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RESEARCH ARTICLE OPEN ACCESS

Lignocellulosic Waste Degradation Potential of Some Cellulolytic Fungal Strains Isolated from Putrid Fruits

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Abstract

Fungi are plentiful in nature and they are found growing on wastes of wood materials. These wastes are equally found in our environment with no usefulness. The aim of this study was to exploit the probability of the isolated fungi from fruits to produce cellulase from wastes of lignocellulosic materials. Cellulase- producing fungi were isolated from fruits (tomato, banana, plantain). The organisms were screened for cellulase production. Culture conditions were optimized with pH, temperature and carbon. Cellulase was produced using lignocellulosic wastes; sawdust, corn cob, sugarcane bagasse. Six cellulase producers were isolated, four of which were selected for synthesis and quantification of the cellulase. The fungi were identified as *Saccharomyces cerevisiae*[4], *Trichoderma* species[1] and *Scopulariopsis brevicaulis*[1]. Of all the tested substrates used in this study, pretreated sugarcane bagasse at 3% w/v concentration with *Scopulariopsis brevicaulis* gave highest cellulase production 18.18 U/mL at 40°C, 5day incubation time and pH 5, followed by *Trichoderma* with 12.39 U/mL. These fungi are good potentials cellulase producers that can be considered at industrial level.

Keywords: Fungi, peptone, substrate, plantain, cellulase

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INTRODUCTION

Cellulolytic enzymes are of three groups, Endo-(1, 4)-β- D glucanase, Exo- (1, 4) β- D glucanase and β glycosidase (Zhang et al., 2006). Profitable productions of cellulase can be achieved through solid or submerged culture fermentation. Media used in cellulase fermentation may contain cellulose in different degree of limpidness or as raw plant waste substrate (Aiello, 1996). Enzymes including cellulases have been reported produced in a wide collection of organisms; fungi, bacteria and invertebrates (Gautam et al*.,* 2010). Cellulases are of industrial importance specially used in the textile (Bhat, 2000), detergent ((Karmakar and Ray, 2011), pulp and paper (Bedford et al., 2003), food (De Castro, 2008) waste treatment (Gupta et al., 2010), as well as improving digestibility of animal feeds (Dhiman et al., 2002)

Cellulose is the most abundant and renewable biopolymer on earth. It is the dominating waste material from agriculture and constitutes the waste generated from both natural and man-made activities. Cellulose is generally degraded by a multi-complex enzyme called cellulases. Synergistic mechanism among three types of enzymes namely endoglucanase, exoglucanase and β-glucosidase are requisite for the inclusive enzymatic hydrolysis of cellulose biopolymer (Gao et al*.,* 2008). Cellulose is a linear polymer of glucose and composed of anhydroglucose units attached together by β-1, 4 glycosidic bonds. The number of glucose units in the cellulose molecules varies and degree of polymerization ranges from 250 to over 10,000 depending on the source and treatment method (Klemm et al., 2005).

Large quantities of lignocellulosic wastes are generated through forestry, agricultural practices and industrial processes, particularly from agro-allied industries such as breweries, paper-pulp, textile and timber industries. These wastes generally accumulate in the environment thereby causing pollution problem (Abu et al*.,* 2000; Gilna and Khaleel, 2011). Lignocellulosic biomass is the most abundant organic material in nature with high biotechnological potential (Rastogi et al., 2010). The biological conversion of lignocellulosic materials into industrial products requires the use of cellulolytic and hemicellulolytic enzymes to release fermentable sugars, microorganisms play an important role in this (Jourdier et al., 2012; Romano et al., 2013). Moreover, the plant biomass regarded as "wastes" can be recycled and transformed into valuable products such as biofuels, chemicals, cheap energy sources for fermentation, enhanced animal feeds and human nutrients (Howard et al*.*, 2003). Microorganisms are the major sources of enzyme production industrially and the production of enzyme from microbes is highly treasured because of simplicity of cultivation, high yield and utilization of various cheapest sources (El- Shora et al., 2014b; Selvaraj and Arvind, 2015) and seasonal fluctuation of raw materials do not occur (El-Shora et al., 2014a),

Techniques are being used to reduce the wastes on the environment with the use of microorganisms to produce beneficial products to mankind (Lynd et al., 2002), hence this study was aimed to produce cellulase using cellulosic wastes with the fungi isolated from different fruits.

