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Isolation and Screening of Cellulolytic Fungi from Cocoa Pod Husks and Optimization Conditions for Cellulase Production

Folake T. Afolabi[1*], Ololade Obisesan[2], Richard O. Igbinadolor[3]

[1] *Industrial Microbiology and Biotechnology Unit, Department of Microbiology, University of Ibadan, Nigeria*
[3] *End Use Research, Cocoa Research Institute of Nigeria, Ibadan, Oyo State, Nigeria*

Abstract

This study aimed at isolating fungi from cocoa pod husks, screen for their ability to degrade cellulose, optimize, produce and purify cellulase. Cocoa pod husks were obtained from Badeku area of Oyo State. The isolates that showed consistent zone of hydrolysis and negative for aflatoxin production were used for cellulase production.

Cellulase activity was measured in terms of FPUase and CMCase. A total of 23 fungal isolates were obtained from cocoa pod husk out of which 12 showed a consistent zone of hydrolysis. The fungal isolates were: *Aspergillus niger* CH2, *A. niger* CH4, *A. flavus* CH5, *Fusarium oxysporium* CH8, *A. niger* CH10, *A. flavus* CH13, *A. niger* CH15, *Phytophthora* sp. CH16, *A. niger* CH18, *A. niger* CH20, *A. flavus* CH21, *A. niger* CH23. *A. niger* CH4 and *F. oxysporium* CH8 were the isolates used in this study. Incubation time, pH, temperature and substrate concentrations were the parameters used for the optimization of cellulase. The preferred optimized conditions determined for the production of cellulase by *A. niger* CH4 were 96 hrs, pH 5.5, 30°C and 5% CPH concentration while for *F. oxysporium* CH8 they were 72 hrs, pH 5.9, 30°C and 4% CPH concentration. Endoglucanase activity was higher in *F. oxysporium* CH8 while exoglucanase was higher in *A. niger* CH4. The purification of the ammonium sulphate purified cellulase enzyme using Sephadex G-200 gel filtration increased *A. niger* CH4 cellulase activity by 1.49-times while that of *F. oxysporium* CH8 increased by 1.63-times. *A. niger* CH4 and *F. oxysporum* CH8 can be used for the production of cellulose.

Keywords: Cellulose, cellulase, cellulolytic fungi, cocoa pod husk

Резюме

Целта на настоящото проучване е да се изолират щамове гъби от какаови шушулки, да се проучи способността им да разграждат целулоза, да се оптимизират условията за култивиране и да се получи пречистена целулаза. Какаовите шушулки са от района на Badeku, щата Oyo, Нигерия. Изолатите, които показват постоянна зона на хидролиза и са отрицателни за производството на афлатоксин, са използване за получаване на целулаза.

Целулазната активност се измерва по отношение разграждането на карбоксиметил целулоза (КМЦ активност) и на филтърна хартия (ФХ активност). Получени са общо 23 фунгални изолати, от които 12 показват постоянна зона на хидролиза на целулозата. Тези изолати са идентифицирани като *Aspergillus niger* CH2, *A. niger* CH4, *A. flavus* CH5, *Fusarium oxysporium* CH8, *A. niger* CH10, *A. flavus* CH13, *A. niger* CH15, *Phytophthora* sp. CH16, *A. niger* CH18, *A. niger* CH20, *A. flavus* CH21, *A. niger* CH23. В следващите експерименти са използвани щамовете *A. niger* CH4 и *F. oxysporium* CH8. Оптимизирани са параметрите на ферментацията - време на инкубация, pH, температура и концентрация на субстратите. Предпочитаните условия, определени за производството на целулаза от *A. niger* CH4 са 96 часа култивиране при pH 5.5, 30°C и 5% концентрация на какаова шушулка, докато за *F. oxysporium* CH8 са 72 ч, pH 5.9, 30°C и 4% концентрация на какаова шушулка. Ендоглюканазната активност е по-висока при *F. oxysporium* CH8, докато екзоглюканазата е по-висока

* Corresponding author: folakeojo1@yahoo.com; folakeojo1@ui.edu.ng

при *A. niger* CH4. За получаване на пречистен целулазен ензим след утаяване с амониев сулфат се използва гел-филтрация със Sephadex G-200, при което активността на целулазата на *A. niger* CH4 се повишава 1.49 пъти, докато тази на *F. oxysporium* CH8 се увеличава с 1.63 пъти. *A. niger* CH4 и *F. oxysporum* CH8 могат да бъдат използвани за производството на целулаза.

