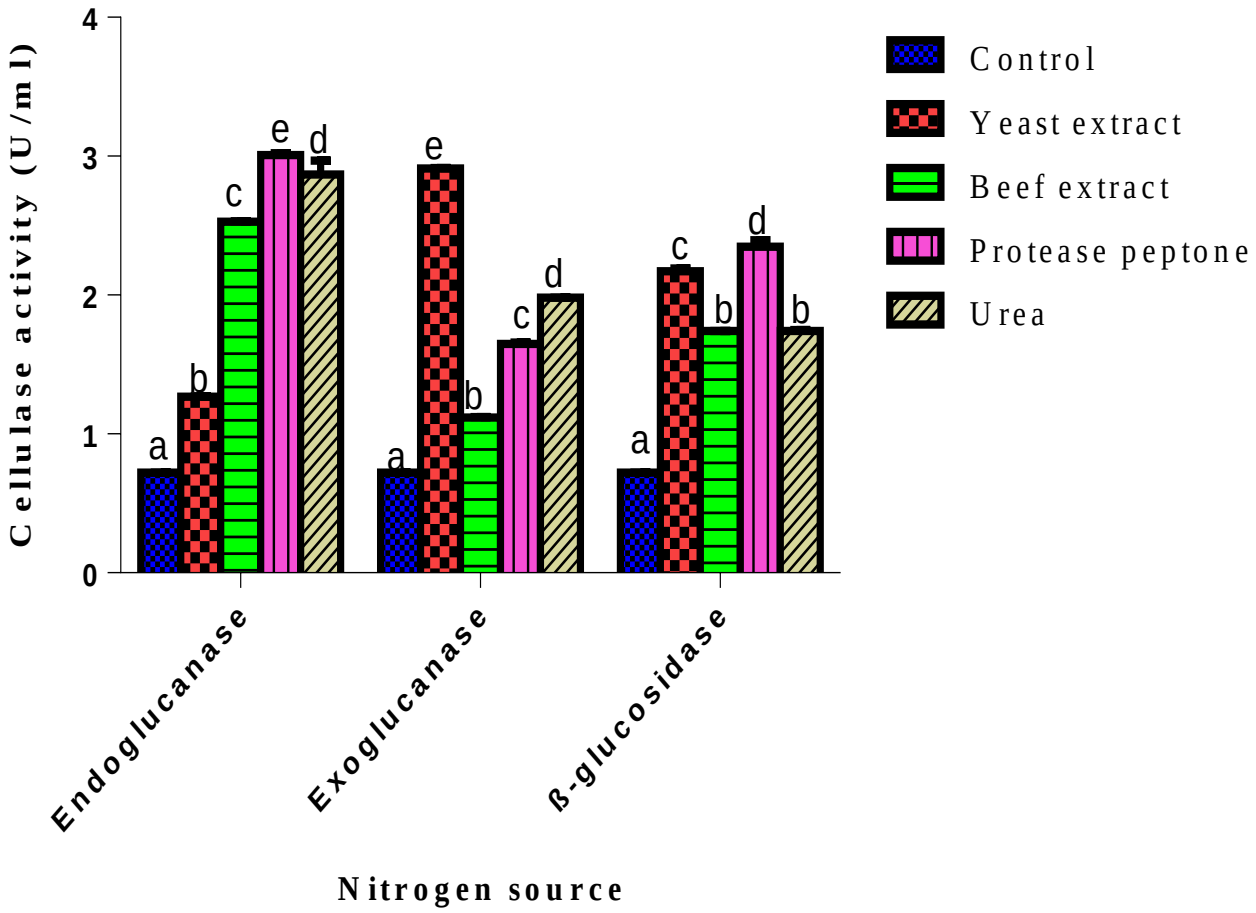


Figure 9: Cellulase activities produced by *A. niger* cultured on *A. hypogaea* shell using different nitrogen source.

Values are expressed as mean ± SEM of three different determinations. Bars with different superscripts for the parameter are significantly different ($p < 0.05$).

4.3 Purification of Cellulase Produced by *Aspergillus niger* Cultured on *Arachis. hypogaea* Shell

The summary of purification processes of cellulase produced by culturing *A. niger* on *A. hypogaea* shell is presented in Table 4. *A. niger* was innoculated in the fermentation medium and only the endo-ß-1,4-glucanase activity was determined under optimum fermentation conditions of 120 hours, pH of 4-5, temperature of 40 °C, substrate concentration of 5%, inoculum size of 10-13 cfu/ml and in the presence of protease peptone as nitrogen source. However, the activities of other 2 enzymes, exoglucanase and β-glucosidase were not expressed during purification. The crude cellulase had a total activity and specific activity of 87.69 U/ml and 7.11 U/mg respectively. As crude enzyme was subjected to each step of purification process, there was reduction in the total activity of the enzyme, which was accompanied by corresponding increase in the specific activity. Partially purified enzymes obtained by precipitation with 80% saturation of ammonium sulphate had a specific activity of 14.68 U/mg and 2.06-fold purification. Upon dialysis with three changes of buffer, the further partially purified cellulase gave specific acitivity of 222 U/mg and 31.22 folds purification. The purified cellulase obtained after sephadex G-100 gel filtration gave 68.12 folds purification and specific activity of 484.3 U/mg respectively (Table 4). Percentage yield of cellulase decreased stepwisely from 100% to 3.87%. The elution profile diagram of *A. hypogaea* shell cellulase on sephadex G-100 is presented in Figure 10.

Table 4: Summary of purification of cellulase produced from culture of *Aspergillus niger* under optimum fermentation conditions on *Arachis hypogaea* treated shells.

S/N	Purification Steps	Total Volume (mL)	Endoglucanase Activity (U/mL)	Total Protein (mg/mL)	Specific Activity (U/mg)	Purification Fold	Percentage Yield (%)
1	**Crude**	120	87.69	12.330	7.11	1	100
`2	**$(NH_4)_2SO_4$ Precipitation**	30	25.99	1.770	14.68	2.06	29.64
3	**Dialysis**	10	6.66	0.030	222.00	31.22	7.59
4	**Gel Filtration**	5	3.39	0.007	484.30	68.12	3.87

Value are expressed as mean of three different determinations.
Optimum fermentation conditions of 120 hours, pH of 4-5, temperature of 40 °C, substrate concentration of 5%, inoculum size of 10-13 cfu/ml and in the presence of protease peptone as nitrogen source.

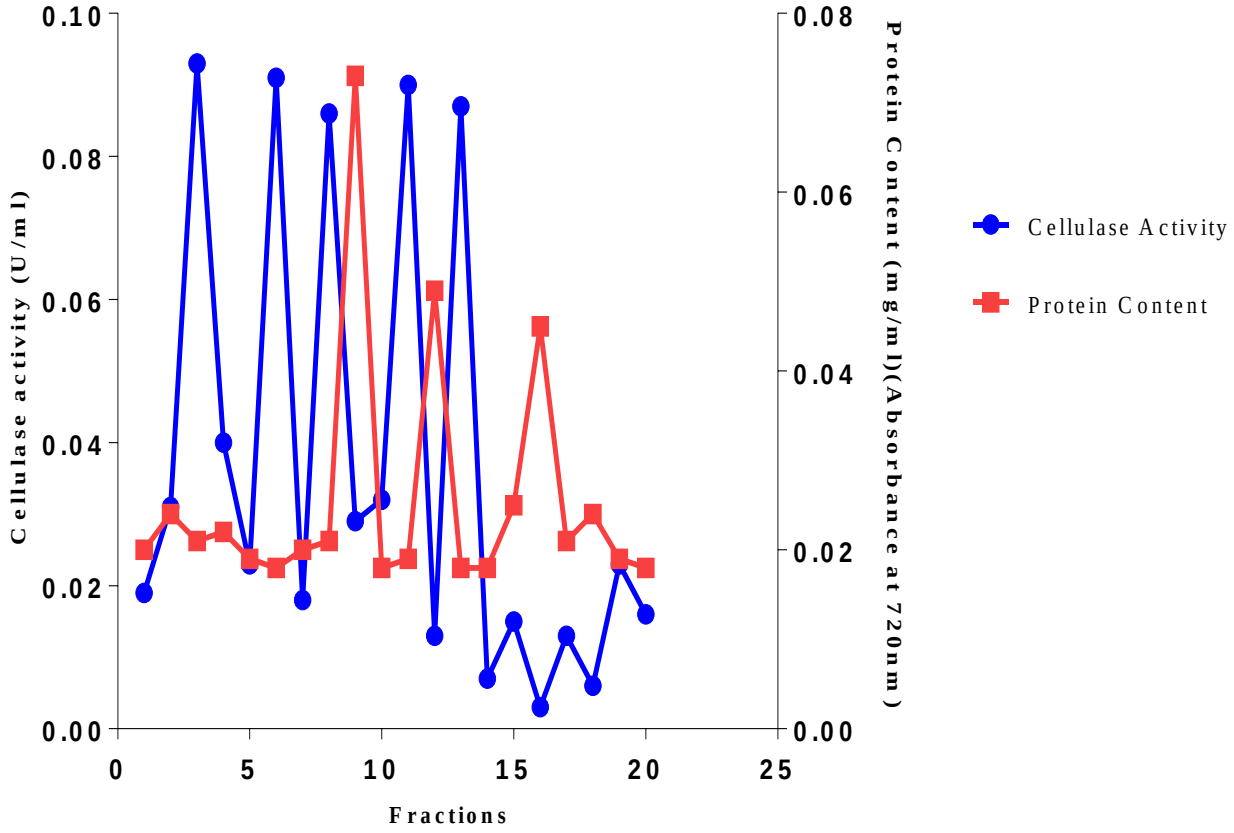


Figure 10: Elution Profile of *A. hypogaea* shell cellulase on sephadex G-100 chromatography.

4.4 Substrate Specificity of Purified Cellulase Produced by *Aspergillus niger* Cultured on *Arachis hypogaea* Shell

The purified cellulase showed highest relative activity with carboxylmethylcellulose as substrate and the least relative activity with filter paper (Table 5). The relative activity of purified cellulase was also low with other substrates like avicel, glucose, sucrose and $_{\rho}$-NPG.

Table 5: Substrate specificity of purified cellulase (endoglucanase)

Substrates	Relative Activity (%)
CMC	100
Avicel	32.60
Glucose	3.30
Sucrose	3.22
$_{P}$-NPG	3.16
Filter Paper	1.16

Each value is expressed as mean of three different determinations.

4.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) Profile of Purified Cellulase Produced by *Aspergillus niger* Cultured on *Arachis hypogaea* Shell

SDS-PAGE profile of cellulase produced by *A. niger* is shown in Figure 11. Lane A is the crude enzyme; B is the dialysed enzyme; C is the ammonium sulphate fraction; D is the enzyme obtained after gel filteration chromatography and E is the standard molecular markers of a known molecular weight. A clear single band was observed on lane D while more than one band were seen on lanes A - C. The purified cellulase observed in lane D has an estimated molecular weight of 13,500 Da based on the relative movement of the protein when compared to the standard marker on the gel from estimation on the calibration curve of the gel filtration on Sephadex G-100. Using SDS-PAGE, the purified cellulase showed a single band.

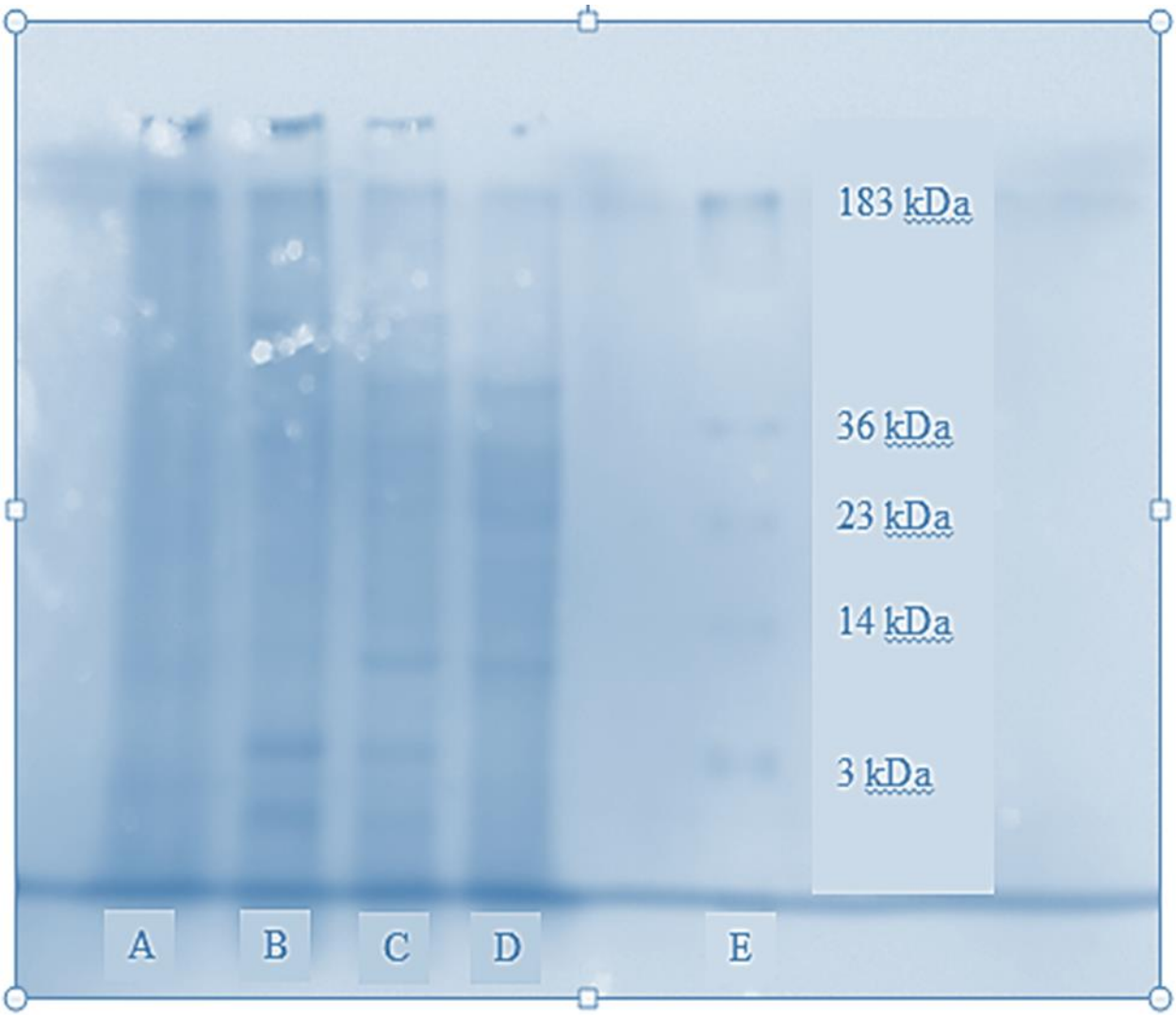


Figure 11: SDS-PAGE profile of cellulase produced by *A. niger* cultured on treated *A. hypogaea* shell.

Lane A, crude cellulase; B, ammonium sulphate precipitation; C, dialyzed enzymes; D, purified enzyme (13.5 kDa) and E, molecular weights in kDa of standard marker (standard protein markers are β-Galactosidase, 183 kDa; Phosphorylase B, 36 kDa; albumin, 23 kDa; ovalbumin, 14 kDa; carbonic anhydrase, 3 kDa).

4.6 Kinetic Analysis of Purified Cellulase

A plot of cellulase activity against concentration of substrate yielded a hyperbolic curve which showed that the purified cellulase obeyed Michaelis-Menten type kinetics (Figure 12). From the Lineweaver-Burk plot (Figure 13), K_m and V_{max} values of purified cellulase from *A. niger* were calculated to be 0.23 mg/ml and 9.26 U/ml respectively. The turn-over number, K_{cat}, for purified cellulase was extrapolated from the graph and it was found to be 0.08 per second.

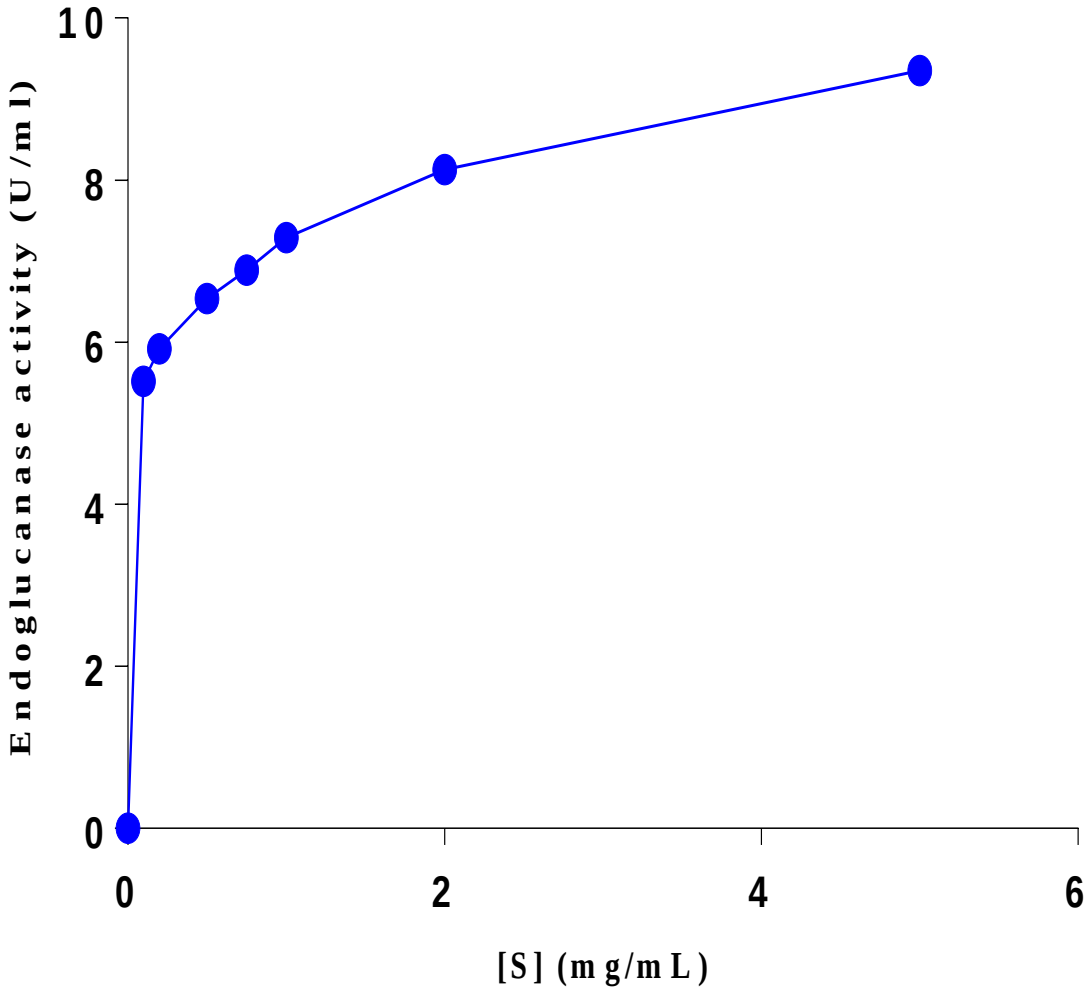


Figure 12: Substrate kinetics of purified cellulase produced by *A. niger*.

Values are expressed as mean of three different determinations.

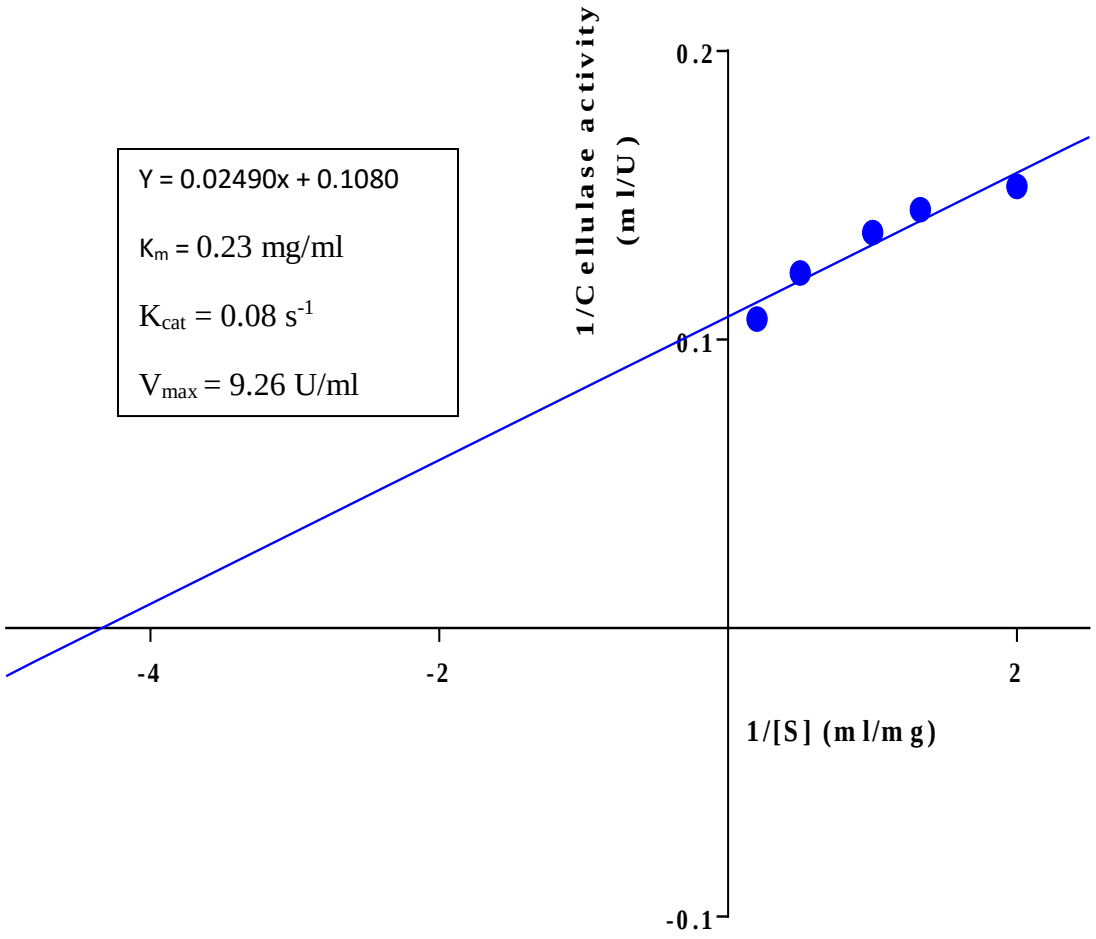


Figure 13: Lineweaver-Burk plot of cellulase hydrolyzing carboxylmethylcellulose.