MATERIALS AND METHODS

Isolation procedure

Ripe fruits (Tomatoes, Plantain, and Banana) were purchased at Timi Market, Ede, Osun State. The fruits were left on the laboratory bench for twenty-one days to allow for colonization by cellulase producing microorganisms. 5mm cock borer was sterilized and used to remove tissue from area close to the edge colonization and attack of Spoiled Tomatoes, Plantains and Bananas to obtain actively growing mycelia. This was aseptically inoculated at the centre of sterile modified GPYA Media.(Glucose 4.0g, Peptone 0.5g,Yeast Extract 0.5g, Agar 1.5g, distilled water 100mls, Chloramphenicol 0.05g). The plates were done in triplicate and incubated at 27±2°C for 5 days (Onilude, 1996; Osho, 2005). This procedure was carried out in air lamina flow chamber. The chemicals used were of analytical grade. Both the chemicals and Glassware were sterilized by autoclaving and hot air oven at temperature of 121°C, for 15 min and 180°C for two hours respectively. Inoculating needles were sterilized by flaming.

Identification of pure fungal cultures of isolates

The isolated fungi were subcultured on sterile culture plates containing PDA medium to obtained pure culture of each isolate. Observation of microscopic characteristics of each isolate was done using sterile forceps to aseptically pick a

strand of mycelium with the fruit body and it was carefully placed on a clean microscopic slide, stained with lactophenol blue and subsequently covered with a clean cover slip. Mycelium was then observed under the x40 objective of the light microscope. Isolates were then subjected to both cultural and morphological characterization and compared with characteristics in the Fungal Compendium (Domsch et al., 1980). Each isolate was code labeled and sub-cultured regularly to maintain viability. Primary and secondary screening for cellulase production and activities were done according to methods described by Adesina and Onilude, (2013). The amount of reducing sugar present in the fermentation medium, cellulase activities were determined (Miller, 1959). This was done in conjunction with the method of Mandels and Webber (1969). One unit of cellulase was defined as the amount of enzyme that released Iμmol reducing sugar as glucose equivalent per minute in the reaction mixture under the specified assay conditions. All enzyme assays were performed in triplicates.

Cellulosic materials

Sawdust of Iroko wood (*Milicia excelsa*) and Araba wood (*Ceiba pentandra*) were obtained at Oke Gada Sawmill, Ede, Osun State, Nigeria. Sugarcane bagasse and corn cob used in this study were obtained at Oje market of Ede, Osun State.

Consequence of early pH on cellulase production

Ten milliliter (10 mL) each of sterile CPY broth (CMC 1.0 g, Peptone 0.5 g, Yeast Extract 0.5 g,) at pH 5, 6, 7, 8 with 0.2M Phosphate buffer in sterile universal bottles were aseptically inoculated with 1ml inoculum of *Trichoderma species, Scopulariopsis brevicaulis* and *Saccharomyces cerevisiae*. Uninoculated sterile CPY at pH 5-8 in universal bottles were used as control. The bottles were incubated at 27±2°C for 5days.

Cellulase assay at pH 5, 6, 7, 8

Cellulase was assayed by centrifuging 5mls of the yeasts culture (broth) at 4000rpm for 30minutes while *Trichoderma* species and *Scopulariopsis brevicaulis* culture (broths) were filtered with Whatman No1 filter paper. 0.5mL supernatant/filtrate of each culture was pipette into sterile test tubes containing 0.5mL of sterile 0.5% CMC at pH 7 in 0.2M Citrate- phosphate buffer (Jeffries, 1996).The mixture was incubated at 50 °C for 30 minutes. 1 mL of DNSA Solution (Dinitrosalicylic acid) was added to the mixture and boiled in the water bath for 5minutes. It was then allowed to cool in water for color stabilization before optical density was measured with spectrophotometer at 540nm (Acharya et al., 2008).

Consequence of diverse concentrations of carboxyl methylcellulose on cellulase production

The effect of different carboxyl methylcellulose concentrations from 0.5 %-1.5 % was studied. 10 mL each of sterile modified CPY broth (CMC, Peptone, Yeast Extract) was inoculated with 1mL inoculum of *Saccharomyces cerevisiae*, spores of *Trichoderma species* and *Scopulariopsis brevicaulis* culture aseptically. Uninoculated 0.5%-1.5% sterile CPY broth in Marcatney bottles were used as control. The culture and control were incubated at 27±2°C for 5days. Cellulase was assayed using method reported by Acharya et al. (2008).

