EXTRACTION AND CHARACTERIZATION OF CELLULASE FROM FOREST AND COMPOST SOIL FUNGAL ISOLATES AND EVALUATION OF ITS APPLICATION FOR DEGRADATION OF FINGER MILLET AND OAT STRAW

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EXTRACTION AND CHARACTERIZATION OF CELLULASE FROM FOREST AND COMPOST SOIL FUNGAL ISOLATES AND EVALUATION OF ITS APPLICATION FOR DEGRADATION OF FINGER MILLET AND OAT STRAW

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As thesis research advisors, we hereby certify that we have read and evaluated this thesis prepared, under our guidance, by Yidnekachew Kifle entitled "**Extraction and Characterization of Cellulase From Forest and Compost Soil Fungal Isolates and Evaluation of Its Application for Degradation of Finger Millet and Oat Straw"**. We recommend that it be accepted as fulfilling the thesis requirement.

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DEDICATION

This thesis manuscript is dedicated to my step father Atle Zegeye who passed away during the period of my research engagement (I was always wondering about his patience and altruistic behavior); may God save his soul! And to my father Kifle Awoke for his love and the sacrifice he paid for my success.

STATEMENT OF THE AUTHOR

I hereby certify that this thesis is my own work and all the sources of materials used for the thesis have been duly acknowledged. This has been submitted in partial fulfilment of the requirements of M.Sc. in Biotechnology at Haramaya University and is deposited at the University Library to be made available to borrowers under the rules of the Library. I solemnly declare that this thesis is not submitted to any other institution, elsewhere, for the award of any academic degree, diploma, or certificate.

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BIOGRAPHICAL SKETCH

The author was born at Kabe Kebele, Woreillu district, South Wollo Zone of Amhara Regional State on April 1989 from his father Mr. Ali Yimam and his mother Mrs. Asnaku Kifle. He attended his elementary school education at Leshete and Elshama Elementary School, his secondary school education at Degollo Secondary School and his preparatory school education at Woreillu Secondary and Preparatory School between 1996 and 2008. In October 2009, he joined Agro Technical and Technology College and graduated in Animal Science in July 2011. After graduation, he was employed by the Ministry of Agriculture on 10th November 2011 as an instructor of Animal Sciences in Agarfa Agricultural College. Three years later, in October 2015, he joined the School of Graduate Studies at Haramaya University to pursue his MSc degree in Biotechnology.

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LIST OF ABBREVIATIONS AND ACRONYMS

CBM	Cellulose Binding Module
CD	Catalytic Domain
CMC	Carboxy Methyl Cellulose
DNS	Dinitrosalicylic acid
EC	Enzyme Commission
EIAR	Ethiopian Institute of Agricultural Research
FMS	Finger Millet Straw
HPLC	High-Performance Liquid Chromatography
IUPAC	International Union of Pure and Applied Chemistry
OD	Optical Density
OS	Oat Straw
MEA	Malt Extract Agar
NABRC	National Agricultural Biotechnology Research Center
PI	Isoelectric Point
rpm	Revolution Per Minute
SmF	Submerged Fermentation
SC	Substrate concentration
SSF	Solid State Fermentation

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EXTRACTION AND CHARACTERIZATION OF CELLULASE FROM FOREST AND COMPOST SOIL FUNGAL ISOLATES AND EVALUATION OF ITS APPLICATION FOR DEGRADATION OF FINGER MILLET AND OAT STRAW

ABSTRACT

Cellulases are complex hydrolytic enzymes working synergistically on the hydrolysis of cellulose to produce smaller sugar components including glucose units. These enzymes have tremendous environmental, industrial and agricultural applications including enhancement of the degradability of lignocellulosic materials. In this research fungal isolates were isolated from forest and compost soil samples and allowed to produce cellulases; and the extent to which the cellulases enhance the degradability of finger millet and oat straw was evaluated. A total of 53 fungal isolates were isolated from forest and compost soil samples. Further screening of the isolates on 1% carboxymethyl cellulose media resulted in 21(40%) cellulolytic fungal species. Out of which, six were selected as efficient cellulolytic fungi and eventually subjected for identification. These isolates belonged to the genera Trichoderma, Aspergillus and Penicillium on the basis of their morphological characteristics. Cultivation of the fungal isolates for cellulase production using Submerged fermentation and Solid-state fermentation was assayed at different growth conditions. Assay for cellulase activity was carried out using Carboxymethyl cellulose (for CMCase/endoglucanase) and Filter paper (for FPase"/ total cellulase) activity. The results showed that the highest cellulase production (46.52±0.44U/dL CMCase and 41.57±0.39 FPU/dL FPase) from SmF was obtained at pH of 5.5. Although, a fungal isolate designated as FSI6 (isolated from forest soil sample) showed maximum CMCase production (83.12 ± 3.18 U/dL) and FPase (44.51± 0.391FPU/dL) in FMS supplemented SSF at 28^{0}C and a pH of 6. All compost soil isolates showed better cellulase production in SSF at 38^{0}C and a pH of 5 than forest soil isolates. For instance, the fungal isolate designated as CSI3 (isolated from compost soil sample) showed maximum CMCase stability (48.37 ± 0.27U/dL) at 60^{0}C and a pH of 5. Partial characterization of the cellulases from the six isolates exhibited differences in terms of temperature, pH and metallic ion effects. The effect of pH and temperature on the activity and stability of cellulase indicated that as the temperature and pH increased, cellulase activity also increased while a further increase beyond the highest values revealed a decrease in activity. More than 50% residual activity of cellulase was obtained at temperatures 50 and 60°C incubated for 30min for all six isolates. On the other hand, the effect of metallic ions (Ca^{2+}, Na^{+}, Co^{2+}, Mg^{2+} and Fe^{2+}) on cellulase activity showed that three of the ions i.e. Mg^{2+}, Co^{2+} and Ca^{2+} decreased the activity while Fe^{2+} enhanced the same as the concentration increased. The percentage saccharification on finger millet (28.9%) was higher than that of oat straw. The findings generally suggest that the isolates had high potential for production of cellulase which can be used in the improvement of degradation of cellulosic biomasses.

Key Words: CMCase and FPase, Filamentous Fungi, Isolation and screening, Lignocellulose, Percentage saccharification, Soil

1. INTRODUCTION

Approximately 70% of the plant biomass is locked up in 5- and 6-carbon sugars. These sugars are found in lignocellulosic biomass comprised of mainly cellulose (a homologous polymer of glucose monomers linked by β-1,4 glycosidic bonds); lesser hemicelluloses (heterologous polymer of 5- and 6-carbon sugars consisting of pentoses D-xylose, D-arabinose and hexoses D-mannose, D-glucose, D-galactose with sugar acids); and least of all lignin (a complex aromatic polymer).

Cellulose is the world's most abundant naturally occurring renewable resource available to man for the production of a 'greener' energy replacement which can meet the high energy demand of the world. It is the most promising biomass for energy source to the current as well as to the future world (Czaja *et al*., 2006).

The biotechnological conversion of this cellulosic biomass is a potentially sustainable approach to develop novel bioprocesses and products. Some of these potentials can be readily exploited to produce sugars, fuels and feed stocks through the use of appropriate enzymes. The enzymatic hydrolysis of cellulose using cellulases leads to the formation of reducing sugars, which can be used as a source of food, as a substrate for single cell protein production and as a raw material for industrial fermentation (Mariamma, 1995).

Enzymatic hydrolysis, unlike acid hydrolysis, holds tremendous promise due to the high specificity and production of high yields of glucose without generation of degradation by-products. Besides, the utility cost is lower than acid hydrolysis as enzymatic hydrolysis occurs under mild reaction conditions. Furthermore, microorganisms that degrade cellulose are both abundant and ubiquitous in nature and hence it is easier to produce cellulases by directly cultivating these organisms in media containing cheaper lignocellulosic substrates. Currently, microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications (Bhat *et al*., 2011).

The production of bio-based products and bioenergy from less costly renewable lignocellulosic materials would bring benefits to the local economy, environment, and the national energy security (Zhang, 2008).

Cellulases are modular enzymes that are composed of independently folding, structurally and functionally discrete units, referred to as either domains or modules (Henrissat *et al*., 1998). These enzymes are responsible for the hydrolysis of the β-1,4 glucosidic bonds in cellulose. They are members of the glycoside hydrolase family of enzymes, which hydrolyze oligosaccharides and/or polysaccharides (Henrissat and Davies, 1997).

Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials (Henrissat *et al.,* 1998). These microorganisms can be aerobic, anaerobic, mesophilic or thermophilic. Among them, the species of *Clostridium, Bacillus, Cellulomonas, Thermomonospora, Trichoderma, Penicillium,* white rot fungi, and *Aspergillus* are the most extensively studied cellulase producers (Sukumaran *et al.,* 2005). The cellulase system in fungi is considered to comprise three hydrolytic enzymes which are endo–1, 4 –β–D – glucanases, exo – 1, 4 –β– D – glucanases and cellobiase(β-glucoside glucohydrolase).

A variety of food crops are produced around the globe, which generate enormous agricultural residues. This lignocellulosic biomass can serve as low cost feed stocks, for production of fuel, ethanol and other value-added commodity chemicals (Parimala *et al.,* 2007). As compared to forage, cereal straws are of poor nutritive value and low digestibility because of their higher lignin, cellulose and hemicellulose content. The presence of binding matrix of each other affects the accessibility of energy rich polysaccharides. To overcome the crisis of feed, several cereal crop residues like wheat straw, Teff Straw, Oat straw, Finger millet Straw, Corn Stover and so on are generally used as livestock feed.

From the biomass conversion point of view, cellulases actively convert the cellulosic renewable resources into glucose and other simple fermentable sugars that can be used as substrates for the production of bioethanol. The massive exploitation and utilization of fossil fuels has considerably reduced these natural reserves and simultaneously caused severe environmental pollutions via the release of greenhouse and toxic gases. As a result, the world economy has now focused on the production of bio-fuel, especially bio-ethanol, from renewable resources and is expected to replace 20% of the fossil fuel consumption by 2020 (Msangi *et al.,* 2007). Hence, at present, the most actively investigated application of cellulase is the production of biofuels, especially bioethanol from cellulosic biomasses.

Applications of exogenous enzymes (cellulases and hemicellulases) in the feed industry have also received considerable attention because of their potential to improve feed value, to increase the digestibility and absorption of nutrients, to remove anti-nutritional factors from feeds and has significant potential to improve feed utilization and animal performance (Arora *et al*., 2011; Kholif *et al.,* 2015). Pretreatment of agricultural silage and grain feed by cellulases or xylanases has been reported to have improved their nutritional values greatly (Godfrey and West, 1996).

In tropical and subtropical farming systems, forage plants are the major sources of energy for livestock's. The fiber is a major component of the forage dry matter. However, it has low energy and low digestibility potential (Azzaz *et al.*, 2012) because the plant cell wall is an interwoven matrix of polymers that form complex and dynamic structures. These dynamic structures are barriers against microbial invasion and limit their access to the digestible cell wall networks of plants (McDonald *et al*., 2011).

This study was conducted to screen and characterize cellulase from forest and compost soil fungi to enhance straw degradability for such types of energy sources with the following specific objectives:

- To isolate cellulase producing fungal isolates from forest decay and compost soil and identify them on the basis of their morphological characteristics
- To determine the activities of the crude enzyme using commercial substrates
- To evaluate the effect of physiochemical parameters (i.e. temperature, carbon source, pH and metal ions) on the production of cellulase in lab scale label
- To evaluate the extent of degradation caused by crude enzyme of the fungal isolates on natural cellulosic substrates (Finger millet and Oat straw)
- To compare the amount of cellulase produced by submerged fermentation and solid-state fermentation under small scale label

2. LITERATURE REVIEW

2.1. Cellulases

Cellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer, i.e. cellulose to smaller sugar components. Cellulases are often modular, containing a catalytic core, a linker and a carbohydrate-binding module (Teter *et al*., 2005). Cellulolytic enzymes can play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa (Khokhar *et al*., 2012).

2.1.1. General classification of cellulase

According to Enzyme Commission (EC) studies (Sharada *et al.,* 2014*;* Beauchemin *et al.,* 2003), there are 'three' major types of cellulases involved in the hydrolysis of native cellulose to glucose (which work synergistically), namely: Endoglucanase, exoglucanase and β–glucosidase / cellobiase. All three have in common the ability of hydrolyzing the 1,4-β-glycosidic bond between the D-glucose molecules, but they differ in their starting point and substrate when hydrolyzing (Goldschmidt, 2008). However, in spite of such differences, in nature complete cellulose hydrolysis is mediated by a combination of these three main types of cellulases.

2.1.1.1. Endoglucanase (E.C.3.2.1.4)

Endoglucanase (endo –β– 1, 4 – glucanase, endo –β– 1, 4 – D – glucan – 4 –glucano hydrolase), often called CMCase, hydrolyzes carboxyl methyl cellulose or swollen cellulose, due to which there is a rapid decrease in chain length along with a slow increase in reducing groups. The endoglucanase randomly hydrolyzes the β–1,4 bonds in the middle portion of the cellulose molecule. It acts randomly on soluble and insoluble 1,4-β-glucan substrates, which are commonly measured by detecting the decrease in viscosity or reducing groups released from carboxymethylcellulose (CMC) (Dashtban *et al*., 2010).

2.1.1.2. Exoglucanase (E.C. 3.2.1.91)

Exoglucanase (Exo–β– 1, 4 – D – glucan – 4 – cellobiohydrolase) degrades cellulose by splitting off the cellobiose units from the non- reducing end of the chain. Cellobiohydrolase does not degrade cotton rapidly, but can affect considerable scarification of micro crystalline celluloses. It is also active against swollen, partially degraded amorphous substrates and cellodextrins, but does not hydrolyze soluble derivatives of cellulose like carboxymethyl cellulose and hydroxyethyl cellulose. Some cellulase systems also contain glucohydrolase (exo-1,4-D-glucan-4-glucohydrolase) as a minor component (Joshi *et al*., 1999). Finally, these oligosaccharides are converted to glucose by β-glucosidase (Bhat and Bhat, 1997).

The "exo-1,4-β-D-glucanases," include both the 1,4-β-D-glucan glucohydrolases (EC 3.2.1.74), which liberate D-glucose from 1,4-β-D-glucans and hydrolyze D-cellobiose slowly, and 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91), which liberates D-cellobiose from 1,4-β-glucans. Differentiation of these enzyme classes requires analytical techniques to distinguish glucose and cellobiose and is usually carried out by HPLC.

2.1.1.3. β – glucosidase / Cellobiase (E.C.3.2.1.21)

β– glucosidase (E.C.3.2.1.21) completes the process of cellulose hydrolysis by cleaving cellobiose and removing glucose from the non – reducing ends of oligosaccharides. Complete degradation of cellulose to glucose requires the synergistic action of all the three components. β-glucosidases are common among plants, fungi and bacteria, and show an identical similarity with respect to their sequences and structures. They can be classified on the basis of their substrate activity or their nucleotide sequence identity (Singh *et al*., 2016). Glucosidases are the essential part of cellulase system (cellulose metabolizing enzymes) and catalyze the last and final step in cellulose hydrolysis. Cellulases hydrolyze the cellulose to produce cellobiose and other short oligosaccharides which are finally hydrolyzed to glucose by β-glucosidase.

Measurement of this activity is carried out either specifically on cellobiose or cello-oligomers with product analysis by Filter paper assay or HPLC or by direct spectrophotometric or fluorometric analysis of various chromogenic and fluorogenic analogues of cellobiose and cello-oligomers (Wyman *et al*., 2005).

2.1.2. Sources of cellulase

Cellulases are distributed throughout the biosphere such as plants, animals and microorganisms. However, cellulases from higher plants such as *Lantana camara*and *Cuscutareflexa*are mostly involved in fruit ripening and senescence. Few animals such as the blue mussel *Mytilus edulis*, the green mussel, the edible snail *Helix pomatia*, the marine cellulo*Littorina brevicula,* termites and protozoa (König *et al.*, 2002; Ram *et al.,* 2014) were reported as cellulase producers. Protozoa such as *Epidinium caudatum* and *Eudiplodinium ostracodinium*. Archaea such as *Sulfolobussolfataricus* and *Pyrococcusfuriosus*are also cellulase producers. However, fungi and bacteria are considered to be the main source for cellulases with novel and high specific activities. Some of the microbial genera and species that are known to produce cellulases are shown in Table 1. Of these, the fungi are the most common producers of cellulases (Arriffin *et al*., 2006; Lynd *et al*., 2002) and as a result they are usually better degraders of plant cell wall constituents.

Lignocellulolytic enzyme-producing fungi are widespread, and include species from the ascomycetes (e.g.*Trichoderma reesei)* and basidiomycetes phyla such as white-rot (e.g. *Phanerochaetechrysosporium)* and brown-rot fungi (e.g. *Fomitopsispalustris).* In addition, a few anaerobic species (e.g. *Orpinomyces sp.)* are found to be able to degrade cellulose in the gastrointestinal tracts of ruminant animals (Ljungdahl, 2008). The species most often used to study the production of cellulase are the two soft-rot fungal species i.e. *T.ressei*and *T. viride*. Other fungal species often used are *Fusarium solani*, *Aspergillus niger*, *Penicillium funicolsum*, and *Cellulomonas sp. (*Maeda *et al*., 2010). The bacterial species *Clostridium thermocellum*and *Clostridium thermosaccharolyticum* also represent promising candidates for cellulase production since they are thermophilic (less contamination problem and faster rate at a high temperature), anaerobic (no oxygen transfer limitation), and ethanologenic (capable of carrying out simultaneous saccharification and fermentation resulting in the production of ethanol from complex lignocellulosic substrates) (Rao *et al.,* 1988).