Each value is expressed as mean of three different determinations.

4.7 Effect of pH on the Activity of Purified Cellulase

The endoglucanase activity of the purified cellulase was optimal at pH 4 with an activity of 48.78 U/mL (Figure 14). There was a drastic decrease in the activity of endoglucanase activity of purified cellulase above or below the pH 4 (Figure 14). A 1.0 change in pH resulted in over 50% loss of activity.

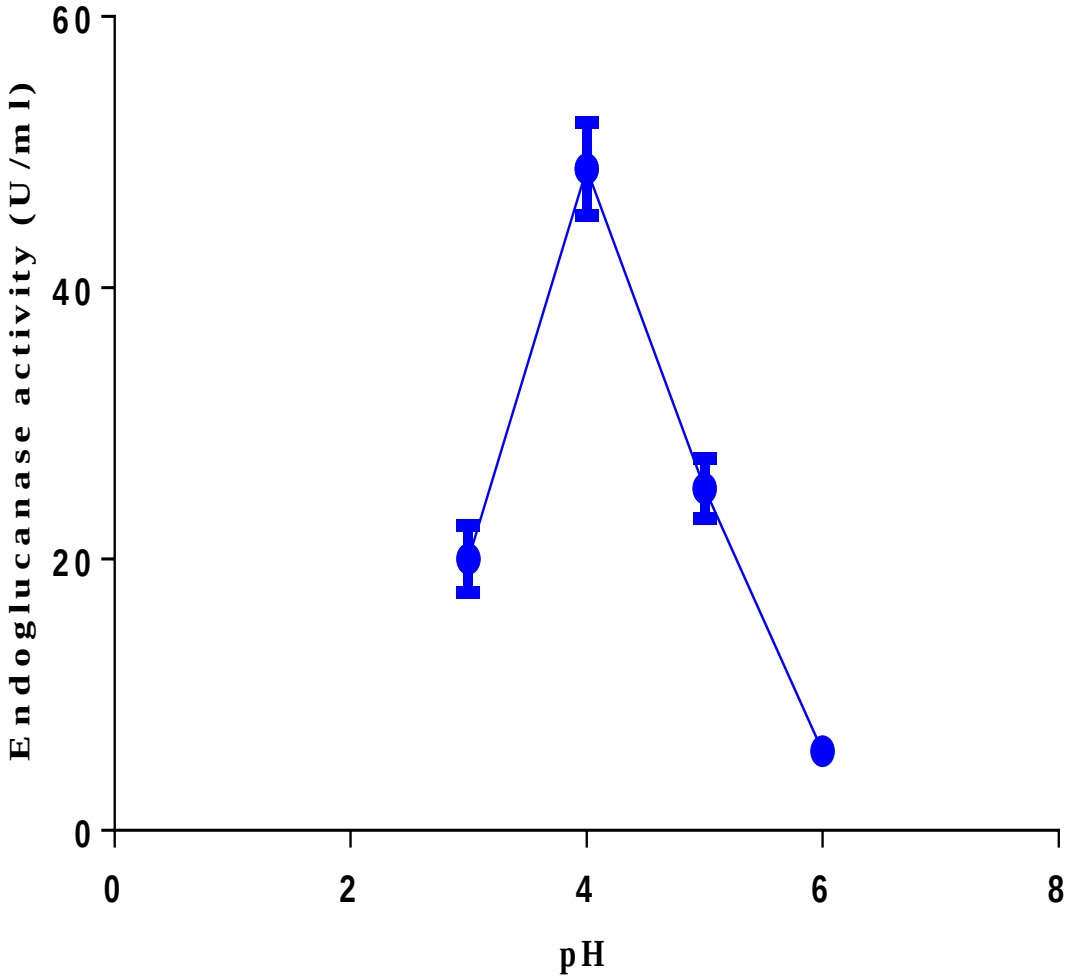


Figure 14: Effect of pH on endoglucanase activity of purified cellulase produced by *A. niger*.

Each value is expressed as mean of three different determinations.

4.8 Effect of Temperature on the Activity of Purified Cellulase

The endoglucanase activity of the purified cellulase was optimal at temperature of 40 ℃ There was a dire decrease in the activity of endoglucanase activity of purified cellulase above or below the temperature of 40 ℃ (Figure 15). A 5 ℃ increase or decrease in temperature resulted in about 64% fall in the activity, while a 10 ℃ increase in temperature resulted in about 158% decrease in activity.

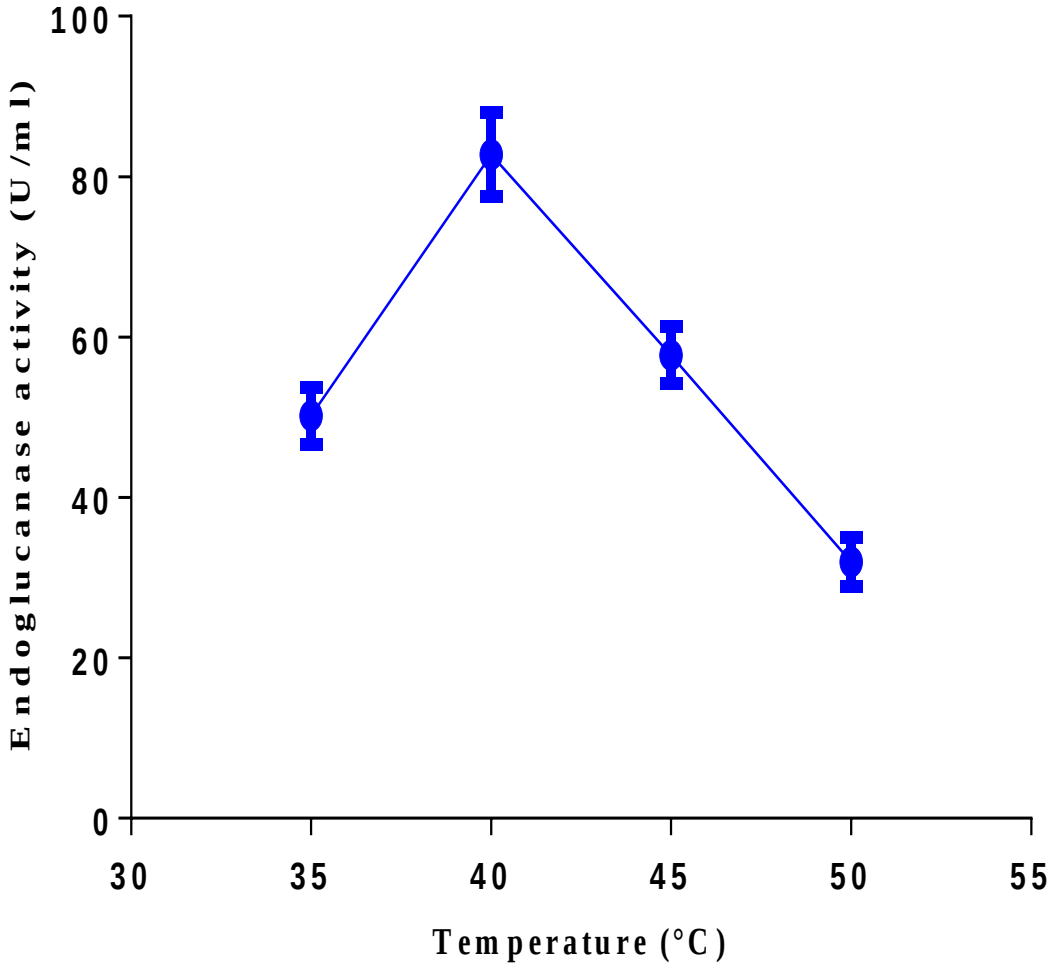


Figure 15: Effect of temperature on endoglucanase activity of purified cellulase produced by *A. niger*.

Each value is expressed as mean of three different determinations.

4.9 Effect of Cations on Endoglucanase Activity of Purified Cellulase

4.9.1 Effect of Monovalent Cations on Endoglucanase Activity of Purified Cellulase

The effect of monovalent cations (Na^+ and K^+) is presented in Figure 16. The Na^+ at concentrations above 0.5 mM stimulates the activity of purified cellulase with about 1.48 folds increase while K^+ at all the concentrations investigated (0.05, 0.1, 0.2, 0.5, 0.75 and 1.0 mM) inhibited endoglucanase activity of purified cellulase.

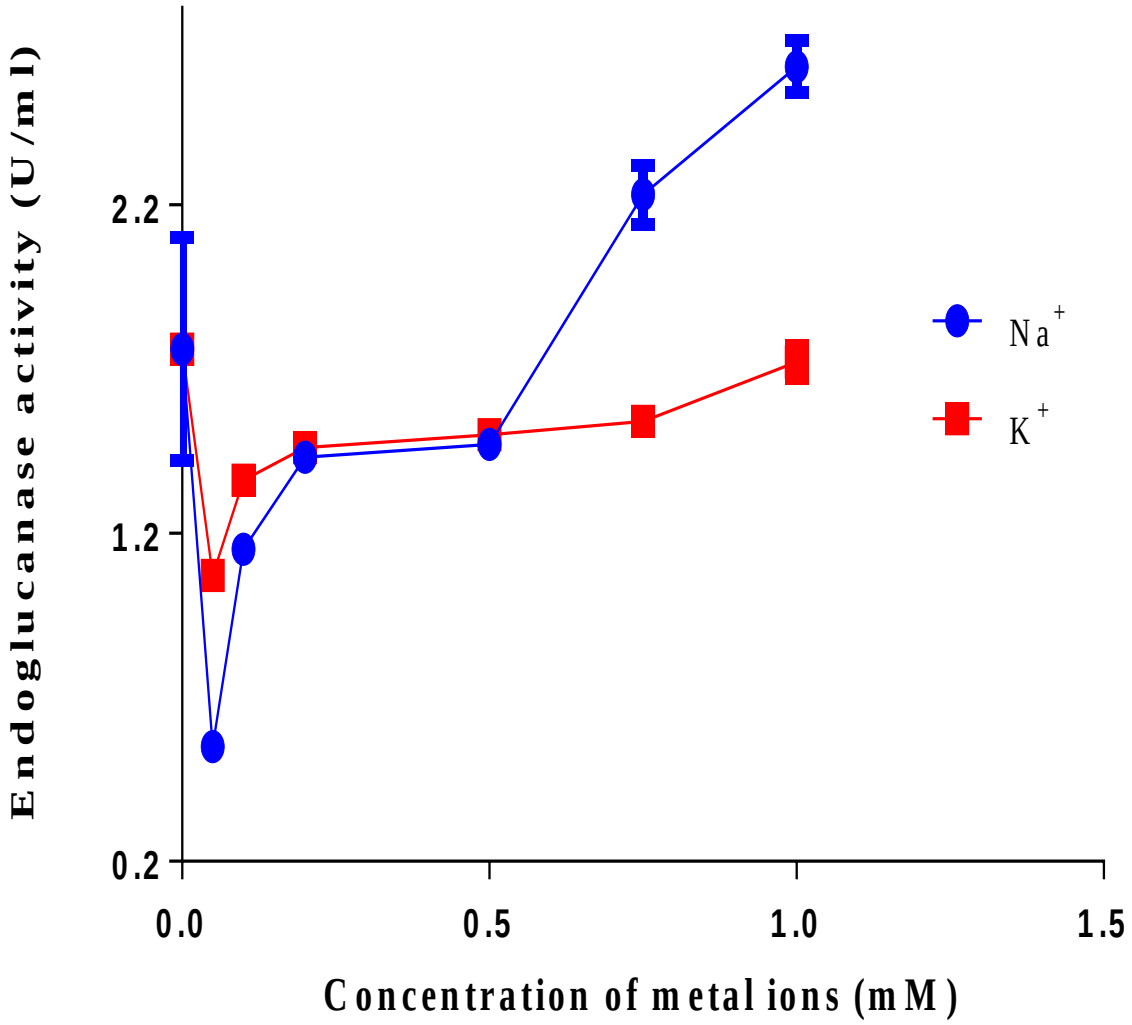


Figure 16: The influence of Na^+ and K^+ on endoglucanase activity.

Each value is expressed as mean ± SEM of three different determinations.

4.9.2 Effect of Divalent Cations on Endoglucanase Activity of Purified Cellulase

Divalent metal ions such as Mg^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} inhibited the activity of purified cellulase at all the concentrations investigated as compared to when divalent cations were not added while Mn^{2+} and Co^{2+} stimulate the activity of purified cellulase at all the concentrations investigated (Figure 17). Addition of Mg^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+} and Fe^{2+} to the enzyme solution resulted to 83%, 64%, 64%, 97% and 99% loss of activity respectively.

The lineweaver-burk plot of purified cellulase catalyzed hydrolysis of carboxylmethylcellulose in the presence and absence of Mg^{2+} is shown in Figure 18. The value of K_m in the presence of Mg^{2+} was 0.1176mg/ml which was greater than the value obtained in the absence of Mg^{2+} which was 0.0212mg/ml. The V_{max} of the purified cellulase in the presence and absence of Mg^{2+} was approximately 1.66 U/ml.

The maximum rate of reaction (V_{max}) was 1.418 U/ml in the presence of Zn^{2+} with K_m of approximately 0.02 mg/ml. However, the V_{max} of purified cellulase was found to be 1.66 U/ml with K_m of 0.02 mg/ml in the absence of Zn^{2+} (Figure 19).

The lineweaver-burk plot of purified cellulase catalyzed hydrolysis of carboxylmethylcellulose in the presence and absence of Cu^{2+} is presented in Figure 20. The V_{max} in the presence of Cu^{2+} was 1.478 U/ml with a K_m of 0.02 mg/ml. The V_{max} obtained in the presence of Cu^{2+}, 1.478 U/ml, was lowered than 1.620 U/ml obtained in the absence of Cu^{2+} though the same K_m of 0.02mg/ml was obtained.

The V_{max} of purified cellulase in the presence of Ca^{2+} was 0.095 U/ml lower when compared with 1.590 U/ml obtained in the absence of Ca^{2+}. The K_m of purified cellulase in the presence and absence of Ca^{2+} was calculated to be 0.012 and 0.017 mg/ml respectively (Figure 21).

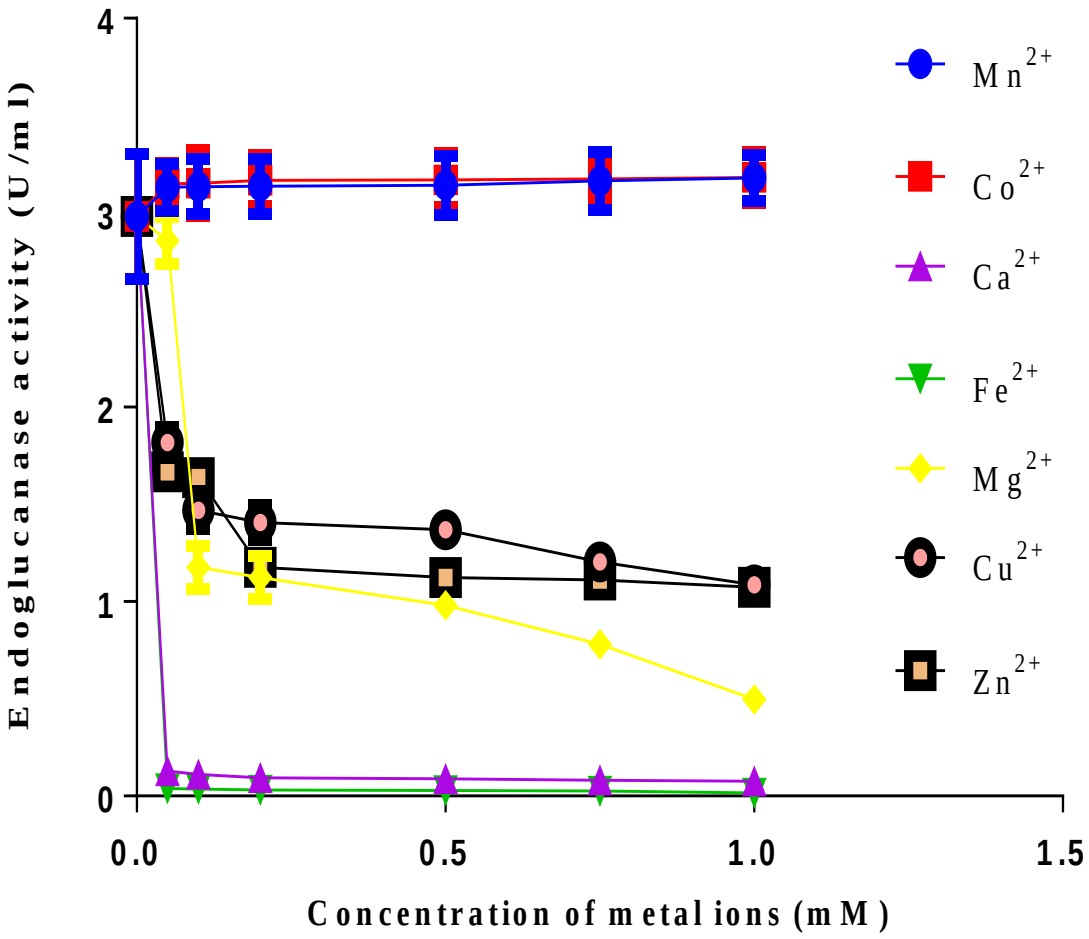


Figure 17: Effect of divalent cations (Mn^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}, and Zn^{2+}) on the activity of purified cellulase.

Each value is expressed as mean ± SEM of three different determinations.

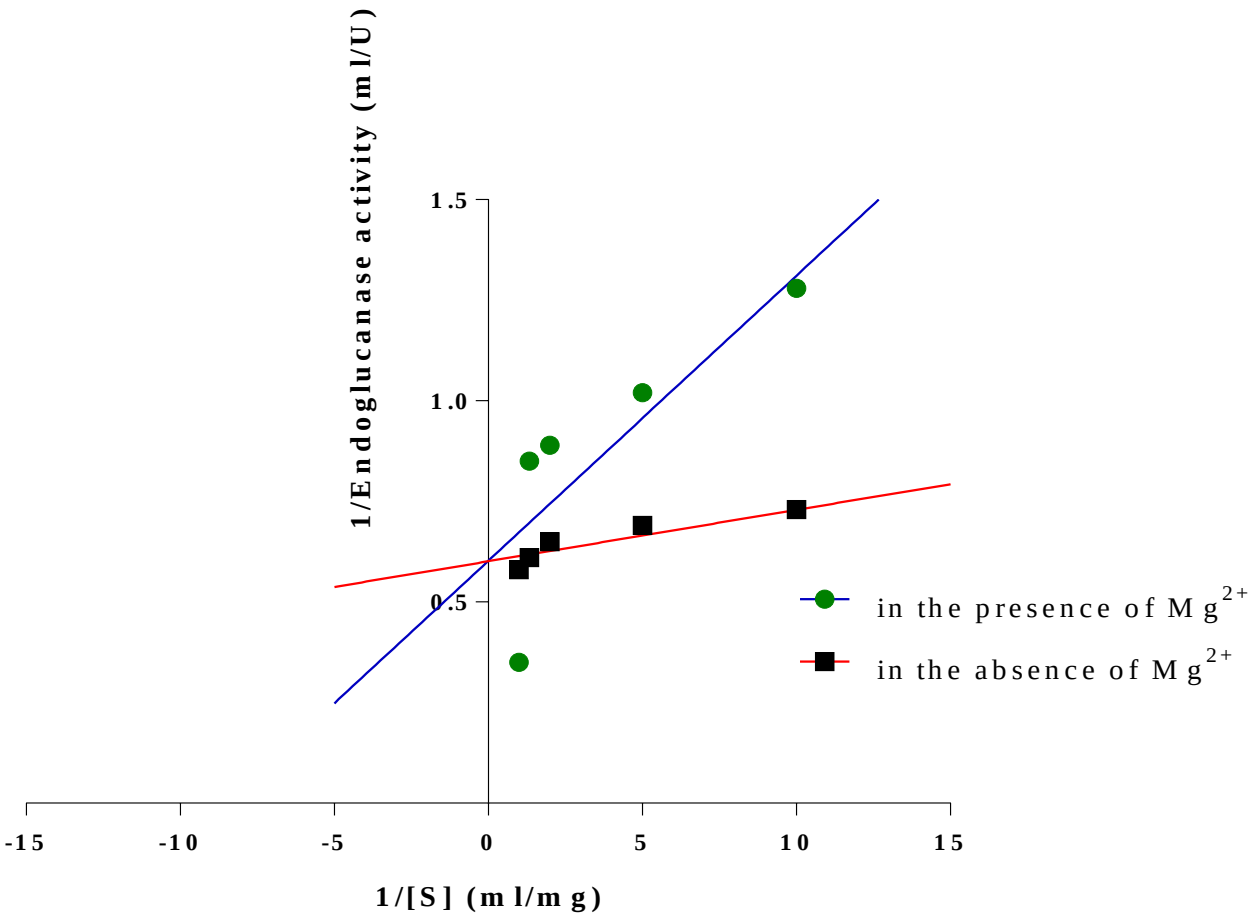


Figure 18: Lineweaver-Burk plot of cellulase catalysed hydrolysis of carboxylmethylcellulose in the presence of Mg^{2+}.