Consequence of diverse temperatures on cellulase production by the isolates

Optimum temperature on cellulase production by the isolates was determined at temperature ranging from 30-50°C. Cellulase was

Table 1. Consequence of diverse initial pHs on cellulase production

	Isolates/cellulase activity			
pH	TRC / U/mL	SAC1/ U/mL	SAC3/ U/mL	SCB/ U/mL
5	2.45	2.45	2.56	2.67
6	2.34	2.34	2.45	2.0
7	2.22	4.11	2.34	1.67
8	1.28	1.28	2.22	1.56

Table 2. Consequence of varying temperatures on cellulase production

Isolates	Temperature (U/mL)				
	30°C	35°C	40°C	45°C	50°C
SAC1	3.0	5.01	5.56	7.01	5.45
SAC3	2.89	2.57	3.22	5.56	4.34
TRC	1.11	1.67	2.67	3.57	1.67
SCB	1.22	2.22	5.56	2.89	2.89

assayed using method reported by Acharya et al. (2008).

Preparation of substrates

The substrates were sundried, ground and sieved with a 2 mm mesh size (Irfan et al., 2011).

Pretreatment of lignocellulosic substrates

The substrates were alkali-treated by autoclaving the washed and dried lignocellulosic substrates at 121°C for 30min with 0.25M NaOH (20mL/g substrate). The substrates recovered by filtration through muslin cloth were thoroughly washed with de-ionised water and neutralized with 0.25M HCl. The substrates were finally washed with many changes of de-ionised water and dried at 65°C to constant weight (Singh et al., 1998)

Fungal inocula preparation

Sterilized molten CPY media (CMC 1.0 g, Peptone 0.5 g, Yeast Extract 0.5 g, Agar 1.2 g, 100 mL distilled water) at pH 6 was inoculated with yeast strains aseptically. Culture discs from *Trichoderma species* and *Scopulariopsis brevicaulis* culture plates cut separately with a cork borer (5mm diameter) were aseptically inoculated separately in 1% sterilized CPY media. After 5days of growth at 27±2°C the cultures were used as inocula for cellulase production.

Outcome of varying concentrations of substrates on cellulase production

Preparation of media using treated and untreated cellulosic substrates

The substrates; soft wood sawdust from Araba wood (*Ceibapen tandra*), corn cob, sugarcane bagasse, hard wood sawdust from Iroko wood (*Milicia excelsa*) serving as carbon sources in the fermentation media were prepared by supplementing each substrates with Peptone and Yeast Extract. The media were prepared using appropriate buffers at pH 6. Each substrate was prepared in the range 1%-3%w/v. 10 mL of each concentration was dispensed in sterile universal bottles, autoclaved at 121°C for 15minutes, cooled and aseptically inoculated separately with 1 mL inocula of *Saccharomyces cerevisiae*, T*richoderma species* and *Scopulariopsis brevicaulis* spores. Uninoculated sterile media at concentration 1%-3% were used as control. The bottles were incubated at 45°C for 5 days.

Cellulase assay by the isolates using lignocellulosic wastes

Cellulase was assayed by centrifuging 5mL of the yeasts culture (broth) at 4000rpm for 30minutes and filtered in the case of T*richoderma species* and *Scopulariopsis brevicaulis* with Whatman No1 filter paper. 0.5mls supernatant/ filtrate of each culture was pipette into test tubes containing 0.5mls of Sterile 0.5% CMC at pH 7 with 0.2M Citrate-phosphate buffer (Jeffries, 1996). The mixture was incubated at 50°C for 30minutes. 1 mL of DNSA (Dinitrosalicylic acid) solution was added to the mixture and boiled in the water bath for 5minutes. It was cooled in water for color stabilization and the optical density was measured with a Spectrophometer at 540nm. (Acharya et al., 2008).