Introduction

Cellulases are a class of enzymes (produced majorly by microorganisms e.g. fungi, bacteria, protozoan) that speed up the degradation of cellulose and some other polysaccharides. Some plants and animals are also able to produce cellulases. Cellulases are hydrolytic enzymes responsible for the decomposition of the natural cellulose polymer by acting on 1, 4 β-D-glucosidic linkages which is finally converted into glucose monomer. The production and applications of cellulases are important in the bioprocessing industries, preparation of medicines, waste treatment, food production, paper industries, perfumes, baking (Picart *et al*., 2007).

Cellulose, the major component of plant cell walls, is the most abundant polysaccharide in nature and a source of renewable bioenergy (Priit *et al*., 2001) and a linear polysaccharide composed of β-1, 4-linked glucose molecules (Olanbiwoninu and Odunfa, 2016).

Fungal cellulases are the most involved in the degradation of lignocellulosic materials. Examples of lignocellulose are cocoa pod husks, corn cobs, rice husks, sugarcane bagasse, citrus zest, mango pills etc. which are mostly agricultural wastes and wastes from various industrial processes. Sometimes, the bulk of these fruits are wastes dumped indiscriminately in the environment; this is responsible for the ready availability of many lignocellulosic materials to be used as substrate for large-scale production of value-added products. Cocoa (*Theobroma cacao* L.) is one of the cash crops in Nigeria cultivated majorly in the south western part of the country (Oyewole *et al*., 2012). Cocoa pod husk as a by-product of cocoa processing has vast quantities of sugars occurring as structural polysaccharide - cellulose and hemicellulose (Igbinadolor, 2012). Cocoa is an important plant in Indonesia, and the country is the second largest producer of cocoa beans in the world after Ivory Coast (FAOStat, 2012). This indicates that a large quantity of cocoa pod husk is also produced in the country as a by-product. The total area of cocoa plantations in 2011 was about 1.68 million hectars (Agricultural Ministry, 2012), which equals at least 4.54 million tonnes of dried cocoa pod husk.

Among the different ligno-cellulosic raw materials, cocoa pod husk is known as a rich source of biomass very much available in Nigeria as agricultural waste residues in farms with vast quantities of sugars occurring as structural polysaccharide, cellulose and hemicellulose (Igbinadolor, 2012). In West Africa, an estimated 6.7 million metric tons of cocoa pod husk is generated as a by-product of cocoa cultivation (Hamsat and Adeola, 2011). These wastes are usually left unattended and later disposed of by burning which increases the problem of greenhouse effect. It is estimated that 0.8 to 1.0 million tonnes of cocoa pod husk (CPH) is generated annually in cocoa farms in Nigeria (Igbinadolor, 2012). Cocoa pod husk (CPH) is an agro-based by-product which can be incorporated into layers diets to reduce the maize requirement. Very few of the potentials locked up in this by-product have been exploited (Egbe and Olubamiwa, 1989). Research conducted in Nigeria has shown that crushed CPH could be integrated into livestock feeds. Also, animal feeding trials have been carried out in Ghana and Brazil (Egbe and Olubamiwa, 1989).

The growing interest in the use of biomass-based materials like cocoa pod husk (CPH) for bio-ethanol production, especially when accruing as wastes from the agricultural sector is generally a welcome development. Biomass feedstocks are one of the most abundant renewable resources in the world; not only as an alternative source of energy but also have remarkable potentials to mitigate greenhouse gas emissions and contribute to the development of organic chemical industries. However, before lingo-cellulosic materials can be effectively utilized, there is a need for some conversion processes.

The indiscriminate disposal of cocoa pod husk has been a major environmental concern because cocoa pod husk cannot be digested efficiently by animals due to its relatively high content of fibre. Also, cellulase of chemical origin is expensive and has been an obstacle for conversion of biomass into value -added products. The use of readily available lignocellulosic materials (e.g. cocoa pod husks) as substrate for the production of cellulase requires less energy, saves cost and significantly reduces environmental pollution. If cellulase is produced directly from CPH and then applied to further degrade the CPH cellulose, the cost of cellulase will be significantly reduced, the cost of livestock feed will drop, and a significant level of waste lying fallow and constituting a nuisance in our en-

vironment will be reduced leading to a significant decrease in the greenhouse effect. Also, there will be an increase in the profit of the local farmers who are the major producers of this by-product because the whole cocoa fruit will be economically significant. Therefore, this present study was aimed to: (i) isolate fungi from cocoa pod husks, screen for their ability to degrade cellulose; (ii) optimize the different conditions for cellulase production using cocoa pod husk as substrate, produce and purify the cellulase.