In general, microbial cellulases are the most economic and easily available because the producer microorganisms can grow on inexpensive media prepared from agricultural and food industry by-products (Korish, 2003).

Table 1. Cellulase producing organisms

Major Group	Genus and Species
Fungi	**Soft rot fungi** *Aspergillus niger; A. nidulans; A. oryzae; A. terreus; Fusariumsolani; F. oxysporum; Penicilliumbrasilianum; P. occitanis; P. decumbans; Trichodermareesei; T. longibrachiatum; T. harzianum;Humicolainsolens; H. grisea; Melanocarpusalbomyces; Chaetomiumcellulyticum; C. thermophilum; Neurosporacrassa; P. fumigosum; Thermoascusaurantiacus;Mucorcircinelloides; P. janthinellum; Paecilomycesinflatus; P. echinulatum; Trichoderma atrovirid*
	Brown rot fungi *Coniophoraputeana; Lanzitestrabeum; Poria placenta; Tyromycespalustris; Fomitopsis sp.*
	White rot fungi *Phanerochaetechrysosporium; Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotusostreatus; Phlebiagigantea*
Bacteria	**Aerobic bacteria** *Acinetobacter junii; A. amitratus; Acidothermuscellulolyticus; Anoxybacillus sp.; Bacillus subtilis; B. pumilus; B. amyloliquefaciens; B. licheniformis; B. circulan; B. flexus; Bacteriodes sp.; Cellulomonasbiazotea; Cellvibriogilvus; Eubacteriumcellulosolvens; Geobacillus sp.; Microbisporabispora; Paenibacilluscurdlanolyticus; Pseudomonas 7ellulose; Salinivibrio sp.; Rhodothermus marinus*
	Anaerobic bacteria *Acetivibriocellulolyticus; Butyrivibriofibrisolvens; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens; Fibrobacter succinogenes; Ruminococcusalbus*
Actinomycetes	*Cellulomonasfimi; C. bioazotea; C. uda; Streptomyces drozdowiczii; S. lividans; Thermomonosporafusca; T. curvata*

Sources; Coughlan (1995)

2.2. Fungal Cellulases

Efficient cellulolytic fungi are represented by aerobic fungal species belonging to *Aspergillus*, *Penicillium*, *Chaetomium*, *Trichoderma, Fusarium*, *Stachybotrys*, *Cladosporium*, *Alternaria*, *Acremonium*, *Ceratocystis*, *Myrothecium*, *Humicola*, etc. (Khokhar *et al.*, 2012). The cellulases produced by these aerobic fungi are usually preferred by the industry, because they are extracellular, adaptive in nature and usually secreted in large quantities during growth. This is in sharp contrast to many bacterial as well as anaerobic fungal cellulases which exist as tight multi-enzyme complexes; often membrane bound as cellosomes, from which it is difficult to recover individual active enzyme species; hence, economically less important (Mathew *et al*., 2008).

Production of cellulolytic enzyme from filamentous fungi is wide spread; among them, species of *Aspergillus, Trichoderma, Penicillium and Sclerotium* are found as highly cellulolytic, and are mainly considered for commercial exploitation (Sivaramanan, 2014.; Milala *et al*., 2005). Structurally, fungal cellulases are simple and modular enzyme with functionally distinct modules or domains (Bayer *et al*., 1998). Some of them possess two domains, catalytic domain and carbohydrate binding domains connected by a serine- and threonine-rich polylinker with varying chain length and structure (Payne *et al*., 2015). The carbohydrate binding modules vary in size ranging from 4 to 20 kDa, and are rich in aromatic and often polar amino acid residues that immobilize the substrate during catalysis. The active site of the catalytic domain may be topologically tunnel, cleft or pocket in shape allowing the hydrolysis of the substrate efficiently.

2.3. Cellulase Production

Fermentation is the technique used for biological conversion of complex substrates into simple compounds by various microorganisms. It has been widely used for the production of cellulase. Over the years, fermentation techniques have gained immense importance due to their economic and environmental advantages. The cellulolytic activities of fungi may vary depending upon the medium as well as the culture conditions. Hence, the formulation of suitable fermentation strategies is the key factor for deciding the efficiency of a fungus in terms of the production of cellulase. Two broad fermentation techniques have emerged as a result of this rapid development and subsequently used in the production of cellulase: Submerged Fermentation (SmF) and Solid-State Fermentation (SSF).

2.3.1. Submerged fermentation (SmF)

Submerged fermentation is the most commonly used technology for large scale production of enzymes. It is generally carried out in the presence of free-flowing liquid, in which soluble substrates are dispersed; however, compared to liquid medium, filamentous fungi like *Aspergillus*, *Penicillium* and *Trichoderma* normally produce large quantity of cellulase in solid medium.

In fact, the easiness in controlling the process parameters, monitoring and downstream process make SmF more attractive (Sukumaran *et al*., 2005). In SmF, various factors such as pH, temperature, substrate concentration, inducer, medium composition, etc., influence the production of cellulase significantly. The main drawback in SmF is the requirement of long time (i.e., gestation period) with less production (Singhania *et al*., 2010). Among microorganisms, bacteria are most commonly used for the production of enzymes by SmF (Smitha *et al*., 2013; Sreedevi *et al*., 2013); but, some species of fungi such as *Aspergillus*, *Penicillium*, *Trichoderma*, etc. are also being cultivated under SmF for the production of cellulase.

In order to obtain better productivity, various synthetic or natural carbon sources are used in SmF; for instance, Acharya *et al.* (2008) studied the production of cellulase by *A. niger* on pretreated (by alkali) sawdust and the maximum cellulase activity was observed under optimized condition, i.e., pH between 4 and 4.5, 120 rpm, at 28°C and peptone as nitrogen source. Similarly, Karthikeyan *et al.* (2010) investigated the production of cellulase by *Penicillium* strain K-P in liquid medium by supplementing various carbon and nitrogen sources at varying pH and temperature; the fungus showed the maximum cellulase activity in the presence of fructose, ammonium nitrate, pH 3.0 and 30°C on day 5.

Production of cellulases was studied by *A. niger* in the presence of various carbon and nitrogen sources at varying pH; and found that the maximum production of cellulase was on Czapek-Dox medium supplemented with 1% CMC or sawdust at pH 5 (Narasimha, 2006). From the aforesaid reports, it is clear that the production of cellulase mainly observed under acidic pH and 20-30°C; in addition to the ability of the fungi in utilizing the carbon and nitrogen sources present in the liquid medium.

2.3.2. Solid State Fermentation (SSF)

Solid-state fermentation (SSF) is defined as the fermentation in near absence of free water (Pandey *et al*., 2000). SSF for production of cellulases is rapidly gaining interest as a cost-effective technology as the microorganisms, especially fungal cultures produce comparatively high volume of cellulase due to the conditions of fermentation which show similarity to the natural environment (Jha *et al*., 1995). However, sufficient moisture is provided to support the

growth of fungi on lignocellulosic substrates (Singhania *et al*., 2009). Due to this, the cost of dewatering in downstream processing can be greatly reduced.

The production cost of SSF can be further reduced down to tenfold compared to submerged fermentation (Shrestha *et al*., 2010) due to the lower energy consumption and the employment of suitable low cost lignocellulosic substrate. SSF also offers advantages in terms of higher concentration of enzymes, higher fermentation productivity and lower demand on the sterility of the equipment (Raghavarao *et al.,* 2003).

The crude enzyme solution obtained from SSF can be applied directly to hydrolyze the lignocellulosic substrate. Apart from the operational advantages, cellulase produced by fungi in SSF has better performance in terms of hydrolysis efficiency compared with those produced from the liquid culture (Tewalt and Schilling, 2010).

2.4. Factors Affecting Cellulase Production

Since all enzymes are made of protein, cellulase production in cultures is highly influenced by physiochemical factors such as the nature of the cellulosic substrate, pH of the medium, nutrient availability, inducer supplementation, inhibitors, fermentation temperature, heavy metal ions etc. As a result, large-scale production of cellulases requires understanding and proper controlling of the growth and enzyme production capabilities of the producers. Enzymes change their shape if the temperature or pH changes, so they have to have the right conditions (Mojsov, 2012).

Optimization of growth conditions and processes has been attempted to a large extent in improving cellulase production. Cellulases produced by compost organisms such as the filamentous fungi –*Trichoderma*, *Penicillium*, *Aspergillus*, *Humicola*, etc., can perform at diverse ranges of pH and temperature. The media formulation for fermentation is of significant concern since no general composition can give the optimum growth and cellulase production. Also, the media used are mostly specific for the organism concerned (Singhania *et al*., 2010).

Characteristically, most cellulases have similar pH optima, solubility and amino acid composition. Thermal stability and exact substrate specificity may vary. However, it should be remembered that cellulase preparations generally contain other enzymatic activities besides

cellulase, and these may also affect the properties of the preparations. Cellulase preparations are effective between pH 3 and 7. The optimum pH generally lies between 4 and 6.

Cellulase is inhibited by its reaction products e.g. glucose, cellobiose (Qing *et al*., 2010). No inhibition up to 7 mM of Ca^{2+}, Zn^{2+} and K^{1+} was observed while Mn^{2+} showed onset of inhibition at 5 mM. On the other hand, Mg^{2+} could not recover the initial activity up to 7 mM while Co^{2+} completely inhibited the activity and Endoglucanase were inhibited by Hg^{2+}, Ag^{1+} while, partial inhibited by 10 mMZn^{2+}, Fe^{2+}, Mg^{2+} but stimulated by Mn^{2+} (Jabbar *et al*., 2008). The activity of cellulase preparations has been found to be completely destroyed after 10-15 minutes at 80°C. Solutions of cellulase at pH 5-7 are stable for 24 hours at 4 °C. These products should be stored at 4°C, in a dry place in tightly closed containers.

2.5. Pretreatment of Lignocellulosic Materials

Due to the recalcitrant nature of lignocellulosic biomass it is necessary to apply some form of physical, chemical and/or biological treatment in order to improve the accessibility of the cellulose to cellulolytic enzymes by removing or modifying the surrounding lignin and xylan (Zhang *et al*., 2009).

Physicochemical and structural factors significantly affect the rate of degradation of native lignocellulosic biomass. The pretreatment step can change the degree of polymerization, crystallinity index, and lignin-carbohydrate linkages and increase the porosity of lignocelluloses (Chandra *et al*., 2010). Thus, the effectiveness of pretreatment depends on how much accessibility to cellulose is obtained. This step is the most important step in the total cost and time reduction of fermentation process. In this process, at high solid loading, when there is release of high amount of sugars then the bottlenecks one has to deal with are mass transfer limitations, increased medium viscosity and generation of high concentration of inhibitors. Cellulosic materials have low density and are highly hygroscopic, thus slurries will become paste like material after pretreatment (Chen *et al.,* 2010).

Pretreatments are numerous and are classified as physical, chemical or biological in nature. Physical methods include mechanical processes such as chopping, ball milling, attrition, and wet disk refining, hydrothermolysis, pyrolysis, irradiation using electron beams and other high-energy radiation as well as microwave heating. Unanalyzed steam explosion is possible

for some feedstocks, and is known as auto hydrolysis; the resulting breakdown of glycosidic linkages is dependent on acids formed within the biomass itself (Fan *et al*.,1982; Ramos, 2003).

Chemical pretreatments include ammonia, solvent, wet oxidation, alkali, acid and others. Chemicals can also be used to catalyze physical pretreatments, as in the case of pH-controlled hydrothermolysis and acid-catalyzed steam explosion (Ewanick, 2007). Biological treatments make use of the agents of wood decay in nature; bacteria, fungi, and soil microflora (Ghosh and Singh, 1993). However, for effective pretreatment for bioconversion purposes, it is important that minimal sugars be consumed and only the lignin affected. The primary organisms that degrade wood are white rot, brown rot, and soft rot fungi. Of these, the most useful for pretreatment is the white rot variety, typically of the group Basidiomycota, which oxidizes and breaks down lignin (Sánchez, 2009). While there is no energy input required during this type of pretreatment, it takes days to weeks and is considered too slow to be technically and economically feasible.

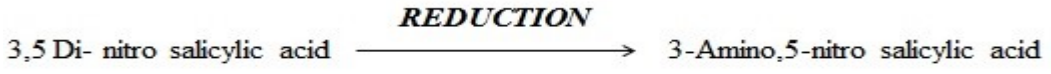
2.6. Assay for Cellulase Activity

2.6.1. Carboxymethyl cellulase activity (CMCase)

CMCase can randomly hydrolyze internal glycosidic bonds in cellulose chains. Endoglucanase activities can be measured using a soluble cellulose derivative with a high degree of polymerization(DP) such as carboxymethyl cellulose (CMC). Carboxymethyl cellulase (CMCase) was mainly evaluated based on the procedure described by Eveleigh *et al.* (2009).

In this method, CMCase activity was measured by determining the amount of free carbonyl group (C=O), the so-called reducing sugar, after 5 minutes of enzyme reaction with 0.5% CMC at pH 5 and a temperature of 50°C (Mandels *et al.*, 1976; Sharmila,2014). The amount of reducing sugar was estimated using the DNS method (Eveleigh *et al.,* 2009). Enzyme activity was expressed in international units (IU) where one unit (IU) of enzyme is defined as the amount of enzyme that liberates 1 µmol of glucose per minute under the assay conditions (Gupta *et a*l., 2012; Kim *et al.,* 2012).

Reducing sugars contain free carbonyl groups that have the property to reduce many reagents including dinitro salicylic acid. When alkaline solution of 3,5-dinitrosalicylic acid reacts with a reducing sugar it gets reduced and changes into 3-amino-5-nitrosalicylic acid that imparts to the reaction mixture an orange color. The intensity of the colour, when measured using a device (spectrophotometer), can be used as an index of the amount of reducing sugar.



It contains sodium potassium tartrate, which decreases the tendency to dissolve oxygen by increasing the ion concentration in the solution. Phenol increases the amount of color produced during the color-developing reaction. Sodium bisulfite stabilizes the color obtained and reacts with any oxygen present in the buffer. Finally, an alkaline buffer is required for the redox reaction between DNS and glucose, or other reducing sugars. DNS will be added at the last step of the enzyme assay to stop the reaction. To promote full color development, samples have to be boiled vigorously and the absorbance of diluted samples will be read at 540 nm (Zhang *et al.*, 2009).

2.6.2. Filter paper assay (FPase activity): total cellulase activity

To compare the efficacy of cellulase activity between microorganisms or their secreted enzymes, techniques for measuring total cellulase activity are required. The FPA is the key method for analysis of total cellulase activity. In 1976, the FPU was developed by Mandels and his collogues. The FPU became widely used since 1984, when the Commission on Biotechnology of the International Union of Pure and Applied Chemistry(IUPAC) proposed a number of standard procedures for the measurement of cellulase activity. Traditionally, the FPU uses a 1 × 6-cm strip of Whatman no. 1 filter paper as the standard substrate because it is readily available and inexpensive (Coward-Kelly *et al.*, 2003). This standard filter paper method has been reviewed by Ghose (1987).

The International Unit(IU) of filter paper activity (Fpase) (FPU) is defined as the micromole of glucose equivalent liberated per minute of culture filtrate under assay conditions, where assay conditions refer to the conditions such as pH and temperature at which the enzymes are held at during the assay and depend largely on the properties of the enzyme, varying widely

between cellulases and microorganisms. Reducing sugar is estimated as glucose by the Miller method. This assay is performed so that 0.5 mL of diluted enzymes releases about 2.0 mg of glucose equivalents in 60 min, as determined by the 3,5-dinitrosalicylicacid (DNS) assay (Miller, 1959; Wood and Bhat, 1988).

2.7. Microbial Cellulases and their Industrial Applications

Microbes are an attractive topic of interest for the production of cellulases and hemicellulases due to their immense potential for cellulase production, enzyme complexity and extreme habitat variability (Karmakar and Ray, 2011).

Structurally fungal cellulases are simpler as compared to bacterial cellulase systems, cellulosomes (Zhang, 2006). Fungal cellulases typically have two separate domains: a catalytic domain (CD) and a cellulose binding module (CBM), which is joined by a short polylinker region to the CD at the N-terminal. The CBM is comprised of approximately 35 amino acids, and the linker region is rich in serine and threonine. The main difference between cellulosomes and free cellulase enzyme is in the component of cellulosomes-cohesin containing scaholding anddockerin containing enzyme. The free cellulase contains cellulose binding domains (CBMs), which are replaced by a dockerin in cellulosomal complex, and a single scaholding-born CBM directs the entire cellulosomes complex to cellulosic biomass (Bayer, 2004).

Microbial cellulases are preferred for their vast industrial applicability and relatively lower cost of production. Cellulases are important enzymes not only for their potential applications in different industries like industries of animal feed, agricultural, laundry, textile, brewery, food processing, olive oil extraction, carotenoid extraction, pharmaceutical & medical sciences, analytical applications, protoplast production, genetic engineering and pollution treatment, but also for the significant role in bio conversion of agriculture wastes into sugar and bio ethanol (Sharada *et al*., 2014).

2.8. Potential Application of Cellulases

Cellulases are used in the textile industry, in detergents, pulp and paper industry, improving digestibility of animal feeds, in food industry (Kirk *et al*., 2002; Singhania *et al*., 2010) and

these enzymes account for a significant share of the world enzyme market (Sukumaran *et al*., 2005). The major industrial applications of cellulases are shown in Table 2.