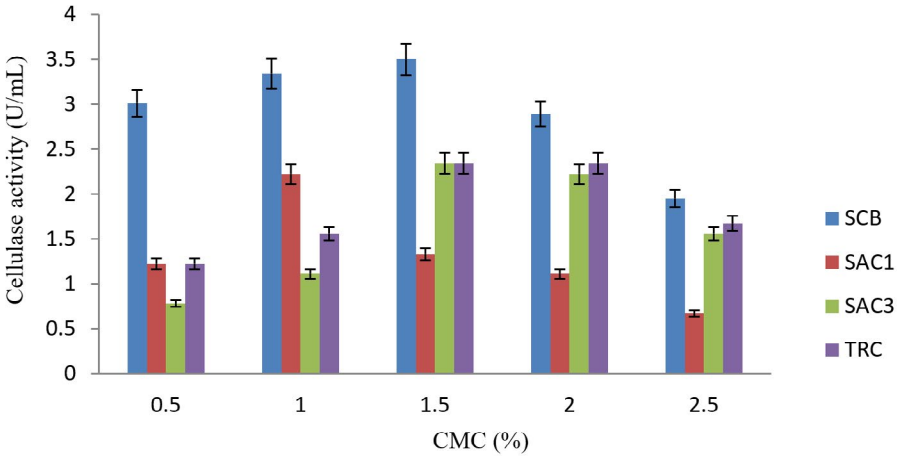

Fig. 1. Consequence of diverse concentrations of carboxylmethyl cellulose on the production of cellulanase by fungi isolates

RESULTS

The six (6) fungi used in this study showed the ability to produce cellulase on carboxyl methyl cellulose. They include *Saccharomyces cerevisiae* (SAC1), *Saccharomyces cerevisiae* (SAC2), *Saccharomyces cerevisiae* (SAC3), *Saccharomyces cerevisiae* (SAC4) *Trichoderma* sp. (TRC) and *Scopulariopsis brevicaulis* (SCB) with relative enzyme activity 2.90, 0.35, 2.80, 0.25, 4.20 and 4.02 respectively.

DISCUSSION

Various substrates such as pineapple pulp, paper, pineapple peel, sawdust, sugarcane bagasse, wheat bran, beech wood have been used to produce cellulase. In this study, ability of cellulase produced by fungal isolates from fruits to degrade treated and untreated softwood sawdust from Araba (*Ceibapen tandra*), hardwood sawdust from Iroko wood (*Milicia excelsa*), corncob and sugarcane bagasse were studied. Parameters such as pH, carbon source, temperature induce the yield of cellulase (Igbal et al., 2010). Maximum cellulase activity of 2.45 U/mL and 2.56 U/mL were noticed for TRC and SAC3 respectively at pH 5 while 4.11 U/mL cellulase activity was for SAC1 at pH 7. The result in this work is in conformity with Omojasola et al. (2008), who observed that