Materials and Methods

Sample collection

Healthy and rotten samples of lignocellulolytic material (cocoa pod husk) were obtained from Badeku area, Ona-ara Local government, Ibadan, Oyo State. Samples were brought to the laboratory in sterile polyethylene bags.

Preparation of lignocellulolytic substrate

Fresh cocoa pod husks were washed to remove dirt. These cocoa pod husks were sun-dried and weighed until consistent weight was obtained. Then, they were cut into small strips with a sterile razor blade. The cocoa pod husks were oven-dried at 150ºC for 2 hrs, milled to powder and kept in sterile ziploc bags prior to the time of use (Sai *et al*., 2015).

Isolation of fungi

Potato Dextrose Agar (PDA, Oxoid) was used as the medium for isolation of fungal isolates and it was prepared according to the manufacturer's description. Isolation was carried out by the method of Aneja (2005) using serial dilution and pour plate methods. One g of cocoa pod husk was used to prepare stock solution by adding it to 9 ml of sterile distilled water in sterile test tubes.

Screening for cellulolytic fungi

This was done using carboxymethyl cellulose agar (CMC) according to the method of Tirthesh and Ramendra (2015). The medium was sterilized at 121ºC for 15 min and allowed to cool before being poured into sterile Petri-dishes and allowed to solidify. Five mm cock borer was flamed then used to bore holes in the solidified CMC-agar plates and in the pure culture of three-day old fungi isolates. Agar blocks from three- day old fungal isolate plates was then transferred to the center of the CMC-agar and incubated at 37ºC for 72 hours. Screening was done by staining the CMC-agar plates with 1% Congo-red dye for 1 hour and then further de-stained using 1M NaCl for 30 min. Clear zones were observed, measured and values were recorded.

Morphological and cultural characterization of isolates

Colonies developing on the plates were grouped on the basis of their morphology. Cultural features observed were front, back, color pattern of mycelia growth, colony diameter, and color of spores and production of color pigment, presence or absence of odor. Microscopic identification was done using 0.1% lactophenol cotton blue stain covered with coverslip and then observed under microscope using x40 objective lens. This was done for the different isolates and observations were recorded with reference to Leslie and Summerell (2006), Sharma and Pandey (2010).

Aflatoxin vapor color change test

This was done according to the method of Abbas *et al*. (2004). Isolates were grown as single colonies on PDA plates. One drop of NH_4OH was put on the cover of the plate and the plate was left inverted. Change in the color of the underside of the plates into brownish/yellowish or pink/red confirmed the aflatoxin producing isolates.

Fermentation and extraction of cellulase

Production of cellulase was done using the submerged fermentation method of Quratu-ul-ain *et al*. (2013) using Vogel's Medium. Five ml of selected fungi spore suspension were inoculated into 250 ml Erlenmeyer flasks which contained 100 ml of Vogel's medium. The spore suspension was prepared by flooding the pure fungal plates with 10 ml of sterile distilled water and filtered to remove the mycelia. The medium was sterilized at 121ºC for 15 min before inoculating with the fungal isolates. After fermentation, the medium was centrifuged at 4000 rpm at 4ºC for 15 min. The supernatant was collected and used for determination of enzyme activity.

Determination of cellulase activity

This was done according to the DNS method of Miller (1959), Ghose (1987), Adney and Baker (2008). The DNSA reagent used for the determination of reducing sugar was composed of dinitrosalicylic acid, Rochelle salt, phenol, sodium bisulphate and sodium hydroxide.

Filter paper assay for saccharifying cellulase

Whatman No. 1 filter paper strip, 1.0 x 6.0 cm (=50mg) was used as substrate. The filter paper was cut into a 1 cm x 6 cm strip. Two dilutions of the crude enzyme were done by adding 1 ml crude

enzyme to 0.5 ml citrate buffer and 1 ml crude enzyme to 1 ml citrate buffer respectively. One unit of FPase activity was defined as the amount of enzyme needed to liberate 1 µmol of glucose/min during hydrolysis reaction.

Purification of cellulase

The cellulase enzymes were partially purified using ammonium sulphate precipitation, also known as "salting out", according to the method of Kotchoni and Shonukan (2002). The supernatant (crude enzyme) was brought to 40% and 80% saturation with $(NH_4)_2SO_4$ over the course of two days overnight at 4°C, and centrifuged at 12 000rpm for 30 minutes. The precipitate was dissolved in 20 mmol L-1 0.1 M sodium citrate buffer pH 4.8. The partially purified enzyme was dialyzed using the same buffer for 24 hrs with three buffer changes. Dialysis was performed using a cellulose membrane with a molecular weight cut-off of 5 kDa. Partially purified cellulase was passed through a Sephadex G-100 column. Fractions were collected at a flow rate of 1 ml 5 min-1 and assayed for enzymatic activity.