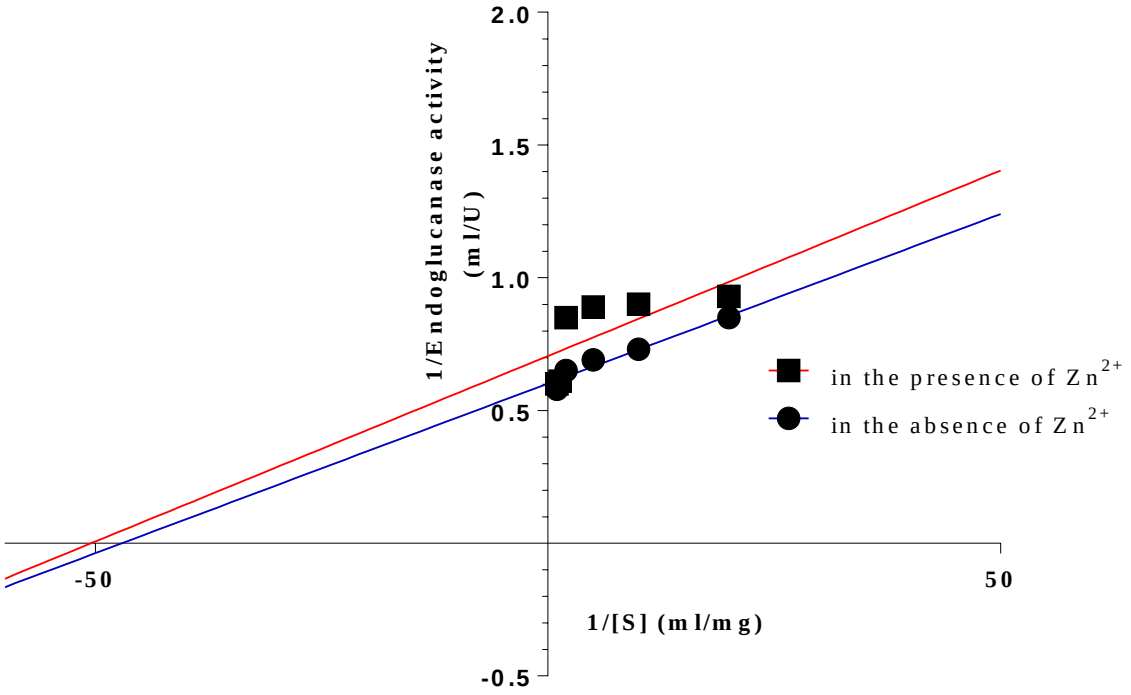


Figure 19: Lineweaver-Burk plot of cellulase catalysed hydrolysis of carboxylmethylcellulose in the presence of Zn^{2+}.

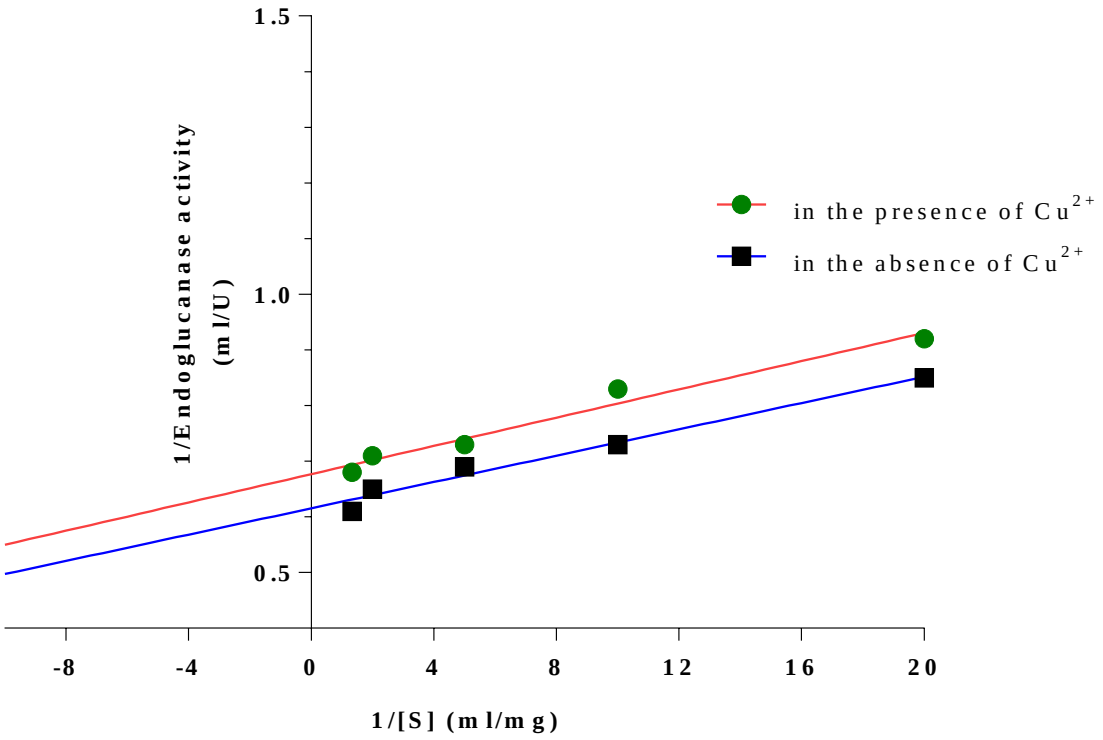


Figure 20: Lineweaver-Burk plot of cellulase catalysed hydrolyzing carboxylmethylcellulose in the presence of Cu^{2+}.

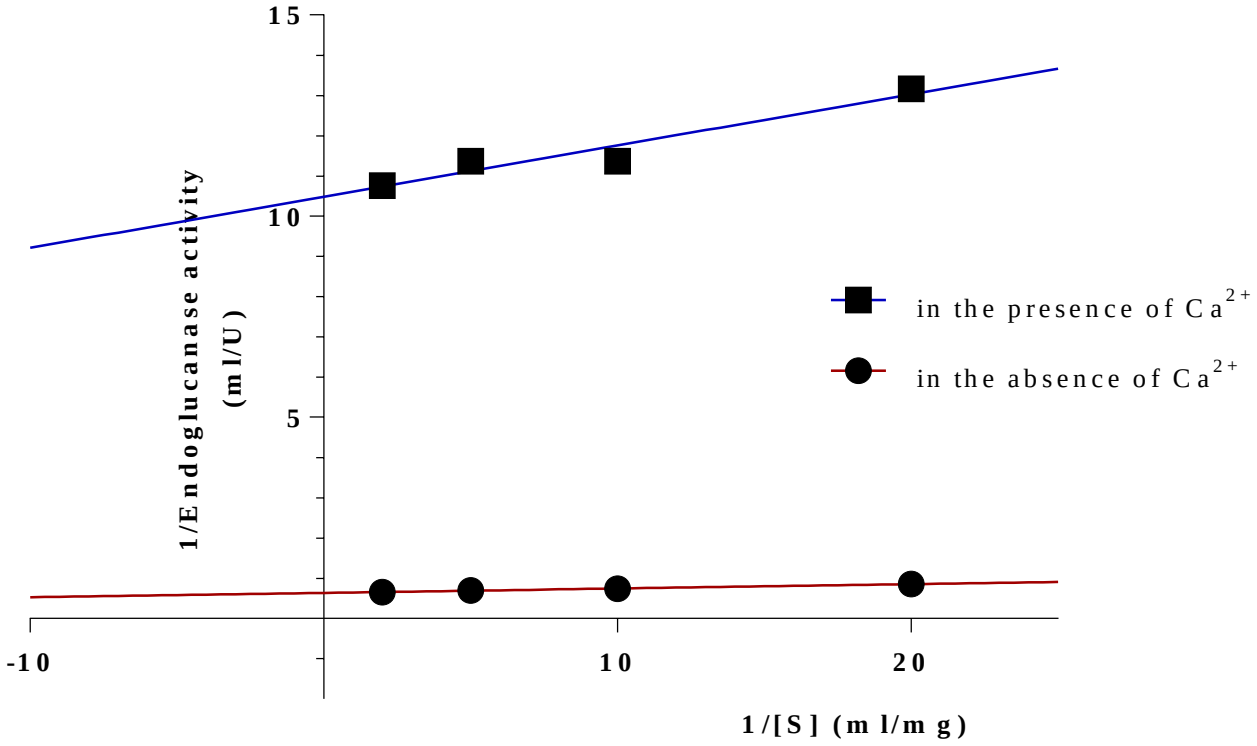


Figure 21: Lineweaver-Burk plot of cellulase catalysed hydrolysis of carboxylmethylcellulose in the presence of Ca^{2+}.

Figure 22 shows the lineweaver-burk plot of purified cellulase catalyzed hydrolysis of carboxylmethylcellulose in the presence and absence of Fe^{2+}. The value of K_m in the presence and absence of Fe^{2+} 0.02 mg/ml respectively. However, the V_{max} of the purified cellulase in the presence and absence of Fe^{2+} differs with values of 0.039 and 1.663 U/ml respectively. The summary of K_m and V_{max} for each of the metal ions is presented in Table 6.

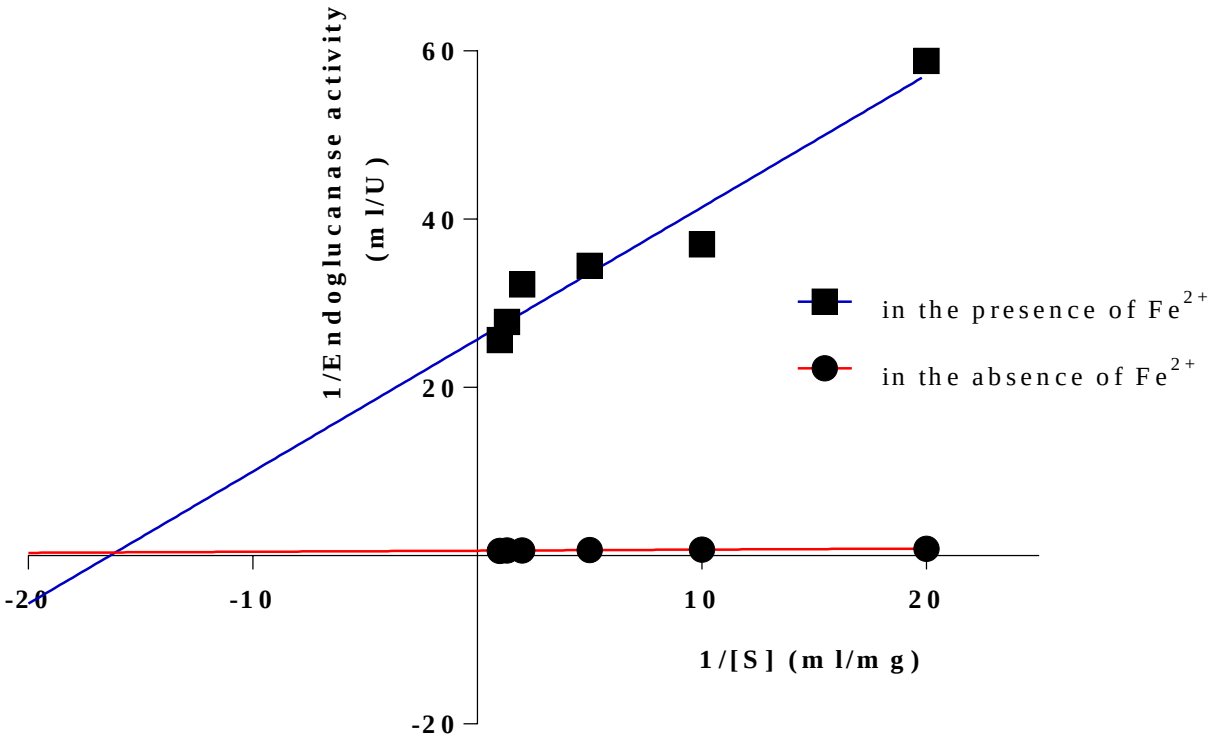


Figure 22: Lineweaver-Burk plot of cellulase catalysed hydrolysis of carboxylmethylcellulose in the presence of Fe^{2+}.

Table 6: Effect of Cations on Kinetic Parameters of Purified Cellulase

Cations	V_{max} (U/ml)	K_m (mg/ml)
Absence of Mg^{2+}	1.663	0.0212
1 mM Mg^{2+}	1.660	0.1176
Absence of Zn^{2+}	1.660	0.020
1 mM Zn^{2+}	1.418	0.020
Absence of Cu^{2+}	1.620	0.020
1 mM Cu^{2+}	1.478	0.024
Absence of Ca^{2+}	1.590	0.017
1 mM Ca^{2+}	0.095	0.012
Absence of Fe^{2+}	1.663	0.020
1 mM Fe^{2+}	0.039	0.0612

4.10 Effect of Anions on Endoglucanase Activity of Purified Cellulase

The effect of some anions (CO_3^{2-}, Cl^- and SO_4^{2-}) on the activity of purified cellulase are presented in Figures 23. The presence of Cl^- and SO_4^{2-} at 0.1 and 0.2 mM respectively activated cellulase enzyme with about 2.9 and 3.2 folds increase in the activity though the activity of cellulase dropped drastically as concentration of Cl^- and SO_4^{2-} increased. There was a loss of activity of about 32% in the presence of 1 mM Cl^-. As observed from the result, there was a decrease in the activity of cellulase in the presence of CO_3^{2-} at all the concentrations (0.05, 0.1, 0.2, 0.5, 0.75 and 1.0 mM) investigated. Addition of CO_3^{2-} inhibited the activity of partially purified cellulase with about 95% loss of activity. Also, the concentrations above 0.2 and 0.5 mM in the presence of Cl^- and SO_4^{2-} respectively inhibited the cellulase (Figure 23).

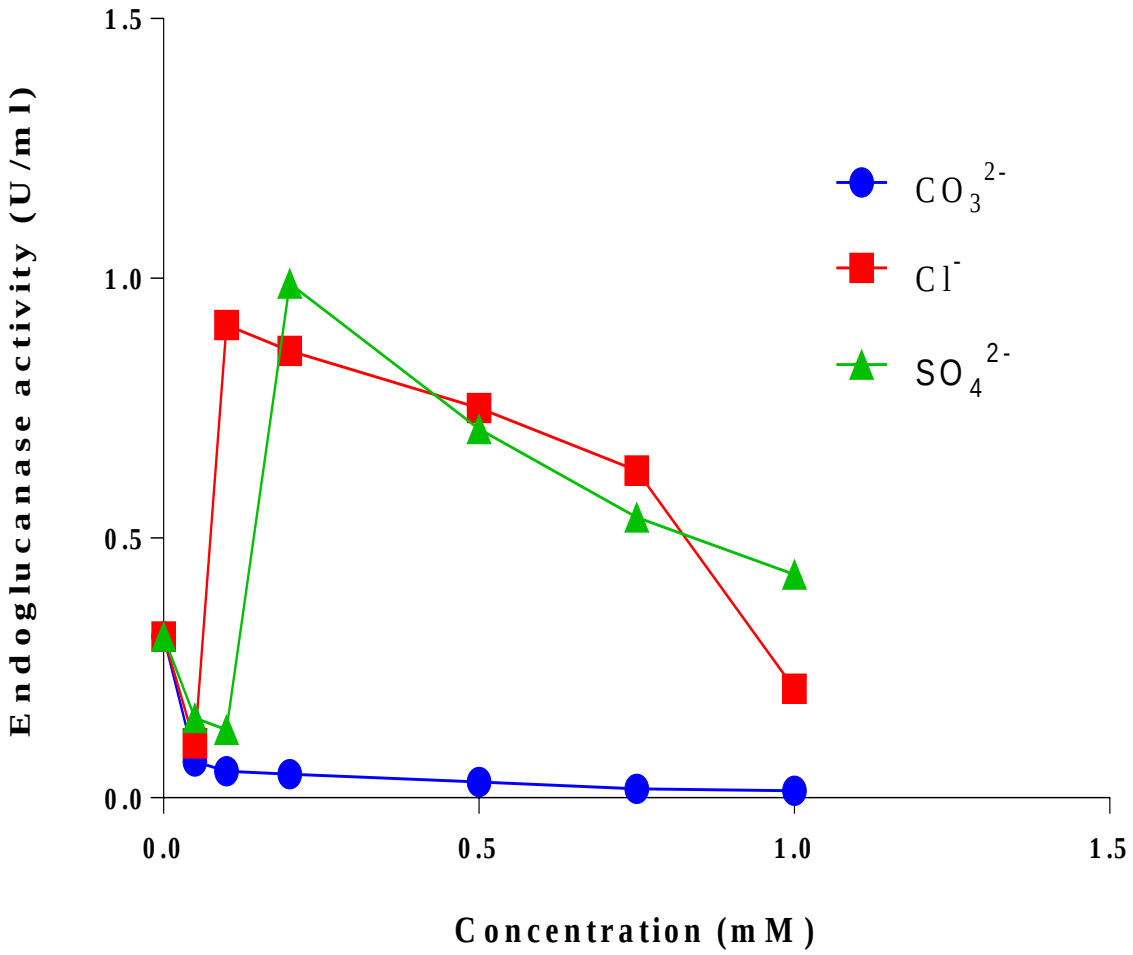


Figure 23: Effect of some anions on endoglucanase activity of purified cellulase.

4.11 Effect of Some Surfactants on Endoglucanase Activity of Purified Cellulase

The effect of DMSO, Trton X, Tween 20 and Mercaptoethanol on endoglucanase activity of purified cellulase is shown in Figure 24. The addition of DMSO decreased the activity of purified cellulase and this resulted in about 49% loss of activity. Also, addition of Triton X, Tween 20 and Mercaptoethanol inhibited the activity of purified cellulase at 0.1 to 1.0 mM concentrations which resulted to 31%, 85% and 98% loss of activity respectively.

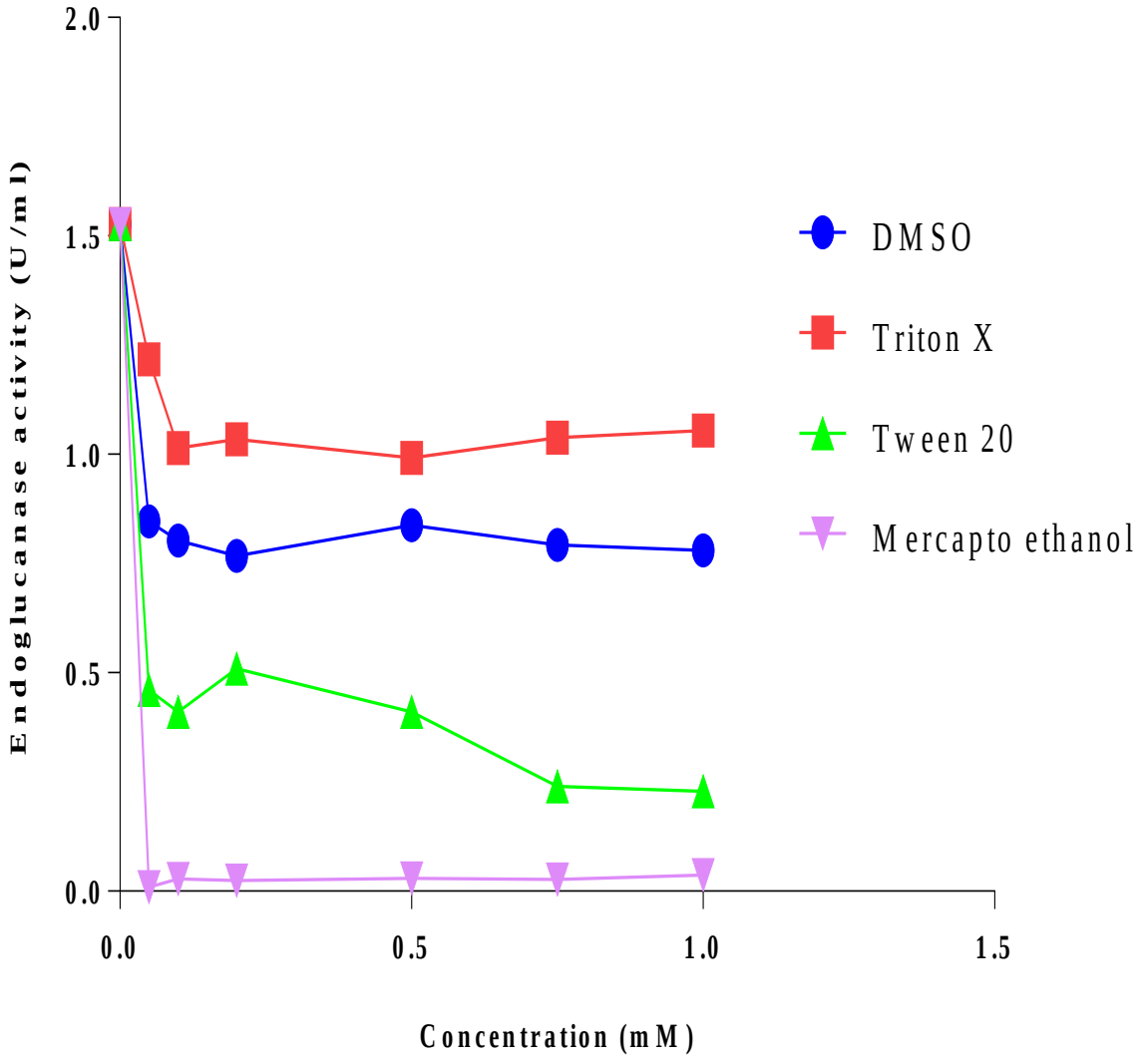


Figure 24: Effect of DMSO, Triton X, Tween 20 and Mercaptoethanol on the activity of purified cellulase.