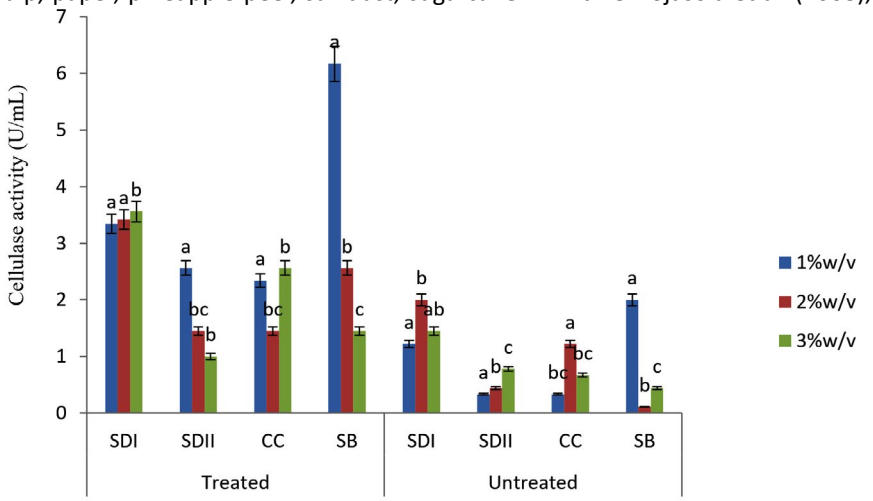

Fig. 2. Degradation ability of the isolates SAC1 on Lignocellulose Substrates

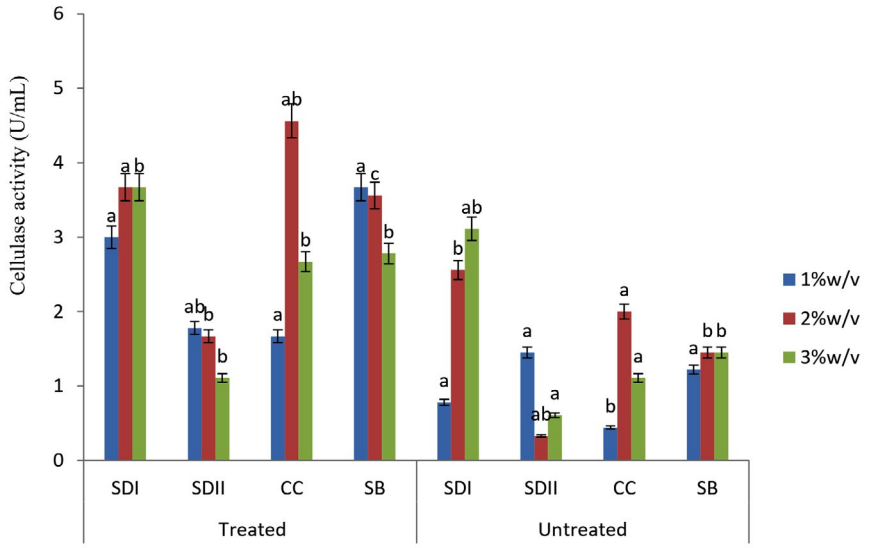

Fig. 3. Degradation ability of the isolates SAC3 on Lignocellulose Substrates

3.0 U/mL of cellulase was produced at pH 5 by *Saccharomyces cerevisiae*. Selvaraj and Arvind, (2015) also reported higher cellulase production at acidic pH. The high activity of the enzymes at acidic condition is an advantage for industrial fermentation.

Cellulase activity was greatly affected by the concentration of carbon source in submerged fermentation. The activity improved as the concentration of carboxyl methyl cellulose (carbon source) increased from 0.5% - 1.5%.w/v The maximum yield of cellulase by isolates SCB, SAC3 and TRC were 3.5 U/mL, 2.34 U/mL and 2.34 U/mL respectively at 1.5% w/v concentration while it was 0.4mg/ml for SAC1 at 1.0% w/v.

The temperature at which enzyme-substrate reaction was incubated plays a significant role in cellulase productivity (Seyis and Aksoz, 2003). Cellulase action was investigated at different temperatures, from 30-50°C. The maximum yield of cellulase by SAC1, SAC3 and TRC were 7.01 U/mL, 5.56 U/mL, 3.67 U/mL respectively at 45°C while the minimum yield of cellulase were 3.0 U/mL, 2.89 U/mL and 1.11 U/mL respectively at