Results

Fungal isolation, screening and characterization

The result of the cellulolytic fungi isolated from cocoa pod husk samples is shown in Table 1. A total of 23 isolates were obtained from cocoa pod husks. They included species of *Aspergillus*, *Fusarium*, *Rhizopus* and *Phytophtora*.

The *Rhizopus* isolate formed no zone of hydrolysis on CMC-agar. Seventeen isolates formed zones of hydrolysis on CMC-agar. Of the 17 isolates only 12 showed consistent zones of hydrolysis of over 10 mm. The dominant genera were: *Aspergillus*, *Phytophtora* and *Fusarium* with *Aspergillus* spp. The percentage of occurrence of these isolates is shown in Fig. 1. *A. niger* had the highest percentage of occurrence of 50%, *A. flavus* 34%, *Phytophtora* sp. 8%, and *F. oxysporum* 8%.

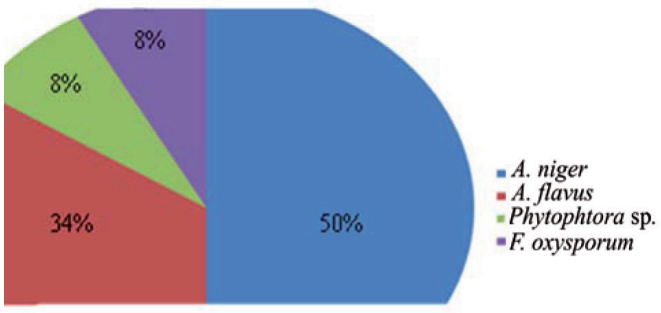

Fig. 1. Percentage occurrence of fungal isolates obtained from cocoa pod husk sample

Table. 1. Fungal isolates obtained from cocoa pod husks showing zones of hydrolysis on CMC-agar

Isolates	R1 (mm)	R2 (mm)	R3 (mm)
A. niger CH 1	12	9	7
A. flavus CH 2	10	13	13
A. flavus CH 3	5	4	8
A. niger CH 4	19	19	19
A. flavus CH 5	14	10	12
Fusarium sp. CH 6	-	-	-
A. niger CH 7	13	9	8
F. oxysporum CH 8	12	12	10
A. flavus CH 9	-	-	-
A. niger CH 10	18	17	19
A. niger CH 11	-	-	-
A. niger CH 12	12	9	10
A. flavus CH 13	12	12	11
A. flavus CH 14	-	-	-
A. niger CH 15		14	`2
Phytophtora sp. CH 16	10	11	11
Rhizopus sp. CH 17	-	-	-
A. niger CH 18	12	10	12
A. flavus CH 19	-	-	-
A. niger CH 20	19	18	19
A. flavus CH 21	12	13	13
A. niger CH 22	8	3	-
A. niger CH 23	17	15	18

Key: R1, zone of hydrolysis repetition one; R2, zone of hydrolysis repetition two; R3, zone of hydrolysis repetition three; (-), noo zone of hydrolysis; CH, isolates from cocoa pod husk sample

Aflatoxin vapor color test

The results of the aflatoxin vapor color change test carried out on the fungal isolates obtained from the cocoa pod husk sample with a consistent zone of hydrolysis above 10 mm on CMC-agar is represented in Table 2. *A. niger* CH4, *F. oxysporum* CH8 and *Phytophtora* sp. CH16 showed no sign of production of aflatoxin. All the consistently cellulolytic *A. flavus* isolates tested positive for production of aflatoxin.

Production of cellulase

A. niger CH4 and *F. oxysporium* CH8 were the isolates used for cellulase production in this study. Isolate CH4 was chosen of all the cellulolytic *A. niger* isolates obtained because it had the most consistent zone of hydrolysis. Control fermentation using CMC as substrate for cellulase production was first carried out.

Table 2. Reaction of fungal isolates obtained from CPH sample to NH_4OH vapor color change

Strains	Aflatoxin
production	
A. flavus CH 2	+
A. niger CH 4	-
A. flavus CH 5	+
F. oxysporum CH 8	-
A. niger CH 10	-
A. flavus CH 13	+
A. niger CH 15	
Phytophtora sp. CH 16	-
A. niger CH 18	-
A. niger CH 20	-
A. flavus CH 21	+
A. niger CH 23	-

Key: CPH, cocoa pod husk; (+), positive; (-), negative; CH, isolate from cocoa pod husk sample

This is represented in Tables 3 and 4. Cellulase produced by isolate *A. niger* CH 4 had the highest cellulase production and activity after 96 hrs with FPUase and CMCase values of 0.357 U/ml and 0.680 U/ml respectively. Cellulase produced by *F. oxysporium* had FPUase activity of 0.172 U/ml and CMCase activity of 0.728 U/ml after 72 hrs.