4.12 Detergent Compatibility of a Purified Cellulase

The enzyme incubated at 55 °C with detergent solution revealed maximum compatibility with Omo, Klin, Wow, Ariel, Sunlight, Mama and Bimbo (Figure 25). Therefore, their suitable controls were also run and their activities were found to be very low as compared to those with cellulase. This revealed that the cellulase is compartible with local detergents and suggesting its potential as suitable additive to detergents.

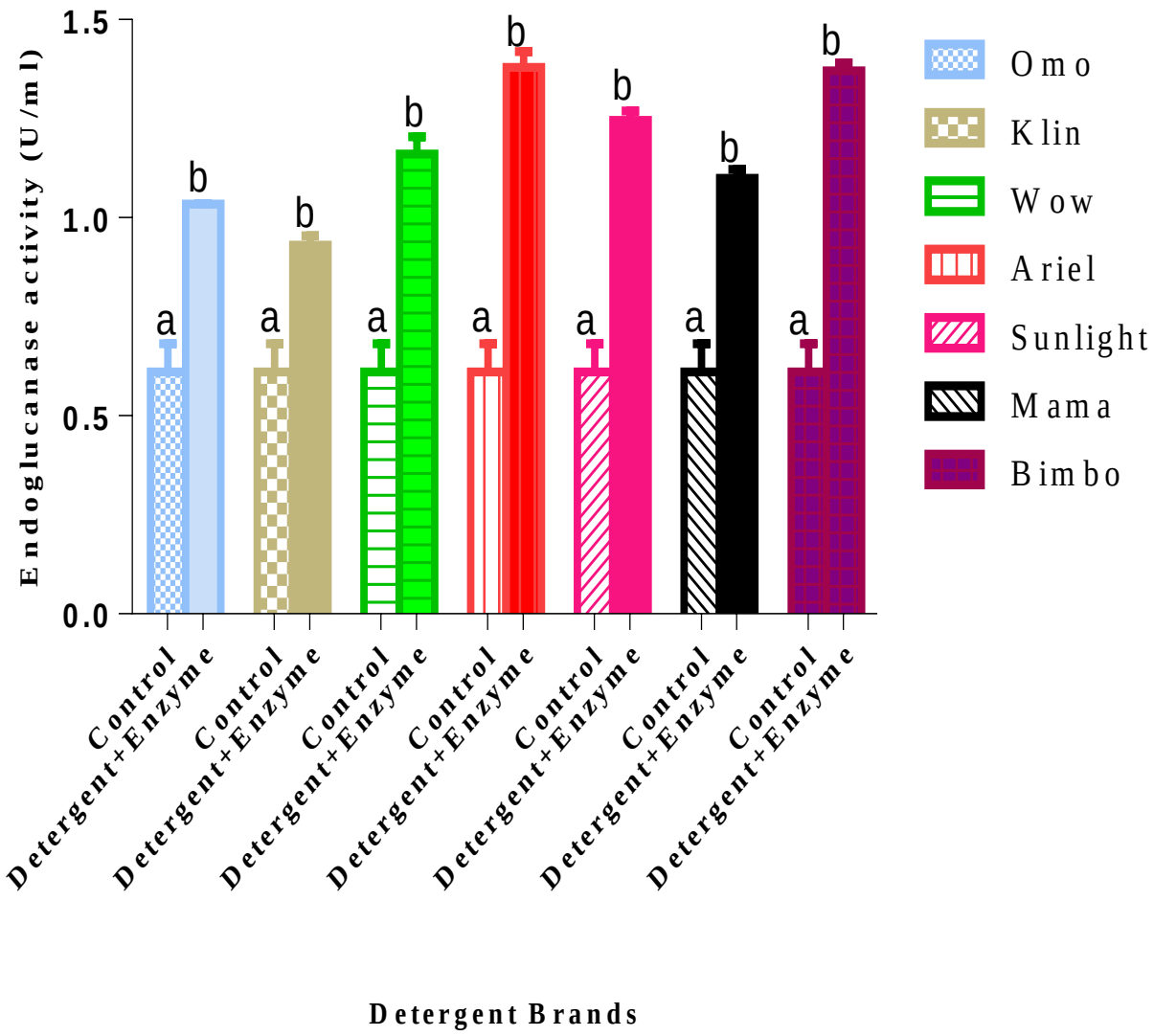


Figure 25: Detergent compatibility of purified cellulase with various detergent brands.

Each value is expressed as mean ± SEM of three determinations. Bars with different superscripts for the parameter are significantly different ($p < 0.05$).

Chapter Five

5.0 Discussion

The substantial amounts of waste materials such as shells, tatters, trunks, peels and seeds are engendered as a result of agricultural practice. Large quantities of these agrowastes obtained are from heavy consumptions of agricultural products. These wastes particularly groundnut shells are copious because once the nuts have been removed, the shells are always discarded. Accumulation of these shells constitutes what we called "wastes" and consequently lead to environmental pollution. Transformation of these wastes to expedient products will not only combat environmental pollution arising from unnecessary discarding of shell but also boast the economy of our country. The uses of different agricultural wastes such as corn cob, rice bran, bagasses, wheat bran, banana trunk for production and characterization of cellulase have been well documented (Yang *et al*., 2006). Therefore, this study addressed the possible use of *Arachis hypogaea* shells as a substrate for cellulase production from *Aspergillus niger*.

5.1 Proximate Analysis of Pretreated *A. hypogaea* Shells

The potential usefulness of agricultural wastes depends on pretreatment approaches as well as chemical composition. An efficient pretreatment distorts cell wall physical obstructions as well as cellulose crystallinity and connotation with lignin so that biomass can be amenable to enzymes hydrolysis (Wyman *et al*., 2005). The pretreatment process has become an vital step, restraining the progress of cellulosic ethanol (Himmel *et al.*, 2007; Yang and Wyman, 2008). Several approaches have been employed for the treatment of lignocellulosic biomass, but only few of them gave encouraging results.

In this study, chemical pretreatment involving acid and alkaline were employed to choose the most suitable pretreated substrates that will serve as inducer of cellulase from *A. niger*. The

results obtained showed that alkaline pretreatment produced the highest percentage of crude fibre as well as highest percentage of cellulose, an indication that it may serve as the best substrate for cellulase production from *A. niger*. The alkaline solutions used in treating *A. hypogaea* shells employed in this study might have removed some considerable amounts of lignins present in the *A. hypogaea* shells and increased it digestibility. This agreed with the findings of Beukes and Pletschke (2011) who reported that alkali removes lignins and make agrowaste more diagestible. Some reports have indicated that alkaline pretreatments make enzyme available and thus ease the hydrolysis of lignocellulosic biomass (Beukes and Pletschke, 2011). One of the benefits of employing alkaline as a pretreatment strategy is the flexibility of the process this is because alkaline pretreatments can be carried out at different conditions of temperatures as well as chemical concentrations (Beukes and Pletschke, 2011). Mosier *et al.* (2005) and Chang (2007) reported that alkali pretreatments solubilized lignin and increase the approachability of the lignocellulose surface by the removal of acetyl and uronic acid substituents on hemicellulose.

The proximate analysis of the treated and untreated *A. hypogaea* was carried out in order to determine the percentage crude fiber as well as cellulose composition in each of the pretreated *A. hypogaea* shells. The result obtained from this study revealed treated, and untreated substrates have high percentage of crude fiber. Crude fiber is a measure of the quantity of undigested carbohydrates such as cellulose, pentosans, lignin, pectins and other components of this type present in foods (Van Soest and Robertson, 1979). It is the residue of plant materials remaining after solvent extraction followed by hydrolysis with dilute acid and alkali. The undigested or dietary fibers are a complex mixture of different materials. The main ones are cellulose, the glucose polymer that is the predominant material of plant cells; hemicellulose, a shorter version of cellulose: pectin, the glue that binds plant cells together with cellulose from the woody cell

walls of plants (Gidenne, 2003). The high crude fibre content obtained from this study for untreated, pretreated shells is an indication that *A. hypogaea* shell is very rich in cellulose and can serve as an inducer for cellulase production. However, alkaline pretreatment gave the highest percentage of cellulose (52.5%) compared to acid pretreatment (39.1%) and unpretreated substrate (23.7%). This observation was in agreement with findings of Gimba *et al*. (2010) who reported the value of 50- 60% cellulose from *A. hypogaea* shells. It can therefore, be said that alkaline treated *A. hypogaea* may be the preferred substrate for cellulase production from *A. niger*.

5.2 Optimization of Cellulase Production

Incubation period plays an important role in the production of enzymes. Enzyme production by microorganisms especially *A. niger* is based on the two factors that are very significant during fermentation processes; these are the specific growth rate of microorganisms as well as synthetic ability of the organisms (Kunamneni *et al*., 2005). In the present study cellulase activity as a measure of cellulase production increased steadily and reached maximum at 120 hours of incubation under submerged fermentation (SmF) (Figure 4). Further extension of incubation time beyond 120 hours resulted in loss of cellulase activity which might be as a result of reduction in enzyme production. This reduction in cellulase activity might be due to the exhaustion of carbon source in the fermentation medium with the lapse in time, which stressed the fungal physiological response to enzyme production resulting in the decrease in the secretory ability of microorganisms (Nochure *et al*., 1993). Reduction in cellulase activity may also be due to depletion of essential nutrients required for the growth of *A. niger* and consequently lead to low enzyme production. The potentials of microorganisms to produce enzymes in a relatively short incubation period have been reported (Sonjoy *et al*., 1995). The result obtained from this

study is similar to that reported by Abu *et al*. (2000) who reported 120 hours of incubation period for cellulase production but differs from that of Devanathan *et al.* (2007) and Acharya *et al.* (2008) who reported 96 hours of incubation period for cellulase production. For maximum cellulase production from *A. niger* cultured on *A. hypogaea* shell, 120 hours of incubation is required.

pH brought morphological changes in microorganisms and as a result, these microorganisms are usually sensitive to the concentration of hydrogen ions present in the fermentation medium. Among optimization parameters for cellulase production, pH of the fermentation medium plays a significant role by inducing morphological changes in microbes and in enzyme production (Mrudula and Murugammal, 2011). Gupta and coworkers (2003) reported that pH is a key machinery that affects enzyme production during fermentation. The pH change observed during the growth of microbes also affects product stability in the medium (Gupta *et al*., 2003). Optimum pH for maximum production of cellulase obtained from this study was 4.0 under SmF (Figure 5). This result was contrary to the observations which was made for cellulase production from *A. terreus* QTC 828 in SmF by Ali *et al.* (1991) and *Trichoderma reesei* in SSF by Doppelbauer *et al.* (1987) who both reported a pH of 6.0 for cellulase production. Also, Krishna (1999) reported a pH of 7.0 for cellulase produced by bacteria using banana peels as a substrate in solid state fermentation. The best pH for producing cellulase from *A. niger* cultured on *A. hypogaea* is 4.0. The differences in the results obtained from this study and the earlier reported work may be as results of using different microorganisms as well as different agricultural wastes.

Incubation temperature which is a function of microbial strain plays a central role in the metabolic activities of a microorganism and should be determined for each set of conditions

(Bhanja *et al*., 2007). As incubation temperature increases, the kinetic energy increases and consequently lead to increase in the rate of reaction as observed in many chemical reactions. However, the stability of the enzyme decreases due to the effect of heat which will denature the enzyme. Holding enzyme at a high enough temperature may denature the enzyme. The results obtained from this study revealed that optimum temperature for maximum enzyme production was recorded at 40 ºC for endoglucanase and β-glucosidase whereas the optimum temperature for exoglucanase was recorded at 50 ºC. The result obtained from this study was in agreement with the findings of Ali *et al.* (1991) who reported maximum yield of cellulase from *A. niger* Z10 strain and *A. terreus* at 40 ºC, respectively in SSF. Loss of cellulase activity observed beyond 40 ºC and 50 ºC may be due to thermal degradation of enzymes. The best temperature for producing cellulase from *A. niger* when cultured on *A. hypogaea* shell is 40 - 50 ºC.

Substrate concentration is an important factor that affects the activity of enzyme and subsequently enzyme production. Enzymes are not passive surfaces on which reactions occurs but rather are complex machines that operate through a great variety of chemical mechanisms. In an enzyme catalyzed reaction, the activity of enzyme increased as substrate concentration increases. Though, at a point, a further increase in substrate concentration will have little or no effect on the activity of enzyme, at that point the enzyme is said to be saturated with its substrate. The results obtained in this study revealed that maximum cellulase activity was achieved at the maximum concentrations investigated.

The size of the inoculum is a measure of the amount of enzyme produced by microorganisms inoculated into the fermentation medium. In the present study, increase in inoculum size correspond to an increase in the production of cellulase. Maximum enzyme production was obtained at inoculum size of 13 cfu/ml. When a single inoculum is employed for

enzyme production during fermentation, it may take a longer time before enzymes are secreted and the quantity of enzymes that will be produced will be very small compared to when the innoculi is double or even thrice. At the inoculum size above 13 cfu/ml, a sharp reduction in cellulase production was observed. This reduction in cellulase production with further increase in innoculum size might be as a result of clumping of cells which could have reduced the macro and micronutrients present in the fermentation medium (Srivastava, *et al*., 1987). This result is in line with earlier work reported by Kunamneni *et al*. (2005) who explained that further increase in innoculum size resulted in decreasing enzyme production owing to nutrients constraint.

Nitrogen is the main component of protoplasm and building block of proteins. In this study, all the nitrogen sources enhanced cellulase production when compared to control. Among them peptone supported maximum enzyme production of endoglucanase and β-glucosidase while yeast extract supported maximum exoglucanase production. Similar reports on stimulation of cellulase by protease peptone had earlier been reported (Kathiresan and Manivannan, 2006; Devanathan *et al.,* 2007). Sun *et al.* (1999) reported that good cellulase yield can be obtained with ammonium compound as the nitrogen source. Though the addition of organic nitrogen sources such as beef extract and peptone resulted in increased growth and enzyme production, they were not an effective replacement for inorganic nitrogen sources because of their higher cost.

5.3 Purification of Cellulase Produced by *A. niger* Cultured on *A. hypogaea* Shell

Enzymes generally can be obtained from three different sources namely plants, animals and microorganisms. These enzymes are usually confined to a diverse compartment in the sources mentioned above; some are localized in the cytosol while others in mitochondria and other organelles. In order to carry out structural elucidation of a particular enzyme and to

characterize it for analytical purpose, the crude enzymes must first be obtained after which it will be subjected to series of purification procedures. Cellulase isolated from *A. niger* when cultured on *A. hypogaea* shells used as substrate gave a specific activity and percentage yields of 484.30 U/mg and 3.87 % respectively after gel filtration chromatography. The percentage yields after gel filtration chromatography was far less than the value obtained for crude cellulase (100%). Crude enzyme contains total protein which include desired and undesirable proteins. Therefore, percentage yield is expressing how much of the desired enzyme was actually recovered. The reduction in percentage yields obtained from this study may be as a result of removal of some undesirable proteins at each purification stage or may as well be due to denaturation of unwanted proteins during purification steps. The percentage yield (3.87%) obtained from this study was more than 2.11% reported by Iqbal *et al*. (2011) when he obtained cellulase from *Trichoderma viride* cultured on wheat straw under SSF. Thus, it can be infered that *A. hypogaea* may be a better inducer of cellulase than wheat straw. Also, Olama *et al*. (1993) reported cellulase purification from *T. viride* and recorded a 99.8% loss of protein with the specific activity increased to about 22.8 folds. Sultana (1997) reported 13.71 U/mg specific activities which were increased by about 32 folds from *Aspergillus* sp. Po-Jui *et al*. (2004) also observed that the specific activity of 38.22 U/ml increased by about 9.04 folds from *Sinorhizobium fredee* by DEAE Sepharose anion-exchange column and followed by Phenyl-Sepharose column purification. The increase in the specific activity of cellulase from crude (7.11 U/mg) to sephadex G-100 column chromatography (484.30 U/mg) as well as a corresponding increase in purification fold from crude (1.0) to sephadex G-100 column chromatography (68.12) obtained from this study may be due to removal of some endogenous inhibitors that may be present in the crude enzyme (Adeleke *et al*., 2012).

The elution profile of *A. hypogaea* shell cellulase on sephadex G-100 chromatography produced 5 distinct peaks. This implies that cellulase enzyme was localized in those 5 peaks produced. This observation was contrary to the findings of Adeleke *et al*. (2012) who reported 3 peaks obtaining from elution profile of rerun of cellulase obtained from *Bacillus coagulans Co4* on CM Sepharose CL-6B.

5.4 Substrate Specificity of Purified Cellulase Produced by *Aspergillus niger* Cultured on *Arachis hypogaea* Shell

Substrate specificity refers to the ability of an enzyme to select the precise substrate from sets of chemical compounds. Specificity is a molecular recognition mechanism and functions through structural and conformational complementarity between the enzyme and substrate. Enzymes show different degree of specificity towards their substrates. The results obtained from this study revealed that the purified cellulase was able to hydrolyze CMC and shows absolute specificity for CMC (Table 5). The purified enzyme displayed little hydrolytic activity against other substrates tested. This finding was in agreement with the work of Yin *et al*. (2010) who reported 100 % relative activity for cellulase against CMC. Therefore, the best substrate for cellulase produced by *A. niger* when cultured on *A. hypogaea* shell is CMC.

5.5 Estimation of Molecular Weight of Purified Cellulase Produced by *A. niger* Cultured on *A. hypogaea* Shell

The electrophoretic pattern obtained from this study give an indication that to some extent, certain degree of purification was attained. This was evidenced with a decreased in the number of bands formed after each purification step. The molecular weight of purified cellulase obtained from this study was found to be 13.5 KDa as determined by SDS polyacrylamide gel electrophoresis (Figure 11). Reports of molecular weight of purified cellulase from different

microorganisms include (i) cellulase purified from *T. viride*, with a molecular weight of 38~54 KDa reported by Ogawa (1989), (ii) cellulase from *T. viride*, with a molecular weight of 58 KDa reported by Olama *et al*. (1993), (iii) cellulase from *Aspergillus sp*, with a molecular weight of 31.2 KDa reported by Sultana (1997) and (iv) Shaojun *et al*. (2001) purified cellulase from *V. volvaceae* and obtained a molecular weight of 42 KDa. In addition, Saha (2004) obtained a molecular weight of 27 kDa from *Mucor circinelloides*, Lucas *et al*. (2001) obtained 35 kDa from *Chalara paradoxa*, Mawadza *et al*. (2000) obtained 40 kDa from *Bacillus* strains, and Akiba *et al*. (1995) also obtained a molecular weight of 40 kDa from *A. niger*. The differences in the molecular weight of purified cellulase obtained from this study and those earlier reported may be as a result of different in microorganisms as well as agricultural wastes used as substrate in this study.

5.6 Kinetic Analysis of Purified cellulase

K_m is a measure of affinity a particular enzyme has for its substrates. It can also be expressed as the concentrations of the substrate when the velocity of the enzyme catalyzed reaction is half maximum. V_{max} on the other hand is the maximum velocity of an enzyme in enzyme catalyzed reaction. These two parameters are very important in enzyme study. Usually, when the K_m value is low, it implies that a particular enzyme has a very strong affinity for its substrate and to achieve maximum rate of reaction, little amounts of substrates will be required. The value obtained from this study for K_m and V_{max} are 0.23 mg/ml and 9.26 U/ml respectively, differ from K_m and V_{max} for different microorganisms that have been reported. Ekperigin (2007) reported K_m values of 0.32 and 2.54 mg/ml from *A. anitratus* and *Branhamella* sp. respectively in the presence of cellobiose as substrate whereas 4.97 and 7.90 mg/ml were reported using CMC as substrate. Also, Bakare *et al*. (2005) and Cascalheira and Queiroz, (1999) got K_m value

of 3.6 mg/ml from *P. fluorescens* and 1.1 *m*M from *T. reesei* respectively. The variation in the value of K_m obtained from this study and other reported studies may be due to genetic variation among different species of microorganisms employed (Iqbal *et al*. 2011). K_{cat} measures the number of substrate molecule each enzyme site converts to product per unit time. The higher the K_{cat}, the more substrates get turned over to products in one seconds and vice-versa. The value obtained for K_{cat}, from this study is 0.08 s^{-1} which implies that more enzyme will be needed to convert substrate to product.