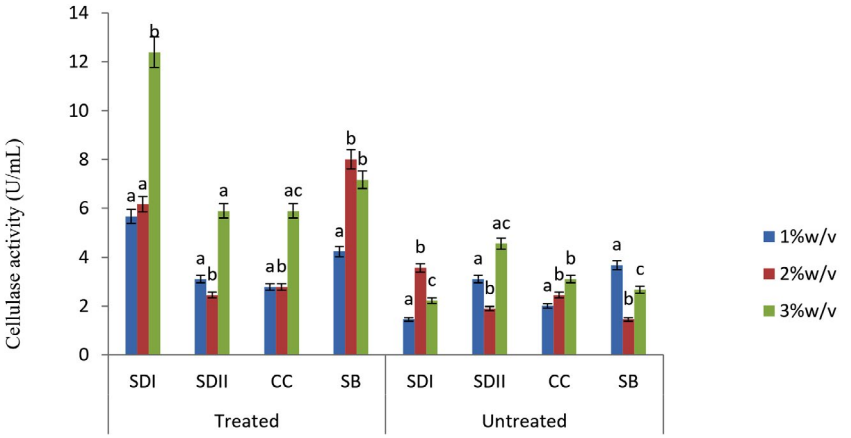

Fig. 4. Degradation ability of the isolates TRC on Lignocellulose Substrates

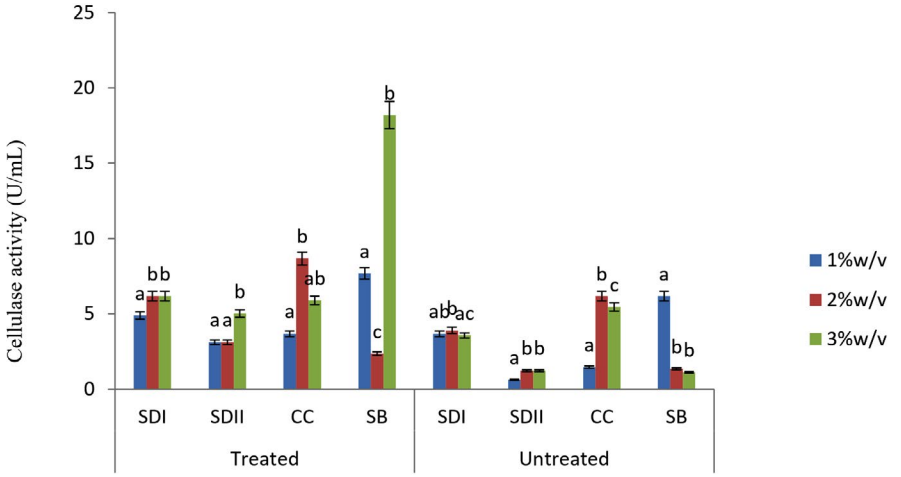

Fig. 5. Degradation ability of the isolates SCB on Lignocellulose Substrates

SDI Araba wood (*Ceibapen tandra*)—Sawdust, SDII Iroko wood (*Milicia excels*) —Sawdust: CC---Corn cob: SB—Sugar cane bagasse; Each value is a mean of two replicates; ± stands for standard deviation among replicates; Means with different letters within each substrate differ significantly ($p \leq 0.05$) using Duncan`s Multiple Range Test (Fig. 2, 3, 4 and 5)

30°C Fortkamp and Knop, (2014) observed that optimum enzyme production by most fungi is at mesophilic range. Increased temperature above 45°C lead to decreased in cellulase activity. This may be due to the fact that most of cellulase-producing fungi are mesohpiles, though there are few thermophilic ones. Gautam et al. (2010) had earlier reported similar results.

In this study there were higher cellulase productions in treated soft wood Araba (*Ceibapen tandra*) than hardwood Iroko(*Milicia excels*) (Fig. 2-5). The fact that softwood has more cellulose than hardwood may be responsible for this. Cellulase activity for SAC3 increased from 1%-3% w/v concentrations of untreated softwood while the increment was up to 2 % w/v for SAC1, TRC and SCB, after which a decrease in activity was noticed (Fig. 2-5). The maximum cellulase activities using treated and untreated softwood sawdust were 12.39 U/mL for treated and 3.89 U/mL for untreated. The treated is isolates TRC and untreated for SCB (Fig. 4 and 5). The higher degree of fermentation of woody substrate in this study by *Trichoderma* sp. has earlier been reported. Adesina and Onilude 2013 observed that between 6.01 U/mL and 10.18 U/mL of glucanase activities were recorded for *Trichoderma* spp. isolated from wood chips. Maximum cellulase activities using treated hardwood sawdust were 5.01 U/mL and 5.89 U/mL for SCB and TRC respectively at 3% w/v. The activity is low when untreated hardwood was used. The variation in the amount of cellulase when treated and untreated hardwood was used may be due to the existence of some components (activators or inhibitors) in these materials (Mabrouk and Anwany, 2008). Result of cellulase production in different concentrations of treated and untreated lignocellulose showed that treated Sugarcane bagasse was more fermented by *Scopulariopsis brevicaulis* (SCB) with activity 18.18 U/mL followed by 12.39U/mL cellulase activity by isolate TRC in *Ceibapen tandra* at 3.0% w/v (Fig. 4 and 5). Leghlimi et al. (2013) also reported the production of hydrolytic enzyme on lignocellulosic substrates by the organism. The higher amount of cellulase activity observed in the treated sugarcane bagasse than the other lignocellulosic materials had earlier been attributed to the increment in surface area and reduce crystallinity in sugarcane bagasse exposed to alkaline pretreatment (Akhtar et al. 2001). The general dissimilarity in the quantity of cellulase formed when treated and untreated lignocellulosic materials were used, especially the higher amount of enzyme produced with the treated is due to the removal of lignin. Lignin hinders the interaction of cellulase with cellulose locked up in lignocellulose ((Lynd et al., 2002; Ja`afaru and Fagade, 2007; Gautam et al., 2010).

CONCLUSION

Fungi are good sources of enzymes especially for industrial purpose. Temperature, pH, nitrogen and carbon sources have substantial influence on cellulase production by the fungi investigated in this study. It was shown in this study that treated *Ceiba pentandra* and sugarcane bagasse gave maximum cellulase by *Trichoderma sp.* and *Scopulariopsis brevicaulis* respectively. These two fungi could be good sources of industrial cellulase.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Authors' Contribution

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ETHICS STATEMENT

Not applicable.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

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