Table 3. Production and saccharification of *A. niger* CH 4 cellulase using CMC as fermentation substrate

Incubation time (hrs)	FPUase		CMCase	
	Ab. Glucose (mg/0.5ml)	Activity (U/ml)	Ab. Glucose (mg/0.5ml)	Activity (U/ml)
24	0.878	0.163	0.801	0.296
48	0.914	0.169	0.849	0.314
72	1.334	0.247	1.034	0.383
96	1.942	0.359	1.839	0.680
120	1.877	0.347	1.502	0.556

Key: CH, isolate from cocoa pod husk sample; CMC, carboxymethyl cellulose; FPUase, filter paper assay for saccharifying cellulose; CMCase, carboxymethyl cellulose assay for endo-β-1,4-glucanase; Ab., absolute

Table 4. Production and saccharification of *F. oxysporum* CH 8 cellulase using CMC as fermentation substrate

Incubation time (hrs)	FPUase		CMCase	
	Ab. Glucose (mg/0.5ml)	Activity (U/ml)	Ab. Glucose (mg/0.5ml)	Activity (U/ml)
24	0.808	0.149	0.819	0.303
48	0.904	0.167	0.894	0.330
72	0.930	0.172	1.968	0.728
96	0.867	0.160	1.826	0.675
120	0.829	0.153	0.951	0.352

Key: CH, isolate from cocoa pod husk sample; CMC, carboxymethyl cellulose; FPUase, filter paper assay for saccharifying cellulose; CMCase, carboxymethyl cellulose assay for endo-β-1,4-glucanase

Optimized cellulase production

Comparison of the control, pilot and the optimized cellulase production is shown in Fig. 2. and Fig. 3. It was observed that FPUase activity of cellulase produced by *A. niger* CH 4 and *F. oxysporum* CH8 rose significantly in the optimized FPUase than the control while there was a downside in the CMC-ase.

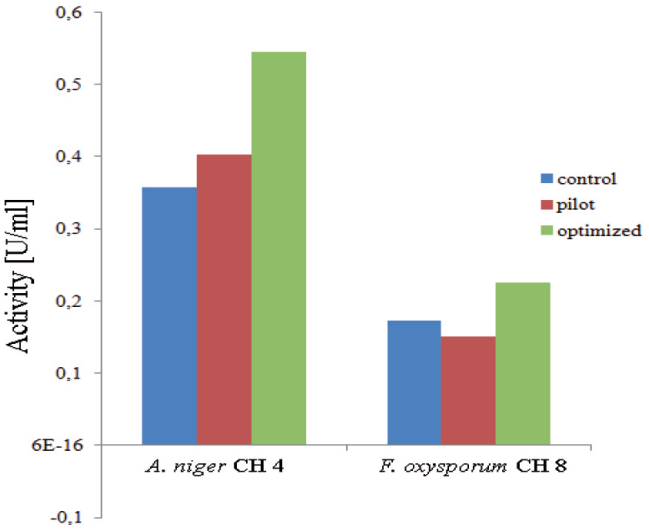

Fig. 2. Difference in the FPUase activity of cellulase produced in the control, pilot and fermentation.

Purification of cellulase

The activity of the fractions obtained from the gel filtration of the partially purified and dialyzed cellulase using Sephadex-200 gel filtration is shown in Fig. 4. with fraction 7 of the *A. niger* CH4 having the highest activity of 2.633 U/ml and fraction 9 of *F. oxysporum* CH8 with 1.716 U/ml.

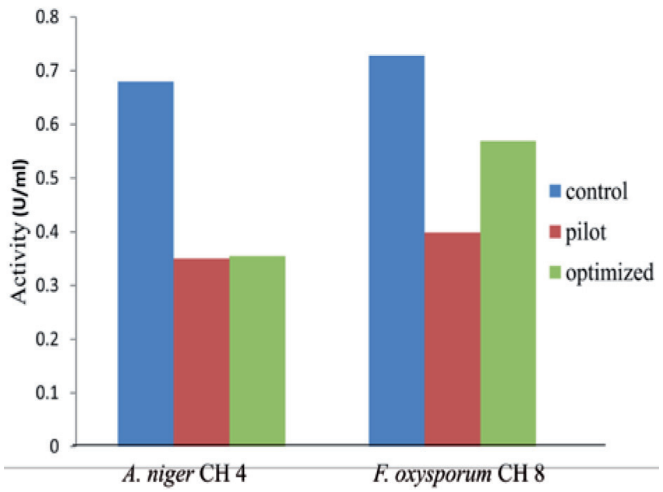

Fig. 3. Difference in the CMCase activity of cellulase produced in the control, pilot and fermentation