5.7 Effect of pH on Endoglucanase Activity of Purified Cellulase

The effect of pH on the activity of purified cellulase was shown in Figure 14. Cellulase activity was found to be affected by change in the concentration of hydrogen ions present in the solution. The optimum enzyme activity was observed at pH 4.0 in this study. This finding was in agreement with Kim (1995) who isolated cellulase from *M. circinelloides* and reported a pH value between 4.0 - 7.0. Fungal cellulases with pH values of 4.5 to 6.0 have been reported and have been obtained from *V. diplasia* (Bhadauria *et al*., 1997) and *T. reesei* QM 9414 (Wang, 1999). From the results obtained from this study, it can be suggested that purified cellulase is moderately active in acidic solution. A sharp decrease in cellulase activity as pH 4 approaching neutral and alkaline regions may be due to destruction of active site as well as alterations in secondary or tertiary structure of cellulase.

5.8 Effect of Temperature on Endoglucanase Activity of Purified Cellulase

The finding of optimum temperature for the purified cellulase (40 °C) in this study differs from that reported in several other studies. The results obtained in this agreed closely with the findings of Thongekkaew *et al*. (2008) who reported between 40 – 50 °C as optimum

temperature during the characterization of CMCase produced from *Cryptococcus sp.* S-2 as against Fadel (2000) who found 55 °C as the optimum temperature at which the enzyme was most active and stable. The optimum temperature of 55 °C was also reported by Saha (2004) for maximum CMCase activity. Loss of activity observed after the optimum temperature may be due destruction of weak bonds such as Van der Waals, and hydrogen bonds that help in maintaining the three-dimensional structure of the enzymes. Temperature optimum for purified cellulase was observed at 40 °C. For temperatures higher than 40 °C, enzyme starts to lose its activity rapidly as the denaturation of the enzymic protein occurs at elevated temperatures (Figure 15). For enzyme to be found applicable in industries, such enzyme must be stable to moderate heat. Therefore, for cellulase obtained from *A. niger* cultured on *A. hypogaea*, the optimum temperature for the maximum activity is 40 °C.

5.9 Effect of Cations on Endoglucanase Activity of Purified Cellulase

Cations may influence the activity of enzymes positively or negatively. Some metal ions may act as cofactor that stimulate the activity of enzymes while some may act as inhibitor. For example, pyruvate kinase, dialkyllglycine decarboxylase, diol dehydrogenase, inosine monophosphate dehydrogenase are all potassium-activated enzymes. Also, alkaline phosphatase is a magnesium-dependent enzyme. In some enzymes, the activated cation is near the active site of the enzyme while in others, it is distance. The activity of purified cellulase was inhibited by K^+ at all the concentrations investigated, however, Na^+ also inhibited cellulase at a concentration below 0.75 mM, while above 0.5 mM, Na^+ stimulated the activity of purified cellulase. This implies that for effective catalysis, concentrations above 0.5 mM will be required for cellulase obtained from *A. niger* cultured on *A. hypogaea* shells. This result contradicts the report of Wang *et al*. (2012) who reported that Na^+ and K^+ showed little or no effect on the activity of purified

cellulase. The activity of purified cellulase was inhibited by Mg^{2+}, Cu^{2+} and Zn^{2+}. Findings in this study agreed with Sanwal (1999) and Wang *et al*. (2012) who both reported inhibitory effect of Mg^{2+}, Cu^{2+} and Zn^{2+} on the activity of cellulase. Also, Ca^{2+} and Fe^{2+} inhibited the activity of cellulase while Mn^{2+} and Co^{2+} stimulated cellulase activity. The ability of Mn^{2+} and Co^{2+} to activate cellulase have been documented (Lucas *et al*., 2001; Saha, 2004). There was a positive correlation between the degree of inhibition and valence of metal ions (Wang *et al*., 2012). The kinetic properties and pattern of inhibition of Mg^{2+} differs from other divalent cations like Cu^{2+}, Zn^{2+}, Ca^{2+} and Fe^{2+}. Based on the findings from this study, Mg^{2+} inhibited cellulase competitively. Mawadza *et al*. (2000) reported that endoglucanase has two conserved glutamate residues at the active site. Divalent cation such as Mg^{2+} may bind to this conserved glutamate residue to influence cellulase activity. Mg^{2+} may bind with electronegative glutamate residues in a reversible manner, giving rise to changes in the enzymatic catalytic activity and compete with substrate for the active site. Unlike Mg^{2+}, other divalent cations like Cu^{2+}, Zn^{2+} and Fe^{2+} inhibited purified cellulase in a non-competitive manner. In the presence of Cu^{2+}, Zn^{2+} and Fe^{2+} the V_{max} were lowered while K_m values remained approximately unchanged. Ca^{2+} inhibited the purified cellulase in an uncompetitive manner.

5.10 Effect of Anions on Endoglucanase Activity of Purified Cellulase

The result obtained from this study (Figure 23) revealed that addition of anions inactivated cellulase. Since the optimum pH for maximum activity of cellulase was 4, addition of anions significantly changed the pH of the reaction system by increasing the pH. This leads to denaturation of cellulase at higher pH. Though, the activity of cellulase was increased at low concentrations of Cl^- and SO_4^{2-}, enzyme activity was completely inhibited in the presence of CO_3^{2-}. It can be proposed that Na_2CO_3 might not be suitable for pretreating lignocellulosic

biomass. In view of the fact that the optimum pH of cellulase is 4.0, the decrease in cellulase activity in the presence of anions can simply be attributed to the change in pH.

5.11 Effect of Some Surfactants on Endoglucanase Activity of Purified Cellulase

The surfactants are believed to be a significant environmental factor that affects the activity of enzyme. Surfactants are used extensively for solubilizing protein from lipid membranes and other biological materials, and for maintaining the solubility of certain proteins in the solution. This study signifies an effort to reveal the effect of non-ionic surfactants on the activity of purified cellulase from *A. niger* cultured on *A. hypogaea* shells. According to present findings, non-ionic detergents such as triton X and tween-20 inhibited the activity of cellulase at all concentrations investigated. Different effect of anionic surfactants on the activity of enzymes has been documented (Shaheen *et al*., 2017). Anionic surfactants such as DMSO and mercaptoethanol showed inhibitory effect on the activity of purified cellulase. This inhibition may be attributed to the inability of proteins to absorb anionic detergent that denatures its structure by disrupting bonds and ultimately obstruct protein function (Bartnik, 1992).

5.12 Detergent Compatibility of a Purified Cellulase

The potential application of cellulase and other enzymes in detergent industry is a more topical invention. The findings from this study revealed high level of compatibility upon the addition of purified cellulase to local detergents of expected use. This revealed that the cellulase is compatible with local detergents and suggesting its potential use as suitable additive for detergent industries.

5.13 Contributions to Knowledge

Cellulases represent one of the largest groups of industrial enzymes that are on sales worldwide. The enzyme has attracted lots of attention with a view to exploiting it for biotechnological and industrial uses. Cellulases are inducible enzymes that can be obtained from microorganisms when cultured on cellulose containing substrates. The study has contributed to the increasing knowledge by providing information on alternative source of cellulase from *A. hypogaea* shells. Though, several reports on the use of different agrowastes in cellulase production have been documented.

The cellulase purified from *A. hypogaea* shell was characterized by estimating its molecular weight, effect of pH and temperature, kinetic parameters, effect of cations and anions, effect of surfactants on the purified cellulase were also well studied. The cellulase produced from *A. niger* cultured on *A. hypogaea* displayed high level of compatibility with local detergents of expected use. This enzyme was activated in the presence of some divalent cations.

The characteristics exhibited by the cellulse purified from this study can be exploited for their applications in the industries such as detergent, pulp and paper industries as well as in waste management.

Conclusion

In this study, the utilization of *A. hypogaea* shells, which normally cause environmental pollution as a result of consumption of groundnuts, for cellulase production was reported. The summaries of the findings are summarized below:

1. By gel filtration the enzyme was purified to 68.12 folds with a yield and specific activity of 3.87% and 484.3 U/mg respectively;
2. The V_{max} value for cellulase was 9.26 U/ml whereas the K_m was 0.23 mg/ml;
3. The estimated molecular weight of cellulase was 13.5 kDa;
4. The optimum pH for the cellulase was 4 and optimum temperature was 40 ^oC;
5. The presence of anions (CO_3^{2-}, Cl^- and SO_4^{2-}) decreased the activity of cellulase. Cations like Na^+ activated cellulase activity at a concentration above 1 mM while K^+ did not affect the cellulase activity Divalent cations such as Mg^{2+}, Zn^{2+} and Cu^{2+}, Ca^{2+} and Fe^{2+} inhibited the activity of enzyme. Mn^{2+} and Co^{2+} enhanced the activity of purified cellulase at all the concentrations investigated;
6. Cellulase was inhibited by DMSO, triton X, tween 20 and mercaptoethanol; and
7. Cellulase from *A. niger* cultured on *A. hypogaea* shell was found to be compatible with various detergents brands of expected use such as omo, klin, wow, ariel, sunlight, mama and bimbo.

The study concluded that production of cellulase from *A. niger* cultured on *A. hypogaea* shells was achieved at incubation period of 120 hours, pH of 4, temperature of 40 ^oC, inoculum size of 13 cfu/ml and this enzyme was activated by Mn^{2+} and Co^{2+}.

Recommendations for Further Studies

One of the important findings in this research is the production of cellulase from *A. niger* cultured on *A. hypogaea* shells. Therefore, the following recommendations can be made:

1. The genes responsible for the production of cellulase from *A. niger* should be cloned into *E.coli* or *Pichia pastoris* in order to produce the enzyme in large quantities;
2. The purified cellulase should be subjected to some analytical techniques in order to establish the structural configuration of the enzyme;
3. The amino acid composition of the purified cellulase should be determined;
4. Other parts of groundnut plant which may be considered as wastes should also be tried for enzyme production;
5. Efforts should be made in transferring the knowledge acquired from the conduct of this research to the various industries where cellulases have been found applicable. This will in turn contribute to the economic growth of this country.

References

Abdulla, H. M. and El-Shatoury, S. A. (2007). Actinomycetes in rice straw decomposition. *Waste Management*, 27(6): 850-853.

Abdul-Rahim, M. (2014). *Evaluation of storage facilities for groundnut and cowpea in the Zabzugu and Saboba Districts in the Northern Region of Ghana* (Doctoral dissertation).

Abu, E. A., Ado, S. A, James, D. B. (2005). Raw starch degrading amylase production by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisae* grown on Sorghum pomace, *Afr. J. Biotechnol.*, 4: 785 – 790.

Adekunle, A., Orsat, V. and Raghavan, V. (2016). Lignocellulosic bioethanol: A review and design conceptualization study of production from cassava peels. *Renewable and Sustainable Energy Reviews*, 64: 518-530.

Adeleke, E. O., Omafuvbe, B. O., Adewale, I. O., and Bakare, M. K. (2012). Purification and characterisation of a cellulase obtained from cocoa (*Theobroma cacao*) pod-degrading *Bacillus coagulans* Co4. *Turkish Journal of Biochemistry/Turk Biyokimya Dergisi*, 37(4):

Adeyeye, E. I. and Ayejuyo, O. O. (1994). Chemical composition of *Cola acuminata* and *Garcinia kola* seeds grown in Nigeria. *International Journal of Food Sciences and Nutrition*, 45(4): 223-230.

Adeyosoye, O. I., Adesokan, I. A., Afolabi, K. D and Ekeocha, A. H. (2010). Estimation of proximate composition and biogas production from *in vitro* gas fermentation of sweet potato (*Ipomea batatas*) and wild cocoyam (*Colocasia esculenta*) peels. *African Journal of Environmental Science and Technology*, 4 (6): 388-391.

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A. and Levin, D. B. (2011). Biomass pretreatment: fundamentals toward application. *Biotechnology advances*, 29(6): 675-685.

Akiba, S., Kimura, Y., Yamamoto, K., and Kumagai, H. (1995). Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*. *Journal of Fermentation and Bioengineering*, 79(2): 125-130.

Ali, S., Hall, J., Soole, K. L., Fontes, C. M., Hazlewood, G. P., Hirst, B. H. and Gilbert, H. J. (1995). Targeted expression of microbial cellulases in transgenic animals. *Progress in Biotechnology*, 10: 279-293.

Ali, S., Sayed, A., Sarker, R. I. and Alam, R. (1991). Factors affecting cellulase production by *Aspergillus terreus* using water hyacinth. *World J. Microbiol. Biotechnol.,* 7 (1): 62-66.

Amadioha, A. C. (1993). Production of cellulolytic enzymes by *Rhizopus oryzae* in culture and Rhizopus-infected tissues of potato tubers. *Mycologia,* 88: 574-578.

Anita, S., Namita, S. and Bishnoi, N. R. (2009). Production of cellulases by Aspergillus heteromorphus from wheat straw under submerged fermentation. *International Journal of Environmental Science and Engineering*, 1(1): 23-26.

Antal, M. J., Leesomboon, T., Mok, W. S. and Richards, G. N. (1991). Mechanism of formation of 2-furaldehyde from D-xylose. *Carbohydrate Research*, 217: 71-85.

Anwar, Z., Gulfraz, M. and Irshad, M. (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *Journal of Radiation Research and Applied Sciences*, 7(2): 163-173.

Babayemi, J. O. and Dauda, K. T. (2009). Evaluation of solid waste generation, categories and disposal options in developing countries: a case study of Nigeria. *Journal of Applied Sciences and Environmental Management*, 13(3): 83-88

Bakare, M. K., Adewale, I. O., Ajayi, A., and Shonukan, O. O. (2005). Purification and characterization of cellulase from the wild-type and two improved mutants of Pseudomonas fluorescens. *African Journal of Biotechnology*, 4(9):

Baker, S. E. and Bennett, J. W. (2007). An overview of the genus Aspergillus. *The Aspergilli: Genomics, Medical Aspects, Biotechnology,* and *Research Methods*, 3-13.

Bakri, Y., Jacques, P. and Thonart, P. (2003). Xylanase production by *Penicillium canescens* 10–10c in solid-state fermentation. In *Biotechnology for Fuels and Chemicals*: 737-748. Humana Press.

Bamforth, C. W. (2009). Current perspectives on the role of enzymes in brewing. *Journal of Cereal Science*, 50(3): 353-357.

Bansal, N., Tewari, R., Soni, R. and Soni, S. K. (2012). Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. *Waste Management*, 32(7): 1341-1346.

Bao, Y., Han, J., Hu, F. B., Giovannucci, E. L., Stampfer, M. J., Willett, W. C. and Fuchs, C. S. (2013). Association of nut consumption with total and cause-specific mortality. *N Engl J Med*, (369): 2001-2011.

Bartnik, F. G. (1992). Interaction of anionic surfactants with proteins, enzymes, and membranes. *Surfactant Science Series*, 43: 1-42.

Baughman, T., Dotray, P., Grichar, J., Black, M., Woodward, J., Trostle, C. and Baumann, P. (2007). Texas peanut production guide. *Online. Coop. Ext. Serv., Texas A and M Univ., College Station, TX*.

Bayer, E. A., Chanzy, H., Lamed, R. and Shoham, Y. (1998). Cellulose, cellulases and cellulosomes. *Current Opinion in Structural Biology*, 8(5): 548-557.

Bayer, E. A., Morag, E. and Lamed, R. (1994). The cellulosome—a treasure-trove for biotechnology. *Trends in Biotechnology*, 12(9): 379-386.

Behera, B. C., Sethi, B. K., Mishra, R. R., Dutta, S. K. and Thatoi, H. N. (2016). Microbial cellulases - diversity and biotechnology with reference to mangrove environment: A review. *Journal of Genetic Engineering and Biotechnology*.

Bello, M. O., Falade, O. S., Adewusi, S. R. A. and Olawore, N. O. (2008). Studies on the chemical compositions and antinutrients of some lesser known Nigeria fruits. *African Journal of Biotechnology*, 7 (21): 3972-3979.

Bennett, A. F. (1980). The metabolic foundations of vertebrate behavior. *Bioscience*, 30(7): 452-456.

Bennett, J. W. (1985). Molds, manufacturing and molecular genetics. In *UCLA Symposia on Molecular and Cellular Biology (USA)*.

Benore, M. (2010). Response to Review of Fundamental Laboratory Approaches for Biochemistry and Biotechnology. *Biochemistry and Molecular Biology Education*, 38(2): 64-64.

Berg, J. M., Tymoczko, J. L. and Stryer, L. (2007). *Biochemistry (Loose-Leaf)*. Macmillan.

Beukes, N. and Pletschke, B. I. (2011). Effect of alkaline pre-treatment on enzyme synergy for efficient hemicellulose hydrolysis in sugarcane bagasse. *Bioresource Technology*, 102(8): 5207-5213.

Bhadauria, A., Sodhi, H. S., Kapoor, S. and Phutela, R. P. (1997). Isolation and characterization of *Volvariella diplasia* enzyme mutants. *Indian J. Exp. Biol*., 35:516-519.

Bhanja, T. S., Rout, R., Banerjee and Bhattacharya. B. C. (2007). Comparative profiles of α-amylase production in conventional tray reactor and growtek biorSeactor. *Bioprocess Biosyst Eng*., 30: 369-376.

Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnology Advances*, 18(5): 355-383.

Bhat, M. K. and Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances*, 15(3): 583-620

Bokhorst, S., Kardol, P., Bellingham, P. J., Kooyman, R. M., Richardson, S. J., Schmidt, S. and Wardle, D. A. (2017). Responses of communities of soil organisms and plants to soil aging at two contrasting long-term chronosequences. *Soil Biology and Biochemistry*, 106: 69-79

Brosse, N., Hussin, M. H. and Rahim, A. A. (2017). Organosolv Processes.

Burgess, R. R. (2009). Refolding solubilized inclusion body proteins. *Methods in Enzymology*, 463: 259-282.

Burgess, R. R. and Deutscher, M. P. (Eds.). (2009). Guide to protein purification. Academic Press, (Vol. 463).

Caf, Y. and Arikan, B. (2017). A Novel Alkaline, Highly Thermostable and Oxidant Resistant Carboxymethyl Cellulase (Cmcase) Produced by Thermophilic *Bacillus sonorensis* CY-3. *Int. J. Curr. Microbiol. App. Sci.*, 6(3): 2349-2362.

Chaabouni, S. E., Belguith, H., Hassairi, I., M'Rad, K. and Ellouz, R. (1995). Optimization of cellulase production by Penicillium occitanis. *Applied Microbiology and Biotechnology*, 43(2): 267-269.

Chukwu, O. and Orhevba, B. A. (2011). Proximate analysis of 'eri' by-product of soyamilk processing. *International Journal of Academic Research*, 3(2).

Clanet, M., Durand, H. and Tiraby, G. (1988). Enzymatic saccharification of municipal wastes. *Biotechnology and Bioengineering*, 32: 930-4.

Coral, G., Arikan, B., Ünaldi, M. N. and Güvenmez, H. (2002). Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain. *Turkish Journal of Biology*, 26(4): 209-213.

Cortez, J. M., Ellis, J. and Bishop, D. P. (2002). Using cellulases to improve the dimensional stability of cellulosic fabrics. *Textile Research Journal*, 72(8): 673-680.

Coughlan, M. P. (1992). Enzymic hydrolysis of cellulose: an overview. *Bioresource Technology*, 39: 107-15.

Cui, J., Katsuno, T., Totsuka, K., Ohnishi, T., Takemoto, H., Mase, N. and Mizutani, K. (2016). Characteristic Fluctuations in Glycosidically Bound Volatiles during Tea Processing and Identification of Their Unstable Derivatives. *Journal of Agricultural and Food Chemistry*, 64(5): 1151-1157.

Dan, S., Marton, I., Dekel, M., Bravdo, B. A., He, S., Withers, S. G. and Shoseyov, O. (2000). Cloning, expression, characterization, and nucleophile identification of family 3, *Aspergillus niger* β-glucosidase. *Journal of Biological Chemistry*, 275(7): 4973-4980.

Dashtban, M., Schraft, H. and Qin, W. (2009). Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. *International Journal of Biological Sciences*, 5(6): 578.

Devanathan, A., Shanmugan, T., Balasubramanian and Manivannan, S. (2007). Cellulase production by *Aspergillus niger* isolated from coastal mangrove debris. *Trends. Appl. Sci. Res*., 2: 23-27.

Dienes, D., Egyhazi, A. and Reczey, K. (2004). Treatment of recycled fiber with Trichoderma cellulases. *Industrial Crops and Products*, 20(1): 11-21.

Ding, J. C., Xu, G. C., Han, R. Z. and Ni, Y. (2016). Biobutanol production from corn stover hydrolysate pretreated with recycled ionic liquid by Clostridium saccharobutylicum DSM 13864. *Bioresource Technology*, 199: 228-234.

Doppelbauer, R., Esterbauer, H., Steiner, W., Lafferty, R. M. and Steinmuller, H. (1987). The use of lignocellulosic wastes for production of cellulase by *Trichoderma reesei. Appl. Environ. Microbiol*., 26 (5): 485-494.