Table 2. Applications of cellulases in various industries

Industry	Applications
Bioconversion	Conversion of cellulosic materials to ethanol, other solvents, organic acids and single cell protein, and lipids; production of energy-rich animal feed; improved nutritional quality of animal feed; improved ruminant performance; improved feed digestion and absorption; preservation of high quality fodder
Detergent	Cellulase-based detergents; superior cleaning action without damaging fibers; improved color brightness and dirt removal; remove of rough protuberances in cotton fabrics; antire deposition of ink particles
Food	Release of the antioxidants from fruit and vegetable pomace; improvement of yields in starch and protein extraction; improved maceration, pressing, and color extraction of fruits and vegetables; clarification of fruit juices; improved texture and quality of bakery products; improved viscosity fruit purees; improved texture, flavor, aroma, and volatile properties of fruits and vegetables; controlled bitterness of citrus fruits
Pulp and paper	Co-additive in pulp bleaching; biomechanical pulping; improved draining; enzymatic deinking; reduced energy requirement; reduced chlorine requirement; improved fiber brightness, strength properties, and pulp freeness and cleanliness; improved drainage in paper mills; production of biodegradable cardboard, paper towels, and sanitary paper
Textile	Bi-ostoning of jeans; biopolishing of textile fibers; improved fabrics quality; Improved absorbance property of fibers; softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics; restoration of colour brightness
Agriculture	Plant pathogen and disease control; generation of plant and fungal protoplasts; enhanced seed germination and improved root system; enhanced plant growth and flowering; improved soil quality; reduced dependence on mineral fertilizers
Others	Improved carotenoids extraction; improved oxidation and colour stability of carotenoids; improved olive oil extraction; improved malaxation of olive paste; improved quality of olive oil; reduced risk of biomass waste; production of hybrid molecules; production of designer cellulosomes

Source: Shweta (2014)

2.8.1. Bioethanol

The massive exploitation and utilization of fossil fuels have insistently reduced its natural reserves and caused severe environmental pollution via the release of green house and toxic gases. So that, the world economy is now focused on biofuels, especially bioethanol from renewable resources, which is expected to replace 20% of the fossil fuel consumption by 2020 (Msangi *et al.,* 2007). The most actively investigated application of cellulase is the production of biofuels, especially bioethanol.

Cellulases actively convert the cellulosic renewable resources in to glucose and other simple fermentable sugars that can be used as substrates for the production of bioethanol. Production of bioethanol from lignocelluloses is a multistep process. Initially, the lignocellulosic biomass is subjected to pre-treatment – either mechanically, chemically or enzymatically to remove lignin and hemicelluloses fractions, followed by the treatment with cellulase to release fermentable sugars (pentoses and hexoses). Then the hydrolyzed cellulosic residue is used for the microbial fermentation to produce bioethanol (Sun and Cheng, 2002).

Agricultural residues such as sugarcane bagasse, straw of wheat, rice and corn; wheat bran, corn Stover, etc. were successfully used as raw materials for the production of bioethanol, employing cellulases produced by various filamentous fungi including *Aspergillus*, *Trichoderma*, and *Penicillium* (Binod *et al.*, 2010; Li *et al*., 2011).

2.8.2. Animal feed industry

Biotechnology of cellulases and hemicellulases began in early 1980s, first in animal feed followed by food applications (Chesson, 1987; Thomke *et al*., 1980; Voragen *et al*., 1992). Ruminants are well adapted to utilize plant cell walls, and the degradation thus by the animals is of vital worth.

However, even under perfect feeding program, cell wall digestibility in the total gastrointestinal tract is less than 65%. Feed enzymes have an important role to play in current farming systems. The use of enzymes as additives in ruminant nutrition has a huge potential (Taghizadeh and Nobari, 2012).

Cellulases (EC 3.2.1.4) can be used to improve forage production for cattle feeding, which involves enhancement of the digestibility of grasses containing large amounts of potentially total digestible nutrients and energy values together with only small amounts of water soluble carbohydrates. The forage diet of ruminants, which contains cellulose, hemicellulose, pectin, and lignin, is more complex than the cereal based diet of poultry and pigs. Enzyme preparations containing high levels of cellulase, hemicellulase, and pectinase have been used to improve the nutritive quality of forages (Kuhad *et al*., 2011).

Exogenous enzymes have been used extensively to remove anti-nutritional factors from feeds, to increase the digestibility of existing nutrients, and to supplement the activity of the endogenous enzymes of poultry (McAllister *et al*.,2001).

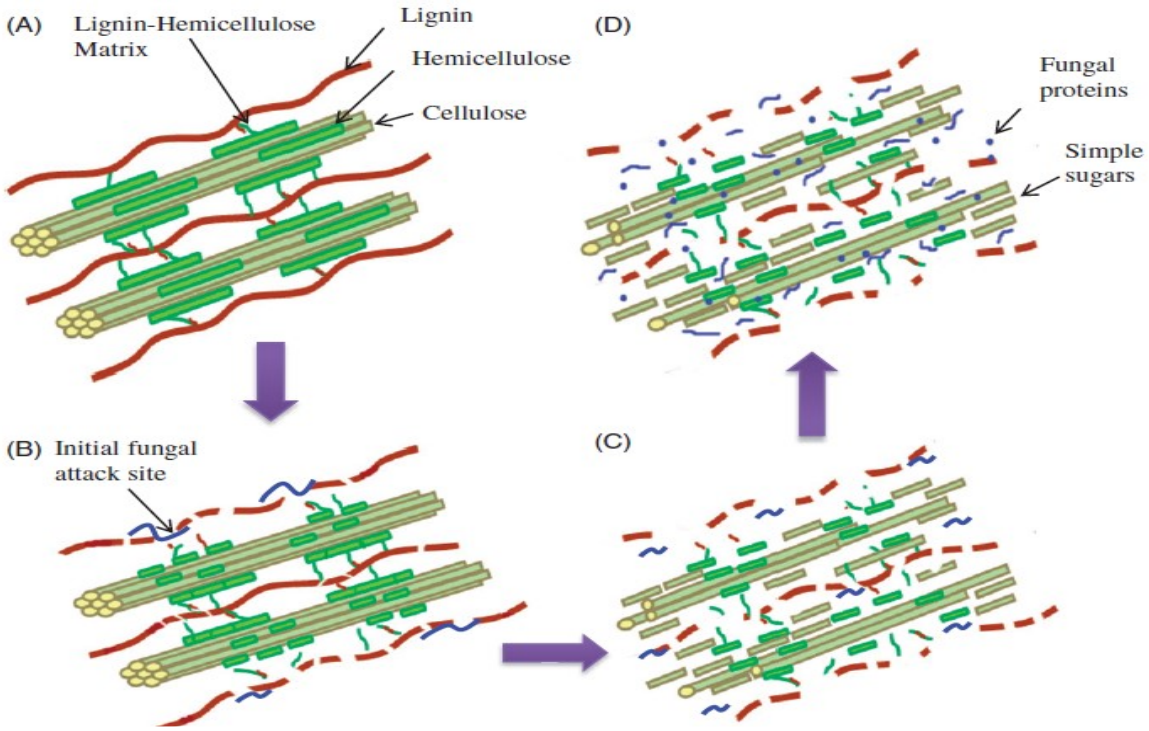


Figure1. Degradation of all three polymers and fungal growth

(Source: McAllister *et al*., 2001)

(A) Structure of a typical lignocellulosic residue, (B) Initial fungal attack on lignin hemicellulose matrix, (C) Degradation of all three polymers and fungal growth, (D) Nutritionally upgraded lignocellulose along with fungal proteins and simple sugars to be used as animal feed.

2.8.3. Food industry

Cellulase had been widely used in food industry. Its use in the pretreatment of brewery mash could increase enzymolysis rate of brewery mash proteins by above 10%. Its use in the production of quality Fen-flavor liquor could increase liquor yield by 13% with no adverse effects on liquor quality and liquor taste. Addition of 0.02%～0.1% cellulase during grains

steeping in the production of Japanese Sake would result in smooth saccharifying fermentation and high liquor yield.

Addition of cellulase in alcohol fermentation could shorten fermentation time and significantly increase alcohol output rate. Currently, the problems in cellulase use mainly included the optimization of microbial species and the use cost (Tolan and Foody, 1999; Zhang *et al*., 2005).

2.8.4. Textile industry

Cellulases have achieved their worldwide success in textile and laundry because of their ability to modify cellulosic fibers in a controlled and desired manner, so as to improve the quality of fabrics. Bio-stoning and bio-polishing are the best-known current textile applications of cellulases. Cellulases are used in the bio- stoning of denim garments for producing softness and brightness (Xia and Cen, 1999).

In the conventional textile processing, the grey fabric has to undergo a series of chemical treatments before it turns into a finished fabric. This includes de-sizing, scouring, mercerization, bleaching and washing. The chemicals used for all these steps are quite toxic. The use of enzymes in the textile industry is an example of white industrial biotechnology, which allows the development of environmentally friendly technologies in fiber processing and strategies to improve the final product quality. The consumption of energy and raw materials, as well as increased awareness of environmental concerns related to the use and disposal of chemicals into landfills, water or release into the air during chemical processing of textiles are the principal reasons for the application of enzymes in finishing of textile materials. Biopolishing is a finishing process that improves fabric quality by mainly reducing fuzziness and pilling property of cellulosic fiber. The objective of the process is elimination of micro fibrils of cotton through the action of cellulose enzyme (Mojsov, 2012).

2.8.5. Environmental waste treatment

Today, white biotechnology is geared towards creating new materials and bio based fuels from agricultural waste and providing alternative bio based routes to chemical processes. These efforts could lead to the development of improved enzymes such as amylases, hemicellulases or cellulases that could be used in the textile industry.

Cellulose is considered as one of the most important sources of carbon on this planet and its annual biosynthesis by both land plants and marine algae occurs in many tones per annum. Numerous agricultural residues generated due to diverse agricultural practices and food processing such as rice straw, yam peels, cassava peels, banana peels among others represents one of the most important energy resources. The major components of these are cellulose and hemicellulose (75-80%) while lignin constitutes only 14%. Agriculture wastes contain a high proportion of cellulosic matter which is easily decomposed by a combination of physical, chemical and biological processes. The bunch consists of 70 % moisture and 30% solid (Thambirajah *et al*. 1995).

The recognition that environmental pollution is a worldwide threat to public health has given rise to a new massive industry for environmental restoration. Biological degradation, for both economic and ecological reasons, has become an increasingly popular alternative for the treatment of agricultural, industrial, organic as well as toxic waste. These wastes have been insufficiently disposed leading to environmental pollution.

Recycling of agricultural residue can be achieved naturally and artificially by microorganisms. This is due to the ability of microorganisms to synthesize high amount of extra cellular exoglucanase within a relatively short period of time, utilizing Agro-wastes that would otherwise cause environmental pollution, could be used for rapid and commercial production of cellulose (Sukumaran *et a*l., 2005).

Aerobic organisms such as fungi, bacteria, and some anaerobic organisms have been shown to be able to degrade some constituents of these residues. Cellulases are important enzymes not only for their potent applications in different industries, like industries of food processing, animal feed production, pulp and paper production, and in detergent and textile industry, but also for the significant role in bioconversion of agriculture wastes in to sugar and bioethanol (Mojsov, 2012).

2.9. Cellulose

Cellulose is the world's most abundant, naturally occurring organic compound, that available to man (Bhat and Hazlewood, 2011). It is the main constituent of the cell wall of plants. The cellulose in each layer of cell wall occurs as long slender bundles composed of long chain of

beta-D-glucopyranose residues linked by 1,4 glycosidic bond, called elementary fibrils with diameter (Mariamma, 1995). The number of glucose units in each chain or molecule of cellulose is known as the degree of polymerization (DP), although it has been shown that the basic structural unit is actually cellobiose. Cellulose chains can aggregate and connect via inter-molecular hydrogen bonds to form units known as elementary fibrils that contain ordered, crystalline, regions as well as disordered, amorphous regions. Crystalline regions are generally more resistant to enzymatic attack, while amorphous regions present more accessible sites (Blackwell, 1982).

Cellulosic biomass includes agricultural (e.g., straws, corn stover and sugarcane bagasse) and forestry (e.g., sawdust, thinnings, and mill wastes) residues, portions of municipal solid waste (e.g., waste paper), and herbaceous (e.g., switchgrass) and woody (e.g., poplar trees) crops. Such materials are abundant and competitive in price with petroleum, and cellulosic biomass can provide a sustainable resource that is truly unique for making organic products (Wyman *et al.,*2005). Lignocellulose is the potential source of biofuels, bio-fertilizers, animal feed and chemicals, besides being the raw material for paper industry (Sukumaran *et al.,* 2005).

Table 3. Composition of various lignocellulosics

	Composition (%)		
	Lignin	Cellulose	Hemicellulose
Softwood	25-35	45-50	25-35
Hardwood	18-25	40-55	24-40
Agricultural residues	10-30	25-45	10-40

Source: Emtiazi *et al*. (2001)

2.10. Fungal Degradation of Cellulosic Residues

A molecule of cellulose is an unbranched polymer of 1 to 1000 million D-glucose units, linked together with beta-1, 4 glycosidic bonds. However, they differ in the crystalline structures and bindings by other biochemical.

Two types of hydrogen bonds are present in cellulose molecules: one kind that forms between the CH_3OH group and the oxygen in the pyranose ring within the same molecule and the other one that forms between the C_6H_5OH group of one molecule and the oxygen of the glucosidic

bond of another molecule. Ordinarily, the beta-1, 4 glycoside bonds easy to break. However, because of these hydrogen bonds, cellulose forms very tightly packed crystallites. These crystals are sometimes so tight that fluids (such as water, cellulase) cannot enter and break or dissolve; only *exogluconase*, a subgroup of cellulase that attacks the terminal glucosidic bond, is able to effectively (Chalamcherla *et al*., 2009).

The two major ways to degrade cellulose to glucose are: Chemical and Enzymatic. Water's inability to penetrate cellulose explains why cellulose is insoluble. On the other hand, amorphous cellulose allows the penetration of endogluconase, another subgroup of cellulase that catalyzes the hydrolysis of internal bonds. This difference in the crystalline structure is that the hydrolysis rate is much faster for amorphous cellulose than crystalline cellulose. The process of breaking the glucosidic bonds that hold the glucose basic units together to form a large cellulose molecule is called hydrolysis because a water molecule must be supplied to render each broken bond inactive.

Fungal ligninolysis and break down of cellulose-hemi-cellulose matrix liberate simple degradable components that can be easily utilized by rumen microflora, thus improving the ruminant digestibility. Bioconversion of these residues into more nutritive animal feed has fascinated many workers to work on the problem and make it practically possible at lab-level fermentation (Bisaria *et al*., 1997).

The use of supplemental exogenous fiber degrading enzymes to improve fiber digestibility and feed utilization was first examined for ruminants in the 1960s, as reviewed by McAllister *et a*l. (2001) and Rode *et al*. (2001).

Applications of cellulases in the feed industry have received considerable attention because of their potential to improve feed value and performance of animals. Pretreatment of agricultural silage and fiber feed by cellulases can improve its nutritional value (Godfrey and West, 1996). With increasing consumer concern about the use of growth promoters and antibiotics in ruminant production, and the magnitude of increased animal performance obtainable using feed enzymes, these products could play an important role in future ruminant production systems (Beauchamin *et al.,* 2003).

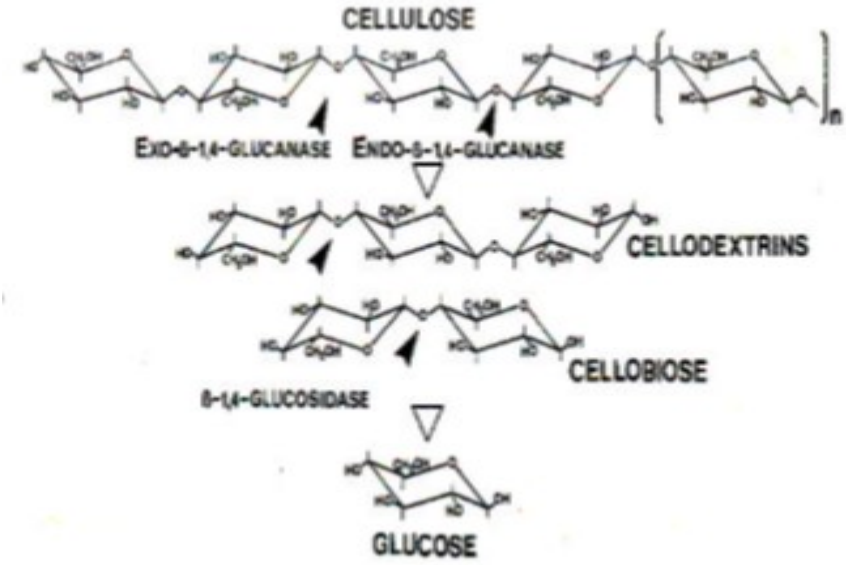


Figure 2. Diagrammatic overview represents the cellulose hydrolysis by cellulase system

Source: Fatani, 2016; Korish, 2003

2.10.1. Finger millet (*Eleusine coracana*)

Millets are one of the cereals besides the major wheat, rice, and maize. Millets are major food sources for millions of people, especially those who live in hot and humid areas of the world. They are grown mostly in marginal areas under agricultural conditions in which major cereals fail to give substantial yields (Adekunle, 2012).