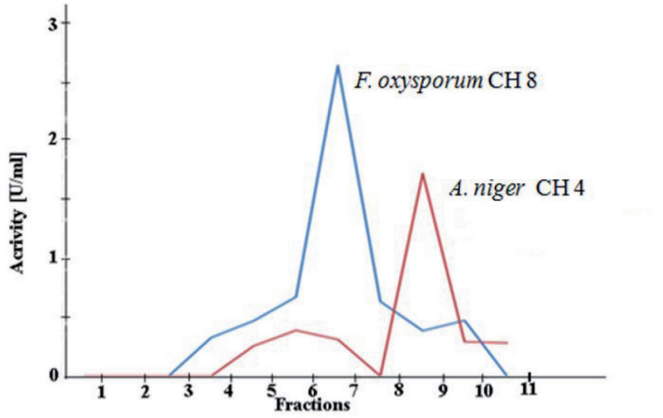

Fig. 4. Activity of fractions from Sephadex-G 200 filtration

Discussion

Twenty three fungal isolates were obtained. This is in agreement with Stone *et al*. (2000) and Arnold (2001) who stated that among terrestrial plants, fungi appear to be quite ubiquitous and every plant species examined to date harbors one or more fungal endophyte species. The dominating genera in this study were: *Aspergillus*, *Phytophtora* and *Fusarium* with *Aspergillus* spp. having the highest occurrence. This is in agreement with the works of Amin *et al*. (2014) that reported the isolation of *Aspergillus* spp. and *Fusarium* spp. from cocoa pod. The successful isolation of *Phytophtora* sp. from cocoa pod husk in this study is in accordance with the fact noted by Bowers *et al*. (2001) that *Phytophtora* spp. is an economically significant microorganism involved in the destruction of cocoa pod. Also, Fagbohun *et al*. (2011) in their research reported that filamentous fungi *A. flavus*, *A. niger* and fungi-like *P. palmivora* are associated with cocoa beans during storage. In agreement with this study, Ogundeji and Olufolaji (2015) confirmed that *Aspergillus* spp. is the predominant fungi of cocoa beans storage in south-west Nigeria. This study also corroborates the data reported by ICCO (2004) that *Aspergillus* spp. is the predominant fungi associated with cocoa pod spoilage in West Africa. This study, however, is in disagreement with the works of Marciano *et al*. (2005), whose fungal isolates from cocoa pod were: *Acremonium*, *Blastomyces*, *Botryosphaeria*, *Cladosporium*, *Colletotrichum*, *Cordyceps*, *Diaporthe*, *Fusarium*, *Geotrichum*, *Gibberella*, *Gliocladium*, *Lasiodiplodia*, *Monilochoetes*, *Nectria*, *Pestalotiopsis*, *Phomopsis*, *Pleurotus*, *Pseudofusarium*, *Rhizopycnis*, *Syncephalastrum*, *Trichoderma*, *Verticillium* and *Xylariato* with the exception of *Fusarium* sp. being the only similar fungal isolate. This may be a result of climatic, environmental and continental differences.

Fungi are the main cellulase producing microorganisms (Narendhirakannan *et al*., 2014). In this study, seventeen of the twenty-three fungal isolates were capable of producing zones of hydrolysis. This is in agreement with Ibatsam *et al*. (2012), who stated that a large number of microorganisms are capable of degrading cellulose, but only a few of these microorganisms produce significant quantities of enzymes capable of completely hydrolyzing cellulose. Of the 17 isolates only 12 showed consistent zones of hydrolysis above 10 mm and they were identified as species *A. niger*, *A. flavus*, *Phytophtora* sp., and *F. oxysporum*. The cellulolytic ability of *A. niger* isolates and *A. flavus* isolates in this study agrees with Ibatsam *et al*. (2012), who reported the species of the genera *Aspergillus* to be very active cellulolytic fungi. Reddy *et al*. (2014) and Mangalanayaki and Madhavan (2015) agreed with this study that *A. niger*, *A. flavus* and *F. oxysporum* are good producers of cellulase. Akinrefon (1968), who studied the production of extracellular enzymes by *P. palmivora* corroborates the cellulolytic activity of *Phytophtora* sp. in this study, although *Phytophtora* spp. is more exacting in its nutrition for extracellular enzymes than its production during growth.