Dotray, P. A., Grichar, W. J., Baughman, T. A., Prostko, E. P., Grey, T. L. and Gilbert, L. V. (2012). Peanut (Arachis hypogaea L.) response to lactofen at various postemergence timings. *Peanut Science*, 39(1): 9-14.

Dourado, F., Bastos, M., Mota, M. and Gama, F. M. (2002). Studies on the properties of Celluclast/Eudragit L-100 conjugate. *Journal of Biotechnology*, 99(2): 121-131.

Dreizen, S., Bodey, G. P., McCredie, K. B. and Keating, M. J. (1985). Orofacial aspergillosis in acute leukemia. *Oral Surgery, Oral Medicine, Oral Pathology*, 59(5): 499-504.

Duchesne, L. G., Lam, J. S., MacDonald, L. A., Whitfield, C. and Kropinski, A. M. (1988). Effect of pH and acrylamide concentration on the separation of lipopolysaccharides in polyacrylamide gels. *Current Microbiology*, 16(4): 191-194.

Dukhin, A. S. (2002). *Ultrasound for Characterizing Colloids Particle Sizing, Zeta Potential Rheology*. Elsevier.

Dukhin, A., and Goetz, P. J. (2002). *U.S. Patent No. 6,449,563*. Washington, DC: U.S. Patent and Trademark Office.

Duong-Ly K. C. and Gabelli, S. B. (2014). Salting out of proteins using ammonium sulphate precipitation. *Methods in Enzymology*, 541: 85-94.

Ekperigin, M. M. (2007). Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. *African Journal of Biotechnology*, 6: 28-33.

Elzbieciak-Wodka, M., Popescu, M. N., Ruiz-Cabello, F. J. M., Trefalt, G., Maroni, P. and Borkovec, M. (2014). Measurements of dispersion forces between colloidal latex particles with the atomic force microscope and comparison with Lifshitz theory. *The Journal of Chemical Physics*, 140(10): 104906.

Escobar, M. O. and Hue, N. V. (2008). Temporal changes of selected chemical properties in three manure–Amended soils of Hawaii. *Bioresource Technology*, 99(18): 8649-8654.

FAOSTAT, Food and Agricultural Organization of the United Nations, Statistics Division. 2013. 9–17.

Festersen, R. M., Nielsen, A. V., Joergensen, C. T. and Christensen, L. L. H. (2016). *U.S. Patent No. 9,279,165*. Washington, DC: U.S. Patent and Trademark Office.

Fockink, D. H., Urio, M. B., Chiarello, L. M., Sánchez, J. H. and Ramos, L. P. (2016). Principles and challenges involved in the enzymatic hydrolysis of cellulosic materials at high total solids. In *Green Fuels Technology*, 147-173.

Fontaine, S., Bardoux, G., Benest, D., Verdier, B., Mariotti, A. and Abbadie, L. (2004). Mechanisms of the priming effect in a savannah soil amended with cellulose. *Soil Science Society of America Journal*, 68(1): 125-131.

Gaborieau, M., Nicolas, J., Save, M., Charleux, B., Vairon, J. P., Gilbert, R. G. and Castignolles, P. (2008). Separation of complex branched polymers by size-exclusion chromatography probed with multiple detection. *Journal of Chromatography A*, 1190(1): 215-223.

Gafar, M. K., Itodo, A. U., Atiku, F. A., Hassan, A. M. and Peni, I. J. (2011). Proximate and mineral analysis of the leaves of hairy indigo (*Indigofera astragalina*). Pakistan *Journal of Nutrition*, 10(2): 168-175.

Galbe, M. and Zacchi, G. (2007). Pretreatment of lignocellulosic materials for efficient bioethanol production. *Adv. Biochem. Eng. Biotechnol*., 108: 41–65.

Galindo, F. G. and Yusof, N. L. (2015). New insights into the dynamics of vacuum impregnation of plant tissues and its metabolic consequences. *Journal of the Science of Food and Agriculture*, 95(6): 1127-1130.

Garg, S. K., and S. Neelakantan. (1982). Studies on the properties of celiulase enzyme from *Aspergillus terreus* GN1. *Biotechnology and Bioengineering,* 24: 737-742.

Garrett, Reginald, H., Grisham, Charles, M. (2013). Biochemistry (5th ed.). Belmont, CA: Brooks/Cole, Cengage Learning. p. 108.

Gharpuray, M. M., Lee, Y. H and Fan, L. T. (1983). Structural modification of lignocellulosic by treatment to enhance enzymatic hydrolysis. *Biotechnol. Bioeng*., 25: 157-170.

Ghildyal, N. P., Lonsane, B. K., Sreekantiah, K. R., Sreenivasa Murthy, V. (1985). Economics of submerged and solid state fermentation for the production of amyloglucosidase. *J. Food Sci.Technol*., 22: 171-176.

Ghose, T.K. (1987). Measurement of cellulase activities. *Pure Appl. Chem*., 59: 257-268.

Gidenne, T. (2003). Fibres in rabbit feeding for digestive troubles prevention: respective role of low-digested and digestible fibre. *Livestock Production Science*, 81(2): 105-117.

Gilbert, H. J. (2014). Developing novel enzyme repertoires for the efficient deconstruction of plant biomass tailored for the bioenergy industry. In *Plants and BioEnergy*, 197-209.

Gimba, C. E., Salihu, A. A., Kagbu, J. A., Turoti, M., Itodo, A. U. and Sariyya, A. I. (2010). Study of pesticide (Dichlorvos) removal from aqueous medium by *Arachis hypogaea* (groundnut shell) using GC/MS. *World Rur Observ*, 2(1): 1-9.

Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S. and Bogel-Łukasik, R. (2010). Hemicelluloses for fuel ethanol: a review. *Bioresource Technology*, 101(13): 4775-4800.

Goma, M., Zein, G. N., Mahmoud, R. M., Gibriel, A. and Abouzied, M. 1982. Characteristics of cellulolytic enzymes of *Aspergillus niger* and *Aspergillus terreus*. *Minufiya J. Agr. Res.*, 5: 299-317.

Gottschalk, L. M. F., Oliveira, R. A. and da Silva Bon, E. P. (2010). Cellulases, xylanases, β-glucosidase and ferulic acid esterase produced by *Trichoderma* and *Aspergillus* act synergistically in the hydrolysis of sugarcane bagasse. *Biochemical Engineering Journal*, 51(1): 72-78.

Goyal, M., Kalra, K.L., Sareen, V.K., Soni, G. (2008). Xylanase production with xylan rich lignocellulasic waste by a local soil isolate of *Trichoderma viride*. *Braz. J. Microbiol*., 39: 535-541.

Graham, H. and Balnavel, D. (2008). 15 Dietary enzymes for increasing energy availability. *Biotechnology in animal feeds and animal feeding*, 295.

Grubisic, Z., Rempp, P. and Benoit, H. (1996). A universal calibration for gel permeation chromatography. *Journal of Polymer Science Part B: Polymer Physics*, 34(10): 1707-1713.

Guenet, J. M. (2010). *Polymer-Solvent Molecular Compounds*. Elsevier.

Gunaseelan, V. N. (2016). Biochemical methane potential, biodegradability, alkali treatment and influence of chemical composition on methane yield of yard wastes. *Waste Management and Research*, 34(3): 195-204.

Gupta, J. K. and Gupta, Y. P. (1979). Properties of cellulase from *Trichodema viride*. *Folia Microbiol*, 24: 269-272.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K. and Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process Biochemistry*. 38: 1599-1616

Gupta, R., Khasa, Y. P. and Kuhad, R. C. (2011). Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. *Carbohydrate Polymers*, 84(3): 1103-1109.

Gupta, R., Mehta, G., Khasa, Y. P. and Kuhad, R. C. (2011). Fungal delignification of lignocellulosic biomass improves the saccharification of cellulosics. *Biodegradation*, 22(4): 797-804.

Gupta, R., Sharma, K. K. and Kuhad, R. C. (2009). Separate hydrolysis and fermentation (SHF) of *Prosopis juliflora*, a woody substrate, for the production of cellulosic ethanol by *Saccharomyces cerevisiae* and *Pichia stipitis*-NCIM 3498. *Bioresource Technology*, 100(3): 1214-1220.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. and Lorito, M. (2004). *Trichoderma* species _ - opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology*, 2(1): 43-56.

Hayashida, S., Ohta, K. and Mo, K. (1988). Cellulases of *Humicola insolens* and *Humicola grisea*. *Methods in Enzymology*, 160: 323-332.

Hebeish, A. and Ibrahim, N. A. (2007). The impact of frontier sciences on textile industry. *Colourage*, 54(4): 41-55.

Hendriks, A. T. W. M. and Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Rechnology*, 100(1): 10-18.

Herbrecht, R., Denning, D. W., Patterson, T. F., Bennett, J. E., Greene, R. E., Oestmann, J. W. and Sylvester, R. (2002). Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *New England Journal of Medicine*, 347(6): 408-415.

Himmel, M. E., Ruth, M. F. and Wyman, C. E. (1999). Cellulase for commodity products from cellulosic biomass. *Current Opinion in Biotechnology*, 10(4): 358-364.

Hobdey, S. E., Knott, B. C., Momeni, M. H., Taylor, L. E., Borisova, A. S., Podkaminer, K. K. and Ståhlberg, J. (2016). Biochemical and structural characterizations of two dictyostelium cellobiohydrolases from the amoebozoa kingdom reveal a high level of conservation between distant phylogenetic trees of life. *Applied and Environmental Microbiology*, 82(11): 3395-3409.

Horwitz, W. (1975). Official methods of analysis. Washington, DC: *Association of Official Analytical Chemists*, 222.

Hromádková, Z., Košťálová, Z. and Ebringerová, A. (2008). Comparison of conventional and ultrasound-assisted extraction of phenolics-rich heteroxylans from wheat bran. *Ultrasonics Sonochemistry*, 15(6): 1062-1068.

Hsu, J. C. and Lakhani, N. N. (2002). *U.S. Patent No. 6,413,363*. Washington, DC: U.S. Patent and Trademark Office.

Hui, L., Wan, C., Hai-Tao, D., Xue-Jiao, C., Qi-Fa, Z. and Yu-Hua, Z. (2010). Direct microbial conversion of wheat straw into lipid by a cellulolytic fungus of *Aspergillus oryzae* A-4 in solid-state fermentation. *Bioresource Technology*, 101(19): 7556-7562.

Humpf, H. U. and Schreier, P. (1991). Bound aroma compounds from the fruit and the leaves of blackberry (Rubus laciniata L.). *Journal of Agricultural and Food chemistry*, 39(10): 1830-1832.

Ibraheem, S. A., Malomo, S. O. and Igunnu, A (2017). Characterization of detergent-stable proteases isolated from *Citrus sinensis* peel. *Biokemistri, 29*(2): 61-66.

Ibrahim, N. A., El-Badry, K., Eid, B. M. and Hassan, T. M. (2011). A new approach for biofinishing of cellulose-containing fabrics using acid cellulases. *Carbohydrate Polymers*, 83(1): 116-121.

Ilmén, M., Den Haan, R., Brevnova, E., McBride, J., Wiswall, E., Froehlich, A. and Thorngren, N. (2011). High level secretion of cellobiohydrolases by Saccharomyces cerevisiae. *Biotechnology for Biofuels*, 4(1): 30.

Immanuel, G., Dhanusha, R., Prema, P. and Palavesam, A. (2006). Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *International Journal of Environmental Science and Technology*, 3(1): 25-34.

Ishikawa, E., Sakai, T., Ikemura, H., Matsumoto, K. and Abe, H. (2005). Identification, cloning, and characterization of a *Sporobolomyces singularis* β-galactosidase-like enzyme involved in galacto-oligosaccharide production. *Journal of Bioscience and Bioengineering*, 99(4): 331-339.

Ishizawa, C. I., Davis, M. F., Schell, D. F. and Johnson, D. K. (2007). Porosity and its effect on the digestibility of dilute sulfuric acid pretreated corn stover. *Journal of Agricultural and Food Chemistry*, 55(7): 2575-2581.

Jouniaux, L. and Pozzi, J. P. (1995). Permeability dependence of streaming potential in rocks for various fluid conductivities. *Geophysical Research Letters*, 22(4): 485-488.

Juturu, V. and Wu, J. C. (2014). Microbial cellulases: engineering, production and applications. *Renewable and Sustainable Energy Reviews*, 33: 188-203.

Kabel, M. A., Bos, G., Zeevalking, J., Voragen, A. G. and Schols, H. A. (2007). Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw. *Bioresource Technology*, 98(10): 2034-2042.

Kamboj, V. P. (2000). Herbal medicine. *Current Science*, 78(1): 35-39.

Kamiya, N., Matsushita, Y., Hanaki, M., Nakashima, K., Narita, M., Goto, M. and Takahashi, H. (2008). Enzymatic in situ saccharification of cellulose in aqueous-ionic liquid media. *Biotechnology Letters*, 30(6): 1037.

Karmakar, M. and Ray, R. R. (2011). Current trends in research and application of microbial cellulases. *Research Journal of Microbiology*, 6(1): 41.

Kataria, R. and Ghosh, S. (2014). NaOH pretreatment and enzymatic hydrolysis of Saccharum spontaneum for reducing sugars production. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, 36(9): 1028-1035.

Kathiresan, K. and Manivannan, S. (2006). Cellulase production by *Penicillium fellutanum* isolated from coastal mangrove rhizosphere soil. *Res. J. Microbiol.,* 1 (5): 438-442.

Kim, C.H. (1995) Characterization and substrate specificity of an endo-beta-1,4-D-glucanase I (Avicelase I) from an extra cellular multienzyme complex of *Bacillus circulans*. *Applied and Environmental Microbiology*, 61: 959-965

Kindt, T. J., Goldsby, R. A., Osborne, B. A. and Kuby, J. (2007). *Kuby immunology*. Macmillan

Kirk, O., Borchert, T.V. and Fuglsang, C.C. (2002) In-dustrial enzyme applications. *Current Opinion in Bio-technology*, 13: 345-351.

Klemm, D., Heublein, B., Fink, H. P. and Bohn, A. (2005). Cellulose: fascinating biopolymer and sustainable raw material. *Angewandte Chemie International Edition*, 44(22): 3358-3393.

Knappert, D., Grethlein, H. and Converse, A. (1981). Partial acid hydrolysis of poplar wood as a pretreatment for enzymatic hydrolysis. In *Biotechnol. Bioeng. Symp., (United States)*, 11, No. CONF-810554.

Koo, Y. M. (2001). Pilot-scale production of cellulase using *Trichoderma reesei* Rut C-30 in fed-batch mode. *Journal of Microbiology and Biotechnology*, 11(2): 229-233.

Krapovickas, A. and Gregory, W. C. (1994). Taxonomia del genero" arachis (leguminosae)". *Bonplandia*, 1-186.

Krapovickas, Antonio; Gregory, Walton C. (2007). Translated by David E. Williams and Charles E. Simpson. "Taxonomy of the genus *Arachis* (Leguminosae)" (PDF). IBONE. 16: 1–205.

Krishna, C. (1999). Production of bacterial cellulases by solid state bioprocessing of banana wastes. *Bioresour. Technol*., 69(3): 231-239.

Kubicek, C. P. and Harman, G. E. (1998). *Trichoderma and Gliocladium. Volume 1: Basic biology, taxonomy and genetics*. Taylor and Francis Ltd

Kuhad, R. C., Gupta, R. and Khasa, Y. P. (2010). Bioethanol production from lignocellulosic biomass: an overview. *Teri Press, New Delhi, India*.

Kuhad, R. C., Gupta, R. and Singh, A. (2011). Microbial cellulases and their industrial applications. *Enzyme Research*.

Kuhad, R. C., Gupta, R., Khasa, Y. P. and Singh, A. (2010). Bioethanol production from lantanacamara (red sage): pretreatment, saccharification and fermentation. *Bioresource Technology*, 101(21): 8348-8354.

Kuhad, R. C., Manchanda, M. and Singh, A. (1999). Hydrolytic potential of extracellular enzymes from a mutant strain of Fusarium oxysporum. *Bioprocess and Biosystems Engineering*, 20(2): 133-135.

Kuhad, R. C., Mehta, G., Gupta, R. and Sharma, K. K. (2010). Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by *Saccharomyces cerevisiae*. *Biomass and Bioenergy*, 34(8): 1189-1194.

Kulshrestha, S. (2012). Much Ado About Nothing: Examining the Curse of Tutankhamun. *Inquiries Journal*, 4(06).

Kumar, A. and Awasthi, A. (2009). *Bioseparation engineering*. IK International Pvt Ltd.

Kumar, P., Barrett, D. M., Delwiche, M. J. and Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & engineering chemistry research*, 48(8): 3713-3729.

Lee, S. M. and Koo, Y. M. (2001). Pilot-Scale Production of Cellulase Using Trichoderma reesei Rut C-30 Fed-Batch Mode. *Journal of Microbiology and Biotechnology*, 11(2): 229-233.

Leggio, L. L. and Larsen, S. (2002). The 1.62 Å structure of *Thermoascus aurantiacus* endoglucanase: completing the structural picture of subfamilies in glycoside hydrolase family 5. *FEBS Letters*, 523(1): 103-108.

Li, J., Henriksson, G. and Gellerstedt, G. (2007). Lignin depolymerization/repolymerization and its critical role for delignification of aspen wood by steam explosion. *Bioresource Technology*, 98(16): 3061-306

Lin, Y. and Tanaka, S. (2006). Ethanol fermentation from biomass resources: current state and prospects. *Applied Microbiology and Biotechnology,* 69: 627-642.

LoPachin, R. M. (2004). The changing view of acrylamide neurotoxicity. *Neurotoxicology,* 25(4): 617-630.

Lorito, M., Hayes, C. K., Di Pietro, A., Woo, S. L. and Harman, G. E. (1994). Purification, characterization, and synergistic activity of a glucan 1, 3-β-glucosidase and an N-acetyl-β-glucosaminidase from *Trichoderma harzianum*. *Phytopathology*, 84(4): 398-405.

Lorsch, J. (2014). Methods in Enzymology. Laboratory methods in enzymology: protein part A. Preface. *Methods in Enzymology*, 536.

Lynd, L. R., Wyman, C. E. and Gerngross, T. U. (1999). Biocommodity engineering. *Biotechnology Progress*, 15(5): 777-793

Ma, H., Wang, K., Gao, Z., Wang, H., Wang, S., Zhang, C. and Bai, J. (2014). Current characteristic signals of aqueous solution transferring through microfluidic channel under non-continuous DC electric field. *AIP Advances*, 4(10): 107-139.

Macris, B. J., Ketos, D., Evangelidou, X., Galiotou-Panayotou, M. and Rodis, P. (1987). Solid state fermentation of straw with *Neurospora crassa* for CMCase and β-glucosidase production. *Biotech. Lett*., 9: 661-664.

Mai, C., Kües, U. and Militz, H. (2004). Biotechnology in the wood industry. *Applied Microbiology and Biotechnology*, 63(5): 477-494.

Mamo, G., Faryar, R. and Karlsson, E. N. (2013). Microbial glycoside hydrolases for biomass utilization in biofuels applications. *Biofuel Technologies*, 171-188.

Mangan, D., McCleary, B. V., Liadova, A., Ivory, R., McCormack, N. (2014). "Quantitative fluorometric assay for the 612-622.

Marchessault, R. H. and Sundararajan, P. R. (1975). Effect of acetate substituents on the conformations of di-and polysaccharides. *Pure and Applied Chemistry*, 42(3): 399-415.

Martin, W. R. and Hayes, C. K. (2017). *U.S. Patent No. 9,681,668*. Washington, DC: U.S. Patent and Trademark Office.

Mawadza, C., Hatti-Kaul, R. and Zvauya, R. Mattiasson, B. (2000). Purification and characterization of cellulases produced by two Bacillus strains. *Journal of Biotechnology*, 83: 177-187.

McCleary, B. V. (1980). New chromogenic substrates for the assay of alpha-amylase and (1→4)-β-D-glucanase. *Carbohydrate Research*, 86(1): 97-104.

McCleary, B. V., Mangan, D., Daly, R., Fort, S., Ivory, R. and McCormack, N. (2014). Novel substrates for the measurement of endo-1, 4-β-glucanase (endo-cellulase). *Carbohydrate Research*, 385: 9-17.

McKee, T. and Mckee, J.R. (2003) Biochemistry: An introduction. 2nd Edition, McGraw-Hill Companies, Inc. and China Science Press, Beijing, 128-130.

McMillan, J. D. (1994). Pretreatment of lignocellulosic biomass.

McMillan, J. D. (1997). Bioethanol production: status and prospects. *Renewable Energy*, 10(2-3): 295-302.

Mishra, A. (1988). Studies a cellulolytic fungi and cellulolytic enzymes with special reference to Aspergilli. Ph.D. Thesis. Department of Botany, Gorakhpur Univ. India.

Mojsov, K. (2010). Application of solid-state fermentation for cellulase enzyme production using *Trichoderma viride*. *Perspectives of Innovations, Economics and Business*, 5(2): 108-110.

Moroz, O. V., Maranta, M., Shaghasi, T., Harris, P. V., Wilson, K. S. and Davies, G. J. (2015). The three-dimensional structure of the cellobiohydrolase Cel7A from *Aspergillus fumigatus* at 1.5 Å resolution. *Acta Crystallographica Section F: Structural Biology Communications*, 71(1): 114-120.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M. and Ladisch, M. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology*, 96(6): 673-686.