Finger millet, *E. coracana L*. is also known as *ragi* and *mandua*(India); *kaddo*(Nepal); *fingerhirse*(Germany); *petit mil, eleusinecultivee, coracan, koracan*(France); *bulo*(Uganda); *ambale, lupoko, mawele, amale, bule*(Zambia); *poho, rapoko, zviyo, njera, mazhovole*(Zimbabwe); finger millet, African millet, koracan (England); *dagussa*, *tokuso, barankiya*(Ethopia); *wimbi, mugimbi*(Kenya). It is an important staple food in parts of eastern and central Africa and India (Utta *et al*., 2015; Shobana, 2009).

Finger millet stands unique among the cereals such as barley, rye and oats with higher nutritional contents and has outstanding properties as a subsistence food crop. It is rich in calcium (0.34%), dietary fiber (18%), phytates (0.48%), protein (6%–13%) minerals (2.5%–3.5%), and phenolics (0.3%–3%). Moreover, it is also a rich source of thiamine, riboflavin,

iron, methionine, isoleucine, leucine, phenylalanine and other essential amino acids. The abundance of these phytochemicals enhances the nutraceutical potential of finger millet, making it a powerhouse of health benefiting nutrients. It has distinguished health beneficial properties, such as anti-diabetic (type 2 diabetes mellitus), anti-diarrheal, antiulcer, anti-inflammatory, antitumerogenic (K562 chronic myeloid leukemia), atherosclerogenic effects, and antimicrobial and antioxidant properties.

The world total production of millet grains in year 2013 was 762,712 metric tons and the top producer was India with an annual output of 334,500 tons contributing 43.85%. Finger Millet straw has a good taste and odour for animals while they are feeding on it but not palatabele and easily degradable (Chandra *et al*., 2016).

2.10.2. Oat (*Avena sativa*)

On the other hand, Oats rank around sixth in the world cereal production statistics following wheat, maize rice, barley and sorghum. In many parts of the world oats are grown for use as grain as well as for forage and fodder, straw for bedding, hay, haylage, silage and chaff. Livestock grain feed is still the primary use of oat crops, accounting for an average of around 74% of the world's total usage in 1991 to 1992. Oat is an important winter fodder, mostly fed as green but surplus is converted into silage or hay to use during fodder deficit periods (Ahmed *et al.,* 2014).

3. MATERIALS AND METHODS

3.1. Research Design

The research was carried out using CRD with two replications. The experiment included five factors as treatments, i.e. six isolates, three levels of temperature, three levels of pH, two types of carbon source, and two types of fermentation techniques (Gomez and Gomez, 1984).

3.2. Soil Samples Collection

Soil samples were collected from two different locations i.e. forest soil sample were brought from Menagesha Suba forest while compost soil samples were collected from Holeta Agricultural Research Center Horticulture department compost site using sterile polyethylene bag from a depth of 15-20cm and were brought to national agricultural biotechnology research center (NABRC)laboratory and then stored at 4°C until use.

3.3. Preparation of Growth Substrates

Oat straw (OS) and Finger Millet straw (FMS) were from Holeta Agricultural Research Center Livestock department forage site and from local farm land in Dangila district, respectively. These substrates were then ground and sieved to a uniform particle size (1.0 – 2mm) and kept in a refrigerator at 4ºC for experimental purposes (Shrivastava *et al*., 2011).

3.4. Isolation and Screening of Cellulolytic Filamentous Fungi

Fungal isolates were isolated from serially diluted soil samples using spread plate technique. 10 gm of soil sample was transferred to 90 ml of sterilized distilled water. Dilutions were made up to 10^{-7} and 0.1 ml of soil suspension was spread plated on to sterile Malt Extract Agar (MEA) medium and incubated at 28ºC for 7 days. The media were amended with chloramphenicol to prevent the growth of bacteria (Mukunda *et al*., 2014). About 53 Fungi isolates were isolated and sub-cultured on MEA to maintain the purity of the cultures through streaking techniques. Stock cultures were maintained at 4^0C.

3.4.1. Screening for cellulolytic fungal isolates

The isolated fungal cultures were screened for their ability to produce cellulase complex following the protocol developed by Mandels (1974). Screening was done through detection

and measurement of the cellulase activities of each fungal isolate on Mandels-Weber medium containing Mandels' mineral salt in grams per litter (g/L) of solution: Urea, 0.3; $(NH_4)_2SO_4$, 1.4, KH_2PO_4, 2.0; $CaCl_2$, 0.3; $MgSO_4$, 0.3; yeast extract, 0.25; peptone, 0.75; carboxymethyl cellulose (CMC), 10; and agar, 15 (Mandels, 1974). Agar blocks (8 mm in diameter) from one-week old fungal colony grown on MEA plates were cut and placed in the center of the basal media plates. The plates were incubated at 28ºC for seven days. Then the plates were then examined for cellulolytic activity using Congo red test.

3.4.2. Congo red test

Cellulolytic fungal species were selected on the basis of the diameter of the hydrolysis zone surrounding the colonies. For observation of the hydrolysis zone, plates were flooded with 0.1% Congo red aqueous solution and shaken at 80 rpm for 30mins then poured off and destained with 1 M NaCl solution at 80 rpm for 20 mins (Khokhar *et al*., 2012).

Clear zones were observed around colonies of the active fungal isolates and the diameters measured. Cellulase activity on CMC agar was then recorded as an Index of Relative Enzyme Activity (I_{CMC}) by calculating the ratio of clear zone diameter and colony diameter (Pečiulytė, 2007).

$$\text{Index of Relative Enzyme Activity } (I_{CMC}) = \frac{\text{Clear zone diameter}}{\text{Colony diameter}}$$

3.5. Identification of the Isolated Fungi

Isolated cellulolytic fungi were identified based on their macroscopic (obverse and reverse pigmentation on the plates) and microscopic (conidia, conidiophore and philaid shapes) appearances; and especially shape of their spore structure. The morphology of the isolates, stained with lactophenol-cotton blue, was studied using a light microscope (Chinedu *et al.,* 2005). Microscopic observation was done under the 40x objective (Domsch *et al*., 1980). Spores, phialides and conidia were observed; and data were recorded and used for identification (Sivaramanan, 2014).

3.6. Preparation of Fungal Inoculum

Cells or mycelia of the fungal isolates were collected from the maintenance cultures (PDA

slants) were taken and inoculated into fresh PDA slants. The cultures were incubated at 28ºC for seven days. After incubation, about two ml of sterile physiological saline solution was introduce into the slant culture and shaken for few minutes. From the resulting spore suspension, measured the OD and adjust through addition of sterile saline or incubated at 28ºC for 72 hrs. Finally, the OD were measured and that resulted in 1 x 10^7 colonies/ml was used as inoculum (Korish, 2003).

3.7. Pretreatment of Lignocellulosic Substrates

Pretreatment of the lignocellulosic substrates was carried out for the purpose of enhancing the rate of hydrolysis and increasing the yield of cellulase. Oat straw (OS) and Finger Millet straw (FMS) were, therefore, pretreated using physical methods such as chopping, milling and steam explosion (100ºC for 15 mins) (Abraham and Kurup, 1997; Sharmila,2014; Dashtban *et al.,* 2009).

3.8. Production and Extraction of Cellulases

3.8.1. Cellulase production and extraction in SmF

The six-efficient cellulolytic fungal isolates were used for cellulase production through submerged fermentation. A volume of 200 ml of Mandels-Weber medium amended with 1% substrate (OS or FMS) was distributed into separate 250 ml conical flasks(CFs). The pH of the medium was adjusted to 5, 5.5 or 6. After autoclaving at 121°C for 20mins the fungal spore suspensions were inoculated into the CFs. The flasks were incubated at 20, 28 and 38 °C on a rotary shaker at 120 rpm for 5 days. After 5 days of incubation, the contents of the flasks were aseptically passed through Whatman No.1 filter paper to separate mycelial mat from culture filtrates after centrifuged using falcon centrifuge model Herareus MulltifugeX3R (Miller, 1959). Then the culture filtrate was centrifuged at 10,000 rpm for 15 mins. The crude enzyme was stored in sterilized test tube at 4^{O}C until used for cellulase assay (Linko *et al*., 2012; Bagewadi *et al*., 2015).

3.8.2. Cellulase production and extraction in SSF

Ten grams of substrate (OS or FMS) was added into separate 250 ml CFs and amended using Mandels-Weber medium adjusted to a moisture content of 65%. The pH of the medium was

also adjusted to 5, 5.5 or 6. After autoclaving at 121°C for 20mins 10^7spores mL^{-1} concentration of the fungal spore suspensions were inoculated into the CFs. The flasks were incubated at 20, 28 and 38 °C for 12 days. After 12 days of incubation, 100ml of 0.5M extraction buffer (sodium acetate with a pH of 5) was added, shaken the CFs for five minutes. Then distributed in to falcon tubes and centrifuged at 10,000 rpm at 4ºC for 10minsusing Herareus MulltifugeX3R centrifuge. Then the contents of the falcons were aseptically passed through Whatman No.1 filter paper to separate mycelial mat from culture filtrates. The culture filtrate was centrifuged at 5,000 rpm for 15 min at 4ºC; and the crude enzyme stored in sterilized test tube at 4^{O}C until used for cellulase assay (Miller, 1959).

3.9. Glucose Standard Curve

A standard curve was used to find the unknown concentrations of reducing sugars in all samples. The optical density readings were plotted against the concentration of glucose. The amount of reducing sugar obtained from the hydrolysis of the cellulosic substrates was estimated from extraplotations made using the standard graph plotted for known glucose concentrations following the methods used by Liming and Xeliang (2004) (Appendix Figure 3).

3.10. Enzyme Assay

3.10.1. Assay for CMCase activity

CMCase was measured as described previously by Ghosh (1987) and Agarwal *et al*. (2014). Briefly, a reaction mixture containing 1 ml of 1 % carboxymethyl cellulose (CMC) in 1ml of 0.5 M acetate buffer (pH 5.0) and 1ml of crude enzyme was prepared and incubated at a mixture at 50 ^{O}C for 30 minutes. A blank was also prepared in the same way and incubated without enzyme. After incubation, the enzyme activity was stopped by adding 3ml of DNS-reagent. At the end of incubation, the tubes were placed again in a boiling water bath for 5 min and then cooled down to room temperature. Then 4ml of distilled water was added into the cooled reaction mixture and finally the OD was immediately measured at 540 nm using a spectrophotometer model ELx800. One unit of cellulase activity (U) was defined as the amount of enzyme that produced reducing sugar equivalent to 1 µmol of CMC min^{-1} under the assay conditions (Bailey *et al*., 1992).

3.10.2. Assay for Fpase activity

Total cellulase activity was determined by a method of Mandels *et al*. (1976). An aliquot of 1 ml of cell-free culture supernatant were transferred to a clean test tube and 1 ml of 0.2M acetate buffer (pH 5) was added. Whatman #1 filter paper stirp (6 cm ×1 cm) was added to each tube (Lee and Kim, 1999). Tubes were vortexed to coil filter paper in bottom of the tube. Tubes were incubated at 50^{o}C for one hour. followed by an addition of DNS reagent (3 ml). Tubes were then placed in a boiling water bath for 5 minutes and then in an ice-bath, followed by the addition of 6 ml distilled water to each tube. Contents of the tube were mixed and absorbance was noted at 540 nm of spectrophotometer model ELx800. Cellulase activity was expressed in term of filter paper unit (FPU) per dL of undiluted crude enzyme. One unit of FPU activity were defined as the amount of enzymes which release 1 µmol of glucose equivalents from Whatman No. 1 filter paper in 1hr (Adney and Baker, 1996).

3.11. Optimization of the Isolates Growth Condition for Cellulase Production

3.11.1. Effect of incubation time on production of cellulase

The determination of the maximum cellulase production time was done by incubating inoculated production media (SmF and SSF) at 28^{O}C on rotary shaker for SmF. The evaluation of enzyme production was undertaken from 72hrs to 192hrs and from 144hrs to 384hrs fermentation period for SmF and SSF, respectively, at an interval of 72 hrs. until significant decline of enzyme activity (Bagewadi *et al*., 2015).

3.11.2. Effect of incubation temperature on the production of cellulase

The effect of temperature on cellulase production of isolates was determined by growing the isolates and assaying for their cellulase activities at different temperatures. Fungal isolates were inoculated into the fermentation media which contain either OS or FMS in both fermentation types (SmF and SSF) and incubated at 20, 28, and 38^{O}C for 5 and 12 days for SmF and SSF, respectively. This was done in duplicates according to the method used by Korish (2003).

3.11.3. Effect of pH on cellulase production

The effect of pH on the growth of isolates was determined at different pH. Fungal isolates were inoculated into fermentation media containing either OS or FMS in both fermentation types (SmF and SSF) and incubated at different pH conditions (5.0, 5.5 and 6.0) for 5 and 12 days for SMF and SSF, respectively. This was done also in duplicates (Mojsov, 2012).

3.11.4. Effect of the type of lignocellulosic on the production of cellulase

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Koo, 2001). In this experiment, two types of growth media were prepared using two different lignocellulosic (i.e. OS and FMS) in separate 250ml capacity CFs. After inoculating the media with fungal isolates and incubating them for 5 and 12 days (for SMF and SSF, respectively), portions of the cultures were withdrawn and centrifuged. The supernatants obtained after centrifugation were saved and used for cellulase assay.

3.12. Partial Characterization of Cellulase

3.12.1. Effect of temperature on the activity and stability of cellulase

The effect of temperature on cellulase activity was evaluated over the range of 20 – 90^{O}C with the intervals of 10°C. The reaction mixture consisted of 1ml of crude enzyme, 1ml of acetate buffer (pH 5.0) and 1ml of 1% CMC as a substrate. Then the mixture was incubated at different temperatures (20 – 90 ^{O}C) for 30mins in the water bath. Then cellulase activity was measured as according to Ghosh (1987). Cellulase stability was evaluated through subjected the crude enzyme to different temperatures (20, 30, 40, 50, 60, 70, 80 & 90) ^{O}C for 24hrs in the incubator. Then after the reaction mixtures were prepared as mentioned above and their activities were measured as an indicator of enzyme stability.

3.12.2. Effect of pH on the activity and stability of cellulase

The effect of pH on cellulase activity was evaluated over the range of 4.5 –9.0 with the interval of 0.5. The reaction mixture consisted of 1ml of crude enzyme and 1ml of 1% CMC as a substrate and the reaction was allowed to precede at different pH conditions 4.5, 5, 5.5, 6, 6.5,

7, 7.5, 8, 8.5 & 9.0. Then the mixture was incubated at 50^{O}C for 30mins for all pH conditions. Then cellulase activity was measured according to the methods followed by Ghosh (1987). Cellulase stability over different pH conditions was also evaluated after withdraw the mixtures of crude enzyme with buffer overnight at different pHs and their activities were measured as an indicator of enzyme stability.

3.12.3. Effect of metallic ions on the activity and stability of cellulase

To determine the effect of metal ions on the cellulase activity, different metal ions or salt solutions were used with final concentration of 10mM (Zeng *et al*., 2016) Na^{+} (NaCl), Ca^{2+} ($CaCl_2$), Mg^{2+} ($MgSO_4$), Fe^{2+} ($FeSO_4$) and Co^{2+} ($CoCl_2$).

The crude enzyme, buffer solution and metal ion solutions were incubated overnight in separate test tubes. The blanks were also incubated without the addition of metal ion solution.

3.13. Treatment Lignocellulosic Substrates with Crude enzyme

FMS and OS were oven dried at 70^{O}C for overnight. 500 mg of each of the dried agricultural residues (lignocellulosic substrates) was taken and subjected to pre-treatments as described by Shankar *et al*. (2014). FMS and OS were ground with a grinder (Coffee grinding machine) and sieved to give particles ranging from 1-2 mm. Then, 5 ml of distilled water was added to the ground lignocellulosic materials separately and the resulting suspension was subjected to autoclaving at 121°C (15 psi) for 15 minutes. The physically pre-treated suspensions were then subjected to crude enzyme treatment for 16hrs at 50ºC. Percentage of saccharification was then calculated using the following equation (Kaniz and Manchur, 2015).

$$\text{Saccharification}\,\% = \frac{\text{Glucose released in mg/ml} * \text{TVH}}{\text{Cellulose content}} \times 100$$

Where, TVH = Total volume of hydrolysate

3.14. Data Analysis

In this study, average results of duplicate experiments were taken and data were statistically analyzed using Analysis of variance (ANOVA) and Tukey's multiple-range test was used to compare treatment means at $P < 0.001$ using SAS Software version 9.2.

4. RESULTS AND DISCUSSION

4.1. Isolation and Screening of Cellulolytic Fungi

A total of 53 fungal isolates were collected (Appendix Table 3) from forest and compost soil samples. Screening for cellulolytic fungal isolates was also carried out using Congo red test (Figure 3). The results indicated that 40% (21) of the total isolates were positive for cellulase production (Table 4).