The production of aflatoxin by *A. flavus* isolates in this study agreed with the report of Fakruddin *et al*. (2015), who found all the strains of *A. flavus* isolated from feed and grains positive for aflatoxin production. Erhlich (2014) also stated that *A. flavus* is the most common species associated with aflatoxin contamination of agricultural crops. Hedayati *et al*. (2007) also agrees that *A. flavus* produces the most toxic and potent hepatocarcinogen-

ic natural compound ever characterized. In contrast to this study, Saito and Machinda (1999) observed no color change in the ammonium hydroxide vapor with *A. flavus* on peptone-mineral salts and Czapek solution media. This may be due to the fact that these media do not support aflatoxin production as reported by Abbas *et al*. (2004).

In this research, the isolates *A. niger* CH4 and *F. oxysporum* CH8 were used for cellulase production based on their cellulolytic activity on CMC-agar. Soma and Rangasamy (2011), similarly produced cellulase under submerged and solid state fermentation using *A. niger* and coir waste as substrate. Also, Parkash *et al*. (2016) produced cellulase by *A. niger* using lignocellulolytic substrates. Ramanathan *et al*. (2010) and Mangalanayaki and Madhavan (2016) successfully produced cellulase by *F. oxysporum* using CMC (carboxyl methyl cellulose) and banana waste respectively. *Phytophtora* sp. CH16 was excluded in this study. According to Rossman and Palm (2006), the genus *Phytophtora* belongs to the class *Oomycota* which cannot be categorically called fungi but rather are called fungi-like organisms because they are filamentous in appearance but have many features that differentiate them from true fungi. Vallance *et al*. (2009) and Ruggiero *et al*. (2015) also support the notion that *Phytophtora* spp. are not true fungi.

The highest cellulase production and activity using *F. oxysporum* CH8 as inoculum was observed after 72 hrs with FPUase activity of 0.172 U/ml and CMCase activity of 0.728 U/ml. The high endoglucanase activity in this study as compared with the exoglucanase activity is in agreement with the reports of Ramanathan *et al*. (2010), who confirmed that *F. oxysporum* produces more endoglucanase than exoglucanase though the highest production days and activity differ probably because isolate sources differ. Similarly, Dar *et al*. (2013) suggested that the cellulase produced by *F. oxysporum* is majorly endoglucanase with little or no activity on Avicel and cellobiose. *A. niger* CH4 produced higher yield of cellulase after 96 hrs using CMC as a sole carbon source with FPUase activity recorded as 0.359 U/ml and CMCase of 0.680 U/ml. The recorded activity in this research supports the works of Oyeleke *et al*. (2012) that isolated *A. niger* from corn cobs and observed highest cellulase production on the 4th day of fermentation with CMC as sole carbon source.

In this study, optimization of cellulase production was done using cocoa pod husk (CPH) as the sole carbon source. Ebabhi *et al*. (2013) made it clear that a huge amount of agricultural waste such as crop residue, herbaceous plants, forest residue and animal waste are produced annually around the world and the need to put this waste to beneficial use cannot be over-emphasized.

The incubation time for the highest cellulase production from CPH by *A. niger* CH4 was observed after 120 hrs while *F. oxysporum* CH8 attained its peak after 72 hrs. There was no visible CMCase activity with *A. niger* CH4 at 24 hrs until after 48 hrs. CMCase was valued at 0.350 U/ml and 0.399 U/ml for *A. niger* CH4 and *F. oxysporum* CH8, respectively. FPUase for *F. oxysporum* CH8 cellulase was calculated as 0.151 U/ml and 0.402U/ml for *A. niger* CH4 cellulase. The difference in the best incubation times of *A. niger* CH4 using CPH (120 hrs) as compared to that of the control (96 hrs) may be a result of the degradability of the different carbon sources. This is in agreement with Lee *et al*. (2002), who noted that there is a possibility for cellulase to degrade CMC cellulose faster than the cellulose polymer. This work is in agreement with the work of Bhoosreddy (2012), who observed the highest cellulase production and activity at 120 hrs using corn cob as fermentation substrate. At 168 hrs, there was no observed activity in FPUase of *F. oxysporum* CH8. The 72-hr window of cultivation and production of cellulase by *F. oxysporum* CH 8 in this study is in disagreement with the works of Olajuyigbe *et al*. (2016), who observed maximum glucose production at activity at 96 hrs with no activity at 48 hrs. Shahzadi *et al*. (2014) agrees that the reduction in enzyme production after the optimum cultivation period could be a result of inactivation or inhibition of the fermentation process due to the exhaustion of nutrients in the media or accumulation of toxic wastes that hinder the growth of the fungus. There is a relationship between the glucose produced and the cellulase activity. This is in agreement with the observations of Lui *et al*. (2012) and Olajuyigbe *et al*. (2016), who suggested a correlation between the production of glucose and β-glucosidase.