Mrudula, S. and Murugammal, R. (2011). Production of cellulase by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate. *Brazilian journal of microbiology*, 42(3): 1119-1127.

Muñoz, I. G., Ubhayasekera, W., Henriksson, H., Szabó, I., Pettersson, G., Johansson, G. and Ståhlberg, J. (2001). Family 7 cellobiohydrolases from Phanerochaete chrysosporium: crystal structure of the catalytic module of Cel7D (CBH58) at 1.32 Å resolution and homology models of the isozymes. *Journal of molecular biology*, 314(5): 1097-1111.

Nägele, G. (2014). Dynamics of charged-particles dispersions. *Proceedings of the 5th Warsaw School of Statistical Physics*, 83.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry*, 153(2): 375-380.

Ninfa, A. J., Ballou, D. P. and Parsons, M. B. P. (2010). *Fundamental Laboratory Approaches for Biochemistry and Biotechnology*. Wiley.

Nochure, S.V., Roberts, M.F. and Demain, A.L. (1993). True cellulase production by *C. thermocellum* grown on different carbon sources. *Biotech. Lett*., 15(6): 641-646.

Ogawa, K. (1989). Fractionation and purification of cellulases from *Trichoderma viride*. Bull. Fac. Agr. Myazaki Univ. 36: 271-280.

Ojumu, T. V., Solomon, B. O., Betiku, E., Layokun, S. K. and Amigun, B. (2003). Cellulase Production by *Aspergillus flavus* Linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob. *African Journal of Biotechnology*, 2(6): 150-152.

Okareh, O.T. Adeolu, A.T., and Adepoju, O.T. (2015). Proximate and mineral composition of plantain (*Musa Paradisiaca*) wastes flour; a potential nutrients source in the formulation of animal feeds. *African Journal Food Science and Technology*, 6(2): 53-57.

Okolo, T. O. and Utoh, N. O. (1999). Groundnut Seed Multiplication and Constraints; FAO's Experience. *2010). Profitability of groundnut production in Michika Local Government Area of Adamawa State, Nigeria. J. Agric. Sci*, 1(1): 25-29.

Olama, Z. A., Hamza, M. A., El-Sayed, M. M. and Abdel-Fattah, M. (1993). Purification, properties and factors affecting the activity of *Trichoderma viride* cellulase. *Food Chem*., 47: 221- 226.

Olsen, C., Arantes, V. and Saddler, J. (2015). Optimization of chip size and moisture content to obtain high, combined sugar recovery after sulfur dioxide-catalyzed steam pretreatment of softwood and enzymatic hydrolysis of the cellulosic component. *Bioresource Technology*, 187: 288-298.

Oluyemi, E.A., Akinlua, A.A., Adenuga, A.A. and Adebayo, M.B. (2006). Mineral contents of some commonly consumed. *Nigerian Foods Science Focus,* 11(1): 153-157.

Omojasola, P. F., Jilani, O. P. and Ibiyemi, S. A. (2008). Cellulase production by some fungi cultured on pineapple waste. *Nature and Science*, 6(2): 64-79.

Ong, L. G. A., Abd-Aziz, S., Noraini, S., Karim, M. I. A. and Hassan, M. A. (2004). Enzyme production and profile by *Aspergillus niger* during solid substrate fermentation using palm kernel cake as substrate. *Applied biochemistry and Biotechnology*, 118(1-3): 73-79.

Orth, A. B. and Tien, M. (2013). 17 Biotechnology of Lignin Degradation. *Genetics and Biotechnology,* 2: 287.

Pakarinen, A. (2012). Evaluation of fresh and preserved herbaceous field crops for biogas and ethanol production.

Parisutham, V., Sathesh-Prabu, C., Mukhopadhyay, A., Lee, S. K. and Keasling, J. D. (2017). Intracellular cellobiose metabolism and its applications in lignocellulose-based biorefineries. *Bioresource Technology*.

Parry, N. J., Beever, D. E., Emyr, O. W. E. N., Vandenberghe, I. and Van Beeumen, J. (2001). Biochemical characterization and mechanism of action of a thermostable β-glucosidase purified from *Thermoascus aurantiacus*. *Biochemical Journal*, 353(1): 117-127.

Pascual, J. J. (2001). Recent advances on early weaning and nutrition around weaning. *Proceedings of the 2nd Meeting of COST*, 848: 31-36.

Paul-Dauphin, S., Karaca, F., Morgan, T. J., Millan-Agorio, M., Herod, A. A. and Kandiyoti, R. (2007). Probing size exclusion mechanisms of complex hydrocarbon mixtures: the effect of altering eluent compositions. *Energy and Fuels*, 21(6): 3484-3489.

Pazarlioğlu, N. K., Sariişik, M. and Telefoncu, A. (2005). Treating denim fabrics with immobilized commercial cellulases. *Process Biochemistry*, 40(2): 767-771.

Penttilä, E., Nenonen, M. and Niemi, J. (1998). Cultural and biological bases of idioms: a crosslinguistic study. *Language Contact Variation and Change. Studies in Languages. University of Joensuu*, 32: 234-24.

Pere, J., Puolakka, A., Nousiainen, P. and Buchert, J. (2001). Action of purified *Trichoderma reesei* cellulases on cotton fibers and yarn. *Journal of Biotechnology*, 89(2): 247-255.

Pere, J., Siikaaho, M., Buchert, J. and Viikari, L. (1995). Effects of purified *Trichoderma reesei* cellulases on the fiber properties of kraft pulp. *Tappi Journal (USA)*.

Po-Jui, C., TaoChun, W., YaoTsung, C. and Liangling, L. (2004). Purification and characterization of carboxylmethyl cellulase from *Sinorhigobium fredii*. Bot. Bull. *Acad. Sinica*. 45:111-118.

Poulsen, O. M. and Petersen, L. W. (1988). Growth of Cellulomonas sp. ATCC 21399 on different polysaccharides as sole carbon source induction of extracellular enzymes. *Applied Microbiology and Biotechnology*, 29(5): 480-484.

Putnam, D. H., Oplinger, E. S., Teynor, T. M., Oelke, E. A., Kelling, K. A., Doll, J. D. (1991). "Peanut". Alternative Field Crops Manual, NewCROP Center, Purdue University.

Rai, P., Majumdar, G. C., Gupta, S. D. and De, S. (2007). Effect of various pretreatment methods on permeate flux and quality during ultrafiltration of mosambi juice. *Journal of Food Engineering*, 78(2): 561-568.

Ramos, L. P. (2003). The chemistry involved in the steam treatment of lignocellulosic materials. *Química Nova*, 26(6): 863-871.

Rath, A., Glibowicka, M., Nadeau, V. G., Chen, G. and Deber, C. M. (2009). Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proceedings of the National Academy of Sciences*, 106(6): 1760-1765.

Rawat, R., Srivastava, N. and Oberoi, H. S. (2014). Endoglucanases: characterization and its role in bioconversion of cellulosic biomass. *Recent Advances in Bioenergy Research*, 3: 135-144.

Reed, R. and Reed, R. H. (2007). *Practical skills in biomolecular sciences*. Pearson education.

Rivers, D. B. and Emert, G. H., Factors affecting the enzymatic hydrolysis of municipal-solid-waste components. *Biotechnology and Bioengineering*, 31: 278-81

Robson, L. M. and Chambliss, G. H. (1989). Cellulases of bacterial origin. *Enzyme and Microbial Technology*, 11(10): 626-644.

Saddiq, A. A. N. (2014). Antiagnostic effect of musk and sidr leaves on some of the opportunistic fungi that cause Lung toxicity. *Life Science Journal*, 11(2): 99-108.

Saha, B. C. (2004) "Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*," *Process Biochemistry*, 39(12): 1871-1876.

Saini, J. K., Patel, A. K., Adsul, M. and Singhania, R. R. (2016). Cellulase adsorption on lignin: A roadblock for economic hydrolysis of biomass. *Renewable Energy*, 98: 29-42.

Sales, J. (1996). Histological, biophysical, physical and chemical characteristics of different ostrich muscles. *Journal of the Science of Food and Agriculture*, 70(1): 109-114.

Saliu, B. K. and Sani, A. (2012). Bioethanol potentials of corn cob hydrolysed using cellulases of *Aspergillus niger* and *Penicillium decumbens*. *EXCLI journal*, 11, 468.

Saloheimo, M. and Niku-Paavola, M. L. (1991). Heterologous production of a ligninolytic enzyme: expression of the *Phlebia radiata* laccase gene in *Trichoderma reesei*. *Nature Biotechnology*, 9(10): 987-990.

Sánchez, Ó. J. and Montoya, S. (2013). Production of bioethanol from biomass: an overview. *Biofuel technologies*, 397-441.

Sandgren, M., Ståhlberg, J. and Mitchinson, C. (2005). Structural and biochemical studies of GH family 12 cellulases: improved thermal stability, and ligand complexes. *Progress in Biophysics and Molecular Biology*, 89(3): 246-291.

Sanwal, G. G. (1999). Purification and characterization of a cellulase from *Catharanthus roseus* stems. *Phytochemistry*, 52(1): 7-13.

Saranraj, P., Stella, D. and Reetha, D. (2012). Microbial cellulases and its applictions. A. *Int. J. Biochem. Biotechnol. Sci*, 1: 1-12.

Saritha, M. and Arora, A. (2012). Biological pretreatment of lignocellulosic substrates for enhanced delignification and enzymatic digestibility. *Indian Journal of Microbiology*, 52(2): 122-130.

Schell, D. J., Farmer, J., Newman, M. and Mcmillan, J. D. (2003). Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor. *Biotechnology for Fuels and Chemicals*, 69-85.

Schwarz, W. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology*, 56(5-6): 634-649.

Scopes, R. K. (2013). *Protein purification: principles and practice*. Springer Science and Business Media.

Scopes, R. K. (2013). Protein purification: principles and practice. Springer Science and Business Media.

Shaheen, S., Aman, A. and Siddiqui, N. N. (2017). Influence of Metal ions, Surfactants and Organic Solvents on the Catalytic Performance of Levansucrase from *Zymomonas mobilis* KIBGE-IB14. *Journal of Basic and Applied Sciences*, 13: 41-46.

Shahriarinour, M., Wahab, M. N. A., Mohamad, R., Mustafa, S. and Ariff, A. B. (2011). Effect of medium composition and cultural condition on cellulase production by *Aspergillus terreus*. *African Journal of Biotechnology*, 10(38): 7459-7467.

Shallom, D. and Shoham, Y. (2003). Microbial hemicellulases. *Current Opinion in Microbiology*, 6(3): 219-228.

Shaojun, D., Wei, G. and Busuell, J. A. (2001). Endoglucanase 1 from the edible straw mushroom, *Volvariella volvaeeae*: Purification, characterization, cloning and expression. *Eur. J. Biochem*., 268: 5687-5695.

Sharada, R., Venkateswarlu, G., Venkateswar, S. and Rao, M. A. (2014). Applications of cellulases-review. *International Journal of Pharmaceutical, Chemical and Biological Sciences*, 4(2).

Sharma, B., Agrawal, R., Singhania, R. R., Satlewal, A., Mathur, A., Tuli, D. and Adsul, M. (2015). Untreated wheat straw: potential source for diverse cellulolytic enzyme secretion by *Penicillium janthinellum* EMS-UV-8 mutant. *Bioresource technology*, 196: 518-524.

Sharmeen, S., Rahman, M. M. and Haque, P. (2016). Recent Updates on Immobilization.

Sharyo, M., Sakaguchi, H., Ohishi, M., Takahashi, M., Kida, K., Tamagawa, H.and Franks, N. (2002). *U.S. Patent No. 6,468,391*. Washington, DC: U.S. Patent and Trademark Office.

Silveira, M. H. L., Aguiar, R. S., Siika-aho, M. and Ramos, L. P. (2014). Assessment of the enzymatic hydrolysis profile of cellulosic substrates based on reducing sugar release. *Bioresource Technology*, 151: 392-396.

Sindhu, R., Gnansounou, E., Binod, P. and Pandey, A. (2016). Bioconversion of sugarcane crop residue for value added products–An overview. *Renewable Energy*, 98: 203-215.

Singh S. K., Singh, S.K. Tripathi, V.R. and Garg, S.K. (2012). Purification, characterization and secondary structure elucidation of a detergent stable, halotolerant, thermoalkaline protease from *Bacillus cereus* SIU1. *Process Biochemistry*, 47: 1479–1487.

Singh, A., Kuhad, R. C. and Ward, O. P. (2007). Industrial application of microbial cellulases. *Lignocellulose Biotechnologgy: Future Prospects*, 345-358.

Singhania, R.R., Sukumarana, R.K., Patelb, A.k., Larrocheb, C. and Pandeya, A. (2010). Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. *Enzyme Microb Tech.*, 46: 541–549.

Skoog, D. A. (2006). Principles of Instrumental Analysis, 6th ed.; Thompson Brooks/Cole: Belmont, CA, 2006, Chapter 28.

Smith, B. W. (1950). Arachis hypogaea. Aerial flower and subterranean fruit. *American Journal of Botany*, 802-815.

Smith, M. T., Coward-Kelly, G., Nilsson, D., Kang, Z., Iyer, P. and Deinhammer, R. (2016). *U.S. Patent No. 9,399,782*. Washington, DC: U.S. Patent and Trademark Office.

Solomon, B. O., Amigun, B., Betiku, E, Ojumu, T. V. and Layokun, S. K. (1999). Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 grown on bagasse. *JNSChE*, 16: 61-68.

Somogyi, M. (1952). "Notes on Sugar Determination." *J. Biol. Chem.*, 195: 19–23.

Soni, R., Nazir, A. and Chadha, B. S. (2010). Optimization of cellulase production by a versatile *Aspergillus fumigatus* fresenius strain (AMA) capable of efficient deinking and enzymatic hydrolysis of Solka floc and bagasse. *Industrial Crops and Products*, 31(2): 277-283.

Sonjoy, S.B., Bex, K. and Honston, H. (1995). Cellulase activity of *Trichoderma reesei* (RUT-C30) on municipal solid waste. *Appl. Biochem. Biotechnol*., 51-52(1): 145-153.

Souza, P. M. D. (2010). Application of microbial α-amylase in industry-A review. *Brazilian Journal of Microbiology*, 41(4): 850-861.

Sreena, C. P., Vimal, K. P. and Sebastian, D. (2016). Production of cellulases and xylanase from bacillus subtilis mu s1 isolated from protected areas of munnar wildlife division. *The Journal of Microbiology, Biotechnology and Food Sciences*, 5(6): 500.

Sreenath, H. K., Shah, A. B., Yang, V. W., Gharia, M. M. and Jeffries, T. W. (1996). Enzymatic polishing of jute/cotton blended fabrics. *Journal of Fermentation and Bioengineering*, 81(1): 18-20.

Sridevi, A., Narasimha, G. and Reddy, B. R. (2009). Production of cellulase by *Aspergillus niger* on natural and pretreated lignocellulosic wastes. *The Internet Journal of Microbiology*, 7(1): 580-592.

Srivastava, S.K., Gopalkrishnan, K.S. and Ramachandran, K.B. (1987). The Production of β-Glucosidase in Shake-flasks by *Aspergillus wentii. J. Ferment. Technol.*, 65(1): 95 -99.

Stein-Chisholm, R. E., Finley, J. W., Losso, J. N. and Beaulieu, J. C. (2017). Not-from-concentrate Blueberry Juice Extraction Utilizing Frozen Fruit, Heated Mash, and Enzyme Processes. *HortTechnology*, 27(1): 30-36.

Stork G. and Puls, J. (1996) "Changes in properties of different recycled pulps by endoglucanase treatments," in Proceedings of the International Conference on Biotechnology in the Pulp and Paper Industry: *Recent Advances in Applied and Fundamental Research*, E. Srebotnik and K. Mesner, Eds., 1: 145–150.

Sukumaran, R. K., Singhania, R. R. and Pandey, A. (2005). Microbial cellulases-production, applications and challenges

Sultana, S. (1997). Isolation of cellulolytic microorganism and their activities. M. Phil Thesis. Biochemistry Department, Rajshahi Univ. Bangladesh.

Sun, T., Chance, R. R., Graessley, W. W. and Lohse, D. J. (2004). A study of the separation principle in size exclusion chromatography. *Macromolecules*, 37(11): 4304-4312.

Sun, T., Liu, B.H., Li, Z.H., Liu, D.M., 1999, Effect of air pressure amplitude on cellulase production by *Trichoderma viride* SL1 in periodic pressure solid state fermentation. *Process Biochemistry*, 34: 25-29.

Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S. and Törnqvist, M. (2000). Acrylamide: a cooking carcinogen. *Chemical Research in Toxicology*, 13(6): 517-522.

Taru, V. B., Kyagya, I. Z. and Mshelia, S. I. (2010). Profitability of groundnut production in michika local government area of adamawa state, Nigeria. *J. Agric. Sci*, 1(1): 25-29.

Taylor, C. L. (2003). Qualified health claims: letter of enforcement discretion–nuts and coronary heart disease (Docket No. 02P-0505).

Tejada, M., Gonzalez, J. L., García-Martínez, A. M. and Parrado, J. (2008). Application of a green manure and green manure composted with beet vinasse on soil restoration: effects on soil properties. *Bioresource Technology*, 99(11): 4949-4957.

Telke, A. A., Zhuang, N., Ghatge, S. S., Lee, S. H., Shah, A. A., Khan, H. and Kim, S. W. (2013). Engineering of family-5 glycoside hydrolase (Cel5A) from an uncultured bacterium for efficient hydrolysis of cellulosic substrates. *PLoS One*, 8(6): 65727.

Trache, D., Hussin, M. H., Haafiz, M. M. andThakur, V. K. (2017). Recent progress in cellulose nanocrystals: sources and production. *Nanoscale*, 9(5): 1763-1786.

Tu, M., Chandra, R. P. and Saddler, J. N. (2007). Evaluating the distribution of cellulases and the recycling of free cellulases during the hydrolysis of lignocellulosic substrates. *Biotechnology Progress*, 23(2): 398-406.

Tucker, I. M., Corbett, J. C. W., Fatkin, J., Jack, R. O., Kaszuba, M., MacCreath, B. and McNeil-Watson, F. (2015). Laser Doppler Electrophoresis applied to colloids and surfaces. *Current Opinion in Colloid and Interface Science*, 20(4): 215-226.

Uhlig, H. (1998). *Industrial enzymes and their applications*. John Wiley & Sons.

United Nation Environmental Progrmme, (2009). Waste not, want not: Converting agricultural biomass waste into energy.

Usman, N. and Said, I. (2012). Technology acceptance model (TAM) in a multicultural developing nation's construction industry's ICT. *European Journal of Scientific Research*, 81(4): 582-588.

van Peij, N. N., Gielkens, M. M., de Vries, R. P., Visser, J. and de Graaff, L. H. (1998). The transcriptional activator XlnR regulates both xylanolytic and endoglucanase gene expression in *Aspergillus niger*. *Applied and Environmental Microbiology*, 64(10): 3615-3619.

Van Soest, P. and Robertson, J. (1979). Systems of analysis for evaluating fibrous feeds. In *Standardization of analytical methodology for feeds: proceedings.* IDRC, Ottawa, ON, CA.

Vyas, A., Vyas, D. and Vyas, K. M. (2005). Production and optimization of cellulases on pretreated groundnut shell by *Aspergillus terreus* AV49.

Wackernagel, M., Kitzes, J., Moran, D., Goldfinger, S. and Thomas, M. (2006). The ecological footprint of cities and regions: comparing resource availability with resource demand. *Environment and Urbanization*, 18(1): 103-112.

Wang, G., Zhang, X., Wang, L., Wang, K., Peng, F. and Wang, L. (2012). The activity and kinetic properties of cellulases in substrates containing metal ions and acid radicals. *Advances in Biological Chemistry*, 2(04): 390.

Wang, J. S. (1999). Cellulase production by a mutant strain of *Trichoderma reesei* from bagasse. 23rd ISSCT Congress, New Delhi, India, 67-76.

Wen, Y. Q., He, F., Zhu, B. Q., Lan, Y. B., Pan, Q. H., Li, C. Y. and Wang, J. (2014). Free and glycosidically bound aroma compounds in cherry (Prunus avium L.). *Food chemistry*, 152: 29-36.

Wen, Z., Liao, W. and Chen, S. (2005). Production of cellulase/β-glucosidase by the mixed fungi culture *Trichoderma reesei* and *Aspergillus phoenicis* on dairy manure. *Process Biochemistry*, 40(9): 3087-3094.

Westermeier, R. (2016). Electrophoresis in practice: a guide to methods and applications of DNA and protein separations. *John Wiley and Sons.*

Wolfenden, R. and Snider, M. J. (2001). The depth of chemical time and the power of enzymes as catalysts. *Accounts of Chemical Research*, 34(12): 938-945.

Wu, J. Q., Guo, J. Y., Tang, W., Yang, C. S., Freel, C. D., Chen, C. and Kornbluth, S. (2009). PP1-mediated dephosphorylation of phosphoproteins at mitotic exit is controlled by inhibitor-1 and PP1 phosphorylation. *Nature Cell Biology*, 11(5): 644-651.

Yang, B. and Wyman, C. E. (2008). Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts and Biorefining*, 2(1): 26-40.