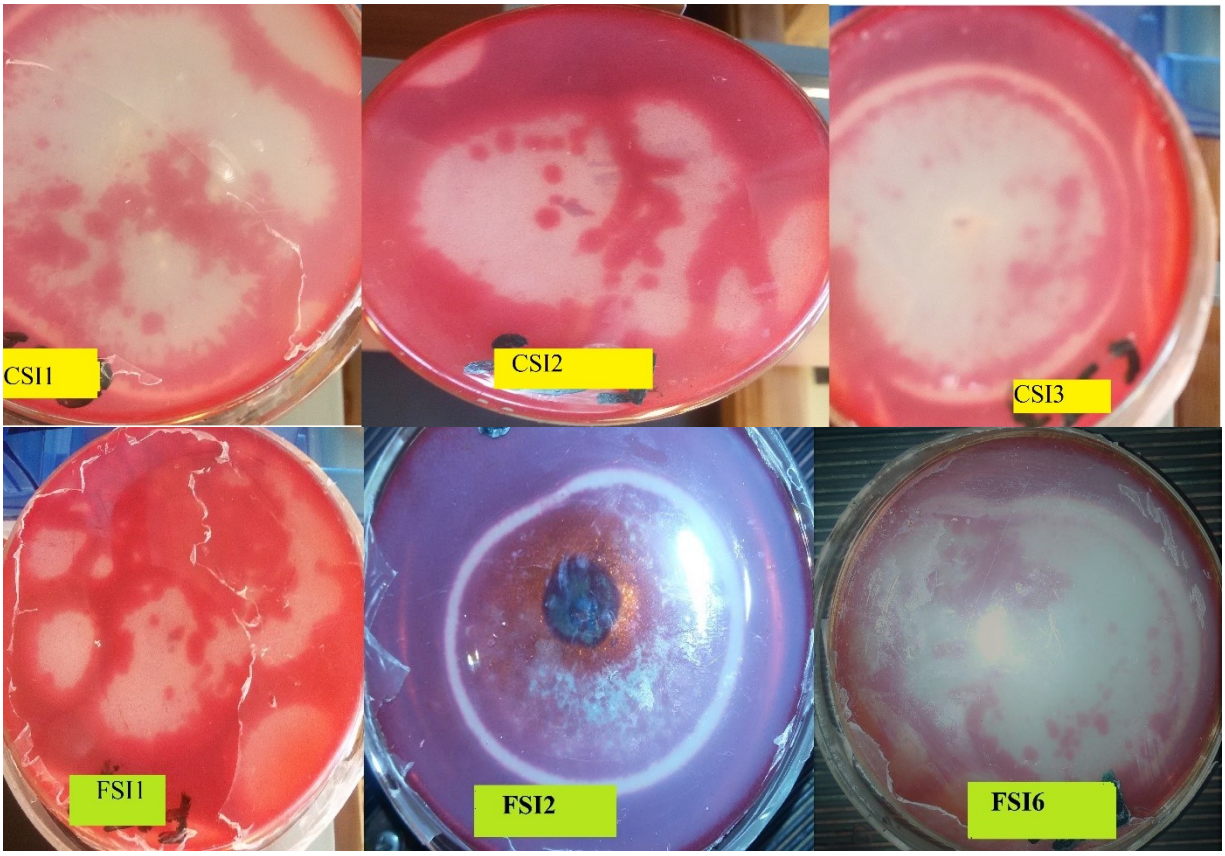


Figure 3. Cellulolytic zone on the CMC rich media

Clear zones were measured around colonies of the active fungal strains and recorded as indicated in Table-4 for analysis. Cellulase activity on carboxymethyl cellulose agar media was expressed as Pečiulytė (2007).

Table 4. Results of the screening test for cellulolytic fungal isolates

S.N.	Isolate's Code Number	Colony diameter (cm)	Diameter of clear zone (cm)	I_{CMC}
1	FSI1	6.7	8	1.19
2	FSI2	5.3	6	1.13
3	FSI5	7.06	6.5	0.92
4	FSI6	4.52	9.5	2.10
5	FSI10	4.6	4.3	0.93
6	FSI11	3.73	3.1	0.83
7	FSI17	1.01	2.9	0.73
8	FSI19	7	6.3	0.97
9	FSI25	10.1	9	0.89
10	FSI27	8.3	7.5	0.90
11	FSI32	6.12	4.9	0.82
12	CSI1	4.34	10	2.30
13	CSI2	3.87	5.5	1.42
14	CSI3	3.38	6.9	2.04
15	CSI7	4.34	4	0.92
16	CSI8	3.5	2	0.57
17	CSI9	3.8	1.8	0.47
18	CSI12	3.5	1.1	0.31
19	CSI13	3	3	1.00
20	CSI15	5	4	0.85
21	CSI17	6.3	1.4	0.22

Efficient cellulase producing fungi isolates were selected based on diameter of the zone of clearing around the fungal colony on carboxy methyl cellulose agar (CMC agar) plates (Bakare *et al.*, 2005). The screening in this experiment allowed the selection of six potential isolates (viz. FSI1, FSI2 and FSI6 from forest soil, and CSI1, CSI2 and CSI3 from compost soil) (Table 4).

4.2. Morphological Characterization of the Isolates

The fungal isolates were characterized by the macroscopic features i.e. the obverse and reverse pigmentation they exhibited when grown on Petri dish containing MEA media. These isolates were also examined for typical microscopic structures, namely: phialides, conidia and spore.

Hence on the basis of these morphological features, isolates FSI1, FSI2 and CSI2 were tentatively identified as members of the genus *Aspergillus* while isolates FSI6 & CSI1 and CSI3 were identified as belonging to the genera *Trichoderma* and *Penicillium*, respectively. The isolates FSI6 and CSI1 showed branched, smooth-walled and colorless hyphae in common with members of the genus *Trichoderma*. Moreover, the conidiophores of these isolates were observed as less complicated and bearing aerial hyphae. They produced smaller branches and ultimately a conifer-like branching system was formed. Their conidiophores are terminated with two or more phialides and primary branches arise at nearly 90 degrees to the main axis. In the genus *Aspergillus*, FSI1, FSI2 and CSI2 had their own morphs as indicated in Appendix Table 4. On the other hand, isolate CSI3 had a rapid growth, dark green obverse pigmentation color with yellowish reverse pigmentation; and their colonies had granular powdery appearance which are all characteristics shared by members of the genus *Penicillium*. Additionally, as in *Penicillium,* the reverse pigmentation of the colony was pale yellow in color and the microscopic features showed septate hyphae with conidiophores attached to them (Tiwari *et al*., 2007).

4.3. Effect of Fermentation Techniques on Cellulase Production

As can be seen from figures 6-15, cellulase production seems to be greatly affected by the type of fermentation technique used. All isolates except FSI1 were a higher level of cellulase record in SSF than in SmF throughout the experiments (Table 5). In line with this result, higher efficiency of enzymatic production in SSF was reported previously by several authors for various types of enzymes and microorganisms (Kamra and Satyanarayana, 2004; Sharma *et al*., 2005; Silva *et al.,* 2005). This could be due the fact that in submerged fermentation (SmF), the fungus is exposed to stressful hydrodynamic forces while in SSF growth is restricted only to the surface of the solid matrix with minimal stress. The other possible explanation for the difference could be the fact that the use of solid systems in SSF provides the fungus with an environment closer to its natural habitat (wood and decayed organic matter), which stimulates the fungus to produce more cellulolytic enzymes (Silva *et al*., 2005).

Table 5. Comparison between SmF Vs SSF

	Treatments					
parameters	T1	T2	T3	T4	T5	T6
SmF	33.71±0.69^{b}	33.44±0.33^{b}	33.77±1.29^{b}	30.47±0.24^{a}	32.99±1.23^{b}	35.5±0.069^{b}
SSF	41.28±1.44^{a}	45.01±0.59^{a}	47.29±1.65^{a}	37.06±0.05^{a}	44.19±1.02^{a}	55.9±0.25^{a}
SEM	0.99	1.23	2.11	0.22	0.89	0.07
SL	**	***	***	ns	**	***

a, b = means with different superscripts in a column are significantly different. *** =($p<0.001$), **=($p<0.01$)*=($p<0.05$; SEM =standard error of the mean, SL=significance level, ns= non-significant,T1 = CSI1 production at(20°C+28°C+38°C), T2= CSI2 production at(20°C+28°C+38°C), T3= CSI3 production at(20°C+28°C+38°C), T4=FSI1 production at(20°C+28°C+38°C), T5= FSI2 production at(20°C+28°C+38°C) and T6= FSI6 production at(20°C+28°C+38°C).

4.4. Effect of Growth Conditions on Cellulase Production

4.4.1. Incubation time

The highest cellulase production was recorded for FSI6 in SmF and SSF at the 5th and 12th days of incubation, respectively (Figure 4 and 5). Enzyme secretion usually occurs maximally during the late exponential and early stationary phases. A prolonged incubation time beyond this period did not increase the enzyme yield rather slightly decline the secretion and reached stationary phase. And at this time the organism could have started producing secondary metabolites with a simultaneous reduction in cellulase yield. The reason for this might have been due to the denaturation of the enzyme caused by the interaction with other components in the medium, deficiency of nutrients, accumulation of toxic substances and proteolysis of cellulase as reported by some authors (Santos *et al.*, 2012; Abdullah *et al*., 2014).

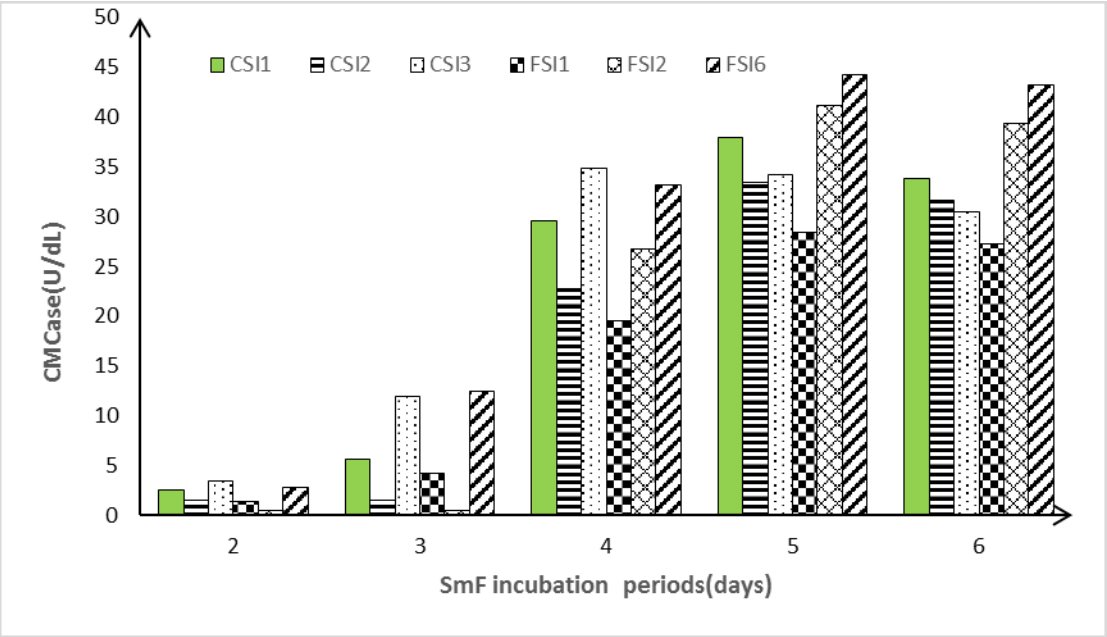


Figure 4.Effect of incubation time on cellulase production of the six fungal isolates in SmF

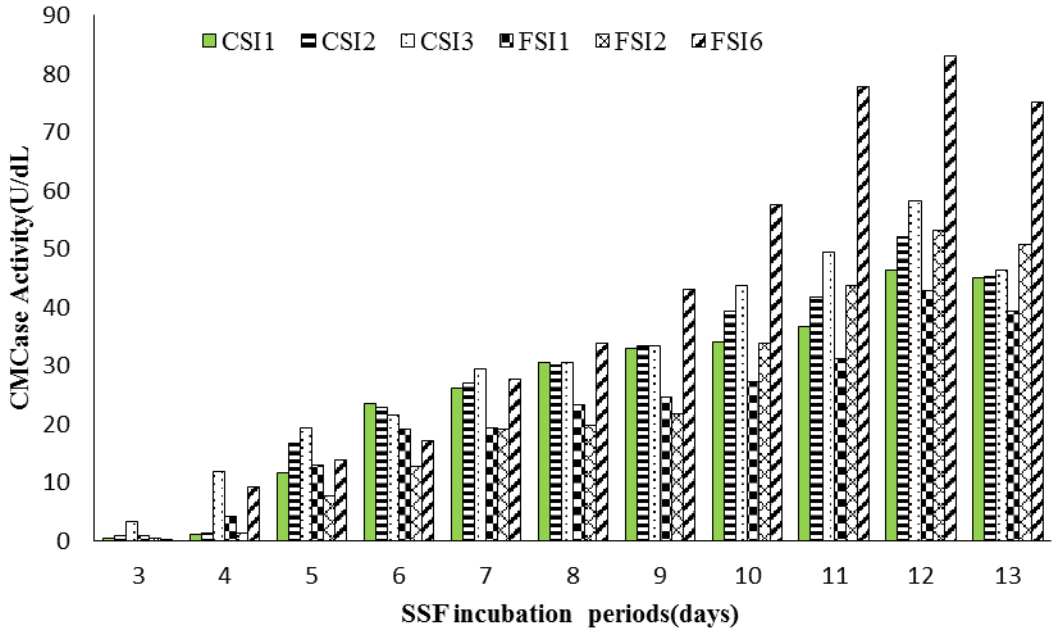


Figure 5. Effect of incubation time on cellulase production of the six fungal isolates in SSF

4.4.2. Incubation temperature

When different incubation temperatures were used in the submerged fermentation, the highest enzyme production was obtained at 28^{O}C for isolates CSI1(CMCase activity of 38.67U/dL; Fpase 23.36FPU/dL), CSI2 (CMCase activity of 34.67U/dL; Fpase 26.4FPU/dL), FSI2(CMCase activity of 41.39 U/dL; Fpase 36.3FPU/dL),and FSI6 (CMCase activity of 46.52 U/dL; Fpase 41.57FPU/dL).Whereas,CSI3 produced maximum at 38^{0}C with activities to 36.8 U/dL, 18.46FPU/dL for CMCase and Fpase, respectively and FSI1 produced maximum cellulase at 20^{0}C with activities corresponding to 41.95U/dL and 20.87FPU/dL for CMCase and Fpase, respectively. This difference might be due to the fact these isolates were collected from different soils which are forest and compost soils and different genera of fungus. It might be due the fact that at high temperature, the growth of the fungi was hindered. (Figure 6 and 7).

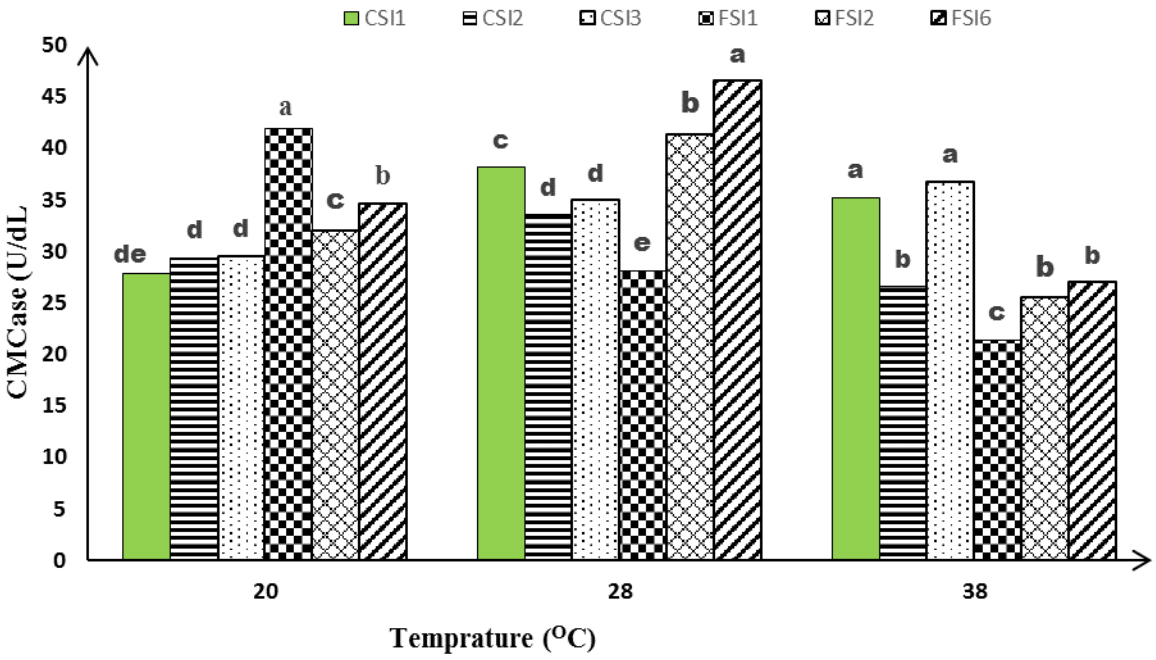


Figure 6. Effect of temperature on the production of cellulase (CMCase) in SmF

Means, in each bar, followed by the same letter are not significantly different(P<0.001) from each other according to Tukey test as showed on Appendix table 5.