Variation in substrate concentration of the fermentation medium showed that 5% concentration best supports the production of cellulase by *A. niger* CH4 while 4% substrate supported *F. oxysporum* CH8 best with a significant decline in production of cellulase at 5%. At 1%, the fermentation medium was not as turbid as those with higher concentrations of substrate with both *A. niger* CH4 and *F. oxysporum* CH8. This research suggests that an increase in the substrate concentration is direct-

ly proportional to an increase in the production of cellulase when using *A. niger* CH4. The drop in the production of cellulase with higher substrate concentrations may be a result of the overpopulation of spores through rapid multiplication of the inoculum or, according to Mohammed *et al*. (2014), a drop in the production with an increase or decrease in substrate concentration may be due to the catabolite repression and/ or the accumulation of the phenolic compounds in the fermentation medium. Worthington (2016) recently suggested that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction rate will increase until it reaches a maximum. After this point, increases in substrate concentration will not increase the rate of production. 0.861 mg/0.5 ml glucose was produced by *F. oxysporum* CH8 at 4% concentration while *A. niger* CH4 produced 1.652 mg/0.5 ml glucose at 5% substrate concentration. This is in agreement with the works of Archaya *et al*. (2012), who observed an increase in production up to 9.6% (0.1813 U/ml) when sawdust was used as the fermentation substrate and *A. niger* as inoculum. Similarly, Mohammed *et al*. (2014) reported that fermentation media supplied with 5% sugar cane bagasse as a sole carbon source was suitable for maximum CMCase (0.582 U/ml), cellulobiase (3.008 U/ml) and FPase (0.129 U/ml) production when *Trichoderma* spp. was used as inoculum.

In this research, *A. niger* CH4 exhibited the highest production and activity at pH 5.5 (Fig. 4 and Fig. 5). This is in disagreement with Akiba *et al*. (1995) who stated that *A. niger* cellulases are active in the pH range 6.0-7.0. However, Bhavsa *et al*. (2015) agrees that the best activity of *A. niger* cellulase from agro-wastes was at pH 5.3. Cellulase production gradually increased until it reached the pH that best suited the production of cellulase by *F. oxysporum* CH8 which was 5.9 although cellulase activity was stable between pH 5.9-7.0. Glucose produced by *A. niger* CH4 at its peak pH of 5.5 was 1.328 mg/0.5 ml with activity of 0.246 U/ml. *F. oxysporum* CH8 produced 1.079 mg/0.5ml of glucose with activity of 0.399 U/ml at pH 5.9, which was the highest for this strain. The ability of *F. oxysporum* CH 8 to produce cellulase at such pH ranges suggests that it is a neutrophile. This is in agreement with Ramanthan *et al*. (2010), who observed the highest cellulase activity at 6.0 and 7.0 with *F. oxysporum* by submerged fermentation. Similarly, this research supports the suggestion of Sethi and Gupta (2014) in the optimization of cultural parameters for cellulase production by fungi that pH plays an important role by inducing morphological changes in microorganisms and in enzyme secretion.

This research showed that the most favorable temperature for both *A. niger* CH4 and *F. oxysporum* CH8 was 30°C. The 72-hour window of cultivation and production of cellulase by *F. oxysporum* CH8 in this study is in disagreement with the works of Olajuyigbe *et al*. (2016), who observed maximum glucose production at activity at 96 hrs with no activity at 48 hrs. Shahzadi *et al*. (2014) agrees that the reduction in enzyme production after the optimum cultivation period could be a result of inactivation or inhibition of the fermentation process due to the exhaustion of nutrients in the media or accumulation of toxic wastes that hinder the growth of the fungus. There is a relationship between the glucose produced and cellulase activity. This is in agreement with the observations of Lui *et al*. (2012) and Olajuyigbe *et al*. (2016), who suggested a correlation between the production of glucose and β-glucosidase.

Conclusion

In conclusion, *A. niger* CH4 and *F. oxysporum* CH8 can be used for the production of cellulase. Optimization of the cultural parameters involved in the production process is essential to achieve maximum output of cellulase. The degradation of lignocellulosic materials by microorganisms is crucial to the sustenance of the world of biotechnology. The utilization of cocoa pod husk combats the problem of waste disposal from agricultural practices and also provides extra income for farmers thereby helping in the alleviation of poverty in present-day Nigeria.

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