Yin, L. J., Lin, H. H. and Xiao, Z. R. (2010). Purification and characterization of a cellulase from *Bacillus subtilis* YJ1. *Journal of Marine Science and Technology*, 18(3): 466-471.

Youn, K. S., Hong, J. H., Bae, D. H., Kim, S. J. and Kim, S. D. (2004). Effective clarifying process of reconstituted apple juice using membrane filtration with filter-aid pretreatment. *Journal of Membrane Science*, 228(2): 179-186.

Yusuf, R. O. and Oyewumi, M. O. (2008). Qualitative assessment of methane generation potential for municipal solid wastes: a case study. *Environmental Research Journal, Medwell Journals*, 2(4): 138-144.

Zeitsch, K. J. (2000). *The chemistry and technology of furfural and its many by-products*, 13.

Zhang, G., Liu, P., Wei, W., Wang, X., Wei, D. and Wang, W. (2016). A light-switchable bidirectional expression system in filamentous fungus *Trichoderma reesei*. *Journal of biotechnology*, 240: 85-93.

Zhang, Y. H. P. and Lynd, L. R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnology and Bioengineering*, 88(7): 797-824.

Zhao, W., Bai, J., McCollum, G. and Baldwin, E. (2015). High incidence of preharvest colonization of huanglongbing-symptomatic Citrus sinensis fruit by *Lasiodiplodia theobromae* (*Diplodia natalensis*) and exacerbation of postharvest fruit decay by that fungus. *Applied and Environmental Microbiology*, 81(1): 364-372.

Appendix I

Preparation of reagents

1. Preparation of Glucose Standard Curve

A stock solution of glucose was prepared by adding 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10mg of glucose to 1ml of citrate buffer in separate test tubes to give a concentration of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10mg of glucose per ml respectively. 3 ml of DNS reagent was added. The mixture was placed in boiling water for 5 minutes and later cooled at room temperature; 3ml of distilled water was added the absorbance was read at 540 nm against the blank using a spectrophotometer.

2. Preparation of 0.15 g/l of ρ-NPG

0.015 g of para-nitrophenyl-β-D-glucopyranoside was weighed and dissolved in small amount of 0.05M citrate buffer (pH 4.8) then make up to 100 ml volumetric flask.

3. Preparation of Potato Dextrose Agar

An amount of 9.75g of potato dextrose agar was diluted in 250 ml distilled water. Solution was autoclaved at 121^0C and 15 psi for 15 minutes until no colloid was seen to ensure the solution is completely diluted. Then the solution was poured into petri-dish until the solution is 1/3 height of the bottles. Lastly, agar was left to solidify.

4. Preparation of 1000 ml of 0.05 M Citrate Buffer pH 4.8

210 g of citric acid monohydrate was weighed and dissolved in about 750 ml of distilled water in a measuring cylinder. Dilute NaOH solution was added until the pH is 4.3. The solution was made up to 1000 ml and pH adjusted to 4.8.

5. Preparation of 3,5-Dintrosalicylic Reagent (DNS)

10.6 g of DNS and 19.8 g of NaOH were weighed and dissolved in 1416 ml of distilled water. The solution was mixed and after which 306 g of sodium potassium tartarate, 7.6 ml and sodium metabisulphite were added to the mixture.

6. Preparation of 1% Carboxymethylcellulose

10g of CMC gum was dissolved in 800ml of 0.05M citrate buffer of pH4.8 (80-90^{0}C) by adding the dry powder slowly with continuous agitation; 100ml of 0.05M citrate buffer at pH 4.8 and 10ml of 1% methiolate were added, it was then made up to a litre with 0.05M citrate buffer and the resulting solution was then refrigerated and warmed to 50^{0}C before use.

7. Preparation of 1% Avicel

10g of avicel was dissolved in 800ml of 0.05M citrate buffer (pH 4.8). The solution was mixed very well to ensure total dissolution of avicel in citrate buufer. The mixture was later made up to 1000 ml.

8. Preparation of 50 ml of 10% Sodium Dodecyl Sulphate (SDS)

5.0 g of SDS was weighed and dissolved in small quantity of distilled water then made up to the mark in 50 ml volumetric flask.

9. Preparation of 10 ml of 30% Bis-Acrylamide Solution

2.9 g of acrylamide and 0.1g of were weighed and dissolved in small quantity of distilled water then made up to 10 ml.

10. Preparation of 1 ml of 10% Fresh Ammonium Persulphate (APS)

0.1 g of ammonium persulphate was dissolved in 1ml distilled water. This was freshly prepared whenever it was needed.

11. Preparation of 100 ml of 0.5% Bovine Serum Albumin

0.5 g of casein powder was weighed and dissolved in 100 ml of 100mM phosphate buffer.

12. Preparation of 100 ml of 0.5 M Na_2CO_3 Solution

5.3 g of anhydrous Na_2CO_3 was weighed and dissolved in small quantity of distilled water and then made up to the mark in 100 ml volumetric flask.

13. Preparation of 100 ml of 0.1 N NaOH solution

0.4 g of NaOH pellet was weighed and dissolved in 100 ml of the solution.

14. Preparation of 50 ml of 1.5 M Tris pH 8.8

9.082 g of Trizma base was weighed and dissolved in small quantity of distilled water then made up to the mark in 50 ml volumetric flask.

15. Preparation of 50 ml of 0.5 M Tris pH 6.8

3.025 g of Trizma base was weighed and dissolved in small quantity of distilled water then made up to the mark in 50 ml volumetric flask.

16. Preparation of 100 mM Glycine-NaOH buffer pH 9.5-12

0.751 g of glycine (aminoethanoic acid) and 0.584 ml of sodium chloride were dissolved in 100 ml solution. 0.4 g of NaOH pellet was weighed and dissolved separately in 100 ml of the

solution. The working solution was made by mixing appropriate volume of glycine solution with appropriate volume of the NaOH and then diluted to 100 ml to produce the desired pH value.

17. Preparation of 100% ammonium sulphate saturation

100% saturated ammonium sulpahate solution was first prepared by weighing about 545g of ammonium sulphate crystals and gently added to 1 litre of distilled water with intermittent stirring until the ammonium sulphate could no longer dissolve in the solution. The undissolved ammonium sulphate particles were then filtered out of the solution after they remained therein for hours. The saturated solution was then kept refrigerated until it was needed for use.

18. Preparation of reagents for Lowry's method of protein determination

The following reagents/chemicals were prepared as follows:

2% Na_2CO_3 was prepared using 0.1N NaOH as solvent

1% Sodium potassium tartrate was prepaed in distilled water.

Reagent A was prepared by mixing 48 litres of alkaline Na_2CO_3, 1 ml of 1% sodium potassium tartarate and 1 ml of 0.5% hydrated copper sulphate together. Reagent B was prepared by mixing folin reagent with distilled water in ratio 1:2.

19. Preparation of Staining Solution

The staining solution was prepared by dissolving 0.25 g of Coomassie Brilliant Blue R-250 in 200 ml of methanol with continuous stirring in a beaker. 50 ml of glacial acetic acid was slowly added while stirring continued. 250 ml of distilled water was added and the resulting solution was stirred for several minutes and then filtered. The dye was stored at room temperature.

20. Preparation of running and loading buffers

The running buffer was prepared by mixing 50 ml of 10x TG (0.25M Tris (pH 8.3), 1.92 M Glycine), 50 ml distilled water and 5 ml of 10% SDS. The mixture was then diluted to 500 ml with distilled water to get 1x TGS running buffer.

Loading buffer (5x) comprises of the following:

1 M Tris (pH 6.8) 2.5 ml

Glycerol 4 ml

SDS 0.8g

β-mercaptoethanol 2 ml

Bromophenol blue 0.05g

The mixture was made up to 10 ml with distilled water.

Appendix II

Protocol for assays

Table 7: Protocol for the assay of endoglucanase activity

Content	Test Sample	Blank
Substrate (1% CMC) (ml)	0.5	0.5
Enzyme source (ml)	0.5	-
Mix well		
Incubate at 50 ^{o}C for 30 mins		
DNS solution (ml)	1	1
Boil for 5 min in boiling water		
Distilled water (ml)	3	3.5
Read the absorbance at 540 nm		

Table 8: Protocol for the assay of exoglucanase activity

Content	Test Sample	Blank
Substrate (1% Avicel) (ml)	0.5	0.5
Enzyme source (ml)	0.5	-
Mix well		
Incubate at 50 ^{o}C for 60 mins		
DNS solution (ml)	1	1
Boil for 5 min in boiling water		
Distilled water (ml)	3	3.5
Read the absorbance at 540 nm		

Table 9: Protocol for the assay of β-glucosidase activity

Content	Test Sample	Blank
Substrate (0.15 g/L ρ-NPG) (ml)	2.0	2.0
Enzyme source (ml)	0.2	-
Distilled water	-	0.2
Mix well		
Incubate at 40 ^{o}C for 30 mins		
Read the absorbance at 420 nm		

Table 10: Protocol for protein determination

Content	Test Sample	Blank
Protein source (ml)	0.5	-
Distilled water (ml)	-	0.5
Reagent D (ml)	5.0	5.0
Mix well		
Reagent E (ml)	0.5	0.5
Mix well		

Read the absorbance at 750 nm after 30 mins.

Table 11: Protocol for determination of effect of Na^+ ion on the activity of purified cellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
NaCl (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 12: Protocol for determination of effect of K^+ ion on the activity of purified cellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
KI (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 13: Protocol for determination of effect of Mg^{2+} ion on the activity of purified cellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
$MgSO_4$ (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 14: Protocol for determination of effect of Zn^{2+} ion on the activity of purified cellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
$ZnSO_4$ (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 15: Protocol for determination of effect of Cu^{2+} ion on the activity of purified cellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
$CuSO_4.5H_2O$ (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 16: Protocol for determination of effect of Ca^{2+} ion on the activity of purified cellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
$CaCl_2$ (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 17: Protocol for determination of effect of Fe^{2+} ion on the activity of purifiedcellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
$FeSO_4$ (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 18: Protocol for determination of effect of Mn^{2+} ion on the activity of purified cellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
$MnCl_2.H_2O$ (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 19: Protocol for determination of effect of Co^{2+} ion on the activity of purified cellulase

	Blank	Test (Absence of metal ion)	Test (Presence of metal ion)
Substrate (1% CMC)	0.5 ml	0.5 ml	0.5 ml
Purified Cellulase	-	0.1 ml	0.1 ml
$CoCl_2.6H_2O$ (0.05 mM)	-	-	0.1 ml
	Mix well and incubate at 50 ^{0}C for 30 min		
DNS Solution	3 ml	3 ml	3 ml
	Boil for 5 min in a boiling water		
Distilled water	5 ml	5 ml	5 ml

Read the absorbance at 540 nm

Table 20: Preparation of PNP standard curve

Test Tubes	Blank	1	2	3	4	5	6	7	8	9	10
PNP (0.1-1.0 mM)	0.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Distilled water	2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Incubated at 37 ℃ for minutes											
100 mM of Na_2CO_3 (ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Read the absorbance at 405 nm.

Table 21: Preparation of protein standard curve

Test tube/Reagents	Blank	1	2	3	4	5	6	7	8	9	10
BSA (1-10 mg/ml)	0.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Distilled water	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Reagent A (ml)	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Allow to stand for 10 minutes											
Reagent B (ml)	0.50	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Total volume (ml)	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0
Allow to stand for 30 minutes for colour development											

BSA - Bovine Serum Albumin; Reagent A - Alkaline copper solution; Reagent B - Folin-ciocalteu.

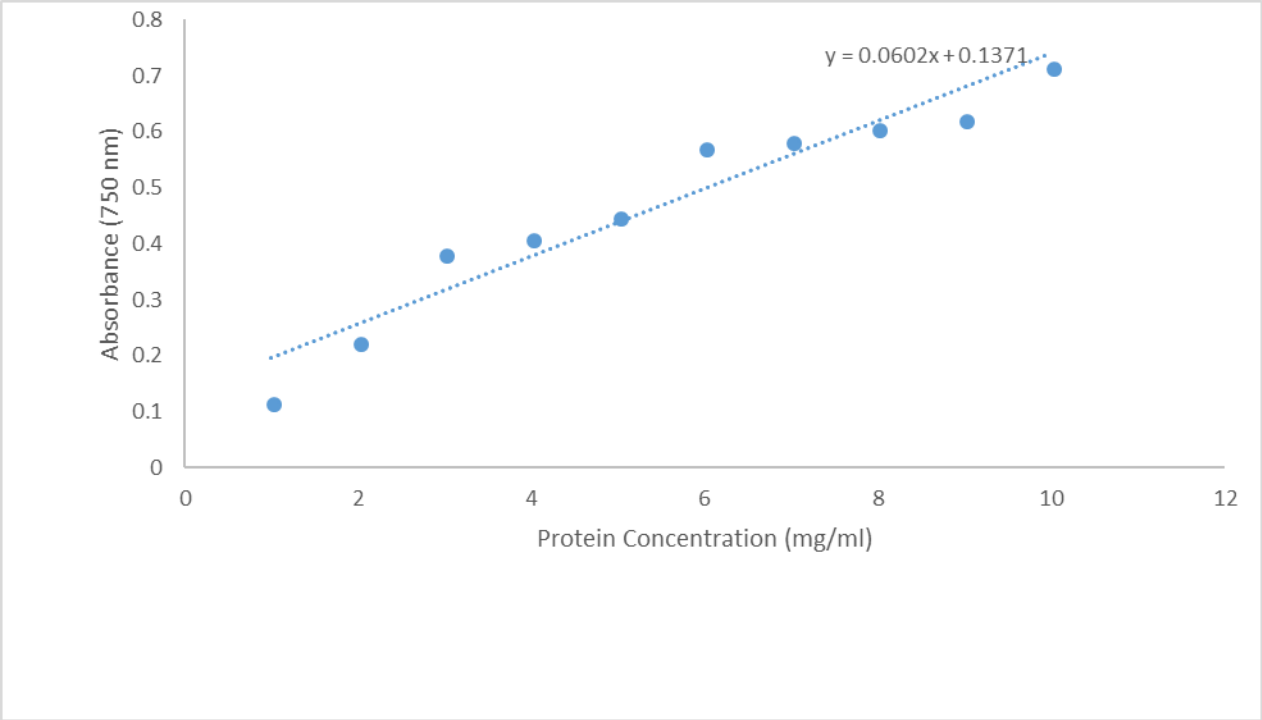


Figure 26: Standard curve for protein

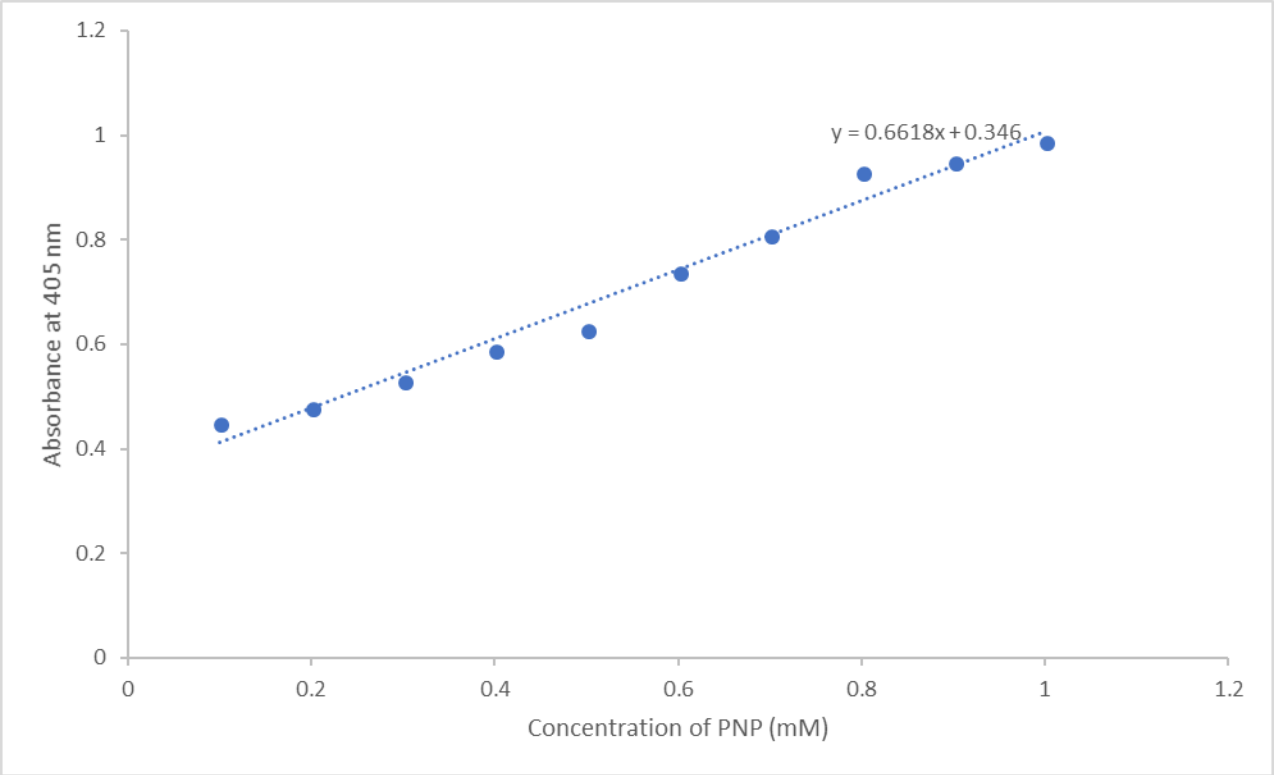


Figure 27: PNP standard curve

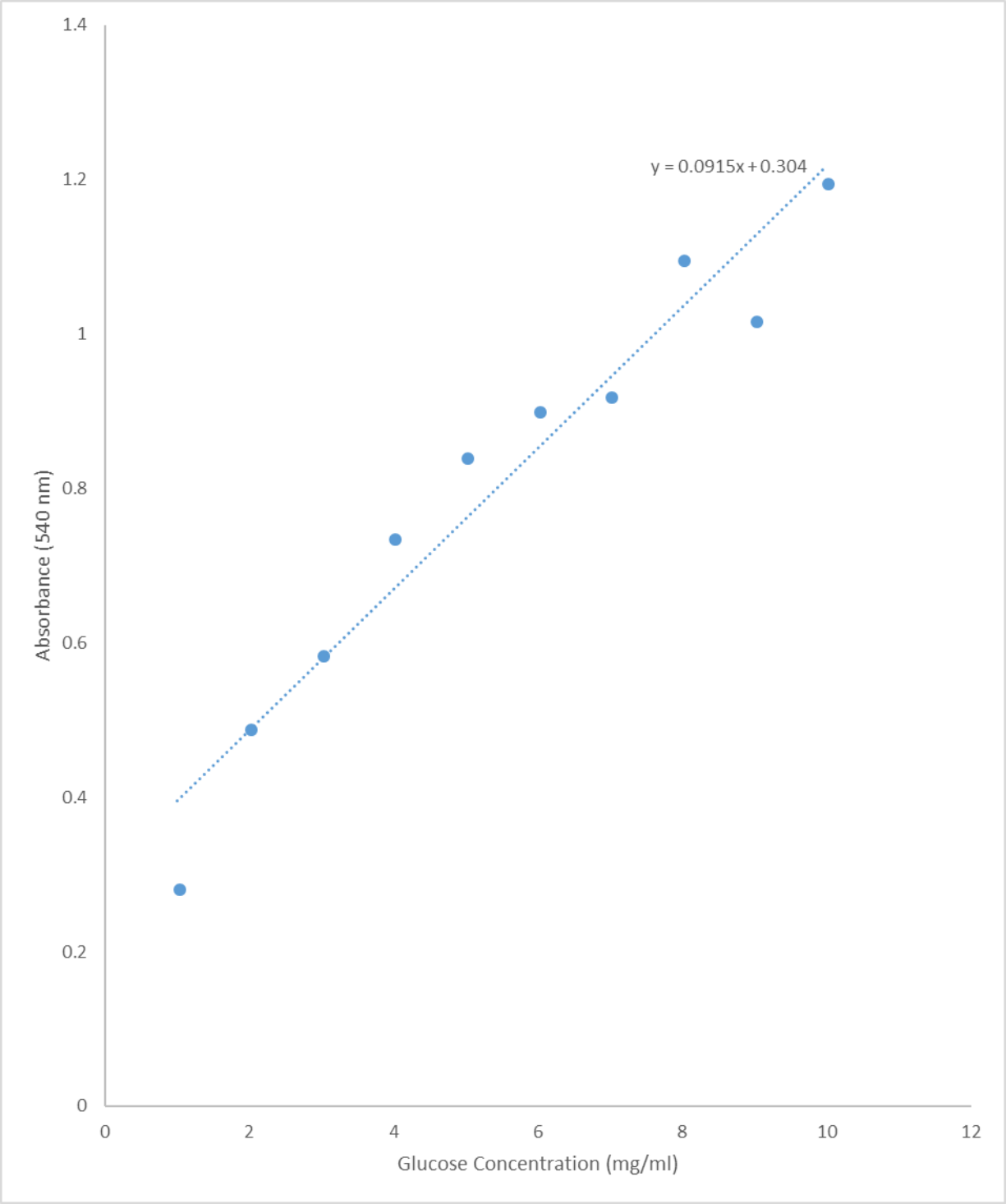


Figure 28: Glucose standard curve