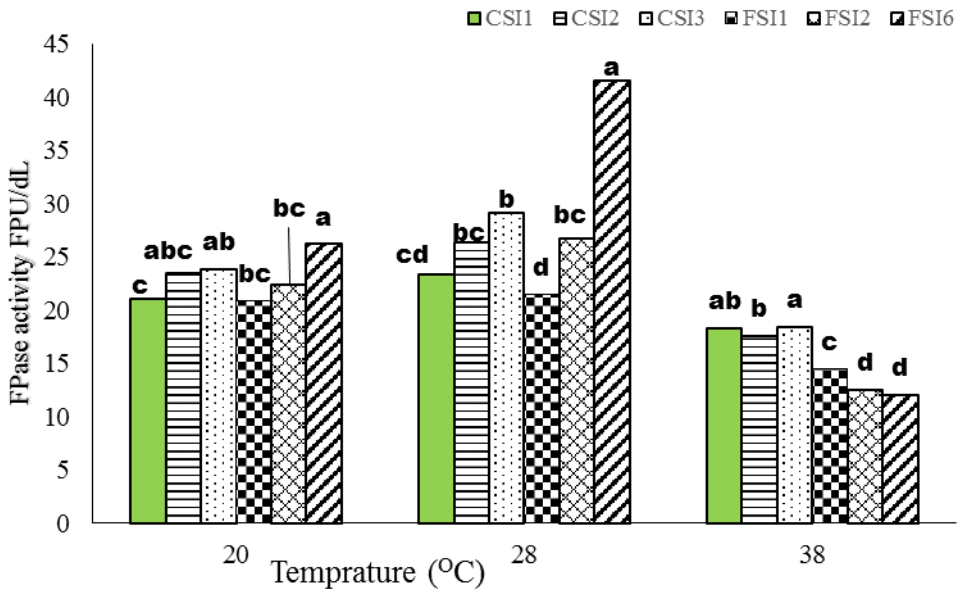


Figure 7. Effect of temperature on the production of cellulase (Fpase) in SmF

In contrast, in solid state fermentation the highest cellulase production was obtained from isolates CSI1, CSI2, CSI3, FSI1, FSI2, and FSI6 with activities corresponding to 46.41 U/dL and 38.34FPU/dL;52.77 U/dL and 32.27 FPU/dL; 58U/dL and 40.45 FPU/dL; 42.78 U/dL and 34.07 FPU/dL; 62.99U/dL and 36.33 FPU/dL; 83.12U/dL and 44.51 FPU/dL for CMCase and Fpase, respectively, at 28^0C (Figure 8 and 9).

The optimum incubation temperature was 28^OC. This was due to the Mesophilic nature of the experimental isolates; Sajith *et al*. (2015) also reported more or less similar findings for *Penicillium* species in which they indicated that cellulase production was higher in SSF than in SmF at a temperature of 28^OC. This might be due to the fact fungi isolates prefer a low water growth conditions; because the conditions of fermentation which show similarity to the natural environment (Jha *et al*., 1995).

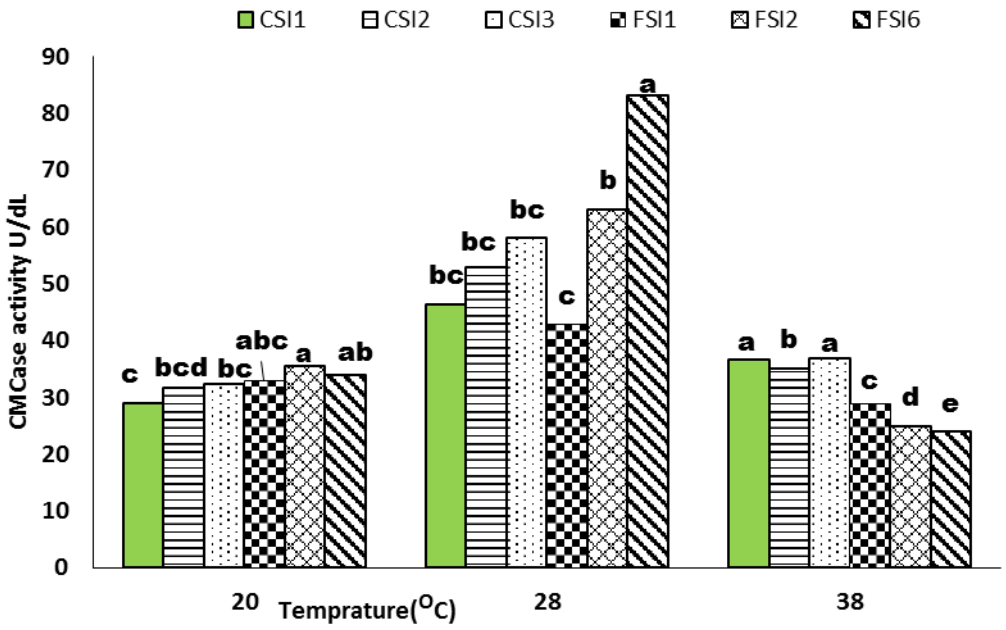


Figure 8. Effect of temperature on the production of cellulase (CMCase) in SSF

Means, in each bar, followed by the same letter are not significantly different(P<0.001) from each other according to Tukey test as showed on Appendix table 5.

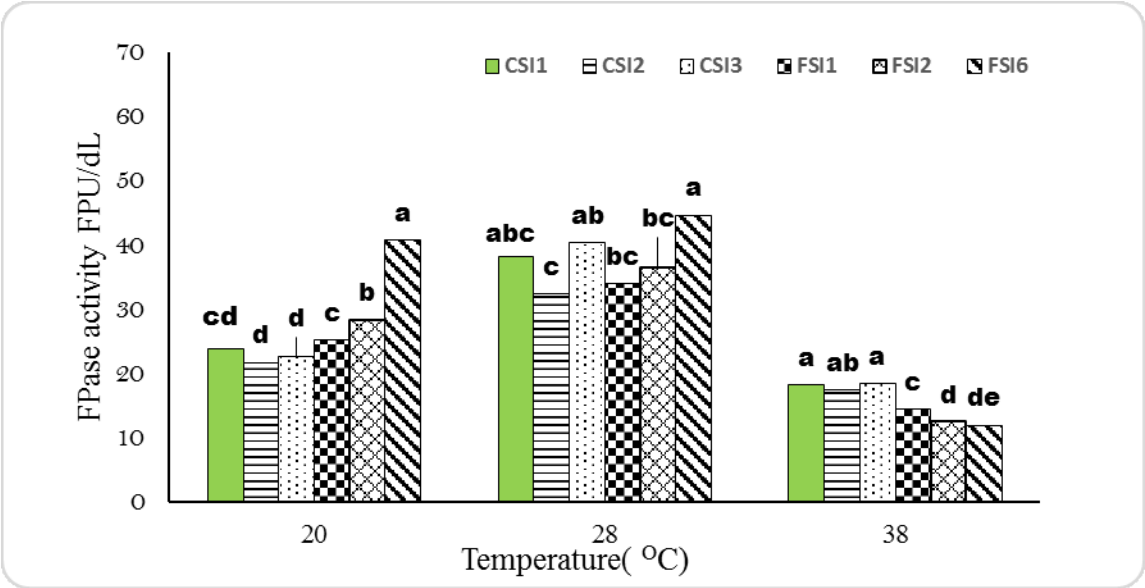


Figure 9. Effect of temperature on the production of cellulase (Fpase) in SSF activity

4.4.3. pH

When the submerged fermentation was subjected to different pH conditions, all isolates resulted in highest cellulase production at a pH of 5.5. Isolates CSI1, CSI2, CSI3, FSI1, FSI2 and FSI6 produced cellulases with activities corresponding to 28.97U/dL &11.62FPU/dL; 26.65U/dL & 9.64FPU/dL; 26.19U/dL & 12.63FPU/dL; 35.65U/dL & 13.09FPU/dL; 36.02U/dL & 11.25FPU/dL; 31.83U/dL &15.1FPU/dL for CMCase and FPase, respectively (Figure 10 and 11). This variation might be due the inherent nature of the isolates.

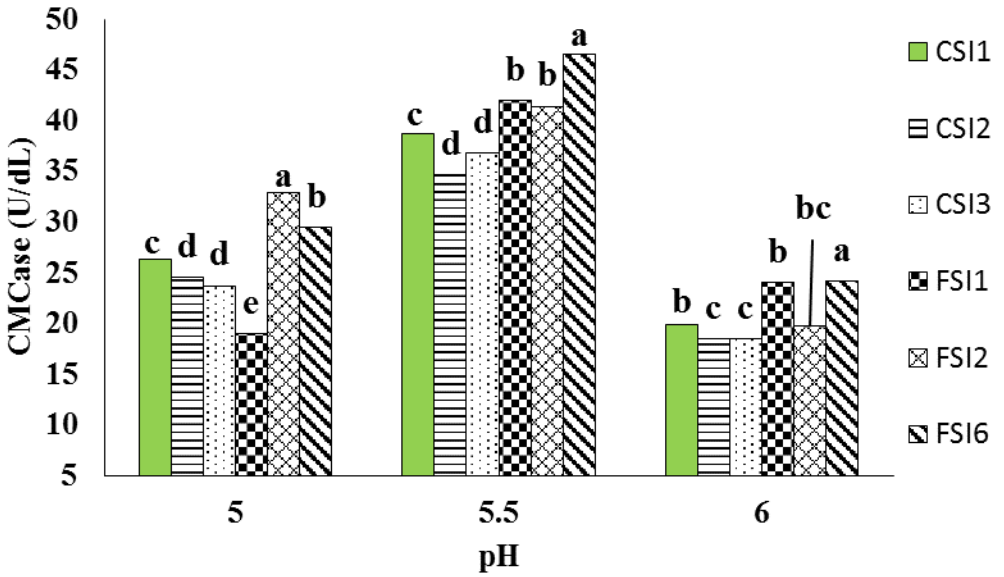


Figure 10. Effect of pH on the production of cellulase (CMCase) in SmF

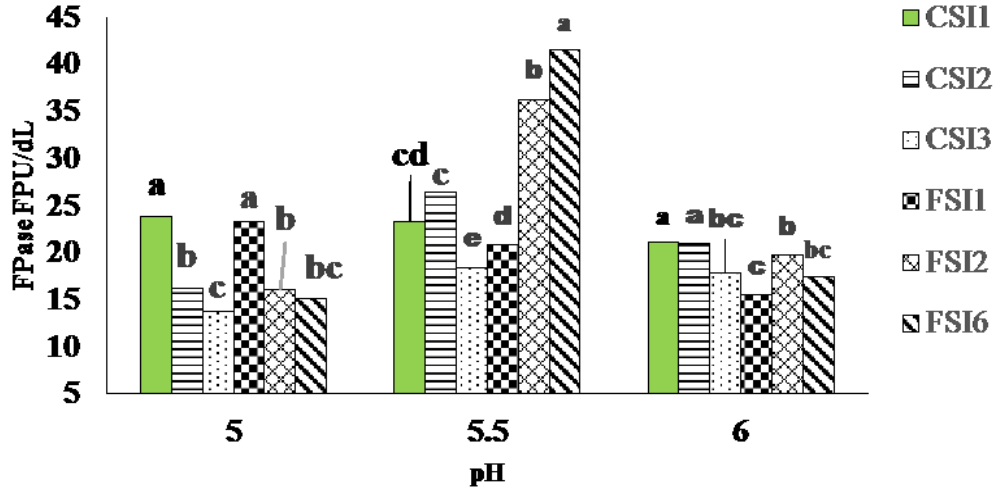


Figure 11. Effect of pH on the production of cellulase (Fpase) in SMF

In SSF, all isolates had the highest cellulase production at pH of six (6); the activities of which corresponded to 46.07U/dL & 38.16FPU/dL; 52.77U/dL & 32.2 FPU/dL; 57.63U/dL & 39.82 FPU/dL; 42.56U/dL & 33.83FPU/dL; 62.99U/dL & 36.33FPU/dL; and 83.12U/dL & 44.51 FPU/dL CMCase and Fpase for CSI1, CSI2, CSI3, FSI1, FSI2 and FSI6, respectively (Figure 15 and 16). These results were in agreement with those of Dutt and Kumar (2014); Vega *et al*. (2005) and Ahmed *et al*. (2009) report; but contradictory to those of Prasanna *et al.* (2016).

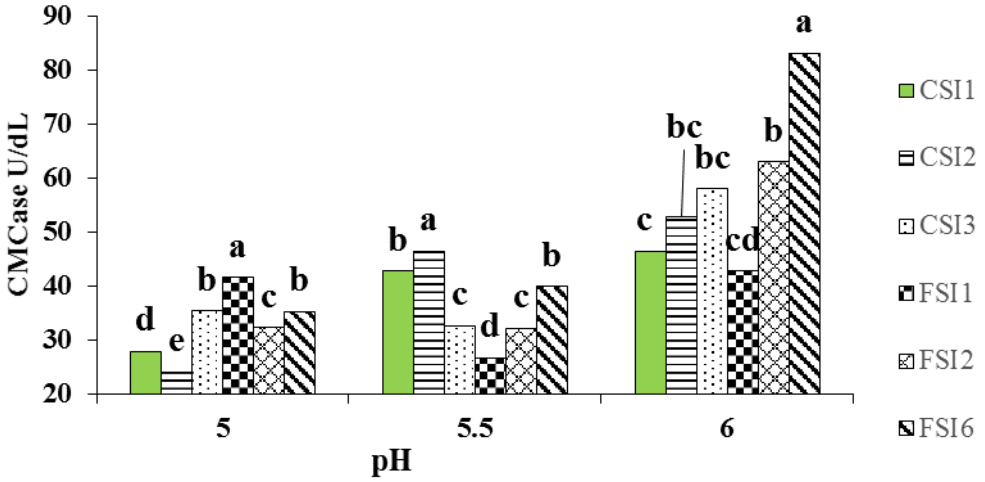


Figure 12. Effect of pH on the production of cellulase (CMCase) in SSF

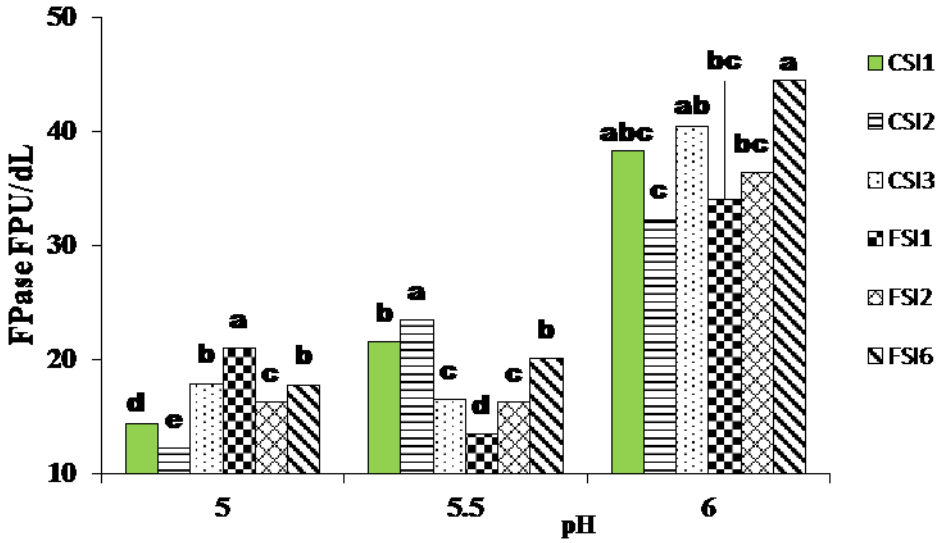


Figure 13. Effect of pH on the production of cellulase (Fpase) in SSF

4.5. Effect of the Type of Lignocellulosic Substrate on the Production of Cellulase

Finger millet straw was observed to induce the highest production of CMCase (44.93U/dL) and FPase (38.8FPU/dL) in submerged fermentation and CMCase (83.12U/dL) and Fpase (44.69FPU/dL) in solid state fermentation. On the other hand, Oat straw resulted in the production of 46.52U/dL and 41.57FPU/dL CMCase and Fpase respectively in submerged fermentation and 82.98U/dL and 44.69 FPU/dL CMCase and Fpase respectively in solid state fermentation as shown in Figures 14 a& b.

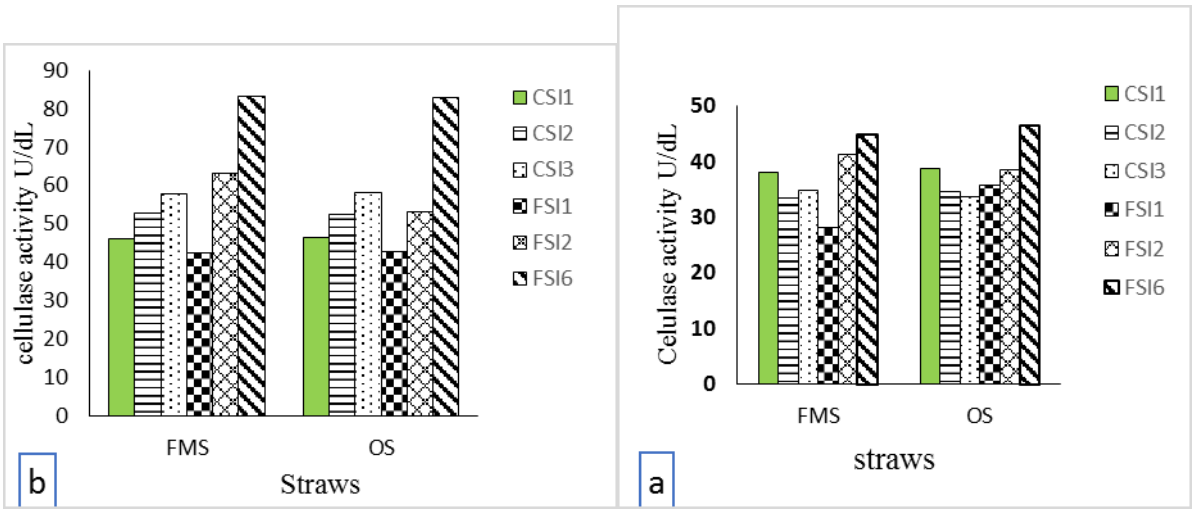


Figure 14. The effect of substrate type on cellulase production in a) SmF and b) SSF

This indicates that FMS is more suitable substrate for cellulase production than OS. Perhaps, this might be due to the Cellulose Crystallinity difference between them. Which means finger millet (*Eleusine coracana*) had low crystallinity index than oat (*Avena sativa*) as described by Rambo and Ferreira, 2015. Cellulolytic fungi can use cellulose as a primary carbon source. Pure, crystalline cellulose, such as Solka Floc, Avicel, and cotton are good cellulase inducers, but are expensive. To keep costs down it is therefore important to use a substrate that is less expensive Wen *et al*., 2005).

4.6. Partial Characterization of Cellulase Produced by the Fungal Isolates

4.6.1. Activity of cellulase at varying temperatures

Effect of temperature on the activity of cellulase was studied, by incubating the crude enzyme with the substrate at temperatures ranging from 20 to 90ºC for 30mins. Maximum cellulase activity recorded for isolates CSI1, CSI2, FSI1, FSI2 and FSI6was at 50ºC, whereas for CSI3 it highest activity was obtained at 60ºC. However, in all cases the activity generally decreased significantly at temperatures below 40°C and above 70°C (Figure 15). Some earlier reports had also indicated varying optimum temperatures (Muñoz *et al*., 2016).

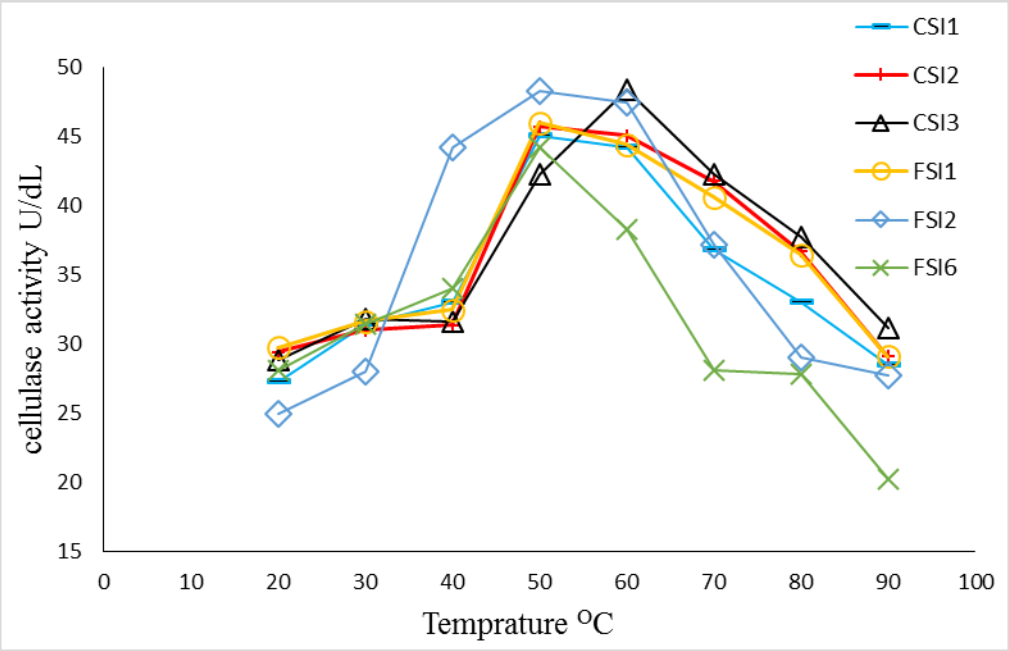


Figure 15. Effect of temperature on cellulase activity

4.6.2. Stability of cellulase at varying temperatures

The effect of temperature on the enzyme stability was determined by pre-exposing the enzyme to various temperatures ranging from 20^{O}C– 90^{O}C for 24hrs. Cellulase activity was determined under the standard enzyme (endo-1,4-β-D-glucanase) assay conditions. The control (activity at optimum temperature) was taken as having 100% activity. The results of this section are depicted in Table 6. As can be seen from the figure, the stability of cellulase slightly decreased with increase in incubation temperature. The stability of cellulase enzyme

could be due to the organisms' genetic adaptability to carry out their biological activities at higher temperatures (Al-Shehri and Mostafa, 2004).

Table 6. Effect of temperature on cellulase stability

Source of cellulase	Activity at optimum temperature	% Residual activity at different temperatures treatments							
		20^{O}C	**30^{O}C**	**40^{O}C**	**50^{O}C**	**60^{O}C**	**70^{O}C**	**80^{O}C**	**90^{O}C**
CSI1	50^{O}C	35.48	44.99	48.63	100	74.59	57.47	48.56	38.16
CSI2	50^{O}C	40.33	44.04	44.88	100	76.54	68.93	57.18	39.59
CSI3	60^{O}C	38.95	45.88	45.57	70.11	100	69.98	59.60	44.37
FSI2	50^{O}C	40.96	45.50	47.38	100	75.09	66.32	56.58	39.59
FSI2	50^{O}C	30.02	37.15	74.49	100	81.95	58.22	39.49	36.43
FSI6	50^{O}C	37.21	45.07	51.02	100	60.95	37.23	36.65	19.10

4.6.3. Activity of cellulase at different pH values

The pH optima for the activity of the cellulase determined through incubated the enzyme on pH range from 4.5 to 9.0 (with 0.5 intervals). In this study as the pH value increase cellulase activity also increased and the highest cellulase activity in CSI1 and FSI2 was shown at pH 5.5. Whereas, CSI2, CSI3, FSI1, and FSI6 highest activity was recorded at pH 7.0, 5.0, 8.0 and 6.0 respectively (Figure16). Further increase in the pH resulted decrease in the activity of cellulase.

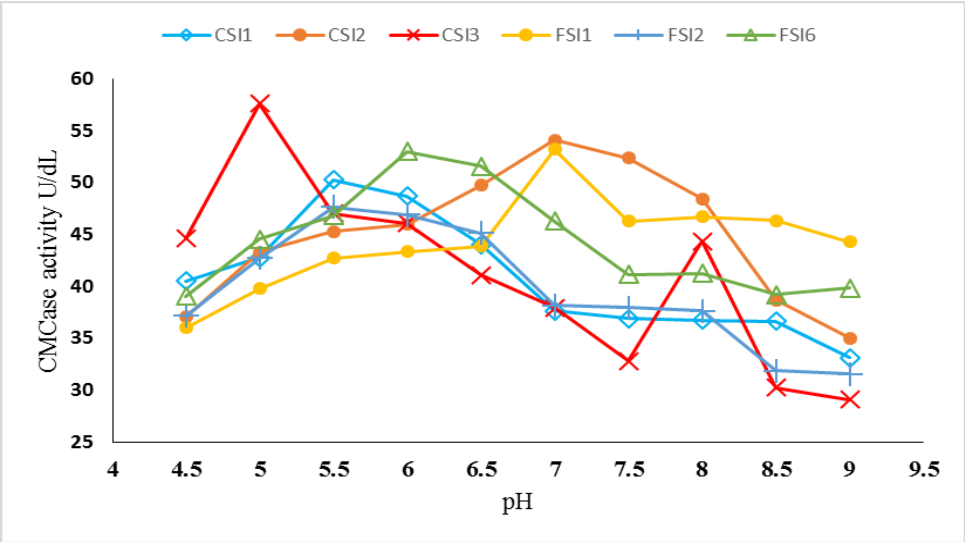


Figure 16. Effect of pH on cellulase activity

This finding suggests that the enzymes would be useful in processes that require a wide pH range from slightly acidic to alkaline medium. The results of the present study were in line with activity of cellulase produced from *A. niger* and *Trichoderma species* at wide range of pH and maximum activity at pH 9.0 (Gautam *et al*., 2011; Salwa *et al.,* 2012).

4.6.4. Stability of cellulase at different pH values

The effect of pH on the enzyme stability was determined by pre-exposing the enzyme at various temperatures ranging from 4.5 to 9.0 for 24hrs. The cellulase activity was determined under standard enzyme assay condition. The control (activity at optimum pH) was taken as having 100% activity.

Table 7. Stability of cellulase at different pH values

Source of cellulase	Activity at optimum pH	% Residual activity for different pH treatments									
		4.5	5	5.5	6	6.5	7	7.5	8	8.5	9
CFI 1	5.5	40.5	42.8	100	48.7	44.0	37.6	36.9	36.8	36.7	33.1
CFI 2	7	37.1	43.3	45.3	46.0	49.8	100	52.4	48.4	38.7	35.0
CFI 3	5	44.6	100	47.0	46.1	41.1	38.0	32.8	44.4	30.3	29.1
FSI 1	7	36.0	39.8	42.7	43.4	43.9	100	46.3	46.7	46.3	44.3
FSI 2	5.5	37.2	42.7	100	46.9	45.1	38.1	37.9	37.7	31.9	31.5
FSI 6	6	39.1	44.5	46.8	100	51.6	46.3	41.1	41.3	39.2	39.9

4.6.5. Effect of metallic ions on cellulase activity

The effect of metal ions on the activity of cellulase of FSI1, FSI2, FSI6, CSI1, CSI2 and CSI3 indicated in figure 17. The results showed that the presence ofFe^{2+} enhances activity of cellulase of CSI1 and CSI2. Whereas, Mg^{2+} and Co^{2+}were highly inhibitory effect on cellulase activity ofFSI6and CSI1 respectively. Among them Mg^{2+} was significantly inhibited the cellulase activity of all studied fungi. Na^{+} showed slightly and similar inhibitory effect to all isolates cellulase followed by Co^{2+}. The experiment was Contrary result to Wen *et al.* (2005) not all metallic ions were inhibitory effect to cellulase enzyme activity. As evidence even at concentration 10mM Fe^{2+} cannot inhibit the activity.

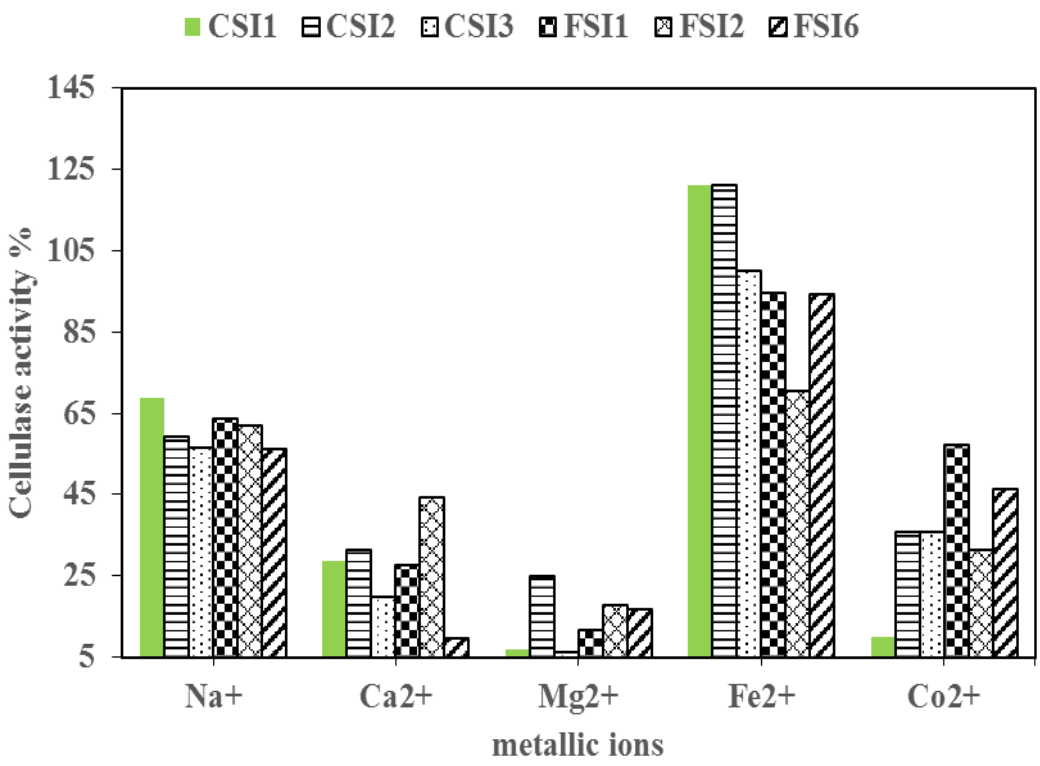


Figure 17. Effect of metallic ions on cellulase activity

4.7. Enzymatic Treatment of Cellulosic Substrates

FMS and OS were pre-treated and subjected to crude enzyme for estimating the saccharification of cellulosic substrates. Followed by physical treatment, enzymatic action was undertaken and the results presented in table 8. Maximum conversion of 22.76 % and 28.9 % were obtained from FMS and OS respectively by using crude enzyme of FSI6 fungal isolates.

Table 8. Percentage Conversion of cellulosic residues into reducing sugars

substrates	Cellulose content	Physical pre-treatment	% of saccharification after enzymatic treatment
OS	35%	Grinding and steaming	28.9%
FMS	29%	Grinding and steaming	29.2%

Percentage of saccharification after enzymatic treatment of straw was relatively higher for finger millet. This variation might be due to crystallinity index of the finger millet is higher as compared to oat straw.

5. SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1. Summary

The study indicates that the forest and compost soil are good sources of filamentous fungi that produced the cellulolytic enzyme. These enzymes depolymerize the cellulose biomass and converted into smaller subunits viz. oligosaccharides, glucans, cellobiose, and glucose. Fermentations by using agro-residual substrates OS and FMS by *Aspergillus, Trichoderma* and *penicillium spps* proved possibility of the enzyme production in economical range. Time courses of cellulase production in all isolates indicates that the production increases as time increases up to the optimum time of incubation and decline after wards. The maximum cellulase production was obtained at five days for SmF and 12 days for SSF of incubation. Beyond this period of incubation relative activity of enzyme was decrease and the maximum activity was obtained from FMS substrate at a moisture content of 65%.

The major physiochemical properties of the cellulase were studied. The effect of pH and temperature on the activity and stability of cellulase indicated that as the temperature and pH increased at a certain point cellulase activity also increased and further increment beyond the highest value showed decreased in activity. Cellulase stability was maintained more than 50% of its residual activity after it was incubated at temperatures below 60°C for 30min. The effect of metallic ions (Ca^{2+}, Na^{+}, Co^{2+}, Mg^{2+} and Fe^{2+}) in the activity of cellulase by fungal strain using SSF and SMF was determined. Among all the selected metallic ions the minimum enzyme activities were occurred in the presence of Mg^{2+}, Ca^{2+} and Co^{2+} ions and subsequently increase in their activity as the iron ion concentration increases. The isolates were produced maximum yield in SSF than SmF and the optimum temperature were recorded at $28^{O}C$ with in a pH of 6.

5.2. Conclusion

The overall finding of the present study shows that it is possible to isolate cellulase producing fungi from forest and compost soil. Additionally, the isolated fungi strains were found and tentatively belong to the genera of *Trichoderma, penicillium and aspergillus*, as these groups of fungi known to produce cellulase. Maximum enzyme was produced from selected isolates using straws as a sole carbon source which is easily available everywhere from agricultural

waste with in inexpensive cost. Enzyme produced from the isolated fungal strain had been stabilized with changes of temperature and pH on the activity and stability of cellulase have taken as an advantage for energy production, waste treatment and animal feed treatment industries at various pH condition. The isolates also showed maximum enzyme production at 28^{O}C. In SmF isolates grew in a short time of incubation. This is important to get the enzyme in very short time and thus reducing the cost of production and risk of contamination. Perhaps, in SSF isolates gave the maximum production of cellulase.

Cellulolytic property of the enzyme helps to convert cellulose into smaller sugar units and value-added product for animal consumption and other energy sources. The enzyme from all isolates had highly active at optimum temperature of 50^{o}C except CSI3 at 60^{O}C. They are also active in broad pH ranges. Iron(II) ion is an inducer for the activity of cellulases. Among the six isolates FSI6 has a higher saccharification potential of lignocellulosic biomasses as compared to other isolates. Saccharification of the finger millet straw was resulted higher percentage than oat biomass in all six experimental fungi isolates. This variation might be due to crystallinity index of the biomasses and the inherent nature of the isolates.

In general, results of this study, suggested that the fungal isolates can be used for the production of physiochemically diversified cellulases using abundant cellulosic substrates; which implies that cellulase can be desirable in various industrial applications.

5.3. Recommendations

The present findings of this study showed that cellulase enzyme was active for cellulose biomass degrading in to their smaller units. Based on the results obtained from this study, the following recommendations should be considered for future work:

- Further research should be conducted on molecular level characterization of CSI1, CSI2, CSI3, FSI1, FSI2 and FSI6.
- Cellulase enzymes should be evaluated for industrial purpose such as textile industry, food industry, animal feed (with in vivo experiment) and bioethanol production.
- This study also strongly recommended that, researches have to be done on large scale production, further purification and complete characterization of cellulases from these isolates.

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7. APPENDIXES

Appendix I: Media preparation

1. **Preparation of Malt Extract Agar (MEA)**

   Dissolve 30g of Malt extract powder, 5g of mycological peptone, 10g of glucose and 15g of agar powder in 1L of distilled water.

2. AppendixTable1. Media composition of the plates used for screening

Composition	**Amount (g/L)**
$NaNo_3$	6.5
K_2HPO_4	6.5
KCl	6.5
$MgSO_4.7H_2O$	3.0
Yeast extract	0.3
Peptone	0.75
CMC	10
Agar	15

0.01g/L of chloramphenicol was added after autoclaved the media.

3. Appendix Table 2. Media composition for submerged fermentation

Composition	**Amount g/L**
Urea	0.3
$(NH_4)_2SO_4$	1.4
$CaCl_2$	0.3
$MgSO_4$	0.3
KH2PO4	2
Yeast extract	0.25
Peptone	0.75
Tween 80	1ml

these were used as supplementary to the straw

4. **Congo Red (0.1%) reagent preparation**

   ❖ Dissolve 0.1 gram of Congo red powder in 100ml of distilled water

Appendix Table 3. Fungal isolates collected from forest and compost soil

S N.	Isolates	sources	cellulolytic test results	S N.	Isolates	sources	cellulolytic test results
1	FSI1	Forest soil	positive	**28**	FSI28	Forest soil	negative
2	FSI2	Forest soil	positive	**29**	FSI29	Forest soil	negative
3	FSI3	Forest soil	negative	**30**	FSI30	Forest soil	negative
4	FSI4	Forest soil	negative	**31**	FSI31	Forest soil	negative
5	FSI5	Forest soil	positive	**32**	FSI32	Forest soil	negative
6	FSI6	Forest soil	positive	**33**	FSI33	Forest soil	negative
7	FSI7	Forest soil	negative	**34**	FSI34	Forest soil	negative
8	FSI8	Forest soil	negative	**35**	FSI35	Forest soil	positive
9	FSI9	Forest soil	negative	**36**	FSI36	Forest soil	negative
10	FSI10	Forest soil	positive	**37**	CSI1	Compost soil	positive
11	FSI11	Forest soil	positive	**38**	CSI2	Compost soil	positive
12	FSI12	Forest soil	negative	**39**	CSI3	Compost soil	positive
13	FSI13	Forest soil	negative	**40**	CSI4	Compost soil	negative
14	FSI14	Forest soil	negative	**41**	CSI5	Compost soil	negative
15	FSI15	Forest soil	negative	**42**	CSI6	Compost soil	negative
16	FSI16	Forest soil	negative	**43**	CSI7	Compost soil	positive
17	FSI17	Forest soil	positive	**44**	CSI8	Compost soil	positive
18	FSI18	Forest soil	negative	**45**	CSI9	Compost soil	positive
19	FSI19	Forest soil	positive	**46**	CSI10	Compost soil	negative
20	FSI20	Forest soil	negative	**47**	CSI11	Compost soil	negative
21	FSI21	Forest soil	negative	**48**	CSI12	Compost soil	positive
22	FSI22	Forest soil	negative	**49**	CSI13	Compost soil	positive
23	FSI23	Forest soil	negative	**50**	CSI14	Compost soil	negative
24	FSI24	Forest soil	negative	**51**	CSI15	Compost soil	positive
25	FSI25	Forest soil	positive	**52**	CSI16	Compost soil	negative
26	FSI26	Forest soil	negative	**53**	CSI17	Compost soil	positive
27	FSI27	Forest soil	positive				

Appendix Table 4. Morphological characteristics of isolates

	Macroscopic observations		Microscopic appearances		
Isolates	Obverse pigmentation	Reverse pigmentation	Conodia	Spore	Phialides
CSI1	Dark green	Creamy	Subglobose to ellipsoidal	Ellipsoidal	Smooth-walled, colourless
CSI2	Brown	Light brown	Biseriate	Aspergillum	Matulae club shaped
CSI3	Light green	Light yellow	Colourless and branched	Round with blue colour	Clustered flask shaped
FSI1	Green	Light brown	Sterigmata biseriates	Round with blue colour	Globose to subglobose
FSI2	Yellowish white	Creamy	Uncolored, coarsely	Smooth and round	Globose
FSI6	Grayish green, with concentric ring	Creamy	Globose to subglobose	Ellipsoidal	Cylindrical

APPENDIX II. Enzyme Extraction and Assay

1) **Enzyme Extraction**

- The Buffer solution was prepared and dissoluted; 30% Na-acetate buffer (0.2M at pH 5) was added to each flask and put them on rotary shaker (150 rpm) for 30minutes at room temperature for maximum enzyme extraction. This step is applicable for SSF merely.
- Centrifugation; Liquid homogenate was then filtered through cotton gauze and centrifuged at 10000rpm for 10 minutes at 4^{o}C to remove solid particulate matter.
- Filtration; cell free culture filtrate was filtered through Whatman no. 1 filter paper and
- Then clear filtrate was centrifuged and the crude enzyme was used for the following experiments.

2) DNS Reagent Preparation

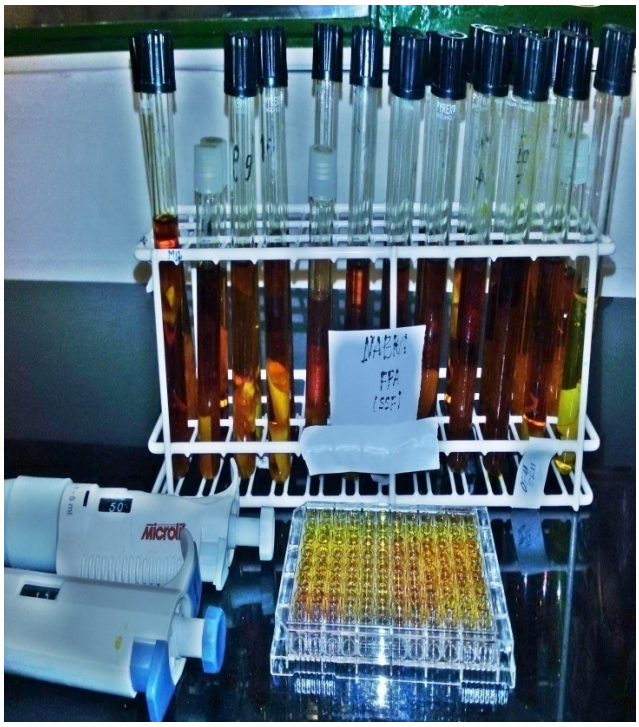
- A 1000 ml beaker was cover with tinfoil to avoid light exposure; I was used black bottle.
- The beaker was Placed on the stir plate and added approximately150 ml of distilled water and a stir bar.
- Continuous magnetic stirring was undertaken
- 5 g of dinitrosalicylic acid was added, and allow to be dissolved.

- Added 4g of NaOH gradually, allow to be dissolved.
- Added 150g of Rochelle salt slowly (sodium potassium tartrate) over a 20-30minutes period.
- Added 2g of Phenol slowly (melted at 60°C) and 2g Sodium meta bisulphate
- The solution was cautiously warmed to a maximum temperature of 50°C to clear the solution.
- The solution may be filtered through a whatman #1 Filter paper was needed.
- Allowed the solution to cool.
- Analytically transferred the solution to a 500ml volumetric flask and diluted to volume with distilled water

3) Carboxymethyl cellulase assay

Substrate; 1% Carboxymethyl cellulose CMC in 0.2 M sodium acetate buffer, pH 5.

1. 0.5 ml enzyme was added, diluted in 1ml acetate buffer, to a test tube.
2. 0.5 ml substrate solution was added, mixed well and incubated at 50°C for 30 mm.
3. 3.0 ml DNS was added and mixed. Then transferred to a rack on the table.
5. Boiled for exactly 5.0 mm in a vigorously boiling water bath containing sufficient water.
6. 20 ml deionized or distilled water was added. Mixed by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion (N.B. This is important!).
7. The color formed against the spectrophotometer was measured at 540 nm. When necessary (i.e., when small dilutions are used), the color formed in the enzyme blank is subtracted from that of the sample tube.
8. The absorbances of the sample tube (corrected by subtraction of the enzyme blank) were translated into glucose production during the reaction using a glucose standard curve.



Appendix Figure 1. Assay for CMCase activity&
Appendix Figure 2. Assay for FPase activity

4) **Filter paper cellulase unit (FPU) assay**

Substrate: Whatman No. 1 filter paper strip, 1.0 x 6.0 cm (= 50mg) was prepared.

1. 1.0 ml 0.2M Na-acetate, pH 5was added to a test tube of which have volume at least 25 ml.

2. Added 0.5 ml enzyme, diluted in citrate buffer. Two dilutions were made of each enzyme sample investigated.

3. One filter paper strip was added, mixed to coil the strip 4. Incubated at 50°C, for 60 min.

5. 3.0 ml DNS was added, mixed and Transferred tubes to a rack on the table.

6. Boiled for exactly 5.0min in a vigorously boiling water bath containing sufficient water. After boiled, transferred to a cold-water bath.

7. 20 ml deionized or distilled water was added. Mixed by completely inverting the tube several times so that the solution separates from the bottom of the tube at each inversion (NB. This is important!).

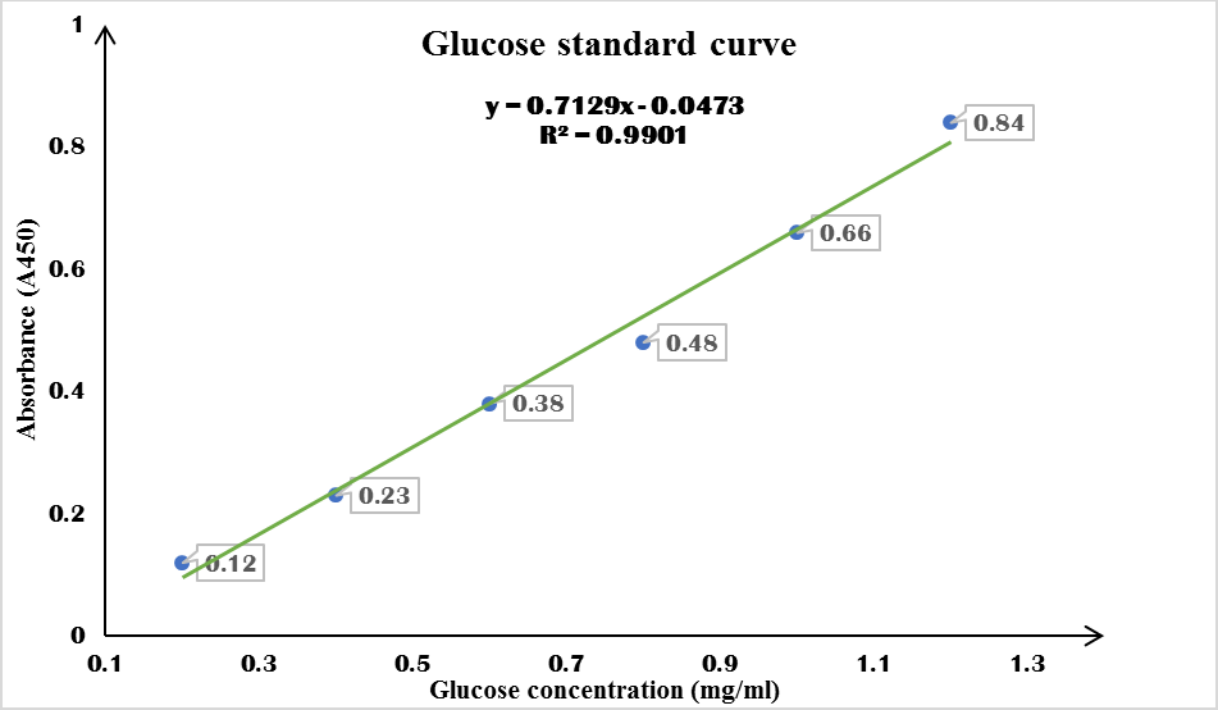
8. When the 'pulp' has settled well, i.e., after at least 20 min, the color formed is measured at 540 nm.

APPENDIX III. Glucose standard curve (GSC)

Appendix Table 4a. Proportion of Glucose to Water to Draw Glucose Standard Curve

Stock: 10 mg/dl

No.	Desired Concentration	Dilution Needed	Volume Stock	Volume DIH_2O (ml)	Total Volume(ml)
1	0 mg/dl	None	0	10.0	10.0
2	25 mg/dl	1:40	0.25	9.75	10.0
3	50 mg/dl	1:20	0.5	9.5	10.0
4	100 mg/dl	1:10	1.0	9.0	10.0
5	200 mg/dl	1:5	2.0	8.0	10.0
6	500 mg/dl	1:2	5.0	5.0	10.0
7	1000 mg/dl	None	10.0	none	10.0



Appendix Figure 3. Standard curve for glucose

APPENDIX IV. ANOVA

Appendix Table 5 Cellulase production from different isolates on different treatment

Para meters	Treatments							
	T1	T2	T3	T4	T5	T6	SEM	SL
CSI1	27.8±0.3^{e}	41.31±0.6^{b}	38.09 ±0.9^{c}	46.07 ± 0.2 ^{a}	35.2±0.3^{d}	36.45±0.23^{cd}	0.533	**
CSI2	29.3±1.0^{d}	47.1±1.2^{b}	34.14±0.8^{c}	52.77±0.24^{a}	36.8±0.67 ^{c}	35.12±0.56 ^{c}	1.37	***
CSI3	29.5±1.4^{d}	47.43±0.25^{b}	34.94±1.02^{c}	57.63±0.09^{a}	36.80±0.8^{c}	36.81±0.63 ^{c}	0.65	***
FSI1	41.1±1^{ab}	39.90±0.71^{b}	28.07±0.64^{c}	42.54±0.24^{c}	21.4±0.3^{d}	28.73±0.02^{c}	0.056	ns
FSI2	32.1±1^{bc}	44.15±0.85^{b}	41.39±0.44^{bc}	62.99±1.24^{a}	25.50±0.3^{c}	25.43±0.25^{c}	5.23	***
FSI6	34.6±0.1^{c}	49.59±1.3^{b}	44.93±0.95b	83.12±3.18 ^{a}	26.9±0.09^{d}	35.21±0.33^{c}	3.68	***

$^{a, b, c}$ = means with different superscripts in a row are significantly different. *** =($p<0.001$), **=($p<0.01$) *=($p<0.05$; SEM =standard error of the mean, SL=significance level, ns= non-significant T1 = 20^{O}C + SmF, T2=20^{O}C +SSF, T3= 28^{O}C +SmF, T4=28^{O}C +SSF, T5 = 38^{O}C + SmF, T6= 38^{O}C + SSF

Appendix Table 6. ANOVA for of CMCase and FPase as affected by temperature

			SMF			
T^{o}(^{O}C)	Source	DF	Sum of Squares	Mean Square	F Value	P
28 (cmcase) In FMS	Model	5	537.47	107.49	210.3***	≤0.001
	Error	12	6.132	0.5		
	Corrected Total	17	543.6			
	R-Square	0.968				
	C.V.	3.16				
28 (fpase) In FMS	Model	5	760.99	152.198	52.99**	0.084
	Error	12	34.467	2.872		
	Corrected Total	17	795.461			
	R-Square	0.956				
	C.V.	6.04				
28 (cmcase) In OS	Model	5	327.548	65.5	131.3***	≤0.001
	Error	12	5.98	0.498		
	Corrected Total	17	333.535			
	R-Square	0.982				
	C.V.	1.86				
28 (fpase) in OS	Model	5	760.944	152.188	52.73***	≤0.001
	Error	12	34.63	2.88		
	Corrected Total	17	798.58			
	R-Square	0.956				
	C.V.	6.04				

			SSF			
T°(°C)	Source	DF	Sum of Squares	MSS	F Value	P
28 (cmcase) in FMS	Model	5	312	314	13.56**	0.059
	Error	12	245	52		
	Corrected Total	17	378			
	R-Square	0.849				
	C.V.	11.92				
28 (fpase) in FMS	Model	5	291.489	58.297	8.20**	0.024
	Error	12	85.35	7.112		
	Corrected Total	17	376.845			
	R-Square	0.777				
	C.V.	7.11				
28 (cmcase) in OS	Model	5	318.8	237.76	10.54**	0.026
	Error	12	56.5	47.09		
	Corrected Total	17	375.3			
	R-Square	0.849				
	C.V.	9.54				
28 (fpase) in OS	Model	5	287.76	57.55	7.99**	0.051
	Error	12	86.38	7.199		
	Corrected Total	17	374.15			
	R-Square	0.769				
	C.V.	7.11				

Appendix table 7. ANOVA for CMCase and FPase as affected by pH

			SMF			
pH	Source	DF	Sum of Squares	MSS	F Value	P
5.5 Cmcase (in FMS)	Model	5	195.92	39.18	699.56***	≤0.001
	Error	12	0.672	0.05		
	Corrected Total	17	199.59			
	R-Square	0.996				
	C.V.	0.83				
5.5 fpase (in FMS)	Model	5	34.26	6.85	42.76***	≤0.001
	Error	12	1.92	0.16		
	Corrected Total	17	36.19			
	R-Square	0.946				
	C.V	3.47				
5.5 Cmcase (in OS)	Model	5	319.92	63.98	70.14***	≤0.001
	Error	12	10.94	0.912		
	Corrected Total	17	330.87			
	R-Square	0.966				
	C.V.	3.52				
5.5 fpase (in OS)	Model	5	15.05	3.01	51.14***	≤0.001
	Error	12	0.706	0.058		
	Corrected Total	17	15.75			
	R-Square	0.955				
	C.V.	2.45				
			SSF			
5.5 Cmcase (in FMS)	Model	5	354.97	70.99	357.54***	≤0.001
	Error	12	0.238	0.019		
	Corrected Total	17	355.21			
	R-Square	0.955				

	C.V.	3.42				
5.5 fpase (in FMS)	Model	5	89.45		357.2***	≤0.001
	Error	12	1.061			
	Corrected Total	17	89.51			
	R-Square	0.989				
	C.V.	1.3933				
5.5 Cmcase (in OS)	Model	5	319.95	63.98	70.14***	≤0.001
	Error	12	10.94	0.912		
	Corrected Total	17	330.87			
	R-Square	0.966				
	C.V.	3.51				
5.5 fpase (in OS)	Model	5	210.11	42.023	121.99***	≤0.001
	Error	12	4.133	0.344		
	Corrected Total	17	214.25			
	R-Square	0.98				
	C.V.	3.15				

***= highly significant at P≤0.001.**= significant at P≤0.001